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Silvia Guillén Morer

Estudio de las relaciones entre resistencia al estrés, capacidad de crecimiento y virulencia en el género Salmonella

Director/es
Cebrián Auré, Guillermo

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Tesis Doctoral

ESTUDIO DE LAS RELACIONES ENTRE RESISTENCIA AL ESTRÉS, CAPACIDAD DE CRECIMIENTO Y VIRULENCIA EN EL GÉNERO SALMONELLA

Autor

Silvia Guillén Morer

Director/es

Cebrián Auré, Guillermo

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Estudio de las relaciones entre resistencia al estrés, capacidad de crecimiento y virulencia en el género *Salmonella*

Memoria para optar al grado de Doctor por la Universidad de Zaragoza presentada por:

Silvia Guillén Morer

Director

Guillermo Cebrián Auré

La presente Tesis Doctoral se ha desarrollado en el laboratorio de Nuevas tecnologías de procesado de los alimentos de la Facultad de Veterinaria de la Universidad de Zaragoza (Instituto Agroalimentario de Aragón—IA2). La realización de la Tesis Doctoral ha sido posible gracias a la financiación del MINECO-CICYT, proyecto AGL2017-84084-R: "La diversidad del género *Salmonella* como modelo para el estudio de las relaciones existentes entre resistencia al estrés, patogenicidad y capacidad de crecimiento microbianas" y del Instituto de Estudios del Huevo, proyecto "Caracterización de la resistencia al estrés y a los tratamientos tecnológicos, de la capacidad de crecimiento y del potencial patógeno de *Salmonella* Heidelberg, *Salmonella* Kentucky, *Salmonella* Livingstone y *Salmonella* Mbandaka" que recibió el premio a la investigación de este instituto en 2018.

Esta Tesis Doctoral está constituida por el compendio de 5 trabajos de investigación previamente publicados en diversas revistas científicas de carácter internacional, que se presentan a continuación en el orden de aparición:

- **Publicación I**: Guillén, S., Nadal, L., Álvarez, I., Mañas, P., Cebrián, G., 2021. Impact of the resistance responses to stress conditions encountered in food and food processing

environments on the virulence and growth fitness of non-typhoidal *Salmonellae*. Foods 10, 617. https://doi.org/10.3390/foods10030617

- **Publicación II:** Guillén, S., Marcén, M., Mañas, P., Cebrián, G., 2020. Differences in resistance to different environmental stresses and non-thermal food preservation technologies among *Salmonella enterica subsp. enterica* strains. Food Res. Int. 132, 109042. https://doi.org/10.1016/j.foodres.2020.109042

- **Publicación III:** Guillén, S., Marcén, M., Fau, E., Mañas, P., Cebrián, G., 2022. Relationship between growth ability, virulence, and resistance to food-processing related stresses in non-typhoidal *Salmonellae*. Int. J. Food Microbiol. 361, 109462. https://doi.org/10.1016/j.ijfoodmicro.2021.109462

- **Publicación IV:** Guillén, S., Marcén, M., Álvarez, I., Mañas, P., Cebrián, G., 2021. Influence of the initial cell number on the growth fitness of *Salmonella* Enteritidis in raw and pasteurized liquid whole egg, egg white, and egg yolk. Foods 10, 1621. https://doi.org/10.3390/foods10071621

- **Publicación V:** Guillén, S., Marcén, M., Álvarez, I., Mañas, P., Cebrián, G., 2020. Stress resistance of emerging poultry-associated *Salmonella* serovars. Int. J. Food Microbiol. 335, 108884. https://doi.org/10.1016/j.ijfoodmicro.2020.108884

Adicionalmente se prevé la publicación de otros cuatro trabajos a partir de los datos obtenidos en la misma, de entre los cuales el siguiente ya ha sido enviado para su publicación a la revista Food Microbiology:

- **Publicación VI:** Guillén, S., Cebrián, G., 2022. Relationship between iron bioavailability and *Salmonella* fitness in raw and pasteurized liquid whole egg. Food Microbiol. (en revision).

Resumen/Abstract

RESUMEN

Los microorganismos del género *Salmonella* constituyen a día de hoy la segunda zoonosis por consumo de alimentos con mayor número de casos confirmados en países occidentales. A pesar de ello, algunos aspectos de su fisiología todavía no son bien conocidos. Entre estos aspectos deficientemente estudiados se encuentra la relación existente entre sus mecanismos de resistencia al estrés, su patogenicidad y su capacidad de crecimiento/competición, lo que resulta especialmente alarmante ya que conocer estos fenómenos es imprescindible para poder diseñar mejores procesos de inactivación y/o planes de actuación a nivel de toda la cadena alimentaria.

Por ello se definió como objetivo general de esta Tesis Doctoral el siguiente: "estudiar y cuantificar la variabilidad intra-específica en resistencia al estrés, capacidad de crecimiento y virulencia dentro del género *Salmonella* y determinar las relaciones existentes entre estas tres características fenotípicas con objeto de profundizar en el conocimiento de la fisiología de este microorganismo y de contribuir al desarrollo de modelos cuantitativos de análisis del riesgo más precisos". Para alcanzar dicho objetivo y verificar estas hipótesis se plantearon los siguientes objetivos parciales: 1) Caracterizar la resistencia frente a diferentes estreses (incluyendo nuevas tecnologías de conservación de los alimentos y estreses ambientales) de diferentes cepas y serovariedades de *Salmonella enterica*; 2) Caracterizar la capacidad de crecimiento y la virulencia (capacidad de adhesión e invasión de cultivos celulares) de diferentes cepas y serovariedades de *Salmonella enterica*; 3) Estudiar la relación existente entre la resistencia al estrés, la capacidad de crecimiento, la virulencia y otros aspectos fenotípicos de las diferentes cepas y serovariedades de *Salmonella enterica*; 4) Validar en condiciones/matrices reales (huevos y ovoproductos) los resultados más relevantes; 5) Explorar los mecanismos moleculares responsables de las diferencias fenotípicas observadas y 6) Sentar las bases para la optimización de la evaluación cuantitativa del riesgo que representa el consumo de un grupo de productos en el que *Salmonella* es el patógeno de referencia: huevos y ovoproductos.

Los resultados obtenidos en esta Tesis Doctoral indican que la variabilidad en resistencia al estrés y capacidad de crecimiento existente entre las 23 cepas de *Salmonella* estudiadas fue

baja, con una diferencia de menos de 3,3 veces en el valor $2D/\mu_{max}$ para todos los agentes y condiciones estudiadas (si se excluye del análisis la resistencia de *S*. Senftenberg 775W frente al calor) y que la variabilidad intra-serovariedad fue similar o mayor que la variabilidad inter-serovar, a pesar de la mayor proximidad genética existente entre las cepas pertenecientes a una misma serovariedad. Además, se observó que, salvo en el caso de la resistencia a los PEAV y al medio osmótico, las cepas de *Salmonella* que mostraron la mayor resistencia frente a un agente/estrés no lo fueron frente a otros agentes/estreses y que una mayor resistencia al estrés no supuso un coste en términos de capacidad de crecimiento para las cepas de *Salmonella* estudiadas. Por otra parte, tampoco se observó ninguna relación entre la resistencia al estrés de las cepas y su virulencia. En relación a este punto, además, se comprobó que las cepas con una mayor capacidad de adhesión no fueron siempre las más invasivas.

Del estudio de los mecanismos responsables de las diferencias en resistencia entre las cepas de *Salmonella* se desprende que RpoS parece desempeñar un papel esencial en la resistencia de *Salmonella* al estrés osmótico, los PEAV y los UV-C. Sin embargo, las diferencias en actividad RpoS existentes entre cepas no explicarían, al menos por sí solas, las diferencias en resistencia (frente a ningún agente) observadas entre las cepas de *Salmonella* aquí estudiadas. Un hecho especialmente relevante que se observó es que en tres de las cinco variantes resistentes aisladas/investigadas (obtenidas mediante la aplicación de ciclos sucesivos de un agente estresante y crecimiento) mostraron mutaciones en el gen *hnr*, un represor de RpoS, lo que sugiere que esto podría constituir una estrategia evolutiva conservada (entre el género *Salmonella*) de adquisición de resistencia.

Por último, al estudiar la capacidad de crecimiento de *Salmonella* en huevo y ovoproductos se observó un fenómeno nunca antes descrito: que la dosis inicial y la historia térmica del huevo líquido entero y la clara determinan la velocidad de crecimiento de *S.* Enteritidis en estos ovoproductos, no ocurriendo esto en yema de huevo. Y siendo un fenómeno que estaría relacionado con la biodisponibilidad del hierro, esta sería mayor en productos pasteurizados y cuanto mayor fuera el número inicial de células. Estos resultados condujeron a que se hiciera una re-evaluación/determinación de la temperatura mínima de crecimiento de *S.* Enteritidis en estos productos, mediante la que se demostró que dicha temperatura mínima también

dependería de la dosis inicial y la historia térmica del huevo entero líquido y la clara, pero no en yema de huevo (manuscrito VIII). Por otra parte, se verificó que, para la mayoría de los agentes/tecnologías de conservación estudiados se observó una correlación significativa entre la resistencia determinada en medios de laboratorio y en matrices reales (alimentos y gallinaza). Finalmente, en un intento de demostrar la utilidad de los datos obtenidos en esta Tesis Doctoral para la mejora de las evaluaciones del riesgo y la toma de decisiones en la industria agroalimentaria se simularon distintos escenarios con objeto de determinar el riesgo relativo asociado a cuatro serovariedades emergentes en ganado aviar en comparación con *S.* Enteritidis, observándose que las primeras no supondrían un riesgo para la salud humana superior a *S.* Enteritidis, al menos en los escenarios estudiados/simulados en esta Tesis Doctoral.

En resumen, los resultados obtenidos en esta Tesis Doctoral contribuyen a una mejor compresión de la fisiología de *Salmonella* y permiten estimar la variabilidad en resistencia, capacidad de crecimiento y virulencia, así como las relaciones existentes entre estas características fenotípicas, que existe a diferentes niveles dentro de este género. Todo ello permitirá, como ya se ha demostrado en esta misma Tesis, desarrollar herramientas más eficaces tanto para la toma de decisiones como para el control de este patógeno en la cadena agroalimentaria.

ABSTRACT

Nowadays, salmonellosis is the second more common food-borne infection in developed countries. In spite of this fact, some aspects related to the physiology of this microorganism are still completely unknown. One of them is the relationship between its mechanisms of stress resistance, virulence and growth fitness. The lack of knowledge on this topic is especially worrying since understanding these relationships is essential for developing and/or improving current inactivation processes and for designing action plans covering/involving the whole food chain.

Therefore, the main objective of this PhD thesis was to study and quantify the intra-specific variability in stress resistance, growth capacity and virulence within *Salmonellae* and to determine the relationships between these three phenotypic characteristics to deepen the understanding of the physiology of this microorganism and to contribute to the development of more accurate quantitative risk analysis models".

The following partial objectives were set to achieve this objective and to verify these hypotheses: 1) To characterize the resistance to different agents (including environmental stresses and food preservation technologies) of different strains and serovars of *Salmonella enterica*; 2) To characterize the growth capacity and virulence (ability to adhere and invade cell cultures) of different strains and serovars of *Salmonella enterica*; 3) To study the relationship between stress resistance, growth capacity, virulence and other phenotypic aspects of different strains and serovars of *Salmonella enterica*; 4) To validate the most relevant results in real conditions/matrices (egg and eggs products); 5) To explore the mechanisms responsible for the phenotypic differences observed and 6) To lay the groundwork for the optimization of the quantitative risk assessment of the consumption of a group of products in which *Salmonella* is the reference pathogen: eggs and egg products.

The results obtained in this PhD thesis indicate that low variability (less than 3.3-fold change in $2D/\mu_{max}$ values for all agents studied) in stress resistance and growth capacity was found among the 23 *Salmonella* strains studied (if *S*. Senftenberg 775W heat resistance is excluded from the analysis) and intra-serovar variability was comparable or higher than inter-serovar variability, despite the similar genetic backgrounds of strains belonging to the same serovar.

Moreover, it was observed that, except for resistance to PEF and osmotic stress, *Salmonella* strains that were the most resistant to given stress were not more resistant to other types of stress and the higher stress resistance of some strains/serovars did not impose a fitness cost to them. On the other hand, no relationship between stress resistance of strains/serovars and virulence was observed either. In addition, strains with a high adhesion ability were not always the most invasive ones. From the study of the mechanisms responsible for the differences in resistance among *Salmonella* strains, it appears RpoS seems to play a crucial role in the resistance of *Salmonella* to osmotic stress, PEF and UV-C. However, RpoS activity alone would not explain the differences in resistance observed among *Salmonella* strains to any of the stressing agents/food preservation technologies here studied. A particularly relevant fact observed is that three out of the five resistant variants isolated/investigated (obtained after repeated rounds of PEF treatment and outgrowth of survivors) showed mutations in *hnr*, a repressor of RpoS, suggesting that this could constitute a conserved evolutionary strategy (within *Salmonellae*) of resistance acquisition.

Finally, a phenomenon never described before was observed when analyzing the growth capacity of *Salmonella* in egg and egg products: the initial dose and the thermal history of the whole liquid egg and egg white determine the growth fitness of *S*. Enteritidis in these egg products, whereas this does not occur in egg yolk.

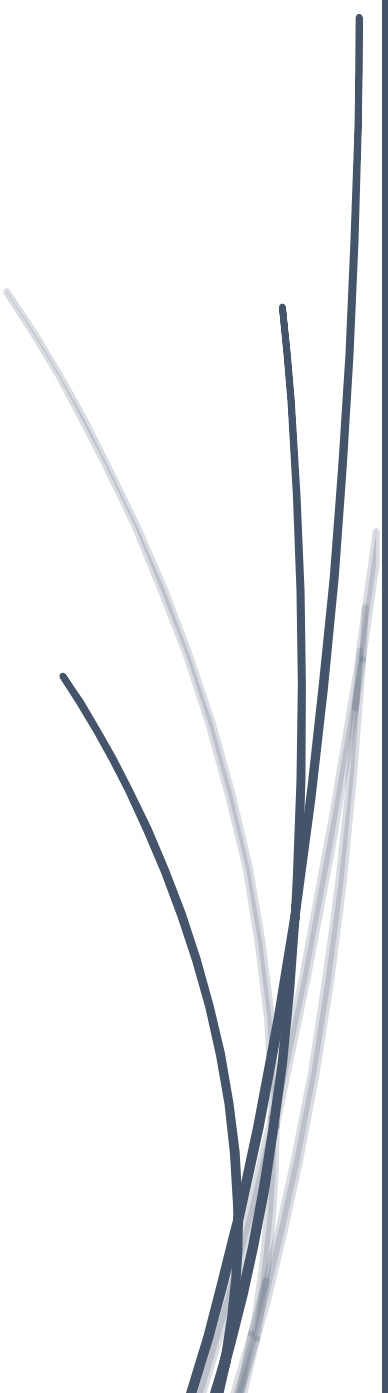
This phenomenon seems to be related to iron bioavailability, which would be higher in pasteurized products and when *Salmonella* cells were inoculated at a high initial dose.

These results led to a re-evaluation/determination of the minimum growth temperature of *S*. Enteritidis in these products, which showed that this minimum growth temperature would also depend on the initial dose and the thermal history of the whole liquid egg and egg white, but not in egg yolk. On the other hand, it was verified that, for most of the agents/technologies studied, a significant correlation was observed between the resistance determined in laboratory media and real matrices (food and poultry manure). Finally, in an attempt to demonstrate the usefulness of the data obtained in this PhD thesis for the improvement of risk assessments and decision making in the agri-food industry, different scenarios were simulated to determine the relative risk associated with four emerging serovars in poultry,

compared to *S*. Enteritidis, and results obtained suggest that emerging poultry serovars/strains would not pose a higher risk for human health than Enteritidis serovar, at least in the scenarios studied/simulated within this this PhD thesis.

Índice

Introducción



1. Enfermedades de transmisión alimentaria

Según la Declaración Universal de los Derechos Humanos el acceso a alimentos seguros, suficientes y nutritivos es un derecho de toda persona (ONU: Asamblea General, 1948) y es esencial para una buena salud. Sin embargo, las enfermedades transmitidas por los alimentos son frecuentes en todo el mundo y se estima que 1 de cada 10 personas en el mundo presenta algún episodio de toxiinfección alimentaria al año (OMS, 2015). Así, en Europa se estima que las enfermedades transmitidas por los alimentos causan aproximadamente 23 millones de enfermos, y alrededor de 5.000 personas mueren a causa de ellas cada año. Ante tal problema de salud pública, los esfuerzos de los diferentes agentes involucrados se han centrado principalmente en estimar el número total de episodios de enfermedades transmitidas por los alimentos para asignar recursos y priorizar las intervenciones. En la figura 1 se muestran las enfermedades zoonóticas más comúnmente transmitidas por los alimentos (casos confirmados) en la Unión Europea (UE). Por orden de frecuencia en las notificaciones, las enfermedades con mayor número de casos confirmados en 2019 fueron la campilobacteriosis y la salmonelosis, seguido de las infecciones por *Escherichia coli* productora de toxina Shiga

(STEC), la yersiniosis y la listeriosis. Sin embargo, esto solo se corresponde a las enfermedades zoonóticas y a los casos confirmados y notificados, el número de toxiinfecciones alimentarias es mucho mayor debido a que sólo una pequeña proporción de las enfermedades se confirman mediante pruebas de laboratorio y se notifican a los organismos de salud pública (Scallan et al., 2011). La Autoridad Europea de Seguridad Alimentaria (EFSA) ha estimado que, en Europa, en concreto para las dos enfermedades con mayor número de casos, el coste de la campilobacteriosis es de unos 2.400 millones de euros y el coste de la salmonelosis más de 3.000 millones de euros anuales, incluyendo la pérdida total de productividad asociada y el coste anual del tratamiento de estas enfermedades transmitidas por los alimentos en Europa (Havelaar, 2011).

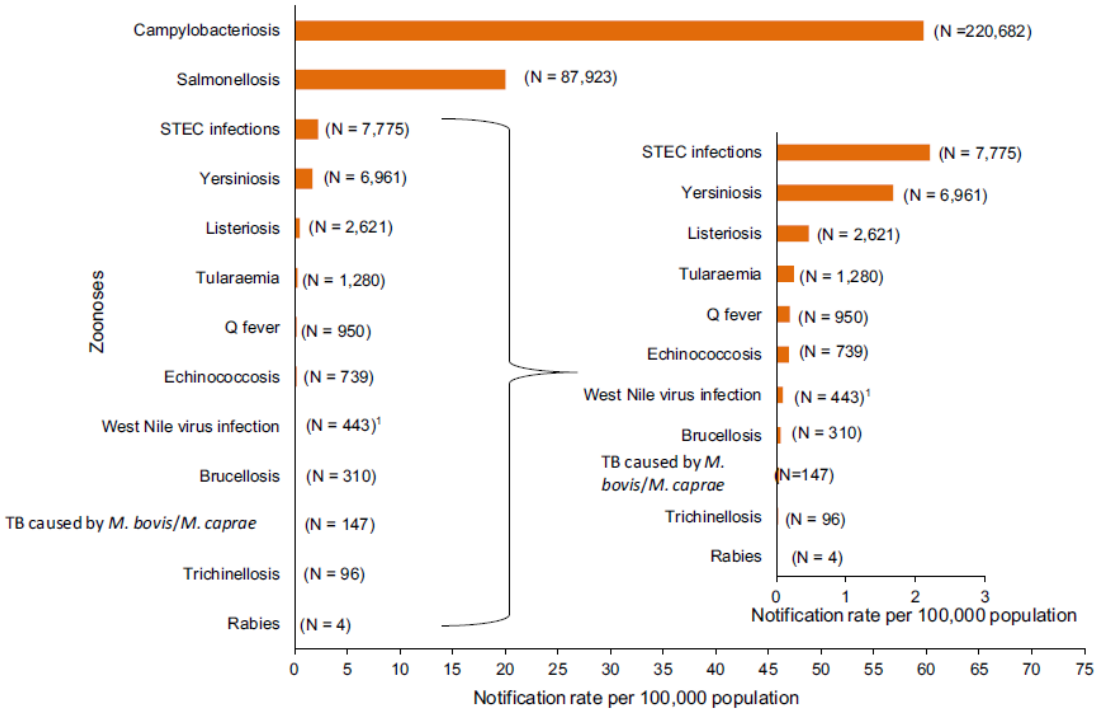


Figura 1. Casos notificados y tasas de notificación de zoonosis humanas confirmadas en la UE, 2019. El número total de casos confirmados se indica entre paréntesis al final de cada barra. [1]Para la infección por el virus del Nilo Occidental se utilizó el número total de casos (EFSA, 2021).

Es más, a pesar de los esfuerzos realizados en el desarrollo de una legislación más estricta, a la concienciación del consumidor, la industria y las administraciones y a los enormes avances científicos y tecnológicos, en las últimas décadas estamos siendo testigos de un incremento en el número de enfermedades de transmisión alimentaria. En este sentido, los factores que

han facilitado este aumento o han contribuido o están contribuyendo a cambiar las tendencias en las enfermedades transmitidas por los alimentos se podrían clasificar en 4 grandes grupos de acuerdo a Skovgaard (2007) y Newell et al. (2010):

1) Cambios sociales y demográficos. Rápido crecimiento de la población y cambio demográfico hacia una población envejecida.

2) Cambios en los sistemas de detección de los patógenos, información y sistemas de vigilancia.

3) Cambios en la cadena agroalimentaria. Estos cambios incluirían el cambio en las prácticas agrícolas, tecnologías emergentes en la producción y conservación de los alimentos, mejora de la logística y condiciones de transporte junto con el cambio de hábitos alimentarios.

4) Continua adaptación microbiana. Un claro ejemplo de esto es la aparición de microorganismos resistentes a los fármacos.

Con el aumento de la globalización, el comercio y los viajes, aumentan los riesgos para la seguridad alimentaria; los peligros transmitidos por los alimentos pueden propagarse fácilmente a países geográficamente distantes y afectar a la salud de las personas en numerosas regiones al mismo tiempo. De la misma forma, el envejecimiento progresivo de la población supone un incremento en el número de personas más susceptible a sufrir determinadas enfermedades debido, entre otras causas, a que disponen de un sistema inmunológico menos robusto y a su propensión a sufrir otras enfermedades, de forma crónica y/o simultánea, que pueden facilitar el desarrollo de enfermedades alimentarias, dificultar su tratamiento o agravar sus causas.

Otro de los factores clave ha sido la vigilancia epidemiológica, esto incluye la mejora en los sistemas de detección e identificación de patógenos, años atrás la aparición de nuevo un patógeno se explicaba fácilmente por el simple hecho de que no se disponía de técnicas de cultivo para su detección. El avance en biología molecular y en métodos independientes de las técnicas de cultivo ha permitido mejorar los sistemas de detección conduciendo a un

incremento en el número de casos notificados. Además, aquí en España existe la Red Nacional de Vigilancia Epidemiológica (RENAVE) que proporciona la información necesaria para la vigilancia y el control de las enfermedades transmisibles en la población, integrando la notificación y la investigación epidemiológica de casos de enfermedades transmisibles, de brotes o de microorganismos.

En cuanto a los cambios sociales y en la cadena alimentaria cabe señalar que, además de la intervención de más personas en la cadena alimentaria, la globalización es también un trampolín para la propagación de enfermedades zoonóticas, promoviendo la exposición a patógenos procedentes de otras partes del mundo, o provocando que nuevos patógenos emerjan por desplazamiento de sus entornos remotos, dándoles acceso a nuevos nichos ecológicos y nuevos hospedadores. Además, la trazabilidad de los alimentos se ha vuelto más compleja, y los ingredientes de muchos alimentos procesados y listos para el consumo pueden proceder de países con diferentes peligros y riesgos de transmisión alimentaria. La urbanización, los cambios en los hábitos de consumo y el cambio climático también afectan a la seguridad alimentaria. En relación a la cadena alimentaria es importante recordar que los alimentos pueden contaminarse a lo largo de las etapas de la cadena alimentaria comenzando por el medio ambiente y la producción primaria, pasando por la fabricación, la distribución y la venta al por menor y terminando con la manipulación y el consumo. Por lo tanto, si no se producen, procesan o manipulan adecuadamente pueden representar un riesgo para el consumidor. Por otra parte, patógenos ya existentes pueden aparecer y resurgir en la cadena alimentaria a consecuencia de la aplicación de nuevas tecnologías. Por ello, es importante conocer el impacto de los nuevos métodos de producción, así como de las nuevas tecnologías de procesado y conservación de alimentos, para determinar si, de alguna manera, esas prácticas pueden suponer la re-emergencia de antiguas amenazas.

Y, finalmente, y en lo que respecta a la continua adaptación microbiana, es necesario señalar que los microorganismos no solo han tenido que desarrollar sistemas de resistencia que les permitan sobrevivir a las diferentes condiciones medioambientales a las que pueden enfrentarse y a los diferentes estreses a los que se pueden ver expuestos antes de ejercer su

efecto patógeno (por ejemplo: limitación de nutrientes y medio ácido en el estómago) (Abee y Wouters, 1999; Dodd y Aldsworth, 2002) sino que, además, aquellas bacterias responsables de las toxiinfecciones alimentarias han tenido que modificar sus mecanismos de virulencia para así adaptarse a los sistemas de defensa de sus hospedadores (Beceiro et al., 2013; Woolhouse et al., 2002), en un proceso co-evolutivo con estos últimos.

2. Análisis de riesgos

Una de las principales herramientas de las que dispone la ciencia y tecnología de los alimentos (y otras disciplinas relacionadas con la salud pública) para el control de los riesgos presentes y futuros y tanto los ya conocidos como los emergentes es la evaluación del riesgo. La evaluación del riesgo emergió ya hace unas décadas como una metodología eficiente para evaluar el impacto en la salud humana de una amplia variedad de riesgos ambientales, entre ellos los de los microorganismos patógenos vehiculados por los alimentos (OMS/FAO, 2007). Además, esta metodología permite determinar el impacto sobre la salud humana que suponen diferentes intervenciones en la cadena alimentaria y resulta una herramienta extremadamente útil para la toma de decisiones y evaluación. Tanto es así que la evaluación del riesgo microbiano en alimentos es habitualmente utilizada, no sólo en el entorno científico sino también por las empresas productoras y comercializadoras de alimentos e incluso para el desarrollo de la legislación alimentaria actualmente en vigor.

El análisis de riesgos es, según la Organización Mundial de la Salud, un proceso estructurado de toma de decisiones que se compone de 3 elementos estrechamente vinculados: gestión del riesgo (GR), evaluación del riesgo (ER) y comunicación del riesgo (CR). Y a su vez, la evaluación del riesgo es el resultado de la consecución de 4 etapas con base científica: la identificación del peligro (IP), la caracterización del peligro (CP), la evaluación de la exposición (EE) y la caracterización del riesgo (CR), donde el resultado de cada etapa es el punto de partida de la siguiente etapa (OMS/FAO, 2007).

Inicialmente, la mayoría de estas evaluaciones eran de tipo cualitativo, usando información categórica y no numérica para las variables (riesgo alto o riesgo bajo) o semi-cuantitativo (probabilidad alta o baja de producir enfermedad). Sin embargo, conforme se han ido

acumulando datos científicos, y dada la mayor flexibilidad de los modelos cuantitativos, la tendencia actual es a trabajar con este último tipo de modelos, asignando valores numéricos a las variables. A su vez estos modelos pueden ser determinísticos, un único valor para las variables del modelo (la media aritmética es el valor puntual más frecuente) o probabilístico, a través del uso de distribuciones de probabilidad que describen la probabilidad asociada a cada valor (Lammerding y Fazil, 2000).

El principal problema para el desarrollo de estos modelos cuantitativos es que es necesario subsanar algunas lagunas de conocimiento que todavía existen a día de hoy, para lo que son necesarios estudios específicos y que posean un adecuado diseño experimental, de tal forma que los datos obtenidos reflejen lo que realmente ocurriría en la cadena alimentaria. Los parámetros del modelo pueden depender de una amplia gama de factores implícitos (por ejemplo, cepa bacteriana), intrínsecos (tipo de medio) y extrínsecos (temperatura) de una manera que aún no se comprende del todo. Por lo tanto, deben estimarse utilizando datos experimentales y, dado que el error experimental es inevitable, sería relevante que se incluyera en el análisis los parámetros de variabilidad e incertidumbre ya que sus valores no pueden conocerse con absoluta certeza. En el contexto de la seguridad alimentaria, la variabilidad incluye fuentes inherentes de variación (por ejemplo, diferencias en la respuesta de células individuales o en la composición de los alimentos) y la incertidumbre abarcaría aquellas fuentes de variación que no se consideran en el sistema (por ejemplo, errores de medición o especificaciones incorrectas del modelo). Por estas razones, los modelos cuantitativos tienen ciertas limitaciones. En cualquier caso, a la vista de los esfuerzos actuales para cuantificar la variabilidad y la incertidumbre de las respuestas microbianas, es probable que los modelos “multinivel” ganen popularidad en los próximos años y sustituyan a los modelos de un solo nivel que ahora son tan relevantes para la microbiología predictiva.

3. *Salmonella*

3.1. Taxonomía y nomenclatura

El género *Salmonella* pertenece al Orden *Enterobacteriales* y a la Familia *Enterobacteriaceae*, y está estrechamente relacionado con otras enterobacterias como *Escherichia coli*, *Shigella* spp. y *Citrobacter* spp (Rogers et al., 2016).

El género *Salmonella* está dividido por dos especies, *Salmonella enterica* y *Salmonella bongori* clasificadas a su vez en varias subespecies (Figura 2). *Salmonella enterica* se subdivide en seis subespecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *Houtenae* (IV) y *S. enterica* subsp. *indica* (VI). A su vez, las subespecies de *Salmonella enterica* y la especie *Salmonella bongori* se dividen en variantes serológicas denominadas serovar, y a día de hoy, se han confirmado al menos 2.659, de las cuales 1.586 pertenecen a *S. enterica* subsp. *enterica* (Grimont y Weill, 2007; Issenhuth-Jeanjean et al., 2014). Estas serovariedades están definidas por la combinación de tres antígenos, el antígeno somático (O), el antígeno flagelar (H) y el antígeno capsular (K) (Grimont y Weill, 2007).

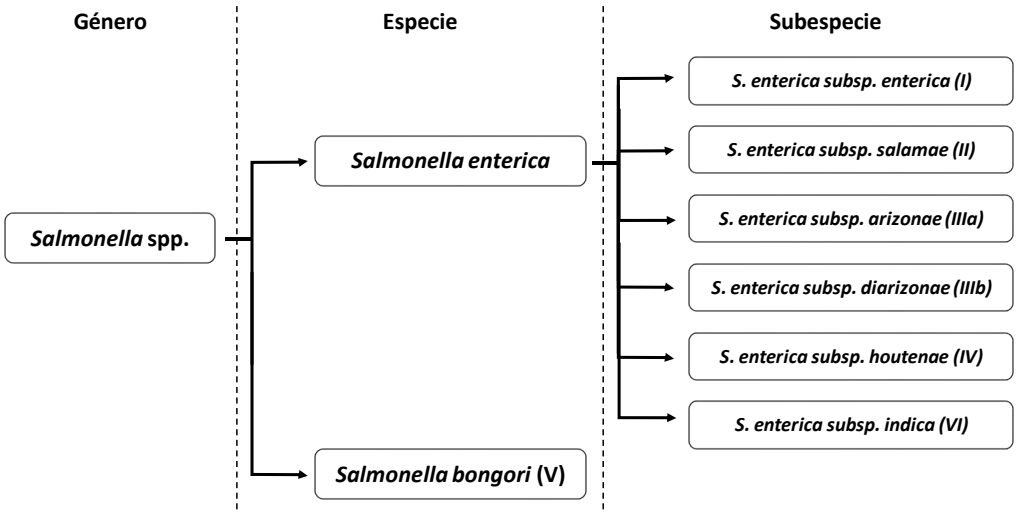


Figura 2. Clasificación actual de los microorganismos del género *Salmonella*.

3.2. Generalidades: morfología, características bioquímicas y requisitos nutritivos

Salmonella se caracteriza por ser un grupo de bacterias Gram negativas, con forma de bacilos, anaerobias facultativas y no formadoras de esporos. La mayoría de las *Salmonellas* son móviles ya que poseen flagelos perítricos, y además poseen fimbrias que les permiten infectar

a una gran variedad de huéspedes tanto mamíferos como reptiles, aves o anfibios (Jajere, 2019).

La mayoría de las cepas de *Salmonella* producen sulfuro de hidrógeno, pero existen algunas serovariedades que no lo producen (por ejemplo, la mayoría de cepas de la serovariedad Paratyphi A y algunas cepas de la serovariedad Choleraesuis). Suelen utilizar el citrato como única fuente de carbono y utilizan la glucosa para la producción de ácido o ácido y gas mediante los procesos de oxidación y fermentación. Además, son catalasa positiva, oxidasa negativa, indol negativa y ureasa negativa, reducen el nitrato a nitrito y tienen un contenido de G+C del 39-59% (Popoff y Le Minor, 2015).

Salmonella tienen unos requisitos nutricionales relativamente sencillos y pueden sobrevivir durante largos periodos de tiempo en los alimentos y en otros sustratos (Tabla 1). El crecimiento y la supervivencia de *Salmonella* spp. están influidos por una serie de factores como son la temperatura, el pH, la actividad de agua (a_w) y la presencia de conservantes. Son bacterias capaces de sobrevivir/crecer en un amplio rango de temperatura, que va desde los 7 a los 46,2 °C, siendo de 35 a 43 °C su rango óptimo (D´Aoust, 1989). En lo relacionado al pH, *Salmonella* es capaz de crecer desde pH 3,8 hasta pH 9,5, con un rango de pH óptimo para el crecimiento alrededor de 7,5. El pH mínimo al que puede crecer *Salmonella* depende de otros factores como la temperatura, la presencia de solutos o el tipo de ácido presente (ICMSF, 1996). La actividad de agua tiene un efecto significativo en el crecimiento de *Salmonella*, es capaz de sobrevivir durante largos periodos en productos de baja humedad, siendo el límite inferior de crecimiento de 0,93 y la a_w óptima de 0,99 (Podolak et al., 2010).

Tabla 1. Condiciones de crecimiento de *Salmonella* (Adaptado de ICMSF., 1996)

Condiciones	Crecimiento
Temperatura (óptima)	7 – 46,2 °C (35 - 43 °C)
pH (óptimo)	3,8 – 9,5 (7,0 - 7,5)
a_w (óptima)	0,93 – 0,99 (0,99)

3.3. Relevancia de *Salmonella* en el entorno alimentario

Como es bien sabido (y se ha indicado anteriormente), los microorganismos del género *Salmonella* son responsables de toxiinfecciones de origen alimentario. De hecho, en 2019 *Salmonella* fue la causa más frecuente de brotes alimentarios de origen conocido en Europa. Se notificaron 926 brotes, un 17.9% del número total de brotes en la Unión Europea en 2019 (EFSA, 2021). Además, como se ha comentado anteriormente, la salmonelosis fue la segunda causa de infección gastrointestinal en humanos, afectando a unas 88.000 personas, solo en España se confirmaron 5.103 casos de salmonelosis en 2019 (EFSA, 2021). La enfermedad se caracteriza por causar una gran variedad de síntomas: fiebre entérica, bacteriemia, enterocolitis, e infecciones focales. Las fuentes de contaminación de los productos alimentarios son diversas, además de una contaminación de manera directa o indirecta de los alimentos a través de los animales, también puede ocurrir a consecuencia de una contaminación cruzada entre comidas ya preparadas, debido a hábitos de higiene inadecuados por parte de los manipuladores de alimentos o ambientes con contaminación fecal, como sería el agua. Así, los alimentos que con mayor frecuencia se identificaron como responsables de los brotes de *Salmonella* de origen alimentario en la Unión Europea en 2019 fueron los huevos y ovoproductos (37.0% de los brotes), seguido de los productos de panadería y pastelería (11,7%) y la carne de cerdo y derivados (9,8%). Otras categorías relevantes son las que incluyen los productos listos para el consumo o de composición mixta (8,7%). No obstante, el rango de productos que pueden vehicular *Salmonella* es mucho más amplio incluyendo también otros productos de origen animal, vegetal, crustáceos o leche (Tabla 2)(EFSA, 2021).

Adicionalmente hay que señalar que, aunque su incidencia es baja, de igual forma que para los zumos de manzana la FDA ha tomado como microorganismo de referencia *E. coli* O157 y exige que los procesos de pasteurización de este tipo de zumo deben inactivar al menos 5 ciclos de este patógeno, *Salmonella* se considera el patógeno de referencia -el más relevante o de riesgo- para los zumos de cítricos (Danyluk et al., 2012).

Tabla 2. Distribución de los brotes de origen alimentario causados por *Salmonella* de fuerte evidencia, clasificados por alimentos, en la Unión Europea en 2019 (EFSA, 2021).

Alimentos	Número de brotes (%)
Huevos y ovoproductos	37,0%
Productos de panadería y pastelería	11,7%
Carne de cerdo y derivados	9,8%
Productos de composición mixta	8,7%
Carne y derivados	5,7%
Carne de pollo (*Gallus gallus)* y derivados	5,3%
Desconocido	4,2%
Carnes rojas o mixtas y derivados	3,4%
Otras, carne de aves de corral mixtas o inespecíficas y derivados	1,9%
Dulces y chocolate	1,9%
Vegetales y zumos y otros productos derivados	1,9%
Crustáceos, mariscos, moluscos y derivados	1,5%
Productos lácteos (distintos de los quesos)	1,5%
Comidas tipo buffet	1,1%
Quesos	1,1%
Otros productos	1,1%
Carne de vacuno y derivados	0,8%
Frutas, bayas y zumos y otros productos derivados	0,8%
Leche	0,4%
Carne de ovino y derivados	0,4%
TOTAL	**100,0%**

Las principales fuentes de *Salmonella* son el tracto gastrointestinal de los seres humanos, los animales domésticos y salvajes, las aves y los roedores. Debido a ello, *Salmonella* está ampliamente extendida en el entorno natural, incluso en el suelo y en el agua, en los que puede sobrevivir durante largos periodos sirviendo estos como reservorio y ayudando a la transmisión entre huéspedes (Winfield y Groisman, 2003). *Salmonella* está principalmente adaptada al tracto intestinal de los vertebrados, a pesar de tener una elevada capacidad de

adaptación (o tal vez precisamente por ello) algunas serovariedades son específicas de un hospedador, por ejemplo, *S*. Typhi se limita estrictamente a los seres humanos y *S.* Gallinarum específicamente a las aves. Por el contrario, existe un gran número de serovariedades (mucho mayor que las anteriores) capaces de colonizar o infectar una amplia gama de huéspedes, siendo estas las que representan un riesgo desde el punto de vista de la microbiología de los alimentos (Jajere, 2019).

En cuanto a las serovariedades responsables de la enterocolitis, aproximadamente unas 2.000, hay que señalar que el responsable de más del 80 % de las toxiinfecciones, es un conjunto más pequeño, de aproximadamente 10-20 serovariedades; de las que destacan *S*. Enteritidis y *S*. Typhimurium (EFSA, 2021). Tan solo las tres serovariedades notificadas con mayor frecuencia, *S*. Enteritidis y *S*. Typhimurium (incluidas las variantes monofásicas), representaron más del 70% de los casos en humanos adquiridos en la Unión Europea en 2019 (EFSA, 2021). *S*. Infantis fue la cuarta serovariedad más frecuentemente notificado en las infecciones humanas adquiridas en Europa y asociadas a viajes y las serovariedades *S*. Derby y *S*. Newport se notificaron casi en igual número, siendo la quinta y sexta serovariedad más frecuentemente notificados en 2019. Además, la tendencia para estas seis serovariedades se ha mantenido estable en los últimos 5 años entre 2015 y 2019 en la Unión Europea (Tabla 3).

Tabla 3. Distribución de las 6 serovariedades de *Salmonella* que más frecuentemente causaron enfermedad alimentaria en 2019 -calculados a partir de los casos notificados de salmonelosis adquirida en la Unión Europea- (EFSA, 2021).

Serovariedad	%
Enteritidis	61,56
Typhimurium	11,62
Typhimurium monofásica	5,17
Infantis	2,34
Derby	0,76
Newport	0,63
Otros	17,93
TOTAL	**100,0%**

En lo que respecta a la identificación de la serovariedad de los aislados de *Salmonella* entre las diferentes fuentes, *S*. Enteritidis se asoció principalmente con fuentes de pollos de engorde (el 67,8% de los aislados de *S*. Enteritidis procedían de manadas de pollos de engorde y de carne) y en segundo lugar con gallinas ponedoras (26,7%). *S*. Typhimurium se asoció principalmente con fuentes de cerdos, pollos de engorde y ponedoras, en un 42%, 34,8% y 13,5%, respectivamente. *S*. Typhimurium monofásica se asoció principalmente con fuentes porcinas (72,1%) y en segundo lugar con pollos de engorde (17,1%). *S*. Infantis se relacionó principalmente con fuentes de pollos de engorde (93,1%). *S*. Derby se asoció principalmente con fuentes de cerdo (72%) y en segundo lugar con fuentes de pavo (19,8%). Para interpretar estos datos, es importante tener en cuenta que la distribución de los aislados serotipados entre las distintas fuentes es muy heterogénea en cuanto al número de aislados por especie (EFSA, 2021).

A pesar de la relevancia de *Salmonella* en aves de corral, de las más de 2.500 serovariedades distintas de *Salmonella* que se han identificado, sólo un 10% de ellas se ha aislado alguna vez en aves de corral, y una proporción aún menor de serovariedades se encuentra habitualmente en las manadas de aves de corral. La distribución de las serovariedades de *Salmonella* procedentes de las aves de corral es variable desde el punto de vista geográfico y cambia con el tiempo, aunque varias serovariedades se detectan sistemáticamente con una alta incidencia en gran parte del mundo (Gast, 2007). Muchas de las serovariedades más prevalentes en el ser humano, como *S.* Typhimurium y *S.* Enteritidis, también son comunes en las aves de corral, lo que sugiere una posible conexión epidemiológica entre los reservorios avícolas y humanos de las *Salmonellas,* por lo que sería necesario estudiar este fenómeno en profundidad.

3.4. Control de *Salmonella* en la cadena alimentaria: estado actual y perspectivas de futuro

La relevancia actual de *Salmonella*, y principalmente de las serovariedades arriba indicadas es evidente y ya se ha discutido. Sin embargo, las razones de que, a pesar de los esfuerzos de la academia, las administraciones y la industria este patógeno alimentario sigue siendo una de las principales causas de enfermedad alimentaria no están del todo claras. Este último hecho es especialmente impactante a la vista de la cantidad y coste de las medidas implementadas, y que a buen seguro constituye el paquete de medidas más importante y/o ambicioso

desarrollado por el sector agroalimentario frente a un microorganismo patógeno. Estas incluyen (entre muchas otras):

- La implementación de una legislación extremadamente exigente (la que más en Europa) en cuanto a los niveles de contaminación admisibles de este patógeno (que prácticamente afecta a todos los productos alimentarios puestos a disposición del consumidor).

- El establecimiento de limitaciones en el uso de productos frescos –como el huevo- para determinados fines -productos no sometidos a tratamiento térmico en establecimientos de restauración colectiva-.

- El desarrollo de planes de control y vigilancia exhaustivos, tanto a nivel de alimentos como de algunos de los sectores productivos más relevantes (avicultura).

- Desarrollo de tratamientos tecnológicos específicos para su inactivación o el control de su crecimiento en determinados productos (ovoproductos o zumos de cítricos en EE.UU).

- Desarrollo y administración de vacunas para avicultura y porcino.

- Campañas de comunicación y formación de consumidores (repetidas casi anualmente en los periodos de mayor riesgo).

La principal justificación de la necesidad de seguir profundizando en el conocimiento de la fisiología de este microorganismo y en el desarrollo de nuevas herramientas de control e inactivación del mismo es precisamente el limitado efecto que todo este conjunto de medidas ha tenido. Además, hay que indicar que al riesgo que ya suponía *Salmonella per se*, hay que añadir el riesgo asociado a la gran capacidad de adquisición de antibio-resistencias de este microorganismo, lo que hace todavía más necesario el desarrollo de las herramientas arriba indicadas.

Finalmente, hay que señalar que si bien la mayoría de las serovariedades de *Salmonella* se pueden considerar como patógenos “persistentes” o “estables” dentro de la cadena agroalimentaria y que incluso ha habido algunas serovariedades cuyo impacto en dicha cadena (aunque no en la salud humana sino animal) se ha conseguido eliminar, hay otras serovariedades que a día de hoy se consideran emergentes (como por ejemplo *S*. Kentucky y

S. Livingstone en aves de corral) y que además no se puede descartar que, por alguna de las razones indicadas al principio de esta introducción, algunas serovariedades -sino todas- puedan re-emerger.

A continuación, se describen y discuten en mayor profundidad algunas de las causas (en las que se centrará esta Tesis Doctoral) que podrían ayudar a explicar el por qué *Salmonella* continúa siendo un problema de seguridad alimentaria de primera magnitud o que podrían conducir a la emergencia de determinadas serovariedades y/o a la re-emergencia de otras.

3.4.1. Cambios en la cadena agroalimentaria: impacto en el riesgo asociado a *Salmonella*

Uno de los ejemplos más claros de cómo la intervención humana en la cadena agroalimentaria puede conducir a la emergencia de nuevos microorganismos o serovariedades es, precisamente el de *Salmonella* en la producción avícola. A principios del siglo XX las serovariedades Gallinarum y Pullorum estaban ampliamente distribuidas en las explotaciones avícolas, causando numerosas bajas en las mismas (Barrow y Freitas Neto, 2011). Por ello se pusieron en marcha campañas de saneamiento con objeto de erradicar estas dos serovariedades, lo que condujo a su casi completa desaparición hacia los años 60-70 (Bäumler et al., 2000). A día de hoy se cree que este fenómeno fue el que condujo a la emergencia de *S*. Enteritidis como principal serovariedad asociada a los huevos y ovoproductos, no obstante, las causas no están aún del todo claras. Una de las teorías más interesantes para explicar este fenómeno postula que esto podría haber sido debido a que inmediatamente tras la erradicación de las serovariedades Gallinarum y Pullorum se produjo un descenso en el nivel de inmunidad de grupo, escenario que aprovechó *S*. Enteritidis (que en un principio se asociaba principalmente a los roedores) para ocupar este nicho ecológico que había quedado vacío (Foley et al., 2011). A esto hay que añadir que estudios realizados mediante el uso de modelos matemáticos, sugieren que la presencia de *S*. Gallinarum sería capaz de excluir por competición a *S*. Enteritidis de las aves (Bäumler et al., 2000). Finalmente, varios autores también han indicado que los cambios en los sistemas productivos que ocurrieron a lo largo de las últimas décadas del siglo XX, como el aumento en la densidad de cría o la integración vertical, podrían haber contribuido también a la diseminación de *S*. Enteritidis (Velge et al., 2005). En cualquier caso, este episodio constituye el primer episodio documentado de

sustitución de serovariedades dominantes de *Salmonella* en el sector aviar y una clara evidencia de que las intervenciones humanas tienen consecuencias que pueden no resultar beneficiosas en todos los sentidos, ya que *S*. Enteritidis representa un peligro para la salud humana mayor que Gallinarum o Pullorum. Sin embargo, esto no ha sido un hecho aislado, en los últimos años, la prevalencia de serovares como *S*. Kentucky y *S*. Heidelberg ha aumentado considerablemente (EFSA, 2021; Kaldhone et al., 2017). Así, en Estados Unidos *S*. Heidelberg sustituyó a *S*. Enteritidis como la serovariedad de aves de corral más frecuentemente aislado entre 1996 y 2006 y desde 2007 ha sido sustituido por *S*. Kentucky (Foley et al., 2011). Del mismo modo, en Europa, *S*. Mbandaka y *S*. Livingstone ya superan a *S*. Enteritidis en frecuencia de aislamiento en pollos de engorde y *S*. Kentucky es la tercera serovariedad más comúnmente encontrado en gallinas ponedoras, después de *S*. Enteritidis y *S*. Infantis (EFSA, 2021). Todos estos datos indican que, en muchos países y entornos avícolas, estas serovariedades emergentes ya han suplantado a *S*. Typhimurium y *S*. Enteritidis como las serovariedades más relevantes asociados a la producción avícola, o al menos desde la perspectiva de la producción de alimentos y la salud animal.

Por otra parte, la aplicación de nuevas tecnologías para la conservación de los alimentos, como los pulsos eléctricos de alto voltaje (PEAV), las altas presiones hidrostáticas (APH), los ultrasonidos (US) o la aplicación de luz ultravioleta (UV-C) es otro de los factores que podría contribuir al resurgimiento de patógenos ya existentes (como *Salmonella*) o la emergencia de nuevos (o de determinadas serovariedades).

Es importante señalar que el objetivo de estas nuevas tecnologías no es simplemente imitar las tecnologías existentes, sino ser superiores en algún aspecto (económico, técnico, calidad de producto, seguridad alimentaria…). Los factores de los que depende la implementación definitiva de las nuevas tecnologías de conservación de los alimentos en la industria son de sobra conocidos. Entre ellos se encuentran factores del tipo económico (coste de equipamiento y operación), otros factores relacionados con el diseño de equipos y/u otros factores importantes como la percepción de estas tecnologías por parte de los consumidores. No obstante, a día de hoy y a pesar de la cantidad de literatura científica publicada, las dudas en relación a la capacidad de estas tecnologías para garantizar la producción de alimentos

seguros desde el punto de vista microbiológico siguen siendo el principal problema de cara a su implementación industrial (Howlett et al., 2003).

Actualmente, existen algunos estudios en los que se compara la resistencia relativa de numerosas cepas y serovariedades de *Salmonella* frente a estas tecnologías que demuestran que aquellas cepas más resistentes frente al calor no lo son frente a otros agentes (Álvarez et al., 2003; Doyle y Mazzotta, 2000; Gayán et al., 2012; Lianou y Koutsoumanis, 2013; Sherry et al., 2004). Esto podría hacer que el riesgo de sufrir salmonelosis se incremente (o descienda) en algunos productos o que, por ejemplo, las serovariedades que actualmente representan un mayor riesgo para la salud del consumidor dejen de serlo y sean sustituidas por otras. Sin embargo, el número de estudios sobre la variabilidad de la resistencia dentro del género *Salmonella* siguen siendo escaso o limitado, y el principal problema es que o se incluye un número reducido de serovariedades/cepas o un pequeño número de agentes estresantes y/o tecnologías de conservación de alimentos. Además, dado que las condiciones experimentales (condiciones de cultivo, cepas, etc.) no son las mismas en la mayoría de los casos, la comparación posterior se hace difícil y/o carece de sentido. Por tanto, sería necesario llevar a cabo estudios que cuantifiquen la resistencia a estas nuevas tecnologías, incluyendo el potencial desarrollo de las respuestas adaptativas al estrés de las diferentes cepas y serovariedades de *Salmonella*, lo que ayudaría a determinar cuáles deberían ser las serovariedades o cepas diana en función de la tecnología y/o producto y facilitaría el diseño de procesos de inactivación y planes de actuación a lo largo de la cadena alimentaria más eficientes lo que, consecuentemente, contribuiría a la implementación definitiva de estas nuevas tecnologías en la industria agroalimentaria.

3.4.2. Adaptación microbiana: la respuesta al estrés y su impacto en otros aspectos de la fisiología de *Salmonella*

Como también se ha indicado anteriormente, las bacterias patógenas como *Salmonella* no solo han tenido que desarrollar sistemas de resistencia que les permitan sobrevivir a las diferentes condiciones medio ambientales, sino que también han tenido que modificar sus mecanismos de virulencia para así adaptarse a los sistemas de defensa de sus hospedadores. Aunque las diferencias entre los procesos evolutivos de desarrollo de resistencia al estrés y el de adaptación al hospedador son obvias, ambos tienen en común algunas características e

implican estructuras y moléculas comunes como la pared celular, las membranas y sus proteínas (porinas, bombas de eflujo, etc.). Además, desde un punto de vista biológico, ambos procesos son necesarios para que las bacterias sobrevivan en condiciones adversas, frente a agentes y estreses medioambientales y frente a los sistemas defensivos del hospedador. De hecho, se ha demostrado una relación estrecha entre la resistencia al estrés, la patogénesis y la complejidad de los sistemas de regulación microbianos. Es decir, la capacidad de patogénesis de los microorganismos depende en gran medida de su capacidad de resistir estreses una vez en el hospedador, y no sólo al estrés ácido del estómago, sino por ejemplo al estrés oxidativo (Felipe-lópez y Hensel, 2011).

A pesar de todo ello, es sorprendente la escasez de investigaciones en las que se ha abordado el estudio del impacto de las respuestas de resistencia en otros procesos celulares, especialmente en la expresión de factores de virulencia y en la patogenicidad de las células microbianas. Una notable excepción es la abundancia de estudios que abordan la relación entre resistencia a los antibióticos y la virulencia/patogenicidad microbiana, excelentemente revisados por Beceiro et al. (2013). En este sentido es necesario indicar que, al igual que se ha descrito que la adquisición de resistencia frente a cada grupo de antibióticos (dada sus diferente estructura y dianas celulares) conduce a la aparición de cepas con características fenotípicas diferentes (y por tanto patogénicas), es más que previsible que los microorganismos desarrollen respuestas de resistencia a estreses diferentes como, por ejemplo, el calor y el medio ácido, y por consiguiente presenten una virulencia notablemente distinta.

Es más, el desarrollo de resistencia al estrés podría afectar a otros aspectos de la fisiología microbiana. Así, se ha demostrado que el desarrollo de respuestas al estrés puede tener un coste en términos de capacidad de crecimiento en condiciones adversas y de competición frente a otros grupos microbianos. Esto se ha observado, por ejemplo, en *Escherichia coli*, microorganismo que tiene un alto grado de similitud (en términos genéticos y fisiológicos) con *Salmonella*. En este microorganismo la expresión/actividad del gen *rpoS* que codifica el factor sigma alternativo de fase estacionaria de la ARN polimerasa, o el desarrollo de algunas respuestas de resistencia frente a los antibióticos se han asociado con un coste en la capacidad

de crecimiento, observándose tasas de crecimiento menores (Andersson y Hughes, 2010; Zambrano et al., 1993).

La relevancia de este fenómeno no debe infravalorarse dado que el desarrollo de respuestas de resistencia, con sus diferentes consecuencias, es un hecho que puede suceder en diferentes puntos de la cadena alimentaria, donde los microorganismos están expuesto a diferentes estreses ambientales o tecnologías de procesado. Asimismo, es conveniente recordar que desde la obtención del producto fresco hasta su procesado y entre esta etapa y su consumo siempre transcurre un cierto tiempo, donde la capacidad de crecimiento y competición también son aspectos muy relevantes desde el punto de vista de la seguridad alimentaria ya que determinarán, junto a la resistencia al estrés, el número de células viables que llegarán al intestino para ejercer su efecto patógeno.

Considerando todo lo anterior y dada la relevancia sanitaria y económica de *Salmonella*, resulta evidente que son necesarios estudios sistemáticos y específicos acerca de la relación existente entre los mecanismos de resistencia microbianos frente a los diferentes agentes y tecnologías, así como otros aspectos de la fisiología celular como la capacidad de crecimiento y competición y en la expresión de factores de virulencia. Dado que contribuir a subsanar esta laguna de conocimiento es uno de los objetivos de esta Tesis Doctoral, en el manuscrito I se ha recopilado y resumido la información existente hasta la fecha acerca de los efectos que el desarrollo de respuestas de resistencia frente a las condiciones de estrés que los microorganismos se pueden encontrar a lo largo de la cadena de obtención y producción de los alimentos (incluyendo el estrés ácido, osmótico y oxidativo, la inanición, las atmósferas modificadas, los detergentes y desinfectantes, la refrigeración, el calor y las tecnologías no térmicas) tendría sobre los diferentes aspectos de la fisiología de las *Salmonellas* no tifoideas, con especial énfasis en la virulencia y la aptitud para el crecimiento. O, dicho de otra forma, se ha tratado de hacer un resumen acerca de la relación entre resistencia al estrés, virulencia y capacidad de crecimiento en *Salmonella*.

3.5. Evaluación del riesgo de *Salmonella*

Son numerosas las evaluaciones del riesgo realizadas y publicadas sobre este microorganismo. De hecho, se podría considerar que es probablemente el riesgo microbiano transmitido por lo alimentos en el que más trabajo se ha realizado en este sentido. Así, se han llevado a cabo numerosas evaluaciones cuantitativas del riesgo microbiológico para *Salmonella* tanto en huevos (Coleman et al., 2005; Hope et al., 2002; OMS/FAO, 2002; USDA-FSIS, 1998; Whiting y Buchanan, 1997), como en carne pollo (Akil y Ahmad, 2019; Lammerding, 2006; OMS/FAO, 2002; Oscar, 2004) o carne de cerdo (Giovannini et al., 2004; Gonzales-Barron et al., 2012; Hill et al., 2010). No obstante, la cantidad de aspectos que todavía se desconocen acerca de su fisiología y que son necesarios para mejorar las evaluaciones del riesgo actuales excede en mucho a los aspectos conocidos.

Así, como se ha comentado antes, un grupo de tan sólo 10-20 serovariedades es el responsable de más del 80% de las toxiinfecciones causadas por *Salmonella*. Las causas de este fenómeno han sido parcialmente exploradas, aunque no del todo identificadas. Los principales enfoques en los que se ha centrado la comunidad científica para explicar este fenómeno son, por una parte, los estudios epidemiológicos y de prevalencia en animales y en las diferentes matrices alimentarias (EFSA, 2021), y por otro, estudios enfocados a determinar la causa de la especificidad de hospedador de las distintas cepas y en los mecanismos de contaminación de los productos alimentarios, como por ejemplo los diferentes mecanismos entre cepas para la contaminación de los huevos (Foley et al., 2013; Sabbagh et al., 2010).

Estos enfoques/estudios permitirían explicar, por ejemplo, las causas de la elevada incidencia de Typhimurium y Enteritidis, serovariedades no específicas de hospedador, y en el caso de Enteritidis se ha demostrado una contaminación directa de los huevos por vía transovárica (Martelli y Davies, 2012). Sin embargo, esto no explicaría por qué algunas serovariedades que se aíslan con frecuencia en gallinas y pollos, como *S*. Mbandaka o *S*. Livingstone (Bellido-Blasco et al., 2006; EFSA, 2021) apenas tienen incidencia en humanos, pese a no ser serovariedades específicas de aves.

Una de las hipótesis de esta Tesis Doctoral es que esto podría ser debido a las diferencias que existen en la resistencia al estrés entre las diferentes serovariedades o su capacidad de crecimiento/competición, aspectos que todavía no han sido estudiados en profundidad o que no se les ha prestado la atención necesaria. Así, si bien es cierto que existen numerosos estudios acerca de la resistencia de *Salmonella* a diferentes agentes de inactivación como los tratamientos térmicos convencionales, el medio ácido o el estrés osmótico (Doyle y Mazzotta, 2000; Sherry et al., 2004), las diferentes condiciones de ensayo o diseños experimentales no adecuados para este fin, incluyendo la limitación en el número de serovariedades estudiadas, una inadecuada elección de las mismas o por centrarse en otros objetivos, no permiten sacar conclusiones a este respecto. Por ello es necesario llevar a cabo estudios específicamente diseñados para determinar con precisión la variabilidad intra-específica en resistencia, también en capacidad de crecimiento y competición, dentro del género *Salmonella*, estudios que, de ser posible, deberían realizarse en aquellos medios y condiciones que mejor reflejen las condiciones reales a los que se enfrentan las células de *Salmonella* en la cadena alimentaria y en tracto gastrointestinal. Por ello, y como se detallará más adelante, el estudio de la variabilidad intra-específica en resistencia al estrés, capacidad de crecimiento y virulencia, es otro de los principales objetivos de esta Tesis Doctoral.

Un ejemplo claro de los efectos que estas lagunas de conocimiento tienen en la toma de decisiones en la industria agroalimentaria es el caso de los criterios microbiológicos que la Unión Europea impone a un gran número de productos alimenticios en relación a *Salmonella*. Así, dada la falta de información disponible a día de hoy en relación a muchos aspectos de la fisiología de *Salmonella* y tomando como base el conocido principio de precaución o cautela de la Comisión Europea (Comisión Europea, 2000) que se aplica de forma general dentro de la legislación alimentaria europea (Reglamento (CE) n° 178/2002, 2002) entre los criterios microbiológicos del Reglamento (CE) nº 2073/2005 (2005) de la Comisión Europea se estableció que *Salmonella* spp. debería estar ausente (ausencia en 25 gramos, salvo algunas excepciones para las que se aplicaba 10 gramos) en un elevado número de productos alimenticios incluyendo carnes, productos lácteos y de la pesca, algunos productos vegetales y tanto productos crudos como cocinados o listos para consumo. Si bien en vista de las ya mencionadas lagunas de conocimiento y de las dificultades para el rápido aislamiento y

tipificación de *Salmonella* a partir de los alimentos, esta medida resultaría, en principio, adecuada, pero es necesario señalar que, a efectos prácticos esta norma equipara el riesgo que suponen serovariedades como *S*. Typhimurium (serovariedad de probada patogenicidad para los humanos) con serovariedades como *S*. Gallinarum (una serovariedad específicamente adaptada a las aves y apatógena para los humanos) en los alimentos.

La depuración (o mejora) de esta normativa, así como de los procesos de toma de decisiones en la industria agroalimentaria requiere de la obtención de nuevos datos y de la mejora de múltiples procesos (de los de detección, por ejemplo) y, probablemente deba hacerse por etapas. Así, un paso intermedio en este proceso de mejora podría ser generar una clasificación de las serovariedades de *Salmonella* similar al modelo de Karmali et al. (2003) para los seropatotipos de *E. coli* para lo que, en primer lugar, sería necesario determinar el riesgo relativo que suponen las diferentes serovariedades. Es más, dado que los datos epidemiológicos y los estudios de prevalencia de *Salmonella* son más abundantes que los de *E. coli,* el modelo o sistema de clasificación podría ser mucho más útil que en el caso de este último.

Por otra parte, un requisito fundamental no sólo para la evaluación del riesgo, sino para la toma de decisiones en cualquier industria y/o para el diseño y optimización de procesos de descontaminación de *Salmonella* es que los estudios deben reflejar o reproducir lo más fielmente los escenarios reales que se pretenden evaluar. En este sentido, en esta Tesis Doctoral se abordan el estudio de dos aspectos claves en relación a este último punto: la existencia de heterogeneidad en las poblaciones microbianas y la necesidad de la validación de los resultados/hipótesis en matrices reales.

En lo que respecta a la heterogeneidad de las poblaciones microbianas, este es un aspecto al que se está prestando cada vez más atención en los últimos años, aunque aún se puede considerar deficientemente estudiado (Ackermann, 2015; Bódi et al., 2017) y cuya relevancia desde el punto de vista de la resistencia a los diferentes agentes estresantes y tecnologías de conservación de los alimentos ha sido ya puesta en evidencia por numerosos autores (Cebrián, 2009; Hauben et al., 1997; Karatzas y Bennik, 2002; Rodríguez-Calleja et al., 2006) y también demostrada para *Salmonella* (Sagarzazu et al., 2013; Sherry et al., 2004).

La diversidad genotípica y fenotípica del género *Salmonella* no sólo es un hecho conocido, sino también uno de sus rasgos más característicos (Cheng et al., 2019). Sin embargo, la información relativa a la capacidad de las diferentes cepas/serovariedades para desarrollar respuestas de resistencia al estrés (ya sean transitorias o permanentes) es mucho más escasa. Del mismo modo que existe variabilidad en la resistencia, esta heterogeneidad también se ve reflejada tanto en la capacidad de crecimiento, como en la patogenicidad como en otros aspectos fenotípicos. De hecho, se ha demostrado que durante la infección las diferentes *Salmonellas* exhiben una expresión heterogénea de genes de virulencia, incluidos los genes flagelares y los genes de las islas de patogenicidad de *Salmonella* (SPI), y que esta diversidad podría ser impulsada por el huésped (Lyu et al., 2021; Tsai y Coombes, 2019).

El estudio de la variabilidad biológica y la cuantificación de su impacto en las evaluaciones del riesgo ha ganado interés durante los últimos años (den Besten et al., 2018; Koutsoumanis y Aspridou, 2017). A menudo, los términos variabilidad e incertidumbre en el contexto de la seguridad alimentaria microbiana suelen confundirse, como anteriormente se ha comentado, la variabilidad incluye las fuentes de variación inherentes (por ejemplo, las diferencias en la respuesta de las células individuales o en la composición de los medios alimentarios) y la incertidumbre, abarca aquellas fuentes de variación que no se tienen en cuenta en el sistema (por ejemplo, errores de medición o errores de especificación del modelo) (Garre et al., 2020). Así, mientras que la incertidumbre puede reducirse reuniendo más datos experimentales de mayor calidad, la variabilidad forma parte del proceso y no puede reducirse simplemente con más y mejores experimentos (Nauta, 2000; Thompson, 2002). Se ha demostrado que la variabilidad de las cepas y la heterogeneidad de la población pueden tener un impacto significativo en la evaluación del riesgo, hasta tal punto de alterar la eficacia de un tratamiento en función de la cepa bacteriana (den Besten et al., 2017; Garre et al., 2020). Por ejemplo, Garre et al. (2020), hallaron una diferencia de aproximadamente 6 reducciones logarítmicas tras 1 minuto de tratamiento a 60 °C entre las cepas de *Listeria* más sensibles y las más resistentes incluidas en el estudio. A la vista de estos resultados es de vital importancia incorporar la variabilidad e incertidumbre a la evaluación cuantitativa del riesgo microbiano, ya que pueden ser igual o más relevantes que los parámetros cinéticos.

Por consiguiente, otro de los principales objetivos de esta Tesis es el estudio y cuantificación de la heterogeneidad fenotípica dentro del género *Salmonella.* Estos estudios servirán, o al menos potencialmente lo harán, para realizar evaluaciones del riesgo más precisas y pueden ayudar a comprender por qué, como se ha comentado anteriormente, algunas serovariedades que se aíslan con frecuencia en los pollos, como *S*. Mbandaka o *S*. Livingstone, tienen una incidencia tan baja en los humanos, a pesar de no ser específicas de las aves de corral.

Adicionalmente, si como se ha hipotetizado antes, existiera una relación entre resistencia al estrés y patogenicidad (tanto directa como inversa) esto supondría que las poblaciones de supervivientes frente a un determinado estrés poseerían una patogenicidad notablemente diferente a la de las poblaciones de origen y que, además, su patogenicidad también podría variar en función del tipo e intensidad del agente estresante al que se enfrentaran las células microbianas, las condiciones de recuperación etc. Es más, la importancia del estudio de la patogenicidad de las subpoblaciones resistentes al estrés se hace más evidente a la vista del siguiente hecho: las células o poblaciones más resistentes al estrés son las que más probabilidades tienen de sobrevivir a los tratamientos tecnológicos y, por lo tanto, de llegar al consumidor a través de los alimentos. En otras palabras, estas células son las más relevantes desde el punto de vista tecnológico por lo que conocer su capacidad de producir enfermedad resulta esencial. Todos estos son aspectos que tienen una gran relevancia en la evaluación del riesgo y que deben de ser estudiados en profundidad, así como los mecanismos subyacentes, lo que permitiría dotar de base biológica a dichas evaluaciones del riesgo y poder desarrollar modelos de predicción del mismo.

Por último es importante señalar que la mayoría de los modelos predictivos disponibles para *Salmonella* se han realizado en medios de laboratorio (Basti y Razavilar, 2004; Koutsoumanis, 2008; Koutsoumanis et al., 2004). Así, muy frecuentemente, a la hora de hacer evaluaciones del riesgo, optimizar procesos y/o tomar decisiones se suelen utilizar modelos generados a partir de datos obtenidos en este tipo de medios. Aunque, en general, estos modelos pueden ofrecer predicciones precisas del crecimiento microbiano en los alimentos, la extrapolación puede resultar compleja ya que los modelos no tienen en cuenta factores significativos para

el crecimiento microbiano, como la estructura de los alimentos, la interacción de los microbios alteradores o el estado fisiológico de las células microbianas.

Dado que desarrollar estos modelos en alimentos es tremendamente costoso una práctica general es validar los modelos generados con medios de laboratorio con resultados obtenidos en alimentos y ya publicados, ya que esto evita el tener que realizar nuevos experimentos con los productos alimentarios. Sin embargo, a menudo esto resulta complicado, ya que, por ejemplo, a veces las condiciones de crecimiento no se describen con precisión o es necesario hacer suposiciones sobre algunos factores o algunos modelos incorporan un nuevo factor para el que se han publicado pocos datos, o incluso ningún dato (Pinon et al., 2004). Así pues, en muchos casos es necesario el desarrollo de nuevos modelos obtenidos usando productos alimentarios. En cualquier caso, el desarrollo de estos últimos o la validación de los modelos obtenidos en medios de laboratorio en alimentos es un requisito imprescindible para hacer evaluaciones del riesgo robustas y significativas o para verificar el impacto que la introducción en la cadena de producción de una nueva tecnología tendría y por ello, una parte de esta Tesis Doctoral se ha centrado, precisamente, en validar los resultados obtenidos en medios de laboratorio en un grupo de alimentos concreto: el huevo y los ovoproductos.

Manuscrito I/Manuscript I

Impact of the resistance responses to stress conditions encountered in food and food processing environments on the virulence and growth fitness of Non-Typhoidal *Salmonellae*

Silvia Guillén, Laura Nadal, Ignacio Álvarez, Pilar Mañas and Guillermo Cebrián

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Review

Impact of the Resistance Responses to Stress Conditions Encountered in Food and Food Processing Environments on the Virulence and Growth Fitness of Non-Typhoidal *Salmonellae*

Silvia Guillén, Laura Nadal, Ignacio Álvarez, Pilar Mañas and Guillermo Cebrián *

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), 50013, Zaragoza, Spain; silviaguillen@posta.unizar.es (S.G.); lnadal@unizar.es (L.N.); ialvalan@unizar.es (I.Á.); manas@unizar.es (P.M.)
* Correspondence: guiceb@unizar.es; Tel.: +34-876554127

Abstract: The success of *Salmonella* as a foodborne pathogen can probably be attributed to two major features: its remarkable genetic diversity and its extraordinary ability to adapt. *Salmonella* cells can survive in harsh environments, successfully compete for nutrients, and cause disease once inside the host. Furthermore, they are capable of rapidly reprogramming their metabolism, evolving in a short time from a stress-resistance mode to a growth or virulent mode, or even to express stress resistance and virulence factors at the same time if needed, thanks to a complex and fine-tuned regulatory network. It is nevertheless generally acknowledged that the development of stress resistance usually has a fitness cost for bacterial cells and that induction of stress resistance responses to certain agents can trigger changes in *Salmonella* virulence. In this review, we summarize and discuss current knowledge concerning the effects that the development of resistance responses to stress conditions encountered in food and food processing environments (including acid, osmotic and oxidative stress, starvation, modified atmospheres, detergents and disinfectants, chilling, heat, and non-thermal technologies) exerts on different aspects of the physiology of non-typhoidal *Salmonellae*, with special emphasis on virulence and growth fitness.

Keywords: *Salmonella*; foodborne pathogen; food preservation; stress resistance responses.

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1. Introduction

Foodborne pathogens have had to develop resistance mechanisms that enable them to withstand stressful environmental and processing conditions they face along the food chain and just before reaching the gut, such as starvation and acidic stomach conditions [1,2]; they have also had to modify and fine-tune their virulence mechanisms in order to evade their host's defense systems [3,4] in a co-evolutionary process with the latter. In this sense, although the differences between those two evolutionary adaptation processes (adaptation to environmental stresses and to hosts) are obvious, they have several characteristics in common. Both processes enable bacteria to survive under adverse conditions, and both involve changes in common molecules and structures such as the cell wall, the bacterial membranes, and their proteins (porins, efflux pumps, etc.). In fact, it has been proved that microbial pathogenesis relies to a great extent on the ability of bacteria to cope with stresses beyond those imposed by the stomach's low pH, such as resistance to oxidative stress [5]. This indicates that there is an intimate relationship between virulence and stress resistance, on the one hand, and highlights the complexity and fine tuning of bacterial gene expression regulation systems on the other. It is also well known that the development of stress resistance can impose a fitness cost to bacteria (as demonstrated for *Escherichia coli rpoS* expression), or as a consequence of the acquisition of resistance to certain antimicrobials [6,7]. This aspect is of utmost relevance for food safety, since the

bacterial ability to grow and compete for nutrients in foods would determine, along with stress resistance, the number of viable cells reaching the gut, and thus, capable of causing illness.

Salmonella is a very good example of successful evolution and adaptation to different niches and hosts. Non-typhoidal *Salmonella* serovars are the second most frequent zoonotic agent in the European Union and the United States [8,9], and are now regarded as a re-emerging pathogen [10]. The *Salmonella* genus includes two species, *S. bongori* and *S. enterica*, whereby the latter is divided into six subspecies. Among those subspecies, *S. enterica* subsp. enterica is a foodborne bacterial pathogen with at least 2600 serotypes [11]. Because of this remarkable genomic diversity, *Salmonella* is found in complex environmental and ecological niches, and survives in harsh environments for long periods [12,13]. This makes it complicated to reduce its overall incidence because *Salmonella* has many sources, which vary according to serotype [14]. Such a degree of genomic, niche, and host diversity makes *Salmonella* a very good model to study the relationships between microbial stress resistance, virulence, and growth fitness.

The objective of this review is therefore to provide a summary of current knowledge regarding the effect that microbial resistance responses to different agents faced by non-Thyphoidal *Salmonella* within the food chain (Figure 1)—including acid, osmotic and oxidative stress, starvation, modified atmospheres, detergents and disinfectants, chilling, heat, and non-thermal technologies—might have on other aspects of microbial physiology, with special emphasis on virulence and growth fitness. The impact of resistance to antibiotics and to other chemical agents such as essential oils and/or natural antimicrobials on *Salmonella* virulence and growth fitness will not be discussed in this review. Information on those particular agents can be found elsewhere [4,15].

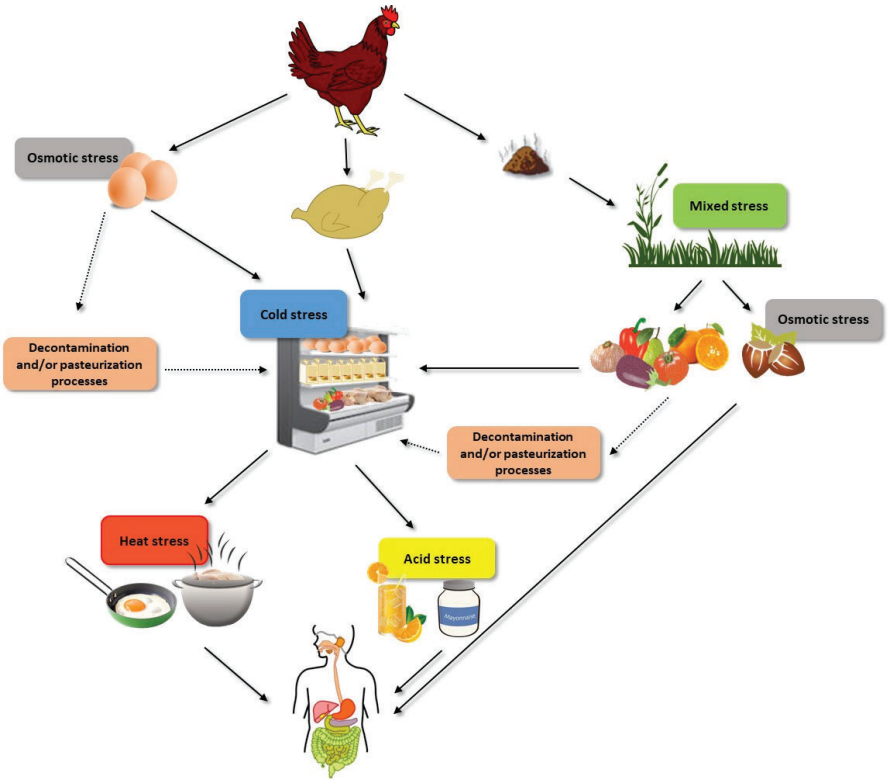

Figure 1. Examples of the different stresses that non-Thyphoidal *Salmonella* cells can face before being ingested with food.2. Bacterial Stress Resistance, Virulence, and Growth Fitness

Environmental stress can be defined as an external factor that exerts an adverse effect on the physiological welfare of bacterial cells, leading to reduction in growth rate, or, in

more extreme circumstances, to inhibition and/or death at individual cell or population levels [16]. In the food processing environment, these stressing factors include agents of a very different nature, including chemical and physical agents such as low pH, low water activity, heat, pulsed electric fields, and ionizing radiation.

As described for antimicrobial resistance, the mechanisms of bacterial resistance to any stress can be classified as innate, adaptive, or acquired [17] (Figure 2). Innate mechanisms include all those structures and potential processes already present and/or active in the bacterial cell when it faces the stressing agent, i.e., they are constitutively and almost continually expressed. Such innate resistance mechanisms include key structures such as the cell envelope robustness, basally expressed homeostatic mechanisms (including membrane pumps, antioxidants, enzymes such as peroxidase, etc.), and damage repair systems (e.g., chaperons and proteases). In the scientific literature, these innate resistance mechanisms are called "classical determinants". Adaptive resistance includes those genotypic and phenotypic changes arising as a consequence of the exposure of bacterial cells to a given environmental stress. Some examples of adaptive resistance mechanisms include changes in cell permeability, overexpression of protective shock proteins, induction of changes in the targets of stressing agents, and also VBNC, biofilm, and persister development [17,18]. Such changes are generally transient: once the stress ceases, they revert. On certain occasions, however, these phenotypic changes become permanent, for instance, through mutation. Moreover, under stressing conditions, a certain decrease in DNA replication consistency may be essential to produce a more heterogeneous population so that some members possessing the new genes or gene combinations can better survive adverse reigning conditions. This process has been described as adaptive or directed mutation. The mechanisms underlying it include stress-induced errors during DNA synthesis, suppression of normal DNA repair checking and repair mechanisms, transient hypermutability, gene amplification, and stress-induced recombination processes [19]. In addition, bacteria can also increase their resistance to stress through horizontal gene transfer, which can be termed as acquired resistance [18].

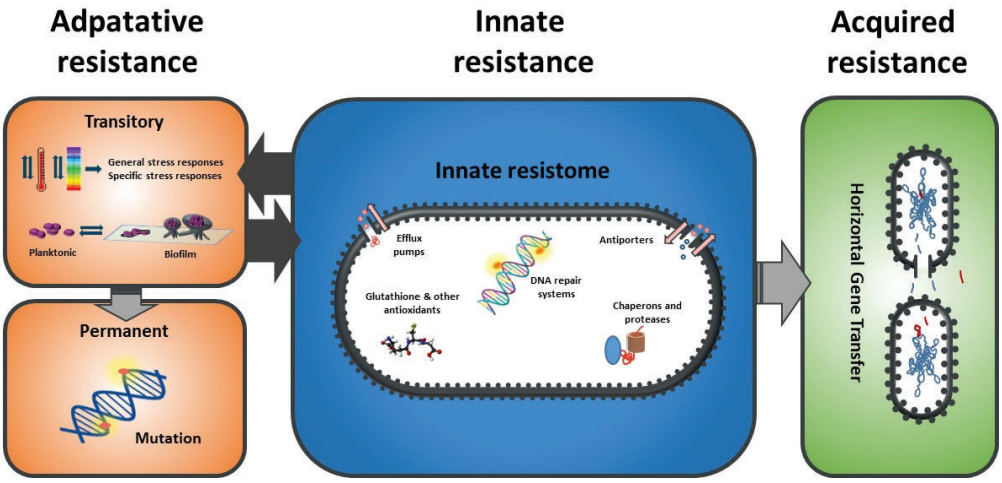

Figure 2. Classification of the mechanisms of stress resistance in bacteria.

Bacterial stress resistance mechanisms can also be classified as specific and/or cross-protective, depending on the range of stressing agents to which the structure or molecule provides protection. In the first case, the triggered response only protects against the same agent that induces it. As described below, general stress response is the major representative of the second type of stress resistance response.

Virulence, on the other hand, can be defined as the ability of a microorganism to cause disease in the host [20]. In this review, we will refer only to humans. The virulence

factors of *Salmonella* have been studied extensively, and include flagella, capsules, plasmids, adhesion systems, hemagglutinins, exotoxins, endotoxins, invasins, and type 3 secretion systems (T3SS) encoded on the *Salmonella* pathogenicity islands (SPI)-1 and SPI-2 and other SPIs [21–24]. The particular role of each of these components, along with their regulation, have likewise been thoroughly investigated, albeit not yet fully elucidated; excellent reviews on the topic can be found elsewhere [25–29]. In order to cause foodborne disease in humans, *Salmonella* cells should survive the (sub)lethal environmental conditions of the gastrointestinal tract (acid, bile salts, low oxygen tension, commensal bacteria, etc.) which are part of the host's defenses against infection. Thus, a virulent strain or cell should not only be capable of invading intestinal epithelial cells, but also of surviving those harsh conditions. However, in this review we shall distinguish, whenever data are available, between phenomena clearly related to stress resistance (e.g., to the acid pH of the stomach, or to ROS within macrophages) and those associated with the mechanisms of cellular/tissue adhesion, invasion, and proliferation.

Finally, the ability to replicate in a given environment is called growth fitness, or bacterial fitness. As indicated for stress resistance, this definition implies that one should determine the environment in which growth is measured: i.e., a strain might be able to grow faster than other strains under certain conditions, but not under others. In general terms, and for the purpose of this review, we shall consider that a strain has lost growth fitness if acquisition of resistance has resulted in a decrease in its growth rate or in its final concentration in either a standard rich medium (such as TSB, BHI, LB) or in a medium with limited nutrient availability but, in both cases, in which no other stressing agent (such as pH, NaCl concentration, elevated or low temperatures, presence of antimicrobials, etc.) is acting. It is evident that situations such as these, including growth at pH or a_w close to the boundaries, are of utmost relevance for food safety, but would make the discussion too complex for the scope of this review and will therefore not be addressed herein.

3. *Salmonella* Stress Resistance Mechanisms: Impact on Virulence and Growth Fitness

3.1. Non-Specific Stress Responses

3.1.1. The General Stress Response (GSR)

Alternative sigma factors are probably the most relevant strategy developed by bacteria when they face adverse conditions [1]. Among them, alternative sigma factor σ^S (also called σ^{38} or RpoS) of RNA polymerase (RNAP) is regarded as the master regulator of the general stress response in many Gram-negative bacteria, including *Salmonella* [30–33]. Expression of RpoS is induced upon exposure of bacterial cells to various stresses such as acid, heat, and oxidative stress, among others, and also upon entry into stationary phase [34]. Recent results have demonstrated that RpoS directly or indirectly modulates the expression of 38% of the observed *S.* Typhimurium proteome [33].

As might be expected, induction of RpoS leads to the synthesis of a number of proteins with a proven role in *Salmonella* resistance to stress, such as Dps, HPII catalase, OstA, OstB, and several acid shock proteins (ASPs). Dps, the most abundant protein in *S.* Typhimurium during stationary growth phase, is a bacterial ferritin involved in the resistance to oxidative stress [35–37]. The *katE* gene, encoding the HPII catalase, is considered to be RpoS-dependent in *Salmonella* [38,39], and it also contributes to the prevention of oxidative stress [40]. RpoS is likewise involved in the transcription of the *otsBA* operon in *S.* Typhimurium [41,42], which plays an important role in countering osmotic stress via regulation of the trehalose synthesis. Under high-osmolarity conditions, trehalose serves as an osmoprotectant [43]. RpoS has also been reported to play an important role in the acquisition of tolerance to organic acid stress by regulating the expression of several acid shock proteins [44].

Nevertheless, it has also been demonstrated that RpoS also controls a broad spectrum of *Salmonella* proteins required for various biological processes, including virulence. According to the data obtained by Rice et al. (2015), RpoS reduces the expression of SPI-1 and SPI-2 at different points in stationary phase cultures [45], which would suggest that RpoS expression might decrease *Salmonella* virulence. However, the transcription SPI-9 operon genes, which contribute to adherence to epithelial cells, increases under low pH and high osmolarity in an RpoS-dependent manner in *S*. Typhi [46] and Nickerson and Curtis observed that a *rpoS* mutant of *S.* Typhimurium exhibited wild-type abilities to attach itself to and invade Int-407 cells and J774 macrophage-like cells [47]. It has also been reported that *rpoS* and RpoS-dependent genes are highly expressed upon entry into macrophages and epithelial cells [38,48], that RpoS induces the expression of SEF14 fimbriae [49], and that RpoS is required for the expression of the plasmid encoded *spv* genes, which, in turn, are required for intracellular growth in deep lymphoid organs such as the spleen and the liver, and which are associated with strains causing non-typhoid bacteremia [50–53]. These latter results would at least partially explain why deletion of *rpoS* or altered *rpoS* alleles leads to a decrease in *Salmonella* lethality in mice [54], and why *S*. Typhimurium *rpoS* mutants demonstrated a decreased ability to colonize murine Peyer's patches after oral inoculation as compared to its wild-type virulent parent strain, which indicates that RpoS-dependent gene expression was required for the initial stages of systemic infection [47].

In summary, contradictory observations have been published with regard to the impact of RpoS expression on *Salmonella* virulence and further work would be needed in order to fully elucidate the role of RpoS in *Salmonella* pathogenesis; this role is undoubtedly a highly complex and tightly regulated multi-step process in which attachment and invasion of epithelial cells is as important as survival to acid, bile, oxidative stresses and other host defense mechanisms, and intra and extra cellular proliferation, and consequently, RpoS might be playing different roles in each of these steps.

In spite of the relevance of RpoS, it should be noted that stress-sensitive *rpoS* mutants are surprisingly common among natural isolates of the closely related *E. coli*. A similar phenomenon has been observed for *S.* Typhi, but not for *S*. Typhimurium [55]. Although it has not yet been demonstrated in *Salmonella*, it is assumed that the natural abundance of mutants in the *rpoS* gene would be due to the so-called Growth Advantage in Stationary Phase (GASP) phenotype that these mutations confer (35, 22). Thus, cells with a reduced RpoS activity can grow better in media with low levels of nutrients, and also seem to possess an advantage in competitive colonization of the intestine [56]. This means that a RpoS-related trade-off between survival potential and nutritional competence would exist: a powerful mechanism that gives rise to phenotypic heterogeneity. The reasons why these *rpoS* mutants are not so common in *S.* Typhimurium remain to be elucidated: one reason might be that *S*. Typhimurium uses RpoS for the expression of important virulence genes [55], although, as pointed out above, the role of RpoS on *Salmonella* virulence seems to be very complex. Finally, it should be noted that RpoS also controls a number of genes involved in other highly relevant cellular processes, such as biofilm formation in *S*. Typhimurium [30,57,58].

3.1.2. The Extracytoplasmic Stress Response (ESR)

Besides RpoS, *Salmonella* encodes other alternative sigma factors, such as RpoH (σ^H) and RpoE (σ^E). The latter is essential for *Salmonella* survival under conditions of extracytoplasmic stress. Inducers of the RpoE pathway include acid stress, oxidative stress, heat shock, carbon starvation, biofilm formation, ultraviolet A (UV-A) radiation, P22 phage, and hypo-osmotic shock, among others [59–61]. Activation of the RpoE pathway is generally due to the accumulation of misfolded and/or mis-translocated outer membrane proteins (OMPs) or lipopolysaccharides (LPS) within the periplasm [60].

The role of RpoE in *Salmonella* pathogenesis has been recently reviewed by Hews et al. (2019) [62]. Early studies of the relationship between RpoE and *Salmonella* virulence

already revealed that deletion of RpoE rendered *Salmonella* cells viable but avirulent in a murine infection model: this was initially thought to be mainly related to their increased susceptibility to a variety of stresses [63–65]. Nevertheless, recent studies have demonstrated the existence of further links between RpoE and coordination of virulence gene expression. Thus, RpoE would also upregulate SPI-2 genes [66,67], and it has also been observed that deletion of the RpoE-dependent genes *skp*, *surA* and/or *degP* (alone or in combination with other genes) results in a significant loss of virulence [68–70]. In addition to these proteins, RpoE-regulated sRNAs have also been linked with virulence [71]. Furthermore, in *S*. Typhi, *rpoE* mutants displayed a reduced expression of the pathogenicity islands SPI-1 and SPI-2 encoding the T3SSs required for invasion and intracellular survival, and, therefore, were attenuated for invasion and intracellular survival [72,73]. It should also be noted that another system regulating the response of *Salmonella* to envelope stress, the Cpx system, has also been proved to be involved in the regulation of virulence in *S.* Typhimurium [74,75].

The relationship between RpoE expression and growth fitness in *Salmonella* has also been investigated. In the study conducted by Shetty et al. (2019), *rpoE S.* Enteritidis mutants grown in Luria-Bertani (LB) broth at room temperature showed an extended lag phase [76]. Similar results have been obtained for *S.* Typhimurium by Testerman et al. (2002) and Humphreys et al. (1999) [63,64]. Supplementation of glucose in LB medium as well as growth in M9-G medium rendered normal growth curves of the *rpoE* mutant. In addition, results obtained in the latter study led to the conclusion that the *rpoE* mutant is not able to properly utilize carbon sources other than glucose, since replacement of glucose with succinate as the carbon source led to a considerably extended lag phase.

3.2. Specific Stress Responses

3.2.1. Acid Stress

Food acidification is one of the most widely used methods to control the growth—or accelerate the inactivation—of undesirable microorganisms, including foodborne pathogens such as *Salmonella*. Thus, fermentation, a naturally occurring form of acidification, has long been used for food preservation, as has acidification by direct addition of organic and other appropriate acids [77]. Exposure of *Salmonella* cells to acid conditions leads to the activation of the acid tolerance response (ATR). As described in Álvarez-Ordoñez et al. (2011), the ATR consists of 3 main systems: pH homeostatic systems, modifications of the membrane composition, and synthesis of acid shock proteins (ASPs) [78]. To date, it has been demonstrated that exposure to acidic conditions results in the induction of at least five regulons leading to the synthesis of ASPs: Fur, PhoPQ, OmpR, RpoE, and RpoS [61,78].

The Fur protein controls a subset of ASPs, which contribute to the *S*. Typhimurium exponential-phase ATR, and confer protection mainly against organic acid stress [79,80]. Deletion of *fur* in *Salmonella* attenuates its virulence [54,81,82], and a new role for Fur as a regulator of the expression of the SPI-1 type III secretion system has been described [83], suggesting a role for this regulator in pathogenicity. Induction of PhoPQ and its regulon by acidic conditions (including inside the gastric tract and within macrophage phagosomes) has also been demonstrated [44,80,84–87]. It should be noted that, as described for Fur, several studies have shown that *Salmonella* strains harboring null alleles of the *phoP* or *phoQ* gene were highly attenuated for virulence [82,88–90]. Acid shock also induces OmpR by means of its phosphorylation from the phosphate donor acetyl phosphate. OmpR, in its phosphorylated state, triggers the expression of various genes involved in the acid-inducible stationary-phase ATR [91–93]. Interestingly, several studies have connected OmpR with *Salmonella* virulence, mainly through the regulation of the SPI-1- and SPI-2-encoded genes, thereby observing that *ompR* mutants are highly attenuated in mice [82,94–97]. Finally, both RpoS and RpoE have also been shown to be involved in the ATR

of *Salmonella* [61,80,98]. The impact of the induction of both sigma factors on *Salmonella* virulence was discussed above.

Although all these data might suggest that exposure to acid stress would lead to an increase in *Salmonella* virulence, results obtained seem to indicate that not all virulence factors/pathways would be equally affected by acid shocks. On one hand, Ryan and coworkers observed that exposure of *S*. Typhimurium to acid conditions led to a downregulation of some SPI-1 genes [99], and, similarly, Kim et al. [100] found that the *invE* gene (SPI-1) was downregulated in lysogenic *S*. Typhimurium when treated at pH 3.0, 4.0, and 5.0 for a short time. On the other hand, acid shock would lead to an upregulation of SPI-2 genes [99,101]. In addition, differences in these responses have been observed when comparing planktonic and biofilm *Salmonella* cells [102,103]. Such differences between activation of SPI-1 and SPI-2 might be explained by the fact that SPI-2 plays a key role in *Salmonella* intracellular pathogenicity where acidification of the phagosome serves as a signal for SPI-2 induction. By contrast, as indicated by Kitamoto et al. (2016), virulence genes will not be required in other acidic environments, such as the stomach, which might explain why other SPI-1's would be repressed upon exposure to acid conditions [104].

In any case, these results and conclusions were all obtained under conditions leading to a transient (acid) stress response; they are therefore not so relevant from a food safety perspective, at least if we assume that all the cells suffer such acid shock in the stomach. The results obtained by Karatzas et al. (2008) indicated that sustainable *Salmonella enterica* acid-resistant variants, obtained upon repeated cycles of acid challenge and growth, displayed an increased expression of SEF17 fimbriae and a reduced virulence [105]. Nevertheless, since these variants' mechanisms of acid adaptation are unknown, it is very difficult to compare these data with the data previously presented. In Table 1, some examples of stable *Salmonella* variants obtained after successive exposure to different selecting agents are listed. The impact of the resistance responses developed on the virulence and growth fitness of these strains is also included.

On the other hand, Karatzas et al. (2008) observed that the acquisition of acid resistance did have a fitness cost (at neutral pH) for *Salmonella* cells [105]. Apart from the overexpression of ASP and of pH homeostasis systems, it is plausible that an increase in the amount of Cyclopropane Fatty Acids (CFA) caused by adaptation to acid shock might also impose some fitness cost, since they contribute to membrane rigidification, and, whereas rigid membranes seem to be associated with stress resistance, exponential growth phase cells tend to display membranes that are more fluid, which is normally related to active growing [106,107].

3.2.2. Osmotic Stress

Hyperosmolarity and desiccation—usually in combination with other agents—are frequently used by the food processing industry as a means to prevent bacterial proliferation, in food products [108]. It is remarkable that although *Salmonella* is not able to grow at water activities below 0.93, the presence of *Salmonella* has been reported in numerous low-moisture foods [108].

The mechanisms of *Salmonella* survival in low a_w environments and the regulation thereof are partially but still not completely understood. Thus, when *Salmonella* cells are exposed to a high osmotic pressure environment, they develop various adaptive responses that include the accumulation of solutes such as K^+ and osmoprotectants (betaine, proline and trehalose), changes in the composition and permeability of their membranes, degradation of ribosomal RNA molecules, and many other consequences of the activation of both RpoS and RpoE [78,108–110].

The transcriptome of *Salmonella* cells after their exposure to low a_w environments has been studied by various researchers [110–114]. In some cases, exposure to low a_w can even provoke *Salmonella* cells to enter into a physiologically dormant state [111]. In general, however, in spite of the different conditions assayed, almost no gene related to virulence was found to be up- or down-regulated in these studies. By contrast, Kröger et al. (2013)

observed that exposure of *S*. Typhimurium cells to a 10 min 0.3 M NaCl shock resulted in an increased expression of several SPI-2 genes and of some effector proteins such as SseK1, AvrA, GtgE or SptP [115]; Huang et al. (2007) observed that a 120 min 0.3 M NaCl shock resulted in an increased expression of SPI-1 genes, although in their case they worked with *S*. Typhi cells [113]. Nevertheless, it should be noted these conditions are not comparable to those applied in the previously indicated studies.

These results, especially from authors who did not observe any change in the expression of virulence factors, contrast with investigations that studied the effect of osmotic shocks/desiccation on the capability of *Salmonella* cells to invade CaCo2 cells, such as the study by Lang et al. (2017), who observed that drying increased the invasion capacity of *S*. Typhimurium and *S*. Senftenberg cells [116], and the study by Yoon et al. (2013), who observed that exposure of *S*. Enteritidis cells to NaCl increased their cell invasion efficiency (Table 1) [117].

These later results might explain why salmonellosis, epidemiologically linked to the ingestion of a contaminated low a_w product, may arise from a low infectious dose (of the order 10–100 CFU) in contrast with the infectious dose following ingestion of other contaminated foods (>10^5 CFU) [118]. Nevertheless, it cannot be discarded that other factors such as an underestimation of actual numbers of bacteria contaminating the low a_w food matrix (because of clumping, filamentation, or entry into VNBC state) or an increase in stress resistance of *Salmonella* cells (due to the development of cross-resistance mechanisms because of low a_w matrixes that could be exerting a protective effect) could also be contributing to this phenomenon.

3.2.3. Oxidative Stress

In order to reduce the total microbial load in surfaces, the food industry often uses disinfectants such as sodium hypochlorite or hydrogen peroxide. Nitrites and nitrates that lead to the formation of Reactive Nitrogen Species (RNS) are also found in some food products and/or are intentionally added to prevent microbial growth. These are nevertheless not the only sources of oxidative damage that *Salmonella* cells can encounter in the course of the food processing chain, since it has been already demonstrated that many agents/technologies lead to the generation of Reactive Oxygen Species (ROS) within microbial cells [119].

Once inside the host, *Salmonella* cells are confronted with professional phagocytic cells, eventually also leading to exposure to ROS and RNS [120]. Furthermore, ROS are formed by bacterial cells themselves through the activity of the respiratory electron transport chain [121]; *Salmonella* has therefore developed a wide range of mechanisms to cope with them. They include the synthesis of enzymes capable of detoxifying them, such as superoxidodismutases, alkyl hydroperoxide reductases, catalases, peroxidases, thioredoxin, periplasmic oxidoreductases, DNA and protein damage repair enzymes (such as RecA, LexA, and SulA), and various chaperons and proteases. Further mechanisms include reductants such as glutathione, but also the induction of changes in membrane permeability as described by van der Heijden et al. (2016) [122]. Furthermore, it has been demonstrated that *Salmonella* induces and makes use of an inflammatory response with its accompanying ROS production, since the latter provides *Salmonella* with a competitive advantage over the gut microbiota [123–125].

The intimate relationship between some of these defense mechanisms and bacterial virulence is well illustrated by the fact that periplasmic superoxide dismutase SodCI becomes upregulated even when *S*. Typhimurium replicates in non-activated murine monocytic cells. One should thereby bear in mind that periplasmic superoxide dismutase SodCI is likewise part of the *Salmonella* PhoP/PhoQ virulence regulon, which also includes the SPI-1, SPI-2, and the *spv* genes [126–128]. However, it should also be noted that the regulation of the oxidative stress response is very complex: a number of regulators are involved, and some of them induce opposite effects on *Salmonella* virulence. Thus, in addition to RpoS and RpoE, the SoxR/SoxS and the OxyR regulons have been shown to play

a major role in *Salmonella* response to oxidative stress [62,129]. Nevertheless, neither the SoxR/SoxS nor the OxyR regulon seem to play a relevant role in *Salmonella* pathogenesis [109,130] although the latter seems to contribute to intestinal colonization and is a target for the immune system [131,132]. *Salmonella* NsrR regulon is activated as a consequence of nitrosative stress, and deletion of some of its genes does result in a reduced virulence in mice; further work shall be required, however, to elucidate whether this regulon plays a role in processes other than stress resistance [133].

Transcriptomic analysis of *Salmonella* responses to chlorine, hydrogen peroxide, and nitric oxide revealed that exposure to chlorine led to a downregulation of SPI-1-regulated genes in *S.* Typhimurium LT4, although almost no differences in the level of expression of these genes were observed between control and chlorine-treated for *S. Enteritidis* LT2 cells [134]. In a similar study, Cadena and co-workers [135] observed that exposure of *S.* Heidelberg to acidified calcium hypochlorite or peroxyacetic acid, only resulted in minor changes in the expression of virulence related genes. On the other hand, Kröger et al. (2013) reported that peroxide and nitric oxide shocks resulted in downregulation of the SPI-2 genes; however, it should be noted that the control cells in their study (non-oxidative stressed cells) were grown in an acidic phosphate-limiting minimal medium specifically designed to induce SPI-2 transcription [115].

To the best of our knowledge, isolation of mutants/clones with an increased resistance to oxidative stresses has only been reported in the works by Karatzas et al. [136,137]. Their results showed that *S.* Typhimurium variants with an increased resistance to an oxidizing compound blend displayed a decreased ability to invade CaCo-2 cells and also lower growth rate and yield.

3.2.4. Starvation

Starvation or nutrient limitation is, paradoxically, quite common along the food processing chain. Thus, bacteria can be under starvation when they are located in food processing equipment or surfaces, but also on the surface of many products (e.g., those possessing a shell) or even inside certain products (e.g., iron availability is limited in egg white). The starvation stress response (SSR) of *S.* Typhimurium encompasses the genetic and physiological changes that occur when they are starved of an essential nutrient, e.g., phosphate (P), carbon (C), or nitrogen (N) [138]. Depending on the limiting nutrient, however, the changes induced by the cell will differ, although certain common regulons are induced by the absence of almost any of them.

The starvation stress response (SSR) is regulated by different proteins/systems including RelA (responsible for the synthesis of guanosine tetraphosphate (ppGpp)), SpoT (a bifunctional synthetase and hydrolase of ppGpp), Crp (the cyclic AMP (cAMP) receptor protein), CsrA (a carbon-storage regulator) [139], and DksA (an RNA polymerase-binding protein) [140–144]. RpoS is also induced by a variety of starvation conditions whereas RpoE has been proven to be induced by carbon starvation [138,145].

Regarding the link between the SSR and *Salmonella* virulence, it has been demonstrated that *Salmonella* cells deficient in ppGpp synthesis display a reduced expression of *hilA* and *invF*, encoding major transcriptional activators required for SPI-1 gene expression, and that they are non-invasive in vitro and highly attenuated in vivo [146]. (p)ppGpp is also required for the activation of SPI-2 gene transcription [147]. Furthermore, it was very recently demonstrated that an *S.* Typhimurium mutant with a SpoT variant without hydrolase activity displayed a decreased expression of SPI-2 genes [143], indicating that RelA and SpoT would play different roles in *Salmonella* pathogenesis. On the other hand, CsrA is a post-transcriptional regulator that controls the expression of SPI-1 and SPI-2 through direct repression of *hilD* translation initiation during growth in rich media [139,148,149]. End products of metabolism and amino acid starvation stimulate *csrB*/*C* transcription of two inhibitory small RNAs that sequester CsrA and, thus, inhibit its repression of *hilD.* Finally, deletion of *crp* has been proven to reduce *Salmonella* pathogenic-

ity of different serovars [150–153] and the deletion of DksA led to a decrease in the virulence of *S.* Typhimurium in mice [154]. The results of Kroger et al. (2013) are thereby consistent with the hypothesis that starvation causes significant changes in *Salmonella* virulence, since they observed that growth in PCN (a minimal medium) resulted in an increased transcription of many SPI-1 genes, as compared to growth in LB [115].

Iron, on the other hand, is not only an essential growth factor for *Salmonella*, but also seems to play a very relevant role in its pathogenicity. Thus, the ability to acquire iron has been suggested as a key factor that determines the ability of *Salmonella* cells to outcompete the commensal microbiota within the gut; it also serves as a signaling element that regulates various genes, including virulence-associated genes [155]. The amount of available iron also seems to determine the adhesion and invasion capability of *Salmonella* cells [156–158]. Starvation for iron occurs within the food chain under conditions such as those described above, but also in some food products such as egg white, which is a very relevant matrix for salmonellosis [159].

The master regulator Fur (ferric uptake regulator) is the main transcription regulator in *Salmonella* in response to iron availability [160,161]. Thus, transcriptional repression by Fur is relieved when iron is limited, leading to the expression of iron acquisition systems, including those that produce and secrete iron-chelating siderophores such as enterobactin and salmochelin [162]. A clear example of the link between Fe metabolism and virulence is the fact that deletion of genes of these iron uptake systems (such as *tonB*) resulted in *Salmonella* with a lower ability to invade Caco-2 [158]. Transcriptomic analysis has revealed that exposure to low Fe concentrations leads to the induction of the *sitABCD* iron transporter located in the SPI-1 [115]. Although no effect of the expression of SPI-2 genes was observed upon exposure to low Fe concentrations in that study, in other studies increased expression of SPI-2-associated virulence genes upon exposure of *Salmonella* cells to these conditions has been reported [155,163]. Finally, Dostal et al. (2014) observed that the number of *Salmonella* cells capable of invading a CaCo2-HT29-MTX co-culture was greater when the media had low iron concentrations [156]. In other studies, however, it has been observed that increasing the iron concentration has the same effect [158].

3.2.5. Modified Atmospheres

Modified-atmosphere technologies are capable of extending the shelf life of food products by minimizing the physiological, chemical, and microbial decomposition of foods in an atmosphere that differs from the normal composition of air [164]. For this reason, such technologies, especially Modified Atmosphere Packaging (MAP), are extensively used nowadays for preserving fresh, minimally processed, and processed food products.

S. enterica is a facultative anaerobe; it thrives in an anaerobic environment by performing fermentations and/or by using alternative electron acceptors, such as nitrate or fumarate [165]. FNR (also called OxrA) is considered to be the main regulator of the adaptive response of *S. enterica* to lack of oxygen, and is a cytoplasmic oxygen sensor that can bind promoter sequences. Upon interaction with the RpoA subunit of RNA, it induces the transcription of a variety of genes required for anaerobic metabolism, whereas it represses many of the genes encoding enzymes involved in aerobic electron transport, oxidative phosphorylation, and some tricarboxylic cycle enzymes [78]. On the other hand, ArcAB is a two-component signal transduction system induced under microaerobic and anaerobic conditions that suppresses the expression of genes encoding enzymes of the tricarboxylic cycle, leading to a decrease in the amount of generated ROS [166].

Apart from their role in regulating the main metabolic and energy pathways of *Salmonella*, both regulators have been shown to affect the expression of virulence factors. Thus, OxrA/FNR regulates numerous virulence genes within *Salmonella* pathogenicity island 1 (SPI-1), the virulence operon *srfABC*, and also some flagellar genes (*mcpAC*, *cheV*). Furthermore, an *oxrA* mutant was shown to be non-motile and attenuated in vivo [167]. ArcA also induces certain virulence genes but represses others. This fact, together with

the low number of genes it regulates and the complexity of their regulation, would explain why an *arcA* mutant showed virulence defect in mice [168,169]. Kroger et al. (2013) also observed an increased expression of SPI-1 genes when *S*. Typhimurium was grown in anaerobiosis as compared to aerobic conditions; not, however, after a 30-minute anaerobic shock [115]. Furthermore, the expression of SPI-1 genes increased even further when anaerobically grown cells were exposed to a 30-minute aerobic shock, which suggests that the relationship between the composition of the atmosphere and the expression of SPI-1 factors would be more complex than was initially expected. In any case, various studies have observed that *Salmonella* cells grown in anaerobiosis do display an increased adhesion and invasion capacity of cultured cells [170–173].

3.2.6. Chemical Stressors: Detergents and Disinfectants

Bile salts, which are released after food intake from the gallbladder into the duodenum, act as detergents and, therefore, exert their effects on bacterial cell membranes; they can also have numerous other effects on further molecules such as RNA, DNA, and proteins [174–176]. Although they are not relevant in terms of food preservation, the response of *Salmonella* to bile salts has been widely studied for obvious reasons, and can also serve as a model for the responses that other detergents can trigger.

The deletion of various proteins involved in *Salmonella* bile resistance such as AcrB, Dam, PhoPQ, and Wec has been proven to reduce the virulence of *Salmonella* cells in in BALB/c mice [88,177–180]; this seems to be more likely, however, due to the decrease in stress (bile) resistance than to a decrease in the expression of virulence factors. Prouty and Gunn (2000) demonstrated that *S.* Typhimurium grown in the presence of bile is able to invade epithelial cells at only 4% of the level of cells grown in the absence of bile, and transcription of invasion gene regulators (*sirC* and *invF*) was shown to be repressed in the presence of bile, resulting in decreased transcription of SPI-1 genes [181,182]. Recently, Urdaneta et al., 2019 observed that 7 out of 10 *Salmonella* bile-resistant mutants recovered from the gallbladder of infected mice showed one or more virulence-related defects; they suggested that resistance to bile would be achieved at the expense of virulence impairment, and that it may involve fitness tradeoffs in certain cases [183]. Nonetheless, most authors agree that *Salmonella* may use bile as an environmental signal to repress its invasive capacity in the intestinal lumen, where bile concentrations are high, and invasion may then be initiated after transiting the mucus layer [104,181,182].

Disinfectants can be broadly grouped into oxidizing agents, surface active compounds, and iodophores [184]. Widely used sanitizers, such as halogen-based compounds, peracetic acid (PAA), ozone, and hydrogen peroxide, fall within the group of oxidizing agents; their impact on *Salmonella* virulence and growth fitness was discussed above. However, surface-active compounds, such as acid anionic compounds and quaternary ammonium compounds (QACs), are also frequently used in food industries. In a recent study by Cadena et al. (2019), the authors observed that the effect of exposing *S.* Heidelberg cells to cetylpyridinium chloride on the transcription of virulence factors depended on the strains studied [135]. Thus, whereas for one of the strains the upregulation of some SPI-1 genes was observed, for the other one no differences were observed. By contrast, Kautz et al. (2013) observed that *S.* Enteritidis strains with reduced susceptibility to dodecyltrimethylammonium chloride were found to be less invasive (Caco-2 cells were used) and had fewer fimbriae, and that the majority had lower expression levels of *fimA*, *csgG*, and *spvR* (3 out of 4) than those of the parental strain, thereby suggesting a generally decreased pathogenicity (Table 1) [185]. Similar results to those of Kautz and co-workers were obtained by Karatzas et al. (2007), who observed that stable variants of *S.* Typhimurium SL1344 exposed to three different commercial disinfectants displayed a decreased invasiveness of Caco-2 cells (Table 1) [136,137]. These authors also observed that acquisition of resistance to these disinfectants also resulted in a decreased growth fitness in LB broth.

3.2.7. Chilling

Although *Salmonella* is a mesophilic microorganism unable to grow below aprox. 7 °C, *Salmonella* cells are often exposed to refrigeration and freezing temperatures within the food chain. The adaptation mechanisms of *Salmonella* cells to cold have been recently reviewed by Ricke et al. (2018), and include changes in the composition of their envelopes (i.e., membrane fluidification), the induction of the synthesis of a series of proteins (Cold Induced, Cold Shock and Cold Acclimatization Proteins), and changes in DNA supercoiling [186].

Regarding the impact of the cold stress response on *Salmonella* virulence, it should be noted that the NusA protein, which has been identified as induced under cold temperature conditions [187,188], was found to impact *hilA* expression, the transcriptional activator of *Salmonella* Pathogenicity Island-1 [189].

On the other hand, whereas Kröger et al. (2013) did not observe any significant change in the expression of SPI-1 and SPI-2 genes after a 10-minute shock at 10 °C [115], Shah et al. (2014) observed that exposure of *S*. Typhimurium cells to a temperature of 5 °C for 48 h resulted in induction of several groups of virulence genes, including T3SS-associated genes located on SPI-1 and SPI-2 [190]. The latter authors also studied the effect of this cold shock on the ability of *Salmonella* to adhere to and invade Caco-2 cells, and observed an increase in both phenomena.

3.2.8. Heat

Heat has been widely used in the food industry as a preservation agent since it is capable of inactivating most microorganisms and enzymes present in foods. Therefore, heat is a method capable of simultaneously guaranteeing food safety and food stability [191]. Given its relevance, the mechanisms of microbial inactivation by heat and the stress responses that bacteria can develop upon exposure to sublethal heat treatments (the heat shock response) have been extensively studied. The heat shock response in *Salmonella* involves activation of various regulons including those controlled by the sigma factors RpoS, RpoE, and RpoH [192]. The role of RpoS and RpoE in *Salmonella* stress resistance, pathogenesis, and growth fitness was discussed above and, therefore, will not be reviewed in this section. On the other hand, the sigma factor $\sigma^{32/H}$ controls more than 30 proteins, most of which are associated chaperones and proteases [31,193–197]. Although both RpoH and RpoE are alternative sigma factors, RpoH regulates Heat Shock Proteins (HSPs) for the cytoplasmic components, whereas RpoE regulates the extracytoplasmic (cell envelope) proteins in response to high temperatures, as it does with other envelope stress factors [60,198–203]. As described for *rpoS, rpoH* gene expression is also regulated by RpoE [204–209].

Increased expression of virulence genes in *Salmonella* upon exposure to sublethal heat shocks or growth at high temperatures has been reported in a number of studies such as that of Yang et al. (2013), who observed that virulence-related genes—*spvR*, *hilA*, *avrA*—were more expressed in *S*. Enteritidis cells the higher the growth temperature (between 10 and 42 °C), while *sefA* maximum expression was observed when cells were grown at 37 °C [210]. Recently, Dawoud et al. (2017) reviewed the potential relationship between heat resistance (or the heat shock response) and virulence [211]. In their review, they indicated that various *Salmonella* heat-shock proteins have been shown to play a role in pathogenesis, including FtsH, FkpA, SurA [68,69,212–216]. Similarly, it has been demonstrated that increasing growth temperature to at least 37 °C leads to a decrease in the binding capacity of H-NS to DNA (AT-rich sequence), which leads, in turn, to virulence gene (SPI-1, 2, 3 and 5) expression [217–224]. The same authors also point out that exposure of *Salmonella* cells to sublethal heating conditions might also exert an influence on their virulence via changes in DNA topology, such as a DNA supercoiling which would lead, in turn, to SPI-1 gene expression [225,226] or could be due to the generation of bends in the AT-rich sequence regions situated in the 5′-end upstream of the promoter region that

would influence the interaction between RNA polymerase and the promoter region, thereby altering gene expression [227,228]. Information concerning the impact of other heat resistance mechanisms, such as membrane rigidification, on *Salmonella* virulence is very scarce; further research is necessary in this domain.

In parallel, various investigations have studied the influence of the development of resistance to heat of *Salmonella* cells on its virulence by using different models. Sirsat et al. (2001) reported that exposing *Salmonella* cells to a heat shock of 42 °C resulted in an improved adhesion to Caco-2 cells, but not in a higher degree of invasion [192]. The same authors reported that upon that heat shock the genes of two *Salmonella* pathogenicity islands (SPI-2 and SPI-5) were upregulated, which would explain their higher adhesion and would confer a higher chance of survival in the host while genes of SPI-1 were downregulated. In another study, Lang et al., 2017 compared the invasion ability of dried *Salmonella* cells after different heat treatments and likewise did not find any differences with the non-heat treated cells, although, in this case, the reduced metabolic activity of dried cells might explain the absence of effects of the heat treatment [116]. To the best of our knowledge there is only one work in which the virulence of stable variants/clones of *Salmonella* cells has been examined and the results of this work indicated that the heat resistant strain displayed a lower pathogenicity after intraperitoneal injections into 20–22 g mice [229].

To sum up, and although the relationship between the heat shock response and virulence in *Salmonella* seems to clearly exist, further work is still required to explain certain (at least apparently) contradictory findings, such as the diverging results obtained regarding expression of SPI-1 genes upon exposure to sublethal heat conditions or why the already demonstrated overexpression of virulence related genes after a heat shock is not directly translated into an increased invasivity or virulence in animal models.

On the other hand, the development of heat resistance would probably have a negative impact on *Salmonella* growth fitness, always considering non stressing conditions. This assumption is based on the fact that, in *Salmonella*'s close relative *E. coli*, overexpression of GroEL/GroES is required in order to enable growth at high temperatures (48.5 °C) [230]. It has nevertheless also been demonstrated that overexpressing GroES imposes a high fitness cost [231]. Similarly, Ezemaduka et al. (2014) observed that expression of a protein from *Caenorhabditis elegans* allowed *E. coli* cells to grow under temperatures up to 50 ºC [232]. Authors have indicated that this protein would help to maintain cell envelope integrity. However, an excessive rigidification of the membrane would also have a negative impact on growth rates unless other compensatory mechanisms were activated [233].

3.2.9. Non-Thermal Technologies

Given the limitations of heat treatments for the preservation of food quality while ensuring food safety, a considerable number of so-called "Non-thermal technologies" have been investigated as alternatives to thermal treatments, with the objective of meeting the required safety or shelf-life demands while minimizing eventual effects on nutritional and quality attributes. However, the amount of information regarding the impact of novel non-thermal technologies on *Salmonella* virulence and growth fitness is quite scarce. This is not surprising, since even the amount of studies that deal with the development of microbial homologous adaptation to technologies such as Cold Atmospheric Plasma, Pulsed Electric Fields, Ultrasound, High Hydrostatic Pressure, and UV light is very limited and, to the best of our knowledge, nonexistent for *Salmonella* except for studies by Maâlej et al. (2014) and Timmons (2018) [234,235].

Some works dealing with the development of resistance to ionizing radiation in *Salmonella* cultures and its impact on other aspects of its physiology were carried out in the late 1960s and early 1970s. Most of the data obtained, included in Table 1, indicate that the increase in resistance to ionizing radiation acquired did not result in major changes in either the growth fitness or the pathogenicity of *Salmonella cells* [229,236–239], although in

some works a decreased growth ability was found in minimal medium [236] or at relatively low temperatures [237,238]. Also a decrease in pathogenicity *S*. Typhimurium cells with repeated exposure to ionizing radiation was observed by Previte et al. (1971) [239].

Regarding resistance to UV light, Maalej, et al. (2014) observed that pre-adaptation of three different *Salmonella* strains led to an increase in the proportion of CFA in their membranes [234], thus consistent with the results obtained by Gayán et al. (2013), who suggested that membrane fluidization would lead to an increased sensitivity to UV in *E. coli* [240]. As described for acid stress, membrane rigidification might impose a fitness cost unless compensatory changes were set up. Since *rpoS* as well as many genes related to the oxidative stress response have been shown to be related to *Salmonella* UV resistance [241], it is plausible that the development of UV resistance might have the same effects, at least to some extent, on virulence and growth fitness as those discussed above. This nevertheless remains to be demonstrated.

Information regarding the impact of the development of resistance to Pulsed Electric Fields (PEF) on *Salmonella* virulence and growth fitness is likewise scarce, but data obtained to date seem to indicate that development of resistance to PEF after several cycles of treatment and growth of survivors would not result in decreased growth fitness [242]. On the other hand, the results obtained by Sanz-Puig and coworkers (2019) indicate that the development of PEF resistance would result in a decreased virulence of *Salmonella* cells in a *Caenorhabditis elegans* model [243,244]. It should also be noted that a potential link between RpoS activity and PEF resistance was suggested by Sagarzazu and coworkers, which would imply that, although still not demonstrated, an increase in PEF resistance might indeed have a fitness cost and imply changes in *Salmonella* virulence [242]. Sanz-Puig et al. (2019) also studied the impact of the development of HHP resistance after repeated exposure to sublethal HHP treatments on *Salmonella* virulence, and observed, as described above for PEF, that this stress resistance response also led to a decrease in virulence in *Caenorhabditis elegans* [244].

Finally, results obtained by Timmons (2018) indicate that exposure of *Salmonella* cells to Cold Atmospheric Plasma (CAP) might lead to the upregulation of SPI-2 genes, although results were inconclusive due to inconsistency between experimental replicates [235]. The same author also observed that even though *Salmonella* cells were not able to increase their resistance to CAP upon five successive treatments with that technology, transcriptomic analysis revealed an upregulation of SPI-2 genes [235].

3.3. Other Stress Responses

3.3.1. Viable but Non-Culturable (VBNC) and Persister Cells

Under some circumstances, exposure to stresses of different types can lead to the induction of the VBNC state in bacterial cells. This phenomenon has also been described for *Salmonella*: although cells maintain some metabolic activity, they can only be cultured (multiply) after a resuscitation step. The stressing conditions that have been shown to induce the VBNC state in *Salmonella* include UV light, osmotic stress, nutrient starvation, and heat, among others [245–247]. Recent results have demonstrated that VBNC cells can arise without the input of an obvious stress, similarly to persisters (see below) [248]. The pathogenicity of these cells is still regarded as controversial: this is of major relevance because it raises uncertainty regarding the health risk posed by such VBNC forms. Thus, although some studies observed that microbial cells can retain some pathogenic effects [249–251], most studies carried out with *Salmonella* cells have shown a simultaneous loss of culturability and pathogenicity [246,247]. Furthermore, it has been hypothesized that the pathogenicity observed in the former studies might be linked to the reversion of cells to a culturable state [252,253]. Further work will be required to fully elucidate this point: in any case, however, it seems clear that the pathogenicity of these cells would be lower than that of the culturable ones, indicating that the major risk associated with VBNC cells would be the difficulty of their detection. Therefore, the existence of this phenomenon (the

VBNC state) really calls into question the suitability of traditional microbiological techniques for enumerating pathogenic microorganisms in foods, along with the validity of risk assessments performed on the basis of these data. On the other hand, one of the major characteristics of VBNC cells is that they require a resuscitation step before they are able to multiply. Thus, although once resuscitated they would display a growth rate similar to non-VBNC cells, the resuscitation step would, in many cases, hamper their multiplication in food products or, at least, delay it significantly.

During growth, genetically clonal bacterial populations contain a small fraction of non-growing, non-dividing cells that arise from transient, reversible phenotype switching. These growth-arrested cells are usually tolerant to antibiotics and are called (antibiotic) persisters [254]. However, it is now known that cells can be induced to become persisters through exposure to stressful conditions, similarly to the VBNC state [254]. The distinction between persisters and VBNC is quite complex, and nowadays it is considered that both survival strategies would represent a continuum between actively growing and dead cells, with VBNC cells being in a deeper state of dormancy than persister cells. Moreover, both phenomena can coexist within the same bacterial population. For a detailed review of the similarities and differences between them, we recommend Ayrapetyan et al. (2018) [248]. Persister cells are thought to be ubiquitous among bacterial species, and have been already described in *S.* Typhimurium [254–256]. It is very important to point out that, in contrast to resistant cells, persisters are genetically identical to susceptible bacteria, thereby constituting phenotypic variants of the wild type. They can be formed in different ways, but, upon removal of the stressing agent, they switch back to a normally growing state [257]. To date, the study of this phenomenon has been almost exclusively restricted to the field of clinical microbiology; however, antimicrobial agents commonly used in the food industry, such as nisin, can also induce their formation [258]. It is thus plausible that these cells could also appear in response to other agents. Further work will be required to determine the relevance of this phenomenon from a food safety perspective (apart from resistance to antibiotics); as pointed out above, although persisters are non-growing cells, this is a transitory state with, predictably, no fitness cost apart from the time required to exit from dormancy, which, moreover, is not as deep as that of VBNC cells. Finally, it should be noted that some microbial persisters (*Porphyromonas gingivalis*) would apparently maintain their ability to invade epithelial cells [259].

3.3.2. Biofilms

Biofilms are defined as a mode of growth where cells aggregate and become embedded in a self-produced extracellular matrix, usually in contact with a physical surface. The exact reasons why bacteria aggregate together are not fully understood, but it has been clearly demonstrated that bacteria in the biofilm are resistant to disinfectants as well as to chemical, physical, and mechanical stresses [260–262]. The major constituents of *Salmonella* biofilms and the complex network regulating their formation have been described elsewhere [263–265]. Although it has been proposed that aggregation could provide *Salmonella* with a mechanism for surviving the harsh conditions of the host intestinal tract to ensure that a "viable and sufficient" inoculum could reach the epithelial layer and, therefore, a lower number of biofilm cells would be required to cause disease, it has been demonstrated that biofilm cells and/or the presence of extracellular matrix factors reduce *Salmonella* virulence in the mouse model of infection [266–268]. In line with these results, Mackenzie et al. (2015) observed that expression of SPI-1 was lower in biofilm than in planktonic *Salmonella* cells, although a direct link between the biofilm regulator CsgD and SPI-1 expression has not yet been established [267]. On the other hand, it should be noted that curli and cellulose (components of the *Salmonella* biofilm extracellular matrix) may also play a highly relevant role in host–pathogen interactions [269]. However, deletion of *csgBA* (formerly *agfBA*), encoding the main curli subunit proteins, caused no noticeable impairment of *Salmonella* virulence [270], and various studies seem to indicate that cellu-

lose would hamper *Salmonella* virulence (summarized in Mackenzie et al. 2019) [269]. Several questions nevertheless remain to be solved in order to elucidate the precise role of curli and cellulose in *Salmonella* pathogenesis and the relevance of biofilm formation in this phenomenon.

Some studies have compared the physiological status of biofilm and planktonic cells by determining their growth rates, with contradictory results. Thus, some investigators [271,272] have reported increased biofilm growth rates in comparison to planktonic growth rates, while others [273] have reported the opposite. Further research is still required in order to clarify this point [274,275].

4. Impact of Stress Resistance Responses on Other Aspects of Salmonella Physiology

Although, as pointed out above, flagella facilitate adherence to surfaces including the host epithelium, their major function is to enable bacteria such as *Salmonella* to swim through liquid environments and on surfaces, thereby enabling them to chemotactically swim towards nutrients or away from harmful substances [276]. Although it can be expected that their synthesis might be somehow related to the stress resistance responses, the relationship between them is not fully understood in *Salmonella* cells. Thus, whereas in *E. coli* K12 it is well established that RpoS downregulates the expression of flagella, results from Lévi-Meyrueis et al. (2014) indicate that the flagellin genes *fliC*, and to a lesser extent *fljB*, were positively controlled by RpoS, resulting in a decreased motility in a rpoS deletion mutant, even though transcription of the *flhDC* genes encoding the master regulator of flagellar synthesis were slightly upregulated in a *Salmonella rpoS* strain [42]. These authors nevertheless also indicated that, given the complexity of regulatory controls affecting motility, it cannot be determined whether the positive regulation of *fliC* accounts for the effect of RpoS on motility, or if RpoS would be acting by other means as well. On the other hand, RpoE seems to be involved in the downregulation of *Salmonella* motility, and it has been suggested that this downregulation of flagellar synthesis might be helpful in host immune evasion, thus increasing bacterial fitness during infection [277]. Likewise, Ryan et al. (2015) observed that genes belonging to the flagellar assembly and chemotaxis modules, as well as FliA, were highly downregulated under the acid tolerance response of *S*. Typhimurium [99]: the results obtained by Sirsat et al. (2011 and 2015) indicate that heat shock would cause a similar effect. By contrast, exposure to a_w 0.11 resulted in an up to threefold increase in the expression of certain genes involved in the biosynthesis of flagella in *S.* Typhimurium cells [278] and the results of Walker and co-workers [279] indicated that particularly surface growth at low pH values, would induce a "hyper-flagellate" phenotype.

Quorum Sensing (QS) regulates numerous important cell functions in both Gram-positive and Gram-negative bacteria, including metabolism, protein synthesis, expression of virulence factors, antibiotic resistance, biofilm formation, biofilm maintenance and dispersal, and entry to stationary phase. It has long been established that in *Salmonella* the production autoinducer 2 (AI-2) (one of the two major bacterial QS systems) is induced by a series of stimuli such as low pH and high osmolarity [280,281]. In addition, a very interesting phenomenon is the ability of CAP to directly disrupt quorum sensing molecules utilized by Gram-negative bacteria (acylhomoserine lactones) [282], which would result in a decreased virulence of quorum-sensing-controlled virulence factors as described for Pseudomonas by Ziuzina et al. (2015) [283].

Finally, the potential impact of the development of stress resistance response to agents and/or technologies commonly used/encountered in the food chain on antimicrobial resistance remains largely unexplored. Given the fact that, in many cases, they share resistance mechanisms (e.g., membrane stability/permeability) it can be expected that a potential link between them can exist. This was already demonstrated by McMahon et al. (2007), who observed that incubation at sublethal high temperature (45 °C) decreased *Salmonella* antibioresistance but by contrast, osmotic (>4.5 NaCl %) and acid (<5.0) shocks resulted in an increase in *Salmonella* resistance to certain antimicrobials [16]. Similarly,

Table 1. Examples of stable *Salmonella* variants obtained after successive exposure to different selecting agents: impact on virulence, growth fitness and other phenotypical characteristics.

Selection Agent	Strain	Effect in Virulence	Effect in Growth Fitness	Other Characteristics	References
		Acid Stress			
pH 2.5	*S.* Enteritidis 66045	Lower colonization of spleens and livers	Reduced growth rate and yields	-	[105]
pH 2.5	*S.* Typhimurium 30	Lower virulence	Reduced growth rate and yields	Increased heat resistance	[105]
		Osmotic stress			
NaCl	*S.* Typhimurium NCCP10812	No changes in invasion	Not determined	Decreased atb resistance	[117]
NaCl	*S.* Enteritidis NCCP12243	Increased invasion	Not determined	Antibiotic susceptibility	[117]
		Oxidative stress, detergents and disinfectants			
Blend of oxidizing compounds	*S.* Typhimurium SL1344	Decreased invasion	Reduced growth rate and yields	Decreased atb resistance Reduced motility	[136,137]
QA + FA + GA	*S.* Typhimurium SL1344	Decreased invasion	Reduced growth rate and yields	Decreased atb resistance Reduced motility	[136,137]
Phenolic tar acids-based disinfectant	*S.* Typhimurium SL1344	Decreased invasion	Reduced growth rate and yields	Decreased atb resistance Reduced motility	[136,137]
DTAC	*S.* Enteritidis ATCC 4931	Decreased invasion	Not determined	Fewer fimbriae	[185]
		Heat stress			
55 °C	*S.* Typhimurium phage type l	Decreased virulence	Not determined	Increased roughness	[229]
		Non-Thermal Technologies			
γ- radiation	*S.* Typhimurium phage type 2c	No change	Not determined	Increased roughness	[229]
γ- radiation	*S.* Typhimurium LT2	No change	Grows poorly in minimal media	Increased cell size	[236]
Ionizing radiation	*S.* Typhimurium ATCC 7823	Not determined	No change	-	[237,238]
Ionizing radiation	*S.* Newport ATCC 6962	Not determined	Reduced growth rate at 10–20 °C.	-	[237,238]
Ionizing radiation	*S.* Thompson ATCC 8391	Not determined	No change	-	[237,238]
Ionizing radiation	*S.* Heidelberg ATCC 8326	Not determined	No change	-	[237,238]
Ionizing radiation	*S.* Typhimurium strain RIA	Decreased virulence	Not determined	-	[239]
UV-C	*S.* Typhimurium (various strains)	Not determined	Not determined	Increased atb resistance	[284]
PEF	*S.* Typhimurium SL1344	Not determined	Not determined	-	[242]
PEF	*S.* Typhimurium CECT 443	Less virulent in *C. elegans*	Not determined	-	[243,244]
HHP	*S.* Typhimurium CECT 443	Less virulent in*C. elegans*	Not determined	-	[243,244]

Álvarez-Molina et al. (2020) reported that repeated exposure of different microorganisms (including *Salmonella*) to UV-C and CAP led to an increase in resistance to certain antimicrobials, a phenomenon that was linked, depending on the case, to changes in antibiotic cellular targets, to membrane transporters probably involved in the nonselective efflux of antibiotics and, very interestingly, to stress response regulators (Table 1) [284].

5. Variability among Salmonella Strains and Serovars

The geno- and pheno-typic diversity of the *Salmonella* genus is not only a well-known fact but also one of its more characteristic features [285]. Many works have studied in different depths the variability in stress resistance among *Salmonella* strains and/or serovars [286–295]. Nevertheless, the information regarding the ability of the different strains/serovars to develop stress resistance responses (either transient or permanent) is much more scarce. In any case, if as described above, the development of stress resistance can have a cost in terms of growth fitness and/or virulence, it would be reasonable to think that strains and/or serovars that had evolutionarily acquired resistance to particular stresses or conditions, will also probably have lower growth fitness and that, at least, will display an altered virulence ability. Similarly, other phenotypical aspects might also be affected. This is of the highest interest since this phenomenon might also help to understand why, for instance, some serovars that are frequently isolated in chickens, such as *S*. Mbandaka or *S*. Livingstone [8] have such a low incidence in humans, despite not being poultry specific.

Unfortunately, not many works have been carried out in order to try to validate, at the species or subspecies level, if the development of increased stress resistance has a fitness and virulence cost for *Salmonella* [296,297]; further work is still required in order to corroborate it. In this sense, the results of Shah (2013), in spite of being obtained only with *S*. Enteritidis strains, are particularly interesting [298]. Thus, result obtained by this author indicate that whereas naturally virulent strains of this serovar (designated as high-pathogenicity or HP strains) were also those displaying a higher oxidative and osmotic stress resistance, low pathogenicity (LP) strains showed increased expression of the *tdc*, *gar*, and *gud* operons suggesting that the primary focus of these later strains could be survival and cell growth through enhanced nutrient acquisition rather than invasion and proliferation.

6. Concluding Remarks

From all that has been discussed in this review, one can conclude that, in most cases, the development of stress resistance responses imposes a fitness cost to *Salmonella* cells. By contrast, the impact of *Salmonella* stress resistance responses on the expression of virulence factors varies widely depending on the stressing agent and the virulence factor studied. In this sense, as discussed above, it should be noted that *Salmonella* possesses a plethora of virulence factors with a particularly tight and complex regulation. Furthermore, each one of these virulence factors plays a very well-defined role in *Salmonella* pathogenesis; since each one is only useful in a particular step during the infection process, virulence factors are induced or repressed sequentially during infection. Thus, for instance, increasing the expression of SPI-2 genes could be regarded as a phenomenon leading to an increase in *Salmonella* pathogenicity. Nevertheless, since the virulence factors encoded in SPI-2 will not help these bacteria to adhere to and invade the epithelial cells of the gut, they will probably not increase the chance of *Salmonella* cells to cause disease in a real scenario. What is more, they may even be counterproductive.

At this point it is important to remark that the impact of transient stress resistance responses to agents or technologies encountered in food and food processing environments on the expression of *Salmonella* virulence factors is normally not as relevant as the development of permanent responses since, once inside the host, *Salmonella* cells will have to sequentially cope with a number of different stresses that will trigger new adaptive responses and will re-define the virulence factors they express. In this sense, the transient stress responses that would probably exert a greater influence on *Salmonella* virulence are those affecting the bacterium's resistance to the acid pH of the stomach, or those limiting the capability of *Salmonella* cells to adapt to other stresses that *Salmonella* might face within the host, such as bile salts or Fe starvation.

Having said this, studying the effect of transient stress responses on *Salmonella* virulence is still of the highest relevance for various reasons. First of all, it is important from a

clinical point of view because *Salmonella* does indeed develop various transient stress resistance responses within the host that have been shown to be highly relevant for its pathogenesis. Secondly, these studies provide interesting and useful clues regarding the connections between different *Salmonella* regulation networks. Finally, they provide us with an idea of how cells that have developed permanent stress responses tend to behave.

From all the data presented above it can be concluded that stable variants/mutants with increased stress resistance display, in most cases, a decreased fitness cost and a lower virulence. It should be noted, however, that deletion mutants in certain genes involved in the development of stress resistance have been proven to be avirulent. This, in turn, reinforces the view that the risk of suffering salmonellosis would not only depend on the virulence of *Salmonella* cells (the amount of virulence factors expressed) but also on their ability to resist certain stresses once inside the host, as well as on the number of cells ingested (which, in turn, depends on the ability of *Salmonella* cells to grow and/or resist stresses in food). Therefore, the question whether the development of a stress resistance response would lead to an increase or decrease in the risk of suffering salmonellosis would not only be defined by the stress resistance response's impact on the expression of virulence factors, but also on how it would impact the resistance to the stress and growth fitness. Depending on the level of induction of a stress response pathway, it is at least theoretically possible that the outcome might be completely different, ranging from no change to either an increase or a decrease in the probability (risk) of causing illness.

In addition, it should be noted that the different stress resistance (or ability to develop it) among *Salmonella* strains and/or serovars, and its impact on other aspects of *Salmonella* physiology, might help to explain why less than 20 serovars are responsible of more than 80% of all the cases reported, why some *S*. Enteritidis strains are more pathogenic that others or why some non-host-specific strains that are frequently isolated from animals and/or food products have a very low incidence of disease in humans.

In view of this, it is clear that more in-depth studies specifically tackling the impact of microbial stress resistance responses on other aspects of microbial physiology—especially, but not exclusively, growth fitness and virulence – are still required. Further studies on the pathogenicity of VBNC/persisters and biofilm cells should also be carried out. These studies will not only contribute to a more detailed knowledge of *Salmonella* physiology but may also improve our understanding of this genera's ecology, while helping to improve current food preservation processes and risk assessment models.

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Objetivos

Como se ha descrito en la introducción son todavía muchos los aspectos que se desconocen acerca de la fisiología de *Salmonella* lo que, probablemente, es una de las principales causas de que a pesar de los enormes esfuerzos realizados por la administración y la industria agroalimentaria la salmonelosis siga siendo un problema sanitario de primera magnitud. Por ello, se definió como objetivo general de esta Tesis Doctoral el siguiente:

Estudiar y cuantificar la variabilidad intraespecífica en resistencia al estrés, capacidad de crecimiento y virulencia dentro del género *Salmonella* y determinar las relaciones existentes entre estas tres características fenotípicas con objeto de profundizar en el conocimiento de la fisiología de este microorganismo y de contribuir al desarrollo de modelos cuantitativos de evaluación del riesgo más precisos.

Asimismo, esta Tesis Doctoral plantea las siguientes hipótesis de partida y que pretende verificar:

- Que el desarrollo de respuestas de resistencia al estrés conlleva, o puede hacerlo, un coste en términos de capacidad de crecimiento o virulencia.

- Que las diferencias en resistencia al estrés y capacidad de crecimiento entre las diferentes serovariedades de *Salmonella* podrían explicar, o contribuir a explicar, la diferente frecuencia con la que dichas serovariedades causan gastroenteritis en humanos.

Para alcanzar dicho objetivo y verificar estas hipótesis se plantearon los siguientes objetivos parciales:

1) Caracterizar la resistencia frente a diferentes estreses (incluyendo nuevas tecnologías de conservación de los alimentos y estreses ambientales) de diferentes cepas y serovariedades de *Salmonella enterica*.

2) Caracterizar la capacidad de crecimiento y la virulencia (capacidad de adhesión e invasión de cultivos celulares) de diferentes cepas y serovariedades de *Salmonella enterica*

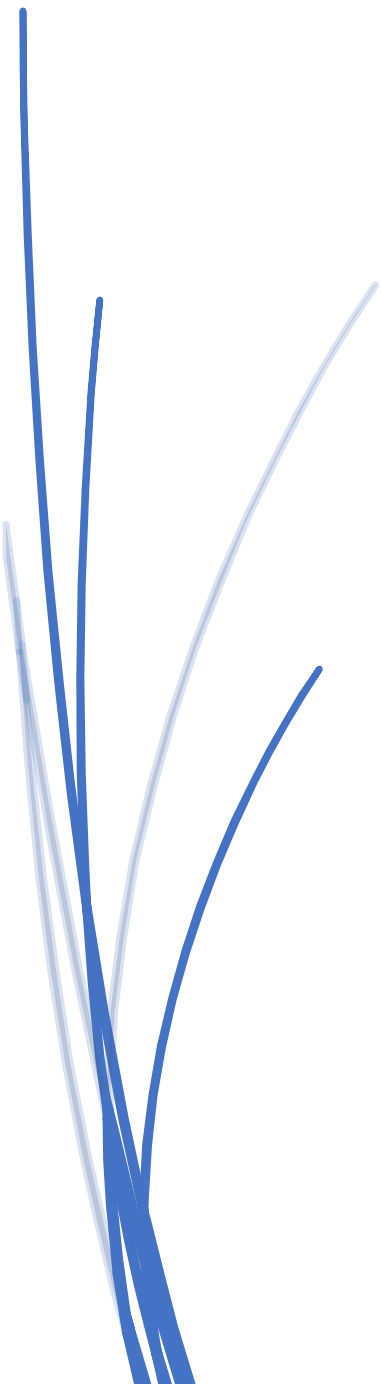
3) Estudiar la relación existente entre la resistencia al estrés, la capacidad de crecimiento, la virulencia y otros aspectos fenotípicos de las diferentes cepas y serovariedades de *Salmonella enterica*.

4) Validar en condiciones/matrices reales (huevos y ovoproductos) los resultados más relevantes.

5) Explorar los mecanismos moleculares responsables de las diferencias fenotípicas observadas.

6) Sentar las bases para la optimización de la evaluación cuantitativa del riesgo que representa el consumo de un grupo de productos en el que *Salmonella* es el patógeno de referencia: huevos y ovoproductos.

Resultados



A continuación, se muestran los resultados obtenidos en el marco de esta Tesis Doctoral. Dichos resultados se podrían clasificar en dos grandes bloques y al menos cinco capítulos.

El **primer bloque** corresponde a todos aquellos estudios llevados a cabo en medios de laboratorio y cuyo objetivo es el estudio de la variabilidad en la resistencia al estrés, la capacidad de crecimiento y la virulencia dentro del género *Salmonella*, la relación entre estas características fenotípicas y los mecanismos responsables de las diferencias en resistencia al estrés. En él se incluyen 4 artículos distribuidos en 3 capítulos:

Capítulo 1.- Estudio de la variabilidad en la resistencia frente a diferentes estreses y tecnologías de conservación dentro del género *Salmonella*.

Como su nombre indica, en este bloque se aborda la caracterización de la resistencia al estrés de las diferentes cepas de *Salmonella*. Incluye un artículo: "Differences in resistance to different environmental stresses and non-thermal food preservation technologies among *Salmonella enterica* subsp. *enterica* strains, 2019" que cubre el estudio de 15 cepas y se vería completado por el artículo "Stress resistance of emerging poultry-associated *Salmonella*

serovars, 2020" en el que se incluye también el estudio de otras 8 cepas y la validación de los resultados en huevos y ovoproductos, motivo por el que se ha incluido dentro del capítulo 5.

Capítulo 2.- Estudio de la variabilidad en la capacidad de crecimiento y la virulencia dentro del género *Salmonella:* relación con la resistencia al estrés.

En él se incluye el artículo "Relationship between growth ability, virulence, and resistance to food-processing related stresses in non-typhoidal *Salmonellae*, 2022" en el que no sólo se aborda el estudio de la variabilidad en la capacidad de crecimiento, la virulencia y otros aspectos fenotípicos dentro del género *Salmonella* sino que además se trata de dar respuesta, al comparar estos datos con los de resistencia al estrés a una de las hipótesis que pretendían verificar: "que el desarrollo de respuestas de resistencia al estrés conlleva, o puede hacerlo, un coste en términos de capacidad de crecimiento o virulencia".

Capítulo 3.- Exploración de los mecanismos de resistencia al estrés dentro del género *Salmonella.*

En este tercer capítulo se incluyen dos artículos (todavía sin enviar a publicar) en los que a través del uso de variantes resistentes a diferentes estreses y obtenidas mediante la exposición sucesiva a ciclos de inactivación-crecimiento se pretende explorar cuáles pueden ser los mecanismos responsables de las diferencias en resistencia observadas entre las diferentes cepas/serovariedades de *Salmonella* estudiadas.

En el **segundo bloque** se incluyen una serie de estudios diseñados con el objetivo de validar los resultados que se consideraron más relevantes de entre los obtenidos en medios de laboratorio (bloque I) en huevo y ovoproductos. Estos a su vez se han dividido en dos capítulos:

Capítulo 4.- Estudio de la influencia de la dosis y la historia térmica del huevo en la velocidad de crecimiento de *Salmonella* Enteritidis.

En los primeros ensayos de validación de los resultados obtenidos en medios de laboratorio en huevos y ovoproductos se observó un fenómeno que no había sido descrito con anterioridad y extremadamente curioso que era que la dosis inicial de inóculo parecía afectar a la velocidad de crecimiento microbiana, un fenómeno que además aparecía o no en función

de la fracción de huevo estudiada y su historia térmica. Así, pues este capítulo se dedica a describir dicho fenómeno ("Influence of the initial cell number on the growth fitness of *Salmonella* Enteritidis in raw and pasteurized liquid whole egg, egg white, and egg yolk, 2021"), explorar los mecanismos responsables del mismo ("Relationship between iron bioavailability and *Salmonella* fitness in raw and pasteurized liquid whole egg, enviado a Food Microbiology") y a, mediante un enfoque probabilístico, redefinir las temperaturas mínimas de crecimiento de *S*. Enteritidis en huevo y ovoproductos no sólo en función de la cepa y fracción investigada sino también de la dosis inicial y la historia térmica del huevo ("Modelling the low temperature growth boundaries of *Salmonella* Enteritidis in raw and pasteurized egg yolk, egg white and liquid whole egg: influence of the initial dose, sin enviar").

Capítulo 5.- Exploración del riesgo asociado a serovariedades emergentes en el sector aviar tales como *S.* Heidelberg, *S.* Kentucky, *S.* Livingstone y *S.* Mbandaka.

Este último capítulo consta de un artículo ("Stress resistance of emerging poultry-associated *Salmonella* serovars, 2020") en el que además de caracterizar la resistencia al estrés de 4 cepas de *S*. Enteritidis y 4 de las serovariedades emergentes arriba mencionadas en medios de laboratorio también se estudia la misma en huevos y ovoproductos, con objeto de verificar si las conclusiones extraídas en los primeros son extrapolables en los segundos. Además, en la segunda parte de este capítulo se muestra, a modo de ejemplo, el potencial que los resultados obtenidos en esta Tesis Doctoral y de la aplicación de nuevos enfoques probabilísticos podrían tener para la estimación del riesgo y la toma de decisiones en la industria agroalimentaria y en el sector de las aves de puesta/huevos y ovoproductos en particular.

Adicionalmente, al final de esta Tesis Doctoral se han incluido dos anexos, cuyo contenido se detalla a continuación:

Anexo I.- Estudio comparativo de la capacidad de adhesión e invasión de diferentes cepas de *Salmonella* en diferentes modelos *in vitro*.

En este anexo se incluye un estudio previo para la selección del modelo *in vitro* para estudiar la capacidad de adhesión e invasión de las diferentes cepas/serovariedades de *Salmonella*. En

él se testaron diferentes tipos de células (Caco-2 y HT29-MTX) y el efecto de la adición de una microbiota sintética (viva o inactivada).

Anexo II.- Development of a predictive growth model of S*almonella* Enteritidis in whole liquid egg using multilevel modelling: a preliminary step.

En este anexo se muestran los resultados del estudio realizado durante la estancia de la doctoranda en la Universidad de Wageningen (Países Bajos). El mismo persigue el desarrollo de un modelo multinivel el cual integrará la incertidumbre del ensayo y la variabilidad entre réplicas y entre cepas y que permitirá determinar con mayor precisión el crecimiento de *S*. Enteritidis en huevo y ovoproductos en función de la cepa, la temperatura de almacenamiento, la historia térmica del huevo y el número inicial con la que el producto resulte contaminado. Estos resultados no se han presentado en forma de manuscrito dentro de la memoria ya que aún se está trabajando en ellos, aunque constituyen parte de la misma, pudiéndose considerar una parte del capítulo 4.

Por último, señalar que dado que en cada uno de los artículos incluidos dentro de estos bloques y/o capítulos, se ha incluido la metodología empleada se ha omitido su presentación de forma independiente dentro de esta Tesis Doctoral.

Capítulo 1

Estudio de la variabilidad en la resistencia frente a diferentes estreses y tecnologías de conservación dentro del género *Salmonella*

Manuscrito II/Manuscript II

Differences in resistance to different environmental stresses and non-thermal food preservation technologies among *Salmonella enterica* subsp. *enterica* strains

Silvia Guillén, María Marcén, Pilar Mañas and Guillermo Cebrián

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Differences in resistance to different environmental stresses and non-thermal food preservation technologies among *Salmonella enterica* subsp. *enterica* strains

Silvia Guillén, María Marcén, Pilar Mañas, Guillermo Cebrián*

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón – IA2 – (Universidad de Zaragoza-CITA), Zaragoza, Spain

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ABSTRACT

In this work the resistance of 15 strains belonging to 11 serovars of *Salmonella enterica* subsp. *enterica* to several different environmental stresses (acid, hydrogen peroxide, NaCl and heat) and non-thermal food preservation technologies (HHP, PEF, UV) was determined and compared. Results obtained showed that differences in resistance among strains, quantified as *2D*-values, varied less than 2.4-fold for all agents, including heat if *S.* senftenberg 775W is excluded from the analysis. These results also indicate that variability in resistance among strains of the same serovar was comparable to inter-serovar variability. *Salmonella* strains that were the most resistant to a given stress were not more resistant to other types of stress. Nevertheless, a positive correlation was observed between the resistance of *Salmonella* strains to oxidative and osmotic stress, as well as between UV and PEF resistance. These results would be especially helpful in defining safe food preservation processes and might be very useful for improving quantitative microbiological risk assessments of *Salmonella* in food products.

1. Introduction

The relevance of *Salmonella* as an agent responsible for food-borne toxiinfections is well known. Currently, the microorganisms of the genus *Salmonella* constitute the second most frequent cause of food-borne disease in Europe and the United States (European Food Safety Authority (EFSA), 2018; Scallan et al., 2011), only surpassed by *Campylobacter*. The main reservoir of *Salmonella* is the intestinal tract of animals; this microorganism can thus contaminate food products of animal and plant origin, directly or indirectly. Food products most frequently identified as responsible for foodborne *Salmonella* infections in the European Union in 2017 were eggs and egg products (36.8% of outbreaks), bakery products (16.7%), and meat and meat products (8.2%). However, the range of products that can vehicle *Salmonella* is much broader, including other products of animal origin, vegetables, crustaceans, or milk (EFSA, 2018).

The microorganisms of the genus *Salmonell*a have evolved to survive in naturally stressful conditions such as high osmolarity, extreme temperatures, and low pHs (Fang, Frawley, Tapscott, & Vazquez-Torres, 2016; Spector & Kenyon, 2012). However, inherent genetic differences among serovars and/or strains can lead to substantial changes in their stress tolerance. Whereas the stress resistance of *S.* Enteritidis and *S.* Typhimurium – the most common serovars associated with human infection worldwide – has been studied in detail, much less information is available regarding most of the other 2500 existing *Salmonella* serovars (Grimont & Weill, 2007).

Previous studies dealing with variability in resistance within the *Salmonella* genus have often been limited, either because they included a low number of serovars/strains or because they only dealt with a small number of stressing agents and/or food preservation technologies (Doyle & Mazzotta, 2000; Gayán, Serrano, Raso, Álvarez, & Condón, 2012; Lianou & Koutsoumanis, 2013; Saldaña et al., 2009; Sherry, Patterson, & Madden, 2004). In addition, since experimental conditions (culture conditions, strains, etc.) were not the same in most cases, subsequent comparison becomes difficult and/or meaningless. The lack of studies dealing with the stress resistance and adaptive stress responses of *Salmonella* strains and serovars is particularly alarming because such studies are not only necessary to understand their physiology, but also to help designing more efficient inactivation processes and/or action plans throughout the food chain with the purpose of preventing the health risk they pose. Such studies would help to improve the accuracy of quantitative microbial risk assessments.

Thus, this study's aim was to determine and compare the resistance of 15 strains belonging to 11 serovars of *Salmonella enterica* subsp.

* Corresponding author.
E-mail address: guiceb@unizar.es (G. Cebrián).

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enterica to different environmental stresses and non-thermal food preservation technologies.

2. Material and methods

2.1. Bacterial strains

15 strains belonging to 11 serovars of *Salmonella enterica* subsp. *enterica* were selected to carry out this investigation: 5 of them corresponded to *S.* Typhimurium. The strains of *S.* Typhimurium (STCC 443, STCC 722, STCC 7162 and STCC 4594), *S.* Enteritidis STCC 4300, *S.* Derby STCC 4397, *S.* Infantis STCC 4373, *S.* Virchow STCC 4154, *S.* Gallinarum STCC 4883, *S.* Senftenberg 775W STCC 4565, *S.* Saintpaul STCC 4153, and *S.* Stanley STCC 4141 were supplied by the Spanish Type Culture Collection. The strains of *S.* Hadar NCTC 13033 and *S.* Newport NCTC 129 were supplied by Public Health England, and the strain of *S.* Typhimurium SL1344 was kindly provided by Tim Brocklehurst from the Institute of Food Research, Norwich. All strains were maintained frozen at −80 °C in cryovials for long-term preservation.

2.2. Growth conditions

Cultures were grown in 96 wells microtiter plates (Thermo Scientific, Roskilde, Denmark). They were prepared by inoculating 100 μl of tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (Oxoid; TSB-YE) with a single colony previously isolated on a plate of tryptone soy agar supplemented with 0.6% w/v yeast extract (Oxoid; TSA-YE). Microtiter plates were sealed with a polyester impermeable film (VWR International, Leuven, Belgium) and incubated overnight at 37 °C under static conditions. One (1) μl of these pre-cultures was inoculated into 100 μl of fresh TSB-YE and incubated for 24 h under the same conditions to obtain the stationary growth phase cultures that were used for stress resistance determinations.

2.3. Acid, hydrogen peroxide, and sodium chloride resistance determinations

The treatment medium for acid-resistance determinations was citrate-phosphate McIlvaine buffer adjusted to different pHs (2.0–3.0) (Dawson, Elliott, Elliott, & Jones, 1974). Hydrogen peroxide resistance was evaluated in 100 mM Tris–HCl buffer (pH 7.0) with hydrogen peroxide added at final concentrations of 10, 30, and 100 mM (Sigma, St Louis, USA). Resistance to osmotic medium was evaluated in TBS-YE supplemented with 25, 30, and 33% w/v of sodium chloride (VWR International; NaCl). In all cases, treatments were performed on microtiter plate, and cells were added to the treatment medium to an initial concentration of 10^7 cells/ml. After inoculation, the suspensions were incubated at a constant temperature of 25 °C throughout the treatment, except for the NaCl determinations, which were carried out at 37 °C due to the low lethality of this agent at room temperature (25 °C). After the selected contact time (up to 50 min, 100 min and 32 h for acid, hydrogen and sodium chloride determinations, respectively) 20 μl samples were withdrawn at preset intervals and transferred into 180 μl of buffered peptone water (Oxoid; BPW). Subsequent serial dilutions were prepared and pour-plated for survival counts as described below.

2.4. Heat treatments

Heat treatments were carried out in a specially designed resistometer (Condón, Arrizubieta, & Sala, 1993). Briefly, this instrument consists in a 400 mL vessel provided with an electrical heater for thermostation, an agitation device to ensure inoculum distribution and temperature homogeneity, and ports for the injection of microbial suspension and for the extraction of samples. Once treatment temperature had attained stability (55, 58, 61, or 64 ± 0.1 °C), 0.1 mL of the microbial cell suspension was injected into the main chamber containing the treatment media, tryptic soy broth. After inoculation, samples were collected at different heating times (up to 16 min) and immediately pour plated and incubated for survival counting.

2.5. High hydrostatic pressure (HHP) treatments

HHP treatments were carried out in a Stansted Fluid Power S-FL-085-09-W (Harlow, London, England) apparatus (Ramos, Chiquirrín, García, Condón, & Pérez, 2015). The pressure-transmitting fluid was a mixture of propylene glycol and distilled water (50/50, v/v). An automatic device was employed to set and/or record pressure and time during the pressurization cycle. Cell suspensions were diluted to a cell concentration of 10^7 cells/ml in citrate-phosphate McIlvaine buffer of pH 7.0, approximately. Samples were packed in plastic bags, which were sealed without headspace and introduced in the treatment chamber. Treatments were applied at 250, 300, and 350 MPa for different treatment times up to 30 min, and temperature never exceeded 40 °C.

2.6. Pulsed electric field (PEF) treatments

The PEF equipment used in this investigation was supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The equipment and treatment chamber have been previously described by Saldaña et al. (2009). Prior to PEF treatments, 0.1 mL of the microbial cell suspension were dissolved in citrate-phosphate McIlvaine buffer (pH 7.0 and 1 mS/cm of conductivity) at a concentration of approximately 10^7 cells/ml. Samples were placed with a sterile syringe in the treatment chamber, which had a gap of 0.25 cm. Treatments were based on square pulses with a width of 3 μs and a frequency of 1 Hz. Electric field strengths were set at 20, 25, and 30 kV/cm. Under these experimental conditions, the energy per pulse was 1.20, 1.88, and 2.70 kJ/kg. Treatments of up to 50 pulses (150 μs) were applied. Under these conditions, the final temperature of the treatment media was always below 35 °C.

2.7. Ultraviolet C light (UV-C) treatments

UV-C treatments were carried out in a microtiter plate under static conditions. Microtiter plates were coated with 0–2 layers of a microplate sealing film (BREATHseal, Greiner bio-one, Frickenhausen, Germany) and located at a distance of 17.50–24.50 cm from a 32W UV-C lamp (VL-208G, Vilber, Germany). Fluence was measured by means of a UVX radiometer (UVP, LLC, Upland, CA). Under these experimental conditions, fluences between 0.20 and 1.10 ± 0.2 mW/cm^2 were attained. The treatment medium was citrate-phosphate McIlvaine buffer of pH 7.0, and the initial concentration was of approximately 10^7 cells/ml. Treatment times of up to 180 s were applied and temperature never exceeded 30 °C.

2.8. Recovery after different treatments and survival counting

After treatments, samples were adequately diluted in Buffered Peptone Water (Oxoid; BPW) and plated in the recovery medium, TSA-YE. Plates were incubated for 24 h at 37 °C, after which the number of colony-forming units (CFU) per plate was counted.

2.9. Curve fitting and statistical analysis

Survival curves were obtained by plotting the logarithm of the survival fraction (Log_{10} N/N_0) versus treatment time (hours for NaCl determinations; minutes for acid, heat, HHP, and peroxide treatments; seconds for UV treatments and μs for PEF treatments). Since deviations from linearity were observed in survival curves to the majority of

Table 1
Resistance (K_{max}. S_l and N_{res}) and goodness of the fit (R^2, *RMSE*) parameters calculated after fitting the survival curves to the 7 agents investigated of the 15 *Salmonella* strains studied to the Geeraerd's model. Values presented correspond to the mean and SD of the means (in parentheses) of the resistance parameters and to the range of values obtained for the goodness of the fit values (all calculated from 3 independent replicates).

	pH					H_2O_2					NaCl					Heat				
	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*
***S.* TM SL1344**	0.217 (0.351)	4.940 (4.281)	-	0.94 - 0.99	0.053 - 0.478	0.126 (0.023)	15.68 (8.795)	-	0.99 - 1.00	0.099 - 0.178	0.523 (0.032)	-	4.28 (0.313)	0.97 - 0.99	0.106 - 0.160	2.823 (0.261)	0.536 (0.279)	-	0.95 - 0.99	0.120 - 0.580
***S.* TM 443**	0.300 (0.165)	8.662 (3.731)	-	0.99 - 1.00	0.011 - 0.087	0.143 (0.027)	21.50 (3.589)	-	0.98 - 0.99	0.000 - 0.233	0.553 (0.073)	-	3.53 (1.348)	0.92 - 0.98	0.194 - 0.296	1.540 (0.358)	0.391 (0.525)	-	0.97 - 0.98	0.149 - 0.250
***S.* TM 722**	0.430 (0.286)	8.633 (10.32)	-	0.97 - 0.99	0.067 - 0.912	0.126 (0.008)	20.92 (0.096)	-	0.99 - 1.00	0.000 - 0.152	0.659 (0.185)	-	2.46 (0.795)	0.98 -0.99	0.122 - 0.146	1.903 (0.272)	0.291 (0.329)	-	0.97 - 0.99	0.161 - 0.312
S. TM 7162	0.433 (0.246)	6.843 (9.650)	-	0.97 - 1.00	0.027 - 0.656	0.125 (0.014)	25.81 (5.560)	-	0.99 - 1.00	0.000 - 0.075	0.577 (0.139)	-	3.14 (0.989)	0.98 - 1.00	0.063 - 0.172	1.812 (0.094)	0.307 (0.457)	-	0.92 - 0.99	0.185 - 0.574
***S.* TM 4954**	0.260 (0.066)	9.339 (4.825)	-	0.99 - 1.00	0.086 - 0.204	0.159 (0.047)	18.14 (16.54)	-	0.92 - 1.00	0.000 - 0.510	0.585 (0.175)	-	3.15 (0.847)	0.97 - 1.00	0.084 - 0.171	1.507 (0.210)	0.094 (0.163)	-	0.96 - 0.98	0.191 - 0.338
***S.* Enteritidis**	0.283 (0.129)	3.339 (3.049)	-	0.93 - 1.00	0.133 - 0.632	0.212 (0.034)	21.74 (5.730)	-	0.99 - 1.00	0.000 - 0.257	0.881 (0.172)	-	2.32 (1.054)	0.96 - 1.00	0.069 - 0.367	1.893 (0.316)	0.028 (0.042)	-	0.97 - 0.99	0.146 - 0.377
***S.* Hadar**	0.186 (0.021)	9.583 (0.962)	-	0.98 - 1.00	0.017 - 0.106	0.184 (0.012)	27.73 (5.766)	-	0.97 - 0.99	0.462 - 0.107	0.748 (0.191)	-	2.82 (0.581)	0.97 - 1.00	0.085 - 0.325	1.883 (0.336)	0.438 (0.290)	-	0.99 - 1.00	0.142 - 0.229
***S.* Derby**	0.177 (0.047)	4.347 (7.529)	-	0.94 - 1.00	0.035 - 0.496	0.160 (0.060)	20.06 (13.43)	-	0.99 - 1.00	0.024 - 0.302	0.568 (0.085)	-	2.66 (0.622)	0.97 - 0.98	0.179 - 0.227	2.540 (0.540)	0.815 (0.363)	-	0.93 - 0.99	0.087 - 0.405
***S.* Infantis**	0.257 (0.051)	5.958 (5.025)	-	0.98 - 1.00	0.000 - 0.292	0.179 (0.029)	21.43 (7.432)	-	0.99 - 1.00	0.192 - 0.267	0.913 (0.320)	-	2.26 (0.446)	0.96 - 0.97	0.252 - 0.383	2.472 (0.234)	0.099 (0.171)	-	0.98 - 0.99	0.229 -0.328
***S.* Virchow**	0.250 (0.070)	4.865 (5.214)	-	0.99 - 1.00	0.067 - 0.319	0.143 (0.035)	15.88 (5.779)	-	0.98 - 0.99	0.084 - 0.348	0.647 (0.069)	-	2.13 (0.229)	0.98 - 0.99	0.140 - 0.206	2.043 (0.132)	0.155 (0.268)	-	0.96 - 0.99	0.128 - 0.557
***S.* Gallinarum**	0.153 (0.023)	1.927 (1.670)	-	0.96 - 0.99	0.078 - 0.303	0.159 (0.049)	24.21 (6.453)	-	0.99 - 1.00	0.071 - 0.228	0.633 (0.327)	-	2.12 (0.214)	0.96 -0.98	0.221 - 0.353	1.693 (0.434)	0.980 (0.638)	-	0.96 - 0.99	0.120 - 0.349
***S.* Senftenberg**	0.297 (0.203)	7.615 (6.951)	-	0.95 - 0.99	0.100 - 0.483	0.122 (0.028)	27.29 (1.240)	-	0.99 - 1.00	0.113 - 0.132	0.894 (0.133)	-	3.24 (0.971)	0.97 - 0.99	0.109 - 0.261	0.196 (0.015)	-	-	0.96 - 0.98	0.028 - 0.146
***S.* Saintpaul**	0.217 (0.006)	7.197 (2.871)	-	0.99 - 1.00	0.013 - 0.115	0.105 (0.007)	15.72 (0.914)	-	0.99 - 1.00	0.142 - 0.188	0.537 (0.094)	-	3.51 (0.389)	0.96 - 0.98	0.087 - 0.360	3.293 (1.104)	0.123 (0.213)	-	0.96 - 0.99	0.223 - 0.535
***S.* Stanley**	0.303 (0.145)	9.762 (3.241)	-	0.99 - 1.00	0.054 - 0.269	0.129 (0.038)	11.81 (0.415)	-	0.98 - 1.00	0.140 - 0.327	0.522 (0.021)	-	2.44 (0.760)	0.94 - 0.98	0.148 - 0.428	1.923 (0.174)	0.330 (0.572)	-	0.96 - 0.97	0.300 - 0.664
***S.* Newport**	0.200 (0.035)	0.136 (0.132)	-	0.99 - 1.00	0.078 - 0.280	0.112 (0.024)	8.489 (8.485)	-	0.96 - 0.99	0.185 - 0.470	0.819 (0.345)	-	2.50 (0.522)	0.97 - 0.99	0.071 - 0.467	2.483 (0.441)	0.480 (0.733)	-	0.93 - 0.99	0.219 - 0.591

	HHP					PEF					UV-C				
	K_{max} (min^{-1})	S_l (min)	N_{res}(CFU/ml)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*
***S.* TM SL1344**	0.027 (0.522)	-	-	0.94 - 0.98	0.177 - 0.316	0.102 (0.018)	-	4.356 (0.226)	0.99 - 1.00	0.031 - 0.251	0.070 (0.002)		-	0.95 - 0.96	0.398 - 0.413
***S.* TM 443**	1.379 (0.932)	2.139 (1.174)	-	0.97 - 0.99	0.113 - 0.253	0.109 (0.012)	-	4.271 (0.529)	0.99 - 1.00	0.076 - 0.128	0.074 (0.003)	5.084 (4.489)	-	0.99 - 1.00	0.011 - 0.035
***S.* TM 722**	0.913 (0.262)	1.502 (1.239)	-	0.94 - 0.99	0.004 - 0.009	0.137 (0.043)	-	4.367 (0.099)	0.99 - 1.00	0.055 - 0.270	0.078 (0.007)	-	-	0.98 - 1.00	0.081 - 0.210
S. TM 7162	0.922 (0.145)	1.304 (0.525)	-	0,92 - 0,99	0.013 - 0.134	0.106 (0.005)	-	4.483 (0.476)	0.99 - 1.00	0.040 - 0.073	0.074 (0.010)	2.677 (4.637)	-	0.98 - 0.99	0.145 - 0.170
***S.* TM 4954**	1.126 (0.674)	1.639 (1.147)	-	0.92 - 0.98	0.029 - 0.231	0.103 (0.019)	-	4.174 (0.346)	0.99 - 1.00	0.028 - 0.183	0.083 (0.022)	10.64 (9.256)	-	0.99 - 1.00	0.062 - 0.147
***S.* Enteritidis**	0.766 (0.233)	0.170 (0.294)	-	0.94 - 0.95	0.380 - 0.419	0.102 (0.018)	-	4.353 (0.204)	0.99 - 1.00	0.062 - 0.216	0.080 (0.006)	3.763 (6.518)	-	0.92 - 1.00	0.087 - 0.196
***S.* Hadar**	0.962 (0.407)	1.322 (1.258)	-	0.92 - 094	0.470 - 0.561	0.168 (0.059)	-	3.542 (0.700)	0.93 - 1.00	0.159 - 0.515	0.093 (0.004)	12.57 (14.46)	-	0.92 - 0.98	0.294 - 0.433

(*continued on next page*)

Table 1 (*continued*)

	HHP					PEF					UV-C				
	K_{max} (min^{-1})	S_l (min)	N_{res}(CFU/ml)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/ml)	R^2	*RMSE*
***S.* Derby**	0.887 (0.220)	1.316 (1.874)	-	0.95- 0.99	0.147 - 0.325	0.152 (0.035)	-	3.645 (0.392)	0.98 -1.00	0.058 - 0.455	0.071 (0.008)	-	-	0.92 - 0.92	0.623 - 0.723
***S.* Infantis**	1.146 (0.292)	1.555 (1.255)	-	0.95- 0.98	0.337 - 0.418	0.129 (0.039)	-	3.780 (0.379)	0.98 - 1.00	0.079 - 0.388	0.067 (0.012)	-	-	0.93 - 0.99	0.137 - 0.232
***S.* Virchow**	1.006 (0.424)	1.985 (2.780)	-	0.94 - 0,99	0.294 - 0.351	0.181 (0.027)	-	3.775 (0.278)	0.98 - 1.00	0.087 - 0.409	0.080 (0.014)	3.739 (5.287)	-	0.98 - 1.00	0.102 - 0.148
***S.* Gallinarum**	0.635 (0.133)	-	-	0.96 - 0.98	0.076 - 0.339	0.180 (0.067)	-	3.059 (0.696)	0.97 - 0.99	0.289 - 0.501	0.112 (0.026)	8.086 (2.254)	-	0.93 - 0.99	0.353 - 0.685
***S.* Senftenberg**	0.650 (0.160)	-	-	0.94 - 0.97	0.045 - 0.234	0.149 (0.052)	-	3.772 (0.428)	0.99 - 1.00	0.022 - 0.285	0.090 (0.007)	5.439 (4.841)	-	0.99 - 1.00	0.048 - 0.090
***S.* Saintpaul**	1.032 (0.698)	1.099 (1.011)	-	0.93 - 0.96	0.063 - 0.336	0.114 (0.014)	-	4.309 (0.202)	0.99 - 1.00	0.099 - 0.223	0.122 (0.048)	13.26 (11.49)	-	0.95 - 1.00	0.129 - 0.463
***S.* Stanley**	0.977 (0.362)	1.494 (1.357)	-	0.93 - 0.97	0.000 - 0.268	0.096 (0.006)	-	4.005 (0.620)	0.99 - 1.00	0.001 - 0.082	0.098 (0.057)	6.793 (11.77)	-	0.95 - 0.99	0.133 - 0.275
***S.* Newport**	0.680 (0.148)	0.748 (1.296)	-	0.93 - 0.99	0.068 - 0.446	0.178 (0.008)	-	4.223 (0.177)	0.95 - 0.99	0.216 - 0.535	0.102 (0.017)	3.463 (5.999)	-	0.96 - 0.98	0.392 - 0.496

*Values in parentheses represent the SD of the means.
** *S.* TM: *S.* Typhimurium

agents/technologies, GInaFiT, the Geeraerd inactivation model-fitting tool was used to fit survival curves and calculate resistance parameters (Geeraerd, Valdramidis, & Van Impe, 2005).

$$Log_{10}(N_t) = Log_{10}\left[(10^{Log_{10}(N_0)} - 10^{Log_{10}(N_{res})}).e^{-k_{max}t}. \left(\frac{e^{k_{max}.S_l}}{1 + (e^{k_{max}.S_l} - 1).e^{-k_{max}.t}}\right) + 10^{Log_{10}(N_{res})}\right] \quad (1)$$

In this equation, N_t represents the number of survivors, N_0 the initial count, and t the treatment time.

This model describes the survival curves by means of three parameters: shoulder length (S_l), defined as the time before exponential inactivation begins; inactivation rate (K_{max}), defined as the slope of the exponential portion of the survival curve; and N_{res} which describes residual population density (tail). Therefore, the traditional decimal reduction time value (D-value) can be calculated from the K_{max} parameter using Eq. (2).

$$D - \text{value} = 2.303/K_{max} \quad (2)$$

Standard deviations (SD), statistical significance of differences ($p < 0.05$), Iterative Grubbs' test (Alpha = 0.05), Pearson's correlation coefficient and statistical analysis (unpaired *t*-test -with and without Welch's correction- and one way ANOVA; $p < 0.05$) were calculated using GraphPad PRISM® statistical software (GraphPad Prism version 7.00 for Windows, GraphPad Software, San Diego, California, USA). Principal component analysis (PCA) was carried out using InfoStat statistical software (InfoStat version 2018, Córdoba, Argentina).

3. Results and discussion

In this study, the variability in resistance of 15 *Salmonella* strains belonging to 11 different serovars against seven different preservation technologies and environmental stresses was studied. The selected serovars included 9 out of the 20 the most common serotypes associated with human infection in Europe throughout the most recent years (EFSA, 2018). The other two serovars (*S.* Gallinarum and *S.* Senftenberg strain 775W) were chosen because of their well-known specific characteristics –avian host-specificity and high heat resistance, respectively- that have been described elsewhere (Eswarappa, Janice, Balasundaram, Dixit, & Chakravortty, 2009; Ng, Bayne, & Garibaldi, 1969). Five strains of *S.* Typhimurium were included in the study to enable comparison between intra-serovar and inter-serovar variability in stress resistance among salmonellae. Among all the strains, *S.* Typhimurium SL1344 was considered as the reference strain throughout the whole study, since it is a well characterized strain (Humphrey, Clark, Humphrey, & Jepson, 2011).

Given the considerable number of determinations to be obtained (more than 450 survival curves), it was decided to obtain the microbial suspensions and to carry out resistance assays to chemical agents in microtiter plates instead of conventional flasks or tubes, as described in the Materials and Methods section. A preliminary study indicated that both the growth kinetics and the resistance of *Salmonella* cells to all chemical agents herein evaluated were comparable for cells grown in microtiter plates and in conventional agitated flaks (data not shown). Once the methodology had been established, survival curves to the 7 agents under study were obtained. These survival curves (representing the Log_{10} of the survival fraction *vs* treatment time) showed different profiles. Thus, for instance, survival curves to hydrogen peroxide and HHP displayed shoulders, whereas those to NaCl and PEF showed tails. Therefore, the non-linear Geeraerd model (Geeraerd, Herremans, & Van Impe, 2000) was required to describe them accurately, and the corresponding resistance parameters (N_0; S_l; K_{max}, N_{res}) were calculated. The mean values of these parameters (and their standard deviation),

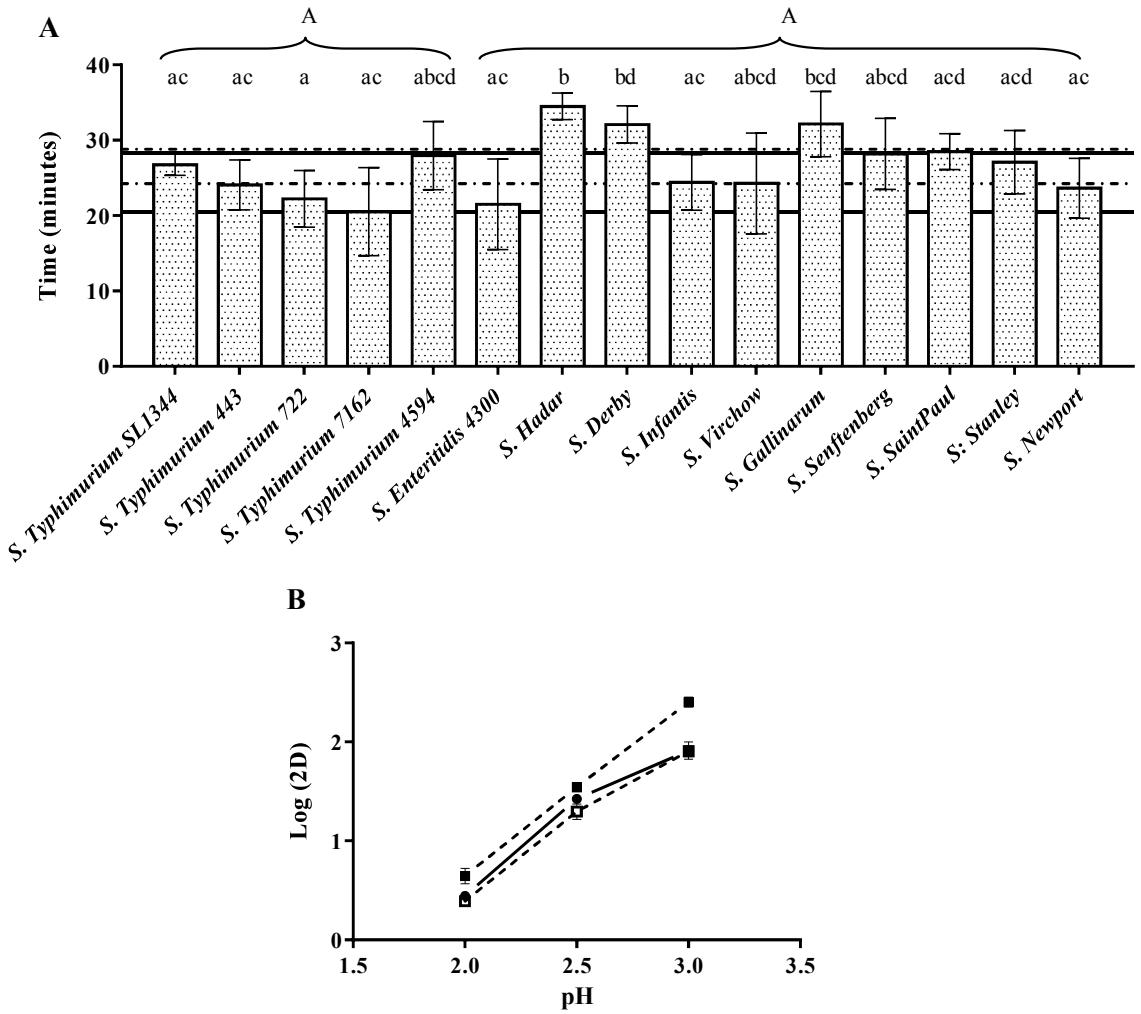

Fig. 1. A) *2D*-values of the 15 strains of *Salmonella* to acid pH (2.5). Discontinuous and continuous lines correspond to the 95% confidence interval of the mean *2D*-value of all the *Salmonella* strains (inter-serovar variability) and of *S.* Typhimurium strains (intra-serovar variability), respectively. Different letters indicate statistically significant differences between strains (lowercase letters) or groups (Typhimurium *vs* Non-Typhimurium; uppercase letters). B) Influence of treatment medium pH on the resistance of the 3 serovars selected: *S.* Typhimurium SL1344 (●, continuous line), *S.* Hadar (■, discontinuous line) and *S.* Typhimurium 7162 (□, discontinuous line). Error bars represent the standard deviations.

together with the goodness-of-fit parameters, are included in Table 1. The traditional decimal reduction time value (*D*) of each survival curve was calculated from its corresponding K_{max} (Eq. (2)). In addition, in order to facilitate comparisons between strains and/or agents, it was decided to use the *2D*-value parameter (the time required to reduce bacterial counts in 2 Log_{10} cycles). This parameter was chosen because it takes into account simultaneously the duration of the shoulder phase and the inactivation rate in the linear portion of the curve and also because not all the treatments (at the intensities here applied) achieved 3 Log_{10} cycles of inactivation (Cebrián, Mañas, & Condón, 2016). Anyway, it should be noted that similar conclusions can be drawn if the *1D*-value (time to inactivate the first Log_{10} cycle) or *3D*-values (when it was possible to calculate it) are compared (data not shown).

3.1. Acid resistance

The *2D*-values of the 15 studied strains when exposed to acid pH (2.5) varied from 20.52 to 34.48 min (average value = 26.52 min). *S.* Hadar was the most resistant and *S.* Typhimurium 7162 the most sensitive strain (Fig. 1A). Fig. 1A also includes the 95% confidence interval of the mean of the calculated *2D*-values for the whole set of strains under study (discontinuous line) as a measurement of inter-serovar variability in resistance, and the 95% confidence interval of the mean of the *2D*-values calculated for the 5 *S.* Typhimurium strains (continuous line) as an intra-serovar variability measurement. Although the number of strains used to determine these confidence intervals is different (15 *vs* 5), it can be observed that the variability in resistance to acid conditions among *S.* Typhimurium strains was greater (at least comparable) than inter-serovar variability. In addition, no significant differences ($p > 0.05$) were found when the acid resistance of the strains belonging to *S.* Typhimurium (5 strains) *vs* that of strains belonging to other serovars (10 strains) was compared (unpaired *t*-test with Welch correction). In other words, the differences in acid resistance observed among *Salmonella* strains would probably be more linked to strain-specific characteristics than to serovar-specific ones. Nevertheless, it should be remarked these conclusions should be taken with caution since the number of strains (15) and serovars (10) studied in this work is quite low and further studies including a higher number of strains and serovars would be required to validate them. While the influence of pH on growth and survival of microorganisms has been widely studied, few studies are available on the variability in acid resistance among multiple strains of *Salmonella enterica*. Rodríguez, Aguirre, Lianou, Parra-Flores, and García de Fernando (2016) studied the influence of the type of substrate and acid, including citric acid as in our study, on microbial resistance to acid conditions, and they found notable differences among bacterial genus. Among the studied microorganisms they

included *S.* Enteritidis 4300, and the calculated *D*-value was in the range of those observed in this study (5.62 min at pH 2.56 *vs* 9.09 at pH 2.5). In other studies, where a larger number of serovars was evaluated, the medium was acidified with HCl. Although this implies that resistance values are not directly comparable with those obtained in this study, it should be noted that both Berk, Jonge, Zwietering, Abee, & Kieboom (2005) and Lianou and Koutsoumanis (2013) reported a considerable variability in acid resistance among the tested strains, greater than that observed in this study. Such differences between our results and those previously reported might be due to the number of strains studied or the chosen strains, yet might also be due to the different type of acid used, since it is well known that the mode of action of organic and inorganic acids and the resistance mechanisms of bacteria against each of them are very different (Spector & Kenyon, 2012).

In order to determine if these conclusions were valid for a wider pH range, we studied the influence of treatment medium pH (from 2.0 to 3.0) on the *2D*-values of the most pH-resistant and pH-sensitive serovars. *S.* Typhimurium SL1344 was likewise included in this set of experiments as a reference strain. As can be observed in Fig. 1B, which represents the Log_{10} of the *2D*-values of each strain *vs* treatment medium pH, the influence of treatment medium pH on the resistance of the three serovars was very similar, strongly suggesting that the conclusions drawn from the experiments carried out at pH 2.5 would be valid for a wider range of pH, at least between 2.0 and 3.0.

3.2. Hydrogen peroxide resistance

Resistance to 30 mM hydrogen peroxide was also determined for the 15 strains, and the obtained results are displayed in Fig. 2A. In this case *S.* Senftenberg was the most resistant strain (*2D*-value 66.52 min), and the least resistant one was *S.* Enteritidis 4300 (*2D*-value 43.83 min). These values are in the range of those reported in previous research works (Sagarzazu, Cebrián, Pagán, Condón, and Mañas (2013); Wahlig et al., 2019). As described for acid resistance, intra-serovar variability in hydrogen peroxide resistance exceeded inter-serovar variability and no significant differences were found when comparing the hydrogen peroxide resistance of the 5 *S.* Typhimurium strains *vs.* the other 10 non-*S.* Typhimurium strains. To the best of our knowledge, no previously published study has dealt specifically with the heterogeneity of hydrogen peroxide resistance within the genus *Salmonella*. On the other hand, as can be deduced from Fig. 2B, a modification of the concentration of H_2O_2 had the same effect on the *2D*-values calculated for the most and the least H_2O_2 resistant strains, as well as for *S.* Typhimurium SL1344.

3.3. NaCl resistance

2D-values in NaCl-added medium for the strains under study varied from 5.39 to 9.03 h, these are values corresponding to *S.* Enteritidis 4300 and *S.* Saintpaul, respectively. According to the results obtained, intra-serovar variability was as large or even larger than inter-serovar variability (Fig. 3A). A similar result was observed by Lianou and Koutsoumanis (2011) when they evaluated the growth capacity (growth rate, μ_{max}) of 60 *Salmonella* strains at different concentrations of NaCl. On the other hand, results obtained here indicate that, despite the observed differences among *2D*-values, there were no significant differences ($p > 0.05$) in NaCl resistance among the studied *S.* Typhimurium strains. A similar result was obtained by Cebrián, Arroyo, Mañas, and Condón (2014), who determined the maximum non-inhibitory concentration of NaCl for four *S.* Typhimurium strains and found hardly any differences among them. Nevertheless, and conversely to what it was observed for acid and hydrogen peroxide resistance, significant differences ($p < 0.05$) were found when comparing the NaCl resistance of *S.* Typhimurium strains *vs.* the other 10 *Salmonella* strains. This would mean that NaCl resistance might be, at least to some extent, serovar-dependent, being that of *S.* Typhimurium strains among the highest of the serovars here studied. Further work would be required in order to validate this conclusion.

Regarding the influence of NaCl concentration on the resistance of *Salmonella* (*2D*-value), increasing the NaCl concentration resulted in a decrease in the *2D*-values in the three strains studied (Fig. 3B). However, whereas in the range between 20 and 30% the magnitude in decrease was similar for all three strains, above that concentration the decrease was much more marked for the more NaCl-resistant ones. This strongly suggests that differences in NaCl resistance among *Salmonella* strains would depend on the NaCl concentration used.

3.4. Heat resistance

Conversely to acid resistance, large differences in heat resistance were observed between the most and the least heat-resistant serovar. Thus, the *2D*-value to heat (58 °C) varied between 1.62 min and 23.46 min for serovars Saintpaul and Senftenberg (strain 775W), respectively (Fig. 4A). In parallel, intra-serovar differences in heat resistance were much smaller than the differences observed when comparing different serovars. However, these observations are mainly due to the extraordinary thermal resistance of *S.* Senftenberg strain 775W, which has already been documented. This particular strain is considered a singularity, not only when compared with other *Salmonella* serovars, but also with other strains belonging to the serovar Senftenberg (Ng et al., 1969). Therefore, if this strain is excluded from the analysis, one can conclude that inter-serovar variability in resistance to heat would be lower than intra-serovar variability. Remarkably, the heat resistance parameters (*D*-values) and the variability in heat resistance determined here are comparable to those previously reported, even though other strains, growth methods, and treatment mediums were used. Thus, Juneja, Eblen, and Ransom (2001) evaluated the heat resistance of 35 *Salmonella* strains in chicken broth at 58 °C, reporting *D*-values between 1.29 and 2.98 min. Similarly, Quintavalla, Larini, Mutti, and Barbuti (2001) reported that the *D*-values of 94 *S. enterica* strains belonging to different serovars determined in culture broth at 58 °C ranged between 0.79 and 2.67 min. The variability in heat resistance among strains obtained in this study is also similar to that determined in the meta-analysis carried out by van Asselt and Zwietering (2006). As pointed out by den Besten, Wells-Bennik, and Zwietering (2018) if *S.* Senftenberg 775W is excluded from analysis, the variability in heat resistance among *Salmonella* serovars is, in general terms, lower than among strains of other species.

The influence of treatment temperature on microbial heat resistance is usually estimated *via* the calculation of the z value (the inverse of the slope of the line obtained when the Log_{10} of the *D*-values is represented *vs* its corresponding treatment temperature). In this case, we calculated the z_{2D} (increase in temperature required to reduce the 2*D*-value 10-fold) for the most and the least heat-resistant strain, as well as for *S.* Typhimurium SL1344, and no significant differences ($p > 0.05$) were found among them (Fig. 4B). Therefore, it is feasible to conclude that the relative resistance of the different *Salmonella* strains would be similar regardless of treatment temperature, within the range studied here.

3.5. HHP resistance

As it can be observed in Fig. 5A, *S.* Typhimurium SL1344 displayed the highest baroresistance (*2D*-value at 300 MPa = 8.83 min), and *S.* Infantis the lowest (*2D*-value at 300 MPa = 5.79 min). The average 2*D*-value was of 6.98 min for all the strains/serovars, and of 7.05 min for the *S.* Typhimurium strains but, in spite of this slightly higher average *2D*-value, no significant differences ($p > 0.05$) were found when comparing the baroresistance of *S.* Typhimurium strains (5 strains) *vs* that of strains belonging to other serovars (10 strains). As for all agents, except heat, the 95% confidence interval of the mean of the *2D*-values calculated for *S.* Typhimurium strains was broader than that

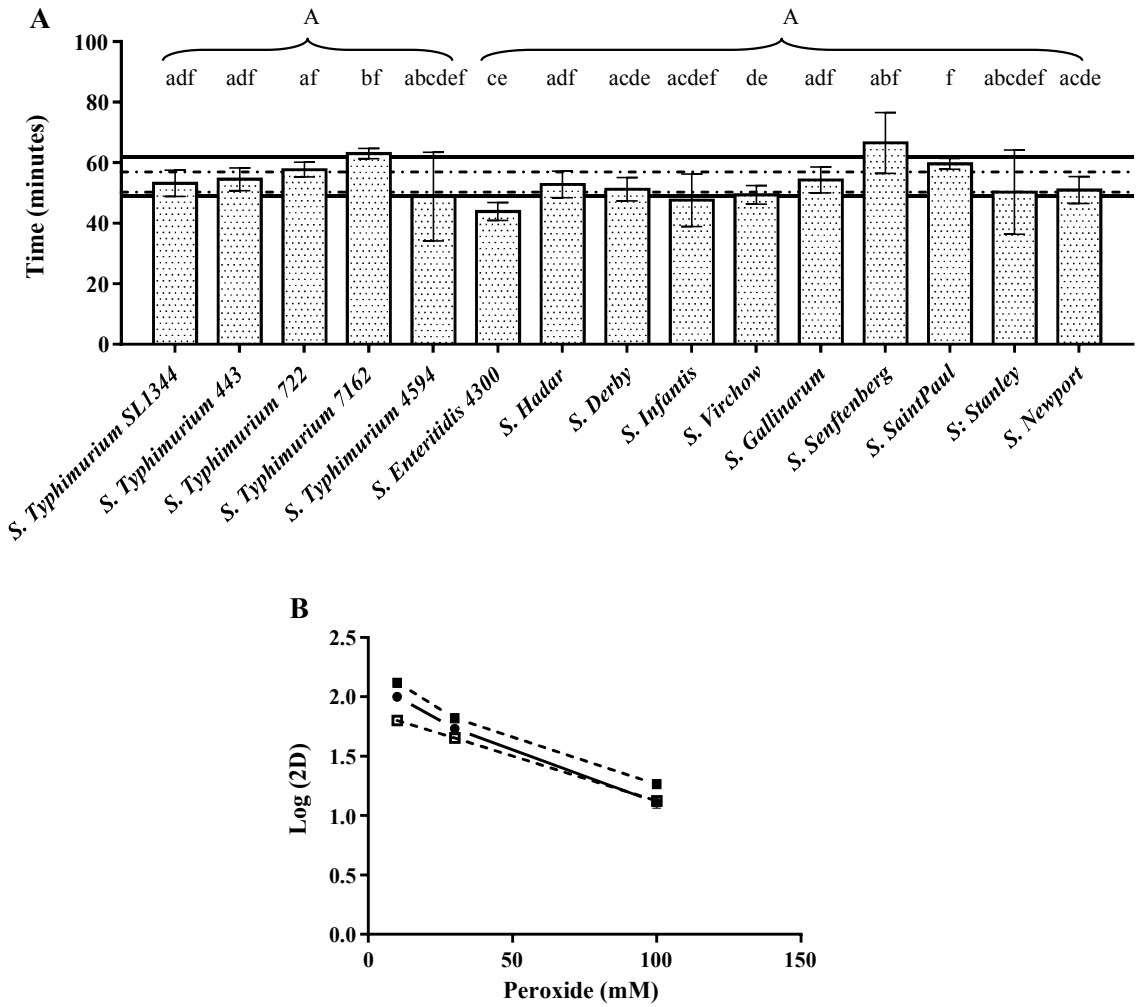

Fig. 2. A) *2D*-values of the 15 strains of *Salmonella* to hydrogen peroxide (30 mM). Discontinuous and continuous lines correspond to the 95% confidence interval of the mean *2D*-value of all the *Salmonella* strains (inter-serovar variability) and of *S.* Typhimurium strains (intra-serovar variability), respectively. Different letters indicate statistically significant differences between strains (lowercase letters) or groups (Typhimurium *vs* Non-Typhimurium; uppercase letters). B) Influence of the hydrogen peroxide concentration on the resistance of the 3 serovars selected: *S.* Typhimurium SL1344 (●, continuous line), *S.* Senftenberg (■, discontinuous line) and *S.* Enteritidis 4300 (□, discontinuous line). Error bars represent the standard deviations.

corresponding to the whole set of strains. These results agree with those obtained by Sherry et al. (2004) who observed that resistance to high pressure was relatively uniform among the serovars studied. In contrast, Tamber (2018), who studied the HHP resistance of 99 *S. enterica* strains from 24 serovars, found that after exposure to 600 MPa for 3 min, differences of up to 5 Log_{10} cycles in the number of survivors were found between the most and the least baroresistant strains. Further work will be required to ascertain whether these differences are a result of differences among process parameters and experimental conditions applied in the studies, or whether they may reflect inherent differences among the tested strains. In any case, Tamber (2018) also observed that, despite the close genetic relationships between the strains of some serovars, the distribution of resistance patterns differed among strains, suggesting that there was no significant relationship between pressure tolerance and the serovar.

Since our reference strain (*S.* Typhimurium SL1344) was already the most HHP-resistant one, we included the second most resistant one, *S.* Newport, in the experiments designed to determine the influence of pressure on the *2D*-values. For the three strains, a marked and similar decrease in resistance was observed after raising pressure from 250 to 300 MPa, but not to 350 (Fig. 5B). This could be attributed to the presence of tails in survival curves to HHP, which may interfere with the estimation and interpretation of the *2D* parameter. Patterson, Quinn, Simpson, and Gilmour (1995) analyzed that, when calculating *D*-values corresponding to high hydrostatic pressure treatments, difficulties could arise due to the surviving tail populations, and this effect was noticeable when pressure was greater than 350 MPa. In any case, the observed trends were similar for all three strains, indicating that, as for acid, peroxide and heat, conclusions drawn for selected pressure would be valid for the entire range under study.

3.6. Resistance to PEF

The estimated *2D*-value (μs) for the tested strains varied from 26.16 to 49.83, for *S.* Virchow and *S.* Stanley, respectively (Fig. 6A), i.e. an approximately 2-fold variation between the most and the least resistant strains. Variability in PEF resistance among *Salmonella* serovars has scarcely been studied. The results obtained for the Typhimurium strains are similar to those obtained by Saldaña et al. (2009). Similarly, up to a 2-fold difference in the calculated *5D*-values was observed when comparing the resistance to PEF of *S.* Senftenberg 775W, *S.* Typhimurium STCC 443 and *S.* Enteritidis STCC 4300 in the range between 19 and 28 kV/cm (Álvarez, Mañas, Condón, & Raso, 2003). As for most of the previously studied agents, the 95% confidence interval of the mean of the *2D*-values calculated for the 5 *S.* Typhimurium strains was similar to that calculated for the whole set of strains (15) but it should be noted

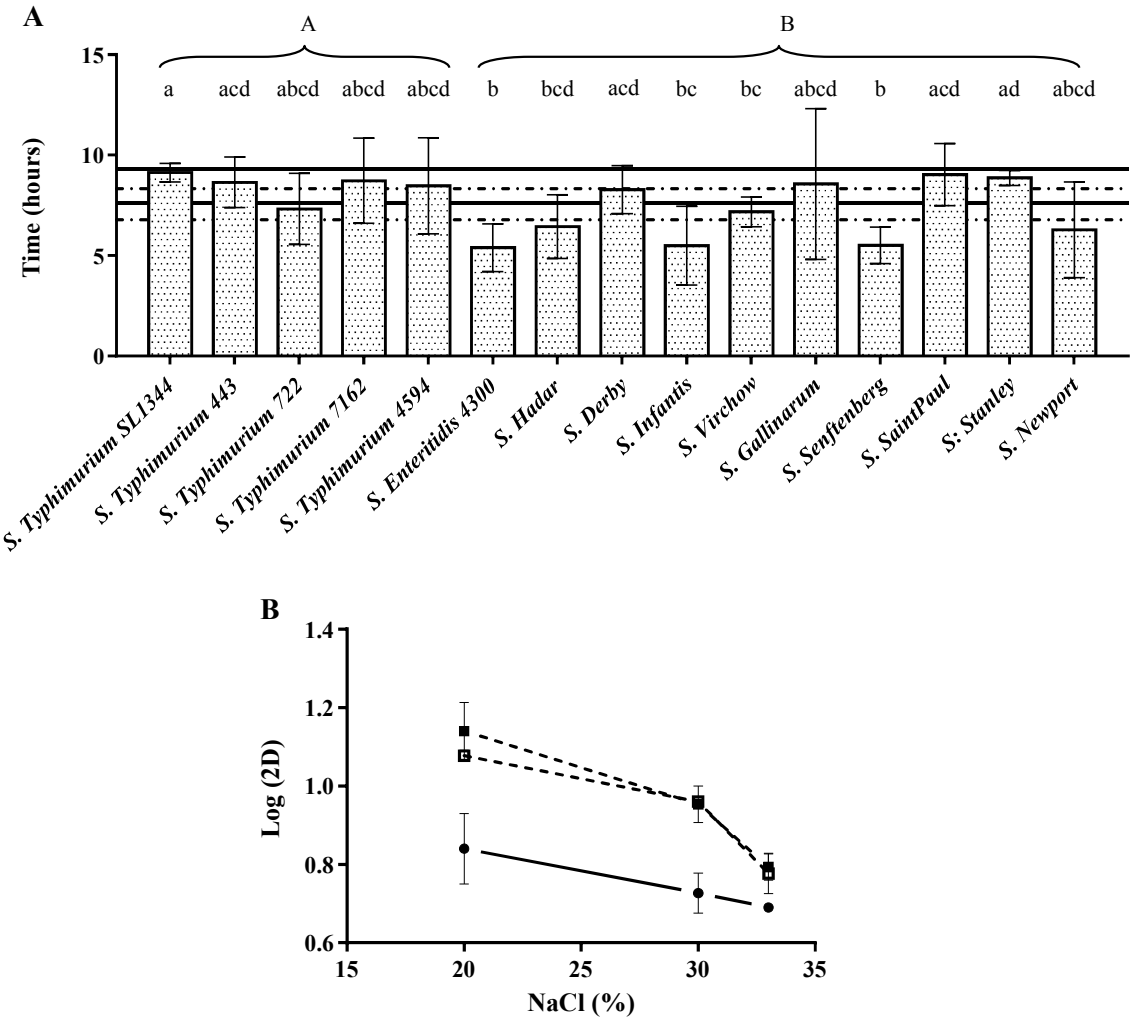

Fig. 3. A) *2D*-values of the 15 strains of *Salmonella* to sodium chloride (30%). Discontinuous and continuous lines correspond to the 95% confidence interval of the mean *2D*-value of all the *Salmonella* strains (inter-serovar variability) and of *S.* Typhimurium strains (intra-serovar variability), respectively. Different letters indicate statistically significant differences between strains (lowercase letters) or groups (Typhimurium *vs* Non-Typhimurium; uppercase letters). B) Influence of sodium chloride concentration on the resistance of the 3 serovars selected: *S.* Typhimurium SL1344 (●, continuous line), *S.* Saintpaul (■, discontinuous line) and *S.* Enteritidis 4300 (□, discontinuous line). Error bars represent the standard deviations.

that the PEF resistance of the *S.* Typhimurium strains was in the upper range. Furthermore, significant differences ($p < 0.05$) were found when comparing the PEF resistance *S.* Typhimurium strains *vs*. the other 10 *Salmonella* strains, thus suggesting that this trait might be both strain and serovar dependent. Finally, as can be seen in Fig. 6B, the influence of electric field strength on the resistance of the three serovars under study (the most and the less resistant ones, along with strain SL1344) was analogous.

3.7. UV-C resistance

The *2D*-value to UV-C (0.47 mW/cm^2) treatments for the tested strains ranged from 49.73 to 70.20 s. *S.* Gallinarum and *S.* Newport were the most sensitive, and *S.* Infantis was the most resistant one. The differences in resistance among strains of *S.* Typhimurium were comparable to those observed when comparing different serovars (Fig. 7A) but statistical analysis suggests that differences in UV resistance might be determined by both the strain and the serovar. In any case, the *2D*-value varied less than 1.5-fold. Gayán et al. (2012) also observed a 1.4-fold difference in the *4D*-values to UV light among five strains of *Salmonella*, revealing that *S.* Typhimurium STCC 878 and *S.* Enteritidis 4300 were the most resistant and the most sensitive strain, respectively, among the strains they studied. Gabriel and Nakano (2009) also reported that in phosphate-buffered saline (PBS) buffer the *S.* Enteritidis strain they tested was less resistant to UV-C than *S.* Typhimurium. Kim and Yuk (2017) similarly tested the resistance of 18 *Salmonella* strains to 405 nm LED light indicating that efficacy of 405 nm LED illumination may depend on serotype and strain within the same serotype. In addition, as can be seen in Fig. 7B, the *2D*-values of the three selected strains showed a similar trend when fluence was modified.

3.8. Comparative study

In order to establish meaningful comparisons among strains and agents/technologies, we applied the iterative Grubbs' test to the obtained data (*2D*-values) in order to identify potential outliers that could exert a disproportionate influence on further data analysis and lead to non-valid conclusions. Grubbs' test detected a single outlier: the *2D*-value to heat of *S.* Senftenberg 775W. This value was therefore excluded from subsequent analysis. This was a true outlier value, since the elevated heat resistance of this strain has been documented elsewhere (Ng et al., 1969).

As described above, one of the major objectives of this investigation was to quantify and compare variability in resistance to different stresses/technologies among different *Salmonella* strains. Since the *2D*-

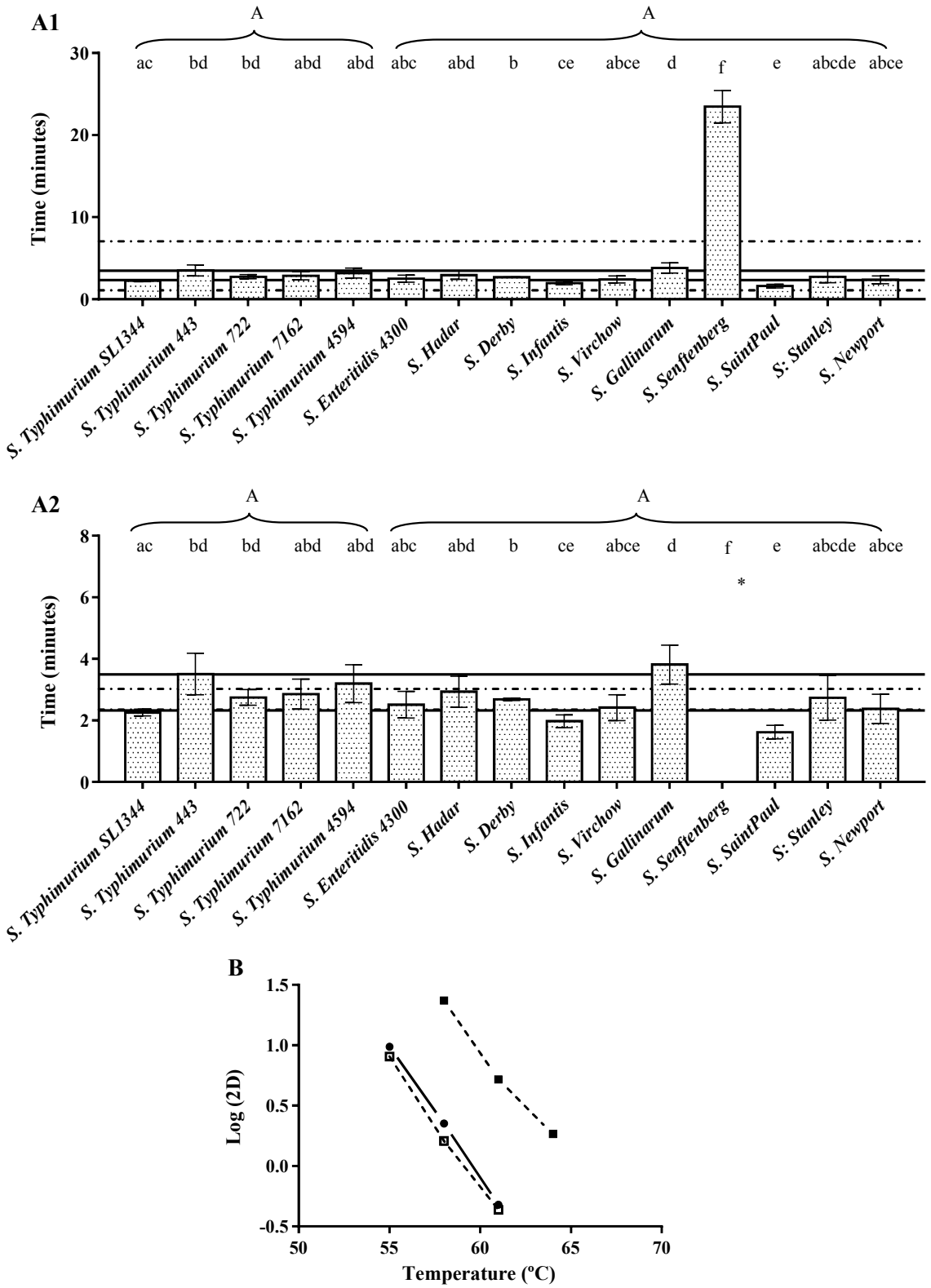

Fig. 4. A1) *2D*-values of the 15 strains of *Salmonella* to heat (58 °C) and A2) *2D*-values excluding *S.* Senftenberg from the analysis. Discontinuous and continuous lines correspond to the 95% confidence interval of the mean *2D*-value of all the *Salmonella* strains (inter-serovar variability) and of *S.* Typhimurium strains (intra-serovar variability), respectively. Different letters indicate statistically significant differences between strains (lowercase letters) or groups (Typhimurium *vs* Non-Typhimurium; uppercase letters). B) Influence of treatment medium temperature on the resistance of the 3 serovars selected: *S.* Typhimurium SL1344 (●, continuous line), *S.* Senftenberg (■, discontinuous line) and *S.* Saintpaul (□, discontinuous line). Error bars represent the standard deviations.

values obtained for each agent/technology cannot be directly compared because of the varying time scale of survival curves, these resistance parameters were normalized by dividing them by the average *2D*-value of the resistance of all the *Salmonella* strains studied. These normalized values were used to build Fig. 8, which illustrates the variability in resistance of the 15 strains studied to each of the 7 agents investigated.

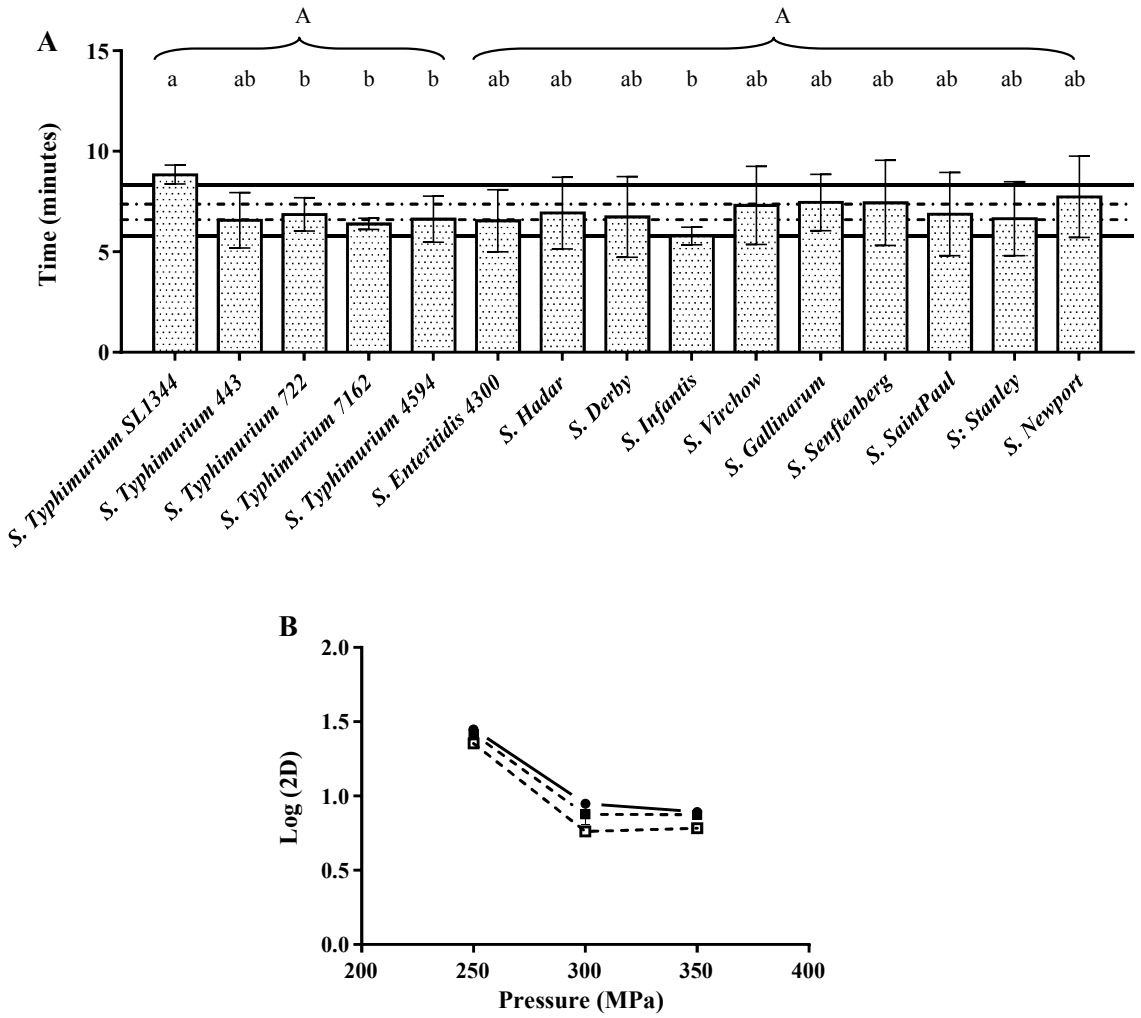

Fig. 5. A) *2D*-values of the 15 strains of *Salmonella* to high hydrostatic pressure (300 MPa). Discontinuous and continuous lines correspond to the 95% confidence interval of the mean *2D*-value of all the *Salmonella* strains (inter-serovar variability) and of *S.* Typhimurium strains (intra-serovar variability), respectively. Different letters indicate statistically significant differences between strains (lowercase letters) or groups (Typhimurium *vs* Non-Typhimurium; uppercase letters). B) Influence of the pressure on the resistance of the 3 serovars selected: *S.* Typhimurium SL1344 (●, continuous line), *S.* Newport (■, discontinuous line) and *S.* Infantis (□, discontinuous line). Error bars represent the standard deviations.

As can be observed in the figure, resistance to UV was the most homogeneous one. Conversely, *Salmonella* resistance to heat and PEF was much more heterogeneous. When comparing these two latter technologies it should be noted that, although the difference between the maximum and the minimum *2D*-values was higher for heat (whiskers length), the 25th and 75th percentiles (box length) were more separated for PEF, thereby indicating that the frequency distribution of heat values would have a higher kurtosis (i.e. a higher probability of including outliers). On the other hand, the dispersion of resistance values of almost all treatments showed a symmetrical distribution around the median, except for NaCl resistance values, for which the dispersion of resistance values displayed a positive asymmetric right-skewed distribution.

These results are similar to those previously reported by Cebrián et al. (2016) who concluded that the differences in resistance among strains of the genus *Salmonella* were smaller for UV than for the other agents studied (heat, PEF, and HHP), and that, conversely to other microorganisms and provided that *S.* Senftenberg 775W is excluded from analysis, variability in resistance to PEF and HHP is comparable to that of heat. Furthermore, as already pointed out by den Besten et al. (2018) for heat, all these data suggest that the variability in stress resistance among *Salmonella* serovars would generally be lower than among strains of other species.

It should be noted that this comparison was established using results obtained under very specific fixed experimental conditions: bacteria were grown to stationary growth phase under optimal conditions, and treatments were applied in buffer/laboratory media at neutral pH, and with a very high water activity. Although results obtained here indicate that the range of experimental conditions under which these conclusions are valid would be broader (pH 2.0–3.0; 55–64 °C; 250–350 MPa; 10–100 mM H_2O_2; 20–30% NaCl; 20–30 kV/cm; 0.2–1.1 mW/cm^2), results already indicate that, for instance, if resistance to NaCl were studied at higher NaCl concentrations (33%), the observed variability in resistance would be of lower magnitude. Similarly, Lianou and Koutsoumanis (2011) already observed that the magnitude of differences in growth rate (μ_{max}) among *Salmonella* strains depended highly on growth conditions (composition of the growth medium). On the other hand, our results indicate that variability among experimental replicates (biological replicates) was lower than intra-serovar and inter-serovar variability, with very few exceptions.

Our experimental design also allowed us to determine whether any positive or negative association between *Salmonella* resistance to the different stresses could be ascertained. For this purpose, Pearson's correlation test was performed (Table 2). Result indicate a positive correlation between resistance to osmotic and oxidative stress (r = 0.565, p-value = 0.035). Further analysis of results corroborated

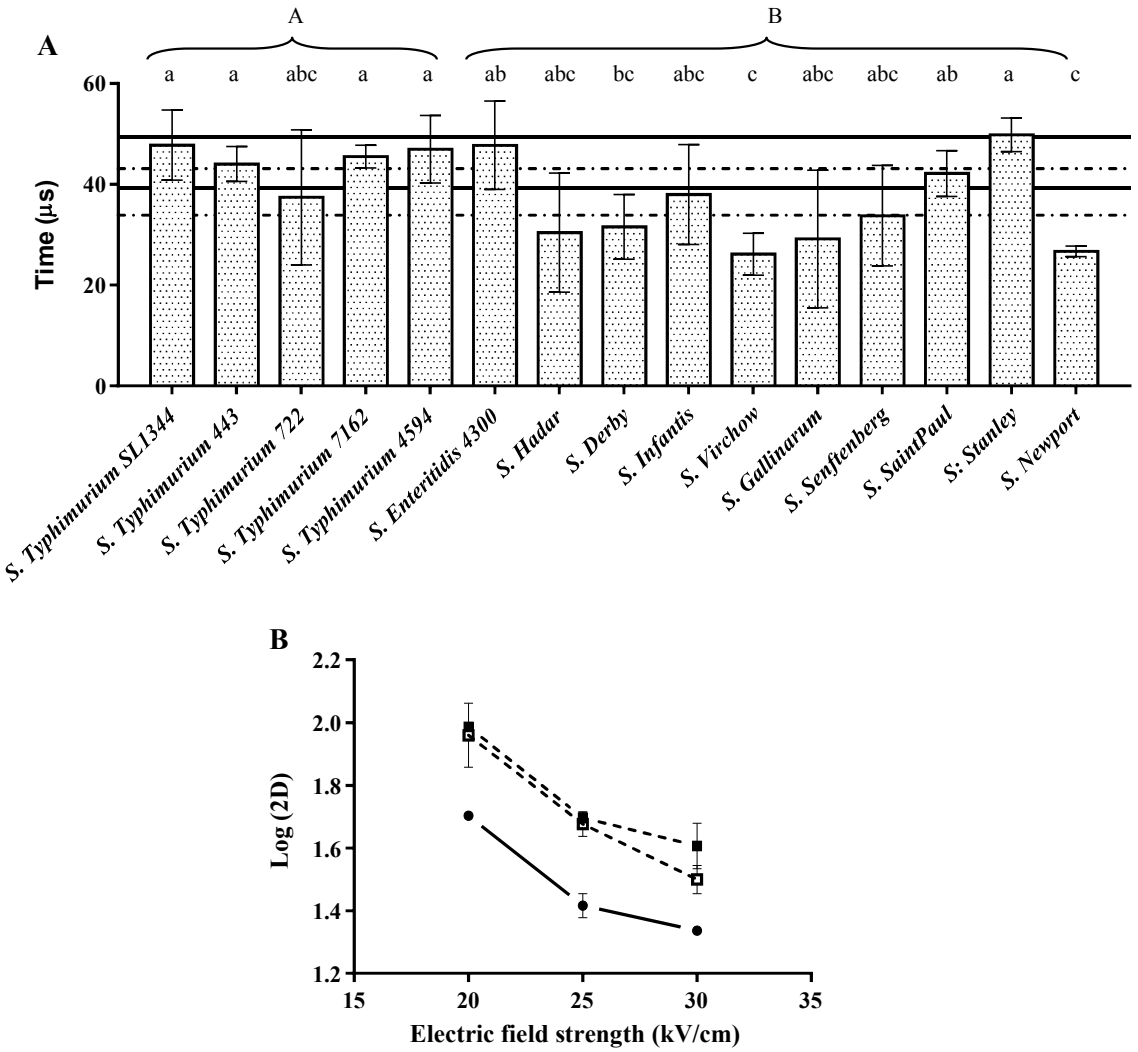

Fig. 6. A) *2D*-values of the 15 strains of *Salmonella* to pulsed electric fields (25 kV/cm). Discontinuous and continuous lines correspond to the 95% confidence interval of the mean *2D*-value of all the *Salmonella* strains (inter-serovar variability) and of *S.* Typhimurium strains (intra-serovar variability), respectively. Different letters indicate statistically significant differences between strains (lowercase letters) or groups (Typhimurium *vs* Non-Typhimurium; uppercase letters). B) Influence of sodium chloride concentration on the resistance of the 3 serovars selected: *S.* Typhimurium SL1344 (●, continuous line), *S.* Stanley (■, discontinuous line) and *S.* Virchow (□, discontinuous line). Error bars represent the standard deviations.

the existence of this relation: *S.* Enteritidis 4300, *S.* Infantis, *S.* Newport, and *S.* Virchow are the most sensitive serovars to the two environmental stresses, and *S.* Saintpaul, *S.* Typhimurium 443 and *S.* Typhimurium 7162 are the most resistant (Table 1 and Fig. 2A and 3A). A positive correlation was also observed between PEF and UV-C resistances (r = 0.558, p-value = 0.038). The most resistant strains to both technologies would be *S.* Typhimurium SL1344 and *S.* Typhimurium 4954, and the most sensitive strains would be *S.* Newport, *S.* Virchow, and *S.* Gallinarum. It should also be noted that, as pointed out above, the same conclusions can be drawn if the *1D* or the *3D*-values are used to establish these comparisons, with the only exception that if *1D*-values are compared a positive correlation between acid and UV resistance is observed.

Based on our results, there would be no correlation between resistance to heat and acid pH (r = 0.233, p-value 0.423). This finding contrasts with the fact that the existence of cross-protection phenomena between pH and heat has already been described in *Salmonella* spp. (Álvarez-Ordóñez, Fernández, López, Arenas, & Bernardo, 2008). It also contrasts with the results of Humphrey, Slater, McAlpine, Rowbury, and Gilbert (1995), who observed that the most heat-resistant *S.* Enteritidis PT4 isolates were also more resistant to acid, H_2O_2, and desiccation. Nevertheless, similar results to those reported herein were obtained by Lianou and Koutsoumanis (2013), and by Gill, Tamber, and Yang (2019).

According to our PCA analysis, the two principal components explain 53.8% of the variability of the data (Fig. 9). CP1 would be positively correlated with UV and PEF resistance, and negatively with pH and HHP, whereas CP2 would be positively correlated with NaCl, H_2O_2 and PEF resistance (Table Fig. 9). Thus, strains with a higher PEF and UV resistance are located more on the right on the x-axis (CP1), whereas those more resistant to NaCl and H_2O_2 are higher on the y-axis (CP2). In this plot, it can also be observed that strains displaying similar resistance profiles are located close to one another (e.g. the *S.* Typhimurium STCC 443 and *S.* Stanley). These observations are very similar to the Pearson's test results, since both indicate an association between UV and PEF, as well as between NaCl and H_2O_2 resistance, along with certain further trends, such as a positive association between PEF and NaCl resistance, and negative correlations between PEF and acid resistance, and between HHP and UV resistance.

Altogether, these results demonstrate that *Salmonella* strains that are the most resistant to a given stress are not necessarily more resistant to other types of stresses, as also has been previously demonstrated for *Salmonella* by other authors such as Sherry et al. (2004), Lianou and Koutsoumanis (2013) and Gill et al. (2019). This can be easily

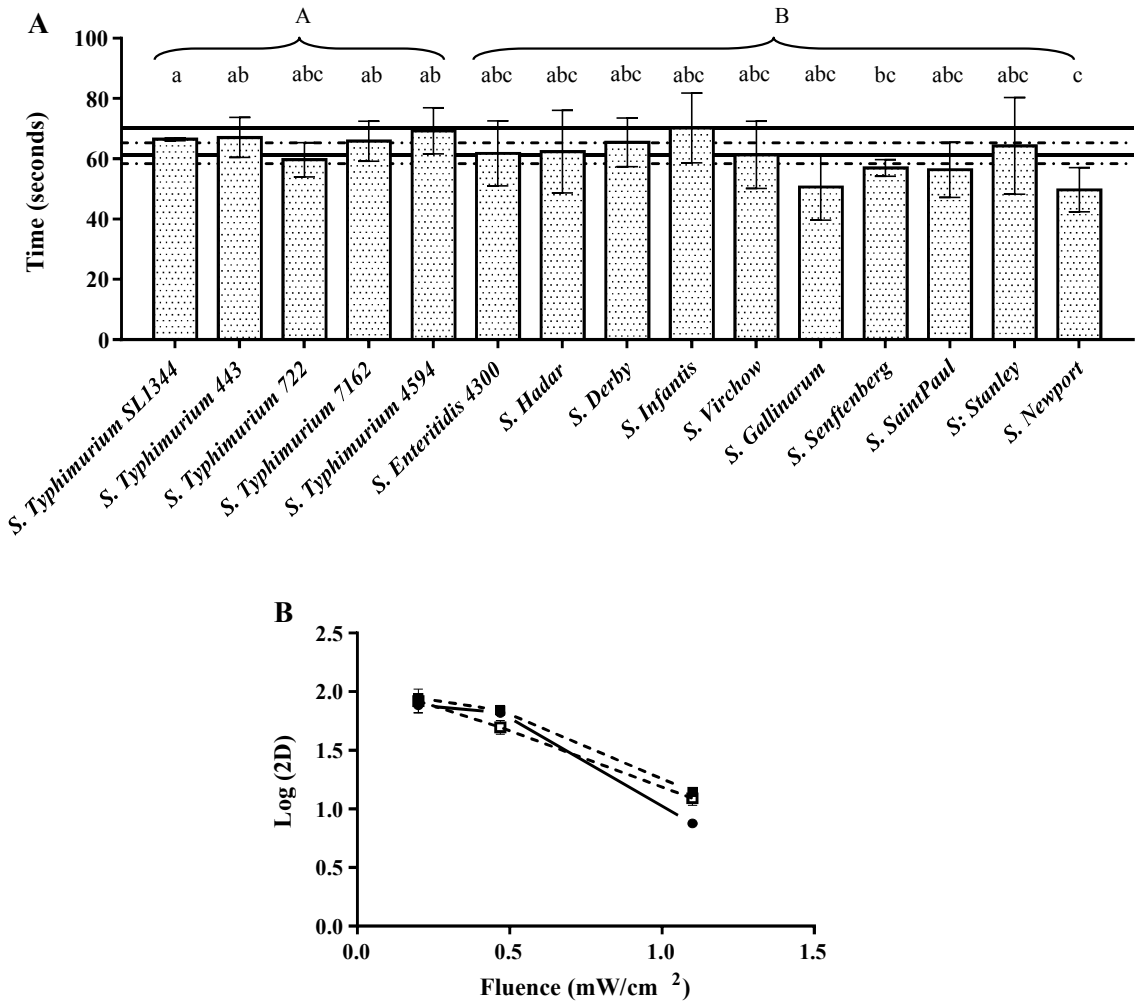

Fig. 7. A) *2D*-values of the 15 strains of *Salmonella* to UV-C (0.47 mW/cm2). Discontinuous and continuous lines correspond to the 95% confidence interval of the mean *2D*-value of all the *Salmonella* strains (inter-serovar variability) and of *S.* Typhimurium strains (intra-serovar variability), respectively. Different letters indicate statistically significant differences between strains (lowercase letters) or groups (Typhimurium *vs* Non-Typhimurium; uppercase letters). B) Influence of UV-C fluence on the resistance of the 3 serovars selected: *S.* Typhimurium SL1344 (●, continuous line), *S.* Infantis (■, discontinuous line) and *S.* Gallinarum (□, discontinuous line). Error bars represent the standard deviations.

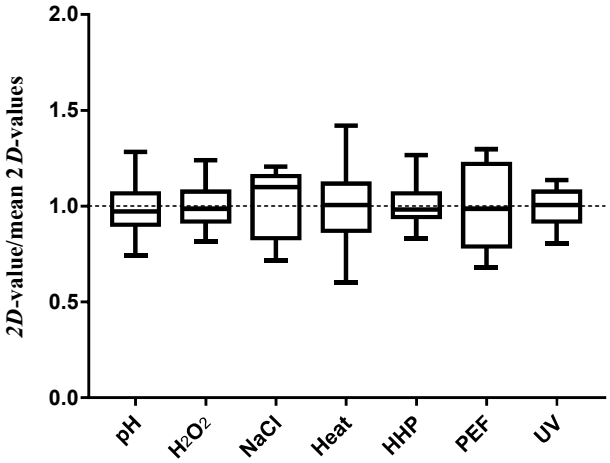

Fig. 8. Variability in resistance to different environmental stresses and non thermal food preservation technologies among the *Salmonella* strains studied. The *2D*-value to heat of *S.* Senftenberg has been excluded from the analysis as described in the results section.

explained by the different modes of action and cellular targets of each of the technologies/agents studied here (Cebrián et al., 2016; Sherry et al., 2004). Nevertheless, since an association between NaCl and hydrogen peroxide resistance, as well as between PEF and UV resistance, was found, further work will be required to elucidate the underlying mechanisms.

It should be noted that the mode of action of NaCl and hydrogen peroxide on bacterial cells are assumed to be quite different. Thus, NaCl is a water-depressing solute that imposes a hyperosmotic stress on cells and that, once inside the cytoplasm, can inhibit enzyme activity by perturbing the hydrophobic–electrostatic balance between the forces maintaining protein structure, and can exert Na^+-specific toxic effects such as the inhibition of certain enzymatic activities and ionic channels of the bacterial cell (Murguía, Bellés, & Serrano, 1996; Stewart, Cole, Legan, Slade, & Schaffner, 2005). Hydrogen peroxide acts indirectly though the generation of oxidative species (such as the hydroxyl radical) via the Fenton reaction, which can cause oxidative damages to various cellular components, including DNA and proteins (Imlay & Linn, 1988; Juven & Pierson, 1996). A potential explanation of the relationship between both agents might be found in the results of Mandal and Kwon (2017), who observed that more than 30% of the

Table 2
Pearson correlation coefficient values calculated for the *2D* resistance values of the 15 *Salmonella* strains to the different environmental stresses and non-thermal food preservation technologies studied. Values in parentheses correspond to the p-value.

	pH	H_2O_2	NaCl	Heat	HHP	PEF	UV
pH		−0.043 (0.883)	0.128 (0.662)	0.233 (0.423)	0.181 (0.537)	−0.340 (0.234)	−0.128 (0.663)
H_2O_2	−0.043 (0.883)		**0.565 (0.035)**	0.061 (0.837)	0.075 (0.799)	0.043 (0.885)	−0.176 (0.548)
NaCl	0.128 (0.662)	**0.565 (0.035)**		0.099 (0.735)	0.233 (0.422)	0.446 (0.110)	0.233 (0.423)
Heat	0.233 (0.423)	0.061 (0.837)	0.099 (0.735)		−0.040 (0.892)	−0.061 (0.836)	−0.043 (0.885)
HHP	0.181 (0.5357)	0.075 (0.799)	0.233 (0.422)	−0.040 (0.892)		−0.184 (0.528)	−0.403 (0.153)
PEF	−0.340 (0.234)	0.043 (0.885)	0.446 (0.110)	−0.061 (0.836)	−0.184 (0.528)		**0.558 (0.038)**
UV	−0.128 (0.663)	−0.176 (0.548)	0.233 (0.423)	−0.043 (0.885)	−0.403 (0.153)	**0.558 (0.038)**	

genes involved in desiccation resistance in *Salmonella* Typhimurium were also involved in hydrogen peroxide (H_2O_2, 1 mM) resistance. Nevertheless, the same authors also indicated that much less genes (15%) were shared between osmotic stress (3% NaCl), and hydrogen peroxide resistance. In any case, the stressor concentrations used in their study are much lower than in ours, and further work would be required to determine if their results are valid under our conditions.

Similarly, whereas the main targets of PEF are the cellular envelopes (Mañas & Pagán, 2005), the effect of UV light on genetic material is the main factor responsible for the latter technology's ability to inactivate microorganisms (Gayán, Condón, & Álvarez, 2014), although other cellular components such as proteins can also undergo damage. Regarding this second association (PEF-UV) it should be noted that membrane fluidity has been proposed as a factor which plays a role in microbial resistance to UV (Gayán, Mañas, Álvarez, & Condón, 2013), in such a way that a more fluid membrane would render a more UV-sensitive cell. However, the role of membrane fluidity in PEF resistance, although widely discussed, still remains to be clarified (Cebrián et al., 2016).

The development of cross-resistance responses is commonly attributed to the activation/induction of general stress sigma factors such as RpoS in the case of *Salmonella* (Hengge, 2011). In the same way, it has been hypothesized that differences in stress resistance among strains could be due, among other factors, to a potential association between stress sensitivity and mutations in the *rpoS* gene, or with a decreased level of expression of RpoS-dependent genes (Jørgensen et al., 2000). Since it has been demonstrated that the deletion of *rpoS* leads to a decrease in resistance of *E. coli* to all the agents tested here (Notley-McRobb, King, & Ferenci, 2002), and a similar role for *rpoS* would be expected in *Salmonella* (Robbe-Saule, Algorta, Rouilhac, & Norel, 2003), if an increased expression of RpoS-controlled genes was the cause for increased resistance to a particular stress, it should be accompanied with an increased resistance to all agents tested because our experiments were carried out with stationary growth phase cells. Most agents exert a plethora of effects on bacterial cells, i.e. most of them are regarded as multi-target agents, and even those that share a cellular target (such as PEF and HHP, for instance) have widely differing mechanisms of action. Our results might also be explained by specific resistance mechanisms playing a greater role than general stress mechanisms in *Salmonella* resistance, thereby masking the influence of general stress response (RpoS) controlled mechanisms. In fact, the combination of these three factors – different mechanisms of action plus multi-target technologies plus specific resistance mechanisms playing a major role in resistance – would probably explain the obtained results, even for agents with very similar modes of action and targets, such as heat and HHP (Sherry et al., 2004). Furthermore, even for a single agent such as HHP, Tamber (2018) indicated that the response of *S. enterica* strains was heterogeneous and multifactorial, making it impossible to identify a unique mechanism capable of explaining the observed differences in resistance, and thereby hampering the prediction of individual *S. enterica* strains' response to HHP.

Finally, it is worth noting that further examination of Fig. 9 reveals that *S.* Typhimurium strains clustered together in the PCA biplot -right on the x axis and high on the y axis- and quite apart from most of the

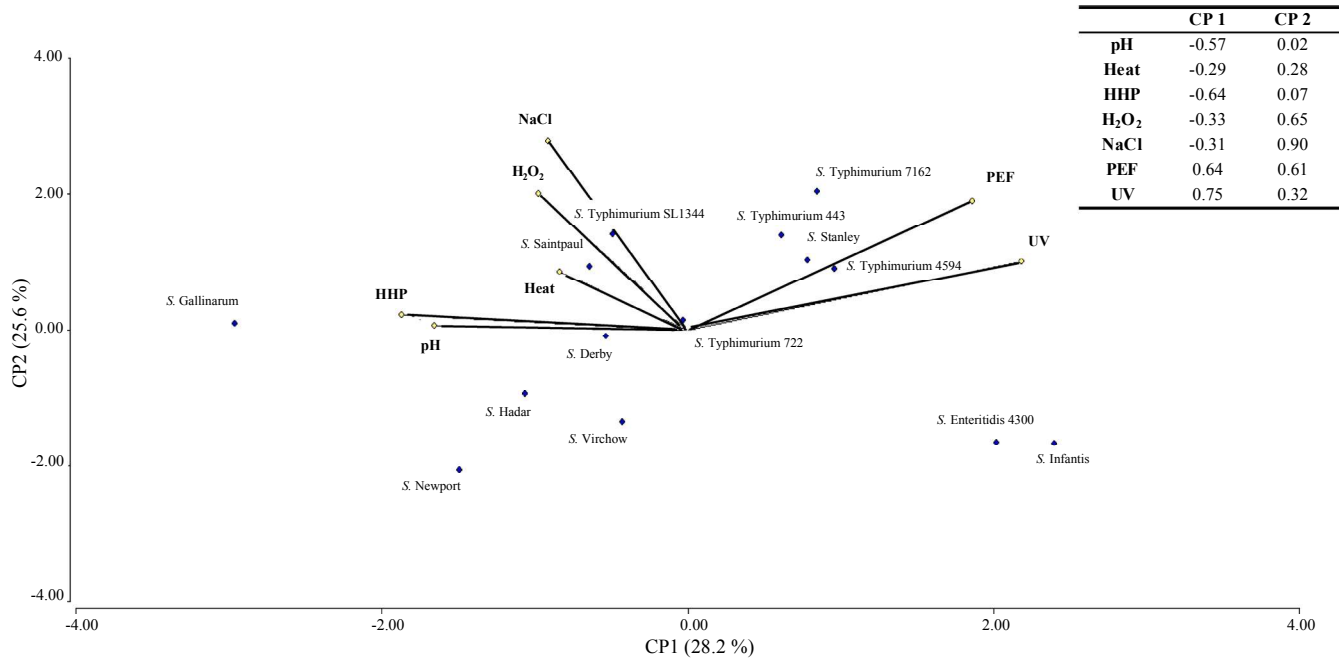

Fig. 9. Bioplot representation of the principal component analysis, showing the distribution of *Salmonella* serovars along components 1 and 2.

strains from other serovars here studied. This would mean that *S.* Typhimuirum strains were among the most PEF, UV, hydrogen peroxide and NaCl resistant *Salmonella* strains and that these strains would be displaying a differentiated stress-resistance phenotype -at least for some agents-, what would be reasonable given their closer genetic background. These conclusions are consistent with those drawn in sections 3.2 to 3.7 and seem to indicate that resistance to some agents such as PEF, UV and NaCl, might be, at least to some extent, a serovar-dependent characteristic. In any case it should be noted that the number of strains here studied is limited and that further studies, including a higher number of strains and from a wide range of *Salmonella* serovars would be required to validate the conclusions drawn from these results.

4. Conclusions

The resistance of 15 strains belonging to 11 serovars of *Salmonella enterica* subsp. *enterica* to several different environmental stresses (acid, hydrogen peroxide, NaCl and heat) and non-thermal food preservation technologies (HHP, PEF, UV) was determined and compared. For most agents tested, intra-serovar (*S.* Typhimurium) variability in resistance was comparable to inter-serovar variability, despite the similar genetic backgrounds of strains belonging to the same serovar. If *S.* Senftenberg 775W is excluded from the analysis, differences in resistance (*2D*-values) among strains varied less than 2.4-fold for all agents, including heat. Results reported herein also indicate that *Salmonella* strains that are the most resistant to a given stress are not necessarily more resistant to other types of stress. Nevertheless, the statistical analysis of the whole set of data reveals a positive correlation between the resistance of *Salmonella* strains to oxidative and osmotic stress, as well as between UV and PEF resistance. Further work will be required to fully elucidate the mechanisms responsible for these two phenomena.

The results obtained in this work would be especially helpful in defining safe food preservation processes and in improving quantitative microbiological risk assessments of *Salmonella* in food products.

CRediT authorship contribution statement

Silvia Guillén: Investigation, Methodology, Formal analysis, Writing - original draft. **María Marcén:** Investigation, Writing - review & editing. **Pilar Mañas:** Conceptualization, Writing - review & editing. **Guillermo Cebrián:** Conceptualization, Writing - review & editing, Supervision.

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Capítulo 2

Estudio de la variabilidad en la capacidad de crecimiento y la virulencia dentro del género *Salmonella:* relación con la resistencia al estrés

Manuscrito III/Manuscript III

Relationship between growth ability, virulence, and resistance to food-processing related stresses in non-typhoidal *Salmonellae*

Silvia Guillén, María Marcén, Ester Fau, Pilar Mañas and Guillermo Cebrián

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Relationship between growth ability, virulence, and resistance to food-processing related stresses in non-typhoidal *Salmonellae*

Silvia Guillén, María Marcén, Ester Fau, Pilar Mañas, Guillermo Cebrián *

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), Zaragoza, Spain

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ABSTRACT

The ability of *Salmonella* to resist and adapt to harsh conditions is one of the major features that have made this microorganism such a relevant health hazard. However, the impact of these resistance responses on other aspects of *Salmonella* physiology, such as virulence and growth ability, is still not fully understood. The objective of this study was to determine the maximum growth rates (in three different media), virulence (adhesion and invasion of Caco-2 cells), and other phenotypic characteristics (biofilm-forming ability and antimicrobial resistance) of 23 *Salmonella* strains belonging to different serovars, and to compare them with their previously determined stress resistance parameters. Significant differences ($p < 0.05$) in growth rates, virulence, and biofilm-forming ability were found among the 23 strains studied. Nevertheless, whereas less than 3-fold change between the lowest and the highest growth rate was observed, the percentage of cells capable of invading Caco-2 cells varied more than 100-fold, that to form biofilms more than 30-fold, and the antibiotic MICs varied up to 512-fold, among the different strains. Results indicate that those strains with the highest cell adhesion ability were not always the most invasive ones and suggest that, in general terms, a higher stress resistance did not imply a reduced growth ability (rate). Similarly, no association between stress resistance and biofilm formation ability (except for acid stress) or antibiotic resistance (with minor exceptions) was found. Our data also suggest that, in *Salmonella*, acid stress resistance would be associated with virulence, since a positive correlation of that trait with adhesion and a negative correlation with invasion was found. This study contributes to a better understanding of the physiology of *Salmonella* and the relationship between bacterial stress resistance, growth ability, and virulence. It also provides new data regarding intra-specific variability of a series of phenotypic characteristics of *Salmonella* that are relevant from the food safety perspective.

1. Introduction

The relevance of *Salmonella* as a foodborne pathogen is undisputed. Together with *Campylobacter*, it has been at the top of the ranking of the most commonly reported causes of foodborne outbreaks and cases for the last 40–50 years in the United States and Europe (Dewey-Mattia et al., 2018; EFSA, 2019; Gould et al., 2013; Omer et al., 2018). The success of *Salmonella* seems to depend on multiple factors, including its ability to withstand multiple stresses encountered in the environment and in the digestive tract, to invade gut cells and survive intracellularly, and to compete for nutrients such as iron, but also to rapidly adapt and evolve (Dandekar et al., 2012; Nilsson et al., 2005; Petrovska et al., 2016; Spector and Cubitt, 1992; Waldner et al., 2012; Winfield and Groisman, 2003).

Nowadays, more than 2500 serovars of *Salmonella* have been described, but those responsible for most human infections are a smaller group. Thus, less than 20 serovars are responsible of more than 80% of all the cases reported (CDC, 2018; EFSA, 2019). The causes underlying this phenomenon have been explored but not fully elucidated. Thus, it is well known that some serovars are host-specific -or have a very narrow range of potential hosts; the epidemiological studies carried out to date have revealed that the incidence of diverse serovars in animals and food products varies widely, also depending on the type of product (Foley et al., 2013; Sabbagh et al., 2010). However, it is also well known that other factors can be even more relevant for explaining this phenomenon such as, for instance, the ability of *S.* Enteritidis to contaminate eggs through the trans-ovarian route (Gantois et al., 2009). Furthermore, it is quite plausible that further factors might also be determining the

* Corresponding author.
E-mail address: guiceb@unizar.es (G. Cebrián).

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differences in prevalence observed among *Salmonella* serovars, such as differences in resistance to stress, or differences in terms of growth fitness and/or competition for nutrients.

The genotypic and phenotypic diversity of *Salmonella* has been widely studied, especially regarding virulence and antibiotic resistance (Gerlach and Hensel, 2007; Jajere, 2019), but also regarding stress resistance (Abdullah et al., 2018; Guillén et al., 2020a, 2020b; Lianou and Koutsoumanis, 2013). Although its ability to resist and adapt to harsh conditions is one of the major features that have made *Salmonella* such a relevant health hazard, the impact of these resistance responses on other aspects of *Salmonella* physiology, such as virulence and growth fitness, are much less known (Guillén et al., 2021). Relevant exceptions should be noted such as, for instance, the proven relationship between bile resistance and expression of virulence factors (Prouty and Gunn, 2000; Urdaneta et al., 2019), between oxidative stress and proliferation or survival in macrophages (Golubeva and Slauch, 2006; Krishnakumar et al., 2004), or the role of heat-shock proteins in pathogenesis (Behrens-Kneip, 2010; Humphreys et al., 2003). By contrast, the trade-off between survival potential and nutritional competence (Notley-McRobb et al., 2002), which has been proven to exist in *Salmonella*'s close relative *E. coli*, still remains to be demonstrated.

In view of the above, it is clear that more in-depth studies that specifically deal with the impact of microbial stress resistance responses on other relevant aspects of microbial physiology, such as growth fitness and/or virulence are still required. The objective of our study was to determine growth fitness (in 3 different growth media), virulence (adhesion and invasion of Caco-2 cells) biofilm-forming ability and antimicrobial resistance of 23 *Salmonella* strains belonging to different serovars, and to compare them with their stress resistance as previously determined in Guillén et al. (2020a, 2020b).

2. Material and methods

2.1. Bacterial strains

23 strains belonging to 15 serovars of *Salmonella enterica* subsp. *enterica* were selected to carry out this investigation. The rationale behind the choice of these strains has already been discussed in previous works (Guillén et al., 2020a, 2020b). The strains of *S.* Typhimurium (STCC 443, STCC 4594, STCC 7162 and STCC 722), *S.* Enteritidis (STCC 4300, STCC 4155, STCC 4396, STCC 7160 and STCC 7236), *S.* Derby STCC 4397, *S.* Infantis STCC 4373, *S.* Virchow STCC 4154, *S.* Gallinarum STCC 4883, *S.* Senftenberg 775W STCC 4565, *S.* Saintpaul STCC 4153, and *S.* Stanley STCC 4141 were supplied by the Spanish Type Culture Collection. The strains of *S.* Hadar NCTC 13033, *S.* Newport NCTC 129, *S.* Kentucky NCTC 5799, *S.* Mbandaka NCTC 7892, and *S.* Livingstone NCTC 9125 were supplied by Public Health England. *S.* Heidelberg DMS 9379 was supplied by German Collection of Microorganisms, and the strain of *S.* Typhimurium SL1344 was kindly provided by Tim Brocklehurst from the Institute of Food Research, Norwich. The source of the strains (for which it is known) is included in Supplementary Material (Table S1). The strains were maintained frozen at −80 °C in cryovials for long-term preservation.

2.2. Growth conditions

Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (TSB-YE, Oxoid) in 96-well microtiter plates (Thermo Scientific, Roskilde, Denmark), and incubated at 37 °C under static conditions as described in Guillén et al. (2020b).

2.3. Maximum growth rate determination assays

The growth rates of the 23 *Salmonella* strains were calculated in three different media: TSB-YE at 37 °C, Luria-Bertani (LB) broth supplemented with 100 μM 2-2′dipyridyl (DPY), an iron chelator, at 37 °C, and minimal medium, M9-broth, supplemented with 20 mM gluconate, which is the principal carbon source in the intestine (Bleibtreu et al., 2013). Precultures of each of the strains were diluted 1: 100 into 100 μL of prewarmed media placed in 96-well microtiter plates. These plates were sealed (under anaerobic conditions for LB-DYP and M9-Gluconate growth curves) with a polyester impermeable film (VWR) and incubated under static conditions at 37 °C for 24 h. Samples were taken at preset intervals, adequately diluted in buffered peptone water (Oxoid), and plated in tryptic soy agar (Oxoid) supplemented with 0.6% w/v yeast extract (Oxoid, TSA-YE). These plates were incubated for 24 h at 37 °C and then colonies were manually counted. Growth curves were obtained by plotting the decimal logarithm of the number of cells (Log_{10} CFU/mL) against time, and were then fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000).

$$Y_t = Y_0 + \mu_{max}\cdot A_t - \frac{Y_{max}-Y_0}{M}\cdot ln\left[1 - e^{-M} + \left(e^{-M\cdot\frac{Y_{max}-Y_0-\mu_{max}\cdot A_t}{Y_{max}-Y_0}}\right)\right] \quad (1)$$

$$A_t = t - \lambda\cdot\left[1 - \frac{1}{h_0}\cdot ln\left(1 - e^{-h_0\cdot\frac{t}{\lambda}} + e^{-h_0\cdot\left(\frac{t}{\lambda}-1\right)}\right)\right] \quad (2)$$

where Y_t is the Log_{10} of cell concentration at time t (CFU/mL); Y_0 is the Log_{10} of the initial cell concentration (CFU/mL); Y_{max} is the Log_{10} of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (Log_{10}/h); λ is the Lag time (h); and M and h_0 are the curvature parameters, that in this study were fixed at a constant value of 10 (Baranyi and Roberts, 2000). Curve fitting was carried out using GraphPad PRISM® (GraphPad Software, San Diego, CA, USA) statistical software.

2.4. Virulence assays

2.4.1. Caco-2 cell maintenance and preparation

The human colon carcinoma Caco-2 cell line (TC7 clone) was kindly provided by Dr. Edith Brot-Laroche (Université Pierre et Marie Curie-Paris 6, UMR S 872, Les Cordeliers, France) at Passage 25 and used in experiments at Passage 30-35. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in 75 cm^2 flasks. Cells were grown in Dulbecco's Modified Eagle's Medium + Gluta-MAX™ (DMEM, Invitrogen, France) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, France), 1% Minimal Essential Medium with Non-Essential Amino Acids (MEM NEAA 100×, Invitrogen, France), and 1% antibiotics (penicillin/streptomycin, Invitrogen). Once the cells reached 80% confluence, they were dissociated with 0.05% Trypsin-1 mM EDTA (Invitrogen) and seeded at a density of approximately 15,000 cells per well in 96-well tissue culture plates (Nunc, France) containing 200 μL of complete medium per well. Plates were incubated in humidified atmosphere containing 5% CO_2 at 37 °C for 15–17 days to attain fully differentiated cell layers. Culture medium was replaced every 2 days, and cell confluence was confirmed by optical microscopy.

2.4.2. Adhesion and invasion in Caco-2 cells

Prior to use for virulence assays, cell layers were washed three times in DPBS (Dulbecco's Phosphate Buffered Saline); 200 μL of complete medium without antibiotics were added. For adhesion assay, suspensions of different *Salmonella* strains were added at an initial concentration of 10^6 CFU/mL on washed Caco-2 cells. Cells were incubated with bacteria for 30 min in humidified atmosphere containing 5% CO_2 at 37 °C. After incubation, non-adhered bacteria were removed by washing the cell cultures twice with DPBS, and the cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. These lysates were adequately diluted and then plated in Xylose Lysine Desoxycholate Agar (XLD, Oxoid). Plates were incubated at 37 °C for 24 h before manual counting of growing colonies. For invasion assays, bacterial inoculation was performed as described for

the adhesion assay, and plates were maintained in 5% CO_2 at 37 °C for 30 min. The infected cells were washed twice with DPBS, after which they were maintained during 1 h in DMEM containing 100 μg/mL of gentamicin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) per well to inactivate extracellular bacteria. After incubation, cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. Lysates were processed for determination of *Salmonella* counts as described above. The adhesion and invasion rates were calculated as percentages of adhered or invading bacteria to initial bacteria added. These percentages were calculated and represented with GraphPad software.

2.5. Biofilm formation ability assay

Biofilm formation ability of the 23 studied strains was evaluated in a 96-well microtiter plate by adapting the protocol of Patel and Sharma (2010). Briefly, overnight pre-cultures of *Salmonella* strains were diluted 1:100 in 100 μL TSB-YE media in wells of a sterile 96-well polystyrene microtiter plate (Fisher Scientific, Newark, DE) and incubated under static conditions at 37 °C. After 24, 48 and 72 h incubation in microplate culture, media was completely removed, and the wells were washed three times by immersing the plate in sterile distilled water tempered to 37 °C. The plates were air-dried for 30 min, and 125 μL crystal violet solution (0.1% w/v, Fisher Scientific) was added per well and incubated at room temperature during 20 min. Crystal violet solution was removed by washing as indicated above. To quantify biofilm formation, 125 μL of acetic acid (30% v/v) were added to each well, and the absorbance of each well at 580 nm was measured (Genios, Tecan, Männedorf, Switzerland). Thus, the concentration of crystal violet remaining in each well is proportional to the number of biofilm forming cells. For each replicate experiment, four wells were inoculated for each strain. According to the criteria suggested by Stepanovic et al. (2000) and based on the OD produced by bacterial films, strains were classified into the following categories: strong, moderate, weak, or no biofilm producers. In order to establish meaningful comparisons the area under the curve (AUC) was calculated as described in Espina et al. (2015). Briefly, the absorbance at 580 nm vs time (up to 72 h; with measurements every 24 h) was plotted for each strain and the AUC values were calculated using GraphPad software and following the trapezoid rule, where the total area is the sum of all rectangular trapezoids, each defined by two adjacent absorbance values with respect to the ground (in the y axis) and the time between those measurements (in the x axis). The formula we applied was:

$$AUC = \sum_{i=1}^{n-1} \frac{x_i \cdot (y_i + y_{i+1})}{2} \quad (3)$$

where x_i is the time between measurements in hours, y_i is the absorbance value at 580 nm for each measurement, and n is the total number of measurements.

2.6. Antibiotic resistance assays

The minimum inhibitory concentration (MIC) of seven antibiotics representative of different classes (ampicillin, chloramphenicol, nalidixic acid, oxytetracycline, rifampicin, streptomycin, and sulfanilamide) against the 23 strains under investigation was determined by Broth Dilution Susceptibility Tests. Briefly, 1 μL of bacterial pre-culture was inoculated into 100 μL of fresh TSB-YE (yielding an initial concentration of approx. 10^7 CFU/mL) with increasing concentrations of the corresponding antibiotic, and incubated for 24 h at 37 °C. The range of concentrations used to determine the MICs of antibiotic was 0 to 512 μg/mL, except for sulfonamides, for which the range was 0 to 4096 μg/mL. MICs were then determined as the lowest concentration of antibiotic that completely inhibited growth (optical absorbance equal or lower than non-inoculated wells) of each strain after 24 h of cultivation at 37 °C.

2.7. Statistical analysis

All the determinations were carried out in triplicate on different working days. Standard deviations (SD) and Pearson's and Spearman correlation coefficients were calculated using GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA). The same software was used to carry out the Iterative Grubbs's test (Alpha = 0.05) and the statistical analyses (Welch's *t*-test, ANOVA, and Tuckey tests; *p*-value < 0.05). In order to quantify and compare experimental, intra-serovar and intra-species variability the Coefficient of Variation (CV, in %) was used (CV = standard deviation × 100 / mean) as described in Lianou and Koutsoumanis (2012).

3. Results and discussion

3.1. Growth rates in different media

Growth curves of the 23 *Salmonella* strains under study were obtained in three different media: in TSB-YE, a nutrient-rich medium, in LB medium with iron limitation caused by the addition of DPY, and in a minimal medium containing gluconate as the sole carbon source. The latter two media were tested because they simulate pathophysiological conditions in the intestine, and therefore anaerobic conditions were used (Bleibtreu et al., 2013). Growth curves obtained were fitted with the Baranyi model (Baranyi and Roberts, 2000). The μ_{max} (Log_{10}/h) values calculated for each strain in the three growth media are shown in Fig. 1 (growth parameters and goodness of the fit parameters are included in Supplementary Table 2). In TSB-YE, the average μ_{max} (Log_{10}/h) for the 23 strains was 0.966 ± 0.204; the highest growth rate was that of *S.* Saintpaul (1.293 ± 0.064), and the lowest that of *S.* Gallinarum (0.457 ± 0.054). In LB-DPY, the average μ_{max} (Log_{10}/h) was 0.697 ± 0.112; the highest growth rate was determined for *S.* Senftenberg (0.862 ± 0.067), and the lowest for *S.* Gallinarum (0.342 ± 0.021). Finally, in M9-Gluconate, the average μ_{max} (Log_{10}/h) was 0.549 ± 0.092; the highest growth rate was that of *S.* Typhimurium (0.713 ± 0.033) and the lowest, as in the case of the other two media tested, that of *S.* Gallinarum (0.310 ± 0.047). None of the strains displayed a statistically significant Lag phase (h) (different from 0; $p > 0.05$) in any of the three media tested, except for *S.* Gallinarum in TSB-YE (1.05 h ± 0.433).

ANOVA analysis of the calculated μ_{max} values revealed a significant effect ($p < 0.05$) of the strain studied in the three growth media/conditions assayed (Supplementary Fig. 1). The obtained data also indicate that, among the three media tested, *Salmonella* strains displayed a higher growth rate ($p < 0.05$) in TSB-YE, a rich medium with no nutrient limitation. In LB-DPY, growth rates were reduced by 26% on average, because in this medium, iron, which is essential for bacterial growth, particularly during infection (Costa et al., 2016; Tan et al., 2019), is chelated by DPY, making it less bioavailable. Growth in M9-Gluconate imposed an even higher fitness cost (also statistically significant; $p < 0.05$), leading to an average reduction of 42% in *Salmonella* growth rates (Fig. 1). Apart from that, a significant correlation ($p < 0.05$) was observed between the maximum growth rates in TSB-YE and those in M9-Gluconate, with a Pearson correlation coefficient of 0.536 ($p = 0.008$) and a Spearman correlation coefficient of 0.437 ($p = 0.037$). A significant correlation was also observed when comparing the μ_{max} values obtained in TSB-YE and LB-DYP, with a Pearson correlation coefficient of 0.593 ($p = 0.003$) although in this latter case the significance of the Spearman correlation test only indicated a trend ($p < 0.1$) ($r_s = 0.382$, $p = 0.072$). Similarly, a significant correlation was found between the maximum growth rates calculated in LB-DPY and M9-Gluconate (Pearson r = 0.522, $p = 0.011$), but according to the Spearman rank correlation coefficient it was only a trend ($r_s = 0.388$, $p = 0.067$). These results would suggest that those strains that display a higher growth rate under non-limiting conditions would also display a higher growth rate in

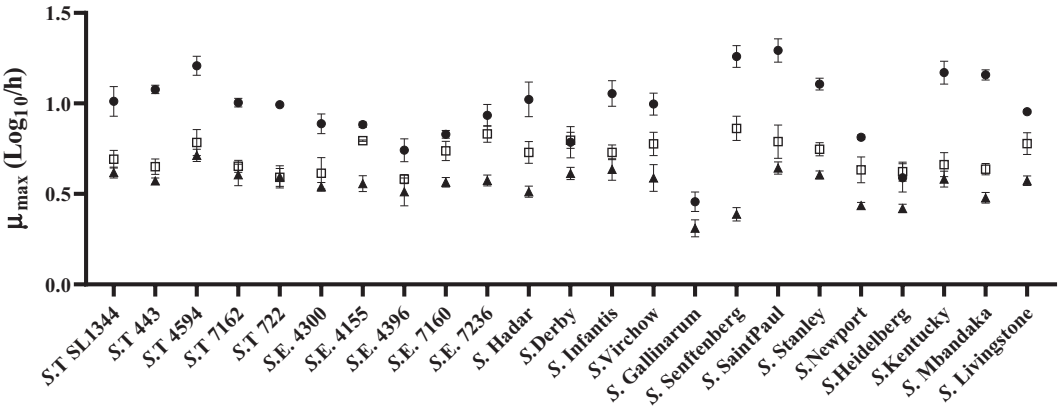

Fig. 1. Maximum growth rates (μ_{max} (Log_{10}/h)) of the 23 *Salmonella enterica* strains studied in TSB-YE (●), LB supplemented with 100 μM 2-2′dipyridyl (DPY) (□) and M9-broth supplemented with 20 mM gluconate (▲) at 37 °C. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between strains.

media with reduced amounts of Fe or with gluconate as the sole carbon source. However, it should be noted that a high degree of experimental variability was observed, and that correlations were not significant if *S.* Gallinarum was excluded from the analysis. These conclusions should be therefore taken with care.

On the other hand, no significant differences were found when comparing the average μ_{max} value in TSB-YE of either the *S.* Enteritidis strains (0.855 ± 0.074; n = 5) or the *S.* Typhimurium strains (1.059 ± 0.090; n = 5) with the average μ_{max} value of the other 13 strains tested (non-Enteritidis and non-Typhimurium strains; 0.974 ± 0.252; n = 13) or with the overall average value of all the 23 strains (0.967 ± 0.204 n = 23). Similar results were obtained for the other two media tested (Fig. 2). Still, in TSB-YE and M9-Gluconate, the average μ_{max} values of *S.* Typhimurium were significantly higher than those of *S.* Enteritidis strains. It should also be noted that inter-serovar variability (CV = standard deviation × 100 / mean) tended to be slightly higher (up to 2.8 times) than intra-serovar variability in these latter two media. These conclusions should nevertheless be taken with caution too, since the number of strains per serovar was low (5), and comparisons were established among groups with a different number of strains (5 vs 13 vs 23). Further work would therefore be required to validate them.

Most of the available studies dealing with the growth ability of *S. enterica* strains/serovars have reported a small variability in growth parameters. Thus, Juneja et al. (2003) concluded that slight variations in kinetic parameters among *Salmonella* strains were not associated with any serovar effect, but merely reflected an experimental variability, while Lianou and Koutsoumanis (2011) observed that differences in growth rate among 60 *Salmonella* strains mainly depended on composition of growth medium; furthermore, this variability was not related to the *Salmonella* serovar under any of the growth conditions tested. Díez-García et al. (2012) observed an up to 4-fold change among the growth kinetic parameters determined for a total of 69 *S. enterica* strains belonging to 10 serovars growing in TSB at 37 °C, conditions very close to one of those studied herein; even in this case, however, these differences can be considered small if compared to the variability of other phenotypical characteristics. Mutations conferring a growth advantage have already been described for *Salmonella* and the closely related *E. coli*. Thus, competitive fitness and/or growth rates can be increased through mutations in genes affecting general gene expression, e.g., mutations in *rpoA/B/S* and *arcA* (Knöppel et al., 2018; Saxer et al., 2014), or in genes directly associated with the use of certain resources, e.g., mutations in pyruvate kinase I (PykF) or *glpK*, which allow a better utilization of glucose or glycerol as a source of carbon and energy. This suggests that, some strains displaying increased growth rates would at least sporadically appear, and/or that some specific serovars might, in the same way, display an increased fitness under very specific growth conditions (e.g. those governing in their particular niche), which, in turn, would contrast with the low variability in growth rate observed in most studies. It should nevertheless be noted that most of these studies have been carried out in non-selective media and under very favorable growth conditions. In addition, this low variability might also be explained, at least partially, because bacteria would make use of several strategies, rather than a single strategy, to optimize their fitness, as suggested by Knöppel et al. (2018).

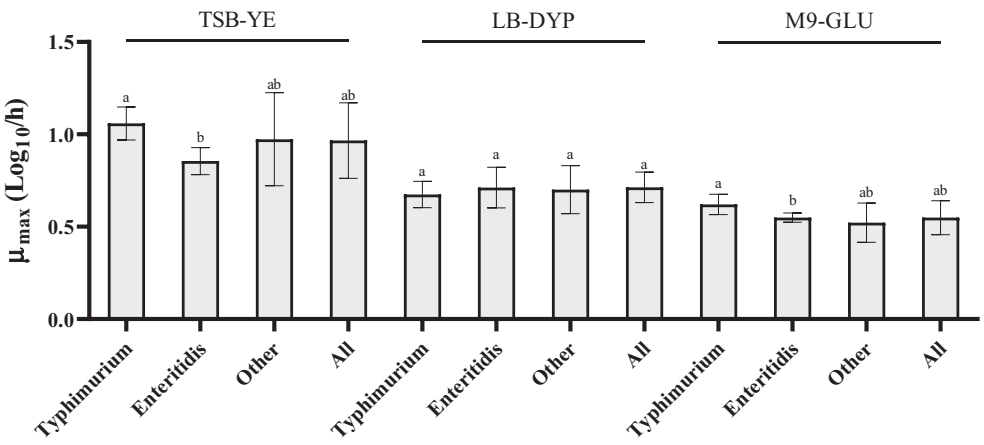

Fig. 2. Average μ_{max} (Log_{10}/h) values of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella enterica* strains studied (All) in TSB-YE, LB supplemented with 100 μM 2-2′dipyridyl and M9 supplemented with 20 mM gluconate at 37 °C. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups.

3.2. Caco-2 cell adhesion and invasion

The percentage of cells capable of adhering to the Caco-2 layer ranged from 0.47% to 6.95%, with *S.* Newport showing the lowest capacity to adhere to Caco-2 cells and *S.* Saintpaul the highest (Fig. 3A). On the other hand, invasion ability varied between <0.01% and 1.02%. *S.* Gallinarum and *S.* Newport showed the lowest capacity to invade Caco-2 and *S.* Enteritidis 7160 the highest (Fig. 3B). Statistical analysis (ANOVA) revealed that the strain under study had a significant effect on the rates of adhesion and invasion ($p < 0.05$) (Fig. 3A and B), and also that significant differences existed between the rate of adhesion of *S.* Enteritidis and *S.* Typhimurium and between the rate of invasion of *S.* Typhimurium and of those strains not belonging to serovars Enteritidis or Typhimurium (Fig. 4). It should also be noted that considerable variation in the rate of adhesion and invasion among strains belonging to the same serovar was observed. Thus, up to 4-fold differences in adhesion ability were found among the five different strains of the Typhimurium serovar, and almost 3-fold changes were found for the five strains of the serovar Enteritidis. On the contrary, the highest intra-serovar variability in invasivity was displayed by the serovar Enteritidis (CV = 124%), which included the strains with the highest and lowest ability to invade enterocytes among all the strains tested, excluding *S.* Gallinarum and Newport.

Nevertheless, it should be noted that experimental variability among biological replicates was very high, with a Coefficient of Variation (3 biological replicates) for invasion assays of 56% on average, and up to 91% for certain strains such as *S.* Enteritidis 7160, a phenomenon that might be masking actual differences among strains/serovars.

The Grubbs's test detected as a possible outlier the invasion value obtained for *S.* Enteritidis 7160; it was therefore eliminated to perform the correlation analysis. No association was found between adhesion and invasion ($r = -0.109$, $p = 0.630$, $r_s = -0.115$, $p = 0.610$). The process of adhesion and invasion of cells by *Salmonella* spp. has been widely studied using the intestinal epithelial cell line Caco-2 (Dostal et al., 2014; Gagnon et al., 2013; McWhorter et al., 2015). The variability in adhesion ability to Caco-2 cells among *Salmonella* strains

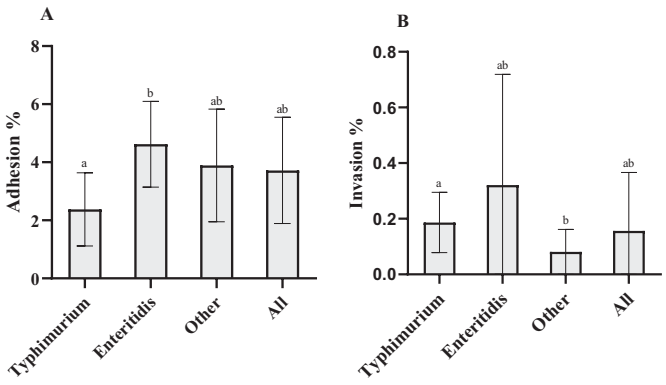

Fig. 4. Adhesion (A) and invasion capacity (B) to Caco-2 cells of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella* strains studied (All). Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups.

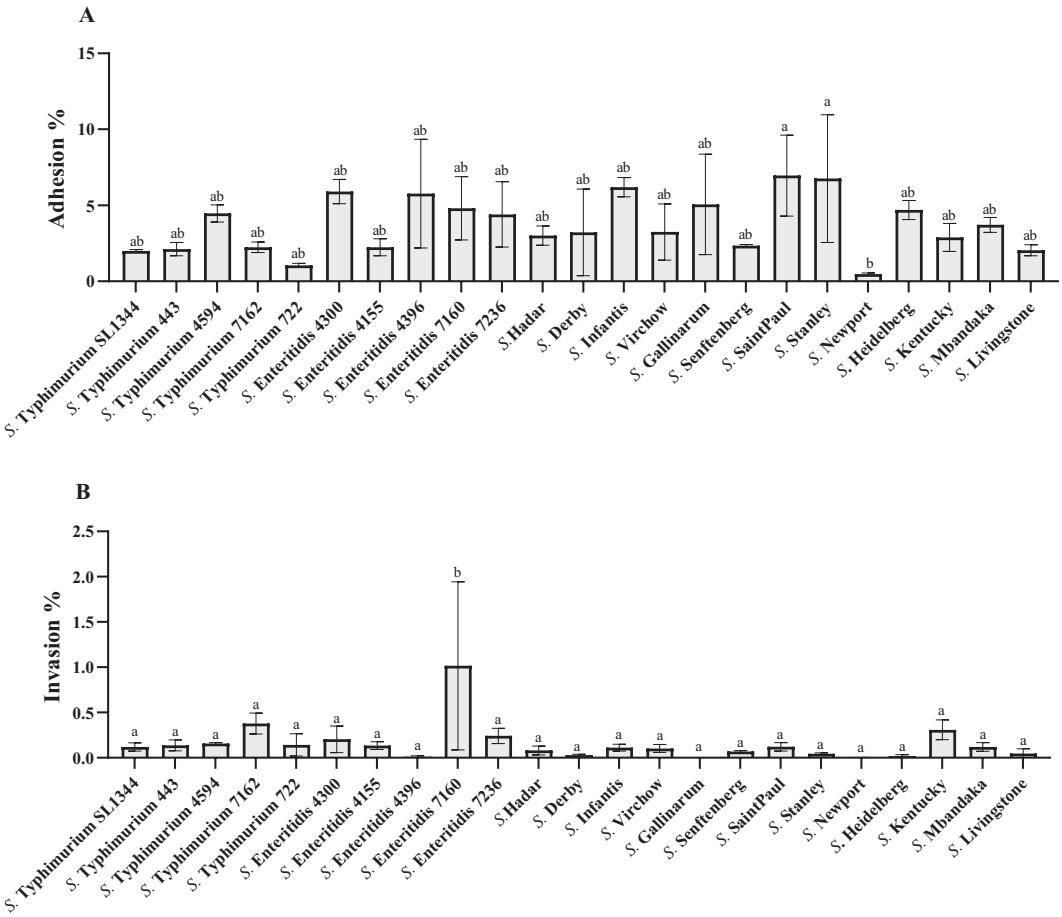

Fig. 3. Adhesion (A) and invasion (B) capacity to CaCo-2 cells of the 23 strains of *Salmonella enterica* studied. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between strains.

observed in this study is in accordance with those reported in the work of Gagnon et al. (2013), who indicated that such differences in adhesion were highly dependent on the serovar and, at the same time, dependent on the expression of genes encoding protein secretion systems, effector proteins and chaperones, and/or transcriptional regulators. Differences in adhesion ability among serovars might be explained by the fact that the majority of *Salmonella* strains possess serotype-specific virulence plasmids, which are low-copy-number plasmids (1 to 2 copies per cell) ranging from 50 to 100 kb, depending on the serovar (van Asten and van Dijk, 2005). In addition, and as observed here, considerable variation in the rate of adhesion and invasion among strains belonging to the same serovar and even possessing the same virulence genes has also been reported (Dostal et al., 2014; McWhorter et al., 2015).

On the other hand, the invasion capacity of the strains included in this study was low, similarly to that observed by McWhorter et al. (2015). Although it has been demonstrated that the adhesion and invasion processes are coordinated, different pathways modulate these separate virulence mechanisms (Velge et al., 2012), which would explain why strains displaying a high adhesion ability are not always the most invasive ones (e.g. *S.* Stanley). Possession of serotype-specific virulence plasmids would explain, at least partially, the differences in invasion ability among serovars, as indicated above for adhesion. Substantial differences in virulence among strains of the same serovar (Enteritidis) have also been reported (Shah, 2014). Transcriptional analysis has revealed that *S.* Enteritidis strains with low pathogenicity displayed reduced expression of several transcriptional regulators, reduced expression of genes involved in virulence (e.g., *Salmonella* pathogenicity island 1 (SPI-1), SPI-5, as well as fimbrial and motility genes), and protection against osmotic, oxidative, and other stresses, such as iron-limiting conditions commonly encountered within the host. It should also be noted that environmental conditions are known to influence the expression of *Salmonella* virulence genes; therefore, growth, pre-invasion and invasion conditions can significantly affect the invasiveness of *S.* Typhimurium as well as the ability of the bacteria to replicate intracellularly (Dostal et al., 2014; Foster et al., 2001; Ibarra et al., 2010; Kortman et al., 2012; Tan et al., 2019). As a way of example, McWhorter et al. (2015) observed substantial differences in the invasion ability of *Salmonellae* depending on the environment in which the *Salmonella* cells were grown – normal saline or LB broth – with greater invasion capacity observed in the latter. Similarly, it has been demonstrated that the concentration of iron, both before and during adhesion and invasion assays, can significantly affect the rates of *Salmonella* adhesion and invasion (Dostal et al., 2014; Foster et al., 2001; Kortman et al., 2012; Tan et al., 2019). Further work will thus be required to determine if the differences in adhesion and invasion ability among strains and serovars reported herein would also exist in more complex media that simulate gut conditions more closely.

Finally, it should be noted that a lower invasion capacity of *S.* Gallinarum, as compared to *S.* Enteritidis, has also been previously reported in chicken and human epithelial cell lines (Rossignol et al., 2014). By contrast, although data regarding the adhesion and invasion ability of *S.* Newport have been reported (Deekshit et al., 2015), further work would be required to elucidate whether the low adhesion and invasion abilities (at least in relative terms) of the *S.* Newport strain used in our study are specific of this strain, or whether they are a common feature of the whole serovar.

3.3. Static biofilm formation ability

The ability to form biofilms is a well-known phenotypic characteristic of *Salmonella* cells. The results of the static biofilm formation assay are shown in Fig. 5. It should be noted that, in order to establish meaningful comparisons, the AUC values calculated as described in materials and methods were compared. The average value for this parameter of the 23 tested strains of *Salmonella* spp. was 4.7 ± 6.0: The *S.* Gallinarum strain was the one with the lowest biofilm formation capacity (0.92 ± 0.94), while the *S.* Senftenberg strain showed maximum biofilm formation capacity (28.9 ± 4.2). This biofilm formation ability of the *S.* Senftenberg strain is remarkable and will be discussed below. Thus, the biofilm formation ability value of *S.* Senftenberg was 6 times higher than the average value of all the 23 *Salmonella* strains, and twice that of the strain with the second highest formation capacity (*S.* Typhimurium 722). These two strains can also be classified – as likewise described in "Materials and methods"– as strong biofilm producers. *S.* Typhimurium STCC 7162, *S.* Derby, *S.* Saintpaul, *S.* Stanley, *S.* Newport, and *S.* Livingstone strains were moderate biofilm producers, and the rest of the strains were weak biofilm producers. On the other hand, intra-serovar variability in biofilm formation was higher for *S. Typhimurium* strains (more than 9-fold difference between the strain with the highest and the lowest ability; CV = 99.2%) than for *S.* Enteritidis strains (less than 2-fold; CV = 21.1%), but still lower than the overall inter-serovar variability (almost 16-fold; CV = 126%, even if *S.* Senftenberg is excluded from the analysis). In spite of this high variability, Welch's *t*-test indicated that Enteritidis strains displayed (on average) a lower biofilm ability than strains belonging to serovars other than Enteritidis and Typhimurium (Fig. 6).

The ability of *Salmonellae* to form biofilms on polystyrene surfaces (a hydrophobic material) is well documented, as is the above-described existence of wide differences among strains and serovars. Furthermore, various authors have already suggested the existence of serovar-

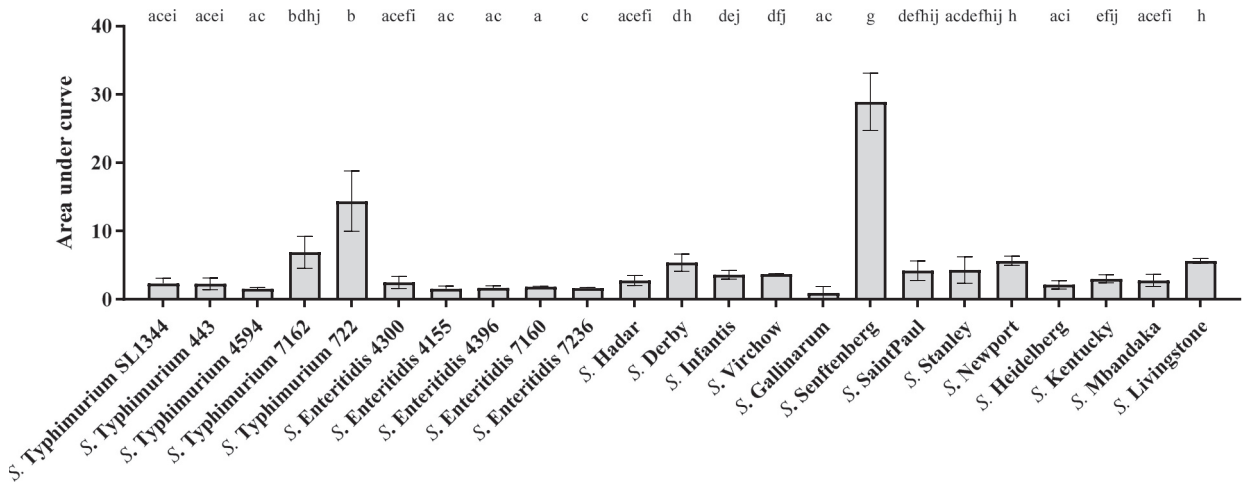

Fig. 5. Biofilm-forming ability of the 23 strains of *Salmonella enterica* studied. Values correspond to the Area under the Curve calculated as described in Material and methods. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between strains.

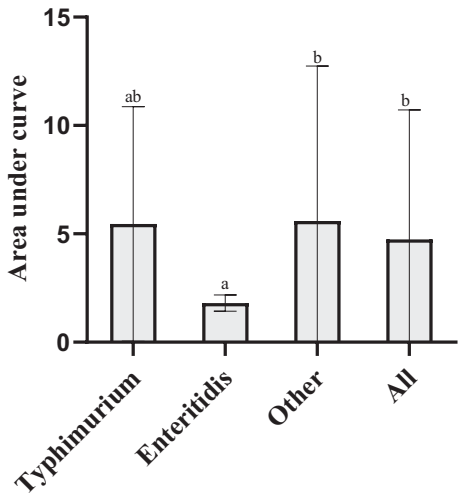

Fig. 6. Biofilm-forming ability of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella* strains studied (All). Values correspond to the Area under the Curve calculated as described in Material and methods. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups.

specific attachment mechanisms (Berger et al., 2009; Klerks et al., 2007), which is the first step of the biofilm formation process, and Patel and Sharma (2010) indicated that attachment to lettuce leaves and subsequent biofilm formation by *Salmonella* strains may differ depending on the specific properties of the serovars. Similarly, Díez-García et al. (2012) observed substantial differences in terms of biofilm forming ability among the *Salmonella* serovars they tested, and reported a variability similar to that found here in the biofilm forming ability of Typhimurium and Enteritidis strains. Furthermore, Vestby et al. (2009) found a clear difference in terms of biofilm-forming capability among 116 strains belonging to four serovars. Their results also suggested that the type of serovar would exert an important influence on biofilm formation. Nevertheless, although the latter authors determined that strains of the Typhimurium serovar were relatively poor biofilm producers, our results indicate that Typhimurium strains display a high variability in biofilm formation capacity, with strain 722 being a strong producer, and 7162 a moderate producer. This wide intra-serovar variability might explain why some other authors did not find any relationship between the biofilm-forming ability of strains and their serovar (Lianou and Koutsoumanis, 2012). On the other hand, and as pointed out above, the strain of Senftenberg used in this study was classified as a strong biofilm former, ranking above all the other strains studied. Unluckily, although currently only limited information is available regarding the ability of *S.* Senftenberg to form biofilms, it should be noted that Xia et al. (2009), who studied biofilm formation in 16 *Salmonella* isolates from retail foods, observed that Senftenberg isolates were most prolific in biofilm formation, and Vestby et al. (2009), using a microtiter plate assay over prolonged incubation periods, reported that serovar Senftenberg strains were the only ones that displayed a significant increase in OD values from day two to four. Our results are also consistent with previous investigations indicating that *S.* Gallinarum, together with other host-specific serovars, is a weak biofilm producer (MacKenzie et al., 2017).

3.4. Resistance to antibiotics

As can be observed in Table 1, substantial differences were observed in MIC values against the seven tested antibiotics depending on the strain, although these differences were considerably more marked for certain groups of antibiotics. *S.* Typhimurium 7162 generally showed the highest resistance to all antibiotics under study: it was the most resistant to ampicillin, chloramphenicol, and oxytetracycline. In contrast, *S.* Enteritidis 4396 can be considered the most sensitive to the antibiotics included in our study. If the results corresponding to each antibiotic are analysed separately, one can observe that all strains except *S.* Typhimurium 7162 had MICs below 20 μg/mL for ampicillin (between 2 and 16 μg/mL). Resistances to chloramphenicol and oxytetracycline laid between 4 and 16 μg/mL. For chloramphenicol, however, three strains had higher MIC values (*S.* Infantis and *S.* Senftenberg, 32 μg/mL and *S.* Typhimurium 7162, 128 μg/mL), while a further three strains had higher MIC values for Oxytetracycline (*S.* Infantis and *S.* Enteritidis 4300 32 μg/mL and *S.* Typhimurium 7162 128 μg/mL). For nalidixic acid, the range of observed MICs was wider (between 1 and 64 μg/mL), with *S.* Typhimurium 722 and *S.* Enteritidis 7160 not inhibited, even at the higher concentration tested (64 μg/mL). The MICs

Table 1
Minimum inhibitory concentrations (MIC) for the different antibiotics of the 23 *Salmonella enterica* strains tested. Units in μg/mL.

	Ampicillin	Chloramphenicol	Nalidixic acid	Oxytetracycline	Rifampicin	Streptomycin	Sulfonamide
*S.*T SL1344	4	4	8	1	16	256	4096
*S.*T 443	8	8	32	8	16	64	4096
*S.*T 4594	8	16	64	8	16	>512	4096
*S.*T 7162	>512	128	64	512	32	512	4096
*S.*T 722	8	8	>64	8	16	>512	4096
S. E. 4300	8	8	8	32	16	128	4096
S. E. 4155	2	4	4	2	16	16	>4096
S. E. 4396	2	4	1	1	8	16	>4096
S. E. 7160	1	4	>64	1	16	16	>4096
S. E. 7236	4	4	16	8	16	16	>4096
S. Hadar	16	8	64	8	16	>512	2048
S. Derby	16	8	64	8	16	256	4096
S. Infantis	16	32	64	32	>64	>512	4096
S. Virchow	8	16	16	4	16	128	4096
S. Gallinarum	2	4	64	2	16	>512	4096
S. Senftenberg	16	32	32	8	32	128	4096
S. SaintPaul	8	8	64	8	16	>512	4096
S. Stanley	8	8	16	8	32	128	4096
S. Newport	2	4	4	2	8	128	4096
S. Heidelberg	8	4	8	4	8	>512	4096
S. Kentucky	8	8	8	4	>64	64	>4096
S. Mbandaka	8	8	8	4	16	128	4096
S. Livingstone	8	8	32	8	>64	64	4096

T: Typhimurium. E: Enteritidis.

determined for rifampicin ranged from 8 to 64 μg/mL, with a particular higher resistance to this antibiotic of Kentucky, Livingstone and Infantis strains; against streptomycin, most of the strains showed values higher than 128 μg/mL, whereby the most sensitive strains belonged to the Enteritidis serovar (STCC 4155, 4396, 7160 and 7236). All strains displayed a MIC equal or superior to 4096 μg/mL against sulfonamides, except for *S.* Hadar, with a MIC of 2048 μg/mL. Regarding the comparison between serovars, and the comparison between intra-serovar and inter-serovar variability, and excluding *S.* Typhimurium 7162 from the analysis because of its high resistance levels, statistical analysis indicates that resistance to all the antibiotics studied would generally be comparable among *S.* Typhimurium, *S.* Enteritidis and all the other strains, as would be intra- and inter-serovar variability. In any case, it should be noted that resistance to ampicillin, chloramphenicol, and oxytetracycline on the part of the *S.* Enteritidis and Typhimurium strains featured in this study tended to be lower than that of the other serovars (analysed together) (Fig. 7).

It is well known that bacterial antibiotic resistance is determined by many factors, such as membrane permeability, the level of expression of various proteins, or the presence and expression of certain genes, among others (Beceiro et al., 2013). The resistance of microbial cells to a particular antibiotic would thus be due to the sum of the strain's intrinsic resistance, the resistance it has developed to attempt to adapt to the new stimulus, and the resistance it may have acquired by horizontal gene transfer (Schwarz et al., 2005). Although this also implies that the presence of a certain antibiotic resistance gene in two different strains does not mean that both will have the same degree of resistance to that antibiotic, the impact exerted by that presence (and expression) of certain genes/mutations upon microbial antibiotic resistance is more than well known, in any case, and has been extensively studied. Therefore, in order to try to determine if the differences in antibiotic resistance among strains were associated with the presence of any of those antibiotic genes, we decided to perform a basic *in-silico* analysis in which we searched for the presence of certain of those *Salmonella* resistance genes and/or mutations conferring resistance to the groups of antibiotics tested in this study, which are the same as those included in Li et al. (2019), in the publicly available genome sequences (NCBI and ENA) of the strains studied here. Unfortunately, to the best of our knowledge, the complete genome sequence (chromosome + plasmids if found) is only publicly available for seven of the 23 strains included in our study: *S.* Typhimurium SL1344, *S.* Typhimurium 443, *S.* Typhimurium 4594, *S.* Typhimurium 722, *S.* Saintpaul, *S.* Stanley and *S.* Newport. Our analysis revealed that none of the β-lactam, chloramphenicol, quinolone, tetracycline, or sulfonamide resistance genes studied was present in any of those seven strains. Similarly, no mutations in the *rpoB* gene, which have been linked to rifampicin resistance, were found (Brandis and Hughes, 2018). Only genes conferring resistance to aminoglycosides (*aac(6′)-Ib* and *aadA1*) were found in some of the strains, but no clear relationship between their presence and an increased resistance to streptomycin was ascertained (Supplementary Table 3). This almost complete absence of antibiotic resistance genes might be related to the fact that all of the strains used in this study were obtained from collections, and it would suggest that the differences in antibiotic resistance of these 7 strains would be more closely associated with differences in their intrinsic resistance. On the other hand, the sizeable differences between *S. Typhimurium* 7162 and all the other strains in terms of resistance to several of the antibiotics studied suggest either that this strain would have acquired this multiple resistance through horizontal gene transfer, or that its physiology/phenotypic characteristics would differ widely from all the other strains. Further work would be required to verify these two hypotheses.

Finally, multiple correlations have been obtained between the MIC values of the different strains (Table 2 and Supplementary Table 4). Multiple antibiotic resistance is usually attributed to the fact that genes conferring antimicrobial resistance in *Salmonella* are usually transported in integrons and plasmids (Chen et al., 2004; Schwarz et al., 2005); however, as previously pointed out, this does not seem to be the case, at least for some of the strains studied here. Despite the interest inherent in these obtained correlations, they lie outside of the scope of this article and will not be further discussed here.

3.5. Relationship between growth rates, virulence, phenotypic characteristics, and resistance to food processing-related stresses

We correlated the growth, virulence, biofilm-forming ability, and antibiotic resistance parameters ascertained in this study with one another and with the resistance parameters (*2D*-values) to different environmental stresses and food technologies as previously determined in Guillén et al. (2020a, 2020b) for the same set of strains. The rationale behind the choice of the *2D*-value parameter (time required to inactivate the first 2-Log10 cycles) was already discussed in Guillén et al. (2020a). The iterative Grubbs's test was applied to identify potential outliers that could exert a disproportionate influence on further data analysis and lead to non-valid conclusions. Grubbs's test detected multiple outliers:

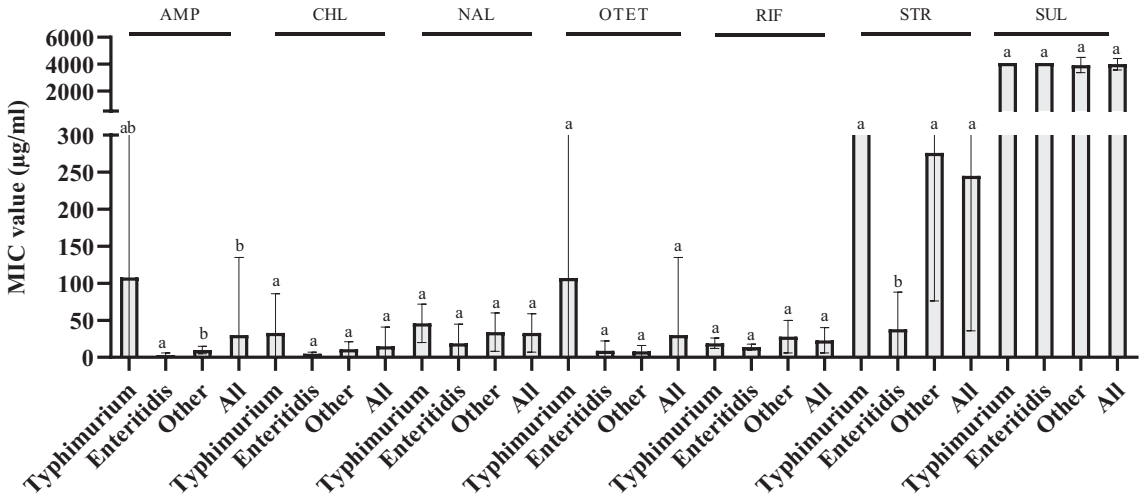

Fig. 7. Minimum inhibitory concentrations (MIC) for the different antibiotics of the *Salmonella enterica* strains belonging to serovar Typhimurium (Typhimurium), serovar Enteritidis (Enteritidis), other serovars (non-Enteritidis and non-Typhimurium strains; Other) and of the 23 *Salmonella* strains studied (All). Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between groups. Ampicillin (AMP); Chloramphenicol (CHL); Nalidixic acid (NAL); Oxytetracycline (OTET); Rifampicin (RIF); Streptomycin (STR); Sulfonamide (SUL).

Table 2
Spearman's correlation coefficient values calculated for the *2D* resistance values (obtained in Guillén et al., 2020a, 2020b), μ_{max} values in the 3 media studied (see text), adhesion and invasion ability (%), biofilm formation capacity (AUC) and MIC values of each antibiotic of the 23 *Salmonella enterica* strains studied. Values in parentheses correspond to the *p*-value.

	pH	H_2O_2	NaCl	Heat	HHP	PEF	UV	TSB-YE	LB-DPY	M9-gluconate	Adhesion	Invasion	Biofilm	AMP	CHL	NAL	OTET	RIF	STR
pH		0.139	0.160	−0.251	**0.454**	0.134	−0.070	−0.194	0.279	−0.138	**0.433**	**−0.484**	**−0.447**	−0.117	−0.256	0.268	−0.163	−0.290	−0.049
		(0.526)	(0.467)	(0.260)	**(0.030)**	(0.541)	(0.750)	(0.376)	(0.197)	(0.530)	**(0.039)**	**(0.022)**	**(0.037)**	(0.603)	(0.250)	(0.215)	(0.467)	(0.179)	(0.826)
H_2O_2	0.139		0.311	−0.041	0.018	0.103	−0.042	−0.042	0.071	−0.282	−0.260	−0.018	0.012	−0.179	−0.294	−0.033	−0.242	0.002	−0.150
	(0.526)		(0.149)	(0.857)	(0.936)	(0.641)	(0.851)	(0.847)	(0.749)	(0.193)	(0.231)	(0.938)	(0.956)	(0.426)	(0.185)	(0.882)	(0.279)	(0.992)	(0.494)
NaCl	0.160	0.311		−0.256	0.009	**0.643**	0.282	0.156	**0.431**	**0.415**	−0.098	0.334	−0.115	−0.417	−0.313	0.009	−0.248	0.109	−0.380
	(0.467)	(0.149)		(0.250)	(0.968)	**(0.001)**	(0.193)	(0.478)	**(0.040)**	**(0.049)**	(0.656)	(0.128)	(0.610)	(0.054)	(0.156)	(0.967)	(0.267)	(0.621)	(0.074)
Heat	−0.251	−0.041	−0.256		−0.133	−0.281	0.324	0.227	−0.253	−0.157	−0.293	0.096	0.143	0.342	0.307	0.196	0.223	0.373	0.185
	(0.260)	(0.857)	(0.250)		(0.554)	(0.206)	(0.142)	(0.310)	(0.256)	(0.484)	(0.185)	(0.678)	(0.526)	(0.129)	(0.176)	(0.381)	(0.330)	(0.088)	(0.411)
HHP	**0.454**	0.018	0.009	−0.133		0.128	−0.140	−0.238	0.171	−0.154	−0.126	−0.290	−0.062	−0.226	−0.347	0.178	−0.216	−0.261	0.103
	(0.030)	(0.936)	(0.968)	(0.554)		(0.559)	(0.523)	(0.274)	(0.434)	(0.484)	(0.567)	(0.191)	(0.785)	(0.312)	(0.113)	(0.417)	(0.334)	(0.228)	(0.641)
PEF	0.134	0.103	**0.643**	−0.281	0.128		0.258	0.021	**0.430**	0.337	0.022	0.350	−0.191	−0.233	−0.163	0.164	0.218	0.239	−0.271
	(0.541)	(0.641)	**(0.001)**	(0.206)	(0.559)		(0.235)	(0.925)	**(0.041)**	(0.116)	(0.920)	(0.111)	(0.393)	(0.296)	(0.468)	(0.455)	(0.329)	(0.272)	(0.210)
UV	−0.070	−0.042	0.282	0.324	−0.140	0.258		0.226	0.255	0.270	−0.049	**0.426**	−0.097	0.187	0.128	0.028	0.193	0.404	−0.191
	(0.750)	(0.851)	(0.193)	(0.142)	(0.523)	(0.235)		(0.299)	(0.240)	(0.212)	(0.823)	**(0.048)**	(0.667)	(0.406)	(0.570)	(0.900)	(0.390)	(0.056)	(0.382)
TSB-YE	−0.194	−0.042	0.156	0.227	−0.238	0.021	0.226		0.382	**0.437**	0.024	**0.455**	0.233	**0.507**	**0.697**	0.222	**0.429**	**0.541**	0.168
	(0.376)	(0.847)	(0.478)	(0.310)	(0.274)	(0.925)	(0.299)		(0.072)	**(0.037)**	(0.914)	**(0.034)**	(0.297)	**(0.016)**	**(0.000)**	(0.309)	**(0.046)**	**(0.008)**	(0.443)
LB-DPY	0.279	0.071	**0.431**	−0.253	0.171	**0.430**	0.255	0.382		0.388	0.004	0.170	0.014	0.296	0.333	0.227	0.289	0.371	−0.192
	(0.197)	(0.749)	**(0.040)**	(0.256)	(0.434)	**(0.041)**	(0.240)	(0.072)		(0.067)	(0.986)	(0.449)	(0.950)	(0.181)	(0.130)	(0.298)	(0.191)	(0.081)	(0.381)
M9-gluconate	−0.138	−0.282	**0.415**	−0.157	−0.154	0.337	0.270	**0.437**	0.388		0.093	**0.436**	0.343	0.254	0.389	0.403	0.352	0.374	0.265
	(0.530)	(0.193)	**(0.049)**	(0.484)	(0.484)	(0.116)	(0.212)	**(0.037)**	(0.067)		(0.674)	**(0.042)**	(0.118)	(0.254)	(0.074)	(0.056)	(0.108)	(0.078)	(0.221)
Adhesion	**0.433**	−0.260	−0.098	−0.293	−0.126	0.022	−0.049	0.024	0.004	0.093		−0.115	−0.315	0.036	0.087	0.143	0.206	−0.021	0.115
	(0.039)	(0.231)	(0.656)	(0.185)	(0.567)	(0.920)	(0.823)	(0.914)	(0.986)	(0.674)		(0.610)	(0.154)	(0.874)	(0.702)	(0.515)	(0.358)	(0.924)	(0.601)
Invasion	**−0.484**	−0.018	0.334	0.096	−0.290	0.350	**0.426**	**0.455**	0.170	**0.436**	−0.115		0.020	0.100	0.256	0.125	0.371	0.338	−0.067
	(0.022)	(0.938)	(0.128)	(0.678)	(0.191)	(0.111)	**(0.048)**	**(0.034)**	(0.449)	**(0.042)**	(0.610)		(0.931)	(0.665)	(0.263)	(0.578)	(0.098)	(0.124)	(0.768)
Biofilm	**−0.447**	0.012	−0.115	0.143	−0.062	−0.191	−0.097	0.233	0.014	0.343	−0.315	0.020		**0.487**	**0.439**	0.206	0.362	0.357	0.273
	(0.037)	(0.956)	(0.610)	(0.526)	(0.785)	(0.393)	(0.667)	(0.297)	(0.950)	(0.118)	(0.154)	(0.931)		**(0.025)**	**(0.047)**	(0.357)	(0.107)	(0.103)	(0.220)
Ampicillin	−0.117	−0.179	−0.417	0.342	−0.226	−0.233	0.187	**0.507**	0.296	0.254	0.036	0.100	**0.487**		**0.814**	0.420	**0.758**	**0.471**	**0.481**
	(0.603)	(0.426)	(0.054)	(0.129)	(0.312)	(0.296)	(0.406)	**(0.016)**	(0.181)	(0.254)	(0.874)	(0.665)	**(0.025)**		**(0.000)**	(0.052)	**(0.000)**	**(0.027)**	**(0.023)**
Chloramphenicol	−0.256	−0.294	−0.313	0.307	−0.347	−0.163	0.128	**0.697**	0.333	0.389	0.087	0.256	**0.439**	**0.814**		**0.433**	**0.697**	**0.598**	0.329
	(0.250)	(0.185)	(0.156)	(0.176)	(0.113)	(0.468)	(0.570)	**(0.000)**	(0.130)	(0.074)	(0.702)	(0.263)	**(0.047)**	**(0.000)**		**(0.044)**	**(0.000)**	**(0.003)**	(0.134)
Nalidixic acid	0.268	−0.033	0.009	0.196	0.178	0.164	0.028	0.222	0.227	0.403	0.143	0.125	0.206	0.420	**0.433**		**0.459**	0.350	**0.566**
	(0.215)	(0.882)	(0.967)	(0.381)	(0.417)	(0.455)	(0.900)	(0.309)	(0.298)	(0.056)	(0.515)	(0.578)	(0.357)	(0.052)	**(0.044)**		**(0.032)**	(0.101)	**(0.005)**
Oxytetracycline	−0.163	−0.242	−0.248	0.223	−0.216	0.218	0.193	**0.429**	0.289	0.352	0.206	0.371	0.362	**0.758**	**0.697**	**0.459**		**0.474**	0.329
	(0.467)	(0.279)	(0.267)	(0.330)	(0.334)	(0.329)	(0.390)	**(0.046)**	(0.191)	(0.108)	(0.358)	(0.098)	(0.107)	**(0.000)**	**(0.000)**	**(0.032)**		**(0.026)**	(0.134)
Rifampicin	−0.290	0.002	0.109	0.373	−0.261	0.239	0.404	**0.541**	0.371	0.374	−0.021	0.338	0.357	**0.471**	**0.598**	0.350	**0.474**		0.019
	(0.179)	(0.992)	(0.621)	(0.088)	(0.228)	(0.272)	(0.056)	**(0.008)**	(0.081)	(0.078)	(0.924)	(0.124)	(0.103)	**(0.027)**	**(0.003)**	(0.101)	**(0.026)**		(0.932)
Streptomycin	−0.049	−0.150	−0.380	0.185	0.103	−0.271	−0.191	0.168	−0.192	0.265	0.115	−0.067	0.273	**0.481**	0.329	**0.566**	0.329	0.019	
	(0.826)	(0.494)	(0.074)	(0.411)	(0.641)	(0.210)	(0.382)	(0.443)	(0.381)	(0.221)	(0.601)	(0.768)	(0.220)	**(0.023)**	(0.134)	**(0.005)**	(0.134)	(0.932)	

Ampicillin (AMP); Chloramphenicol (CHL); Nalidixic acid (NAL); Oxytetracycline (OTET); Rifampicin (RIF); Streptomycin (STR).
Significant correlations are indicated in bold.

the *2D*-value to heat of *S.* Senftenberg 775W, invasion percentage of *S.* Enteritidis 7160, biofilm percentage of *S.* Senftenberg 775W, and the MIC values of *S.* Typhimurium 7162 for ampicillin, chloramphenicol, and oxytetracycline. These values, together with the MICs for sulfonamides, were therefore excluded from subsequent analysis. Several correlations ($p < 0.05$) and trends ($p < 0.10$) were found using the Pearson's and Spearman's tests (Table 2 and Supplementary Table 4); the most relevant ones will be discussed below.

It is widely assumed that stress resistance implies a fitness cost for bacteria, although its magnitude seems to depend on the nature of the stressing agent/microbial response triggered (Karatzas et al., 2008a, 2008b; Urdaneta et al., 2019). However, in our case, no significant (inverse) correlation between stress resistance and fitness cost was observed for the 23 strains studied here. Moreover, a correlation was found between Pulsed Electric Fields (PEF) and NaCl resistance and the growth parameters in LB-DYP and M9-Gluconate, but not in TSB-YE (Table 2 and Supplementary Table 4). Besides the fact that the results obtained here -with only 23 strains- cannot be directly extrapolated to the whole *Salmonellae*, we should also recall that variability in stress resistance and in growth rates among the strains of *Salmonella* were both low (less than 3-fold). This, together with the experimental variability inherent to these types of determinations, might be hindering the existence of such a relationship or might be leading to the appearance of casual, but not causal, relationships. Thus, further work would be required to elucidate the actual nature of such relationships, especially regarding the relationship between PEF, NaCl resistance, and growth rates in limited but non-selective media. In addition, it should be noted that although it has been demonstrated that deletion or overexpression of certain genes involved in stress resistance does have an impact on growth ability/rates (Sabater-Muñoz et al., 2015; Shetty et al., 2019; Spector and Cubitt, 1992), and, similarly, since a fitness cost has been observed in stress-resistant strains selected through repetitive exposure to stressing agents (Karatzas et al., 2008a, 2008b; Licciardello et al., 1969), one can expect that strains in real food scenarios with mutations causing a high fitness cost would soon be outcompeted if such genetic changes were not counterbalanced by compensatory mutations (Levin et al., 2000), unless the bacteria found a particular niche in which the alteration turned out to be profitable on a medium and long-term basis.

Regarding the relationship between stress resistance and virulence, results concerning acid resistance stand out. Thus, a positive correlation was found between acid resistance and adhesion rates (Spearman $r_s = 0.433$, $p = 0.039$), as well as a negative correlation with invasion rates (Spearman $r_s = -0.484$, $p = 0.022$). Exposure to acidic conditions results in the induction of different regulons, including Fur, PhoPQ, OmpR, RpoE, and RpoS, some of which play a role in the regulation of *Salmonella* virulence (Álvarez-Ordóñez et al., 2011; Muller et al., 2009), leading to the down-regulation of certain SPI-1 genes such as *SirA*, *HilA*, *HilC*, *HilD*, and *InvF* (Ellermeier et al., 2005; Kim et al., 2014; Ryan et al., 2015). Previous studies have reported that, as observed herein, exposure to acidic conditions can lead to a decreased invasion ability of *Salmonella* cells (Kim et al., 2014), which would probably be linked to that down-regulation of SPI-1 genes. On the other hand (as mentioned above), despite the fact that these virulence mechanisms are co-regulated, this latter phenomenon would not exert an influence on adhesion, which could be mediated by adhesion factors including pili, fimbriae, flagella, non-fimbrial adhesins such as SiiE, or other adhesins (Barlag and Hensel, 2015; Horstmann et al., 2020). Similarly, Karatzas et al. (2008a) observed that sustainable *Salmonella enterica* acid resistant variants, obtained through repeated cycles of acid challenge and growth, displayed an increased expression of SEF17 fimbriae, but a reduced virulence. Nevertheless, *S.* Enteritidis strains possessing a low pathogenicity (both in vitro and in vivo) as well as a low acid resistance have also been reported (Shah, 2014). Conversely to acid resistance, a correlation was found between *Salmonella* resistance to PEF ($r = 0.363$, $p = 0.097$) and to UV ($r_s = 0.426$, $p = 0.048$), on the one hand, and its invasion ability, on the other hand, although in the former case statistical analysis only indicated a trend. The resistance mechanisms of *Salmonella* to these technologies are still not very well known; therefore, it is difficult to hypothesize about the potential causes underlying this correlation. From the few data available, it can be remarked that RpoS has been suggested to play a role in *Salmonella* PEF and UV resistance (Child et al., 2002; Rice et al., 2015; Sagarzazu et al., 2013) and virulence, which might explain the data we have obtained. A more detailed review on the relationship between stress resistance, growth ability and virulence in non-typhoidal *Salmonellae* can be found elsewhere (Guillén et al., 2021).

Apart from acid stress, it is remarkable that no correlation between any of the studied stressing agents or growth ability and biofilm formation was observed. Similarly, Lianou and Koutsoumanis (2012) did not observed any relationship between the biofilm-forming ability and the growth kinetic behavior of the *Salmonella* strains they studied. No correlation among resistance to any of the food-related stresses and any of the studied antibiotics was found using Spearman's test. We found a negative correlation, however, between acid resistance and biofilm formation ability (Spearman $r_s = -0.447$, $p = 0.037$). It has been demonstrated that the acid-induced PhoPQ system would be a repressor of biofilm formation, which would explain the results obtained herein, although the precise role of PhoPQ in PhoPQ is still not fully understood (Prouty and Gunn, 2003; Steenackers et al., 2012; Tsai et al., 2020). By contrast, other authors have reported opposite results regarding the relationship between acid resistance and biofilm formation in *Salmonella*. Thus, Shah et al. (2011, 2012) found that, within a set of six *S.* Enteritidis strains, those not capable of forming biofilms were also the most acid-sensitive ones. Further studies will be required to explain these apparently contradictory results.

In addition to the comparisons established between *Salmonella* resistance to different food-related stresses and the other phenotypic characteristics, other relevant correlations were observed. We found a positive correlation between invasion and maximum growth rates in TSB-YE (Spearman $r_s = 0.455$, $p = 0.034$) and in M9-Gluconate ($r_s = 0.436$, $p = 0.042$; $r = 0.447$, $p = 0.037$); surprisingly, however, no association was found between maximum growth rates in LB-DYP and virulence, even though the relationship between Fe availability (both after and during invasion) and *Salmonella* virulence ability has already been demonstrated, and even though, in our study, a correlation between growth rates in the three media was observed (see above). It is well known that in a nutrient-rich medium, *Salmonella* activates the expression of genes encoding effector proteins such as HilD and HilC, thereby activating *hilA* expression, or InvF, which controls the expression of SPI-1 and SPI-2 (Ellermeier et al., 2005); it is also well known that nutrient limitation is a key regulatory signal for certain virulence genes in *Salmonella* (O'Neal et al., 1994). Thus, for instance, the expressions of SPI-1 and SPI-2 are induced in vitro by limiting concentrations of potassium or magnesium (Bustamante et al., 2008; Kröger et al., 2013). Although it seems clear that an increased growth rate and/or ability to compete for nutrients would provide *Salmonella* cells with an advantage once inside the gut and would therefore make these strains more virulent, we know of no previous study indicating a correlation between growth ability/rates and invasivity in vitro.

A negative correlation between adhesion rate and biofilm formation capacity ($r = -0.434$, $p = 0.043$) was also found. Even though adhesion to Caco-2 cells and to a polystyrene surface are analogous processes, the characteristics of the surface and the structures/metabolic pathways involved are not the same (although some participate in both phenomena); in addition, the formation of biofilms is a multi-step process in which adhesion is only one of the phases (Peng, 2016). Other correlations were found, such as the one between ampicillin ($r_s = 0.487$, $p = 0.025$) or chloramphenicol ($r_s = 0.439$, $p = 0.047$) and biofilm formation capacity, or the one between growth rates and antibiotic resistance (Table 2 and Supplementary Table 4), but the discussion thereof lies outside the scope of this article.

Finally, it should be noted that the results obtained would be similar if the outliers are included in the analysis, with only some minor

exceptions (Supplementary Table 5). The most relevant consequence from including them is, probably, that a correlation between heat and hydrogen peroxide resistance and biofilm formation would be found ($r = 0.875$, $p = 0.000$; $r = 0.490$, $p = 0.048$, respectively). This is due to the fact that the *S.* Senftenberg 775W strain here studied is the most heat and hydrogen peroxide resistant and also the one with the highest ability to form biofilms. The high heat resistance of this particular *S. Senftenberg* 775W strain is well known (Ng et al., 1969), but this is a strain-specific and not serovar-specific trait, whereas the high ability to form biofilms seems to be a serovar-specific characteristic (Xia et al. (2009)) what suggest that this correlation –between heat resistance and biofilm formation ability- is just casual. In any case, the mechanisms responsible for the high heat and hydrogen peroxide resistance of this strain, as well as for the high capability to form biofilms of the serovar Senftenberg deserve further study. Including this strain in the analysis also changed the significance of the correlations between acid pH resistance and biofilm forming ability (from $r_s = -0.447$, $p = 0.037$ to $r_s = -0.365$, $p = 0.087$) and between the later and adhesion (from $r_s = -0.434$, $p = 0.043$ to $r_s = -0.346$, $p = 0.106$). On the other hand, the highly invasive *S.* Typhimurium STCC 7160 strain is also one of the most acid-resistant ones and, therefore, including it in the analysis results in the disappearance of the correlation between acid resistance and invasion ability. Also some changes in the correlations between antibiotic resistance and 1) stress resistance (appearance of a significant correlation between ampicillin and heat resistance), 2) growth ability (a positive correlation between oxytetracycline resistance and growth in M9-broth supplemented gluconate) and 3) biofilm forming ability (a positive correlation between resistance to oxytetracycline and biofilm forming ability) occurred –mainly- due to the inclusion in the analysis of the antibiotic multi-resistant strain *S.* Typhimurium STCC 7162.

4. Conclusions

In this study we determined the maximum growth rates in different media, the ability to adhere to and invade Caco-2 cells, biofilm formation capacity, and antibiotic resistance of 23 *Salmonella* strains belonging to 15 different serovars, and compared these traits with the resistance of the 23 strains to different food-related stresses.

Significant differences in growth rates among strains, as well as depending on the growth medium/condition assayed, were observed. Our results suggest that those strains displaying a higher growth rate under non-limiting conditions also display a higher growth rate in media with reduced amounts of Fe, or with gluconate as the only carbon source. However, less than a 3-fold difference between the lowest and the highest growth rate (determined in the same medium) was observed. Statistical analysis also revealed that the type of strain under study had a significant effect on the rate of adhesion and the rate of invasion; however, no correlation was found, i.e. strains with a high adhesion ability were not always the most invasive ones.

Conversely to that described for growth rates, the ability to form biofilms varied widely: more than 30-fold among the different strains studied. Finally, regarding antibiotic resistance, and if *S.* Typhimurium 7162 is excluded from the analysis, variability among strains in terms of resistance depended on the antibiotic studied, with 8-fold differences in the MIC of ampicillin, chloramphenicol, and rifampicin, but more than 64-fold for nalidixic acid.

On the other hand, our results suggest that, in general terms, the higher stress resistance of some strains/serovars did not impose a fitness cost to them. Similarly, no association was found between stress resistance and biofilm formation ability (except for acid stress) or antibiotic resistance (except for two cases: UV-rifampicin and H_2O_2-oxytetracycline, inverse in the latter case). Our data also suggest that acid stress resistance is associated with virulence in *Salmonella*, since a positive correlation of acid stress resistance with adhesion and a negative one with invasion was found. In any case we remind the reader that this is only an observational study, and that further work would be required to verify the existence (or absence) of these relationships, along with associated underlying mechanisms.

Data reported herein would also be helpful in developing predictive models of *Salmonella* growth, and for improving quantitative microbiological risk assessments (QMRA) of *Salmonella* in food products. Thus, results here reported not only provide an estimation of the intra and inter-serovar variability in growth, stress resistance and virulence within non-typhoidal *Salmonellae*, which is of the highest relevance for QMRA, but they also help to identify strains that might potentially suppose a higher risk for food safety because of their higher fitness, stress resistance or virulence (e.g. *S.* Senftenberg 775w because of its high heat resistance and biofilm forming ability). Further work will be required in order to determine the mechanisms responsible for the differences in fitness, stress resistance and virulence among *Salmonellae,* especially those conferring some strains very particular characteristics, and to determine if these strains suppose a higher risk or not.

CRediT authorship contribution statement

Silvia Guillén: Investigation, Methodology, Formal Analysis, Writing-Original draft preparation. **María Marcén**: Investigation, Writing - Review & Editing. **Ester Fau**: Investigation, Writing - Review & Editing. **Pilar Mañas**: Conceptualization, Writing - Review & Editing. **Guillermo Cebrián**: Conceptualization, Writing - Review & Editing, Supervision.

Declaration of competing interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109462.

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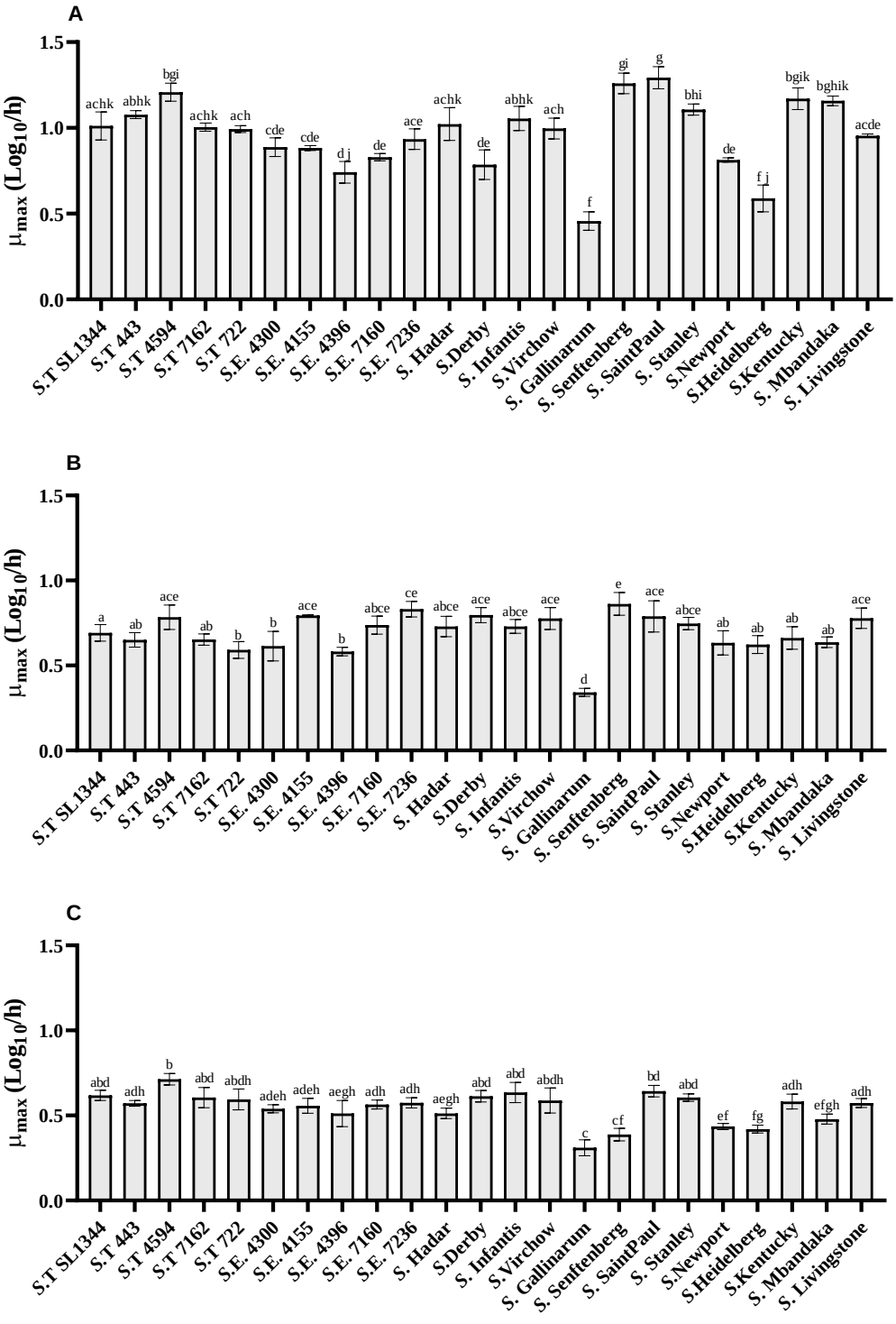

Figure S1. Maximum growth rates (μ_{max} (Log$_{10}$/h)) of the 23 *Salmonella enterica* strains in TSB-YE (A), LB supplemented with 100 µM 2-2'dipyridyl (B) and M9-broth supplemented with 20 mM gluconate (C) at 37 °C. Error bars correspond to the standard deviation of the means and letters indicate statistically significant differences between strains.

Table S1. *Salmonella enterica* serovars of known source used in this study.

Strain	**Source**
S. Typhimurium STCC 443	Human gastroenteritis
S. Typhimurium STCC 4594	Bovine, liver
S. Typhimurium STCC 7162	Faeces
S. Enteritidis STCC 4396	Human gastroenteritis
S. Enteritidis STCC 7160	Chicken, digestive tract
S. Enteritidis STCC 7236	Faeces
S. Infantis STCC 4373	Sea water
S. Stanley STCC 4141	Human gastroenteritis
S. Newport NCTC 129	Human gastroenteritis
S. Livingstone NCTC 9125	Faeces

Table S2. Growth parameters (Y_0, Y_{max}. λ and μmax) and goodness of the fit (R^2, *RMSE*) calculated with the Baranyi and Roberts model of the 23 *Salmonella* strains growth in TSB-YE, LB supplemented with 100 µM 2-2'dipyridyl and M9-broth, supplemented with 20 mM gluconate. Values presented correspond to the mean and SD of the means (in parentheses).

	TSB-YE						LB-DYP					
	Y_0 (Log(CFU/ml))	μmax (Log_{10}/h)	λ (h)	Y_{max} (Log(CFU/ml))	R^2	RMSE	Y_0 (Log(CFU/ml))	μmax (Log_{10}/h)	λ (h)	Y_{max} (Log(CFU/ml))	R^2	RMSE
***S*.T SL1344**	6.16 (0.117)	1.012 (0.082)	-	8.97 (0.016)	0.98 - 1.00	0.058 - 0.100	6.21 (0.067)	0.692 (0.049)	-	8.77 (0.071)	0.93 - 1.00	0.044 - 0.255
***S*.T 443**	6.12 (0.033)	1.077 (0.023)	-[o]	9.03 (0.037)	0.99 - 1.00	0.058 - 0.088	6.22 (0.042)	0.651 (0.042)	-	9.04 (0.061)	0.93 - 0.99	0.077 - 0.269
***S*.T 4594**	5.83 (0.128)	1.208 (0.053)	-	8.98 (0.027)	0.97 - 1.00	0.051 - 0.080	5.91 (0.083)	0.784 (0.072)	-	8.73 (0.136)	0.92 -0.97	0.154 - 0.255
***S*.T 7162**	6.04 (0.017)	1.005 (0.023)	-	9.01 (0.033)	0.98 - 0.99	0.115 - 0.121	6.16 (0.064)	0.653 (0.033)	-	8.69 (0.123)	0.95 - 0.96	0.151 - 0.190
***S*.T 722**	6.13 (0.019)	0.993 (0.020)	-	8.83 (0.038)	0.99 - 1.00	0.023 - 0.093	6.20 (0.106)	0.591 (0.050)	-	8.75 (0.196)	0.96 - 0.99	0.071 - 0.172
***S*. E. 4300**	6.23 (0.047)	0.887 (0.054)	-	8.94 (0.028)	0.97 - 0.98	0.055 - 0.079	6.11 (0.049)	0.614 (0.087)	-	8.82 (0.069)	0.95 - 0.99	0.060 - 0.188
***S*. E. 4155**	6.20 (0.047)	0.883 (0.014)	-	9.03 (0.060)	0.97 - 0.99	0.118 - 0.174	6.10 (0.096)	0.795 (0.004)	-	8.77 (0.070)	0.97 - 0.99	0.068 - 1.38
***S*. E. 4396**	6.16 (0.123)	0.741 (0.064)	-	9.02 (0.051)	0.99 - 1.00	0.059 - 0.133	5.96 (0.068)	0.582 (0.025)	-	8.69 (0.011)	0.96 -1.00	0.024 - 0.172
***S*. E. 7160**	6.19 (0.013)	0.829 (0.021)	-	8.98 (0.038)	0.99 - 0.99	0.086 - 0.129	6.15 (0.072)	0.738 (0.053)	-	8.77 (0.070)	0.98 - 1.00	0.053 - 0.123
***S*. E. 7236**	6.26 (0.093)	0.934 (0.061)	-	9.05 (0.027)	0.97 - 0.99	0.118 - 0.195	6.06 (0.084)	0.831 (0.046)	-	8.79 (0.122)	0.98 -1.00	0.041 - 0.110
***S*. Hadar**	6.20 (0.017)	1.022 (0.096)	-	9.01 (0.046)	0.94 - 1.00	0.039 - 0.099	6.19 (0.050)	0.729 (0.060)	-	8.85 (0.083)	0.95 - 1.00	0.056 - 0.208
***S*. Derby**	5.86 (0.067)	0.785 (0.086)	-	9.02 (0.091)	0.96 - 0.98	0.178 - 0.226	6.16 (0.027)	0.797 (0.045)	-	8.77 (0.035)	0.97 - 0.99	0.102 - 0.138
***S*. Infantis**	6.01 (0.072)	1.054 (0.071)	-	9.02 (0.057)	0.99 - 0.99	0.080 - 0.119	6.11 (0.084)	0.729 (0.041)	-	8.77 (0.119)	0.95 - 1.00	0.039 - 0.225
***S*. Virchow**	5.99 (0.065)	0.996 (0.060)	-	9.08 (0.030)	0.93 - 0.99	0.095 -0.286	6.00 (0.073)	0.776 (0.064)	-	8.74 (0.049)	0.95 - 1.00	0.016 - 0.212
***S*. Gallinarum**	5.74 (0.007)	0.457 (0.054)	1.05 (0.433)	8.91 (0.087)	0.99 - 1.00	0.054 - 0.090	6.04 (0.035)	0.342 (0.024)	-	8.09 (0.077)	0.99 - 1.00	0.053 - 0.084
***S*. Senftenberg**	5.87 (0.025)	1.259 (0.060)	-	8.91 (0.034)	0.97 - 0.99	0.101 - 0.180	5.83 (0.057	0.862 (0.067)	-	8.64 (0.028)	0.95 - 0.97	0.159 - 0.252
***S*. Saintpaul**	5.81 (0.053)	1.293 (0.064)	-	8.90 (0.032)	0.97 - 0.99	0.099 - 0.180	6.02 (0.017)	0.789 (0.092)	-	8.95 (0.064)	0.99 - 0.99	0.130 - 0.152
***S*. Stanley**	5.76 (0.083)	1.107 (0.032)	-	8.94 (0.021)	0.97 - 0.98	0.177 - 0.211	5.92 (0.051)	0.747 (0.036)	-	8.86 (0.083)	0.93 - 0.96	0.210 - 0.241
***S*. Newport**	6.01 (0.049)	0.813 (0.013)	-	9.05 (0.042)	0.92 - 0.98	0.147 - 0.321	6.18 (0.066)	0.634 (0.071)	-	8.97 (0.088)	0.98 - 0.99	0.093 - 0.132
***S*. Heidelberg**	6.00 (0.089)	0.589 (0.078)	-	8.98 (0.044)	0.93 - 0.95	0.248 - 0.361	5.71 (0.056)	0.623 (0.052)	-	8.69 (0.087)	0.93 - 0.99	0.082 - 0.232
***S*. Kentucky**	5.91 (0.043)	1.170 (0.063)	-	8.97 (0.059)	0.98 - 1.00	0.046 - 0.163	6.05 (0.084)	0.662 (0.066)	-	8.85 (0.117)	0.93 - 1.00	0.043 - 0.259
***S*. Mbandaka**	5.84 (0.057)	1.158 (0.027)	-	8.89 (0.041)	0.99 - 0.99	0.078 - 0.126	6.21 (0.041)	0.637 (0.032)	-	8.96 (0.094)	0.97 - 0.99	0.077 - 0.140
***S*. Livingstone**	5.85 (0.023)	0.954 (0.010)	-	9.05 (0.076)	0.99 - 1.00	0.078 - 0.116	6.02 (0.065)	0.779 (0.060)	-	8.78 (0.044)	0.93 - 0.99	0.062 - 0.226

T: Typhimurium. E: Enteritidis.

Table S1. Continuation

	M9-Gluconate					
	Y_0 (Log(CFU/ml))	μmax (1/h)	λ (h)	Y_{max} (Log(CFU/ml))	R^2	RMSE
***S*.T SL1344**	6.12 (0.017)	0.618 (0.030)	-	7.90 (0.104)	0.99 - 1.00	0.021 - 0.070
***S*.T 443**	6.20 (0.069)	0.571 (0.017)	-	8.75 (0.011)	0.97 - 0.99	0.066 - 0.153
***S*.T 4594**	5.89 (0.075)	0.713 (0.033)	-	8.75 (0.065)	0.97 - 0.98	0.111 - 0.241
***S*.T 7162**	6.06 (0.021)	0.605 (0.058)	-	8.74 (0.033)	0.98 - 0.99	0.088 - 0.126
***S*.T 722**	6.11 (0.075)	0.595 (0.061)	-	7.99 (0.075)	0.97 - 0.99	0.051 - 0.148
***S*. E. 4300**	6.12 (0.063)	0.540 (0.024)	-	8.70 (0.113)	0.99 - 1.00	0.054 - 0.094
***S*. E. 4155**	6.19 (0.076)	0.557 (0.044)	-	8.63 (0.079)	0.96 - 1.00	0.033 - 0.158
***S*. E. 4396**	6.10 (0.082)	0.512 (0.077)	-	8.48 (0.176)	0.96 - 0.99	0.072 - 0.149
***S*. E. 7160**	6.22 (0.038)	0.564 (0.026)	-	8.72 (0.099)	0.96 - 0.99	0.080 - 0.136
***S*. E. 7236**	6.11 (0.047)	0.574 (0.030)	-	8.71 (0.055)	0.97 - 0.99	0.089 - 0.133
***S*. Hadar**	6.26 (0.053)	0.512 (0.031)	-	8.70 (0.006)	0.93 - 0.97	0.137 - 0.180
***S*. Derby**	5.83 (0.062)	0.613 (0.033)	-	8.72 (0.064)	0.97 - 0.99	0.080 - 0.118
***S*. Infantis**	6.10 (0.055)	0.635 (0.059)	-	8.70 (0.012)	0.98 - 0.99	0.112 - 0.161
***S*. Virchow**	6.01 (0.022)	0.588 (0.074)	-	8.69 (0.006)	0.97 - 0.99	0.098 - 0.151
***S*. Gallinarum**	6.00 (0.028)	0.310 (0.047)	-	8.28 (0.040)	0.96 - 1.00	0.067 - 0.124
***S*. Senftenberg**	5.92 (0.065)	0.388 (0.037)	-	8.61 (0.111)	0.98 - 0.99	0.106 - 0.116
***S*. Saintpaul**	5.98 (0.087)	0.643 (0.034)	-	8.69 (0.019)	0.95 - 0.99	0.097 - 0.185
***S*. Stanley**	5.92 (0.069)	0.605 (0.022)	-	8.74 (0.029)	0.95 - 0.98	0.123 - 0.206
***S*. Newport**	6.23 (0.057)	0.435 (0.018)	-	8.58 (0.126)	0.97 - 0.98	0.111 - 0.129
***S*. Heidelberg**	5.80 (0.092)	0.419 (0.023)	-	8.46 (0.056)	0.98 - 0.99	0.052 - 0.106
***S*. Kentucky**	5.91 (0.067)	0.582 (0.044)	-	8.23 (0.117)	0.97 - 0.99	0.097 - 0.261
***S*. Mbandaka**	6.20 (0.026)	0.478 (0.029)	-	8.70 (0.020)	0.96 - 0.98	0.099 - 0.157
***S*. Livingstone**	5.88 (0.097)	0.574 (0.026)	-	8.73 (0.018)	0.99 - 1.00	0.047 - 0.73

T: Typhimurium. E: Enteritidis.

Table S3. MIC values (µg/mL) of streptomycin and genes conferring resistance to aminoglycosides present in *Salmonella* strains whose complete genome sequence is available in NCBI and ENA.

	streptomycin *MIC*	**Aminoglycosides resistance genes**	
***S*.T SL1344**	256	*aac(6')*	*aadA1*
***S*.T 443**	64	*aac(6')*	*aadA1*
***S*.T 4594**	> 512	*aac(6')*	*aadA1*
***S*.T 722**	> 512	-	*aadA1*
***S*. Saintpaul**	> 512	*aac(6')*	*aadA1*
***S*. Stanley**	128	-	-
***S*. Newport**	128	-	-

T: Typhimurium

Table S4. Pearson's correlation coefficient values calculated for the *2D* resistance values (obtained in Guillén et al., 2020a, b), μ_{max} values in the 3 media studied (see text), adhesion and invasion ability (%), biofilm formation capacity (AUC) and MIC values of each antibiotic of the 23 *Salmonella enterica* strains studied. Values in parentheses correspond to the *p*-value.

	pH	**H_2O_2**	**NaCl**	**Heat**	**HHP**	**PEF**	**UV**	**TSB-YE**	**LB-DPY**	**M9-gluconate**	**Adhesion**	**Invasion**	**Biofilm**	**AMP**	**CHL**	**NAL**	**OTET**	**RIF**	**STR**
pH		0.113 (0.607)	0.225 (0.301)	-0.366 (0.094)	0.338 (0.115)	0.247 (0.256)	-0.047 (0.831)	-0.317 (0.140)	0.130 (0.554)	-0.126 (0.567)	0.334 (0.119)	-0.393 (0.070)	-0.364 (0.096)	-0.201 (0.370)	-0.222 (0.320)	0.234 (0.282)	-0.274 (0.218)	-0.357 (0.094)	-0.143 (0.515)
H_2O_2	0.113 (0.607)		0.202 (0.357)	-0.057 (0.800)	-0.123 (0.576)	0.054 (0.806)	0.059 (0.789)	0.070 (0.750)	0.118 (0.591)	-0.284 (0.190)	-0.270 (0.213)	0.027 (0.905)	0.194 (0.387)	-0.083 (0.712)	-0.012 (0.959)	0.007 (0.974)	**-0.486 (0.022)**	0.006 (0.980)	-0.052 (0.814)
NaCl	0.225 (0.301)	0.202 (0.357)		-0.089 (0.693)	-0.102 (0.645)	**0.545 (0.007)**	0.277 (0.201)	0.256 (0.239)	0.237 (0.276)	**0.491 (0.017)**	-0.048 (0.828)	0.244 (0.273)	-0.030 (0.894)	-0.402 (0.064)	-0.384 (0.078)	0.078 (0.724)	-0.413 (0.056)	-0.049 (0.824)	-0.324 (0.132)
Heat	-0.366 (0.094)	-0.057 (0.800)	-0.089 (0.693)		0.002 (0.993)	-0.263 (0.237)	0.371 (0.089)	0.049 (0.829)	-0.336 (0.127)	-0.205 (0.361)	-0.301 (0.174)	0.140 (0.546)	0.070 (0.756)	0.202 (0.380)	-0.028 (0.905)	0.060 (0.793)	-0.076 (0.742)	0.406 (0.061)	0.079 (0.726)
HHP	0.338 (0.115)	-0.123 (0.576)	-0.102 (0.645)	0.002 (0.993)		0.312 (0.147)	-0.050 (0.820)	-0.126 (0.566)	0.165 (0.453)	-0.043 (0.846)	-0.125 (0.570)	0.016 (0.944)	0.018 (0.936)	-0.063 (0.780)	-0.077 (0.733)	0.205 (0.349)	-0.080 (0.724)	-0.121 (0.582)	0.116 (0.598)
PEF	0.247 (0.256)	0.054 (0.806)	**0.545 (0.007)**	-0.263 (0.237)	0.312 (0.147)		0.297 (0.169)	0.162 (0.460)	**0.417 (0.048)**	**0.466 (0.025)**	0.108 (0.624)	0.363 (0.097)	-0.092 (0.685)	-0.277 (0.213)	-0.141 (0.532)	0.082 (0.710)	0.159 (0.481)	0.070 (0.752)	-0.241 (0.268)
UV	-0.047 (0.831)	0.059 (0.789)	0.277 (0.201)	0.371 (0.089)	-0.050 (0.820)	0.297 (0.169)		0.276 (0.203)	0.321 (0.236)	0.314 (0.145)	-0.065 (0.769)	0.327 (0.137)	-0.090 (0.690)	0.118 (0.599)	-0.012 (0.957)	-0.140 (0.525)	0.073 (0.746)	**0.484 (0.019)**	-0.257 (0.237)
TSB-YE	-0.317 (0.140)	0.070 (0.750)	0.256 (0.239)	0.049 (0.829)	-0.126 (0.566)	0.162 (0.460)	0.276 (0.203)		**0.593 (0.003)**	**0.536 (0.008)**	-0.031 (0.888)	0.421 (0.051)	0.162 (0.470)	0.418 (0.053)	**0.488 (0.021)**	0.085 (0.698)	0.182 (0.417)	0.345 (0.107)	-0.027 (0.903)
LB-DPY	0.130 (0.554)	0.118 (0.591)	0.237 (0.276)	-0.336 (0.127)	0.165 (0.453)	**0.417 (0.048)**	0.321 (0.236)	**0.593 (0.003)**		**0.522 (0.011)**	-0.043 (0.847)	0.139 (0.536)	-0.031 (0.892)	0.389 (0.073)	0.380 (0.081)	0.010 (0.963)	0.081 (0.720)	0.199 (0.364)	-0.263 (0.225)
M9-gluconate	-0.126 (0.567)	-0.284 (0.190)	**0.491 (0.017)**	-0.205 (0.361)	-0.043 (0.846)	**0.466 (0.025)**	0.314 (0.145)	**0.536 (0.008)**	**0.522 (0.011)**		0.115 (0.603)	**0.447 (0.037)**	0.215 (0.337)	0.151 (0.503)	0.096 (0.671)	0.239 (0.273)	0.250 (0.262)	0.238 (0.275)	0.061 (0.784)
Adhesion	0.334 (0.119)	-0.270 (0.213)	-0.048 (0.828)	-0.301 (0.174)	-0.125 (0.570)	0.108 (0.624)	-0.065 (0.769)	-0.031 (0.888)	-0.043 (0.847)	0.115 (0.603)		-0.109 (0.630)	**-0.434 (0.043)**	0.022 (0.921)	0.089 (0.694)	0.107 (0.626)	0.383 (0.078)	0.004 (0.987)	0.143 (0.514)
Invasion	-0.393 (0.070)	0.027 (0.905)	0.244 (0.273)	0.140 (0.546)	0.016 (0.944)	0.363 (0.097)	0.327 (0.137)	0.421 (0.051)	0.139 (0.536)	**0.447 (0.037)**	-0.109 (0.630)		0.103 (0.655)	-0.001 (0.997)	0.042 (0.858)	0.067 (0.767)	0.269 (0.238)	0.284 (0.200)	0.010 (0.965)
Biofilm	-0.364 (0.096)	0.194 (0.387)	-0.030 (0.894)	0.070 (0.756)	0.018 (0.936)	-0.092 (0.685)	-0.090 (0.690)	0.162 (0.470)	-0.031 (0.892)	0.215 (0.337)	**-0.434 (0.043)**	0.103 (0.655)		0.229 (0.318)	0.079 (0.732)	0.304 (0.169)	0.070 (0.764)	0.126 (0.577)	0.287 (0.196)
Ampicillin	-0.201 (0.370)	-0.083 (0.712)	-0.402 (0.064)	0.202 (0.380)	-0.063 (0.780)	-0.277 (0.213)	0.118 (0.599)	0.418 (0.053)	0.389 (0.073)	0.151 (0.503)	0.022 (0.921)	-0.001 (0.997)	0.229 (0.318)		**0.681 (0.000)**	0.394 (0.069)	**0.497 (0.019)**	0.370 (0.090)	0.372 (0.088)
Chloramphenicol	-0.222 (0.320)	-0.012 (0.959)	-0.384 (0.078)	-0.028 (0.905)	-0.077 (0.733)	-0.141 (0.532)	-0.012 (0.957)	**0.488 (0.021)**	0.380 (0.081)	0.096 (0.671)	0.089 (0.694)	0.042 (0.858)	0.079 (0.732)	**0.681 (0.000)**		0.281 (0.205)	**0.514 (0.015)**	**0.472 (0.027)**	0.206 (0.358)
Nalidixic acid	0.234 (0.282)	0.007 (0.974)	0.078 (0.724)	0.060 (0.793)	0.205 (0.349)	0.082 (0.710)	-0.140 (0.525)	0.085 (0.698)	0.010 (0.963)	0.239 (0.273)	0.107 (0.626)	0.067 (0.767)	0.304 (0.169)	0.394 (0.069)	0.281 (0.205)		0.185 (0.410)	0.105 (0.633)	**0.661 (0.001)**
Oxytetracycline	-0.274 (0.218)	**-0.486 (0.022)**	-0.413 (0.056)	-0.076 (0.742)	-0.080 (0.724)	0.159 (0.481)	0.073 (0.746)	0.182 (0.417)	0.081 (0.720)	0.250 (0.262)	0.383 (0.078)	0.269 (0.238)	0.070 (0.764)	**0.497 (0.019)**	**0.514 (0.015)**	0.185 (0.410)		0.369 (0.092)	0.214 (0.340)
Rifampicin	-0.357 (0.094)	0.006 (0.980)	-0.049 (0.824)	0.406 (0.061)	-0.121 (0.582)	0.070 (0.752)	**0.484 (0.019)**	0.345 (0.107)	0.199 (0.364)	0.238 (0.275)	0.004 (0.987)	0.284 (0.200)	0.126 (0.577)	0.370 (0.090)	**0.472 (0.027)**	0.105 (0.633)	0.369 (0.092)		-0.042 (0.848)
Streptomycin	-0.143 (0.515)	-0.052 (0.814)	-0.324 (0.132)	0.079 (0.726)	0.116 (0.598)	-0.241 (0.268)	-0.257 (0.237)	-0.027 (0.903)	-0.263 (0.225)	0.061 (0.784)	0.143 (0.514)	0.010 (0.965)	0.287 (0.196)	0.372 (0.088)	0.206 (0.358)	**0.661 (0.001)**	0.214 (0.340)	-0.042 (0.848)	

Ampicillin (AMP); Chloramphenicol (CHL); Nalidixic acid (NAL); Oxytetracycline (OTET); Rifampicin (RIF); Streptomycin (STR)

Table S5. Spearman's correlation coefficient values calculated for the *2D* resistance values (obtained in Guillén et al., 2020a, b), μ_{max} values in the 3 media studied (see text), adhesion and invasion ability (%), biofilm formation capacity (AUC) and MIC values of each antibiotic of the 23 *Salmonella enterica* strains studied without excluding the outliers detected by the Grubs' test. Values in parentheses correspond to the *p*-value.

	pH	**H_2O_2**	**NaCl**	**Heat**	**HHP**	**PEF**	**UV**	**TSB-YE**	**LB-DPY**	**M9-gluconate**	**Adhesion**	**Invasion**	**Biofilm**	**AMP**	**CHL**	**NAL**	**OTET**	**RIF**	**STR**	**SUL**
pH		0.139 (0.526)	0.160 (0.467)	-0.192 (0.380)	**0.454 (0.030)**	0.134 (0.541)	-0.070 (0.750)	-0.194 (0.376)	0.279 (0.197)	-0.138 (0.530)	**0.433 (0.039)**	-0.298 (0.167)	-0.365 (0.087)	-0.224 (0.304)	-0.345 (0.107)	0.268 (0.215)	-0.258 (0.234)	-0.290 (0.179)	-0.049 (0.826)	-0.257 (0.236)
H_2O_2	0.139 (0.526)		0.311 (0.149)	0.089 (0.685)	0.018 (0.936)	0.103 (0.641)	-0.042 (0.851)	-0.042 (0.847)	0.071 (0.749)	-0.282 (0.193)	-0.260 (0.231)	-0.025 (0.911)	0.136 (0.536)	-0.024 (0.913)	-0.122 (0.578)	-0.033 (0.882)	-0.087 (0.695)	0.002 (0.992)	-0.150 (0.494)	0.096 (0.662)
NaCl	0.160 (0.467)	0.311 (0.149)		-0.307 (0.154)	0.009 (0.968)	**0.643 (0.001)**	0.282 (0.193)	0.156 (0.478)	**0.431 (0.040)**	**0.415 (0.049)**	-0.098 (0.656)	0.387 (0.068)	-0.188 (0.390)	-0.33 (0.121)	-0.241 (0.268)	0.009 (0.967)	-0.173 (0.429)	0.109 (0.621)	-0.380 (0.074)	0.193 (0.378)
Heat	-0.192 (0.380)	0.089 (0.685)	-0.307 (0.154)		-0.072 (0.745)	-0.284 (0.190)	0.217 (0.319)	0.302 (0.161)	-0.096 (0.662)	-0.260 (0.231)	-0.306 (0.156)	-0.059 (0.790)	0.143 (0.250)	**0.413 (0.050)**	0.400 (0.059)	0.190 (0.384)	0.260 (0.231)	**0.429 (0.041)**	0.146 (0.507)	-0.129 (0.559)
HHP	**0.454 (0.030)**	0.018 (0.936)	0.009 (0.968)	-0.072 (0.745)		-0.131 (0.559)	-0.019 (0.523)	-0.264 (0.274)	-0.374 (0.434)	-0.154 (0.484)	-0.126 (0.567)	-0.290 (0.550)	-0.062 (0.930)	-0.226 (0.223)	-0.347 (0.079)	0.178 (0.417)	-0.247 (0.257)	-0.261 (0.228)	0.103 (0.641)	-0.096 (0.662)
PEF	0.134 (0.541)	0.103 (0.641)	**0.643 (0.001)**	-0.284 (0.190)	-0.131 (0.559)		0.258 (0.235)	0.021 (0.925)	0.430 (0.041)	0.337 (0.116)	0.022 (0.920)	**0.420 (0.046)**	-0.188 (0.390)	-0.190 (0.286)	-0.120 (0.586)	0.164 (0.455)	0.226 (0.300)	0.239 (0.272)	-0.271 (0.210)	0.129 (0.559)
UV	-0.070 (0.750)	-0.042 (0.851)	0.282 (0.193)	0.217 (0.319)	-0.019 (0.523)	0.258 (0.235)		0.226 (0.299)	0.255 (0.240)	0.270 (0.212)	-0.049 (0.823)	**0.432 (0.040)**	-0.160 (0.466)	0.171 (0.435)	0.121 (0.583)	0.028 (0.900)	0.177 (0.419)	0.404 (0.056	-0.191 (0.382)	0.096 (0.662)
TSB-YE	-0.194 (0.376)	-0.042 (0.847)	0.156 (0.478)	0.302 (0.161)	-0.264 (0.274)	0.021 (0.925)	0.226 (0.299)		0.382 (0.072)	**0.437 (0.037)**	0.024 (0.914)	0.372 (0.081)	0.322 (0.134)	**0.484 (0.019)**	**0.651 (0.001)**	0.222 (0.309)	0.404 (0.056)	**0.541 (0.008)**	0.168 (0.443)	-0.096 (0.662)
LB-DPY	0.279 (0.197)	0.071 (0.749)	**0.431 (0.040)**	-0.096 (0.662)	-0.374 (0.434)	**0.430 (0.041)**	0.255 (0.240)	0.382 (0.072)		0.388 (0.067)	0.004 (0.986)	0.171 (0.435)	0.137 (0.532)	0.237 (0.275)	0.273 (0.208)	0.227 (0.298)	0.277 (0.299)	0.371 (0.081)	-0.192 (0.381)	-0.016 (0.942)
M9-gluconate	-0.138 (0.530)	-0.282 (0.193)	0.415 (0.049)	-0.260 (0.231)	-0.154 (0.484)	0.337 (0.116)	0.270 (0.212)	**0.437 (0.037)**	0.388 (0.067)		0.093 (0.674)	0.388 (0.067)	0.197 (0.369)	0.304 (0.158)	**0.421 (0.046)**	0.403 (0.056)	0.386 (0.069)	0.374 (0.078)	0.265 (0.221)	0.177 (0.419)
Adhesion	**0.433 (0.039)**	-0.260 (0.231)	-0.098 (0.656)	-0.306 (0.156)	-0.126 (0.567)	0.022 (0.920)	-0.049 (0.823)	0.024 (0.914)	0.004 (0.986)	0.093 (0.674)		-0.059 (0.790)	-0.341 (0.111)	-0.022 (0.919)	0.020 (0.927)	0.143 (0.515)	0.121 (0.583)	-0.021 (0.924)	0.115 (0.601)	0.064 (0.771)
Invasion	-0.298 (0.167)	-0.025 (0.911)	0.387 (0.068)	-0.059 (0.790)	-0.290 (0.550)	**0.420 (0.046)**	**0.432 (0.040)**	0.372 (0.081)	0.171 (0.435)	0.388 (0.067)	-0.059 (0.790)		-0.095 (0.931)	0.063 (0.775)	0.235 (0.279)	0.196 (0.371)	0.283 (0.191)	0.297 (0.168)	-0.172 (0.433)	0.096 (0.662)
Biofilm	-0.365 (0.087)	0.136 (0.536)	-0.188 (0.390)	0.143 (0.250)	-0.062 (0.930)	-0.188 (0.390)	-0.160 (0.466)	0.322 (0.134)	0.137 (0.532)	0.197 (0.369)	-0.341 (0.111)	-0.095 (0.931)		**0.593 (0.003)**	**0.566 (0.005)**	0.203 (0.354)	**0.448 (0.032)**	**0.419 (0.047)**	0.236 (0.278)	0.032 (0.884)
Ampicillin	-0.224 (0.304)	-0.024 (0.913)	-0.33 (0.121)	**0.413 (0.050)**	-0.226 (0.223)	-0.190 (0.286)	0.171 (0.435)	0.484 (0.019)	0.237 (0.275)	0.304 (0.158)	-0.022 (0.919)	0.063 (0.775)	**0.593 (0.003)**		**0.814 (0.027)**	**0.461 (0.000)**	**0.791 (0.000)**	**0.517 (0.011)**	**0.521 (0.011)**	-0.291 (0.178)
Chloramphenicol	-0.345 (0.107)	-0.122 (0.578)	-0.241 (0.268)	0.400 (0.059)	-0.347 (0.079)	-0.120 (0.586)	0.121 (0.583)	**0.651 (0.001)**	0.273 (0.208)	**0.421 (0.046)**	0.020 (0.927)	0.235 (0.279)	**0.566 (0.005)**	0.814 (0.027)		**0.472 (0.023)**	**0.739 (0.000)**	**0.628 (0.001)**	0.386 (0.069)	-0.052 (0.815)
Nalidixic acid	0.268 (0.215)	-0.033 (0.882)	0.009 (0.967)	0.190 (0.384)	0.178 (0.417)	0.164 (0.455)	0.028 (0.900)	0.222 (0.309)	0.227 (0.298)	0.403 (0.056)	0.143 (0.515)	0.196 (0.371)	0.203 (0.354)	**0.461 (0.000)**	**0.472 (0.023)**		**0.495 (0.016)**	0.350 (0.101)	**0.566 (0.005)**	-0.234 (0.283)
Oxytetracycline	-0.258 (0.234)	-0.087 (0.695)	-0.173 (0.429)	0.260 (0.231)	-0.247 (0.257)	0.226 (0.300)	0.177 (0.419)	0.404 (0.056)	0.277 (0.299)	0.386 (0.069)	0.121 (0.583)	0.283 (0.191)	**0.448 (0.032)**	**0.791 (0.000)**	**0.739 (0.000)**	**0.495 (0.016)**		**0.516 (0.012)**	0.389 (0.067)	-0.118 (0.592)
Rifampicin	-0.290 (0.179)	0.002 (0.992)	0.109 (0.621)	**0.429 (0.041)**	-0.261 (0.228)	0.239 (0.272)	0.404 (0.056	**0.541 (0.008)**	0.371 (0.081)	0.374 (0.078)	-0.021 (0.924)	0.297 (0.168)	**0.419 (0.047)**	**0.517 (0.011)**	**0.628 (0.001)**	0.350 (0.101)	**0.516 (0.012)**		0.019 (0.932)	0.055 (0.803)
Streptomycin	-0.049 (0.826)	-0.150 (0.494)	-0.380 (0.074)	0.146 (0.507)	0.103 (0.641)	-0.271 (0.210)	-0.191 (0.382)	0.168 (0.443)	-0.192 (0.381)	0.265 (0.221)	0.115 (0.601)	-0.172 (0.433)	0.236 (0.278)	**0.521 (0.011)**	0.386 (0.069)	**0.566 (0.005)**	0.389 (0.067)	0.019 (0.932)		-0.249 (0.251)
Sulfonamide	-0.257 (0.236)	0.096 (0.662)	0.193 (0.378)	-0.129 (0.559)	-0.096 (0.662)	0.129 (0.559)	0.096 (0.662)	-0.096 (0.662)	-0.016 (0.942)	0.177 (0.419)	0.064 (0.771)	0.096 (0.662)	0.032 (0.884)	-0.291 (0.178)	-0.052 (0.815)	-0.234 (0.283)	-0.118 (0.592)	0.055 (0.803)	-0.249 (0.251)	

Ampicillin (AMP); Chloramphenicol (CHL); Nalidixic acid (NAL); Oxytetracycline (OTET); Rifampicin (RIF); Streptomycin (STR) Sulfonamide (SUL

Capítulo 3

Exploración de los mecanismos de resistencia al estrés dentro del género *Salmonella*

Manuscrito IV/Manuscript IV

Geno- and phenotypic characterization of a *Salmonella* Typhimurium strain resistant to pulsed electric fields

Silvia Guillén, Laura Nadal, Nabil Halaihel, Pilar Mañas and Guillermo Cebrián

Geno- and phenotypic characterization of a *Salmonella* Typhimurium strain resistant to Pulsed Electric Fields

Silvia Guillén[1], Lauda Nadal[1], Nabil Halaihel[2], Pilar Mañas[1] and Guillermo Cebrián[1]*

[1] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón—IA2—(Universidad de Zaragoza-CITA), 50013, Zaragoza, Spain;
[2] Departamento I+D+i, Alquizvetek S.L, Zaragoza, 50013 Zaragoza, Spain
* Correspondence: guiceb@unizar.es (G.C); Tel.: +34-876554127

Abstract: Pulsed Electric Fields (PEF) technologies is regarded as one on the most interesting alternatives to current food preservation methods due to its capability to inactivate vegetative microorganisms while maintaining the organoleptic and nutritional properties roughly unchanged. However, many aspects regarding the mechanisms of bacterial inactivation by PEF are still not fully understood. Thus, the aim of this work was to get further insight into the mechanisms responsible for the increased resistance of a *Salmonella* Typhimurium SL1344 variant resistant to PEF (SL1344-RS), as well as to quantify the impact that the development of PEF resistance had on other aspects of *Salmonella* physiology, such as growth fitness, biofilm formation ability and virulence. Results obtained indicate that the increased PEF resistance of the SL1344-RS variant was due to increased RpoS activity, caused by a mutation in the *hnr* gene. Thus, RNA-seq analysis demonstrated that RpoS activity was increased, since 5 out of the 6 up-regulated genes displaying a significant different expression (after applying the Bonferroni correction) in the PEF resistant strain were RpoS-dependent. This increased RpoS activity also resulted in increased resistance to multiple stresses (acidic, osmotic, oxidative, ethanol and UV-C, but not for heat and HHP), decreased growth rate in M9-gluconate (but not in TSB-YE or LB-DPY) and increased ability to adhere to Caco-2 cells (but no significant change in invasiveness). This work significantly contributes to the understanding of the mechanisms of stress resistance development in *Salmonellae* and points to the crucial role played by RpoS in this process. However, further studies are needed to determine whether this PEF-resistant variant would represent a higher, equal or lower associated risk than the parental strain and in which foods/scenarios.

Keywords: foodborne pathogen, *rpoS*, transcriptomic analysis, stress resistance, growth fitness, virulence

1. Introduction

Pulsed Electric Fields (PEF) consists of the application of short-duration (1–100 μs) high electric field pulses (10–50 kV/cm) to food products placed between two electrodes (Heinz et al., 2001). This technology has been under research for decades as a potential alternative to thermal treatments for the preservation of different food products, and data obtained to date demonstrate that PEF can inactivate vegetative cells of bacteria and yeasts at temperatures below those used in thermal processing (Álvarez et al., 2006), thus potentially enabling to obtain safe foods while minimizing quality and nutritional losses.

However, despite decades of effort and the considerable body of information available concerning microbial inactivation by PEF, the mechanisms of microbial inactivation and, especially, of microbial resistance to this technology still remain largely unknown. Thus, although permeabilization of cellular envelopes as a result of PEF application (also called "electroporation" or "electropermeabilization"), is believed to be the principal mechanism of microbial inactivation by this technology (Barbosa-Cánovas

et al., 1999; Ho and Mittal, 1996; Kinosita et al., 2012; Mañas and Pagán, 2005; Pavlin et al., 2007; Tsong, 1991; Weaver and Chizmadzhev, 1996) the molecular mechanisms of pore formation and resealing in bacteria are almost completely unknown. Therefore, it is assumed that bacterial membrane electroporation occurs essentially as depicted for artificial membranes, eukaryotic cells or in molecular dynamic simulations (El Zakhem et al., 2006; Pavlin et al., 2008; Sözer et al., 2017; Tarek, 2005) and little is known, for instance, about how the complex structure of bacterial envelopes influences the electroporation phenomenon or about the potential contribution of other phenomena to microbial death after exposure to PEF, such as oxidative damage (Marcén et al., 2019; Pakhomova et al., 2012).

Similarly, the factors determining the differences in resistance to PEF among bacterial species and/or strains or depending of the growth and treatment conditions are far from being completely understood. Membrane and envelope structure, composition and physical state characteristics are supposed to play a major role in microbial resistance to PEF but results obtained to date are, in many cases, inconclusive. Thus, various authors have hypothesized that the differences in PEF resistance among cells grown and treated at different temperatures would be related to differences in membrane fluidity but contradictory results can be found in the literature (Cebrián et al., 2016a). Similarly, Chueca et al. (2015) demonstrated that PEF-treated cells activate a response involving components and functions directly associated to cytoplasmic membrane, which confirms the cellular requirement for energy to repair sublethal damage to the cytoplasmic membrane caused by PEF treatments and the reduction of energy needed, probably related to the synthesis of new lipids, as described by García et al. (2005), indicating that mainly the cell envelope is affected during the inactivation process. The role of the outer membrane and other envelope characteristics (such as surface charge) has also been studied (Arroyo et al., 2010; Golberg et al., 2012) but it is still far from being completely understood.

In addition, little is known, at least as compared to other technologies such as heat or High Hydrostatic Pressure, about the ability of bacterial cells to develop homologous and cross-resistance to PEF and on the impact that the development of PEF resistance responses on others aspects of bacterial physiology. Regarding the first, only a few papers have dealt with this topic (Arroyo et al., 2012; Cebrián et al., 2012) although it seems that this phenomenon would be of much less relevance that for other technologies (Cebrián et al., 2016b). The information available regarding the second aspect is even more scarce, although some recent works have investigated, for instance, the impact of PEF of *Salmonella* virulence (Sanz-Puig et al., 2019).

In a previous work we reported the isolation of a PEF resistant *Salmonella* Typhimurium strain (SL1344-RS) obtained after repeated rounds of PEF treatment and outgrowth of survivors (Sagarzazu et al., 2013). This increased PEF resistance was accompanied by an increased resistance to some other agents, such as hydrogen peroxide and acid pH, and was linked to the entry into stationary growth phase of the *Salmonella* cells. Altogether results obtained strongly suggested that the higher PEF-resistance of the variant SL1344-RS could be related to the general stress sigma factor RpoS, since this factor is preferentially expressed in the stationary phase of growth and higher RpoS activity leads to an acquisition of tolerance to a variety of stresses (Hengge-Aronis, 1996). However, this hypothesis remained to be validated. Thus, the aim of this work was to get further insight into the mechanisms responsible for the increased stress resistance of *Salmonella* Typhimurium strain SL1344-RS as well as to quantify the impact that the development of PEF resistance had on other aspects of *Salmonella* physiology, such as growth fitness, biofilm formation ability and virulence.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium SL1344 and a PEF-resistant variant of this strain (SL1344-RS) obtained after repeated rounds of PEF treatment and outgrowth of survivors (Sagarzazu et al., 2013) were used in this study. Strains were maintained frozen at −80 °C in a cryovial for long-term preservation. Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with

0.6% w/v yeast extract (Oxoid, TSB-YE) in 96 wells microtiter plates and incubated at 37 °C under static conditions as described in Guillén et al. (2020).

2.2 Resistance determinations

2.2.1 Sodium chloride resistance determinations

Resistance to osmotic medium was evaluated in TBS-YE supplemented with 30% w/v of sodium chloride (VWR International; NaCl). The treatments were carried out at 37 °C due to the low lethality of this agent, and the initial concentration was of approximately 10^7 CFU/mL. After the selected contact time, up to 32 h, subsequent serial dilutions were prepared in buffered peptone water (Oxoid; BPW) and pour-plated for survival counts as described below.

2.2.2 High hydrostatic pressure (HHP) treatments

HHP treatments were carried out in a Stansted Fluid Power S-FL-085-09-W (Harlow, London, England) apparatus (Ramos et al., 2015). The pressure transmitting fluid was a mixture of propylene glycol and distilled water (50/50, v/v). An automatic device was employed to set and/or record pressure and time during the pressurization cycle. Cell suspensions were prepared at a cell concentration of 10^7 CFU/mL, approximately, in citrate-phosphate McIlvaine buffer of pH 7.0. Samples were packed in plastic bags, which were sealed without headspace and introduced in the treatment chamber. Treatments were applied at 300 MPa for different treatment times up to 30 min, and temperature never exceeded 40 °C.

2.2.3 Ultraviolet C light (UV-C) treatments

UV-C treatments were carried out in a microtiter plate under static conditions. Microtiter plates were coated with 1 layer of a microplate sealing film (BREATHseal, Greiner bio-one, Frickenhausen, Germany) and located at a distance of 22.50 cm from a 32 W UV-C lamp (VL-208G, Vilber, Germany). Radiation intensity was measured by means of a UVX radiometer (UVP, LLC, Upland, CA). Under these experimental conditions, intensity of 0.47 ± 0.02 mW/cm^2 was attained. The treatment medium was citrate-phosphate McIlvaine buffer of pH 7.0, and the initial concentration was of approximately 10^7 CFU/mL. Treatment times of up to 180 seconds were applied and temperature never exceeded 30 °C.

2.2.4 Recovery after different treatments and survival counting

After treatments, samples were adequately diluted in Buffered Peptone Water (Oxoid; BPW) and plated in the recovery medium, TSA-YE. Plates were incubated for 24 h at 37 °C, after which the number of colony-forming units (CFU) per plate was counted.

2.2.5 Survival curves and fitting of data

Survival curves were obtained by plotting the logarithm of the survival fraction (Log_{10} N/N_0) versus treatment time (hours for NaCl determinations; minutes for acid, heat, HHP, and peroxide treatments; seconds for UV-C treatments and μs for PEF treatments). Since deviations from linearity were observed in survival curves to the majority of agents/technologies, GInaFiT, the Geeraerd inactivation model-fitting tool was used to fit survival curves and calculate resistance parameters (Geeraerd et al., 2005).

$$N_t = N_0 \cdot \quad exp^{-K_{max} \cdot t} \cdot \quad \left[\frac{exp^{K_{max} \cdot S_l}}{1 + (exp^{K_{max} \cdot S_l} - 1) \cdot \quad exp^{-K_{max} \cdot t}}\right] \qquad \text{Eq. 1}$$

$$\mathrm{N_t} = (\mathrm{N_0} - \mathrm{N_{res}}) \cdot \quad \exp^{-\mathrm{K_{max}} \cdot \mathrm{t}} + \mathrm{N_{res}} \qquad \text{Eq. 2}$$

In these equations, N_t represents the number of survivors, N_0 the initial count, and t the treatment time.

This model describes the survival curves by means of three parameters: shoulder length (S_l), defined as the time before exponential inactivation begins; inactivation rate (K_{max}), defined as the slope of the exponential portion of the survival curve; and N_{res} which describes residual population density (tail). Therefore, the traditional decimal reduction time value (D-value) can be calculated from the K_{max} parameter using equation 3.

$$D\text{-value} = ln(10)/K_{max} \quad \text{Eq. 3}$$

2.3 Maximum growth rate determination assays

Growth fitness characterization assays were carried out in three different media: TSB-YE at 37 °C, Luria-Bertani (LB) broth supplemented with 100 μM 2-2'dipyridyl (DPY), an iron chelator, at 37 °C, and minimal medium, M9-broth, supplemented with 20 mM gluconate, as the principal carbon source in the intestine as described in Bleibtreu et al. (2013). Pre-cultures of the resistant variant and wild-type strain were diluted 1:100 into 100 μL of prewarmed media placed in 96-well microtiter plates. These plates were sealed (under anaerobic conditions for LB-DYP and M9-Gluconate growth curves) with a polyester impermeable film (VWR) and incubated under static conditions at 37 °C for 24 hours. Samples were taken at preset intervals, adequately diluted in buffered peptone water (Oxoid), and plated in tryptic soy agar (Oxoid) supplemented with 0.6 % w/v yeast extract (Oxoid, TSA-YE).

These plates were incubated for 24 h at 37 °C and then manually counted. Growth curves were constructed by plotting the decimal logarithm of the number of *Salmonella* versus time under the different conditions assayed. Each point in the growth curve corresponds to the average value of all samples analyzed (at least three replicates). The curves obtained were fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000):

$$Y_t = Y_0 + \mu_{max} \cdot A_t - \frac{Y_{max} - Y_0}{M} \cdot ln\left[1 - e^{-M} + \left(e^{-M} \cdot \frac{Y_{max} - Y_0 - \mu_{max} \cdot A_t}{Y_{max} - Y_0}\right)\right] \quad \text{Eq. 4}$$

$$A_t = t - \lambda \cdot \left[1 - \frac{1}{h_0} \cdot ln\left(1 - e^{-h_0 \cdot \frac{t}{\lambda}} + e^{-h_0 \cdot \left(\frac{t}{\lambda} - 1\right)}\right)\right] \quad \text{Eq. 5}$$

where Y_t is the Log_{10} of cell concentration at time t (CFU/mL); Y_0 is the Log_{10} of the initial cell concentration (CFU/mL); Y_{max} is the Log_{10} of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (Log_{10}/h); λ is the lag phase (h); and M and h_0 are curvature parameters, taking them as constant values, and with both set at a value of 10. Curve fitting was carried out using GraphPad PRISM® (GraphPad Software, San Diego, CA, USA) statistical software.

2.4 Virulence assays

2.4.1 Caco-2 cell maintenance and preparation

The human colon carcinoma Caco-2 cell line (TC7 clone) was kindly provided by Dr. Edith Brot-Laroche (Université Pierre et Marie Curie-Paris 6, UMR S 872, Les Cordeliers, France) at Passage 25 and used in experiments at Passage 30-35. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in 75 cm^2 flasks. Cells were grown in Dulbecco's Modified Eagle's Medium + GlutaMAX™ (DMEM, Invitrogen, France) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, France), 1 % Minimal Essential Medium with Non-Essential Amino Acids (MEM NEAA 100X, Invitrogen, France), and 1% antibiotics (penicillin/streptomycin, Invitrogen). Once the cells reached 80% confluence, they were dissociated with 0.05% Trypsin-1 mM EDTA (Invitrogen) and seeded at a density of approximately 15,000 cells per well in 96-well tissue culture plates (Nunc, France) containing 200 μL of complete medium per well. Plates were incubated in humidified atmosphere containing 5% CO_2 at 37 °C for 15-17 days to attain fully differentiated cell layers. Culture medium was replaced every 2 days, and cell confluence was confirmed by optical microscopy.

2.4.2 Adhesion and invasion in Caco-2 cells

Prior to use for virulence assays, cell layers were washed three times in DPBS (Dulbecco's Phosphate Buffered Saline); 200 µL of complete medium without antibiotics were added. For adhesion assay, suspensions of different *Salmonella* strains were added at an initial concentration of 10^6 cells/mL on washed Caco-2 cells. Cells were incubated with bacteria for 30 min in humidified atmosphere containing 5% CO_2 at 37 °C. After incubation, non-adhered bacteria were removed by washing the cell cultures twice with DPBS, and the cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. These lysates were adequately diluted and then plated in Xylose Lysine Desoxycholate Agar (XLD, Oxoid). These plates were incubated at 37 °C for 24 h before being manually counted. For invasion assays, bacterial inoculation was performed as described for the adhesion assay, and plates were maintained in 5% CO_2 at 37 °C for 30 min. The infected cells were washed twice with DPBS, after which they were maintained during 1 hour in DMEM containing 100 µg/mL of gentamicin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) per well to inactivate extracellular bacteria. After incubation, cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. Lysates were processed for determination of *Salmonella* counts as described above. The adhesion and invasion rates were calculated as percentages of adhered or invading bacteria to initial bacteria added.

2.5 Biofilm formation ability assay

Biofilm formation ability was evaluated in a 96-well microtiter plate by adapting the protocol of Patel and Sharma (2010). Briefly, overnight pre-cultures of both *Salmonella* strains were diluted 1:100 in 100 µL TSB-YE media in wells of a sterile 96-well polystyrene microtiter plate (Fisher Scientific, Newark, DE) and incubated under static conditions at 37 °C. After 24, 48 and 72 h incubation in microplate culture, media was completely removed, and the wells were washed three times by immersing the plate in sterile distilled water tempered to 37 °C. The plates were air-dried for 30 min, and 125 µL crystal violet solution (0.1% w/v, Fisher Scientific) was added per well and incubated at room temperature during 20 minutes. Crystal violet solution was removed by washing as indicated above. To quantify biofilm formation, 125 µL of acetic acid (30% v/v) were added to each well, and the absorbance of each well at 580 nm was measured (Genios, Tecan, Männedorf, Switzerland). Thus, the concentration of crystal violet remaining in each well is proportional to the number of biofilm forming cells. For each replicate experiment, four wells were inoculated for each strain. In order to establish meaningful comparisons the area under the curve (AUC) was calculated as described in Espina et al. (2015)). Briefly, the absorbance at 580 nm *vs* time (up to 72 hours; with measurements every 24 hours) was plotted for each strain and the AUC values were calculated using GraphPad software and following the trapezoid rule, where the total area is the sum of all rectangular trapezoids, each defined by two adjacent absorbance values with respect to the ground (in the y axis) and the time between those measurements (in the x axis). The formula we applied was:

$$AUC = \sum_{i=1}^{n-1} \frac{x_i \cdot (y_i + y_{i+1})}{2} \quad \text{Eq. 6}$$

where x_i is the time between measurements in hours, y_i is the absorbance value at 580 nm for each measurement, and n is the total number of measurements.

2.6 Congo Red (CR) assay

Cells from a culture in TSB-YE were inoculated into low salt Luria agar plates (tryptone 10 g/L, yeast extract 5 g/L, technical agar 12 g/L) containing 0.001% Congo Red. Plates were inoculated with 1 µL of pre-cultures and incubated at 37°C for up to 5 d and their colony morphologies were observed.

2.7 Quantification of catalase activity

The catalase activity of each strain was quantified as described in Iwase et al. (2013). Each bacterial suspension (100 µL) was added in a Pyrex tube (13 mm diameter x 100 mm height, borosilicate glass;

Corning, USA). Subsequently, 100 µL of 1% Triton X-100 and 100 µL of undiluted hydrogen peroxide (30%) were added to the solutions and mixed thoroughly and were then incubated at room temperature. Following completion of the reaction, the height of O_2 forming foam that remained constant for 15 min in the test tube was finally measured using a ruler.

2.8 Quantification of glycogen levels

The glycogen levels of each strain was quantified by adapting the protocol of Iwase et al. (2018). Bacteria were cultured for 24 h at 37 °C on Kornberg agar (1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, 1.5% agar, and 1% glucose) (Govons et al., 1969). Bacterial colonies on agar plates were harvested in 500 µL Phosphate Buffered Saline (Oxoid; PBS) and heated at 95 °C for 15 min for enzyme denaturation. Following heat treatment, cells were lysed with 0.1 mm glass beads in a bead-beater instrument (BioSpec product, Oklahoma, USA) and then centrifuged at 10,000xg at 4 °C for 30 min to remove bacterial debris. The resulting supernatants were further filtered to obtain clarified samples using a 13-mm polypropylene 0.20 µm filter (Asahi Glass Co., Ltd., Japan). Iodine solution (3 µL) was added to the filtered supernatants (100 µL) and absorbance was measured at 492 nm using a spectrophotometer (BMG Labteck, Ortenberg, Alemania; CLARIOstar®). Before measuring glycogen levels, 1 µL of 1 M HCl was added to the sample solutions because alkaline samples are colorless.

2.9 Whole genome sequencing (WGS) and identification of mutations

The total genomic DNA (gDNA) from PEF-resistant variant (SL1344-RS) and parental *S.* Typhimurium SL1344 was extracted using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

The genomes of the resistant variant and parental strain investigated were sequenced by the company STAB VIDA (Portugal) in an Illumina Hiseq 4000 platform, using 150bp paired-end. The resulting reads were then subjected to a trimming process using the CLC Genomic Workbench version 12.0. The quality of the produced data was determined by Phred quality score at each cycle using the FastQC program (v3.4.1.1) (Andrews, 2010). Then, the high quality sequencing reads were mapped (length and similarity fractions of 0.8 each) against the *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 reference genome (Kröger et al., 2012). After the mapping, a variant calling algorithm was applied to detect the variants that satisfy the requirements specified by the following filters: minimum Frequency = 35%, minimum Quality (Phred) = 20, minimum coverage = 20, minimum count = 5 and direction filtering. Detection of Insertions and Deletions was also performed by means of a InDels detection tool using the following criteria: minimum number of reads = 5 and P-value threshold = 0.0001.

2.10 RNA extraction and cDNA synthesis

RNA of PEF-resistant variant (SL1344-RS) and parental *S.* Typhimurium SL1344 strain was isolated by phenol-chloroform extraction with a subsequent cleanup procedure using the RNeasy Mini Kit (Qiagen) (Atshan et al., 2012). Cells were pelleted by centrifugation at 8000×g for 3 min in a 4 °C refrigerated centrifuge. The pellet was re-suspended in 100 µL of RNase free water. The tube was vigorously vortexed for 3 min and 100 µL of acid phenol was added with chloroform (1:1). It was vortexed for 1 min and incubated at 70 °C for 30 min. The vortex process was repeated periodically every 5 min. Subsequently, the tube was centrifuged at 12,000×g for 10 min and 100 µL from the aqueous (top only) phase was transferred into a new tube. Seven hundred microliters of lysis buffer were added into the aqueous phase and the subsequent steps were done according to the manufacturer's protocol of RNeasy Mini Kit (Quiagen). The samples, once purified, were treated with DNase to remove residual DNA using the Rapidout Removal Kit (Thermo Fisher Scientific, Massachusetts, USA), also following the manufacturer's instructions and extracted RNA samples were frozen at −80 °C until complementary DNA (cDNA) synthesis.

The RNA previously isolated was converted to cDNA using the Superscript IV Reverse Transcriptase kit (Invitrogen, Carlsbad, USA) using random hexamer primers following the protocol

described by the manufacturer. Once the cDNA was obtained, it was stored at -80 °C until RNAseq and qPCR assays were carried out.

2.11 RNA sequencing (RNA-seq)

RNAseq assays were performed by the company STAB VIDA (Portugal) in an Illumina Novaseq platform, using 150bp paired-end. The library construction of cDNA molecules was carried out using a Ribosomal Depletion Library Preparation Kit. After the sequenciation, the high quality sequencing reads were mapped (length and similarity fractions of 0.8 each) against the *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 reference genome (GenBank: Accession No. FQ312003.1) (Kröger et al., 2012) and analyzed using CLC Genomics Workbench 12.0.3. Gene expression was normalized by calculating reads per kilobase per million mapped reads (RKPM), given by dividing the total number of reads by the number of mapped reads (in millions) x the length in kilobases (Mortazavi et al., 2008). Differential expression analysis (parental *vs* SL1344-RS) was carried out using a multi-factorial statistical analysis tool based on a negative binomial model that uses a generalized linear model approach influenced by the multi-factorial EdgeR method (Robinson et al., 2010). The differentially expressed genes were filtered using standard conditions (Fold change (≥ 2 or ≤ -2 and FDR p-Value ≤ 0.05) (Raza and Mishra, 2012; van Iterson et al., 2010). Further control of the Family-Wise Error Rate (FWER, false positives) was carried out by applying the Bonferroni corrections (p <0.05).

2.12 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Then, expression of three RpoS-dependent genes (*katE*, *katN* and *otsB*) of SL1344-RS and the parental strain was determined using Quantitative Reverse Transcriptase PCR (qRT-PCR). *rpoZ* was used as a reference gene for qPCR normalization (Lévi-Meyrueis et al., 2014).

qPCR amplification was performed using the GoTaq qPCR Master Mix (Promega, Madison, USA) and the primers described in Table 1. The qPCR assays were carried out with a CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, USA) using a protocol with 5 min at 94 °C for GoTaq enzyme activation, followed by 44 cycles of 94 °C for 10 s and 40 s at temperature of 55 °C for annealing, elongation and fluorescence data acquisition. A melting curve between 65 °C and 90 °C was obtained after the last amplification cycle, and at a temperature transition rate of 0.5 °C/s. All amplification reactions were run in triplicate.

The mRNA levels for the genes of interest were quantified from the C_t value, which is the PCR cycle number that generated a common signal for each gene in the exponential phase of amplification. To correct for sampling errors, the levels of expression of each gene, as determined from their C_t values, were normalized to the level of *rpoZ* gene. The relative expression of the genes investigated in each resistant variant was compared to that for parental cells and the fold-change in transcription was calculated using $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

Table 1. Primer sequences used in Real-Time PCR to quantify the expression of RpoS-dependent genes.

Gene target	Forward primer (5′ -to- 3′)	Reverse primer (5′ -to- 3′)
rpoZ	CGAAGAAGGTCTGATTAAC	GACGACCTTCAGCAATA
katE	GGCGTCTGTTCTCTTAT	CTGGAAGTTATGGTAGGG
katN	TGAGTCATCTGGAAATTAT	CGATAAAGTTCCGCTTC
otsB	TTAACCGTATCCCCCGAACTC	CCGCGAGACGGTCTAACAAC

2.13 Statistical analysis

All the determinations were carried out in triplicate on different working days. Standard deviations (SD) and statistical analyses (ANOVA and Tukey tests; p-value < 0.05) were calculated using GraphPad

PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA).

3. Results

3.1 Genetic characterization of the PEF-resistant variant

First, a WGS analysis of strain SL1344-RS was performed to identify the genetic changes responsible for the phenotypic changes observed by Sagarzazu et al. (2013). In parallel, the parental strain was also sequenced. This allowed us to identify 4 SNVs when comparing our parental strain with the reference genome of this same strain (GenBank: Accession No. FQ312003.1) (Kröger et al., 2012). These SNVs are listed in Table S1.

Then we compared the genomes of strains SL1344 and SL1344-RS, and, as can be observed in Table 2, two SNVs were found.

Table 2. Mutations identified in the *S*. Typhimurium SL1344-RS strain (as compared to our *S*. Typhimurium SL1344 strain) by whole genome sequencing (WGS). All detected mutations were single nucleotide variations (SNV).

Region	Genes	Locus tag	Mutation type	Amino acid change	Description
1805904	*hnr*	SL1344_1684	c.902T>C	Leu301Pro	hypothetical regulatory protein
3284620	*yggW*	SL1344_3079	c.804T>C	No change	possible oxygen-independent coproporphyrinogen III oxidase

The first SNV was found in the *hnr* gene at position 902 bp, resulting in the substitution of a Leucine (Leu) by a Proline (Pro). This protein regulates the turnover of the alternative sigma factor σ^S (RpoS) by promoting its proteolysis (Zhou and Gottesman, 1998), and it also controls which mRNAs are destroyed by stimulating polyadenylation (Carabetta et al., 2009). RpoS is regarded as the master regulator of the general stress response in many Gram-negative bacteria, including *Salmonella* (Battesti et al., 2011; Hengge, 2011; Lago et al., 2017; Österberg et al., 2011). Therefore, any change in *hnr* has the potential to affect its RpoS activity and consequently, the stress resistance of *Salmonella* Typhimurium cells. Given the fact that this variant displayed a higher resistance to different stresses it was already speculated in Sagarzazu et al. (2013) that this might be linked to an increased RpoS activity, something that, as will be described below, has been verified though transcriptomic and phenotypic assays.

The second SNV was a reversion of a mutation found when comparing the genome of the parental strain, *S*. Typhimurium SL1344, with that of the reference strain in *yggW* gene at position 804 bp, resulting a silent mutation. YggW also named HemW in *E. coli*, although its function is poorly understood, it probably acts as a heme chaperone (Haskamp et al., 2018). Since this variant was isolated almost ten years ago but WGS sequencing was carried out in fresh cultures in 2020 and the parental strain is extensively used in our lab it cannot be excluded that, instead of a reversion, this difference between the parental and the PEF resistant strain would be due to the fact that this variant was isolated from a culture of the parental strain that did not harbor this mutation. In any case, it should be noted that, since this was a silent mutation no phenotypic change would be associated to it and, therefore, the changes observed would be solely linked to the mutation observed in *hnr*.

3.2 Comparative global gene expression analysis (RNAseq)

In order to determine the impact of the genetic changes observed on *Salmonella* physiology and get a deeper insight into the mechanisms leading to its increased stress resistance we studied and compared the transcriptomes of the parental and the PEF-resistant variant using RNA-sequencing. Using this

approach, we identified a total of 147 genes differentially expressed in the parental strain and the PEF-resistant variant (p <0.05), of which 22 were highly significant (p <0.001). After applying the Bonferroni correction factor -which, despite reducing the number of true discoveries, reduces the number of false positives- 6 genes showed differential expression levels (>2 fold, p <0.05) when comparing the parental and the PEF-resistant variant (Table 3).

Table 3. Genes differential expression levels (>2 fold, p <0.05) in the parental strain, *S*. Typhimurium SL1344, and the PEF-resistant variant.

Gene	Fold change	RpoS-dependent	Reference
SL1344_1197 (YhjQ)	30.50	Yes*	Dong (2010)
SL1344_1443 (YmdF)	154.31	Yes	Oguri et al. (2019)
yciF	91.61	Yes	Beraud et al. (2010)
yciE	204.80	Yes	Beraud et al. (2010)
katN	159.04	Yes	Beraud et al. (2010)
zraP	-19.37	-	Appia-Ayme et al. (2012)

*In E. Coli

Among these 6 genes 5 of them were up-regulated in the PEF-resistant variant, and one of them was down-regulated. In addition, 5 of them have been shown to be RpoS-dependent. These results strongly suggest that the mutation found resulted in a decrease in *hnr*-dependent RpoS proteolysis and indicate that this PEF resistant variant would have an increased RpoS activity.

3.3 qRT-PCR analysis of RpoS activity

In order to verify the results obtained by RNAseq the expression of three well-known RpoS-regulated genes was quantified and used as reporters of their activity: *katE*, *katN* and *otsB* by qRT-PCR. The *katE* gene, encoding the HPII catalase, and *katN* gene, encoding a non-haem catalase, both are considered to be RpoS-dependent in *Salmonella* (Chen et al., 1996; Ibañez-Ruiz et al., 2000; Robbe-Saule et al., 2001), and they also contribute to the prevention of oxidative stress (Visick and Clarke, 1997). RpoS is likewise involved in the transcription of the *otsBA* operon in *S*. Typhimurium (Balaji et al., 2005; Lévi-Meyrueis et al., 2014), which plays an important role in countering osmotic stress via regulation of the trehalose synthesis. Under high-osmolarity conditions, trehalose serves as an osmoprotectant.

Figure 1 shows the relative expression of the three genes studied ($2^{-\Delta\Delta Ct}$) in the PEF-resistant variant as compared to the parental strain. As can be observed in the graph the three RpoS-regulated genes were overexpressed in PEF-resistant variant, especially *ostb* gene, confirming the results obtained by RNAseq.

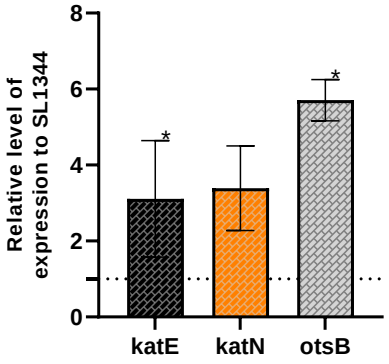

Figure 1. Relative expression of the three genes studied ($2^{-\Delta\Delta Ct}$) in the PEF-resistant variant as compared to the parental strain. Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences (p <0.05) between the parental and the variant.

3.4 Measurement of catalase activity and glycogen levels

RpoS activity is usually indirectly determined by measuring the enzymatic activity of HPII, which catalyzes the dismutation of hydrogen peroxide to water and oxygen, the glycogen levels of *Salmonella* cells (Iwase et al., 2018; Schellhorn, 1995; Tanaka et al., 1997) and also using the dye Congo red (CR) to develop the typical rdar morphotype (Robbe-Saule et al., 2006). Catalase activity was measured by quantifying the trapped oxygen gas, which is visualized as foam. The height of the foam generated by the oxygen gas produced by the catalase-hydrogen peroxide reaction in test tubes is shown in Figure 2. As can be observed the PEF-resistant variant showed a higher foam height (p <0.05) than the parental strain, 10.33 ± 1.528 and 5.67 ± 0.577, respectively. Conversely, we did not observe significant differences in the glycogen levels or visual ones in Congo red staining. Although these two later results might suggest that no differences in RpoS activity would exist between the strains it should be noted that these techniques are especially useful for distinguishing RpoS positive and negative (absence or very low expression) phenotypes and the parental strain already exhibited a RpoS positive phenotype. Therefore, it is reasonable to hypothesize that the absence of differences would be probably due to the inability of these two techniques to distinguish between the different levels of RpoS activity in these strains and not because these differences did not really exist (as all the other results obtained in this work clearly indicate).

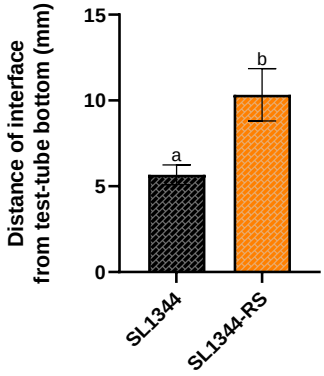

Figure 2. The height of foam generated after mixing catalase, Triton X-100, and H_2O_2 following the methodology described in material and methods of *S*. Typhimurium SL1344 (SL1344) and PEF-resistant variant (SL1344-RS). Letters indicate statistically significant differences (p <0.05) and error bars represent the standard deviations.

3.4 Further characterization of the stress resistance of S. Typhimurium SL1344-RS

In the paper in which its isolation was reported, the resistant variant SL1344-RS showed the same heat resistance as the parental strain. However, survival in acidic pH, and most especially in hydrogen peroxide and ethanol was increased in this resistant variant (Sagarzazu et al., 2013). In this study, we investigated whether the PEF resistant variant was also more tolerant to other technologies or agents. The resistance to osmotic stress, high hydrostatic pressure and UV-C of the SL1344-RS strain was determined and compared with that of the parental strain, *S*. Typhimurium SL1344 (SL1344) as described Guillén et al. (2020). Survival curves were obtained by plotting the logarithm of the survival fraction versus treatment time, and then, the non-linear Geeraerd model (Geeraerd et al., 2000) was used to calculate the corresponding resistance parameters (N_0; S_1; K_{max}, N_{res}). The mean values obtained for these parameters and their standard deviation, together with the goodness-of-fit parameters, are included in Table S2. The traditional decimal reduction time (D) value of each survival curve was calculated from its corresponding K_{max} (Eq. 3). It was decided to use the *2D*-value parameter (time required to inactivate the first 2 Log_{10} cycles) to establish meaningful comparisons between *S.* Typhimurium SL1344 and PEF-resistant variant SL1344-RS, as described in Guillén et al. (2020).

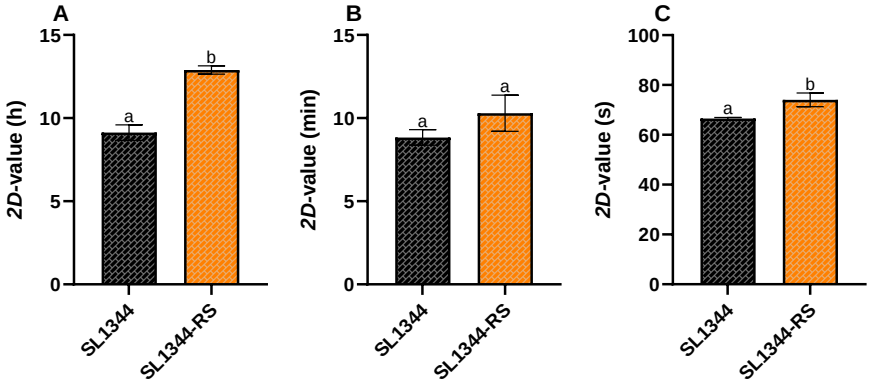

Figure 3. *2D*-values of the parental *S.* Typhimurium SL1344 (SL1344) and PEF-resistant variant (SL1344-RS) to sodium chloride (30% w/v, A), to high hydrostatic pressure (300 MPa, B) and to UV-C (0.47 mW/cm^2, C). Letters indicate statistically significant differences (p <0.05) and error bars represent the standard deviations.

As can be deduced from Figure 3, the SL1344-RS variant showed a higher tolerance to osmotic medium (12.90 ± 0.252 vs 9.12 ± 0.468 h) and UV-C (74.02 ± 2.749 vs 66.51 ± 0.431 s) (p <0.05), but no significant differences (p >0.05) in HHP resistance were found among the two strains. Thus, this PEF resistant variant would not only be more PEF, acid, hydrogen peroxide and ethanol resistant but also more osmotic and UV-C resistant than the parental one. This seems reasonable considering the role of RpoS as a master regulator of the general stress response in *Salmonella* and the fact that RpoS has already been shown to be essential for optimal desiccation, starvation, and acid tolerance in this microorganism (Lee et al., 1995; Loewen et al., 1998). The role of RpoS on *Salmonella* UV-C and PEF resistance has not been explored yet, but results here obtained suggest that RpoS expression/activity might contribute to increase *Salmonella* resistance to these agents. On the other hand, it is not surprising that the two agents to which no change in resistance was observed were heat and HHP since they share various cellular targets and that there is a substantial overlap in the microbial responses to these agents (Cebrián et al., 2016b). In any case, it should be noted that these results do not directly imply that RpoS activity does not influence the resistance of *Salmonella* to these technologies, since results obtained here might be also explained by the fact that the change in RpoS activity required to induce significant changes in resistance to them might be higher than that existing between these two strains or because, once an RpoS activity threshold is reached, subsequent increases would not have any effect on heat and HHP resistance. Even more, other potential explanations cannot be discarded.

Further work would be required to fully elucidate the role of RpoS in *Salmonella* resistance to all these agents but specially against UV-C and PEF. Similarly, new experiments will be required in order to determine what member/s of the *rpoS* regulon is/are responsible for the increase in resistance to each of these agents.

3.5 Characterization of S. Typhimurium SL1344 resistant to pulsed electric fields growth rates in different media

Growth curves of SL1344-RS and the parental strain were obtained in three different media: in TSB-YE, a nutrient-rich medium, LB medium with iron limitation caused by the addition of DPY, and a minimal medium containing gluconate as the sole carbon source. The selection of this growth media has been previously discussed in Guillén et al. (2022). The μ_{max} (Log_{10}/h) values calculated in the three growth media for SL1344 and SL1344-RS are shown in Figure 3 and growth parameters and goodness of the fit parameters are included in Supplementary Table S3. None of the strains displayed a statistically significant Lag phase (h) (different from 0; p > 0.05) in any of the three media tested and significant differences between the strains were only found in M9-Gluconate, 0.618 ± 0.030 vs 0.533 ± 0.024 (p <0.05), which is the poorest/more restrictive medium. In LB-DYP, the growth parameters were similar, 0.681 ± 0.057 Log_{10}/h for SL1344-RS and 0.692 ± 0.049 Log_{10}/h.

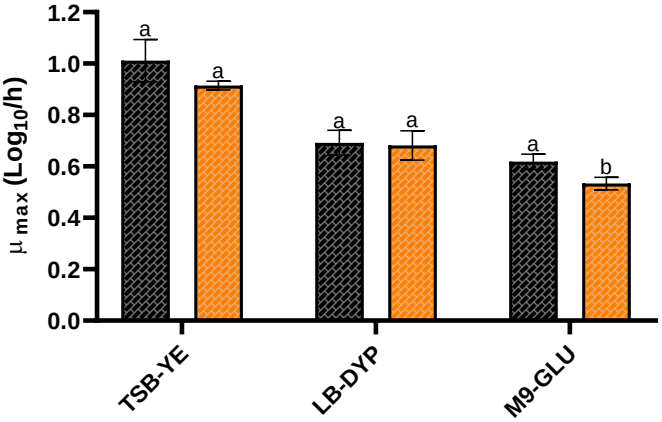

Figure 4. Maximum growth rates (μ_{max} (Log_{10}/h)) of *S.* Typhimurium SL1344 (SL1344, black) and PEF-resistant variant (SL1344-RS, orange), in TSB-YE, LB supplemented with 100 μM 2-2'dipyridyl (DPY) and M9-broth supplemented with 20 mM gluconate at 37 ˚C. Error bars correspond to the standard deviation of the means.

These results are consistent with that reported for *E. coli* and *S.* Typhi, microorganisms in which it has been observed that cells with a reduced RpoS activity can grow better in media with low levels of nutrients, and also seem to possess an advantage in competitive colonization of the intestine (Altuvia et al., 1994; Krogfelt et al., 2000; Sabbagh et al., 2010). In any case it should be noted that the differences in growth rate were lower than a 15%, indicating that the cost of the acquisition of resistance for strain SL1344-RS was not very large (although it should also be noted that the increase in resistance was also quite limited).

3.6 Characterization of virulence capacity of S. Typhimurium SL1344 resistant to pulsed electric fields

The virulence capacity of the parental and stress resistant variant was evaluated by determining the percentage of cells capable to adhere and invade Caco-2 cells (Figure 5). Results obtained indicate that the PEF-resistant strain, SL1344-RS, displayed a higher adhesion capacity to Caco-2 cells than the parental strain, SL1344, 5.21 vs 2.00%, respectively but no significant differences in invasion ability between them were observed (0.19 vs 0.12%; p >0.05). In any case, given the high variability of these assays (already discussed in (Mellor et al., 2009)) these conclusions should be taken with care.

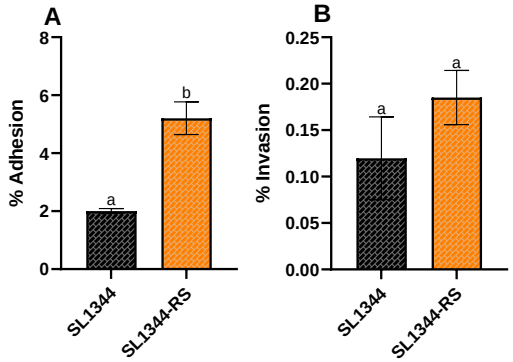

Figure 5. Adhesion (A) and invasion (B) capacity to Caco-2 cells of *S.* Typhimurium SL1344 (SL1344) and PEF-resistant variant (SL1344-RS). Letters indicate statistically significant differences (p <0.05) and error bars represent the standard deviations.

These apparently contradictory results would be probably related to the very complex the role that RpoS has on *Salmonella* virulence (Guillén et al., 2021). Thus, RpoS seems to reduce the expression of some virulence factors while inducing other ones and, therefore, depending on the particular phenotypical trait and the level of expression/activity the outcome might be completely different.

3.7 Static biofilm formation ability

Finally, the results of the static biofilm formation assay are shown in Figure 6. It should be noted that, in order to establish meaningful comparisons, the AUC values calculated as described in materials and methods were compared. Biofilm formation capacity was not altered by the development of PEF resistance in SL1344-RS, since no statistically significant differences (p >0.05) were found between the resistant variant and the parental strain, 2.56 ± 1.125 and 2.29 ± 0.799, respectively. RpoS plays an important role in biofilm formation by regulating the central regulator CsgD (Simm et al., 2014), nevertheless it has also been shown that CsgD regulation is serovar-specific and may be partially independent of RpoS, since other sigma factors can maintain a low level of biofilm formation after removing RpoS (Feng et al., 2020; Römling et al., 2003). This contrasts with the high adhesion capacity to Caco-2 cells shown by SL1344-RS, however, despite being analogous processes, the surface characteristics and structures/ metabolic pathways involved are not the same (although some are involved in both phenomena) (Peng, 2016).

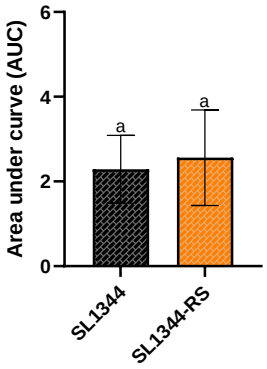

Figure 6. Biofilm-forming ability of *S*. Typhimurium SL1344 (SL1344) and PEF-resistant variant (SL1344-RS). Values correspond to the Area under the Curve calculated as described in Material and Methods. Letters indicate statistically significant differences (p <0.05) and error bars represent the standard deviations.

4. Conclusions

Results obtained in this work indicate that the increased PEF resistance of the *S*. Typhimurium PEF-resistant variant SL1344-RS would be due to an increased RpoS activity, caused by a mutation in the *hnr* gene. This increased RpoS activity also resulted in an increased resistance to multiple stresses (acid, osmotic, oxidative, ethanol and UV-C but not for heat and HHP), a decreased growth rate in M9-gluconate (but not in TSB-YE or LB-DPY) and an increased adhesion ability to Caco-2 cells (but without significant changes in the invasion ability). This work significantly contributes to understand the mechanisms of stress resistance development in *Salmonellae* and point out the crucial role that RpoS plays in this process. Further work should also be done to precisely determine if this PEF-resistant variant has a higher, equal or lower risk associated than the parental strain in different scenarios given the fact that its increase in stress resistance had a fitness cost (at least in M9-gluconate media, which is was designed to resemble conditions in the gut).

Author Contributions: Conceptualization, P.M., and G.C.; methodology, S.G., N.H., P.M., and G.C.; formal analysis, S.G., N.H. and G.C.; investigation, S.G. and L.N.; writing—original draft preparation, S.G.; writing—review and editing, L.N., N.H., P.M., and G.C.; supervision, G.C.; project administration, G.C.; funding acquisition, P.M., and G.C.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Table S1. Genetic variations detected by whole genome sequencing (WGS) between *S*. Typhimurium SL1344 and the reference genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 (GenBank: Accession No. FQ312003.1). All detected mutations were single nucleotide variation (SNV).

Region	Genes	Locus tag	Mutation Type	Aminoacid Change	Description
2411272	*menC*	SL1344_2275	c.444A>G	No coding	O-succinylbenzoate synthase
3284620	*yggW*	SL1344_3079	c.804T>C	No change	possible oxygen-independent coproporphyrinogen III oxidase
3611524	*rpsN*	SL1344_3394	c.89A>G	Asn30Ser	30S ribosomal subunit protein S14
4850544	SL1344_4501	SL1344_4501	c.400T>C	Phe134Leu	hypothetical outer membrane protein

Table S2. Resistance (K_{max}. S_l and N_{res}) and goodness of the fit (R^2, RMSE) parameters calculated after fitting the survival curves to the osmotic stress, high hydrostatic pressure and UV-C treatments investigated of *S*. Typhimurium resistant to PEF and the parental strain to the Geeraerd's model. Values in parentheses represent the SD of the means.

NaCl			HHP			UV-C		
	SL1344	**SL1344-RS**		**SL1344**	**SL1344-RS**		**SL1344**	**SL1344-RS**
K_{max} **(h^{-1})**	0.523 (0.032)	0.392 (0.013)	K_{max} **(min^{-1})**	0.522 (0.027)	0.451 (0.046)	K_{max} **(s^{-1})**	0.070 (0.002)	0.076 (0.020)
S_l **(h)**	-	-	S_l **(min)**	-	-	S_l **(s)**	1.12 (1.940)	11.28 (15.921)
N_{res} **(CFU/mL)**	4.28 (0.313)	4.53 (0.067)	N_{res} **(CFU/mL)**	-	-	N_{res} **(CFU/mL)**	-	-
R^2	0.97 - 0.99	0.97 - 0.98	R^2	0.94 - 0.98	0.99 - 0.99	R^2	0.95 - 0.96	0.99 - 1.00
RMSE	0.106 - 0.160	0.139 - 0.209	**RMSE**	0.177 - 0.316	0.096 - 0.132	**RMSE**	0.398 - 0.413	0.106 - 0.203

Table S3. Growth parameters (Y_0, Y_{max}. λ and μ_{max}) and goodness of the fit (R^2, RMSE) calculated with the Baranyi and Roberts model of of *S.* Typhimurium resistant to PEF and the parental growth in TSB-YE, LB supplemented with 100 μM 2-2'dipyridyl and M9-broth, supplemented with 20 mM gluconate. Values presented correspond to the mean and SD of the means (in parentheses).

	TSB-YE		**LB-DYP**		**M9-Glu**	
	SL1344	**SL1344-RS**	**SL1344**	**SL1344-RS**	**SL1344**	**SL1344-RS**
Y_0 (Log(CFU/mL))	6.16 (0.117)	6.12 (0.049)	6.21 (0.067)	6.04 (0.066)	6.12 (0.017)	6.02 (0.034)
μ_{max} (h^{-1})	1.012 (0.082)	0.914 (0.017)	0.692 (0.049)	0.681 (0.057)	0.618 (0.030)	0.534 (0.025)
Lag (h)	-	-	-	-	-	-
Y_{max} (Log(CFU/mL))	8.97 (0.016)	8.95 (0.011)	8.77 (0.071)	8.56 (0.068)	7.90 (0.104)	8.09 (0.393)
R^2	0.98 - 1.00	0.99 - 0.99	0.93 - 1.00	0.98 - 0.99	0.99 - 1.00	0.99 - 0.99
RMSE	0.058 - 0.100	0.053 - 0.089	0.044 - 0.255	0.048 - 0.082	0.021 - 0.070	0.035 - 0.061

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Isolation and characterization of *Salmonella* Typhimurium SL1344 variants with increased resistance to different stressing agents and food processing technologies

Silvia Guillén, Laura Nadal, Nabil Halaihel, Pilar Mañas and Guillermo Cebrián

Isolation and characterization of *Salmonella* Typhimurium SL1344 variants with increased resistance to different stressing agents and food processing technologies

Silvia Guillén[1], Lauda Nadal[1], Nabil Halaihel[2], Pilar Mañas[1] and Guillermo Cebrián[1]*

[1] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón—IA2—(Universidad de Zaragoza-CITA), 50013, Zaragoza, Spain;
[2] Departamento I+D+i, Alquizvetek S.L, Zaragoza, 50013 Zaragoza, Spain
* Correspondence: guiceb@unizar.es (G.C); Tel.: +34-876554127

Abstract: In this study, resistant variants of *Salmonella enterica* serovar Typhimurium SL1344 to different stressors were selected. In addition, a genetic and phenotypic study was performed to explore the mechanisms underlying the acquisition of resistance. We isolated 4 variants with increased stable resistance to acid, osmotic, high hydrostatic pressure (HHP) and Ultraviolet-C light (UV-C) after repeated rounds of these agents and outgrowth of survivors, and a PEF-resistant variant (SL1344-RS) isolated by Sagarzazu et al. (2013) was also included in the analysis. The results indicated that the isolated variants showed resistance to at least one agent, other than the selective one. This increased resistance, in general terms, had a fitness cost in growth, and had a variable impact on virulence (much greater and significant in adhesion than in invasion). The WGS analysis permitted us to identify the genetic changes, and revealed that in 3 of the 5 variants (including SL1344-RS) a mutation was found in *hnr* gene, an anti-sigma factor that promotes RpoS proteolysis and hence the expression of several *rpoS*-regulated genes was quantified. This increase in RpoS activity would explain the lower growth rates observed in these 3 variants, as it would lead to increased transcription of genes involved in growth arrest and resistance to various types of stress. However, our results indicate that a direct relationship between RpoS activity and stress resistance would not exist within *Salmonellae* with the exceptions of PEF and, maybe, NaCl resistance where RpoS activity could play a major role.

Keywords: foodborne pathogen, *rpoS*, WGS, stress resistance, growth fitness, virulence

1. Introduction

Salmonella are the second most frequent zoonotic agent in the European Union and the United States (EFSA, 2021; Marder, et al., 2018), and are now considered a pathogenic reemerging pathogen (De Cesare, 2018). In Europe, about 88,000 confirmed cases of salmonellosis in humans were reported in 2019, with the cost of salmonellosis estimated at more than €3 billion annually (EFSA, 2021; Havelaar, 2011). The microorganisms of the genus *Salmonella* are a successful example of evolution and adaptation to different niches and hosts. The emergence of variants and the generation of population heterogeneity are factors that may contribute to the survival and adaptive capacity of *Salmonella*. In fact, these emerging resistant variants constitute an excellent model to deepen the understanding of mechanisms involved in cell survival and resistance to the different technologies and stressors. There are several approaches to obtain such variants with stable tolerance, either after several cycles of treatment and

growth of survivors (Karatzas et al., 2008; Sagarzazu et al., 2013), or after a single stressor treatment or food processing technology (Karatzas and Bennik, 2002; Metselaar et al., 2013).

The ability of *Salmonella* cells to resist and adapt to adverse conditions is one of the main characteristics that have made this microorganism such a relevant health hazard, but the impact of these resistance responses on other aspects of *Salmonella* physiology, such as virulence and growth fitness, is much less well known (Guillén et al., 2021). In Guillén et al. (2021), the impact to the resistance responses to stress conditions encountered in food and food processing environments of stable variants exerts on different aspects of non-typhoidal *Salmonellae* physiology, with special emphasis on virulence and growth fitness was reviewed. Nevertheless, further studies are needed to characterize the phenotype of these variants to understand the variability of pathogen populations and the impact of stress resistance on the overall phenotype, as for some food technologies or agents there is insufficient information. And also, the emergence of variants with increased resistance to food processing technologies may have important implications for the application of such technology in the food industry. Therefore, genetic and physiological studies to characterize emerging variants would greatly facilitate the design of efficient processes for industrial applications.

In previous work, a Pulsed Electric Field (PEF)-resistant variant of *Salmonella* Typhimurium SL1344 (SL1344-RS) was obtained after repeated rounds of PEF treatment and outgrowth of survivors (Sagarzazu et al., 2013). This increased PEF resistance was accompanied by an increased resistance to some other agents, such as hydrogen peroxide and acid pH, and was linked to the entry into stationary growth phase of the *Salmonella* cells. Considering the relevance of this, in this study, other environmental stressors and food processing technologies, included in Guillén et al. (2020b), were explored for the isolation of a variant of *Salmonella enterica* serovar Typhimurium SL1344 with increased resistance to them after a selection procedure consisting of alternating rounds of exposure to the stressors or food technologies and outgrowth of the surviving population. In addition, a genetic and phenotypic study was performed to explore the mechanisms underlying the acquisition of resistance.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium SL1344 was used in this study and the model/parental strain. Strain was maintained frozen at −80 °C in a cryovial for long-term preservation. Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (Oxoid, TSB-YE) in 96 wells microtiter plates and incubated at 37 °C under static conditions as described in Guillén et al. (2020b). For some experiments also an additional set of 22 *Salmonella* strains (listed in supplementary Table S1) was used. The same preservation and growth conditions described for *Salmonella* Typhimurium SL1344 were used for the preservation and growth of the variants isolated in this study (see below) and the set of 22 *Salmonella* strains.

2.2 Selection and isolation of resistant cells

Resistant variants were selected by subjecting a *S*. Typhimurium SL1344 culture to successive rounds of the agents and food technologies described below and growth of the surviving cells in liquid medium. The methodology used for the isolation of the different variants was described by Sagarzazu et al. (2013) for the selection of *S.* Typhimurium SL1344 cells resistant to PEF. Briefly, one hundred microliters of the cell suspension treated to the different environmental stresses and food technologies were directly inoculated into 50 mL of sterile TSB-YE which was incubated at 37 °C for 24 h. Simultaneously, plate counts were performed after each treatment to monitor the acquisition of tolerance to the different stressors or food technologies.

The conditions to which the *Salmonella* cells were exposed to the different agents studied are shown in Table 1. The treatment media, initial cell concentrations of the assays and the equipment used will be described in section 2.3. Only in the case of heat treatments, treatments were carried out in glass tubes, which contained tryptic soy broth as a treatment medium, submerged and prewarmed at 58 ± 0.2 °C in

a thermostated water bath, and at initial concentrations of 10^9 CFU/mL, instead of the thermoresistometer described below was used for methodological reasons. Thus, *S*. Typhimurium SL1344 cells were exposed to successive cycles of the different stressors at an intensity leading to an inactivation of around 5 logarithmic cycles. To verify that no contamination had occurred during the screening process, counts were performed in Xylose Lysine Deoxycholate agar (Oxoid, XLD), a *Salmonella* selective medium.

Table 1. Conditions used for the isolation of variants resistant to different environmental stresses and food preservation technologies. The initial concentration of the treatments was 10^9 CFU/mL. Treatment temperature was room temperature (25 °C) but for heat (58 °C) and NaCl (37 °C).

Environmental stresses and food technologies	Conditions	Treatment time
Acid	pH 2.5	180 min
Hydrogen peroxide	50 mM	80 min
Sodium chloride	30% (w/v) NaCl	24 h
Heat	58 °C	5 min
HHP	300 MPa	10 min
UV-C	0.09 mW/cm^2	150 sec

2.3. Environmental stress and food preservation technologies treatments

Environmental stress treatments and food preservation technologies are described in detail in Guillén et al. (2020b). The following is a brief description of the treatments performed.

2.3.1 Acid, hydrogen peroxide, and sodium chloride resistance determinations

The treatment medium for acid-resistance determinations was citrate-phosphate McIlvaine buffer adjusted to pH 2.5. Hydrogen peroxide resistance was evaluated in 100 mM Tris–HCl buffer (pH 7.0) with hydrogen peroxide added at final concentrations of 30 mM (Sigma, St Louis, USA). Resistance to osmotic medium was evaluated in TBS-YE supplemented with 30% w/v of sodium chloride (VWR International; NaCl). In all cases, cells were added to the treatment media at room temperature (25 °C), except for the NaCl determinations, which were carried out at 37 °C due to the low lethality of this agent, to an initial concentration of 10^7 or 10^9 CFU/mL. After the selected contact time, which ranged from 50 min to 32 h, depending on the agent, subsequent serial dilutions were prepared in buffered peptone water (Oxoid; BPW) and pour-plated for survival counts as described below.

2.3.2 Heat treatments

Heat treatments were carried out in a specially designed resistometer (Condón et al., 1993). Once treatment temperature had attained stability (58 ± 0.1 °C), 0.1 mL of the microbial cell suspension was injected into the main chamber containing the treatment media, tryptic soy broth. After inoculation, samples were collected at different heating times up to 20 minutes and immediately pour plated and incubated for survival counting.

2.3.3 High hydrostatic pressure (HHP) treatments

HHP treatments were carried out in a Stansted Fluid Power S-FL-085-09-W (Harlow, London, England) apparatus (Ramos et al., 2015). The pressure transmitting fluid was a mixture of propylene glycol and distilled water (50/50, v/v). An automatic device was employed to set and/or record pressure and time during the pressurization cycle. Cell suspensions were prepared at a cell concentration of 10^7

or 10^9 CFU/mL, approximately, in citrate-phosphate McIlvaine buffer of pH 7.0. Samples were packed in plastic bags, which were sealed without headspace and introduced in the treatment chamber. Treatments were applied at 300 MPa for different treatment times up to 30 min, and temperature never exceeded 40 °C.

2.3.4 Pulsed electric field (PEF) treatments

The PEF equipment used in this investigation was supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The equipment and treatment chamber have been previously described by Saldaña et al. (2009). Prior to PEF treatments, 0.1 mL of the microbial cell suspension were dissolved in citrate-phosphate McIlvaine buffer (pH 7.0 and 1 mS/cm of conductivity) at a concentration of approximately 10^7 CFU/ml. Samples were placed with a sterile syringe in the treatment chamber, which had a gap of 0.25 cm. Treatments were based on square pulses with a width of 3 μs and a frequency of 1 Hz. Electric field strength was set at 25 kV/cm, being the energy per pulse 1.88 kJ/kg. Treatments of up to 50 pulses (150 μs) were applied. Under these conditions, the final temperature of the treatment media was always below 35 °C.

2.3.5 Ultraviolet-C light (UV-C) treatments

UV-C treatments were carried out in a microtiter plate under static conditions or in a petri dish under agitation (300 rpm). Microtiter plates were coated with 1 layer of a microplate sealing film (BREATHseal, Greiner bio-one, Frickenhausen, Germany) or the petri dish lid and located at a distance of 22.50 cm from a 32 W UV-C lamp (VL-208G, Vilber, Germany). Radiation intensity was measured by means of a UVX radiometer (UVP, LLC, Upland, CA). Under these experimental conditions, intensities of 0.47 or 0.09 ± 0.02 mW/cm^2 were attained. The treatment medium was citrate-phosphate McIlvaine buffer of pH 7.0, and the initial concentrations were of approximately 10^7 or 10^9 CFU/mL. Treatment times of up to 180 seconds were applied and temperature never exceeded 30 °C.

2.2.4 Recovery after different treatments and survival counting

After treatments, samples were adequately diluted in Buffered Peptone Water (Oxoid; BPW) and plated in the recovery medium, TSA-YE. Plates were incubated for 24 h at 37 °C, after which the number of colony-forming units (CFU) per plate was counted.

2.3.7 Survival curves and fitting of data

Survival curves were obtained by plotting the logarithm of the survival fraction (Log_{10} N/N_0) versus treatment time (hours for NaCl determinations; minutes for acid, heat, HHP, and peroxide treatments; seconds for UV-C treatments and μs for PEF treatments). Since deviations from linearity were observed in survival curves to the majority of agents/technologies, GInaFiT, the Geeraerd inactivation model-fitting tool was used to fit survival curves and calculate resistance parameters (Geeraerd et al., 2005).

$$N_t = N_0 \cdot \quad exp^{-K_{max} \cdot t} \cdot \quad \left[\frac{exp^{K_{max} \cdot S_l}}{1 + (exp^{K_{max} \cdot S_l} - 1) \cdot \quad exp^{-K_{max} \cdot t}}\right] \qquad \text{Eq. 1}$$

$$\mathrm{N_t} = (\mathrm{N_0} - \mathrm{N_{res}}) \cdot \quad \exp^{-\mathrm{K_{max}} \cdot \mathrm{t}} + \mathrm{N_{res}} \qquad \text{Eq. 2}$$

In these equations, N_t represents the number of survivors, N_0 the initial count, and t the treatment time.

This model describes the survival curves by means of three parameters: shoulder length (S_l), defined as the time before exponential inactivation begins; inactivation rate (K_{max}), defined as the slope of the exponential portion of the survival curve; and N_{res} which describes residual population density

(tail). Therefore, the traditional decimal reduction time value (D-value) can be calculated from the K_{max} parameter using equation 3.

$$D\text{-value} = ln(10)/K_{max} \quad \text{Eq. 3}$$

2.4 Growth fitness characterization assays

Growth fitness characterization assays were carried out in three different media: TSB-YE at 37 °C, Luria-Bertani (LB) broth supplemented with 100 μM 2-2'dipyridyl (DPY), an iron chelator, at 37 °C, and minimal medium, M9-broth, supplemented with 20 mM gluconate, as the principal carbon source in the intestine as described in Bleibtreu et al. (2013). Pre-cultures of each of resistant variant and the parental strain were diluted 1:100 into 100 μL of prewarmed media placed in 96-well microtiter plates. These plates were sealed (under anaerobic conditions for LB-DYP and M9-Gluconate growth curves) with a polyester impermeable film (VWR) and incubated under static conditions at 37 °C for 24 hours. Samples were taken at preset intervals, adequately diluted in buffered peptone water (Oxoid), and plated in tryptic soy agar (Oxoid) supplemented with 0.6 % w/v yeast extract (Oxoid, TSA-YE).

These plates were incubated for 24 h at 37 °C and then manually counted. Growth curves were constructed by plotting the decimal logarithm of the number of *Salmonella* versus time under the different conditions assayed. Each point in the growth curve corresponds to the average value of all samples analyzed (at least three replicates). The curves obtained were fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000):

$$Y_t = Y_0 + \mu_{max} \cdot A_t - \frac{Y_{max} - Y_0}{M} \cdot ln\left[1 - e^{-M} + \left(e^{-M} \cdot \frac{Y_{max} - Y_0 - \mu_{max} \cdot A_t}{Y_{max} - Y_0}\right)\right] \quad \text{Eq. 4}$$

$$A_t = t - \lambda \cdot \left[1 - \frac{1}{h_0} \cdot ln\left(1 - e^{-h_0 \cdot \frac{t}{\lambda}} + e^{-h_0 \cdot \left(\frac{t}{\lambda} - 1\right)}\right)\right] \quad \text{Eq. 5}$$

where Y_t is the Log_{10} of cell concentration at time t (CFU/mL); Y_0 is the Log_{10} of the initial cell concentration (CFU/mL); Y_{max} is the Log_{10} of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (Log_{10}/h); λ is the lag phase (h); and M and h_0 are curvature parameters, taking them as constant values, and with both set at a value of 10. Curve fitting was carried out using GraphPad PRISM® (GraphPad Software, San Diego, CA, USA) statistical software.

2.5 Virulence assays

2.5.1 Caco-2 cell maintenance and preparation

The human colon carcinoma Caco-2 cell line (TC7 clone) was kindly provided by Dr. Edith Brot-Laroche (Université Pierre et Marie Curie-Paris 6, UMR S 872, Les Cordeliers, France) at Passage 25 and used in experiments at Passage 30-35. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in 75 cm^2 flasks. Cells were grown in Dulbecco's Modified Eagle's Medium + GlutaMAX™ (DMEM, Invitrogen, France) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, France), 1 % Minimal Essential Medium with Non-Essential Amino Acids (MEM NEAA 100X, Invitrogen, France), and 1% antibiotics (penicillin/streptomycin, Invitrogen). Once the cells reached 80% confluence, they were dissociated with 0.05% Trypsin-1 mM EDTA (Invitrogen) and seeded at a density of approximately 15,000 cells per well in 96-well tissue culture plates (Nunc, France) containing 200 μL of complete medium per well. Plates were incubated in humidified atmosphere containing 5% CO_2 at 37 °C for 15-17 days to attain fully differentiated cell layers. Culture medium was replaced every 2 days, and cell confluence was confirmed by optical microscopy.

2.5.2 Adhesion and invasion in Caco-2 cells

Prior to use for virulence assays, cell layers were washed three times in DPBS (Dulbecco's Phosphate Buffered Saline); 200 µL of complete medium without antibiotics were added. For adhesion assay, suspensions of different *Salmonella* strains were added at an initial concentration of 10^6 cells/mL on washed Caco-2 cells. Cells were incubated with bacteria for 30 min in humidified atmosphere containing 5% CO_2 at 37 °C. After incubation, non-adhered bacteria were removed by washing the cell cultures twice with DPBS, and the cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. These lysates were adequately diluted and then plated in Xylose Lysine Desoxycholate Agar (XLD, Oxoid). These plates were incubated at 37 °C for 24 h before being manually counted. For invasion assays, bacterial inoculation was performed as described for the adhesion assay, and plates were maintained in 5% CO_2 at 37 °C for 30 min. The infected cells were washed twice with DPBS, after which they were maintained during 1 hour in DMEM containing 100 µg/mL of gentamicin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) per well to inactivate extracellular bacteria. After incubation, cell layers were lysed with 0.1% (v/v) Triton X-100 for 10 min. Lysates were processed for determination of *Salmonella* counts as described above. The adhesion and invasion rates were calculated as percentages of adhered or invading bacteria to initial bacteria added.

2.6 Whole genome sequencing (WGS) and identification of mutations

The total genomic DNA (gDNA) from each *S*. Typhimurium SL1344 resistant variant and the parental was extracted using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

The genomes of the strains/variants investigated were sequenced by the company STAB VIDA (Portugal) in an Illumina Hiseq 4000 platform, using 150bp paired-end. The resulting reads were then subjected to a trimming process using the CLC Genomic Workbench version 12.0. The quality of the produced data was determined by Phred quality score at each cycle using the FastQC program (v3.4.1.1) (Andrews, 2010). Then, the high quality sequencing reads were mapped (length and similarity fractions of 0.8 each) against the *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 reference genome (Kröger et al., 2012). After the mapping, a variant calling algorithm was applied to detect the variants that satisfy the requirements specified by the following filters: minimum Frequency = 35%, minimum Quality (Phred) = 20, minimum coverage = 20, minimum count = 5 and direction filtering. Detection of Insertions and Deletions was also performed by means of a InDels detection tool using the following criteria: minimum number of reads = 5 and P-value threshold = 0.0001.

2.7 RNA extraction

RNA of each resistant variants and the parental strain was isolated by phenol-chloroform extraction with a subsequent cleanup procedure using the RNeasy Mini Kit (Qiagen) (Atshan et al., 2012). Cells were pelleted by centrifugation at 8000×g for 3 min in a 4 °C refrigerated centrifuge. The pellet was re-suspended in 100 µL of RNase free water. The tube was vigorously vortexed for 3 min and 100 µL of acid phenol was added with chloroform (1:1). It was vortexed for 1 min and incubated at 70 °C for 30 min. The vortex process was repeated periodically every 5 min. Subsequently, the tube was centrifuged at 12,000×g for 10 min and 100 µL from the aqueous (top only) phase was transferred into a new tube. Seven hundred microliters of lysis buffer were added into the aqueous phase and the subsequent steps were done according to the manufacturer's protocol of RNeasy Mini Kit (Quiagen).

The samples, once purified, were treated with DNase to remove residual DNA using the Rapidout Removal Kit (Thermo Fisher Scientific, Massachusetts, USA), also following the manufacturer's instructions and extracted RNA samples were frozen at −80 °C until complementary DNA (cDNA) synthesis.

2.8 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

First, the RNA previously isolated was converted to cDNA using the Superscript IV Reverse Transcriptase kit (Invitrogen, Carlsbad, USA) using random hexamer primers following the protocol

described by the manufacturer. Once the cDNA was obtained, it was stored at -80 °C until qPCR assays were carried out.

Then, expression of three RpoS-dependent genes (*katE*, *katN* and *otsB*) of the different strains/variants was determined using Quantitative Reverse Transcriptase PCR (qRT-PCR). *rpoZ* was used as a reference gene for qPCR normalization (Lévi-Meyrueis et al., 2014).

qPCR amplification was performed using the GoTaq qPCR Master Mix (Promega, Madison, USA) and the primers described in Table 2. The qPCR assays were carried out with a CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, USA) using a protocol with 5 min at 94 °C for GoTaq enzyme activation, followed by 44 cycles of 94 °C for 10 s and 40 s at temperature of 55 °C for annealing, elongation and fluorescence data acquisition. A melting curve between 65 °C and 90 °C was obtained after the last amplification cycle, and at a temperature transition rate of 0.5 °C/s. All amplification reactions were run in triplicate.

The mRNA levels for the genes of interest were quantified from the C_t value, which is the PCR cycle number that generated a common signal for each gene in the exponential phase of amplification. To correct for sampling errors, the levels of expression of each gene, as determined from their C_t values, were normalized to the level of *rpoZ* gene. The relative expression of the genes investigated in each resistant variant was compared to that for parental cells and the fold-change in transcription was calculated using $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

Table 2. Primer sequences used in Real-Time PCR to quantify the expression of RpoS-dependent genes.

Gene target	Forward primer (5′ -to- 3′)	Reverse primer (5′ -to- 3′)
rpoZ	CGAAGAAGGTCTGATTAAC	GACGACCTTCAGCAATA
katE	GGCGTCTGTTCTCTTAT	CTGGAAGTTATGGTAGGG
katN	TGAGTCATCTGGAAATTAT	CGATAAAGTTCCGCTTC
otsB	TTAACCGTATCCCCCGAACTC	CCGCGAGACGGTCTAACAAC

2.13 Statistical analysis

All the determinations were carried out in triplicate on different working days. Standard deviations (SD) and statistical analyses (ANOVA, Tukey tests and Pearson's correlation coefficients; p-value <0.05) were calculated using GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA).

3. Results

3.1 Isolation of variants with increased resistance to environmental stresses and food processing technologies

S. enterica serovar Typhimurium SL1344 cells were exposed to different stresses and food processing technologies, including acid, oxidative and osmotic stress, heat, HHP and UV-C treatments, during successive treatments at lethal doses and subsequent growth of surviving cells in liquid medium. An acid resistant variant (SL-Acid), an osmotic resistant variant (SL-NaCl), a variant resistant to high hydrostatic pressure (SL-HHP) and a variant resistant to Ultraviolet-C light (SL-UV) were obtained. The resistant variants isolated after the application the different stresses/agents are summarized in Table 3, where the treatment applied and the number of cycles in which they were isolated are indicated. *Salmonella* cells were also exposed to lethal oxidative and heat treatments but no resistant variants were isolated.

Figure 1 shows the survival curves of *S. enterica* serovar Typhimurium SL1344 (parental strain) and resistant variants to acid and osmotic stress, HHP and UV-C treatments. As can be observed in the figures, the isolated variants were more resistant than the parental culture but the differences were relatively small. Thus, the inactivation reached after 360 minutes at pH 2.5 was approx. 0.54 Log_{10} cycles higher for the parental than in the SL-Acid variant (Figure 1A). Similarly, a difference in the Log_{10} cycles

of cells inactivated between the parental and the resistant variants of 0.84 (parental vs SL-NaCl; 1B), 0.82 (parental vs SL-HHP; 1C) and 1.15 (parental vs SL-UV; 1D) Log cycles, after an exposure to osmotic medium (30% (w/v) NaCl; 48 hours), a 300 MPa treatment and a UV-C treatment of 600 s at 0.09 mW/cm^2, respectively, was observed.

Table 3. Resistant variants of *S*. Typhimurium SL1344 and conditions under which were obtained.

Resistant variant	Environmental stresses and food technologies	Conditions	N° cycles
SL-Acid	Acid	pH 2.5	5
SL-NaCl	Sodium chloride	30% (w/v) NaCl	6
SL-HHP	HHP	300 MPa	8
SL-UV	UV-C	0.09 mW/cm^2	8

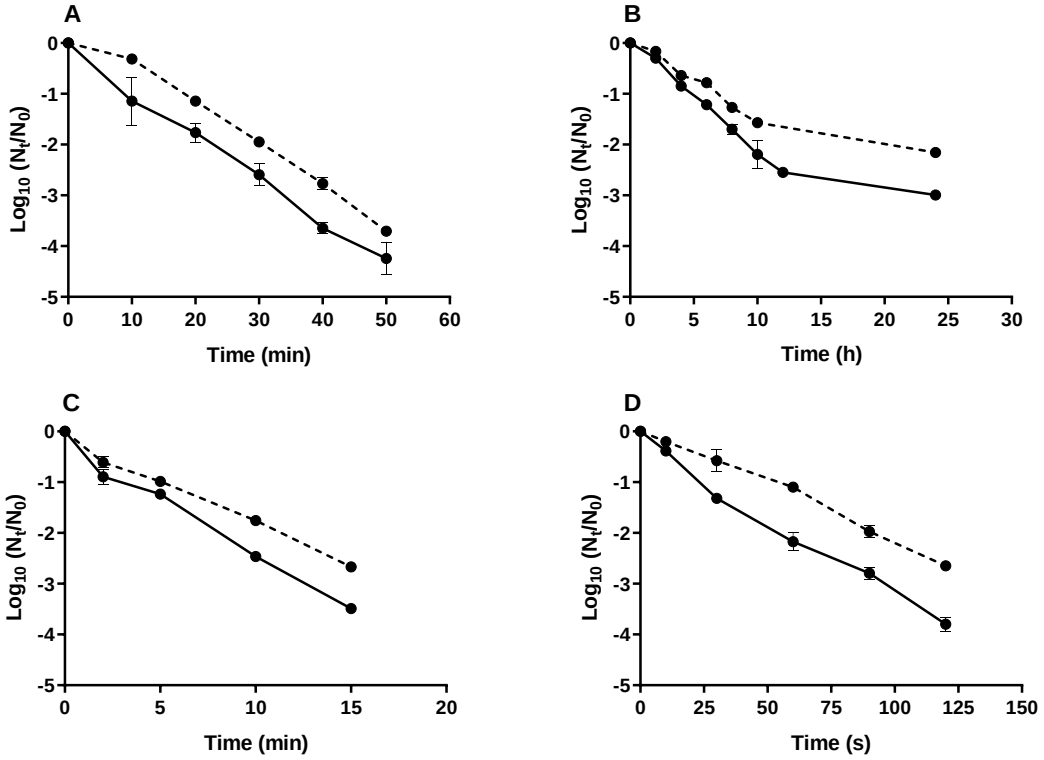

Figure 1. Survival curves of parental *S*. Typhimurium SL1344 (continuous line) and the different variants (discontinuous line) obtained resistant to A) acid pH (variant SL-Acid, pH 2.5), B) osmotic medium (variant SL-NaCl, TSB-YE + 30% NaCl + 37 °C), C) high hydrostatic pressure (variant SL-HHP, 300 MPa), and D) UV-C light (variant SL – UV, 0.09 mW/cm^2).

Since, it has been reported that the acquisition of resistance to an environmental stress might lead to the development of cross-resistance responses and often results in a loss in the growth fitness or virulence of the cells (Guillén et al., 2022), in the following sections the resistance to other stressors, the growth capacity and the virulence capacity of these resistant variants isolated were determined.

3.2 Characterization of S. Typhimurium SL1344 variants to different environmental stresses and food processing technologies

First, the resistance to seven different preservation technologies and environmental stresses of the 4 variants of *S.* Typhimurium SL1344 was determined and subsequently compared to that of the parental strain, *S.* Typhimurium SL1344. The resistance of the PEF-resistant variant (SL1344-RS) isolated by Sagarzazu et al. (2013) to these agents (Guillén et al., to be submitted for publication) is included in the figures for comparison purposes. The adequacy of the methodology used has already been discussed in Guillén et al. (2020b). Survival curves at 7 agents were obtained by plotting the logarithm of the survival fraction versus treatment time, showing different profiles. These profiles showed deviations from linearity; for example, the survival curves to acid and hydrogen peroxide showed shoulders, whereas those to NaCl and PEF showed tails. Therefore, to describe them accurately, the nonlinear Geeraerd model (Geeraerd et al., 2000) was used to calculate the corresponding resistance parameters (N_0; S_l; K_{max}, N_{res}). The mean values obtained for these parameters and their standard deviation, together with the goodness-of-fit parameters, are included in Supplementary Table 2. The traditional decimal reduction time (D) value of each survival curve was calculated from its corresponding K_{max} (Eq. 3). It order to establish meaningful comparisons the *2D*-value parameter (time required to inactivate the first 2 Log_{10} cycles) was used, as described in Guillén et al. (2020b). Since the *2D*-values obtained for each agent/technology cannot be directly compared due to the different time scale of the survival curves, for comparison purposes the calculated resistance parameters were normalized by dividing them by the mean *2D*-value of resistance of parental strain, *S.* Typhimurium SL1344.

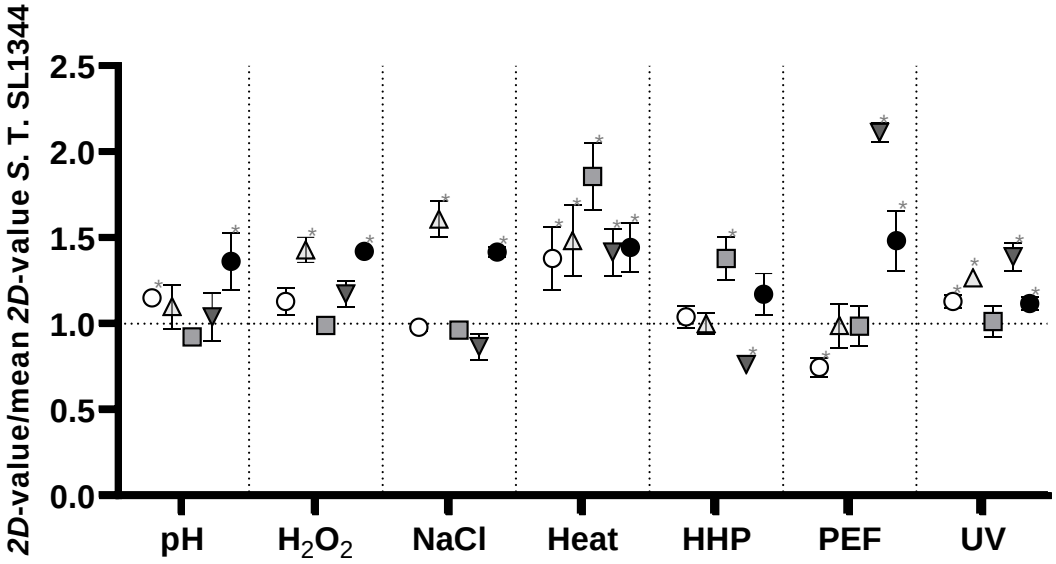

Figure 2. Resistance to seven different preservation technologies and environmental stresses of the 5 variants of *S.* Typhimurium SL1344 isolated, SL-Acid (○), SL-NaCl (▲), SL-HHP (■), SL-UV (▼) and SL1344-RS (●). The normalized resistance parameters are plotted, the mean *2D*-value for each strain divided by the mean *2D*-resistance value of the parental strain, *S.* Typhimurium SL1344. Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences (p <0.05) between the parental and the variants.

As can be observed in Figure 2 and supplementary Table S2 all the variants displayed, to some extent cross-resistance responses. Thus, the SL-Acid variant displayed and increased resistance (as compared to the parental strain) to 4 of the agents/technologies tested, the SL-HHP to two (HHP and heat), the SL-NaCl variant to 5, the UV-C selected variant to 4 and the PEF-selected variant to all of them. Some sensitizations to other agents were also found in some cases, such as towards PEF for the acid-selected one. It should also be noted that, in some cases a variant selected by a different stressor

was more resistant to an agent that one selected with the same (e.g. the resistance to PEF of the UV-C selected variant was higher than that of the PEF-selected one). In any case the differences in resistance observed were low, with a change in the *2D*-value lower than 2.8 fold in all the cases. Furthermore, results obtained for all the variants and agents studied were in the range of those previously obtained following the same methodology (Guillén et al., 2020a, 2020b) for strains obtained from culture collections, and although for some agents (such as NaCl, HHP and PEF) some variants displayed higher resistance than those laboratory strains, differences can be considered small.

3.3 Characterization of the growth capacity of S. Typhimurium SL1344 variants in different media

Growth curves of the 4 resistant variants and the parental strain were obtained in three different media: in TSB-YE, a nutrient-rich medium, LB medium with iron limitation caused by the addition of DPY, and a minimal medium containing gluconate as the sole carbon source. The selection of this growth media has been previously discussed in Guillén et al. (2022). The μ_{max} (Log_{10}/h) values calculated in the three growth media for each variant and the parental strain are shown in Figure 3 (growth parameters and goodness of the fit parameters are included in Supplementary Table 3). In general, the isolated variants showed an impaired growth compared to the parental strain but this was highly influenced by the growth medium studied. In TSB-YE, the highest growth rate of the variants was that of SL1344-RS (0.914 ± 0.017), and the lowest that of SL-UV (0.873 ± 0.014). No statistically significant differences (p >0.05) were observed among the resistant variants, but there were when compared with the parental strain, except for SL1344-RS. In LB-DPY, the highest growth rate was determined for SL1344-RS (0.681 ± 0.057), and the lowest for SL-HHP (0.578 ± 0.043), and significant differences (p <0.05) were found only between the parenteral strain and SL-HHP. Finally, in M9-Gluconate, the highest growth rate was that of SL-Acid (0.573 ± 0.012) and the lowest was that of SL-UV (0.479 ± 0.018) and significant differences (p <0.05) were found between the parenteral strain and SL-NaCl, SL1344-RS and SL-UV. None of the variants displayed a statistically significant Lag phase (h) (different from 0; p >0.05) in any of the three media tested.

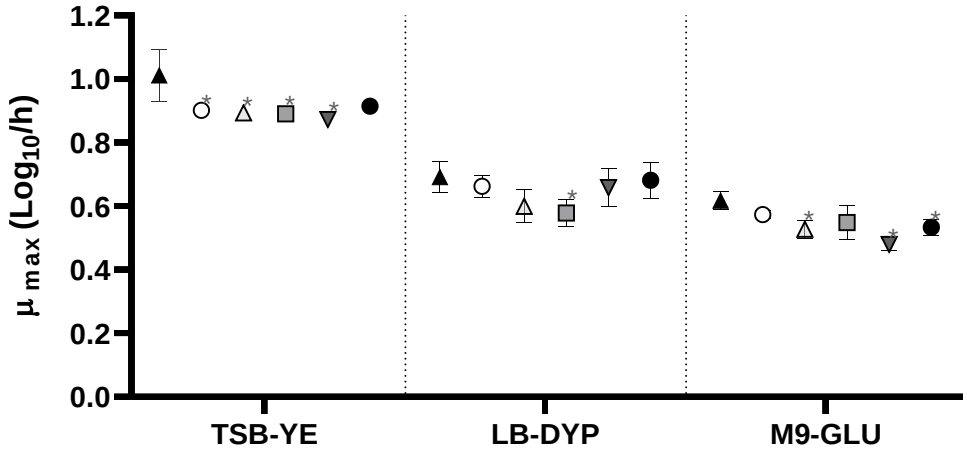

Figure 3. Maximum growth rates (μ_{max} (Log_{10}/h)) of *S.* Typhimurium SL1344 (▲), SL-Acid (◯), SL-NaCl (▲), SL-HHP (■), SL-UV (▼) and SL1344-RS (●) in TSB-YE, LB supplemented with 100 μM 2-2'dipyridyl (DPY) and M9-broth supplemented with 20 mM gluconate at 37 °C. Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences (p <0.05) between the parental and the variants.

As reported for the stress resistance parameters, the growth parameters obtained for all the resistant variants were in the range of those obtained previously following the same methodology (Guillén et al., 2022).

3.4 Characterization of virulence capacity of S. Typhimurium SL1344 variants

The virulence capacity of the resistant variants was evaluated by calculating the percentage of cells capable to adhere to and invade Caco-2 cells (Figure 4). The SL-NaCl, SL-HHP and SL1344-RS variants showed a superior adhesion capacity than the parenteral strain, 5.10, 4.01 and 5.21 vs 2.00%, respectively, while the SL-UV variant showed a lower adhesion capacity (0.83%) than the parental strain. Despite the variability observed in the adhesion, the invasion was not found to be significant. Except for the SL-NaCl variant, which had a higher invasion capacity than the parental strain, 0.31 vs 0.12%, the rest of the variants displayed a similar profile and no differences were found with the parental strain (p >0.05).

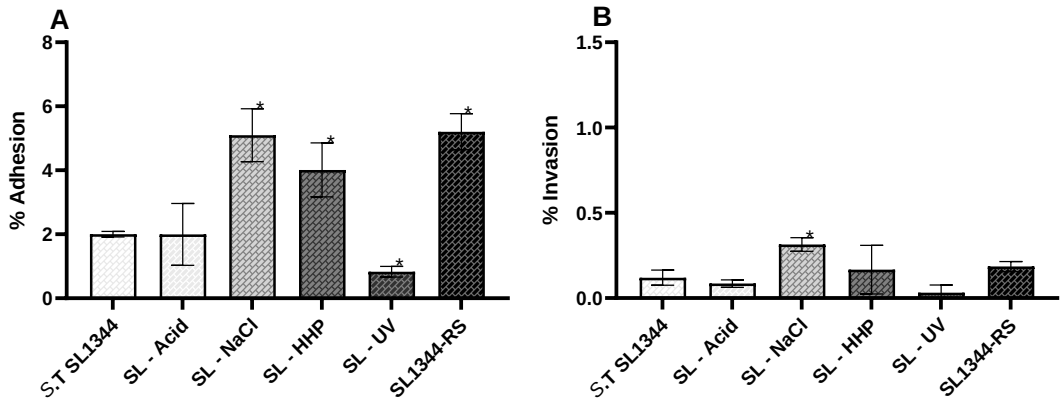

Figure 4. Adhesion (A) and invasion (B) capacity to Caco-2 cells of the 5 resistant variants and the parental strain, *S.* Typhimurium SL1344. Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences (p <0.05) between the parental and the variants.

3.5 WGS of S. Typhimurium SL1344 variants

In addition to the phenotypical characterization of these variants a WGS analysis was performed to identify mutations associated with increased resistance to the exposed agents and possible cross-tolerance to other agents.

As already pointed out in our earlier work (Guillén et al, to be submitted for publication) when comparing the strain we used as the parental one (*S*. Typhimurium SL1344) with the reference genome of this same strain (GenBank: Accession No. FQ312003.1) (Kröger et al., 2012) we found that our strain had suffered 4 mutation (SNPs). These changes can be found in that work and are also included as supplementary material (Table S4). Thus, here we will report and discuss the differences between our parental strain and the variants isolated from it, i.e., the mutations fixed throughout the selection process applied. As can be observed in Table 4, sequenciation of the genomes of these variants revealed that in SL-Acid, SL-NaCl and SL-HHP there was a single SNV in each of them, as compared to the parental strains, whereas in SL-UV 2 SNVs were found.

The genes in which mutations were observed are also included in the Table 4. As can be observed the SL-Acid variant displayed a SNV in *yhfk* gene at position 959 bp, resulting in the substitution of a histidine (His) by a Leucine (Leu). This gene encodes a putative inner membrane protein which, in *E. coli*, *yhfK* expression is regulated by Crp. The *crp* gene encodes cAMP receptor protein (CRP), which regulates the transcription of a magnitude of operons related to sugar transport and catabolic functions (Zheng et al., 2004). In addition, it has been shown that *yhfK* is required in moderate iron-restricted conditions for *S*. Typhimurium growth (Bjarnason et al., 2003; Karash et al., 2021).

Table 4. Mutations identified in the resistant variants (obtained by cyclic exposure to different environmental stressors and food processing technologies) by whole genome sequencing (WGS). All detected mutations were single nucleotide variations (SNV).

Variant	Region	Genes	Locus tag	Mutation type	Amino acid Change	Description
SL-Acid	3638767	*yhfk*	SL1344_3434	c.959A>T	His320Leu	conserved membrane protein
SL-NaCl	1806813	*hnr*	SL1344_1684	c.-8G>A	Change in the Shine-Dalgarno sequence	hypothetical regulatory protein
SL-HHP	14430	*dnaJ*	SL1344_0013	c.836T>G	Leu279Arg	chaperone protein DnaJ
SL-UV	82316	*caiD*	SL1344_0071	c.198A>T	Leu66Phe	carnitine racemase
	1806313	*hnr*	SL1344_1684	c.493C>T	Gln165*	hypothetical regulatory protein

In SL-HHP variant the *dnaJ* gene was mutated at position 826 bp, resulting in the substitution of a Leucine (Leu) by an Arginine (Arg). DnaJ, also known as Hsp40, is a cochaperone that, together with DnaK contributes to protein quality control by facilitating folding of nascent proteins, polypeptides emerging through the Sec system, partially unfolded proteins, and protein aggregates (Kim et al., 2021; Rosenzweig et al., 2019). Furthermore, DnaJ plays an oxidoreductase activity, catalyzing the formation and reduction of disulfide bonds (de Crouy-Chanel et al., 1995), and facilitates the interactions of oxidized DksA with RNA polymerase (Kim et al., 2018).

The SL-UV variant displayed two SNPs. One was found in the *caiD* gene at position 198 bp, replacing a Leucine (Leu) with a Phenylalanine (Phe). This protein is involved in the pathway carnitine metabolism, catalyzes the reversible dehydration of L-carnitinyl-CoA to crotonobetainyl-CoA (Meadows and Wargo, 2015). CaiD was initially suggested to function as the racemase, as the *caiD* gene is required for racemase activity (Eichler et al., 1994), to convert D-carnitinyl-CoA to L-carnitinyl-CoA after being activated by CaiC (Bernal et al., 2008). The other one was found in the *hnr* gene, being a missense mutation at position 493 bp, causing the substitution of a Glutamine (Gln) for a premature stop codon. A SNP was also found in this gene in the SL-NaCl variant, located 8 bp upstream from the AUG start codon, which corresponds to the Ribosome Binding Site (RBS) or Shine-Dalgarno sequence, and, therefore, this change might potentially lead to changes in the level of transcription of *hnr* mRNA. It should be noted that in the SL1344-RS variant, SNPs at position 902 bp, resulting in the substitution of a Leucine (Leu) by a Proline (Pro) was found (to be submitted for publication). This protein regulates the turnover of the alternative sigma factor σ^S (RpoS) by promoting its proteolysis in exponentially growing cells, and it also controls which mRNAs are destroyed by stimulating polyadenylation (Carabetta et al., 2009). *hnr* expression levels increase modestly during entry into stationary phase in an RpoS-dependent manner. The modest induction of *hnr* in stationary phase has been suggested to be important for the rapid destruction of RpoS, act as an anti-sigma factor, when cells exit from stationary phase, although this remains to be demonstrated (Battesti et al., 2011).

3.6 Quantification of RpoS activity in S. Typhimurium SL1344 variants

Since results obtained indicated that the 4 isolated variants (as was the case for the PEF resistant one) were multi-resistant and in 3 of them (including the PEF resistant one) mutations in *hnr*, which

regulates RpoS activity it was hypothesized that, especially in the case of variants SL-UV and SL-NaCl its phenotypic characteristics might be linked to an increased RpoS activity, i.e., that the mutations found resulted in a decreased *hnr*-dependent RpoS proteolysis. Thus, as described earlier on qPCR was used to quantify the expression of several genes regulated by RpoS, that were used as reporters of its activity: *katE*, *katN* and *otsB* (Table 5).

Table 5. Average of the C_t values normalized to the *rpoZ* level gene (ΔC_t) in select stress response genes, *katE, katN* and *otsB*, of resistant variants and the parental strain, *S*. Typhimurium SL1344. Values in parentheses represent the SD of the means and letters indicate statistically significant differences between variants/parental.

Variant	*katE*	*katN*	*otsB*
SL1344	3,31 (1.69)[a]	0,22 (1.91)[ab]	-3,92 (0.50)[a]
SL-Acid	3,18 (1.22)[a]	1,67 (0.86)[a]	-3,46 (1.16)[a]
SL-NaCl	1,75 (0.68)[a]	-1,51 (3.41)[ab]	-4,28 (0.27)[a]
SL-HHP	2,82 (0.49)[a]	0,01 (0.31)[b]	-3,48 (0.63)[a]
SL-UV	1,68 (0.27)[a]	-5,93 (0.29)[c]	-8,70 (0.86)[b]
SL1344-RS	1,84 (0.79)[a]	-1,07 (1.09)[b]	-6,43 (0.14)[c]

As can be observed in Figure 5, whereas for the SL-Acid and the SL-HHP variants, none of the 3 genes studied was overexpressed (what would indicate that their rpoS activity would be similar) in strains SL-NaCl, SL-UV, as it was observed in the SL1344-RS strain, the three genes were overexpressed, especially in the case of the SL-UV variant.

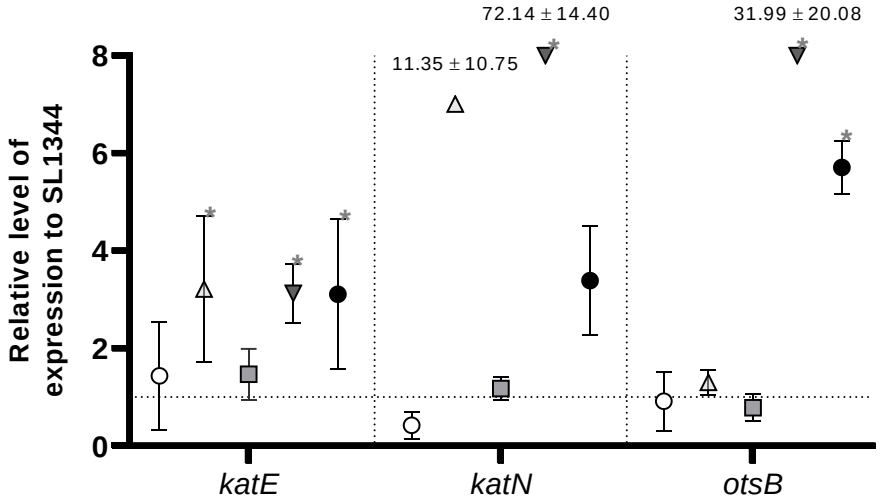

Figure 5. Relative expression of the three genes studied ($2^{-\Delta\Delta Ct}$) in the resistant variants as compared to the parental strain. SL-Acid (○), SL-NaCl (▲), SL-HHP (■), SL-UV (▼) and SL1344-RS (●). Error bars correspond to the standard deviation of the means and the asterisk (*) indicates statistically significant differences (p <0.05) between the parental and the variants.

3.7 Relationship between RpoS and stress resistance in non-typhoidal Salmonellae

Various authors have suggested that the differences in stress resistance among *Salmonella* strains and serovars might be linked to differences in RpoS activity, and similar hypothesis have been done for other microorganisms (and their correspondent alternative sigma factors) (Ait-Ouazzou et al., 2012; Cebrián et al., 2016; Hengge-Aronis, 1996). Therefore, the expression of *katE* and *otsB* (as reporters of RpoS activity), in 22 additional *Salmonella* strains (Table S5) was determined and then compared to its already known stress resistance, growth capacity and virulence (Guillén et al., 2020a, 2020b, 2022). The expression of *katE* and *otsB* of each strain was expressed as the C_t values normalized to the *rpoZ* level gene (ΔC_t). For this purpose, resistant variants were excluded and only the parental strain was included in the analysis. The iterative Grubbs' test was applied to identify potential outliers that could exert a disproportionate influence on further data analysis and lead to non-valid conclusions. Grubbs' test detected multiple outliers: the $2D$-value to heat of *S*. Senftenberg 775W, the maximum growth rate of *S*. Gallinarum in LB-DYP and the invasion percentage of *S*. Enteritidis 7160.

No significant correlation (p >0.05) between stress resistance and expression of RpoS-dependent genes, *katE* and *ostB*, was observed for the 22 *Salmonella* strains, the resistant variants and the parental, *S*. Typhimurium SL1344 (data not shown). There was also no significant correlation between the expression levels of *katE* and *ostB* and fitness cost. And finally, no relationship was found between the expression levels of either *katE* or *ostB* with virulence parameters. Therefore, in this dataset, no association with the level of RpoS-dependent genes expression was found.

4. Discussion

In this study we report the isolation and pheno- and genotypical characterization of 4 resistant variants derived from *S. enterica* serovar Typhimurium SL1344 after its exposure to several rounds of acid stress, osmotic stress, high hydrostatic pressure and UV-C light treatments. A PEF-resistant variant was previously isolated in our laboratory following the same protocol (Sagarzazu et al., 2013), and we also attempted to isolate stable variants resistant to heat and oxidative stress, but without success. This latter phenomenon might be due to several reasons, including the low number of growth-inactivation cycles here applied or also that maybe insufficient selective pressure for their isolation was applied, among others. This technique has been used to isolate other *Salmonella* variants resistant to different stressors, after several cycles of treatment and growth of survivors. Examples of stable variants of *Salmonella* obtained after successive exposure to different selection agents are reported in Guillén et al. (2021). At this point it should be noted that the increase in resistance of the variants here isolated was relatively small, if compared to other ones reported in the bibliography. There are also various possible explanations for this phenomenon but the fact that they were isolated after only a few cycles would probably be one of them, as hypothesized for explaining our unsuccessful isolation of variants after the application of heat and hydrogen peroxide. Nevertheless, this approach has also some advantages. Thus, 3 of the variants isolated only displayed a SNP and the other one 2, making much easier the study of the mechanisms of acquisition of stress resistance (see below).

Our results indicate that all the variants isolated displayed resistance to at least, another agent that was not the selective one. However, whereas for the SL-HHP variant it only displayed an increase resistance to HHP and heat, some of the strains displayed an increased resistance to up to 5 out of the 7 agents tested. This increase in resistance did had a fitness cost, at least in general terms, and had a variable impact in virulence (much higher and significant in adhesion than in invasion).

It has been reported that pressure resistant mutants and/or acid resistant mutants of *L. monocytogenes*, *S. enterica* and *S. aureus* are frequently more tolerant to other stresses, particularly to heat treatments (Greenacre and Brocklehurst, 2006; Karatzas and Bennik, 2002; Karatzas et al., 2007). Similarly, the PEF resistant variant isolated by Sagarzazu and co-workers in 2013 also displayed an increased resistance to acid pH, hydrogen peroxide and ethanol. The occurrence of cross-tolerance between two stresses may be attributed to altered expression levels of proteins involved in the general stress response, such as RpoS, the master regulator of the general stress response (Hengge, 2011), as will be discussed later and might be the case for the SL-NaCl and SL-UV variants, or to similarities between

the cellular targets affected by both agents, as might be the case for the SL-HHP variant and its increased resistance to heat. It is also known that the development of stress resistance can impose a fitness cost on bacteria (as demonstrated in the case of *Escherichia coli* RpoS expression), or as a consequence of the acquisition of resistance to certain antimicrobials (Andersson and Hughes, 2010; Zambrano et al., 1993). However, although it is widely assumed that stress resistance implies a fitness cost for bacteria (Karatzas et al., 2008; Urdaneta et al., 2019), there is not always a relationship between resistance and fitness costs and it is also possible that bacteria can reverse fitness costs by acquiring compensatory mutations (Andersson and Hughes, 2010; Guillén et al., 2021). Finally, although in view of these data, it can be concluded that the virulence capacity is affected as a function of the stressor to which resistance has been acquired, although the processes of adhesion and invasion are coordinated, different pathways modulate these virulence mechanisms separately, and both or only one system may be affected. The differences observed with the parental strain may be due to the ability to adhere, as it is highly dependent on the expression of genes encoding protein secretion systems, effector and chaperone proteins, and/or transcriptional regulators, which are considered susceptible to be modified for the acquisition of resistance to the exposed agent.

On the other hand, the WGS analysis enabled us to identify the genetic changes responsible for these phenotypic changes. Regarding the SL-Acid variant, a SNP was found in the *yhfK*, that encodes a putative inner membrane protein. Although its functions are not known it is known that *yhfK* is required in moderate iron-restricted conditions for *S*. Typhimurium growth (Bjarnason et al., 2003; Karash et al., 2021) and that in *E. coli*, its expression is associated with Crp, which regulates the transcription of a magnitude of operons related to sugar transport and catabolic functions (Zheng et al., 2004). *Salmonella* acid tolerance mechanisms, reviewed in Álvarez-Ordoñez et al. (2011), include a number of transport systems and therefore, it is plausible that this protein might be a part, regulate of affect the activity of one of them. Results obtained also indicated that this mutation also led to an increase in heat resistance. Since this strain displayed a higher heat resistance both when cells were recovered in selective (XLD) and non-selective media (TSA-YE) it can be speculated that this increase would not be related to an increased ability to repair sublethal damages caused by heat but still there is a handful of potential heat protective changes that this mutation can trigger, including, for instance, an increase in membrane stability. On the other hand, it should also be noted that results obtained in growth assays indicate that this mutation would not negatively affect *Salmonella*'s ability to grow in low iron media, what might had been the outcome given the role in this phenotypical trait that this gene plays. Finally, it should also be noted that the SL-Acid variant, showed a significant feature, since its resistance to acid medium was dependent on whether growth occurred under agitation or in static culture conditions, with differences of up to 1.3-fold (data not shown), a phenomenon that was not observed for any other variant and or stress studied. This could be because there are transcriptional factors that are only activated under specific conditions, for example under agitation. Thus, for instance, Lim et al. (2012) noted that the *invF-2* promoter was not activated when cells were grown in static culture conditions. Further work will be required in order to fully elucidate all these questions regarding this mutation in the *yhfK* gene.

In the case of the SL-HHP variant the mutation was found in the *DnaJ* gene. It is well known that DnaJ participates actively in the response to heat stress shock by preventing the aggregation of stress-denatured proteins and by disaggregating proteins (Kim et al., 2021). A similar hypothesis has been proposed for explaining the fact that its deletion also lead to a decreased baro-resistance of *E. coli* cells (Gänzle and Liu, 2015). This will mean that the allele found in this variant will probably be more efficient in preventing and/or repairing protein aggregation induced by these two agents (which, in addition, have a lot of common cellular targets; Cebrián et al. (2016). Nevertheless, it should be noted that DnaJ seems also to play a relevant role in protecting *Salmonella* against oxidative and hyperosmotic stress (de Crouy-Chanel et al., 1995; Kim et al., 2021) although in pour case we did not find differences between the parental and the mutant strain. In any case it should be noted that in this case we are also comparing two alleles (both of them functional) whereas in the mentioned studies the comparison is established with a knock-out strain, in which the protein, and therefore, its activity, is completely absent. DnaJ is also required for growth at high temperatures in *E. coli* (Sell et al., 1990) and for *Salmonella* invasion of

epithelial cells (Takaya et al., 2004). However, in our case the DnaJ allele providing a higher stress resistance also led to a decreased growth rate and although it resulted in a higher adhesion did not significantly change invasion of Caco-2 cells. Again, these discrepancies might be attributed to different factors that should be further investigated.

In any case the most relevant finding of this study is that half of the strains isolated (2, SL-NaCl and SL-UV) displayed mutations in the *hnr* gene, the same gene presenting a mutation in the PEF resistant strain isolated in Sagarzazu et al., (2013). As pointed out above *hnr* regulates RpoS proteolysis and, therefore, these mutations resulted in an altered RpoS activity in these strains. Thus, they led to an increased RpoS activity in these strains, as demonstrated by the higher expression (as compared to the parental strain) of the two reporters of RpoS activity (*katE* and *otsB*) that they displayed. Since the different location of these SNPs within the *hnr* gene they would be expected to have different impacts on hnr activity. Thus, whereas in the *hnr* protein of the SL-UV mutant would be truncated, a protein would be synthesized with 172 amino acids less, the mutation in the Shine Dalgarno sequence of the NaCl strain would only result in a weaker promoter. In fact, the results obtained by qRT-PCR indicate that RpoS activity would be higher in the SL-UV variant than in the SL-NaCl one. Meanwhile, the SL1344-RS variant displaying intermediate values between these two.

It is generally acknowledged that alternative sigma factors are probably the most relevant strategy developed by bacteria when they face adverse conditions (Abee and Wouters, 1999) and the alternative sigma factor σ^S (also called σ^{38} or RpoS) of RNA polymerase (RNAP) is regarded as the master regulator of the general stress response in many Gram-negative bacteria, including *Salmonella* (Battesti et al., 2011; Hengge, 2009; Lago et al., 2017; Österberg et al., 2011). Thus, as it would be expected, the higher RpoS activity of these three strains led to the development of resistance to several agents in all of them. The differences observed in the pattern of multi-resistance (e.g. the SL-NaCl strain was not more PEF resistant than the parental strain but the other two were, and the SL-NaCl and the SL1344-RS were more NaCl resistant than the parental but the SL-UV did not) might be probably due to the combination of several factors including, the level of RpoS activity in each strain, the relevance of RpoS in resistance to each particular agent and, in the case of the SL-UV strain, the presence of an additional mutation in *CaiD*. Thus, CaiD is involved in the pathway carnitine metabolism, a molecule that has several functions in bacterial cells. Thus, it provides osmotolerance, cryotolerace, bile tolerance and barotolerance to bacterial cells but also can serve as a nutrient or as an electron acceptor (Meadows and Wargo, 2015). Thus, this mutation, alone, might have caused a reduction in *S.* Typhimurium NaCl and HHP tolerance, and when combined with the overexpression of RpoS to the particular stress resistance pattern of this strain (the highest UV-C and PEF resistance but a decreased HHP and NaCl resistance). The precise role of RpoS and RpoS-dependent gene expression on *Salmonella* stress resistance, virulence, growth fitness and other phenotypic characteristics was recently reviewed by Guillén et al. (2021). However, its role in *Salmonella* resistance to some agents, such as PEF, UV-C and HHP, still remains to be fully elucidated. In this sense, results here obtained strongly suggest that RpoS would play a relevant role in *Salmonella* resistance to osmotic stress, UV-C and PEF, as it was previously suggested in Guillén et al. (2021). It is noteworthy that not only using these three different agents led to mutations in a RpoS repressor, and therefore, led to the selection of variants with a higher RpoS activity but that in Guillén et al. (2020b) a positive correlation between UV-C and PEF resistance was found among *Salmonella* strains and between PEF and NaCl in Guillén et al. (2022).

In addition, this increased RpoS activity would explain the lower growth rates observed for these 3 variants since it will lead to and increased transcription of genes involved in growth arrest and resistance to a variety of stresses (Bearson et al., 1996) at least if we assume that, as in *E. coli* and *S.* Typhi, cells with a reduced RpoS activity can grow better in media with low levels of nutrients, and also seem to possess an advantage in competitive colonization of the intestine (Altuvia et al., 1994; Krogfelt et al., 2000; Sabbagh et al., 2010). However, it should also be noted that whereas stress-sensitive *rpoS* mutants (also called GASP phenotype: Growth Advantage in Stationary Phase) are surprisingly common among natural isolates of the closely related *E. coli* and *S.* Typhi, they are not in *S*. Typhimurium (Robbe-Saule et al., 2003) a phenomenon that has not been yet clarified.

Explaining the results obtained in the virulence assays for these three strains would be much more difficult since they displayed very different adhesion and invasion abilities. However, it does fit with the fact that the role of RpoS on *Salmonella* virulence seems to be very complex as reviewed in Guillén et al. (Guillén et al., 2021). Thus, RpoS seems to reduce the expression of some virulence factors while inducing other ones and, therefore, predicting what would be the virulence of a strain on the basis of its RpoS activity results extremely difficult.

As pointed out above it has been suggested that the differences in stress resistance among *Salmonella* strains and serovars might be linked to differences in RpoS activity (Abdullah et al., 2018; Wang et al., 2021), and similar hypothesis have been done for other microorganisms (and their correspondent alternative sigma factors) (Lianou and Koutsoumanis, 2013; Robey et al., 2001). However, our results indicate that a direct relationship between RpoS activity and stress resistance would not exist within *Salmonellae* with the exceptions of PEF and, maybe, NaCl resistance. In any case this conclusion should be taken with care given the low number of strains tested (23). It should also be taken into account that, given the complexity of *Salmonella* stress responses -always involving various and in many cases independent pathways-, the influence of RpoS activity might result masked for instance because there are other mechanisms/phenomena more relevant for the resistance to a given particular stress. Thus, our results do not imply either that RpoS activity does not play any roles in *Salmonella* resistance to other agents than PEF and NaCl or that it not contributes to generate the differences in resistance observed among strains but, again highlight NaCl and PEF as agents against which RpoS will be playing a major role.

Finally, the fact that 3 out of 5 f the strains displayed mutations in *hnr* also suggest that this might constitute a conserved strategy within *Salmonellae* in order to (rapidly) acquire stress resistance trough the appearance of a subpopulation with increased stress resistance but also with reduced growth ability. Furthermore, it can also be hypothesized that the absence of GASPs in *S.* Typhimurium might be due to the fact that the generation subpopulations with different RpoS activity in this microorganism would be achieved through changes in RpoS-regulated genes and not in *rpoS* itself. Further work is being carried right now in order to validate these hypotheses as well as to study the reversibility of these genetic changes.

5. Conclusions

In this study, 4 resistant variants derived from *S. enterica* serovar Typhimurium SL1344 after its exposure to several rounds of acid stress, osmotic stress, high hydrostatic pressure and UV-C light treatments were isolated, and at least showed multi-resistance to at least one other stressor. This increased resistance, in general terms, had a fitness cost in growth, and had a variable impact on virulence (much greater and significant in adhesion than in invasion). The increase in resistance could be due to an increase in RpoS activity, since the WGS analysis revealed that in 3 of the 5 variants (including SL1344-RS) a mutation was found in the *hnr* gene, an anti-sigma factor that promotes RpoS proteolysis, in addition to elevated *katE, katN* and *ostB* expression levels. However, extrapolating our results to a larger set of serovars (23) indicates that there is no direct relationship between RpoS activity and stress resistance within *Salmonellae*. Because of the complexity of *Salmonella* stress responses, the influence of RpoS activity could be masked, for example, because there are other mechanisms/phenomena more relevant to resistance to a given stress. In any case, this conclusion should be taken with caution given the low number of strains analyzed.

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Table S1. Additional set of 22 *Salmonella enterica* strains used in this study.

Strains	
S. Typhimurium STCC 443	*S*. Hadar NCTC 13033
S. Typhimurium STCC 4594	*S*. Heidelberg DMS 9379
S. Typhimurium STCC 7162	*S*. Infantis STCC 4373
S. Typhimurium STCC 722	*S*. Kentucky NCTC 5799
S. Enteritidis STCC 4155	*S*. Livingstone NCTC 9125
S. Enteritidis STCC 4300	*S*. Mbandaka NCTC 7892
S. Enteritidis STCC 4396	*S*. Newport NCTC 129
S. Enteritidis STCC 7160	*S*. Saintpaul STCC 4153
S. Enteritidis STCC 7236	*S*. Stanley STCC 4141
S. Derby STCC 4397	*S*. Senftenberg 775W STCC 4565
S. Gallinarum STCC 4883	*S*. Virchow STCC 4154

Table S2. Resistance (K_{max}. S_l and N_{res}) and goodness of the fit (R^2, *RMSE*) parameters calculated after fitting the survival curves to the 7 agents investigated of the 5 *S.* Typhimurium resistant variants and the parental strain to the Geeraerd's model.

pH						
	***S.* T SL1344**	**SL - Acid**	**SL - NaCl**	**SL - HHP**	**SL - UV**	**SL1344-RS**
K_{max} (min^{-1})	0.215 (0.037)	0.203 (0.004)	0.176 (0.017)	0.192 (0.008)	0.217 (0.054)	0.189 (0.029)
S_l (min)	4.94 (4.281)	8.02 (1.282)	2.97 (2.979)	0.68 (1.171)	5.82 (4.803)	8.19 (3.064)
N_{res} (CFU/mL)	-	-	-	-	-	-
R^2	0.94 - 0.99	0.99 - 0.99	0.96 - 0.99	0.95 - 0.99	0.97 - 0.99	0.96 - 0.99
RMSE	0.053 - 0.478	0.066 - 0.121	0.121 - 0.484	0.196 - 0.492	0.110 - 0.308	0.099 - 0.290
H_2O_2						
K_{max} (min^{-1})	0.126 (0.023)	0.115 (0.026)	0.077 (0.009)	0.126 (0.009)	0.130 (0.008)	0.087 (0.011)
S_l (min)	15.68 (8.795)	18.14 (6.843)	15.80 (3.503)	16.123 (1.300)	26.96 (2.654)	21.64 (5.844)
N_{res} (CFU/mL)	-	-	-	-	-	-
R^2	0.99 - 1.00	0.97 - 0.99	0.98 - 0.99	0.98 - 0.99	0.99 - 1.00	0.95 - 1.00
RMSE	0.099 - 0.178	0.057 - 0.250	0.066 - 0.193	0.170 - 0.232	0.025 - 0.135	0.085 - 0.280
NaCl						
K_{max} (h^{-1})	0.523 (0.032)	0.530 (0.013)	0.379 (0.008)	0.532 (0.021)	0.598 (0.051	0.392 (0.013)
S_l (h)	-	-	-	-	-	-
N_{res} (CFU/mL)	4.28 (0.313)	3.84 (0.172)	4.70 (0.007)	3.65 (0.344)	3.95 (0.027)	4.53 (0.067)
R^2	0.97 - 0.99	0.98 - 0.99	0.98 - 0.99	0.97 - 0.99	0.95 - 0.99	0.97 - 0.98
RMSE	0.106 - 0.160	0.075 - 0.172	0.105 - 0.184	0.119 - 0.249	0.035 - 0.319	0.139 - 0.209
Heat						
K_{max} (min^{-1})	2.697 (0.261)	1.603 (0.152)	1.800 (0.298)	1.163 (0.068)	1.727 (0.085)	1.411 (0.146)
S_l (min)	0.56 (0.288)	0.07 (0.109)	0.78 (0.872)	0.26 (0.226)	0.56 (0.190)	-
N_{res} (CFU/mL)	-	-	-	-	-	-
R^2	0.94 - 0.99	0.98 - 0.99	0.98 - 0.99	0.96 - 0.99	0.95 - 0.99	0.97 - 0.99
RMSE	0.076 - 0.523	0.113 - 0.148	0.146 - 0.260	0.107 - 0.233	0.223 - 0.364	0.058 - 0.226
HHP						
K_{max} (min^{-1})	0.522 (0.027)	0.503 (0.030)	0.523 (0.032)	0.380 (0.034)	0.685 (0.030)	0.451 (0.046)
S_l (min)	-	-	-	-	-	-
N_{res} (CFU/mL)	-	-	-	-	-	-
R^2	0.94 - 0.98	0.94 - 0.96	0.98 - 0.99	0.93 - 0.99	0.97 - 0.99	0.99 - 0.99
RMSE	0.177 - 0.316	0.327 - 0.422	0.011 - 0.141	0.052 - 0.254	0.169 - 0.280	0.096 - 0.132
PEF						
K_{max} (μs^{-1})	0.102 (0.018)	0.136 (0.008)	0.104 (0.010)	0.102 (0.012)	0.062 (0.011)	0.074 (0.008)
S_l (μs)	-	-	-	-	-	-
N_{res} (CFU/mL)	4.36 (0.226)	4.51 (0.359)	4.35 (0.256)	4.34 (0.288)	5.59 (0.224)	5.63 (0.243)
R^2	0.99 - 1.00	0.99 - 1.00	0.99 - 1.00	0.99 - 1.00	0.99 - 1.00	0.98 - 1.00
RMSE	0.031 - 0.251	0.056 - 0.238	0.012 - 0.209	0.065 - 0.066	0.043 - 0.061	0.005 - 0.195

Table S2. Continuation

UV-C						
	***S.* T SL1344**	**SL - Acid**	**SL - NaCl**	**SL - HHP**	**SL - UV**	**SL1344-RS**
K_{max} (s^{-1})	0.070 (0.002)	0.077 (0.017)	0.060 (0.008)	0.078 (0.023)	0.063 (0.015)	0.076 (0.020)
S_l (s)	1.12 (1.940)	13.75 (14.688)	6.95 (10.839)	5.42 (9.382)	25.34 (4.031)	11.28 (15.921)
N_{res} (CFU/mL)	-	-	-	-	-	-
R^2	0.95 - 0.96	0.94 - 1.00	0.99 - 1.00	0.98 - 1.00	0.96 - 1.00	0.99 - 1.00
RMSE	0.398 - 0.413	0.123 - 0.413	0.044 - 0.099	0.144 - 0.243	0.054 - 0.304	0.106 - 0.203

Table S3. Growth parameters (Y_0, Y_{max}. λ and μ_{max}) and goodness of the fit (R^2, RMSE) calculated with the Baranyi and Roberts model of the 5 *S.* Typhimurium resistant variants and the parental strain growth in TSB-YE, LB supplemented with 100 μM 2-2'dipyridyl and M9-broth, supplemented with 20 mM gluconate. Values presented correspond to the mean and SD of the means (in parentheses).

TSB-YE						
	***S.* T SL1344**	**SL - Acid**	**SL - NaCl**	**SL - HHP**	**SL - UV**	**SL1344-RS**
Y_0 (Log(CFU/mL))	6.16 (0.117)	6.16 (0.079)	6.12 (0.180)	6.14 (0.261)	5.93 (0.065)	6.12 (0.049)
μ_{max} (h^{-1})	1.012 (0.082)	0.901 (0.019)	0.893 (0.018)	0.890 (0.017)	0.873 (0.014)	0.914 (0.017)
Lag (h)	-	-	-	-	-	-
Y_{max} (Log(CFU/mL))	8.97 (0.016)	9.017 (0.017)	9.05 (0.034)	9.02 (0.029)	9.00 (0.026)	8.95 (0.011)
R^2	0.98 - 1.00	0.98 - 0.99	0.99 - 0.99	0.98 - 0.99	0.98 - 0.99	0.99 - 0.99
RMSE	0.058 - 0.100	0.069 - 0.095	0.078 - 0.096	0.051 - 0.101	0.074 - 0.098	0.053 - 0.089
LB-DYP						
Y_0 (Log(CFU/mL))	6.21 (0.067)	6.04 (0.053)	5.99 (0.107)	6.19 (0.047)	5.94 (0.033)	6.04 (0.066)
μ_{max} (h^{-1})	0.692 (0.049)	0.663 (0.034)	0.600 (0.051)	0.578 (0.043)	0.658 (0.060)	0.681 (0.057)
Lag (h)	-	-	-	-	-	-
Y_{max} (Log(CFU/mL))	8.77 (0.071)	8.60 (0.019)	8.65 (0.093)	8.59 (0.039)	8.57 (0.040)	8.56 (0.068)
R^2	0.93 - 1.00	0.98 - 0.99	0.99 - 1.00	0.98 - 0.99	0.98 - 1.00	0.98 - 0.99
RMSE	0.044 - 0.255	0.057 - 0.089	0.021 - 0.086	0.046 - 0.076	0.016 - 0.082	0.048 - 0.082
M9-Glu						
Y_0 (Log(CFU/mL))	6.12 (0.017)	6.04 (0.011)	6.10 (0.039)	6.07 (0.015)	5.97 (0.128)	6.02 (0.034)
μ_{max} (h^{-1})	0.618 (0.030)	0.573 (0.012)	0.527 (0.028)	0.548 (0.054)	0.479 (0.018)	0.534 (0.025)
Lag (h)	-	-	-	-	-	-
Y_{max} (Log(CFU/mL))	7.90 (0.104)	8.10 (0.340)	8.11 (0.381)	8.03 (0.304)	8.07 (0.400)	8.09 (0.393)
R^2	0.99 - 1.00	0.99 - 1.00	0.99 - 1.00	0.99 - 1.00	0.99 - 1.00	0.99 - 0.99
RMSE	0.021 - 0.070	0.023 - 0.058	0.027 - 0.075	0.025 - 0.049	0.026 - 0.048	0.035 - 0.061

Table S4. Genetic variations detected by whole genome sequencing (WGS) between *S*. Typhimurium SL1344 and the reference genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 (GenBank: Accession No. FQ312003.1). All detected mutations were single nucleotide variation (SNV).

Region	Genes	Locus tag	Mutation Type	Aminoacid Change	Description
2411272	*menC*	SL1344_2275	c.444A>G	No coding	O-succinylbenzoate synthase
3284620	*yggW*	SL1344_3079	c.804T>C	No change	possible oxygen-independent coproporphyrinogen III oxidase
3611524	*rpsN*	SL1344_3394	c.89A>G	Asn30Ser	30S ribosomal subunit protein S14
4850544	SL1344_4501	SL1344_4501	c.400T>C	Phe134Leu	hypothetical outer membrane protein

Table S5. Average of the C_t values normalized to the *rpoZ* level gene (ΔC_t) in select stress response genes, *katE*, and *otsB* of 22 *Salmonella* strains

Strain	*katE*	*otsB*
S. Typhimurium STCC 443	4.93	-1.52
S. Typhimurium STCC 4594	3.71	-1.39
S. Typhimurium STCC 7162	-0.38	-5.30
S. Typhimurium STCC 722	2.35	-3.46
S. Enteritidis STCC 4155	4.83	-3.17
S. Enteritidis STCC 4300	2.01	-4.25
S. Enteritidis STCC 4396	2.69	-4.98
S. Enteritidis STCC 7160	3.69	-4.11
S. Enteritidis STCC 7236	2.67	-4.43
S. Derby STCC 4397	2.77	-3.59
S. Gallinarum STCC 4883	1.69	-7.38
S. Hadar NCTC 13033	2.92	-1.63
S. Heidelberg DMS 9379	1.46	-4.02
S. Infantis STCC 4373	2.10	-4.06
S. Kentucky NCTC 5799	1.27	-5.07
S. Livingstone NCTC 9125	1.08	-4.29
S. Mbandaka NCTC 7892	1.47	-5.15
S. Newport NCTC 129	1.22	-5.16
S. Saintpaul STCC 4153	0.92	-4.15
S. Stanley STCC 4141	0.18	-5.06
S. Senftenberg 775W STCC 4565	1.78	-4.76
S. Virchow STCC 4154	1.35	-8.67

Capítulo 4

Estudio de la influencia de la dosis y la historia térmica del huevo en la velocidad de crecimiento de *Salmonella* Enteritidis

Manuscrito VI/Manuscript VI

Influence of the initial cell number on the growth fitness of *Salmonella* Enteritidis in raw and pasteurized liquid whole egg, egg white, and egg yolk

Silvia Guillén, María Marcén, Ignacio Álvarez, Pilar Mañas and Guillermo Cebrián

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Influence of the Initial Cell Number on the Growth Fitness of *Salmonella* Enteritidis in Raw and Pasteurized Liquid Whole Egg, Egg White, and Egg Yolk

Silvia Guillén, María Marcén, Ignacio Álvarez, Pilar Mañas and Guillermo Cebrián *

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto, Agroalimentario de Aragón—IA2—(Universidad de Zaragoza-CITA), 50013 Zaragoza, Spain; silviaguillen@unizar.es (S.G.); mariamarcenterraza@gmail.com (M.M.); ialvalan@unizar.es (I.Á.); manas@unizar.es (P.M.)

* Correspondence: guiceb@unizar.es; Tel.: +34-876554127

Abstract: *Salmonella* growth in egg and egg products has been widely studied, but there are still some aspects that are not fully known. The objective of this work was to study the influence of the initial cell number on the growth fitness of *Salmonella* Enteritidis in raw and pasteurized egg products. Growth curves of five *Salmonella* Enteritidis strains in raw and pasteurized egg products, starting from different initial numbers, were obtained and fitted to the Baranyi and Roberts model. The results revealed that lower initial numbers led to longer lag phases (λ) and lower maximum specific growth rates (μ_{max}) in raw liquid whole egg. Similar results were observed in raw egg white (except for one strain). Conversely, no influence ($p > 0.05$) of the initial concentration on *Salmonella* growth parameters in raw egg yolk was observed. On the other hand, no influence of the initial number of cells on *Salmonella* growth fitness in commercial pasteurized liquid whole egg was observed. The results obtained demonstrate that the disappearance of this initial-dose dependency phenomenon was dependent on the intensity of the thermal treatment applied. Finally, the influence of the initial number was, in general, lower in pasteurized than in raw egg white, but large differences among strains were observed.

Keywords: egg products; inoculum dose; growth rate; thermal treatments; foodborne pathogen

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1. Introduction

The microorganisms of the genus *Salmonella* are the second most commonly reported causative agent of foodborne outbreaks in the European Union [1] and constitute one of the major public health challenges worldwide. One of the most important sources of *Salmonella* contamination is eggs and egg products. Thus, raw and undercooked eggs are still the products most frequently identified as responsible for foodborne *Salmonella* infections in the European Union (37.0% of *Salmonella* outbreaks in Europe in 2019) [1].

Intact eggs can be contaminated by *Salmonella* using two possible routes, named vertical transmission and horizontal transmission. The first one (vertical transmission or the transovarian route) is due to the infection of the hen's reproductive organs with *S.* Enteritidis and leads to the contamination of the yolk and/or the albumen during egg formation, i.e., before oviposition. The second possible route (horizontal transmission) is by penetration through the eggshell from the colonized gut or from contaminated feces during or after oviposition [2–6].

Salmonella growth and survival in egg and egg products has been widely studied, in particular, that of the serovar Enteritidis, because it is the predominant serovar in foodborne diseases associated with the consumption of these products [7–9]. It is well-known that there are wide differences in *Salmonella* growth ability depending on the egg fraction (yolk vs. white), growth temperature, and other factors [10–12]. *Salmonella* growth fitness is

much greater in egg yolk than in the egg white due to the particular composition and physico-chemical characteristic of the later, which is particularly efficient in the inhibition on microbial growth of microorganisms. Thus, egg white has a high viscosity, an alkaline pH, and a number of antimicrobial components, including lysozyme, ovotransferrin, and several vitamin chelating proteins [13,14]. On the other hand, the influence of growth temperature on *Salmonella* growth in egg yolk and white as well as in liquid whole egg has been thoroughly studied, and there are many works dealing with this topic. Growth models have been developed in order to predict the growth of *Salmonella* in egg products in an effort to establish the optimal temperature and time for their preservation and distribution and, therefore, to ensure their safety through appropriate quantitative microbial risk assessments [11,15,16].

Nevertheless, there are some aspects, such as the influence of the initial cell number and/or the physiological state of cells on *Salmonella* growth fitness (as well as in resistance to stress), that are still not fully known. Indeed, these factors have been highlighted as future research needs in order to improve current *Salmonella* growth models [17]. In addition, in some recent works, it has been reported that *Salmonella* growth in raw egg is slightly slowed down in comparison with pasteurized egg [9,18]. A similar finding was recently reported by Kang and co-workers when studying *Salmonella* growth kinetics in raw and pasteurized egg white [19]. This is a phenomenon of the highest relevance since it might imply a potential food safety risk if data obtained in raw products are extrapolated to heat-treated ones. Unfortunately, data regarding this particular phenomenon are still scarce, and the underlying mechanisms remain to be elucidated.

Therefore, the aim of this work was to study the influence of the initial cell number on the growth fitness of *S*. Enteritidis in raw and pasteurized liquid whole egg, egg white, and egg yolk.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Five strains belonging to *Salmonella enterica* serovar Enteritidis were used in this study. The strains of *S*. Enteritidis (STCC 4300, STCC 4155, STCC 4396, STCC 7160, and STCC 7236) were supplied by the Spanish Type Culture Collection. Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% *w*/*v* yeast extract (Oxoid, TSB-YE) in 96-well microtiter plates and incubated at 37 °C under static conditions, as described by Guillén et al. [20].

2.2. Growth Curves

Growth experiments were carried out in raw liquid whole egg, egg white, and yolk obtained from medium-sized raw eggs (53–63 g) purchased from a local supermarket and in commercial pasteurized liquid whole egg, egg white, and egg yolk (Pascual, Aranda de Duero, Spain). For some experiments (see below), raw liquid whole egg was exposed to heat treatments simulating pasteurization conditions. Absence (25 g) of *Salmonella* in each batch of eggs and/or egg products used was checked following standard protocols (ISO 6579-1:2017).

The different egg products were inoculated with different initial doses (between 10^2 (low dose) and 10^6 (high dose) CFU/mL) of *Salmonella*, and were then incubated at 37 °C. Samples were taken at preset time intervals from 0 to 30 h, adequately diluted in buffered peptone water (Oxoid, BPW), and plated in Xylose Lysine Deoxycholate agar (Oxoid, XLD), which was used as the recovery medium. XLD plates were incubated for 48 h at 37 °C, and the number of colony-forming units (CFU) per plate was counted.

2.3. Thermal Treatments

Thermal treatments (the pasteurization of raw liquid whole egg) were carried out in a specially designed thermoresistometer implemented with a compatible control thermostat that allowed the performance of heating ramps at different rates [21]. Briefly, this instru-

ment consists of a 400 mL vessel provided with an electrical heater for thermostation, a cooling system, an agitation device to ensure distribution and temperature homogeneity, and ports for the injection of microbial suspension and for the extraction of samples. The thermoresistometer was programmed to perform a linear temperature profile from 25 °C to the target temperature at a rate of 1 °C/min and held at that temperature (±0.1 °C). After treatments, pasteurized liquid whole egg was cooled and stored at 4 °C. The raw liquid whole egg was exposed to different treatment conditions simulating different pasteurization conditions: 60 °C for 3.5 min and 70 °C for 1.5 min.

2.4. Growth Curve Fitting and Statistical Analysis

Growth curves were constructed by plotting the logarithm of the number of *Salmonella* vs. time at the different condition assays. Each point of the growth curve corresponds to the average value of all the samples analyzed (at least three replicates). The curves obtained were fitted with the Baranyi and Roberts model (Equations (1) and (2)) [22]:

$$Y_t = Y_0 + \mu_{max} \cdot A_t - \frac{Y_{max} - Y_0}{M} \cdot ln\left[1 - e^{-M} + \left(e^{-M} \cdot \frac{Y_{max} - Y_0 - \mu_{max} \cdot A_t}{Y_{max} - Y_0}\right)\right] \quad (1)$$

$$A_t = t - \lambda \cdot \left[1 - \frac{1}{h_0} \cdot ln\left(1 - e^{-h_0 \cdot \frac{t}{\lambda}} + e^{-h_0 \cdot \left(\frac{t}{\lambda} - 1\right)}\right)\right] \quad (2)$$

where Y_t is the Log_{10} of cell concentration at time t (CFU/mL), Y_0 is the Log_{10} of the initial cell concentration (CFU/mL), Y_{max} is the Log_{10} of maximum cell concentration (CFU/mL), μ_{max} is the maximum growth rate (h^{-1}), λ is the lag phase (h), and M and h_0 are curvature parameters, taking them as constant values and with both set at a value of 10. For this purpose, GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, CA, USA) was used. The same software was used for calculating the goodness of fit parameters (R^2 and RMSE) and to carry out the statistical analysis (Student's *t*-tests and ANOVA). Differences were considered significant for $p \leq 0.05$.

3. Results

3.1. Influence of the Initial Concentration on the Growth Fitness of Salmonella Enteritidis STCC 4300 in Raw Liquid Whole Egg, Egg White, and Egg Yolk

First, the influence of the initial contamination dose on the growth fitness of *Salmonella* Enteritidis STCC 4300 (used as a model strain) in raw liquid whole egg, egg yolk, and egg white was determined. In order to do so, growth curves starting at different concentrations between 10^2 (low dose) and 10^6 (high dose) CFU/mL were obtained in the three egg products. As can be observed in Figure 1A and Table 1, the initial inoculum dose significantly affected the growth parameters calculated in raw liquid whole egg. The maximum growth rate in raw liquid whole egg determined for growth curves starting at a concentration of 10^2 CFU/mL (0.663 ± 0.015 h^{-1}) was significantly ($p < 0.05$) lower than curves starting at a concentration of 10^6 CFU/mL (0.845 ± 0.038 h^{-1}). Similarly, significant differences were also found among the lag values calculated in raw liquid whole egg. In this case, lag values increased as the dose inoculated in raw liquid whole egg decreased, with values of 6.32 ± 0.549 and 1.97 ± 0.038 h for the curves starting at 10^2 and 10^6 CFU/mL, respectively. A similar trend was observed in egg white, the egg product in which the lowest growth rates were obtained. Thus, in egg white, maximum growth rates of 0.164 ± 0.030 and 0.292 ± 0.066 h^{-1} were calculated from the growth curves corresponding to initial doses of 10^2 and 10^6 CFU/mL, respectively. The lag values in egg white also showed a similar trend to those obtained in raw liquid whole egg, with curves starting at 10^6 CFU/mL not displaying any lag phase (not significantly different from 0; $p > 0.05$) and curves starting at 10^2 CFU/mL showing the highest lag value (0.71 ± 0.193 h). It should be noted that in the particular case of raw egg white, the initial number also determined the growth yield (Y_{max}). Furthermore, not only did it determine the growth yield (which was higher

the higher the initial concentration) but also the difference between the initial and the final number of microorganisms (Y_{max}–Y_0), which was also higher the higher the initial number of microorganisms (1.18 Log_{10} cycles for growth curves starting at 10^6 CFU/mL vs. 0.61 Log_{10} cycles for growth curves starting at 10^2 CFU/mL). By contrast, no significant differences were found among the growth parameters calculated in raw egg yolk regardless of the inoculum dose. *S.* Enteritidis STCC 4300 maximum growth rates in egg yolk ranged from 0.837 ± 0.057 to 0.882 ± 0.047 h^{-1}, corresponding to curves that started at 10^6 and 10^3 CFU/mL, respectively. Similarly, the lag value varied between 1.60 ± 0.140, for curves starting at 10^5 CFU/mL, and 2.21 ± 0.240 h for curves starting at 10^3 CFU/mL.

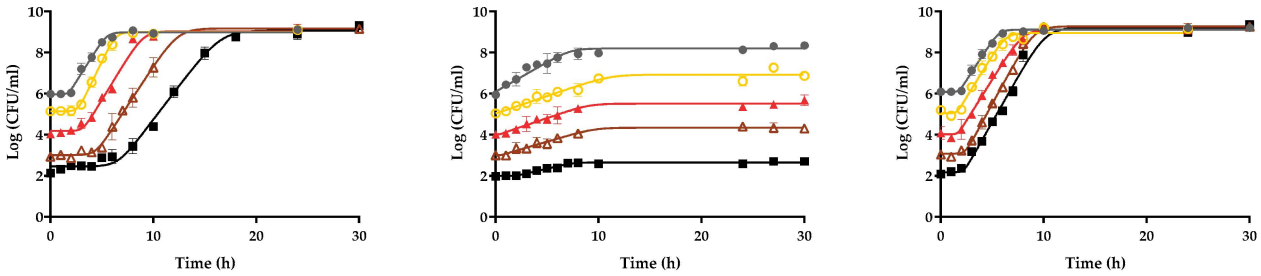


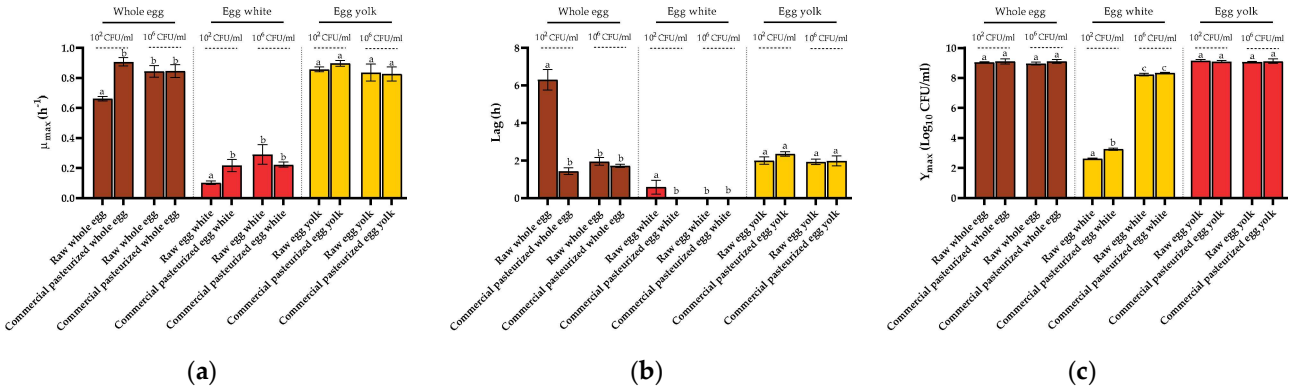
Figure 1. Effect of the inoculum dose on the growth fitness of Salmonella in raw liquid whole egg, egg white, and egg yolk: (**a**) Growth curves of *S.* Enteritidis STCC 4300 in raw liquid whole egg, (**b**) in raw egg white, and (**c**) in raw egg yolk. Initial dose 10^6 CFU/mL ●, 10^5 CFU/mL o, 10^4 CFU/mL ▲, 10^3 CFU/mL Δ, 10^2 CFU/mL ■. Lines correspond to the fit of the Baranyi model to the experimental data. Error bars represent the standard deviation.

Table 1. Growth (Y_0, Y_{max}, λ, and μ_{max}) and goodness of fit (R^2, RMSE) parameters (Baranyi and Roberts model) calculated for *S.* Enteritidis STCC 4300 in raw liquid whole egg, egg white, and egg yolk at 37 °C. Values presented correspond to the mean and standard deviations (SD, in parentheses). Letters indicate statistically significant differences.

Y_0 (CFU/mL)	μ_{max} (h^{-1})	λ (h)	Y_{max} (CFU/mL)	R^2	RMSE
Raw whole egg					
6.00 (0.061)	0.845 (0.038) ^{a}	1.97 (0.038) ^{a}	8.96 (0.093)	0.99–1.00	0.115–0.166
5.15 (0.066)	1.056 (0.142) ^{a,b}	2.71 (0.230) ^{b}	8.99 (0.013)	0.99–1.00	0.127–0.201
4.21 (0.086)	0.982 (0.039) ^{b}	3.49 (0.128) ^{c}	9.01 (0.079)	0.99–0.99	0.287–0.316
3.01 (0.035)	0.836 (0.018) ^{a}	4.54 (0.505) ^{d}	9.16 (0.038)	0.99–1.00	0.133–0.284
2.22 (0.070)	0.663 (0.015) ^{c}	6.32 (0.549) ^{e}	9.05 (0.030)	0.99–1.00	0.225–0.274
Raw egg white					
6.06 (0.116)	0.292 (0.066) ^{a}	- ^{a}	8.24 (0.084)	0.97–0.99	0.131–0.172
5.03 (0.026)	0.193 (0.022) ^{a,b}	0.29 (0.082) ^{b}	6.97 (0.051)	0.96–0.97	0.172–0.187
4.02 (0.023)	0.205 (0.014) ^{a}	0.43 (0.094) ^{b}	5.45 (0.045)	0.96–0.98	0.097–0.144
2.98 (0.059)	0.158 (0.010) ^{b}	0.40 (0.211) ^{b}	4.42 (0.120)	0.98 -0.99	0.055–0.096
2.03 (0.013)	0.104 (0.012) ^{c}	1.83 (0.106) ^{c}	2.64 (0.037)	0.95–0.99	0.067–0.138
Raw egg yolk					
6.10 (0.028)	0.837 (0.057) ^{a}	1.94 (0.147) ^{a}	9.07 (0.038)	0.99–0.99	0.121–0.181
5.07 (0.073)	0.829 (0.027) ^{a}	1.60 (0.140) ^{a}	8.93 (0.139)	0.98–0.99	0.249–0.257
4.05 (0.381)	0.838 (0.018) ^{a}	1.89 (0.403) ^{a}	9.09 (0.071)	0.99–1.00	0.163–0.264
3.09 (0.067)	0.882 (0.047) ^{a}	2.21 (0.240) ^{a}	9.27 (0.106)	0.99–1.00	0.134–0.235
2.15 (0.081)	0.857 (0.016) ^{a}	2.01 (0.195) ^{a}	9.23 (0.056)	0.98–0.99	0.271–0.414

3.2. Growth Fitness of Salmonella Enteritidis STCC 4300 Cells in Raw and Pasteurized Egg Products

Once the influence of the initial concentration on the growth fitness of *S.* Enteritidis STCC 4300 in raw egg products had been clearly established, the influence of the application of thermal (pasteurization) treatments to the three egg products on *Salmonella* growth fitness was studied. For this purpose, growth curves starting at 10^2 (low dose) or 10^6 CFU/mL (high dose) of *S.* Enteritidis STCC 4300 were obtained in commercial pasteurized liquid whole egg, egg white, and egg yolk, and compared with those obtained in raw (untreated) samples (Figure 2).



(**a**) (**b**) (**c**)

Figure 2. Growth parameters of *Salmonella* Enteritidis STCC 4300 cells in raw and commercial liquid whole egg, egg white, and egg yolk when inoculated at 10^2 and 10^6 CFU/mL. (**a**) μ_{max} (h^{-1}), (**b**) lag (h), and (**c**) Y_{max} Log_{10} (CFU/mL) values calculated with the Baranyi model. Error bars represent the standard deviation, and letters indicate statistically significant differences.

As can be observed in Figure 2, when *S.* Enteritidis STCC 4300 cells were inoculated at the lowest concentration (10^2 CFU/mL), their maximum growth rate in commercial pasteurized liquid whole egg was higher than that obtained in raw liquid whole egg at the same concentration, with values of 0.908 ± 0.028 and 0.663 ± 0.015 h^{-1}, respectively. In parallel, the lag value also decreased considerably in commercial liquid whole egg, from 6.32 ± 0.549 h for raw liquid whole egg to 1.44 ± 0.178 h for commercial pasteurized egg. At these low inoculation doses, differences were also found between the estimated growth parameters in raw and commercial pasteurized egg white (Figure 2). Thus, the maximum growth rate in egg white was higher in commercial pasteurized egg white (0.219 ± 0.040 h^{-1}) than in raw egg white, and no lag phase (not significantly different from 0; $p > 0.05$) was found in the former. In addition, significant differences in the Y_{max} value (2.64 ± 0.037 vs. 3.28 ± 0.074 Log_{10} CFU/mL) for raw and commercial pasteurized egg white were also found. These results clearly indicate that prior application of thermal treatments to liquid whole egg and egg white would improve the growth fitness of the cells of this *Salmonella* strain in both egg products when inoculated with a low dose.

On the other hand, no significant differences ($p > 0.05$) were found in the growth parameters (μ_{max} and λ) of *S.* Enteritidis STCC 4300 obtained in commercial pasteurized liquid whole egg and egg white, regardless of the initial dose. Furthermore, growth parameters determined in commercial whole egg and egg white, regardless of the initial dose, were not significantly different ($p > 0.05$) to those determined in raw products when inoculated with 10^6 CFU/mL, but were significantly different ($p < 0.05$) to those obtained in raw products inoculated with 10^2 CFU/mL. Therefore, it can be concluded that pasteurization treatments would not influence the growth of *S.* Enteritidis STCC 4300 cells inoculated at the highest dose in these two egg products, and, what it is much more relevant, that the application of these commercial pasteurization treatments would abolish the initial-dose dependence of *S.* Enteritidis STCC 4300 growth parameters in liquid whole egg and egg white.

Finally, no significant differences ($p > 0.05$) were obtained in egg yolk regardless of the initial dose or thermal history (raw vs. commercial) of the egg product, which is consistent with the results indicated in the previous section.

To further investigate the influence of heat treatment, raw liquid whole egg was exposed to thermal treatments of different intensity at different temperatures and times: 60 °C for 3.5 min and 70 °C for 1.5 min. Figure 3 shows the growth curves of *S.* Enteritidis STCC 4300 when inoculated with an initial dose of 10^2 CFU/mL. Growth curves at the same initial dose in raw and commercial pasteurized liquid whole egg are also included for comparison purposes. As can be observed in the figure, the growth parameters calculated for *Salmonella* Enteritidis STCC 4300 cells in egg exposed to 60 °C for 3.5 min showed intermediate values between those calculated in commercial and raw liquid whole egg. In contrast, no significant differences ($p < 0.05$) were found between the growth parameters determined for *Salmonella* Enteritidis STCC 4300 cells in liquid whole egg pasteurized at 70 °C for 1.5 min and commercial pasteurized liquid whole egg. It was also found that when inoculated with high doses (10^6 CFU/mL), no differences were found in the growth parameters determined, regardless of the thermal treatment, and that these values were not significantly different from those obtained in commercial liquid whole egg and liquid whole egg pasteurized at 70 °C for 1.5 min and inoculated with low doses (data not shown). In summary, it can be concluded that increasing the intensity of the thermal treatment led to a progressive increase in the growth rate and a decrease in the lag phase for *Salmonella* Enteritidis STCC 4300 inoculated with 10^2 CFU/mL in liquid whole egg, and consequently, to the progressive disappearance of the initial-dose dependence of *S.* Enteritidis STCC 4300 growth fitness in this medium.

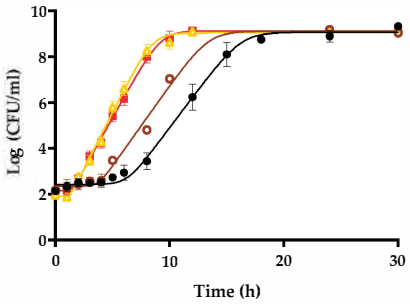


Figure 3. Influence of the intensity of pasteurization treatment on the growth fitness of *Salmonella* Enteritidis STCC 4300 cells inoculated at 10^2 CFU/mL. Growth curves obtained in raw liquid whole egg (●), pasteurized whole egg at 60 °C 3.5 min (o), pasteurized whole egg at 70 °C 1.5 min (Δ), and commercial pasteurized liquid whole egg (■). Lines correspond to the fit of the Baranyi model to the experimental data. Error bars represent the standard deviation.

3.3. Variability in Growth Fitness in Raw Liquid Whole Egg, Egg White, and Egg Yolk among S. Enteritidis Strains

In order to determine if the conclusions drawn above could be extrapolated to the whole *S.* Enteritidis serovar, the growth fitness of another four *S.* Enteritidis strains in the same media and conditions described above was studied. Therefore, growth curves of these four *S.* Enteritidis strains, starting at concentrations of 10^2 and 10^6 CFU/mL, were obtained in raw and commercial pasteurized liquid whole egg, egg yolk, and egg white.

Regarding liquid whole egg, the results obtained indicate that all the *S.* Enteritidis strains studied displayed a similar behavior to that described for strain STCC 4300 (Figure 4). Consequently, the lower growth rates (ranging from 0.456 ± 0.002 h^{-1} for *S.* Enteritidis STCC 4396, to 0.663 ± 0.015 h^{-1} for *S.* Enteritidis STCC 4300) and the highest lag values (ranging from 2.76 ± 0.244 h to 8.93 ± 0.080 h for *S.* Enteritidis STCC 7236 and *S.* Enteritidis STCC 4396, respectively) were observed in curves starting at 10^2 CFU/mL in raw liquid whole egg. Additionally, as also described for strain STCC 4300, the highest growth rates and shorter lag times were observed, on average, in commercial liquid whole

egg and raw egg when curves started at high concentrations (10^6 CFU/mL). However, it should be noted that whereas for four of the five strains (STCC 4300, STCC 4155, STCC 7162, and STCC 7236), growth in raw liquid whole egg starting at low concentrations (10^2 CFU/mL) resulted in significantly ($p < 0.05$) lower growth rates and longer lag times than in the other three conditions tested, with no significant differences ($p > 0.05$) in the growth parameters calculated among the later conditions, for strain STCC 4396, this lower growth fitness in raw liquid whole egg at low initial concentrations was mainly evidenced as an increase in the length of the lag phase. This latter strain, in contrast to the other four, also displayed a lower growth fitness in raw liquid whole egg than in pasteurized liquid whole egg, regardless of the initial dose. No significant differences were found in the Y_{max} values regardless of the strain, initial dose, and/or heat treatment of the liquid whole egg studied (data not shown).

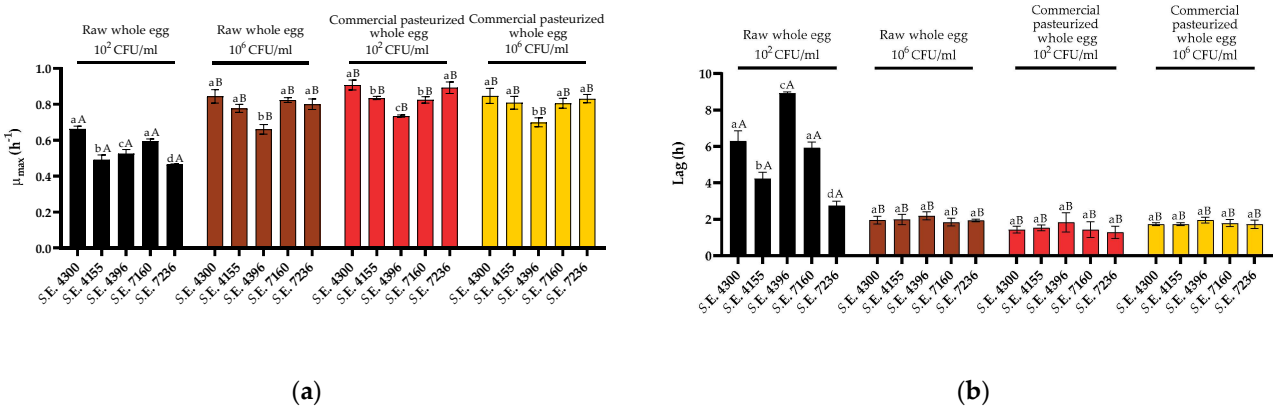
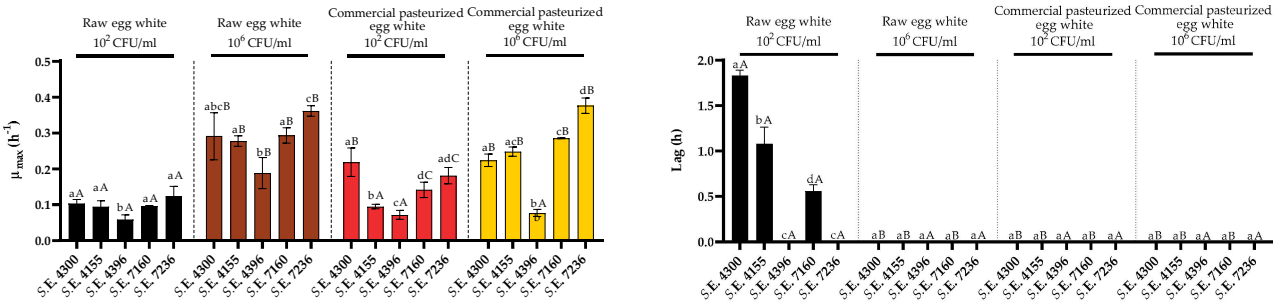


Figure 4. Growth parameters of five strains of *Salmonella* Enteritidis inoculated with 10^2 and 10^6 CFU/mL in raw liquid whole egg and commercial pasteurized liquid whole egg. (**a**) μ_{max} (h^{-1}) and (**b**) lag (h) values obtained after the fit of the growth curves to the Baranyi model. Error bars represent the standard deviation. Differences in the lower-case letters indicate statistically significant differences ($p < 0.05$) between strains grown on the same media and conditions (starting dose). Differences in the upper-case letters indicate statistically significant differences ($p < 0.05$) among growth conditions (raw vs. pasteurized and initial dose) for each strain.

Overall, the differences in growth fitness among the different strains studied were higher in raw egg and, especially, at low inoculation doses. Thus, whereas significant differences were found among the growth rates and lag times of four out of five of the *S.* Enteritidis strains when they were grown in raw liquid whole egg inoculated with low doses (and more than a 3-fold difference in the lag times), only strain STCC 4396 displayed a significantly lower growth rate (only 15% lower than the average) in pasteurized liquid egg inoculated with high doses. In addition, it should be remarked that strain STCC 4396 was the one displaying the lowest growth fitness in all the conditions assayed.

Growth curves starting at high and low doses of the five *S.* Enteritidis strains were also obtained in raw egg white and commercial pasteurized egg white. As can be observed in Figure 5b, significant lag phases (significantly different from 0) were only observed for three strains and only when grown in raw egg white and starting from 10^2 CFU/mL. As described for liquid whole egg, the lowest growth rates (on average) and highest lag phases were observed when cells were grown in raw egg white and starting from 10^2 CFU/mL, and the highest growth rates were observed in egg white inoculated with 10^6 CFU/mL (regardless of its prior thermal history) (Figure 5a). As also described for liquid whole egg, increasing the initial dose resulted in an increase in the growth rates in raw egg white; however, in this case, pasteurization did not abolish the initial-dose dependence of *Salmonella* growth. Thus, although the growth fitness of most *Salmonella* strains (3/5) starting at 10^2 CFU/mL was higher in pasteurized than in raw egg white, it did not reach the values observed when 10^6 CFU/mL was inoculated initially, i.e., the

initial-dose dependence of *S*. Enteritidis growth was more relevant in egg white than in whole egg.

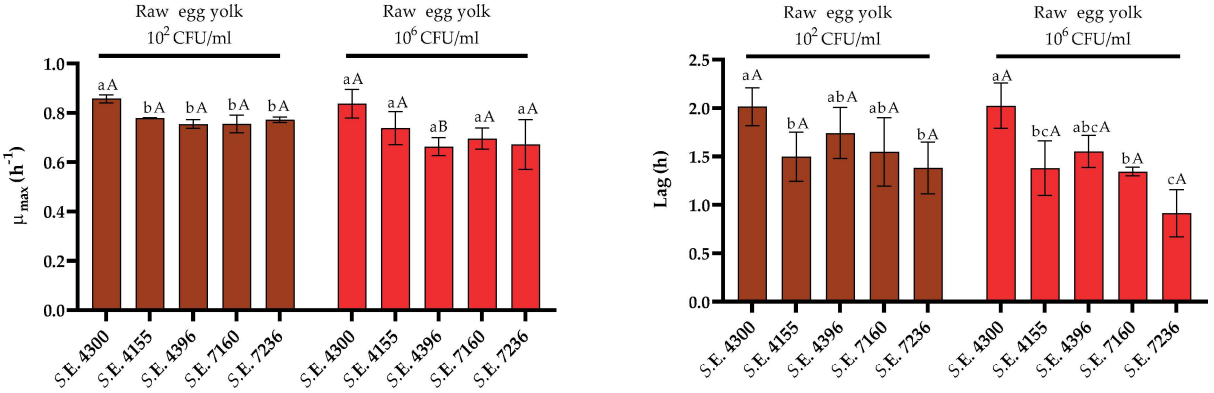


(**a**) (**b**)

Figure 5. Growth parameters of five strains of *Salmonella* Enteritidis inoculated with 10^2 and 10^6 CFU/mL in raw egg white. (**a**) μ_{max} (h^{-1}) and (**b**) lag (h) values obtained after the fit of the growth curves to the Baranyi model. Error bars represent the standard deviation. Differences in the lower-case letters indicate statistically significant differences ($p < 0.05$) between strains grown on the same media and conditions (starting dose). Differences in the upper-case letters indicate statistically significant differences ($p < 0.05$) among growth conditions (raw vs. pasteurized and initial dose) for each strain.

S. Enteritidis STCC 4396 was once again the strain displaying the lowest maximum growth rates in all the conditions studied, with growth rates between 0.059 ± 0.013 h^{-1} and 0.188 ± 0.044 h^{-1}. It also displayed significantly ($p < 0.05$) lower Y_{max} values than the other four strains in three out of four of the conditions studied (data not shown). It should also be noted that, conversely to that observed in liquid whole egg, the variability in growth fitness (growth rates) among strains was higher in commercial than in raw egg white.

Finally, as can be seen in Figure 6, the behavior of all the strains in raw egg yolk was similar to that of *S*. Enteritidis STCC 4300, with no changes in growth fitness regardless of the initial dose. It was also found that these parameters did not significantly change in pasteurized egg yolk (data not shown). In addition, it should be noted that the variability among the growth parameters calculated for *S*. Enteritidis strains in egg yolk was lower than that obtained in whole egg and egg white.



(**a**) (**b**)

Figure 6. Growth parameters of five strains of *Salmonella* Enteritidis inoculated with 10^2 and 10^6 CFU/mL in raw egg yolk. (**a**) μ_{max} (h^{-1}) and (**b**) lag (h) values obtained after the fit of the growth curves to the Baranyi model. Error bars represent the standard deviation. Differences in the lower-case letters indicate statistically significant differences ($p < 0.05$) between strains grown on the same media and conditions (starting dose). Differences in the upper-case letters indicate statistically significant differences ($p < 0.05$) among growth conditions (raw vs. pasteurized and initial dose) for each strain.

4. Discussion

The results presented in this paper demonstrate that the growth fitness of *Salmonella* Enteritidis cells in liquid whole egg and its fractions depends on the egg product (whole egg vs. white vs. yolk), its thermal history (except for egg yolk), the strain studied, and, in the case of raw liquid whole egg and egg white (both raw and pasteurized), the initial concentration of *Salmonella* cells.

As expected, the lowest *S.* Enteritidis growth rates were observed in egg white and the highest in egg yolk. Nevertheless, in raw liquid whole egg at high initial concentrations and pasteurized (70 °C for 1.5 min) liquid whole egg (regardless of the initial concentration in the latter), the growth rates of most (4/5) *Salmonella* strains studied here were comparable to those in egg yolk. These differences in microbial growth fitness depending on the egg fraction studied have already been demonstrated by numerous authors, such as Kang et al., who reported that *Salmonella* growth in egg white was slower than that in liquid egg yolk and liquid whole egg [23], and Kim et al., who demonstrated that there is a difference in *Salmonella* growth fitness in unpasteurized liquid eggs depending on the type of liquid egg products (liquid whole egg, egg yolk, or egg white) and storage temperature [11]. As indicated in the introduction, *Salmonella* growth fitness is much greater in egg yolk than in egg white because the latter has a high viscosity, an alkaline pH, and a number of antimicrobial components, including lysozyme, ovotransferrin, and several vitamin chelating proteins [13,14]. Since liquid whole egg is a mix of both fractions, one would expect that *Salmonella* cells would display intermediate growth fitness, as reported here.

In contrast, the influence of the initial dose on microbial (and particularly *Salmonella*) growth fitness has hardly been studied. Our results demonstrate that the initial number of cells can highly influence the growth fitness of *S.* Enteritidis cells in egg white and also in raw liquid whole egg. In this sense, it should be noted that Zaher and Fujikawa also observed a similar initial-dose effect phenomenon on *Salmonella* growth in raw ground chicken [24]. These authors attributed their results to the competition between *Salmonella* and natural microbiota, but this would likely not be the case for raw egg given the extremely low concentrations of microorganisms in this latter product. Regarding egg products, Kang et al. already determined that the bactericidal activity of egg albumen was dependent on the initial bacterial concentration. Thus, at low initial cellular concentrations, the antimicrobial factors of the egg albumen would be capable of inhibiting the growth of *S.* Enteritidis cells but would fail to control them when the bacterial concentration was higher [25]. Furthermore, in another study, it was reported that low increases in inoculum size, from 2 to 250 cells per egg, had a high impact on the ability of *S.* Enteritidis to migrate from egg white to yolk [26]. The authors established 250 cells as a critical level for *Salmonella* growth in the albumen and indicated that iron was the growth-limiting factor. In this sense, they suggested that over this threshold, *Salmonella* cells would be able to grow because they would be able to synthesize enough enterochelin, a bacterial siderophore that is able to compete with ovotransferrin for iron, or because the death of some cells in the albumen would allow the others to use them as a source of iron and/or energy [26].

Our results are consistent with these findings, although they show that *S.* Enteritidis cells would be able to grow (though very slowly and to very low yields) at concentrations as low as 10^2 CFU/mL in egg white (slightly below the 250 limit previously determined). They also show that this initial-dose dependence would not be found in egg yolk and that, again, *Salmonella* cells would display an intermediate behavior in liquid whole egg. Thus, in raw liquid whole egg, *S.* Enteritidis maximum growth rates and lag times, but not the yields (final number of cells), would be initial-dose dependent; while this dose dependency is maintained in egg white upon its pasteurization, it will be abolished if the heat treatment applied to liquid whole egg is intense enough, as described below. These results can also be explained on the basis of the hypotheses already proposed [26], since this initial-dose dependence would be abolished (at least in liquid whole egg) upon the addition of iron to the medium [18]. Further work would be required in order to determine the growth fitness of *Salmonellae* in egg and egg products at lower initial doses than those studied here, since

that might be the case in many cases/scenarios. This would be of the highest interest for the future development of improved growth models and risk assessments.

The results reported here also demonstrate that *Salmonella* growth fitness in some egg products and conditions would also depend on the thermal history of those egg products. Thus, the application of thermal treatments on egg yolk would not impact *Salmonella* growth fitness regardless of the initial dose, likely because the growth rates in the "least favorable conditions" (i.e., raw product and low initial dose) would already be the highest achievable at this temperature for *Salmonella* cells as they were comparable to those obtained for these strains under the same incubation conditions in rich media, such as TSB-YE [27] (Guillén et al., 2021, submitted for publication). On the other hand, the application of thermal treatments on liquid whole egg led to an increase in *Salmonella* fitness in this product, but only when the initial number of cells was very low. Furthermore, it was demonstrated that this effect was higher the more intense the thermal treatments applied. This is consistent with the results of Sakha et al., who reported that a *S.* Enteritidis cocktail proliferated more rapidly in pasteurized liquid whole egg than in unpasteurized liquid whole egg, although it should be noted that they did not observe any influence of the initial dose on the growth parameters [9]. The lack of influence of thermal treatments on *Salmonella* growth fitness at high concentrations can be explained on the same basis as that of egg yolk. Thus, the growth rate of these *Salmonella* strains at high initial concentrations in this product is comparable to that in egg yolk and also in rich laboratory media. Finally, application of thermal treatments to egg white also resulted in an increase in growth fitness for *Salmonella* when inoculated at low concentrations. Nevertheless, in this case, and even after the application of the harsher treatment conditions tested (up to 120 min at 56.0 °C; data not shown), the growth rate in pasteurized egg white of cells inoculated at 10^2 CFU/mL did not reach that of samples inoculated with 10^6 CFU/mL. Similar results to those obtained here were obtained by Kang et al., who observed that the maximum growth rate of a cocktail of four *Salmonella* strains inoculated in pasteurized egg white at a dose of 3.5 ± 0.5 Log_{10} CFU/mL was much higher than in raw egg white [19].

Classical pasteurization treatments (1–10 min at 60–72 °C) applied in the industry to pasteurize whole egg are limited because of the sensitivity of the egg white proteins to heat treatments, which might lead to egg coagulation. Furthermore, pasteurization conditions should be even milder in egg white (<60 °C). Therefore, it can be speculated that the differences in growth rates between raw and pasteurized liquid whole egg and egg white would be due to the thermal denaturation of egg white proteins with antimicrobial properties, such as ovotransferrin and lysozyme [14,28]. Furthermore, since the concentration of these antimicrobial proteins would be lower in liquid whole egg than in egg white, this would explain why egg pasteurization treatments would be able to completely abolish the initial-dose dependence of *Salmonella* growth fitness in whole egg but not in egg white. Other causes that might also contribute to explaining this different behavior between egg white and whole liquid egg include the lower content of iron and other nutrients in egg white, its particular physical–chemical characteristics, and the lower intensity that can be applied in pasteurization treatments to egg white. Nonetheless, further work will be required to fully elucidate all the components of the egg with antimicrobial activity, including those limiting iron bioavailability, as indicated by Guillén et al. [18]. In this sense, recent studies also suggest that low-weight components (<10 kDa) of egg white are largely responsible for the bactericidal activity of egg white at high treatment temperatures [29].

Regarding the variability in growth fitness among strains, it should be noted that *S.* Enteritidis displays a higher survival capacity in egg white than other species, higher than other *Salmonella* serovars [30]. Nevertheless, it has also been reported that *S.* Enteritidis strains vary in their ability to grow or survive in egg white, indicating that some variants are better adapted to egg white [31–33]. Nonetheless, this advantage over other serovars in the growth and survival ability of *S.* Enteritidis in egg white only existed when the inoculum size was below 10^7 CFU/mL [34]. Previous studies have reported that genes involved in iron acquisition, cell envelope structure, osmotic and oxidative protection,

amino acid and carbohydrate metabolism, motility, and stress responses may contribute to the survival of *S.* Enteritidis in egg albumen [31,33–35]. Egg white is a very unfavorable medium for bacteria, and the major factor limiting bacterial growth in it is iron restriction, having a concentration of iron between 3.6 and 18 μM [36]. *Salmonella* has the ability to produce siderophores in iron-restricted media to cope with this restriction, as in egg white, where iron is assumed to be chelated by ovotransferrin [37]. However, little is known regarding the variability in the ability to synthesize these siderophores among *Salmonella* strains. In any case, it can be speculated that the differences in growth fitness observed among the different strains might be associated with a different siderophore-production ability. This will explain why little or no differences among strains were found in egg yolk and pasteurized liquid whole egg, whereas the highest ones were observed in egg white. Further work will be required in order to verify this hypothesis.

The risk of suffering salmonellosis is related to one or several of the following scenarios: the existence of a very high population of *Salmonella* in raw egg prior to pasteurization, the application of inadequate/insufficient treatment, or post-pasteurization contamination. The potential impact of post-processing contamination of liquid whole egg, egg white, and egg yolk was the one assessed in this study. According to our results, post-pasteurization recontamination of pasteurized liquid whole egg and egg white would represent a greater risk for consumers than that of the unpasteurized products. This is of the highest relevance since commercially pasteurized egg products are often used as ingredients in foods without any further heat treatment during food preparation. Furthermore, if only the risk associated with a potential recontamination is considered, the data obtained here indicate that the lowest intensity pasteurization treatments (such as 60 °C 3.5 min for liquid whole egg) would be more recommendable. It is noteworthy that low-intensity pasteurization treatments have been proposed to increase the digestibility and reduce the allergenicity of whole egg proteins [28].

Finally, it should also be noted that it is generally acknowledged that the initial dose does not determine the microbial maximum growth rates, which will only be determined by the microbial genotype and the growth conditions (intrinsic and extrinsic factors). However, although our results seem to contradict this affirmation, this is not the case. Thus, this general rule is based upon the assumption that the concentration (or bioavailability) of the limiting nutrient/s in batch cultures is fixed, but this is not the case here since the synthesis of siderophores and/or cell death (the two theories proposed for explaining the results obtained here) would increase the amount of iron and energy available in a dose-dependent manner.

5. Conclusions

In summary, our results demonstrate that the initial dose and thermal history of liquid whole egg and egg white can determine the growth fitness of *Salmonella* Enteritidis cells in these products, whereas this does not occur in egg yolk. In addition, our results also indicate that the variability in growth fitness among strains highly depends on the conditions in which it is studied. Further work will be required in order to fully elucidate the mechanisms underlying the results obtained here—particularly how the initial dose determines *Salmonella* growth fitness in some egg products/fractions and why pasteurized egg products provide more favorable conditions to *Salmonella* cells—and to develop novel predictive growth models and perform improved risk assessments of *Salmonella* in egg products, including all the factors that have been proven to affect its growth fitness in them.

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Relationship between iron bioavailability and *Salmonella* fitness in raw and pasteurized liquid whole egg

Silvia Guillén and Guillermo Cebrián

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Relationship between iron bioavailability and *Salmonella* fitness in raw and pasteurized liquid whole egg

Silvia Guillén[1] and Guillermo Cebrián[1]*

[1] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón—IA2—(Universidad de Zaragoza-CITA), 50013, Zaragoza, Spain;
* Correspondence: guiceb@unizar.es (G.C); Tel.: +34-876554127

Abstract: *Salmonella* Enteritidis growth rates in liquid whole egg have been shown to be dependent on the initial inoculum dose and on the egg product's thermal history. This study's objective is to obtain further insight into the mechanisms underlying both phenomena. First we verified that *Salmonella* Typhimurium ATCC 14028s cells displayed the behavior already described for *S.* Enteritidis cells. Then, we carried out supplementation assays by adding different concentrations of egg-white antimicrobial proteins, iron, or siderophores to the growth media (raw or pasteurized liquid whole egg, depending on the assay). These experiments revealed that addition of lysozyme (at the concentration at which it is present in liquid whole egg) did not affect *Salmonella* growth in pasteurized liquid whole egg, but that ovotransferrin as well as Ex-FABP caused a significant (p <0.05) reduction in *Salmonella* growth rates in whole egg pasteurized at 70 °C for 1.5 minutes. Furthermore, we observed that the inactivation of ovotransferrin was dependent on treatment intensity within the range studied. On the other hand, addition of iron or siderophores to raw or low temperature (60 °C/3.5 min) pasteurized liquid whole egg increased the growth rate of *Salmonella* cells inoculated at a low initial dose until reaching that of cells inoculated at a high dose whereby the concentration required for this was lower for pasteurized than for raw egg. Finally, growth of a set of deletion mutants in genes coding for proteins related to different iron uptake systems, along with supplementation assays using spent medium revealed the key role of Salmochelin in growth of *S.* Typhimurium in raw whole egg. In summary, our results strongly suggest that iron bioavailability determines the fitness (growth rates) of *Salmonella* cells in liquid whole egg. Thus, the higher the intensity of the thermal treatment applied to liquid egg, the more iron would be available, a phenomenon that would be linked to the denaturation of iron and/or siderophore binding egg proteins. Further work is still required to fully elucidate why lower *Salmonella* initial doses lead to lower growth rates, but it can be hypothesized that this might be related to a lower amount of siderophores being released to the medium (especially Salmochelin), which would also limit iron bioavailability.

Keywords: *Salmonella*; egg; inoculum dose; thermal treatments; ovotransferrin; Ex-FABP; siderophores.

1. Introduction

Foodborne diseases are an acknowledged public health challenge worldwide. In the European Union, salmonellosis is the second most commonly reported gastrointestinal infection in humans after campylobacteriosis (EFSA, 2021). The microorganisms of the genus *Salmonella* were the

most frequently detected causative agent in foodborne outbreaks (FBOs) in 2019, accounting for 17.9% of total FBOs. One of the most important sources of *Salmonella* contamination are eggs and egg products, principally raw and undercooked eggs (37.0% of *Salmonella* outbreaks in Europe in 2019) (EFSA, 2021). Eggs are contaminated by *Salmonella* through two main routes: vertical transmission, i.e., before oviposition by infection of the reproductive organs, and horizontal transmission, i.e., during or after oviposition upon penetration of the eggshell (De Reu et al., 2006; Gantois et al., 2009; Keller et al., 1995; Messens et al., 2005; Timoney et al., 1989). Apart from presenting several physical barriers (eggshell, cuticle, testaceous membranes), egg, and more particularly egg white, contains a potent complex of antimicrobial molecules that limit bacterial growth and migration into the egg yolk (Baron et al., 2016). The antimicrobial activity of egg white is ensured, on the one hand, by its harsh physicochemical properties, including its alkaline pH as well as its high viscosity, which impair bacterial mobility and accessibility to nutrients (Baron et al., 2017, 2016), Egg white likewise contains a series of compounds (proteins) with specific antimicrobial properties, including lysozyme (a cell wall hydrolase), proteinase inhibitors, and ovotransferrin (an iron chelator that limits iron bioavailability), among others (Baron et al., 1999; Chart and Rowe, 1993). Although these well-known and widely characterized proteins are naturally abundant in egg white, high-throughput approaches have recently revealed that less abundant proteins (such as the siderophore-sequestering "lipocalin" protein Ex-FABP) and peptides might also play a role in the defense against bacterial contamination (Baron et al., 2016; Julien et al., 2020).

The particular composition and physicochemical characteristics of egg white make *Salmonella* growth ability in egg and egg products highly dependent on the egg fraction (yolk *vs* white *vs* whole) (Guillén et al., 2021; Kim et al., 2018; Messens et al., 2004; Moon et al., 2016). However, egg fraction is not the only factor determining *Salmonella* growth ability/rate in egg products. Incubation/storage temperature and time, as well as the supplementation of additives such as NaCl or sucrose to control water activity, also determine *Salmonella* growth fitness in egg and egg products (Gurtler and Conner, 2009; Jakočiūnė et al., 2014; Ng et al., 1979).

Recent studies have demonstrated the influence of the initial cell number and of thermal history (raw *vs* pasteurized) on the growth fitness of *Salmonella* in egg white and liquid whole egg (Guillén et al., 2021, 2020a; Kang et al., 2021); these authors hypothesized that the lower growth rates observed for *Salmonella* cells in raw products inoculated at low initial doses could be related to the antimicrobial activity of egg white proteins, such as ovotransferrin and lysozyme (Baron et al., 2016; Lechevalier et al., 2017). Further research is necessary to verify this hypothesis. Therefore, the aim of the present study is to obtain further insight into the mechanisms responsible for the initial-dose-dependence and thermal-history-dependence of *Salmonella* growth in whole liquid egg.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The strains used in this study are listed in Table 1. They were obtained from the Single-Gene Deletion Mutant Library of *Salmonella enterica* subsp. *enterica*, Strain 14028s (Serovar Typhimurium) through BEI Resources (www.beiresources.org); the parental strain was provided by the Spanish Type Culture Collection (STCC). Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (Oxoid, TSB-YE) in 96-well microtiter plates, and incubated at 37 °C under static conditions as described in Guillén et al. (2020b).

2.2. Growth curves

Growth experiments were carried out in raw liquid whole egg obtained from medium-sized raw eggs (53-63 grams) purchased from a local supermarket either before (raw) or after the application of a pasteurization treatment (see below, section 2.3). For some experiments, commercial pasteurized liquid whole egg (Pascual, Aranda de Duero, Spain) was also used.

The egg products were inoculated with different initial *Salmonella* doses, 10^2 (low dose) and 10^6 (high dose) CFU/mL, and were then incubated at 37 °C. Samples were taken at preset time intervals, from 0 to 30 h, unless otherwise noted, adequately diluted in buffered peptone water (Oxoid, BPW), and plated on Xylose Lysine Deoxycholate agar (Oxoid, XLD), the recovery medium. XLD plates were incubated for 48 h at 37 °C, and the number of colony forming units (CFU) per plate was counted.

Table 1. Strains used in this study.

Strain	Source
S. Typhimurium ATCC 14028s	STCC (strain 4594)
S. Typhimurium ATCC 14028s *ΔentC:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *Δfes:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔfeoB:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔfhuC:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔiroB:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔiroN:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔmntH:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔsitC:Kan*	BEI Resources (NR-29399)
S. Typhimurium ATCC 14028s *ΔzupT:Kan*	BEI Resources (NR-29399)

2.3. Thermal treatments

Thermal treatments (pasteurization of raw liquid whole egg) were carried out in a specially designed thermoresistometer implemented with a compatible control thermostat that allowed for the performance of heating ramps at different rates (Conesa et al., 2003). Briefly, this instrument consists in a 400 mL vessel provided with an electrical heater for thermostation, a cooling system, an agitation device to ensure distribution and temperature homogeneity, and ports for the injection of microbial suspension as well as for the extraction of samples. The thermoresistometer was programmed to perform a linear temperature profile from 25 °C to the target temperature at a rate of 1 °C/min, and then to hold at that temperature (± 0.1 °C). After treatments, pasteurized liquid whole egg was cooled and stored at 4 °C. Raw liquid whole egg was exposed to two different treatment conditions simulating different pasteurization conditions: 60 °C 3.5 min and 70 °C 1.5 min.

For some experiments (application of thermal treatments to ovotransferrin and to Ex-FABP in liquid whole egg, along with "re-pasteurization" treatments), a T100 thermal cycler (Bio-Rad, CA, USA) was used. This thermal cycler was programmed with the same heating ramps and used to apply the same treatment conditions as described for the thermoresistometer. We verified that the liquid whole egg temperature attained after the heating ramp was correct (data not shown).

2.4. Supplementation assays

Supplementation assays were carried out by adding different concentrations of proteins, iron, or siderophores to the growth media (liquid whole egg). In another set of assays, supplementation with spent media obtained from different strains was also studied. Then, liquid whole egg (raw or pasteurized, depending on the assay) supplemented with those molecules or extracts was inoculated with different concentrations of *Salmonella* cells and incubated as described above in order to determine its effect on *Salmonella* growth fitness.

2.4.1. Proteins

Lysozyme (Sigma-Aldrich, St. Louis, USA) up to a concentration of 1.93 mg/mL, ovotransferrin (Sigma-Aldrich) up to 8.80 mg/mL, extracellular fatty-acid-binding protein (Ex-FABP), and a

recombinant egg white lipocalin from *Coturnix coturnix japonica* (CSB-EP878006DXJ; Cusabio Biotech Co. Ltd., Wuhan, China) up to 0.22 mg/mL were added to the growth media (liquid whole egg). Concentrations of those compounds were selected based on their quantity proportion in egg white (Baron et al., 2016; Julien et al., 2020), and assuming a 2:1 ratio between egg white and egg yolk in liquid whole egg. The commercial protein Ex-FABP was desalted before supplementation using an Amicon ultra-0.5 mL centrifugal filter (MWCO = 3 kDa; Millipore, Billerica, MA, USA).

2.4.2. Iron and siderophores

Ferric citrate (Sigma-Aldrich) was added to the growth media up to a concentration of 0.1 mg/mL. Enterobactin (ENT) and Salmochelin S4 (SAL), two siderophores in iron-free form, were purchased from EMC Microcollections (Tuebingen, Germany) and supplemented at concentrations up to 5.00 μg/mL.

2.4.3. Spent medium

Cells of *S*. Typhimurium ATCC 14028s (wild type) and the *ΔiroB* and *ΔentC* mutants were inoculated (approx. 10 UFC/ml) in M9-broth (Sigma-Aldrich, M9) supplemented with magnesium sulfate (Panreac-AppliChem, Darmstadt, Germany) and glucose (Panreac-AppliChem), as indicated by the manufacturer, until cultures reached 10^2 and 10^6 UFC/ml. Bacterial suspensions were then centrifuged at 12000 g for 20 min at 4 °C and the supernatant, the spent medium, was first filtrated with a 0.22 ◎m sterile cellulose filter and then through an Amicon ultra-15 mL centrifugal filter (MWCO = 3 kDa; Millipore). The filtrate was then dyalized using a previously washed cellulose ester (CE) dialysis membrane with a nominal cutoff of 100-500 Da (Spectra/Por®, Biotech CS, US). The sealed membrane was immersed in sterile distilled water (1:1000 ratio) and dialyzed for 9 hours at 4 °C with gentle stirring (water was changed every three hours). Before supplementation, the filtered and dialyzed spent medium was concentrated in a centrifuge vacuum dryer (GeneVac, Ltd., United Kingdom) at 30 °C until liquid was completely evaporated. The obtained pellet was resuspended in sterile distilled water to the desired concentration (1/100 of the initial volume), and was added to the growth media (liquid whole egg). For certain experiments, non-filtered/dialyzed spent M9 medium or liquid whole egg spent filtered and dialyzed medium (following the same protocol) was used.

2.5. Growth curve fit and statistical analysis

Growth curves were constructed by plotting the decimal logarithm of the number of *Salmonella* versus time under the different conditions assayed. Each point in the growth curve corresponds to the average value of all samples analyzed (at least three replicates). The curves obtained were fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000):

$$Y_t = Y_0 + \mu_{max} \cdot A_t - \frac{Y_{max} - Y_0}{M} \cdot ln\left[1 - e^{-M} + \left(e^{-M} \cdot \frac{Y_{max} - Y_0 - \mu_{max} \cdot A_t}{Y_{max} - Y_0}\right)\right] \quad (1)$$

$$A_t = t - \lambda \cdot \left[1 - \frac{1}{h_0} \cdot ln\left(1 - e^{-h_0 \cdot \frac{t}{\lambda}} + e^{-h_0 \cdot \left(\frac{t}{\lambda} - 1\right)}\right)\right] \quad (2)$$

where Y_t is the Log_{10} of cell concentration at time t (CFU/mL); Y_0 is the Log_{10} of the initial cell concentration (CFU/mL); Y_{max} is the Log_{10} of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (Log_{10}/h); λ is the lag phase (h); and M and h_0 are curvature parameters, taking them as constant values, and with both set at a value of 10. For this purpose, we used GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA). The same software was used to calculate the goodness of fit parameters (R^2 and RMSE)

and to carry out the statistical analysis (Student's t tests and ANOVA). Differences were considered significant for $p \leq 0.05$.

3. Results

3.1. Effect of initial concentration on the growth rates of Salmonella in liquid whole egg

The influence of the initial contamination dose of *Salmonella* Typhimurium ATCC 14028s on its growth rates in raw and pasteurized (60 °C/3.5 min or 70 °C/1.5 min) liquid whole egg was analyzed by obtaining growth curves starting at 10^2 (low dose) or 10^6 CFU/mL (high dose) in the three media (Figure 1). The growth parameters calculated after fitting the curves with the Baranyi model are shown in Figure 2. As can be observed in Figures 2A and 2B, the initial inoculum dose significantly (p <0.05) affected the growth parameters λ and μ_{max} calculated in raw liquid whole egg and in egg pasteurized at 60 °C for 3.5 min, but not in egg pasteurized at 70 °C for 1.5 min. Thus, the maximum growth rate in raw liquid whole egg determined for growth curves starting at a concentration of 10^6 CFU/mL (0.743 ± 0.022 log/h) was significantly (p <0.05) higher than for curves starting at a concentration of 10^2 CFU/mL (0.605 ± 0.024 log/h). Significant differences (p <0.05) were also found among the lag values calculated in raw liquid whole egg (4.62 ± 0.228 h *vs* 1.53 ± 0.123 h, for the curves starting at 10^2 and 10^6 CFU/mL, respectively). By contrast, no significant differences were found among the growth parameters μ and μ_{max} calculated in egg pasteurized at 70 °C for 1.5 minutes, regardless of the inoculum dose. The results obtained also indicate that increasing the intensity of the heat treatment applied to liquid whole egg led to a progressive decrease in the lag values and an increase in the μ_{max} for curves starting at 10^2 CFU/mL. Thus, when *S*. Typhimurium ATCC 14028s cells were inoculated (10^2 CFU/mL) into whole egg exposed to 60 °C for 3.5 min, they displayed intermediate values (0.689 ± 0.010 log/h and 2.23 ± 0.099 h for the μ_{max} and λ parameters, respectively) lying between those calculated in raw whole egg and egg pasteurized at 70 °C for 1.5 minutes (significantly different from both of them; p <0.05). However, when inoculated with high doses (10^6 CFU/mL), no significant differences were found in the growth parameters determined for *S*. Typhimurium ATCC 14028s cells, regardless of the heat treatment applied to the liquid whole egg. Neither were significant differences found among the Y_{max} values, regardless of the type of whole egg studied (raw *vs* pasteurized), and regardless of the inoculation dose (Figure 2C). Results obtained in commercial pasteurized liquid whole egg were similar to those obtained in egg pasteurized at 70 °C for 1.5 minutes (data not shown).

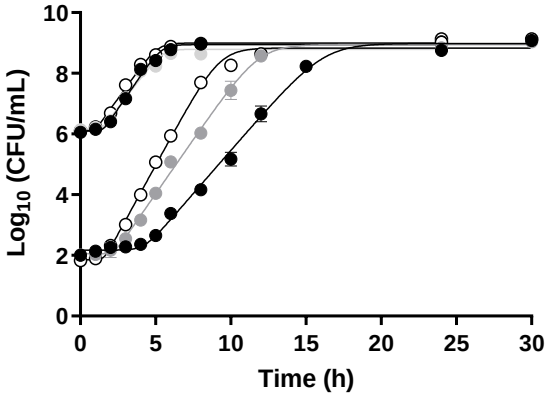

Figure 1. Influence of the intensity of whole liquid egg pasteurization treatment and of the inoculum dose (102 and 106 CFU/mL) on the growth fitness of Salmonella Typhimurium ATCC 14028s cells. Growth curves obtained in raw liquid whole egg (●), pasteurized whole egg at 60 °C 3.5 min (●), and pasteurized whole egg at 70 °C 1.5 min (o) at 102 CFU/mL. Lines correspond to the fit of the Baranyi model to the experimental data. Error bars represent the standard deviation.

These results indicate that increasing the intensity of the heat treatment applied to liquid whole egg leads to a progressive disappearance of the initial dose dependence of *S*. Typhimurium ATCC 14028s growth fitness observed in the raw product.

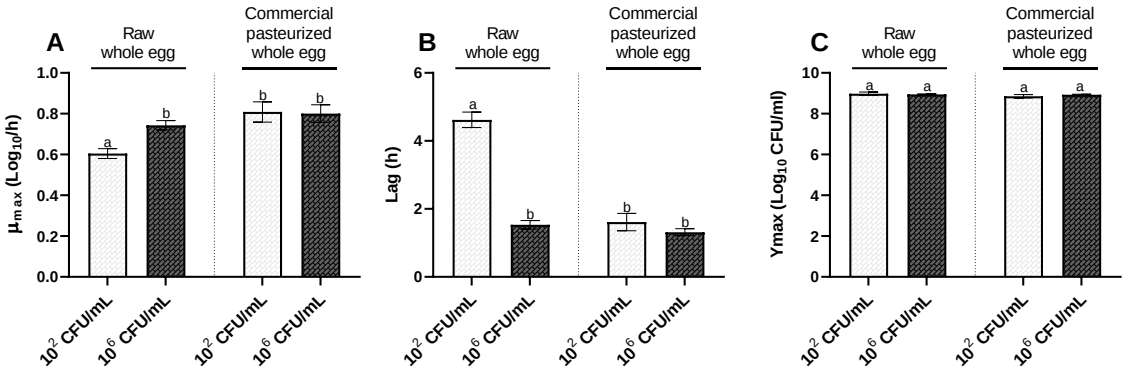

Figure 2. Growth parameters of *Salmonella* Typhimurium ATCC 14028s cells in raw and pasteurized (60 °C/3.5 and 70 °C/1.5 min) liquid whole egg when inoculated at 10^2 and 10^6 CFU/mL. **(a)** μ_{max} (Log_{10}/h); (**b**) lag (h); (**c**) Y_{max} Log_{10} (CFU/mL) values calculated with the Baranyi model. Differences in the lower-case letters indicate statistically significant differences (p <0.05) indicate statistically significant differences (p <0.05) among the parameters determined in each growth medium (raw or pasteurized at 60 °C/3.5 or 70 °C/1.5 min). Differences in the upper-case letters indicate statistically significant differences (p <0.05) among the parameters determined in each growth medium as a function of the starting dose (10^2 vs 10^6 CFU/mL).

3.2. Effect of the supplementation of commercial pasteurized liquid whole egg with egg white proteins with known antimicrobial properties

Since, as suggested in the introduction, the thermal inactivation of egg white proteins with antimicrobial activity might be the cause for the increased growth fitness of *Salmonella* cells in pasteurized liquid whole egg (as compared to raw egg), we designed a series of experiments to verify this hypothesis.

Raw and liquid whole egg pasteurized at different temperatures (60 °C/3.5 min and 70 °C 1.5 min) was supplemented with different antimicrobial egg white proteins (lysozyme, ovotransferrin, and Ex-FABP) at the concentrations at which they are usually present in liquid whole egg (see Material and Methods). The fitness of *S.* Typhimurium ATCC 14028s cells was subsequently determined and compared with that of a raw and pasteurized liquid whole egg without added proteins. Figure 3 shows the effect of supplementation of those proteins in pasteurized liquid whole egg, measured as the increase in the number (in Log_{10}) of cells after 6 hours of incubation at 37 °C ($LogN_6$-$LogN_0$). This length of time was selected because it was the one in which the difference in *Salmonella* counts between raw and pasteurized liquid whole egg (70 °C/1.5 min) was the greatest. Addition of lysozyme to pasteurized liquid whole egg (regardless of thermal treatment) did not cause a significant effect (p >0.05) on the growth fitness of *Salmonella* cells (data not shown). By contrast, the addition of ovotransferrin or Ex-FABP to pasteurized liquid whole egg resulted in a significantly lower increase (p <0.05) compared to control (no protein added) in the number of *S.* Typhimurium ATCC 14028s cells after 6 hours of incubation at 37 °C when eggs were inoculated at the lower dose (10^2 CFU/mL). Thus, for instance, in samples of liquid whole egg pasteurized at 70 °C for 1.5 min with added ovotransferrin or Ex-FABP, the increase in the number of cells after 6 hours of incubation at 37 °C was 2.36 and 0.84 log cycles, respectively, lower than the increase in the same medium without the addition of one of those proteins. Similar results were obtained in egg pasteurized at 60 °C for 3.5 min, although in this case the effect of Ex-FABP was not statistically significant (p =0.1). Results nevertheless also indicated that *Salmonella* growth in raw liquid whole egg was still more restricted than in liquid whole egg pasteurized at 70 °C for 1.5 min supplemented with ovotransferrin or Ex-FABP. We therefore carried out a series of assays

in which those antimicrobial proteins were supplemented together (ovotransferrin + lysozyme, ovotransferrin + Ex-FABP and ovotransferrin + EX-FABP + lysozyme), but results were not significantly different ($p > 0.05$) from those obtained when only ovotransferrin was added (data not shown). Finally, it should be noted that supplementation of any of these proteins did not have any significant effect ($p > 0.05$) on *Salmonella* growth fitness when inoculated at the high dose of 10^6 CFU/mL (data not shown).

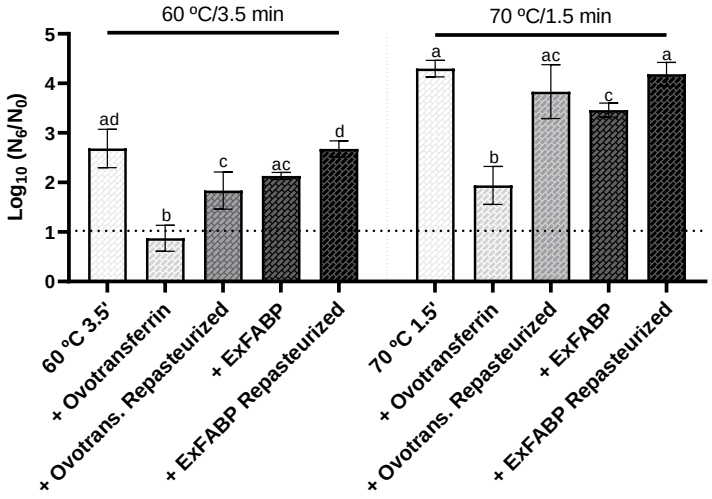

Figure 3. Effect of the supplementation of ovotransferrin (8.80 mg/mL) or Ex-FABP (0.022 mg/mL) to whole liquid egg pasteurized under different conditions (60 °C/3.5 min and 70 °C/1.5 min) inoculated with 10^2 CFU/mL on the growth fitness of *Salmonella* Typhimurium ATCC 14028s. Values correspond to the number of decimal log cycles of growth after 6 hours at 37 °C (Log_{10} N_6/N_0). Figure also includes the values for raw whole liquid egg (Dashed line). Error bars represent the standard deviation, and letters indicate statistically significant differences ($p < 0.05$) among the different conditions assayed for each type of pasteurized whole egg (60 °C/3.5 min and 70 °C/1.5 min).

In order to ascertain the impact of thermal treatments on these proteins' antimicrobial activities, we also evaluated the growth ability of *S.* Typhimurium ATCC 14028s in pasteurized liquid whole egg (at 60 °C/3.5 min and at 70 °C/1.5 min) under each of the assayed conditions, supplemented with the same concentrations of proteins, and subsequently re-pasteurized (applying the same conditions). These additional assays were included because they might help to quantify the impact of thermal treatments of different intensity – and applied in the matrix itself – on the antimicrobial activity of ovotransferrin and Ex-FABP. Under most of the conditions assayed, the antimicrobial effect of both proteins was annulled when they were exposed to the pasteurization treatment, as can be observed in Figure 3. This was true for both proteins treated at 70 °C/1.5 min, and also for Ex-FABP exposed to treatments at 60 °C/3.5 min, but not for ovotransferrin under the latter conditions. Thus, although a heat treatment at 60 °C for 3.5 min did reduce the antimicrobial activity of ovotransferrin (*Salmonella* growth rate increased), growth rate was still lower than in non-supplemented pasteurized egg at the same temperature ($p < 0.05$). Altogether, these results indicate that pasteurization treatments at 70 °C/1.5 min would inactivate ovotransferrin as well as Ex-FABP in liquid whole egg (at least to a high extent), but treatments at 60 °C/1.5 min would not. This strongly suggests that the observed differences in *Salmonella* fitness depending on the intensity of the thermal treatments applied to liquid whole egg might be related, at least to some extent, to the different effect that these thermal treatments would have on those two proteins: mainly, as will be discussed later, on ovotransferrin. Results obtained after carrying out the same experiments in commercial liquid whole egg (re-pasteurization conditions: 70 °C/1.5 min) were similar to those described for whole egg pasteurized at 70 °C for 1.5 (data not shown).

In summary, these supplementation assays indicate that, at the concentrations found in whole liquid egg, ovotransferrin and Ex-FABP, but not lysozyme, would limit *Salmonella* Typhimurium growth rates in liquid whole egg, and that this effect would only be observed when *Salmonella* cells are inoculated at a low dose. In addition, these results strongly suggest that the differences in *Salmonella*

growth rates observed when it grows in raw or in pasteurized liquid whole egg might be linked, at least to some extent, to the thermal inactivation of ovotransferrin and Ex-FABP and consequently, to the existence of differences in iron bioavailability between raw and pasteurized egg.

3.4. Effect of iron and siderophore supplementation on liquid whole egg

In view of the previous results, we studied the influence of the addition of iron to raw and pasteurized liquid whole egg. Supplementation of raw liquid whole egg and whole liquid egg pasteurized at 60 °C for 3.5 min with increasing concentrations of ferric citrate resulted in an increase in the growth rate of *Salmonella* cells when inoculated at low dose (10^2 CFU/mL; Figure 4). Nevertheless, results obtained also indicate that a lower amount of ferric citrate was required for *Salmonella* cells to match their growth rate in egg treated for 1.5 min at 70 °C when inoculated in liquid whole egg pasteurized at 60 °C for 3.5 min (approx. 0.0125 mg/mL) than when inoculated in raw liquid whole egg (>0.025 mg/mL).

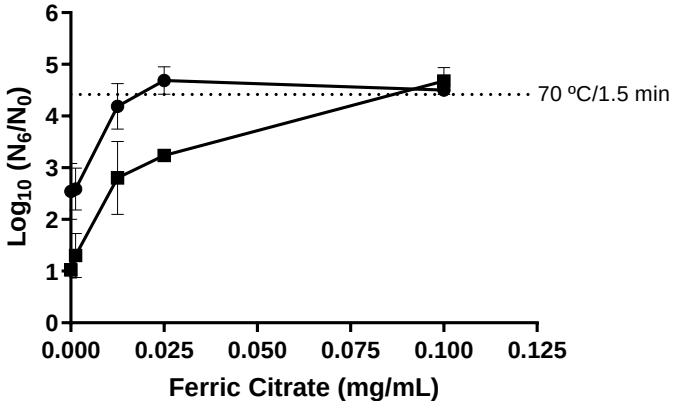

Figure 4. Effect of the supplementation of different concentrations of ferric citrate (from 0.005 mg/mL to 0.1 mg/mL) on the growth fitness of *Salmonella* Typhimurium ATCC 14028s in raw (■) and pasteurized (60 °C/3.5 min; ●) liquid whole egg inoculated with 10^2 CFU/mL. Values correspond to the number of decimal log cycles of growth after 6 hours at 37 °C (Log_{10} N_6/N_0). Dashed line correspond to the values attained after the same incubation time/conditions in liquid whole egg pasteurized at 70 °C for 1.5 minutes (no ferric citrate added). Error bars represent the standard deviation.

On the other hand, no significant change in growth rates (p >0.05) was observed when *Salmonella* cells were inoculated at the high dose (10^6 CFU/mL) in any of the three media, or when ferric citrate was added to liquid whole egg treated for 1.5 min at 70 °C, regardless of the initial dose (data not shown) and even at the highest concentration of ferric citrate tested (0.1 mg/mL; approximately the same amount of iron naturally present in raw whole egg).

These results reinforced the hypothesis that the lower growth rates of *Salmonella* cells in raw liquid whole egg when inoculated at the lower doses would be related to iron bioavailability. Thus, since ovotransferrin and Ex-FABP seemed to be playing a significant role in this phenomenon, we proceeded to study the influence of the addition of different concentrations of two siderophores, Enterobactin and Salmochelin, to raw and pasteurized liquid whole egg.

Figure 5 shows the effect of supplementation of different concentrations of Salmochelin and Enterobactin (from 0.05 ng/mL to 5.0 μg/mL) on the growth fitness of *Salmonella* Typhimurium ATCC 14028s in raw liquid whole egg as well as in liquid whole egg pasteurized at 60 °C for 3.5 min inoculated at 10^2 CFU/mL. For both types of liquid whole egg, increasing the concentration of either Enterobactin or Salmochelin beyond a certain threshold resulted in an increased growth rate of the *Salmonella* cells. It should be noted that in both cases this threshold was lower for Salmochelin than for Enterobactin and that, as described for ferric citrate, the siderophore concentration required for *Salmonella* cells to reach a growth rate similar to that which they would display in egg pasteurized at 70 °C for 1.5 min was lower

when *S.* Typhimurium ATCC 14028s cells were grown in egg pasteurized at 60 °C for 3.5 min than when grown in raw egg. As likewise described for ferric citrate, no effect was observed when siderophores were supplemented in either raw egg or in egg pasteurized at 70 °C for 1.5 min at the high dose, or in egg pasteurized at 70 °C for 1.5 min at the low dose (data not shown). Similarly, addition of ferric citrate, Enterobactin, or Salmochelin to commercial liquid egg did not significantly change the observed growth rates, regardless of the initial number of *Salmonella* cells inoculated (data not shown).

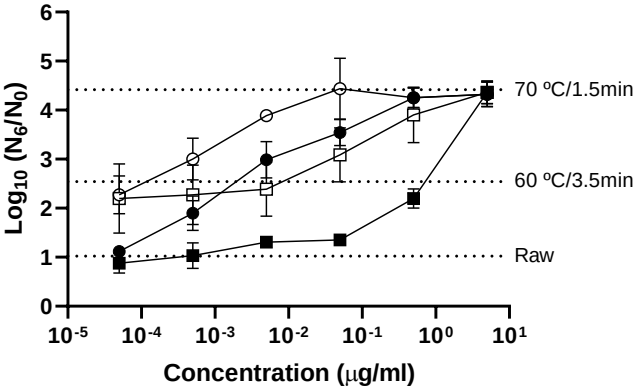

Figure 5. Effect of the supplementation of different concentrations of Salmochelin (circles: ●, ○) and Enterobactin (squares; ■, □) (from 0.05 ng/mL to 5.0 μg/mL) on the growth fitness of *Salmonella* Typhimurium ATCC 14028s in raw (filled symbols; ●, ■) and pasteurized (60 °C/3.5 min; empty symbols; ○, ◙) liquid whole egg inoculated with 10^2 CFU/mL. Values correspond to the number of decimal log cycles of growth after 6 hours at 37 °C (Log_{10} N_6/N_0). Dashed lines corresponds to the values attained after the same incubation time/conditions in raw and pasteurized liquid whole egg (60 °C/3.5 min and 70 °C/1.5 min) with no Salmochelin/Enterobactin added. Error bars represent the standard deviation.

3.5. Effects of deletions in iron-uptake-related genes on the fitness (growth rate) of Salmonella Typhimurium in liquid whole egg

In view of the results previously obtained, we selected 8 strains (*ΔentC, Δfes, ΔfeoB, ΔfhuC, ΔiroN, ΔmntH*, *ΔsitC* and *ΔzupT*) of *S.* Typhimurium ATCC 14028s with deletions in genes coding for proteins involved in different iron uptake systems, determined their growth rate in raw and pasteurized liquid whole egg starting at two different initial doses (10^2 CFU/mL and 10^6 CFU/mL), and compared them to that of the parental (wild type) strain.

The biosynthesis of the siderophore molecule Enterobactin, and thus the synthesis of Salmochelin molecule, is regulated by six enzymes (EntA-EntF) (Raymond et al., 2003; Rusnak et al., 1991). Therefore, deletion of *entC* makes *Salmonella* cells unable to produce Enterobactin or Salmochelin. The *iroN* gene encodes the outer membrane receptor of Fe^{3+} bound to Salmochelin; if it is deleted, iron uptake by Salmochelin will be inhibited, or at least reduced (Hantke et al., 2003). The Enterobactin esterase Fes is an enzyme that cleaves iron-free Enterobactin, since this mutant uses Salmochelin as sole siderophore. In addition to these catecholate siderophores, *Salmonella* has ferric hydroxamate-type siderophores. The protein fhuC forms a complex with the other operon proteins, which, in turn, mediates the translocation of ferrichrome from the periplasm into the cytoplasm (Mademidis and Köster, 1998). Under anaerobic or reducing conditions, Fe^{2+} is the predominant form and is acquired via Fe^{2+} uptake systems (Ratledge and Dover, 2000), such as the FeoABC and MntH transporters. The *feoB* gene encodes an ATP-driven high-affinity transporter that pumps Fe^{2+} across the cytoplasmic membrane (Kammler et al., 1993; Lau et al., 2016); MntH is a divalent cation transport system with high affinity for Mn^{2+} and other divalent metal ions, including ferrous iron (Zaharik et al., 2004). Other complementary systems are the *sitABCD* operon, induced under iron-deficient conditions, which encodes a periplasmic binding protein-dependent ABC transport system that is specific for metal ions (Janakiraman and Slauch, 2000), and the ZupT transporter, a permease that preferentially allows entry of zinc, although the ability to uptake manganese, copper and iron has also been demonstrated (Cerasi et al., 2014).

As can be observed in Figure 6, the *ΔiroN* and *ΔentC* strains displayed lower growth rates in raw liquid whole egg (p <0.05) than the parental strain in raw liquid egg, and also displayed longer lag phases, although in this case only when egg was inoculated with 10^2 CFU/mL. By contrast, no significant differences (p >0.05) among the growth parameters determined in raw egg for the other mutant strains and the parental one were observed (data not shown). It should also be noted that no significant differences were found in the Y_{max} values regardless of strain, initial dose, and/or type of liquid whole egg. On the other hand, no significant differences (p >0.05) between the growth parameters determined for any of the tested strains and the parental strain were observed when grown in egg pasteurized at 70 °C for 1.5 min, regardless of the initial dose (data not shown).

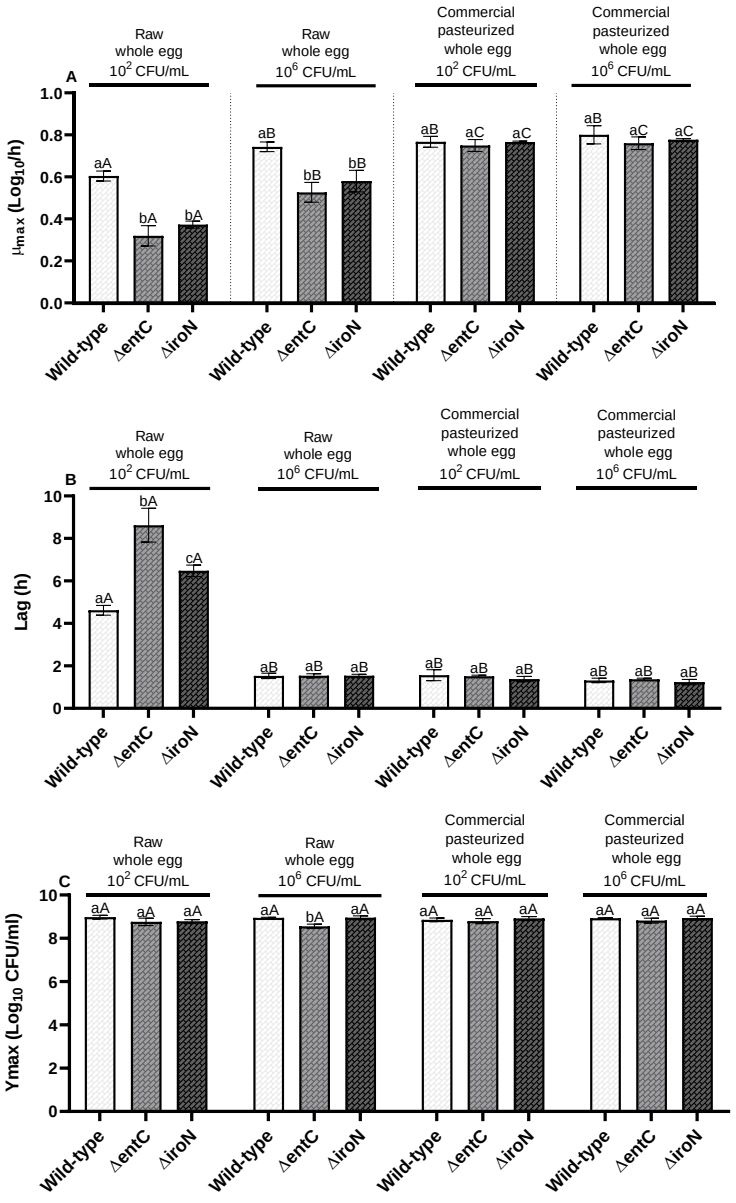

Figure 6. Growth parameters of *Salmonella* Typhimurium ATCC 14028s (Wild-type) cells and its isogenic mutants *ΔentC* and *ΔiroN*, in raw and pasteurized liquid whole egg (70 °C/1.5 min) and when inoculated at different doses (10^2 CFU/mL and 10^6 CFU/mL). (A) μ_{max} ($\log_{10}$/h), (B) lag (h) and (C) Y_{max} Log_{10} (CFU/mL) values obtained after the fit of the growth curves to the Baranyi model. Error bars represent the standard deviation. Differences in the lower-case letters indicate statistically significant differences (p <0.05) between strains grown on the same media and conditions (starting dose). Differences in the upper-case letters indicate statistically significant differences (p <0.05) among growth conditions (raw vs. pasteurized and initial dose) for each strain.

In the light of these results, the fitness of those strains which displayed a reduced growth rate in raw liquid whole egg – i.e., those with deletions in genes related to Salmochelin (*ΔiroN*) or Salmochelin and Enterobactin (*ΔentC)* uptake/synthesis – was also studied in liquid whole egg pasteurized under different conditions, and with or without the addition of Ex-FABP and ovotransferrin (Figure 7). Results obtained indicate that 1) deletion of *iroN* only affected *Salmonella* growth in those conditions in which Ex-FABP was naturally present (raw) or added to the pasteurized egg, and 2) deletion of *entC* caused a decrease in *Salmonella* growth rates under all the conditions assayed, but when grown in egg pasteurized at 60 °C for 3.5 min supplemented with ovotransferrin or in egg pasteurized at 70 °C for 1.5 min.

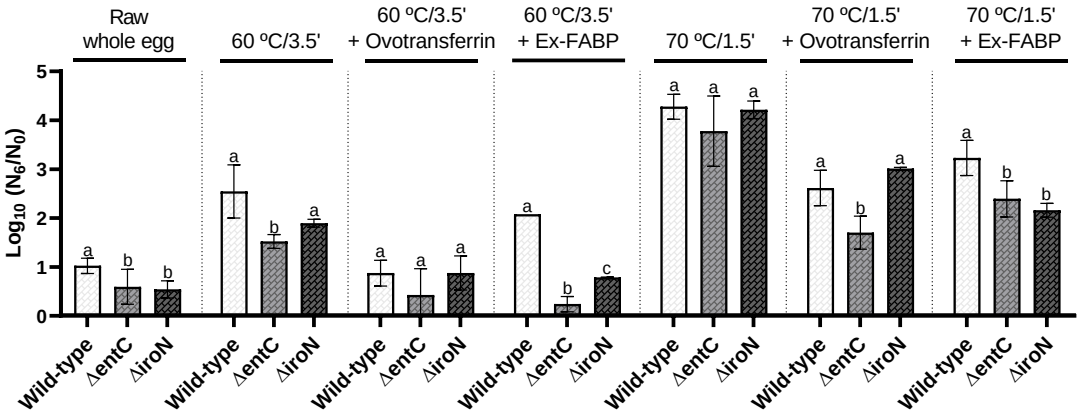

Figure 7. Effect of the supplementation of ovotransferrin (8.80 mg/mL) or Ex-FABP (0.022 mg/mL) to whole liquid egg pasteurized under different conditions (60 °C/3.5 min and 70 °C/1.5 min) inoculated with 10^2 CFU/mL on the growth fitness *Salmonella* Typhimurium ATCC 14028s (Wild-type) cells and its isogenic mutants *ΔentC* and *ΔiroN*. Values correspond to the number of decimal log cycles of growth after 6 hours at 37 °C (Log_{10} N_6/N_0). Figure also includes the values for raw whole liquid egg. Error bars represent the standard deviation, and letters indicate statistically significant differences ($p < 0.05$) among the different strains for each growth medium.

These results will be discussed in detail below, but they reinforce the hypothesis of the existence of a relationship between iron bioavailability and the dependence of *Salmonella* growth rates in liquid whole egg on its thermal history and the dose. They likewise highlight the relevant role played by egg white antimicrobial proteins (ovotransferrin and Ex-FABP), on the one hand, and *Salmonella* siderophores (Salmochelin and Enterobactin), on the other, in the growth of *Salmonella* in liquid whole egg.

3.6. Effect of supplementation with spent medium on the growth rates of Salmonella Typhimurium in liquid whole egg.

In order to obtain further insight into the dependence of *Salmonella* growth rates on initial dose in liquid whole egg, we performed a set of experiments in which different strains (ATCC 14028s and its isogenic mutants *Δfes* and *ΔiroN*) were grown in raw whole egg supplemented with filtered and dialyzed M9 spent medium (prepared as described in Materials and Methods) from parental, *ΔentC,* and *ΔiroB* cultures. Previous experiments showed that the filtered/dialyzed M9 spent medium obtained from *S.* Typhimurium ATCC 14028s increased the growth rates of this same strain inoculated to raw whole egg at a dose of 10^2 CFU/mL to the same extent as the non-filtered/dialyzed one, and that the same was true when the filtered/dialyzed spent medium was obtained from *S.* Typhimurium ATCC 14028s cells grown in raw liquid whole egg (data not shown). Therefore, we carried out all the experiments with filtered/dialyzed M9 spent medium. These experiments also revealed that supplementation with spent medium to samples inoculated at 10^6 CFU/mL did not significantly ($p > 0.05$) modify *S.* Typhimurium ATCC 14028s growth rates.

As can be observed in Figure 8, supplementation of raw whole egg with M9 spent medium obtained from *S.* Typhimurium ATCC 14028s cultures increased the growth rates of the three strains under study when inoculated at 10^2 CFU/mL. Thus, supplementation with that spent medium resulted, after 6 hours, in an increase in 2.2, 2.4 and 1.8 Log_{10} cycles of growth for strain *S.* Typhimurium ATCC 14028s and its isogenic mutants *Δfes* and *ΔiroN*, respectively. This increase was significantly (p <0.05) higher than that observed when the spent medium had been obtained from a *ΔiroB* culture, which cannot synthesize Salmochelin, and also when it had been obtained from a *ΔentC* culture, which is unable to synthesize either Salmochelin or Enterobactin. In addition, no significant differences (p >0.05) were found when strains where supplemented with spent medium only lacking Salmochelin (from *ΔiroB* cultures) or also lacking Enterobactin (from *ΔentC* cultures). However, the growth of the *Salmonella Δfes* and *ΔiroN* strains in raw whole egg supplemented with *ΔentC* and *ΔiroB* spent media was still significantly higher (p <0.05) than without supplementation.

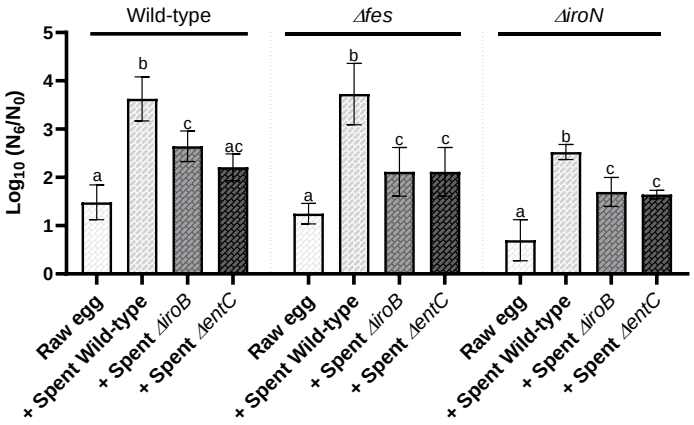

Figure 8. Effect of the supplementation of filtered and dialyzed M9 spent medium (see text) obtained from *Salmonella* Typhimurium ATCC 14028s (Wild-type), *ΔiroB* and *ΔentC* mutants to raw whole liquid egg inoculated with 10^2 CFU/mL on the fitness *Salmonella* Typhimurium ATCC 14028s (wild-type) cells and its isogenic mutants *Δfes* and *ΔiroN*. Values correspond to the number of decimal log cycles of growth after 6 hours at 37 °C (Log_{10} N_6/N_0). Figure also includes the values for raw whole liquid egg. Error bars represent the standard deviation, and letters indicate statistically significant differences (p <0.05) among the different growth conditions for each strain.

4. Discussion

Iron restriction has long been considered the major factor limiting bacterial growth in egg (Garibaldi, 1970; Schade and Caroline, 1944). Thus, although the concentration of iron in egg has been estimated to be between 3.6 to 18 μM, it is assumed that there would be no free iron in egg white, since it would be chelated by ovotransferrin. Ovotransferrin is a monomeric glycoprotein, a member of the transferrin family. It is composed of two homologous lobes: each lobe has an iron-binding site, the amino-terminal lobe (N-lobe) and the carboxyl-terminal lobe (C-lobe). Therefore, one mole of ovotransferrin is able to bind two moles of iron (Baron et al., 2016; Legros et al., 2021). It is thereby believed that main antimicrobial mechanism of egg against *Salmonella* is this chelator effect of ovotransferrin (Baron et al., 1997). It has been recently demonstrated that one of the three lipocalins identified in egg white, the Extracellular Fatty Acid-Binding Protein (Ex-FABP), is able to sequester some siderophores (molecules that bind and transport iron in microorganisms) (Guérin-Dubiard et al., 2006; Julien et al., 2020). Thus, Ex-FABP would be able to sequester Enterobactin, but would display no activity against Salmochelin (Julien et al., 2020). Therefore, Ex-FABP would also limit iron bioavailability through its ability to bind to siderophores.

Our results are consistent with this assumption, since they indicate that while lysozyme supplementation (at a concentration similar to that present in raw whole egg) did not have any impact on *Salmonella* growth rates in pasteurized liquid egg at 70 °C for 1.5 min, supplementation of

ovotransferrin or EX-FABP (at their concentrations found in raw egg) to egg pasteurized under the same conditions did slow down *Salmonella* growth, at least when cells were inoculated at the lower dose (10^2 CFU/mL), whereby the effect of ovotransferrin was higher than that of Ex-FABP. In this sense, it can be assumed that none of the components present in liquid whole egg pasteurized at 70 °C for 1.5 min (as well as in the commercial liquid whole egg studied herein) are limiting *Salmonella* growth, since the growth rates reported here are similar (no significant differences; $p > 0.05$) to those obtained in a rich medium such as TSB-YE (Guillén et al., 2021). This would be either because their concentration is too low, or because they are denatured/inactivated. We should take into account that the same medium might still be limiting the growth of other microorganisms and/or other conditions (e.g. at a different temperature), but, as far as this investigation is concerned, the growth rate of *Salmonella* in liquid whole egg pasteurized at 70 °C for 1.5 min would be considered from here on as the maximum growth rate *Salmonella* can achieve under the conditions studied herein, and will be used as a reference. Furthermore, although it can be argued that the antimicrobial effect observed for ovotransferrin and Ex-FABP might be due to the presence of a higher (up to twofold) concentration of both proteins after supplementation (those naturally present + those supplemented), our results demonstrate that the application of a heat treatment of 1.5 min at 70 °C to both proteins and in liquid whole egg does suppress its antimicrobial activity (see below); therefore, our affirmation can be considered valid. Finally, these results also indicate that lysozyme activity would not be the cause of the differences in *Salmonella* growth rates observed between raw and pasteurized liquid egg, or between low and high initial doses in raw whole egg.

To overcome this limitation in iron bioavailability which is not exclusive to egg white (Dostal et al., 2014), *Salmonella* has developed different systems for the acquisition of iron from the host environment and from host-chelating proteins. These systems have been excellently reviewed in Andrews et al. (2003) and Wellawa et al. (2020). Basically, they can be classified into three groups: ferrous iron transport systems (which include the FeoABC transporter), metal-type ABC transporters (mainly the SitABCD, MntH, and ZupT transporters), and the ferric iron uptake system via siderophores. The main *Salmonella* siderophores are Salmochelins, Enterobactins and, to a minor extent, ferrichromes, all of which are characterized by their high affinity to iron. Thus, while the affinity constant (with iron) of ovotransferrin is in the range between 10^{14} and 10^{18} M^{-1} (Lin et al., 1994), most of the siderophores secreted by *Salmonella* have affinity constants between 10^{30} and 10^{52} M^{-1} (Andrews et al., 2003). It has been demonstrated that all *Salmonella* serovars produce the catecholate siderophore Enterobactin as well as the C-glucosylated Enterobactin derivative Salmochelin (Müller et al., 2009; Raymond et al., 2003) and that siderophore production genes are overexpressed in egg white (Clavijo et al., 2006; Huang et al., 2019). The relevance of those genes (specifically in egg) is associated with the fact that Ex-FABP can only sequester ferric-Enterobactin, and is unable to bind Salmochelin (Correnti et al., 2011). Thus, as observed by Julien et al., 2020, the addition of Ex-FABP at the concentration found in egg white (5 μM) in M9 medium caused a modest impact on growth; in contrast, the *ΔiroBC* mutant, which relied on Enterobactin as the sole siderophore, showed a slower growth rate and yield (Julien et al., 2020). Our results also indicate that Salmochelin plays a key role in *Salmonella* growth in egg since, among all the mutant strains tested (each of them with an impaired ability to use one of *Salmonella*'s major iron import systems), only those mutant strains (*ΔiroN* and *ΔentC*) not able to use Salmochelin for importing iron displayed reduced growth rates in raw egg. This view is likewise supported by our results obtained from supplementation assays with Salmochelin and Enterobactin, which demonstrated that the concentration of Salmochelin required by *Salmonella* to overcome the iron limitation imposed by egg white proteins was lower than that of Enterobactin. Nevertheless, those two mutant strains were capable of growing in raw liquid whole egg, and of reaching the same yields, although more slowly. This indicates that other iron import systems can compensate for a lack of Salmochelin or Salmochelin + Enterobactin synthesis/import and sustain *Salmonella* growth in raw whole egg. Conversely, they also indicate that the lack of any other individual iron import system would be entirely compensated (no effect on growth rates in raw whole egg) by *Salmonella* cells through the use of other ones.

Returning to our study's main objective, which was to study the mechanisms underlying the differences in growth rate of *Salmonella* cells in liquid whole egg as a function of the initial inoculum dose (in raw egg) and the egg's thermal history, our results strongly suggest that both phenomena would be linked to differences in iron bioavailability, as shall be discussed below.

Regarding the cause of the growth rate differences in raw whole egg depending on the inoculum dose, our supplementation experiments with spent M9 medium strongly suggest that the amount of siderophores released to the medium would substantially contribute to explain the differences in growth rate as a function of the initial dose in raw egg, since: 1) spent medium lacking siderophores (generated by a *ΔentC* mutant strain) had a significantly lower growth-promoting effect in raw whole egg and, 2) spent medium filtration + dialyzation assays indicate that the molecules responsible for the observed effect would have an approximate molecular weight between 100 and 3000 Da, consistent with the molecular weight of the siderophores Salmochelin (1016 Da for S4) and Enterobactin (669 Da) (Bister et al., 2004). They also indicate, once more, that Salmochelin would play a predominant role, because, in addition to all we have indicated above, supplementation with the spent medium generated by the parental strain to *Salmonella Δfes* cells (unable to use Enterobactin-bounded Fe) raised growth rates to the same extent as those of the parental strain, and supplementation of the parental strain with spent medium generated by a *ΔiroB* strain (unable to glycosylate Enterobactin, and therefore unable to synthesize Salmochelin) only led to a limited increase in *Salmonella* growth rates. Furthermore, supplementation with a spent medium lacking Salmochelin as well as Enterobactin (generated by a *ΔentC* mutant strain) induced a similar effect than that of a spent medium only containing Enterobactin. In any case, our results also seem to indicate that in *Salmonella* spent medium there were molecules other than those two siderophores, which were capable of accelerating *Salmonella* growth in egg. Further work will be required in order to fully elucidate this point.

Therefore, and assuming that siderophore synthesis and uptake would be determining the growth rate of *Salmonella* cells in raw liquid whole egg, the results obtained in this study might be explained on the basis of the theory/model proposed by Scholz and Geenberger (2015). They observed a phenomenon quite similar to the one observed in our study: in their case, the growth of *E. coli* in a low-iron medium supplemented with the iron chelator transferrin was slower when cells were inoculated at low cell densities than at high ones. They also observed that when *Escherichia coli* and an isogenic Enterobactin synthesis mutant (*ΔentF*) were inoculated in a low-iron medium at high cell densities, the *ΔentF* mutant could compete equally well with the wild type, but that at low cell densities it could not. This led them to propose a model in which at least a certain amount of Enterobactin would remain associated with the cells that produce it, enabling iron acquisition even at very low cell density; whereby Enterobactin that was not retained by producing cells at low density would be lost to dilution. Conversely, at high cell densities, cell-free Enterobactin could accumulate and be shared by all cells in the group, which would lead to an increase in overall fitness (growth rates). Our scenario is admittedly somewhat more complex, with Salmochelin and Enterobactin as the siderophores used differently depending on the amount of inoculum, and with ovotransferrin and Ex-FABP limiting iron bioavailability; nevertheless, the fact that the growth-promoting effect of spent medium was observed when it was obtained from M9 cultures with a high amount of cells (10^6 CFU/mL), and not from cultures having achieved a cell density of only 10^2 CFU/mL, seems to indicate that the model proposed by Scholz and Geenberger (2015) would also be valid in explaining the results we obtained for the growth of *S.* Typhimurium ATCC 14028s in liquid raw egg. In any case, the *ΔiroN* and *ΔentC* mutants grew even more rapidly in raw whole egg when inoculated at 10^6 CFU/mL than at 10^2 CFU/ml, thereby indicating that other factors/molecules might also be at least partially responsible for the initial dose dependency of *Salmonella* growth in raw liquid whole egg. Further work will be required in other to fully elucidate this point.

On the other hand, and with regard to the influence of pasteurization on *Salmonella* growth rates in liquid whole egg, we wish to point out that the classic pasteurization treatments applied in the industry, 1-10 min at 60-72 °C, are limited due to the sensitivity of egg white proteins to heat treatments, which can lead to egg coagulation. Depending on a treatment's intensity, it can denature egg white

proteins with antimicrobial properties such as ovotransferrin and lysozyme (Baron et al., 2016), as was already suggested as the potential cause of the higher growth fitness of *Salmonella* cells in pasteurized liquid whole egg as compared to raw egg (Guillén et al., 2021). Although we have only estimated the denaturation/inactivation of ovotransferrin and Ex-FABP indirectly through the study of their antimicrobial activity and have not calculated the percentage of denaturation/inactivation, our results indicate that both compounds would be denatured by heat (at least when treated at 70 °C for 1.5 min). This is consistent with previously published data reporting denaturation temperatures for ovotransferrin in the range between 60 and 75 °C (Li-Chan et al., 1995), and the melting temperature predicted for Ex-FABP using the SCooP algorithm (Pucci et al., 2017), which is in the same range (T_m = 67.9 °C).

In any case, our results also indicate that *Salmonella* growth in pasteurized liquid egg supplemented with ovotransferrin or Ex-FABP, or both, was still slightly faster than in raw egg. This suggests that there would be other components with antimicrobial activity that would result affected/denatured upon exposure of whole liquid egg to pasteurization conditions. However, other potential explanations cannot be ruled out, such as the existence of significant differences in antimicrobial activity between recombinant *Coturnix japonica* Ex-FABP (the one used for supplementation assays) and *Gallus gallus* Ex-FABP, or between native and commercially available ovotransferrin. Our results nevertheless clearly demonstrate that the addition of iron or siderophores to raw egg is sufficient for *Salmonella* cells to reach their maximum growth rates in whole liquid egg, indicating that if there are other proteins/components contributing to the differences in *Salmonella* growth rates in raw and pasteurized liquid whole egg, they would also be related to iron bioavailability. Given the functions of ovotransferrin and Ex-FABP (one as an iron scavenger and the second one as a siderophore sequester), the effect of their simultaneous addition (at the same concentrations as above) to liquid whole egg pasteurized at 70 °C for 1.5 min would be expected to be additive, but we did not observe that effect. Further work will be required to elucidate this point, taking into account that the effect of the addition of Ex-FABP alone was relatively small (even non-significant in egg pasteurized at 60 °C), and that addition of ovotransferrin alone led to *Salmonella* growth rates very similar to those in raw egg, which, together with the limitations of the plate count technique (it is estimated that the standard deviation of three replicates can account for considerably more than 10% of the mean value (Cebrián et al., 2015)) might also explain the results obtained.

Regarding the effect of the different pasteurization treatments on the antimicrobial activity of these proteins (ovotransferrin and Ex-FABP), results obtained for egg pasteurized at 60 °C (3.5 min) should be viewed with care, due to methodological reasons: mainly because of the low difference in growth rates between raw egg and egg pasteurized at 60 °C for 3.5 min (less than 12.27%), and also in view of the high variability of the plate count technique (see above). In any case, our results strongly suggest that a 60 °C/3.5 min treatment would only denature a fraction of ovotransferrin, since the growth rate of *Salmonella* cells in pasteurized egg (60 °C/3.5 min) supplemented with ovotransferrin exposed to the same treatment conditions did not reach the growth rate in non-supplemented pasteurized (60 °C/3.5 min) whole egg, thereby implying that ovotransferrin still retained a certain degree of antimicrobial activity. This is consistent with the results reported by Baron et al. (2003), who observed that the loss of bacteriostatic activity of transferrin depended on the intensity of the treatment, although the ones they studied were more intense (15 days at 67 or 75 °C). This would imply that the amount of active ovotransferrin might be one of the causes, if not the major one, for the observed differences in *Salmonella* growth rates between raw and pasteurized liquid whole egg at different temperatures. On the other hand, results obtained in our study suggest that a heat treatment of 60 °C for 3.5 min would significantly affect Ex-FABP antimicrobial activity, yet they also indicate that the effect of the supplementation of Ex-FABP on egg pasteurized at 60 °C for 3.5 min was non-significant (p >0.05). These contradictory results might be explained on the basis of the methodological limitations discussed above, but the fact that the concentration of Salmochelin required by *Salmonella* to overcome the iron limitation imposed by egg white proteins in whole egg pasteurized at 60 °C for 3.5 min was lower than that of Enterobactin strongly suggests that Ex-FABP was not denatured after treatments at 60 °C for 3.5 min. Alternatively,

it can be hypothesized that egg might contain other proteins (still not reported) capable of sequestering Enterobactin. Further work will be required to clarify the effect of these low-intensity pasteurization treatments on Ex-FABP activity. Nevertheless, this is, to the best of our knowledge, the first time that the effect of heat treatments on the antimicrobial activity of Ex-FABP has been explored; from our results it can be clearly established that at least pasteurization treatments at 70 °C for 1.5 do affect its antimicrobial activity against *S.* Typhimurium ATCC 14028s.

Altogether, these results strongly suggest that denaturation of proteins limiting iron bioavailability (including ovotransferrin and Ex-FABP) would be the cause of the faster growth of *Salmonella* cells in pasteurized whole egg, and that the different degree of denaturation they would suffer after pasteurization treatments of different intensity would explain the differences in growth observed in liquid whole egg pasteurized under different conditions, as pointed out above. Regarding the first point, it should be noted that although ovotransferrin has an additional antibacterial activity independent of its iron-restricting activity (Aguilera et al., 2003; Ellison et al., 1988), Baron et al. (2003) already reported that, at least in the range of temperatures they studied (67 to 75 °C, which is close to ours), the loss of bacteriostatic activity attributable to thermal denaturation of ovotransferrin was due to a reduction of its iron-chelating activity. This provides further support for our hypothesis that the increased growth rate obtained in pasteurized egg would mainly be linked to an increase in iron bioavailability. Regarding the second point, our supplementation assays with iron and siderophores also demonstrated that iron bioavailability progressively increases as the intensity of the treatment is raised, since the maximum growth rates in liquid whole egg were achieved in egg pasteurized at 70 °C for 1.5 min without requiring the addition of any of those compounds, and the concentrations required in egg pasteurized at 60 °C for 3.5 min were lower than in raw egg.

Furthermore, on the basis of all that has been discussed above, the following model would simultaneously explain the all results here reported:

Iron bioavailability would be limited in raw liquid whole egg mainly due to the presence of ovotransferrin and Ex-FAPB. In this medium, *Salmonella* cells require the use of siderophores (especially Salmochelin) for iron uptake and in order to reach their maximum growth potential (rate). When *Salmonella* cells are present at low cell density in raw whole egg, those siderophores have a privative effect, and growth would be slow. However, if they are present/inoculated at a sufficiently high density, siderophores are released to the medium; the antimicrobial (iron limiting) systems present in raw liquid whole egg would be overcome, and *Salmonella* would grow at their maximum growth rates (in this case, raw liquid whole egg would not be a growth-limiting medium). Application of heat treatments of increasing intensity would cause a progressive denaturation of those iron-restricting proteins (mainly of ovotransferrin), which would allow *Salmonella* cells to uptake iron by other systems, leading to the disappearance of the initial dose dependence of *Salmonella* growth rates.

5. Conclusions

In summary, our results strongly suggest that iron bioavailability determines the fitness (growth rates) of *Salmonella* cells in liquid whole egg. Thus, the higher the intensity of the thermal treatment applied to the liquid egg, the more iron will be available, a phenomenon that would be linked to the denaturation of iron and/or siderophore-binding egg proteins. On the other hand, further work is still required to fully elucidate why lower *Salmonella* initial doses lead to lower growth rates in raw whole egg, but this might be related to the different use (private vs shared) of siderophores on the part of *Salmonella,* depending on the number of cells present in the medium. The present study contributes to a better understanding of the physiology of *Salmonellae,* as well as of the effect of thermal treatments on food products.

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Manuscrito VIII/Manuscript VIII

Modelling the low temperature growth boundaries of *Salmonella* Enteritidis in raw and pasteurized egg yolk, egg white and liquid whole egg: influence of the initial dose

Silvia Guillén, Pilar Mañas, Ignacio Álvarez and Guillermo Cebrián

Modelling the low temperature growth boundaries of *Salmonella* Enteritidis in raw and pasteurized egg yolk, egg white and liquid whole egg: influence of the initial dose

Silvia Guillén[1], Pilar Mañas[1], Ignacio Álvarez[1] and Guillermo Cebrián[1]*

[1] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón—IA2—(Universidad de Zaragoza-CITA), 50013, Zaragoza, Spain;
* Correspondence: guiceb@unizar.es (G.C); Tel.: +34-876554127

Abstract: *Salmonella* is the most frequently reported cause of foodborne outbreaks with known origin in Europe, with eggs and egg products standing out and the most frequent food source (when it was known). The growth and survival of *Salmonella* in eggs and egg products have been extensively studied and, recently, it has been reported that factors such as the initial dose and thermal history (raw or pasteurized) of the egg product can also influence its growth capability in them. Therefore, the objective of this study was to define the boundary zones of the growth/no growth domain of *Salmonella* Enteritidis (4 strains) as a function of temperature (low temperature boundary) and dose in different egg products. A series of polynomial logistic regression equations were successfully built, allowing the study the interactions between these factors on the probability of growth of *S*. Enteritidis in these products. Results obtained indicate that the minimum growth temperatures of *Salmonella* Enteritidis are higher in egg white (9.47 - 18.25 °C) than in egg yolk (7.10 - 7.75 °C) and whole liquid egg (7.15 - 7.78 °C). Results obtained also demonstrate that in some products, raw whole liquid egg and raw and pasteurized egg white, the minimum growth temperature of *Salmonella* Enteritidis cells does depend on the initial dose. Similarly, the previous thermal history of the egg product only influenced the minimum growth temperature in some of them. On the other hand, wide differences in the minimum growth temperatures among strains were observed in some products (up to approx. 6 °C in egg white). Our experimental approach has allowed us to provide a more accurate prediction of *Salmonella* minimum growth temperatures in egg products by taking into account additional factors (dose and thermal history) while also providing a quantification of the intra-specific variability. This would be of the highest relevance for improving the safety of egg products.

Keywords: *Salmonella*; egg; inoculum dose; growth/no growth; logistic regression

1. Introduction

Salmonella is the most frequently reported cause of foodborne outbreaks with known origin in Europe, and in 2019 44.0% of the human cases of salmonellosis associated with a known food source in this region were linked with eggs and egg products (EFSA, 2021). Among the more than 2,500 *Salmonella* serovars, Enteritidis serovar is the most commonly isolated from eggs and egg products, so the growth and survival of this serovar in eggs and egg products have been extensively studied (De Vylder et al., 2013; Gantois et al., 2009). Thus, it is well known that there are large differences in the growth capacity of *Salmonella* depending on the egg fraction (yolk vs. white), growth temperature and other factors. One of the most important environmental factors influencing bacterial population growth is the

temperature. *Salmonella* is capable to grow in a wide range of temperatures, ranging from 5 to 47 °C with an optimum of 35 to 42 °C (D´Aoust, 1989). In egg products the minimum growth temperature of *S*. Enteritidis is around 6–8°C (Kang et al., 2021; Kim et al., 2018; Whiting and Buchanan, 1997). The egg fraction also influences the growth rates and yields of *Salmonella*, being much higher in egg yolk than in egg white (Guillén et al., 2021; Kim et al., 2018; Messens et al., 2004; Moon et al., 2016), what has been attributed to the particular composition and physico-chemical characteristic of the later, which is particularly efficient inhibiting/slowering microbial growth (Baron et al., 1997, 2016). Recently it has been reported that other factors such as the initial dose and thermal history (raw or pasteurized) also influence the growth capability of *Salmonella* in egg and egg products (Guillén et al., 2020a, 2021; Kang et al., 2021). These phenomena seem to be related to the effect of heat treatments on the antimicrobial activity of some egg white proteins (such as ovotransferrin and lysozyme) and on the dose-dependent ability of *Salmonella* cells to uptake iron (Baron et al., 2016; Lechevalier et al., 2017; Scholz and Greenberg, 2015).

Kinetic growth models of *Salmonella* have been developed to predict the growth in egg products in order to establish the optimal temperature and time for their preservation and distribution (Kang et al., 2021; Kim et al., 2018; Li et al., 2017; Singh et al., 2011). In addition to these predictive kinetic models, probabilistic growth/no growth (G/NG) interface models have been obtained as they are more appropriate for defining factor combinations and because they provide more detailed information on microbial growth limits –which is also required for defining the range of applicability of kinetic models. Several growth/no growth models have been developed for *Salmonella* (Basti and Razavilar, 2004; Koutsoumanis et al., 2004; Lanciotti et al., 2001; Pin et al., 2011). Thus, models defining *Salmonella* growth boundaries as a function of the major environmental factors influencing microbial growth, such as temperature, pH and a_w have been developed. However, there are still many other factors potentially affecting/defining *Salmonellae* growth boundaries, such as the influence of the initial number of cells, the thermal history of the food product (e.g. egg), or the physiological state of the cells that have only been scarcely, if ever, investigated. In fact, these factors were already pointed out as future research needs to improve current *Salmonella* growth models by Carrasco and co-workers (Carrasco et al., 2012). In addition, it should be noted that most of the available G/NG models for *Salmonella* were developed using data obtained in laboratory media. Although, in general, these models can give accurate predictions of microbial growth in foods, the models might not take into account significant factors for microbial growth, such as food structure, competition/interaction with other microorganisms and the physiological state of microbial cells, among others. Therefore, either the validation of models obtained in laboratory media in real foods systems or the development of new G/NG models directly derived from data obtained in food products would be required.

Recent studies have demonstrated the influence of the initial cell number and of thermal history (raw vs pasteurized) on the growth fitness of *Salmonella* in egg white and liquid whole egg (Guillén et al., 2020a, 2021; Kang et al., 2021). Therefore, the objective of this study was to determine if these factors also influence the minimum growth temperature of *Salmonella* Enteritidis in whole liquid egg and its fractions. Intraspecific variability was also investigated through the inclusion in the study of four *S.* Enteritidis strains. A series of polynomial logistic regression equations were built (including one for each combination of strain and egg fraction) in order to study the interactions of these factors on the probability of growth of *S*. Enteritidis in these products. The mechanisms underlying the differences among strains observed were also explored.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Four strains belonging to *Salmonella enterica* serovar Enteritidis were used in this study. The strains of *S*. Enteritidis (STCC 4155, STCC 4300, STCC 4396 and STCC 7160) were supplied by the Spanish Type Culture Collection (STCC). Strains were maintained frozen at −80 °C in cryovials for long-term preservation. Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with

0.6% w/v yeast extract (Oxoid, TSB-YE) in 96 wells microtiter plates and incubated at 37 °C under static conditions as described in Guillén et al. (2020b).

2.2. Growth media

Growth experiments were carried out in raw liquid whole egg, egg white and yolk obtained from medium-sized raw eggs (53-63 grams) purchased from a local supermarket and in commercial pasteurized liquid whole egg, egg white and egg yolk (Pascual, Aranda de Duero, Spain).

2.3. Experimental design

The effect of initial dose, incubation temperature and egg fraction on the growth/no growth of *S*. Enteritidis were determined. The selection was based on delimiting the levels of the mentioned factors to the G/NG domain of *S*. Enteritidis, based on previous studies in raw and pasteurized egg and eggs products (Guillén et al., 2021; Kang et al., 2021; Kim et al., 2018; Sakha and Fujikawa, 2012). Since preliminary results showed that no growth was detected at 5 °C or below, data were collected at 6, 6.5, 7, 7.5, 8 and 8.5 °C for whole liquid egg and egg yolk. For egg white the following temperatures were studied: 7, 8, 9, 10, 12, 15, 17.5 and 20 °C. The initial doses were 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/mL. The experimental conditions are summarized in Table 1. The number of conditions per strain was 164, divided between the different egg products: raw and pasteurized liquid whole egg, raw and pasteurized egg yolk and raw and pasteurized egg white. The number of replicates per condition was 24.

Table 1. Experimental conditions and levels of temperature, initial dose and egg product considered for growth/no growth model for *S*. Enteritidis.

Egg product	Temperature (°C)	Initial dose (CFU/mL)
Raw whole egg	6.0	10^2, 10^3, 10^4, 10^5 and 10^6
Raw whole egg	6.5	10^2, 10^3, 10^4, 10^5 and 10^6
Raw whole egg	7.0	10^2, 10^3, 10^4, 10^5 and 10^6
Raw whole egg	7.5	10^2, 10^3, 10^4, 10^5 and 10^6
Raw whole egg	8.0	10^2, 10^3, 10^4, 10^5 and 10^6
Raw whole egg	8.5	10^2, 10^3, 10^4, 10^5 and 10^6
Pasteurized whole egg/Egg yolk	6.0	10^2, 10^4 and 10^6
Pasteurized whole egg/Egg yolk	6.5	10^2, 10^4 and 10^6
Pasteurized whole egg/Egg yolk	7.0	10^2, 10^4 and 10^6
Pasteurized whole egg/Egg yolk	7.5	10^2, 10^4 and 10^6
Pasteurized whole egg/Egg yolk	8.0	10^2, 10^4 and 10^6
Pasteurized whole egg/Egg yolk	8.5	10^2, 10^4 and 10^6
Egg white	7.0	10^2, 10^3, 10^4, 10^5 and 10^6
Egg white	8.0	10^2, 10^3, 10^4, 10^5 and 10^6
Egg white	9.0	10^2, 10^3, 10^4, 10^5 and 10^6
Egg white	10.0	10^2, 10^3, 10^4, 10^5 and 10^6
Egg white	12.0	10^2, 10^3, 10^4, 10^5 and 10^6
Egg white	15.0	10^2, 10^3, 10^4, 10^5 and 10^6
Egg white	17.5	10^2, 10^3, 10^4, 10^5 and 10^6
Egg white	20.0	10^2, 10^3, 10^4, 10^5 and 10^6

2.4. Inoculation procedure

Appropriate dilutions of stationary phase cultures in buffered peptone water (Oxoid, BPW) were used to inoculate each well at the different initial inoculum dose for each of the four Enteritidis strains. 24 replicate microtiter wells per condition and strain were filled with 297 µL of the different egg products, and inoculated with 3 µL of the corresponding dilution, achieving the desired initial dose. After inoculation, the microtiter plates were sealed with a polyester impermeable film (VWR International, Leuven, Belgium) and placed in a FX Incubator (Zeulab, Zaragoza, Spain) at the

corresponding temperatures. Temperature was recorded using a calibrated type K thermocouple temperature sensor (Almemo, Ahlborn, Germany), with an accuracy of ± 0.2 °C, connected to a data logger (Data logger 710, Almemo Ahlborn, Germany).

2.5. Growth/no growth evaluation and selection data

The occurrence of growth was tested after 30 days of incubation in all the conditions and products assayed. For egg white it was also tested after 15 days. The bacterial population in each well was determined by surface plating on Xylose Lysine Deoxycholate agar (Oxoid, XLD), and compared with the initial count. Growth was confirmed when a difference of more than 0.5 Log CFU/mL with the initial dose was detected. When growth was confirmed, it was noted as "1", and "0" if it was not. The classification criterion was carried out at a cut point of $p = 0.5$, being p = probability of growth. Before fitting the data to the model, they were examined in order to detect possible outliers (i.e. decrease of the probability of growth when environmental conditions are less severe, or vice versa). The procedure followed was that proposed by Gysemans et al. (2007), an outlier condition was an unusual change of more than 10% in the observed growth probability compared to the neighboring data point. This was tested by comparing neighboring data points in the temperature and dose directions separately.

2.6. Development of growth/no growth models

The observed data set was fitted to a polynomial logistic regression equation. The equation of the logistic regression model consists of a polynomial (right-hand side) and "logit p", which is equal to ln (p / (1 − p)) (left-hand side). The equation used in this study was a second-order linear logistic regression model, as follows:

$$\mathbf{logit}(\ \boldsymbol{p}) = \boldsymbol{a_0} + \boldsymbol{a_1} \cdot \boldsymbol{T} + \ \ \boldsymbol{a_2} \cdot \boldsymbol{D} + \ \ \boldsymbol{a_3} \cdot \boldsymbol{T} \cdot \boldsymbol{D} \ \ + \ \ \boldsymbol{a_4} \cdot \boldsymbol{T^2} \ \ + \ \boldsymbol{a_5} \cdot \boldsymbol{D^2} \qquad \text{Eq. 1}$$

where p is the probability of growth (p takes values between 0 and 1), a_0 – a_5 are the coefficients to be estimated, T is temperature, and D is initial dose.

This model was fitted in XLSTAT® software (version 2019.2.2, Addinsoft, Boston) by using the forward stepwise procedure and applying Firth's correction. This means that variables were entered one by one into the model. Stepwise entry and removal followed, based on the significance of the Wald criterion ($p = 0.05$, and $p = 0.015$ respectively). Model building stopped when no more variables meet entry or removal criteria or when the current model was the same as the previous one. With this procedure a biologically consistent model was obtained, in accordance to data observed.

2.7. Evaluation of models performance

Once models were obtained, for each model, goodness of fit statistics and predictive performance indexes were calculated in XLSTAT® software. In accordance with Valero et al. (2009), the maximum rescaled R^2 statistic, Pearson residuals and the receiver operating characteristic (ROC) curve were used as measures of goodness of fit of the models.

R^2-Nagelkerke is a modification of the Cox–Snell coefficient to assure that it can vary from 0 to 1, and a better fit of the model entails higher values of R^2 (Nagelkerke, 1991). As the Hosmer–Lemeshow statistic does not give information about the nature of the lack of fit, the Pearson residuals were calculated. They measure the difference between observed and predicted events, taking into account the number of observations (Gysemans et al., 2007). The area under ROC (Receiver Operating Characteristic) curve, c, is a measure of discrimination, obtained from a plot sensitivity (the proportion of observed events that was correctly predicted to be events), against the complement of specificity (the proportion of observed non-events that was correctly predicted to be non-events). The closer the value of c is to 1, the greater is the discrimination (Agresti, 2002). In addition, to compare various models, the Akaike Information Criterion (AIC) was calculated, which estimates the out-of-sample prediction error and thus the relative quality of the statistical models for a given data set.

To better illustrate the fit of the developed model to the observed data, the predicted probabilities at 0.1, 0.5 and 0.9 were calculated holding temperature and dose constant in GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA).

2.8. Validation

The performance of the logistic models developed was compared with growth data from independent experiments. Internal validation of the model was performed by selecting a data set within the interpolation region, and was a total of 48 conditions for the different Enteritidis strains and egg products. The experimental conditions investigated are summarized in Table 2. The procedure followed, inoculation and evaluation, was as described above. To evaluate the fit, goodness of the fit (R^2, *RMSE*) parameters and Pearson's correlation coefficients were calculated using GraphPad PRISM® statistical software.

Table 2. Experimental conditions and levels of temperature, initial dose and egg product considered for validation of growth/no growth models for *S.* Enteritidis strains.

Egg product	Temperature (°C)	Initial dose (CFU/mL)
Raw and pasteurized whole egg	7.25	$10^{4.5}$
Raw and pasteurized whole egg	7.75	$10^{2.5}$
Raw and pasteurized egg white	15.0	$10^{4.5}$
Raw and pasteurized egg white	17.5	$10^{2.5}$
Raw and pasteurized egg yolk	7.25	$10^{4.5}$
Raw and pasteurized egg yolk	7.75	$10^{2.5}$
Raw and pasteurized whole egg	7.25	$10^{4.5}$
Raw and pasteurized whole egg	7.75	$10^{2.5}$
Raw and pasteurized egg white	15.0	$10^{4.5}$
Raw and pasteurized egg white	17.5	$10^{2.5}$

2.9 Estimation of the amount of siderophores produced by the different Salmonella strains

Bacterial strains were checked for siderophore-producing ability by means of the CAS Assay (Schwyn and Neilands, 1987). A modified CAS assay solution prepared according to Alexander and Zuberer (1991), was used for this purpose. Briefly, 21.9 mg of HDTMA was dissolved in 25 mL water while stirring constantly over low heat. In a separate container 1.5 mL of 1 mM $FeCI_3 \cdot 6H20$ (in 10 mM HCl) was mixed with 7.5 mL of 2 mM CAS. This solution was slowly added to the HDTMA solution while stirring, and the mixture was transferred to a 100 mL volumetric flask. Buffer solution was prepared by dissolving 9.76 g MES in 50 mL water. The pH was adjusted to 5.6 with 50% KOH, and the buffer solution was then added to the volumetric flask containing the dye solution. Water was added to bring the volume to 100 mL and the solution was sterilized before further use. Quantitative estimation of siderophore was done by taking supernatant of bacterial cultures grown in M9 broth (Arora and Verma, 2017). After incubation at 37 °C for 24 h, bacterial cultures were centrifuged at 10,000 x g for 10 min, cell pellets were discarded, and supernatant was used to estimate siderophore. Supernatant (0.5 mL) of each bacterial culture was mixed with 0.5 mL CAS reagent and after 20 min optical density was taken at 630 nm (Spectrophotometer: Thermo Scientific, Evolution 201). Siderophore produced by strains was measured in percent siderophore unit (psu) which was calculated according to the following formula (Payne, 1994):

$$\textbf{Siderophore production (psu)} = \frac{(A_r - A_s)\cdot 100}{A_r} \qquad \text{Eq. 2}$$

where A_r = absorbance of reference (CAS solution and un inoculated broth), and A_s = absorbance of sample (CAS solution and cell-free supernatant of sample).

3. Results

In this study, the low temperature growth boundary of four *S*. Enteritidis strains in different egg products was determined. First, the occurrence of growth at different temperatures was determined in 6 different egg products/fractions of 4 *S*. Enteritidis strains when starting at different initial doses. In order to determine the experimental conditions to be assayed, some preliminary assays were performed using *S*. Enteritidis STCC 4300 at two concentrations (10^2 CFU/mL and 10^6 CFU/mL). Thus, for those media for which no effect of the starting dose was observed (egg yolk and pasteurized liquid whole egg) only 3 concentrations were assayed whereas for the rest (raw liquid whole egg and egg white) 5 concentrations were tested. Figure 1 shows, as a way of example, the results obtained for *S*. Enteritidis STCC 4300, in the 6 media tested when inoculated with 10^2 and 10^6 CFU/mL.

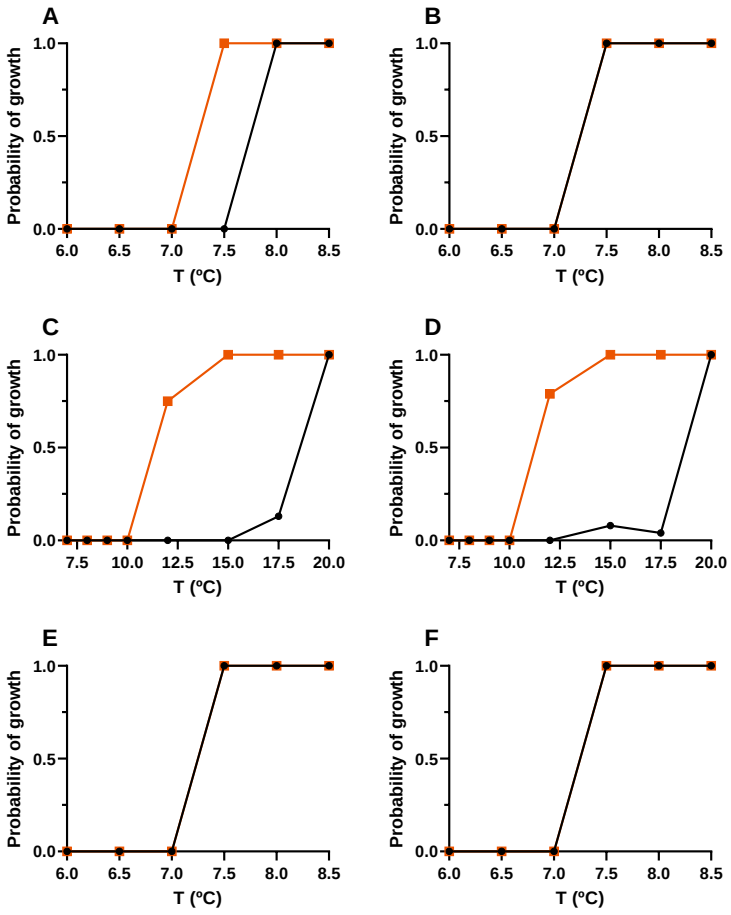

Figure 1. Probability of growth of *Salmonella* Enteritidis STCC 4300 as a function of temperature, at the lowest initial dose, 10^2 CFU/mL (black circles and line), and at the highest dose tested, 10^6 CFU/mL (orange squares and line), and in the different egg products: raw liquid whole egg (A), pasteurized liquid whole egg (B), raw egg white (C), pasteurized egg white (D), raw egg yolk (E) and pasteurized egg yolk (F).

After determining whether growth occurred or not in the different conditions assayed the data were fitted to a polynomial logistic equation as described above. Since, as can be also observed in Figure 1, transition from no growth to growth occurred in very narrow temperature interval (around 0.5 °C), leading to the generation of perfect (or almost perfect) separations in most of the study scenarios -making consequently the maximum likelihood estimate infinite- so the Firth penalty term was added to the log-likelihood function (Firth, 1993). A total of 30 logistic regression models were obtained in raw and pasteurized liquid whole egg, egg white and egg yolk. Once the models for each

strain were obtained, another model was developed for each egg product by fitting altogether the data corresponding to the four strains (Global).

Table 3. Equations obtained by fitting the logistic regression model to the growth/no growth data observed for *Salmonella* Enteritidis strains in the different egg products. The table also includes the equations obtained after fitting simultaneously the data obtained for the four strains (global). The predictors of goodness of fit (R^2-Nagelkerke, the percentage of Pearson residuals values between -1 and 1 and the area under ROC curve) are also included.

Strain	Equation	R^2-Nagelkerke	Pearson residuals	AUC
	Raw liquid whole egg			
4155 STCC	logit (p) = - 72.438 + 1.193 · T^2 + 0.266 · D^2	0.967	98.3%	0.998
4300 STCC	logit (p) = - 63.779 + 1.073 · T^2 + 0.193 · D^2	0.942	97.2%	0.994
4396 STCC	logit (p) = - 70.656 + 1.033 · T^2 + 0.128 · D^2	0.933	97.2%	0.994
7160 STCC	logit (p) = - 79.878 + 1.305 · T^2 + 0.168 · D^2	0.972	98.3%	0.998
Global	logit (p) = - 26.658 + 0.422 · T^2 + 0.077 · D^2	0.850	92.4%	0.979
	Pasteurized liquid whole egg			
4155 STCC	logit (p) = - 52.340 + 0.967 · T^2	0.958	98.15%	0.993
4300 STCC	logit (p) = - 44.849 + 0.812 · T^2	0.923	94.91%	0.988
4396 STCC	logit (p) = - 46.026 + 0.711 · T^2	0.873	93.75%	0.979
7160 STCC	logit (p) = - 44.239 + 0.799 · T^2	0.920	94.44%	0.986
Global	logit (p) = - 19.817 + 0.347 · T^2	0.820	88.02%	0.962
	Raw egg white			
4155 STCC	logit (p) = - 3.809 - 0.395 · T - 3.178 · D + 0.291 · T · D + 0.021 · T^2 + 0.118 · D^2	0.918	94.6%	0.991
4300 STCC	logit (p) = 10.349 - 2.131 · T - 4.890 · D + 0.334 · T · D + 0.074 · T^2 + 0.294 · D^2	0.892	95.5%	0.988
4396 STCC	logit (p) = 0.556 - 3.406 · T + 3.344 · D + 0.089 · T · D + 0.155 · T^2 - 0.343 · D^2	0.923	97.6%	0.993
7160 STCC	logit (p) = 15.995 - 3.250 · T - 4.765 · D + 0.326 · T · D + 0.117 · T^2 + 0.299 · D^2	0.909	96.7%	0.990
Global	logit (p) = - 27.700 + 0.974 · T + 3.888 · D - 0.049 · T · D + 0.015 · T^2 - 0.183 · D^2	0.874	95.0%	0.986
	Pasteurized egg white			
4155 STCC	logit (p) = 30.101 - 3.325 · T - 16.313 · D + 1.106 · T · D + 0.059 · T^2 + 0.763 · D^2	0.951	98.09%	0.996
4300 STCC	logit (p) = 18.907 - 2.397 · T - 9.458 · D + 0.369 · T · D + 0.080 · T^2 - 0.806 · D^2	0.917	97.45%	0.991
4396 STCC	logit (p) = 40.063 - 4.334 · T - 16.525 · D + 0.410 · T · D + 0.152 · T^2 + 1.579 · D^2	0.965	99.55%	0.997
7160 STCC	logit (p) = 67.770 - 5.009 · T - 33.970 · D + 0.720 · T · D + 0.157 · T^2 - 3.433 · D^2	0.948	98.87%	0.996
Global	logit (p) = 0.504 - 0.791 · T - 4.209 · D + 0.170 · T · D + 0.044 · T^2 - 0.431 · D^2	0.878	94.47%	0.987
	Raw and pasteurized egg yolk			
4155 STCC	logit (p) = -72.243 + 1.373 · T^2	0.997	100.0%	0.999
4300 STCC	logit (p) = -72.243 + 1.373 · T^2	0.997	100.0%	0.999
4396 STCC	logit (p) = - 45.233 + 0.704 · T^2	0.873	92.4%	0.975
7160 STCC	logit (p) = - 42.394 + 0.755 · T^2	0.911	91.9%	0.981
Global	logit (p) = - 20.475 + 0.363 · T^2	0.830	87.3%	0.959

Table 3 shows the equations obtained for each strain and egg product and the goodness of the fit, including the R^2-Nagelkerke and the area under ROC curve (AUC) value. Hosmer-Lemeshow statistic should be interpreted with caution as its value can be largely influenced by a single bad prediction, and therefore in this study the Pearson residuals were calculated. These performance statistics indicate a reasonable goodness of fit of the models obtained, even when the data of the 4 strains were modeled together –although this later was worse than when each strain was modeled alone-. Additionally, the estimated coefficients of the variables of the logistic regression models developed for the different Enteritidis strains and egg products, together with their corresponding standard errors can be found in Table S1. In Table S1 only the coefficients of the significant variables (p <0.05) included in each model are shown.

3.1. Estimation of Salmonella Enteritidis minimum growth temperature in liquid whole egg

As can be observed in Table 3, the R^2-Nagelkerke values of the models obtained for raw liquid whole egg varied between 0.933 and 0.972, AUC values between 0.994 and 0.998 and most of Pearson residuals were between -1 and 1, i.e., the differences between the observed and predicted probabilities were small in relation to the number of observations. Thus, the percentage of values between -1 and 1 were 98.3, 97.2, 97.2 and 98.3% for 4155, 4300, 4396 and 7160 STCC respectively. As described above, the goodness of the fit obtained when modelling altogether the data of the 4 strains was poorer, as it would be expected, than the fit of models developed individually (R^2-Nagelkerke 0.850 vs 0.957 in average; concordance index 0.979 vs 0.997 in average; percentage of values of Pearson residual between -1 and 1 92.4 vs 97.8%, in average). Accordingly, in the classification table of observed versus predicted model conditions for Enteritidis strains that is shown in Supplementary Table 2 (Table S2) it can be observed that for all the models developed for raw liquid whole egg, high values of c were obtained, -this value indicating the percentage of data that were correctly classified by the model- ranging from 92.36% obtained for the global model to 98.33% of the model data for the *S*. Enteritidis 7160 STCC strain.

Models described the growth of *S.* Enteritidis strains in raw liquid whole egg as a function of the quadratic terms of initial dose and temperature, indicating that the growth limits of *S.* Enteritidis strains in raw liquid whole egg would be dependent on both inoculum size and temperature. The effect of these two variables (temperature and dose) on the probability of growth of Enteritidis strains in raw liquid whole egg is depicted in Figure 2. Figure 2A shows the growth limits predicted for each strain, as a function of temperature and initial dose, by the logistic regression models obtained by setting the probability at 0.1 (10%) and Figure 2B the growth limits calculated for the model built by modeling altogether the data obtained for the four strains (global) and the model obtained after calculating the average coefficients from the equations developed for each strain and fixing the probability of growth at 0.1, 0.5 and 0.9 (10, 50 and 90%, respectively). An inverse relationship was obtained between the initial dose and the minimum growth temperature; thus, the larger the inoculum size, the lower the minimum growth temperature. These minimum temperatures, around 7.5 °C, for *S*. Enteritidis strains were similar to the mean values reported in literature (ICMSF, 1996). The highest minimum growth temperatures were obtained for *S.* Enteritidis 4396 STCC. In contrast, the lowest minimum growth temperatures, were obtained for strains 4155 and 4300 STCC, for which the influence of temperature and initial dose was similar (Figure 2A). Figure 2B shows the different outcome resulting from using two different ways of estimating the low temperature growth boundaries for *Salmonella* Enteritidis (the set of 4 strains here analyzed). Thus, it can be observed that, as it would be expected, whereas the minimum temperatures calculated from a probability of growth of 0.1 were lower for the model developed when modelling all the data altogether, the opposite happened for those required to ensure a probability of 0.9, or in other words, that the transition zone was much wider for this model than when just calculating the averages of these probabilities for the 4 strains.

Regarding pasteurized liquid whole egg, the goodness of the fit of the models developed was comparable to those developed for raw whole egg and also the goodness of the fit was better for the individual (strain) models than for the model developed using all the data set (4 strains). However, in liquid pasteurized whole egg the models described the growth of *S*. Enteritidis strains only as a function

of the quadratic term of temperature, that is, the growth limit temperatures were the same for all doses. The model that provided the best fit for the whole set of data analyzed altogether included both temperature and the quadratic temperature term as significant parameters but, in order to simplify and facilitate comparisons, the temperature term was eliminated. Despite this change in the equation, the R^2-Nagelkerke value was the same, and the AIC value only increased by 2.78%. Figure 2C shows the growth limit temperatures, which as pointed out above in this case are not dose-dependent. In this case, and even in a clearer way than in raw whole egg, two groups of strains were observed. On one hand, strains 4155, 4300 and 7160 STCC showed similar minimum growth temperatures, around 7.18 °C, whereas strain 4396 STCC required higher temperatures to be able to grow in pasteurized liquid whole egg, with more than half a degree of difference, 7.78 °C.

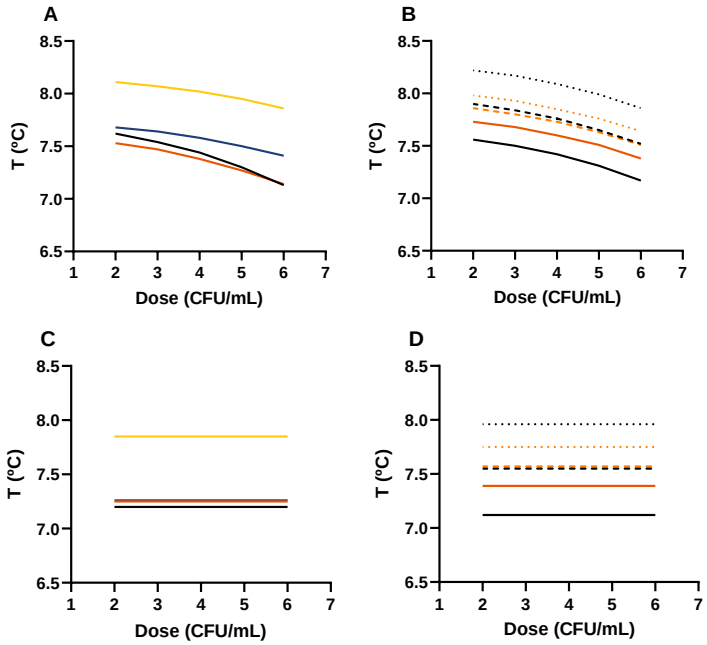

Figure 2. The effect of temperature and initial dose on the predicted probability of *Salmonella* Enteritidis strains in raw liquid whole egg (A-B) and in pasteurized liquid whole egg (C-D). Figures A and C show the predicted growth limit temperatures determined with the logistic regression models developed for each of the different strains, *S*. Enteritidis 4155 STCC (black), *S*. Enteritidis 4300 STCC (orange), *S*. Enteritidis 4396 STCC (yellow) and *S*. Enteritidis 7160 STCC (blue), at a fixed probability of 0.1. Figures B and D show the predicted growth limit temperatures estimated for a probability of growth of 0.1 (continuous line), 0.5 (dashed line) and 0.9 (dotted line) when using the model obtained when fitting altogether data (global; black) and corresponding to the average of the temperatures calculated for each of the 4 strains (average; orange).

If the minimum growth temperatures are compared it can not only be observed that in raw liquid whole egg this temperature was dose dependent and in pasteurized liquid whole egg not but also that the growth temperatures at the higher doses in raw liquid whole egg are comparable to those in pasteurized liquid whole egg. By contrast at low initial doses, which is the most probable scenario in the food industry/chain, differences in the minimum temperature of up to 0.60 °C between the two media were observed (lower in pasteurized egg).

The effect of inoculum size of these same strains, on the growth rates at 37 °C in these same products has been previously studied in Guillén et al. (2021). These authors demonstrated that the initial dose and thermal history of liquid whole egg also determined the growth rate of *Salmonella* at 37 °C and their results strongly indicated that the differences observed would be linked to differences in iron bioavailability. It was also demonstrated that the growth parameters of these same strains were similar in raw and pasteurized liquid whole egg at high doses (Guillén et al., 2021), which would be consistent with the results reported here, where the minimum growth temperatures at the highest dose tested were

comparable in both media, being the average minimum temperature of the strains 7.38 and 7.39 °C (p = 0.1) in raw and pasteurized liquid whole egg respectively at an initial dose of 10^6 CFU/mL.

3.2. Estimation of Salmonella Enteritidis minimum growth temperature in egg white

Following the same methodology, a similar set of models defining the minimum growth temperature of *S*. Enteritidis in egg white (raw and pasteurized) was obtained. The goodness of the fit of these models was also good although slightly worse than those obtained for whole egg (Tables S1 and S2). As can be observed in the Table S1 in all the cases the growth of *Salmonella* in egg white (both raw and pasteurized) was significantly influenced by inoculum size and temperature. However, since the equations did not include the same variables for all the strains they were further homogenized, in this case by including all the variables (the temperature and dose terms, the quadratic terms of temperature and dose and the interaction of temperature and dose) to establish comparisons (Table 3). The minimum growth temperatures (p = 0.1) calculated using these equations for each strain in raw and pasteurized egg white are shown in Figures 3A and 3C. The growth limits calculated for the model built modeling altogether the data obtained for the four strains (global) and calculated using the average (average) of the minimum temperatures of the strains fixing the probability of growth at 0.1, 0.5 and 0.9 (10, 50 and 90%, respectively), are shown in Figures 3B and 3D for comparison purposes.

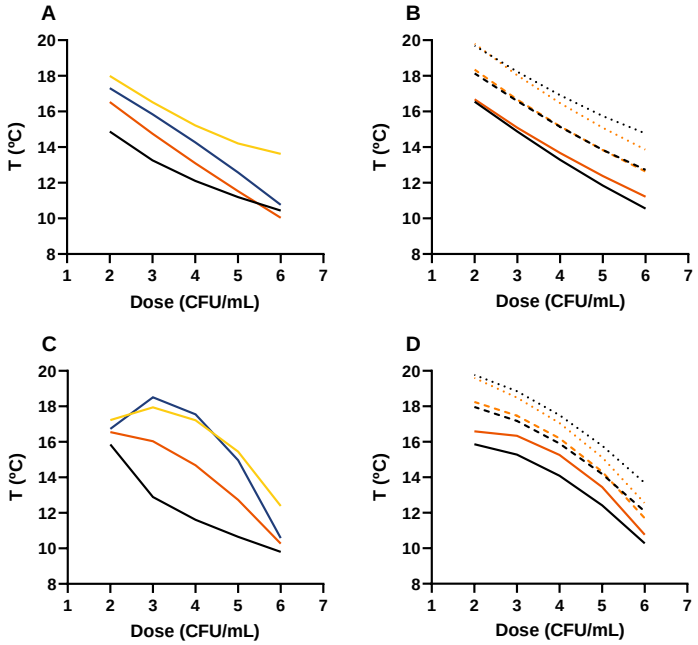

Figure 3. The effect of temperature and initial dose on the predicted probability of *Salmonella* Enteritidis strains in raw egg white (A-B) and in pasteurized egg white (C-D). Figures A and C show the predicted growth limit temperature determined with the logistic regression models developed for each of the different strains, *S*. Enteritidis 4155 STCC (black), *S*. Enteritidis 4300 STCC (orange), *S*. Enteritidis 4396 STCC (yellow) and *S*. Enteritidis 7160 STCC (blue), at a fixed probability of 0.1. Figures B and D show the predicted growth limit temperatures estimated for a probability of growth of 0.1 (continuous line), 0.5 (dashed line) and 0.9 (dotted line) when using the model obtained when fitting altogether data (global; black) and corresponding to the average of the temperatures calculated for each of the 4 strains (average; orange).

As can be observed the minimum growth temperatures in egg white were higher, above 10 °C, than in whole egg, what could be attributed to the higher content in antimicrobial proteins, higher viscosity and more alkaline pH of the former, among other factors. It was also observed that the minimum growth temperatures in pasteurized egg white, although still were dose-dependent, were slightly lower than those in raw egg white, being the magnitude of this difference depended highly on the strain studied. These data are in agreement with those obtained by Kang et al. (2021), who reported

that no growth of *Salmonella* spp. was observed at 5 °C in both raw and pasteurized egg white, and when the samples were stored at 10 °C, growth was also not observed in raw egg white, but slight growth was observed in pasteurized egg white. In addition, it should be noted that the influence of the dose on the minimum growth temperatures in both raw and pasteurized egg white, were much higher than in raw liquid whole egg, with differences of more than 6.0 °C between the lowest and highest initial doses (Figure 3). On the other hand, it has also been indicated that, conversely to pasteurized liquid whole egg, the minimum growth temperatures of *Salmonella* cells in pasteurized egg white was dependent on the initial dose. It is well-known that pasteurization treatments of egg white are at lower temperature (<60 °C) than treatments to pasteurize whole egg (60 – 72 °C) due to the sensitivity of egg white proteins (Baron et al., 2016) and, therefore, the thermal denaturation of egg white proteins with antimicrobial properties, such as ovotransferrin and lysozyme during pasteurization treatments (Lechevalier et al., 2017) would be lower in egg white than in whole egg. Alternatively (or additionally) it can also be speculated that since the amount of these antimicrobial compounds is higher in egg white than in whole egg the treatments applied might not be enough to achieve the degree of denaturation required for abolishing this dose-dependency, as already proposed elsewhere (Guillén et al., 2021).

3.3. Estimation of Salmonella Enteritidis minimum growth temperature in egg yolk

Since in preliminary experiments no influence of the dose or of thermal history of the egg yolk on the minimum growth temperatures of *Salmonella* Enteritidis was found a single model was constructed for this egg product. Again, good goodness of the fit values were obtained (Table 3 and S2). For the models developed in egg yolk the significant parameters were for some strains the temperature term, and for another the quadratic temperature term, therefore for simplification purposes the quadratic temperature term was selected as it was for the pasteurized whole liquid egg (Table 3). Despite this change in the equation, the R^2-Nagelkerke value did not change, and the AIC value only increased by 0.03% for STCC 4300 and 4155 strains. In the case of the model obtained with when fitting the data of the 4 strains altogether (global), the R^2-Nagelkerke changed from 0.839 to 0.830, and the AIC value increased by 4.2%.

Minimum growth temperatures determined were similar to those obtained in pasteurized eggs for the different strains, with values between 7.10 and 7.75 °C (Figure 4). This is consistent with the data obtained in our previous article in which we observed that the growth rates of *Salmonella* cells were similar in raw yolk and pasteurized yolk (Guillén et al., 2021) and also similar to those in pasteurized whole egg and even in raw whole egg inoculated at the higher doses.

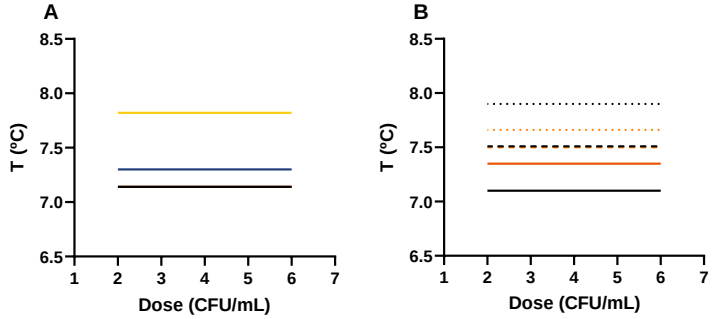

Figure 4. The effect of temperature and initial dose on the predicted probability of *Salmonella* Enteritidis strains in raw and pasteurized egg yolk (A-B). Figure A shows the predicted growth limit temperature determined with the logistic regression models developed for each of the different strains, *S*. Enteritidis 4155 STCC (black), *S*. Enteritidis 4300 STCC (orange), *S*. Enteritidis 4396 STCC (yellow) and *S*. Enteritidis 7160 STCC (blue), at a fixed probability of 0.1. Figure B shows the predicted growth limit temperatures estimated for a probability of growth of 0.1 (continuous line), 0.5 (dashed line) and 0.9 (dotted line) when using the model obtained when fitting ltogether data (global; black) and corresponding to the average of the temperatures calculated for each of the 4 strains (average; orange).

3.2. Validation

In order to evaluate the logistic regression models obtained, several boundary conditions were selected, these were conditions in which not all replicates showed 100% growth or no growth (0<*p* observed <1) (Table 4).

Table 4. Conditions under which boundary responses (0<p observed <1) were obtained for *S*. Enteritidis strains and their corresponding predicted probability (p_{pred}) for the developed models.

Strain	T (°C)	Dose (Log(CFU/mL)	P_{obs}	P_{pred}	Strain	T (°C)	Dose (Log(CFU/mL)	P_{obs}	P_{pred}
Raw liquid whole egg					**Pasteurized liquid whole egg**				
4155 STCC	7.5	3	0.08	0.05	4155 STCC	7.5	2	1.00	0.88
4155 STCC	7.5	4	0.21	0.25	4155 STCC	7.5	4	0.67	0.88
4155 STCC	7.5	5	0.79	0.79	4300 STCC	7.5	2	0.67	0.69
4300 STCC	7.5	3	0.29	0.16	4300 STCC	7.5	4	0.50	0.69
4300 STCC	7.5	4	0.33	0.42	4300 STCC	7.5	6	0.92	0.69
4300 STCC	7.5	5	0.79	0.80	4396 STCC	8.0	2	0.29	0.38
4396 STCC	8.0	4	0.13	0.08	4396 STCC	8.0	4	0.69	0.38
4396 STCC	8.0	5	0.21	0.21	4396 STCC	8.0	6	0.50	0.38
4396 STCC	8.0	6	0.50	0.52	7160 STCC	7.5	2	0.67	0.66
7160 STCC	7.5	5	0.13	0.09	7160 STCC	7.5	4	0.54	0.66
7160 STCC	7.5	6	0.38	0.40	7160 STCC	7.5	6	0.88	0.66
Raw egg white					**Pasteurized egg white**				
4155 STCC	17.5	2	0.33	0.53	4155 STCC	15.0	2	0.25	0.06
4155 STCC	15.0	3	0.63	0.42	4155 STCC	17.5	2	0.04	0.32
4155 STCC	15.0	4	0.75	0.85	4155 STCC	15.0	3	0.79	0.78
4155 STCC	15.0	5	0.88	0.98	4155 STCC	10.0	6	0.21	0.22
4155 STCC	12.0	6	0.79	0.66	4300 STCC	17.5	2	0.04	0.24
4300 STCC	17.5	2	0.13	0.24	4300 STCC	15.0	3	0.08	0.03
4300 STCC	12.0	4	0.13	0.03	4300 STCC	15.0	4	0.17	0.15
4300 STCC	15.0	4	0.42	0.56	4300 STCC	12.0	6	0.79	0.64
4300 STCC	12.0	5	0.38	0.17	4396 STCC	17.5	2	0.04	0.15
4300 STCC	15.0	5	0.92	0.95	4396 STCC	12.0	6	0.04	0.05
4300 STCC	12.0	6	0.75	0.68	7160 STCC	17.5	2	0.17	0.31
4396 STCC	15.0	4	0.04	0.07	7160 STCC	15.0	6	0.04	0.11
4396 STCC	12.0	5	0.13	0.01	7160 STCC	12.0		0.92	0.87
4396 STCC	15.0	5	0.21	0.27	**Raw and pasteurized egg yolk**				
4396 STCC	15.0	6	0.50	0.48	4396 STCC	8.0	2	0.46	0.46
7160 STCC	15.0	3	0.04	0.03	4396 STCC	8.0	4	0.46	0.46
7160 STCC	15.0	4	0.25	0.25	4396 STCC	8.0	6	0.46	0.46
7160 STCC	12.0	5	0.13	0.05	7160 STCC	7.5	2	0.50	0.51
7160 STCC	15.0	5	0.75	0.85	7160 STCC	7.5	4	0.54	0.51
7160 STCC	12.0	6	0.46	0.38	7160 STCC	7.5	6	0.50	0.51

Table 5. Model validation conditions for *S*. Enteritidis strains in the different egg products and their corresponding observed probability (P_{obs}) and the different predicted probabilities for the developed models: models corresponding to each strain (P_{pred} strain) and model developed by fitting altogether the data corresponding to the four strains (P_{pred} global).

Strain	T (°C)	Dose (Log(CFU/mL))	P_{obs}	P_{pred} strain	P_{pred} global	Strain	T (°C)	Dose (Log(CFU/mL))	P_{obs}	P_{pred} strain	P_{pred} global
Raw liquid whole egg						**Pasteurized liquid whole egg**					
4155 STCC	7.25	4.5	0.00	0.01	0.05	4155 STCC	7.25	4.5	0.00	0.18	0.17
	7.75	2.5	0.38	0.70	0.31		7.75	2.5	1.00	1.00	0.74
4300 STCC	7.25	4.5	0.00	0.03	0.06	4300 STCC	7.25	4.5	0.46	0.10	0.17
	7.75	2.5	0.29	0.87	0.32		7.75	2.5	0.96	0.98	0.74
4396 STCC	7.25	4.5	0.00	0.00	0.06	4396 STCC	7.25	4.5	0.00	0.00	0.17
	7.75	2.5	0.00	0.00	0.32		7.75	2.5	0.21	0.04	0.74
7160 STCC	7.25	4.5	0.00	0.00	0.06	7160 STCC	7.25	4.5	0.25	0.09	0.17
	7.75	2.5	0.29	0.39	0.32		7.75	2.5	0.88	0.98	0.74
Raw egg white						**Pasteurized egg white**					
4155 STCC	7.25	4.5	0.67	0.94	0.65	4155 STCC	7.25	4.5	0.88	1.00	0.47
	7.75	2.5	0.50	0.79	0.55		7.75	2.5	0.83	0.92	0.46
4300 STCC	7.25	4.5	0.96	0.83	0.65	4300 STCC	7.25	4.5	0.92	0.44	0.47
	7.75	2.5	0.54	0.49	0.55		7.75	2.5	0.67	0.30	0.46
4396 STCC	7.25	4.5	0.04	0.16	0.65	4396 STCC	7.25	4.5	0.13	0.00	0.47
	7.75	2.5	0.33	0.16	0.55		7.75	2.5	0.50	0.05	0.46
7160 STCC	7.25	4.5	0.50	0.56	0.65	7160 STCC	7.25	4.5	0.63	0.00	0.47
	7.75	2.5	0.63	0.32	0.55		7.75	2.5	0.71	0.02	0.46
Raw egg yolk						**Pasteurized egg yolk**					
4155 STCC	7.25	4.5	0.08	0.48	0.20	4155 STCC	7.25	4.5	0.08	0.48	0.20
	7.75	2.5	0.33	1.00	0.95		7.75	2.5	0.58	1.00	0.95
4300 STCC	7.25	4.5	0.04	0.48	0.20	4300 STCC	7.25	4.5	0.08	0.48	0.20
	7.75	2.5	1.00	1.00	0.95		7.75	2.5	0.75	1.00	0.95
4396 STCC	7.25	4.5	0.00	0.00	0.20	4396 STCC	7.25	4.5	0.00	0.00	0.20
	7.75	2.5	0.00	0.05	0.95		7.75	2.5	0.00	0.05	0.95
7160 STCC	7.25	4.5	0.25	0.06	0.20	7160 STCC	7.25	4.5	0.25	0.06	0.20
	7.75	2.5	0.92	0.95	0.95		7.75	2.5	0.67	0.95	0.95

Afterwards, a validation of the model was performed by selecting a data set within the interpolation region (Table 5). In Table 5, the fit of each model was evaluated, including the model constructed from all the data. In general terms, a good agreement was obtained between the values obtained in the validation and those predicted by the models for each strain and with the model with the data set, although with some exceptions. The best fits were observed for the whole egg, and the worst fits were for pasteurized egg white. Despite the differences between observed and predicted values, in most conditions, if not all, the predicted values were still within the transition zone. In any

case, when analyzing these discrepancies, the complexity of the medium and the very narrow temperature ranges of the transition zone, <0.5 °C, should be taken into account.

3.3. Quantification of the amount of siderophores produced by the different *Salmonella* strains

Figure 5 shows the capacity of siderophore synthesis (in percentage of siderophore units) of each strain, determined as described in material and methods. As can be observed strain 4300 STCC showed significantly higher Fe-siderophore synthesis capacity (65.65 ± 6.905 units) than the other Enteritidis strains. In contrast, strain 4396 STCC showed the lowest Fe-siderophore synthesis activity, 25.69 ± 3.561. The other two strains, 4155 and 7160 STCC, displayed intermediate siderophore production, 38.77 ± 5.543 and 38.43 ± 5.650, respectively.

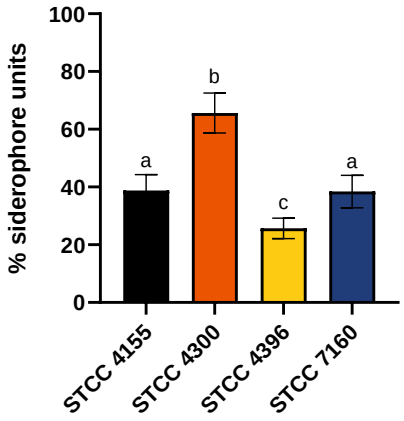

Figure 4. Quantification of the amount of siderophores produced by each *Salmonella* Enteritidis strain. The experimental values are presented as mean ± standard error of mean. Differences in the letters indicate statistically significant differences (p <0.05).

4. Discussion

In this work we provide a new estimation of the minimum growth temperatures of *S*. Enteritidis in raw and pasteurized whole egg, egg white and egg yolk by through the use of probabilistic growth/no growth (G/NG) interface models and the inclusion of new variables/factors such as the initial dose and the thermal history of the egg products.

Regarding the influence of the egg fraction, it is a very well-known that egg white is a much more restrictive medium than whole egg and egg yolk (Guillén et al., 2021; Kim et al., 2018; Moon et al., 2016). The minimum growth temperatures for these egg products are in the range of those already published in the literature (ICMSF, 1996; Kang et al., 2021; Kim et al., 2018; Whiting and Buchanan, 1997) although it should be noted that, for both whole egg and egg white our results indicate that pasteurization enable growth of *Salmonella* cells at lower temperatures. It should also be pointed out that differences (in the minimum growth temperatures) between whole egg and egg yolk will only be found in some scenarios (see below). Thus, the minimum growth temperatures of *S.* Enteritidis cells would be similar in egg yolk (regardless of its thermal history), pasteurized whole egg and in raw whole egg when inoculated at high cell densities, i.e., will only be higher in raw whole egg inoculated at low cell densities. The potential causes for these later two phenomena will be discussed below but the practical implications are obvious: while pasteurization treatments do ensure *Salmonella* inactivation, thus reducing the risk associated to this microorganisms, if these products are later contaminated the probability (and rate) of *Salmonella* growth in pasteurized products would be higher than in raw ones.

Our results also demonstrate that in three of these products (raw whole egg and raw and pasteurized egg white) this temperature depends on the initial dose. This is especially relevant because in most of the studies a fixed inoculum level is used, without considering the potential effect of the initial dose even though, there is evidence suggesting that it may affect microbial growth

(Koutsoumanis and Sofos, 2005; Masana and Baranyi, 2000; Pascual et al., 2001; Robinson et al., 2001). Thus, Koutsoumanis and Sofos indicated that cell density would have a significant impact on the growth/no growth interface and that the probability of growth would be significantly lower at lower cell densities (Koutsoumanis and Sofos, 2005). Similarly, Vermeulen et al. (2009) developed a growth/no growth model for *L. monocytogenes* that incorporated the influence of cell density, and validated with these data a model developed for high cell concentrations and found to be invalid for lower cell densities. Results obtained in this study provide new evidences of the influence of the initial cell density on microbial growth (in this case on the minimum growth temperature of *Salmonella* Enteritidis) in some food products (in this case egg products). This is of the highest relevance from an applied point of view since it indicates that models developed using high initial densities might not be applicable to low density scenarios, which are, on the other hand, the more common in real food scenarios and also because it reinforces the perception that the initial dose should be considered when developing and using G/NG models to identify the conditions necessary to ensure an acceptably low-risk level. In any case, our results also indicate that this phenomenon (the influence of dose on microbial growth) would only happen in some food products, as will be discussed below.

Another relevant and differential aspect of our work/experimental design is that the experiments were carried out with monocultures (in this case 4 Enteritidis strains) while most of the models are constructed on the basis of results obtained with strain cocktails (Koutsoumanis et al., 2004; Valero et al., 2009; Vermeulen et al., 2007). Besides other potential advantages, this experimental design was chosen because it provides very valuable data regarding the intra-specific (in this case intra-serovar) variability in the low temperature growth boundaries. Thus, as has already been indicated throughout the manuscript our results reveal the existence of substantial differences in the minimum growth temperatures depending on the strain studied, with differences of up to 5-6 °C in some conditions when growing in egg white. In addition of the models developed for each strain we also built a model modelling the data of the 4 strains altogether and we compared the results obtained with those of the strains when modelled individually and also to the average values of the 4 strains. As expected, the minimum growth temperature values calculated for a probability of 0.1 for this model obtained by modelling the data of the 4 strains together were similar to those of the strain displaying the lowest minimum growth for the same probability temperatures and those required to reach a probability of 0.9 to those of strain STCC 4396, the one needing higher temperatures for growth (also at this probability; 0.9). In fact, this model would probably be very close to the one we would had obtained if we had inoculated a cocktail of these strains provided that no interaction/interference between the cells of different strains would have occurred. However, as will be discussed below, this might not be the case. Finally, and from a practical point of view, if maximizing food safety is the target either using the strain capable of growing at the lowest temperatures or a cocktail including it (in our case the model developed fitting the data altogether) would be the best options since both will provide similar results.

Another important aspect of the models developed here is that they were obtained in the food itself. Most of the existing G/NG models for *Salmonella* have been obtained in laboratory media (Basti and Razavilar, 2004; Koutsoumanis et al., 2004; Lanciotti et al., 2001), although subsequently validated in food. However, the complex composition of egg, specially egg white, makes difficult to develop laboratory media/models mimicking it and that is why we opted to work in real foods, although this implied that we had to determine if growth had occurred or not on the basis of single (or two in the case of egg white) measurements instead of using whole growth kinetics. It should also be noted that, in addition to food structure and composition, the number and type of microorganisms present in food (competitive microbiota) has been proved to significantly affect *Salmonella* growth ability/rate (Oscar, 2008). However, it should be noted that the internal contamination of the eggs used in this study was extremely low (data not shown) and always much lower that the lower dose inoculated and, therefore, this would not be affecting the estimations done, or help to explain the differences between strains, batches and/or products.

The main factor limiting bacterial growth in egg has long been considered to be iron restriction (Garibaldi, 1970; Schade and Caroline, 1944). Thus, although the concentration of iron in egg has been

estimated to be between 3.6 to 18 μM, it is assumed that there would be no free iron in egg white, since it would be chelated by ovotransferrin. To overcome this limitation in iron bioavailability (Dostal et al., 2014), *Salmonella* has developed different systems for the acquisition of iron including, among others, the ferric iron uptake system via siderophores. The main siderophores of *Salmonella* are Salmochelins and Enterobactins, which are characterized by their high affinity for iron. According to previous studies it seems that the synthesis of these siderophores is key for the growth of *Salmonella* in egg (Correnti et al., 2011; Julien et al., 2020) and furthermore, our previous studies suggest that the bioavailability of iron would explain the differences in growth rate between fractions, depending on the dose and the thermal history of the egg (Guillén et al., 2021).

Results obtained in our previous work (Guillén et al., 2021) demonstrated that the growth rate of *S.* Enteritidis cells depends on both the thermal history of the egg product for egg white and liquid whole egg but not for egg yolk and also that in depends on the initial dose for egg white (both raw and pasteurized) and raw liquid whole egg. In addition, we recently have observed that the differences in iron bioavailability existing between these media and also depending on the initial dose, as described by Scholz and Geenberger (2015), and more specifically the amount of siderophores released to the medium together with the concentration of active iron chelating proteins (ovotransferrin) and/or siderophore sequestering ones (Ex-FABP) would probably be the major cause determining the differences in growth rate observed (Guillén et al., submitted for publication). Results obtained in this work highly resemble those observed previously since the minimum growth temperature of the 4 *Salmonella* strains here studied was only dose dependent in egg white (both raw and pasteurized) and raw liquid whole egg, no differences between raw and pasteurized egg yolk were observed and the minimum growth temperature in raw whole liquid egg inoculated at high doses was similar to that in pasteurized whole liquid egg. Therefore, it is reasonable to speculate that iron bioavailability would also be determining the minimum growth temperatures of *Salmonella* in egg and egg products. Furthermore, results obtained in this work also suggest that the differences in the minimum growth temperature among the different strains observed might also be related to their ability to synthesize these siderophores, since strain STCC 4300 which displayed the highest siderophore production ability was one of the strains (together with STCC 4155) with the lowest minimum growth temperatures whereas strain STCC 4396 was the one producing less siderophores and the one displaying a lower ability to grow at low temperatures. Nevertheless, the results obtained also strongly suggest that this will not be the only cause determining the differences in growth ability at low temperatures since strain STCC 4155, displaying a lower ability to synthesize siderophores than strain STCC 4300, was capable of growing at lower temperatures, at least in egg white. Similarly, in egg yolk (in which no iron restriction is supposed to be found) strain STCC 4396 also displayed higher minimum growth temperatures. In any case it should also be noted that the CAS method only gives an estimation of the total amount of siderophores secreted but that the relevance of the different siderophores seems to be not equal (e.g. Salmochelin would be much more relevant than Enterobactin for *Salmonella* growth in egg products because of the presence of Ex-FABP). Therefore, further work will be required to fully elucidate the causes for the differences in minimum growth temperatures among *Salmonella* Enteritidis strains.

5. Conclusions

The objective of this work was to define the boundary zones of the growth/no growth domain of *Salmonella* Enteritidis as a function of temperature (low temperature boundary) and dose in different egg products.

Results obtained indicate that, as already widely demonstrated, the minimum growth temperatures of *Salmonella* Enteritidis are higher in egg white (9.47 - 18.25 °C) than in egg yolk (7.10 - 7.75 °C) and whole liquid egg (7.15 - 7.78 °C). Results obtained also demonstrate that in some products raw whole liquid egg and raw and pasteurized egg white) the minimum growth temperature of *Salmonella* Enteritidis cells depends on the initial dose. Similarly, the previous thermal history of the egg product only influenced the minimum growth temperature in some of them (particularly to whole liquid egg). Thus in egg yolk no influence of dose and/or between raw and pasteurized products was

observed. In egg white the minimum growth temperatures were dose dependent in both raw and pasteurized products and the influence of the thermal history would be small, if existing. Finally, pasteurization of whole liquid egg abolished the dose dependency of the minimum growth temperature, and therefore, differences in this parameter between raw and pasteurized whole liquid egg were only found when low inoculation doses were compared.

On the other hand, it should be noted that our experimental design (with monocultures) significantly increased the amount of essays that had to be performed but it allowed us to quantify the intra-specific variability in minimum growth temperatures among *Salmonella* Enteritidis strains. Wide differences between strains were observed in some products (up to approx. 6 °C in egg white), with one of them, STCC 4396, showing the highest minimum growth temperatures.

In summary, the models here developed stand out because they would provide a more accurate prediction of *Salmonella* minimum growth temperatures in egg products by taking into account additional factors (dose and thermal history) while also providing a quantification of the intra-specific variability. This is of the highest relevance for improving the safety of egg products since it would allow to develop more precise *Salmonella* quantitative risk assessments and also to improve their production, storage and distribution processes. It might also help to revise or refine current guidelines for its management for both the industry and the consumers.

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Table S1. Estimated coefficients of the logistic regression models for the best model fit obtained. with their corresponding standard errors for the different Enteritidis strains and egg products.

Strain	Coefficient	Estimate	S. E.	Strain	Coefficient	Estimate	S. E.
Raw liquid whole egg				**Pasteurized liquid whole egg**			
4155 STCC	**Constant (a_0)**	-72.438	12.106	**4155 STCC**	**Constant (a_0)**	-52.340	11.272
	T^2 (a_4)	1.193	0.208		**T (a_1)**	-	-
	D^2 (a_5)	0.266	0.046		**T^2 (a_4)**	0.967	0.202
4300 STCC	**Constant (a_0)**	-63.779	11.583	**4300 STCC**	**Constant (a_0)**	-44.849	10.839
	T^2 (a_4)	1.073	0.203		**T (a_1)**	-	-
	D^2 (a_5)	0.193	0.032		**T^2 (a_4)**	0.812	0.193
4396 STCC	**Constant (a_0)**	-70.656	12.356	**4396 STCC**	**Constant (a_0)**	-46.026	11.099
	T^2 (a_4)	1.033	0.187		**T (a_1)**	-	-
	D^2 (a_5)	0.128	0.001		**T^2 (a_4)**	0.711	0.173
7160 STCC	**Constant (a_0)**	-79.878	14.284	**7160 STCC**	**Constant (a_0)**	-44.239	10.77
	T^2 (a_4)	1.305	0.237		**T (a_1)**	-	-
	D^2 (a_5)	0.168	0.046		**T^2 (a_4)**	0.799	0.192
Global	**Constant (a_0)**	-26.658	1.224	**Global**	**Constant (a_0)**	-178.418	33.897
	T^2 (a_4)	0.422	0.019		**T (a_1)**	41.526	8.720
	D^2 (a_5)	0.077	0.008		**T^2 (a_4)**	-2.366	0.560
Raw egg white				**Pasteurized egg white**			
4155 STCC	**Constant (a_0)**	-19.216	1.935	**4155 STCC**	**Constant (a_0)**	-11.750	3.478
	T (a_1)	0.841	0.089		**T (a_1)**	-	-
	D (a_2)	-	-		**D (a_2)**	-3.477	0.405
	T · D (a_3)	0.137	0.016		**T · D (a_3)**	0.519	0.065
	T^2 (a_4)	-	-		**T^2 (a_4)**	-	-
	D^2 (a_5)	-	-		**D^2 (a_5)**	-	-
4300 STCC	**Constant (a_0)**	-13.826	1.275	**4300 STCC**	**Constant (a_0)**	18.907	6.784
	T (a_1)	-	-		**T (a_1)**	-2.397	0.738
	D (a_2)	-	-		**D (a_2)**	-9.457	2.221
	T · D (a_3)	0.150	0.016		**T · D (a_3)**	0.369	0.074
	T^2 (a_4)	0.024	0.002		**T^2 (a_4)**	0.080	0.023
	D^2 (a_5)	-	-		**D^2 (a_5)**	0.806	0.190
4396 STCC	**Constant (a_0)**	-11.121	4.277	**4396 STCC**	**Constant (a_0)**	-12.600	3.512
	T (a_1)	-2.526	0.832		**T (a_1)**	-	-
	D (a_2)	5.467	1.988		**D (a_2)**	-7.060	2.478
	T · D (a_3)	-	-		**T · D (a_3)**	-	-
	T^2 (a_4)	0.139	0.034		**T^2 (a_4)**	0.066	0.008
	D^2 (a_5)	-0.421	0.212		**D^2 (a_5)**	1.193	0.333

Table S1. Continuation

Strain	Coefficient	Estimate	S. E.	Strain	Coefficient	Estimate	S. E.
Raw egg white				**Pasteurized egg white**			
7160 STCC	**Constant (a_0)**	-3.192	4.023	**7160 STCC**	**Constant (a_0)**	67.770	13.334
	T (a_1)	-1.945	0.675		**T (a_1)**	-5.009	0.935
	D (a_2)	-	-		**D (a_2)**	-33.970	6.562
	T · D (a_3)	0.169	0.021		**T · D (a_3)**	0.720	0.165
	T^2 (a_4)	0.095	0.025		**T^2 (a_4)**	0.157	0.028
	D^2 (a_5)	-	-		**D^2 (a_5)**	3.433	0.607
Global	**Constant (a_0)**	-14.338	1.017	**Global**	**Constant (a_0)**	-7.935	1.734
	T (a_1)	-	-		**T (a_1)**	-	-
	D (a_2)	0.813	0.288		**D (a_2)**	-2.845	0.871
	T · D (a_3)	0.057	0.018		**T · D (a_3)**	0.108	0.025
	T^2 (a_4)	0.033	0.003		**T^2 (a_4)**	0.026	0.004
	D^2 (a_5)	-	-		**D^2 (a_5)**	0.374	0.073

Strain	Coefficient	Estimate	S. E.
Raw and pasteurized egg yolk			
4155 STCC	**Constant (a_0)**	-144.315	29.307
	T (a_1)	19.906	4.040
	T^2 (a_4)	-	-
4300 STCC	**Constant (a_0)**	-144.315	29.307
	T (a_1)	19.906	4.040
	T^2 (a_4)	-	-
4396 STCC	**Constant (a_0)**	-45.233	11.127
	T (a_1)	-	-
	T^2 (a_4)	0.704	0.174
7160 STCC	**Constant (a_0)**	-42.394	10.465
	T (a_1)	-	-
	T^2 (a_4)	0.755	0.186
Global	**Constant (a_0)**	-210.943	34.764
	T (a_1)	49.977	8.935
	T^2 (a_4)	-2.909	0.573

Table S2. Classification table of observed vs predicted conditions of the developed models for Enteritidis strains in different egg products.

Raw liquid whole egg						Pasteurized liquid whole egg					
4155 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**4155 STCC**	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	425	5	430	98.84%	**P_{obs}**	**No growth**	216	22	238	90.76%
	Growth	7	283	290	97.59%		**Growth**	0	194	194	100.00%
	Total	432	288	720	98.33%		**Total**	216	216	432	94.91%
4300 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**4300 STCC**	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	417	5	422	98.82%	**P_{obs}**	**No growth**	216	8	224	96.43%
	Growth	15	283	298	94.97%		**Growth**	0	208	208	100.00%
	Total	432	288	720	97.22%		**Total**	216	216	432	98.15%
4396 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**4396 STCC**	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	568	12	580	97.93%	**P_{obs}**	**No growth**	333	0	333	100.00%
	Growth	8	132	140	94.29%		**Growth**	27	72	99	72.73%
	Total	576	144	720	97.22%		**Total**	360	72	432	93.75%
7160 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**7160 STCC**	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	468	0	468	100.00%	**P_{obs}**	**No growth**	216	24	240	90.00%
	Growth	12	240	252	95.24%		**Growth**	0	192	192	100.00%
	Total	480	240	720	98.33%		**Total**	216	216	432	94.44%
Global	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**Global**	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	1800	100	1900	94.74%	**P_{obs}**	**No gowth**	990	45	1035	95.65%
	Growth	120	860	980	87.76%		**Growth**	162	531	693	76.62%
	Total	1920	960	2880	92.36%		**Total**	1152	576	1728	88.02%

Table S2. Continuation

Raw egg white						Pasteurized egg white					
4155 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**4155 STCC**	**P_{pred}**	540	5	545	99.08%
P_{obs}	**No growth**	534	30	564	94.68%	**P_{obs}**	**No growth**	12	331	343	96.50%
	Growth	18	306	324	94.44%		**Growth**	552	336	888	98.09%
	Total	552	336	888	94.59%		**Total**	**No growth**	**Growth**	**Total**	**% correct**
4300 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**4300 STCC**	**P_{pred}**	614	12	626	98.08%
P_{obs}	**No growth**	629	22	651	96.62%	**P_{obs}**	**No growth**	10	228	238	95.80%
	Growth	19	242	261	92.72%		**Growth**	624	240	864	97.45%
	Total	648	264	912	95.50%		**Total**	**No growth**	**Growth**	**Total**	**% correct**
4396 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**4396 STCC**	**P_{pred}**	716	0	716	100.00%
P_{obs}	**No growth**	723	0	723	100.00%	**P_{obs}**	**No growth**	4	168	172	97.67%
	Growth	21	144	165	87.27%		**Growth**	720	168	888	99.55%
	Total	744	144	888	97.64%		**Total**	**No growth**	**Growth**	**Total**	**% correct**
7160 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**7160 STCC**	**P_{pred}**	688	2	690	99.71%
P_{obs}	**No growth**	673	6	679	99.12%	**P_{obs}**	**No growth**	8	190	198	95.96%
	Growth	23	186	209	89.00%		**Growth**	696	192	888	98.87%
	Total	696	192	888	96.73%		**Total**	**No growth**	**Growth**	**Total**	**% correct**
Global	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**	**Global**	**P_{pred}**	2523	54	2577	97.90%
P_{obs}	**No growth**	2575	42	2617	98.40%	**P_{obs}**	**No gowth**	141	810	951	85.17%
	Growth	137	822	959	85.71%		**Growth**	2664	864	3528	94.47%
	Total	2712	864	3576	94.99%		**Total**	540	5	545	99.08%

Table S2. Continuation

Raw and pasteurized egg yolk					
4155 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	216	0	216	100.00%
	Growth	0	216	216	100.00%
	Total	216	216	432	100.00%
4300 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	216	0	216	100.00%
	Growth	0	216	216	100.00%
	Total	216	216	432	100.00%
4396 STCC	**P_{pred}**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	327	0	327	100.00%
	Growth	33	72	105	68.57%
	Total	360	72	432	92.36%
7160 STCC	**Ppred**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	216	35	251	86.06%
	Growth	0	181	181	100.00%
	Total	216	216	432	91.90%
Global	**Ppred**	**No growth**	**Growth**	**Total**	**% correct**
P_{obs}	**No growth**	971	39	1010	96.14%
	Growth	181	537	718	74.79%
	Total	1152	576	1728	87.27%

Capítulo 5

Exploración del riesgo asociado a serovariedades emergentes en el sector aviar tales como *S.* Heidelberg, *S.* Kentucky, *S.* Livingstone y *S.* Mbandaka

Manuscrito IX/Manuscript IX

Stress resistance of emerging poultry-associated *Salmonella* serovars

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Stress resistance of emerging poultry-associated *Salmonella* serovars

Silvia Guillén, María Marcén, Ignacio Álvarez, Pilar Mañas, Guillermo Cebrián*

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), Zaragoza, Spain

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ABSTRACT

In recent years, the on-farm prevalence of some poultry-related *Salmonella* serovars such as *S.* Kentucky, *S.* Heidelberg, *S.* Livingstone and *S.* Mbandaka has increased significantly, even replacing *S.* Enteritidis and *S.* Typhimurium as the most frequently isolated serovars in some production settings and countries. For this reason, the aim of this work was to determine the resistance to several stressing agents and food preservation technologies, in laboratory media and in egg products, of 4 strains of these emerging *Salmonella* serovars associated to poultry and poultry products and to make comparisons with 4 *S.* Enteritidis strains. First, the resistance to acid pH, hydrogen peroxide, NaCl, heat, HHP, PEF and UV of the 8 *Salmonella* strains studied was determined and compared in laboratory media. From this part of the study, it was concluded that variability in resistance to stress among the 8 studied strains varied depending on the investigated agent/technology. However, differences in resistance (*2D*-values) were always lower than 3.3-fold. Results obtained also indicated that the strains of the emerging serovars studied would display lower acid and NaCl resistance, higher heat resistance and similar oxidative, HHP, PEF and UV resistance than *S.* Enteritidis. Then, the resistance of these 8 strains was evaluated and compared in egg, egg products and poultry manure. For some agents -including osmotic stresses, UV and PEF- there was a very good correspondence between the results obtained in laboratory media and in real food matrices and poultry manure ($r > 0.85$; $p < 0.01$). A significant relationship was also found for acid and HHP resistance ($p < 0.05$) and a trend for heat resistance ($p < 0.10$). Therefore, in general terms, conclusions drawn from the study carried out in laboratory media - regarding intraspecific variability and the relative resistance of the different strains - might be extrapolated, although with caution, to real food scenarios. Results obtained in this investigation would help to better understand the physiology and ecology of *Salmonella* and to design better egg preservation strategies.

1. Introduction

Salmonella is the most commonly reported causative agent of foodborne outbreaks in the European Union (EFSA, 2019) and constitutes one of the greatest public health concerns worldwide. The sources of *Salmonella* contamination are relatively diverse, but one of the most important sources is poultry and poultry products. Thus, eggs and egg products stand out as the most frequently identified source of foodborne *Salmonella* infections (45.6% of *Salmonella* outbreaks in Europe in 2018), followed by various types of meat and meat products (16.8%) (EFSA, 2019).

The serovars most frequently implicated in non-typhoid salmonellosis in humans are *S.* Typhimurium and *S.* Enteritidis. Approximately 71% of confirmed human cases in Europe in 2018 are attributed to *S.* Typhimurium and *S.* Enteritidis, almost 50% of them corresponding to Enteritidis (EFSA, 2019). Similarly, they are also the most prevalent serovars, among the five included in the European National Control Programmes, 2007–2017, in *Gallus gallus* breeding flocks, with a prevalence of 0.25% and 0.12% positive flocks for *S.* Enteritidis and *S.* Typhimurium, respectively. Regarding eggs and egg products, it should be noted that most of the strong-evidence *Salmonella* food-borne outbreaks in the European Union involving them were linked to *S.* Enteritidis (66.7% of cases *versus* 6.5% of *S.* Typhimurium) (EFSA Panel on Biological Hazards (BIOHAZ), 2014). However, in spite of the predominant role that these two serovars play nowadays, it should be noted that this has not always been the case (Foley et al., 2011; Hennessy et al., 2004; Martelli and Davies, 2012). In fact, it is believed that the niche created by the eradication through sanitation efforts of the widespread serovars *Salmonella enterica* Pullorum and Gallinarum in the 1960s, conducted to the emergence of *S.* Enteritidis as the main serovar associated to eggs and egg products (Foley et al., 2011).

In recent years, the prevalence of serovars such as *S.* Kentucky and

* Corresponding author.
E-mail address: guiceb@unizar.es (G. Cebrián).

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S. Heidelberg has increased significantly (EFSA, 2019; Kaldhone et al., 2017). Thus, in the United States of America *S.* Heidelberg replaced *S.* Enteritidis as the most frequently isolated poultry serovar from 1996 to 2006 and since 2007 it has been replaced by *S.* Kentucky (Foley et al., 2011). Similarly, in Europe, *S.* Mbandaka and *S.* Livingstone already exceed *S.* Enteritidis in frequency of isolation in broilers and *S.* Kentucky is the third most commonly found in laying hens, after *S.* Enteritidis and *S.* Infantis (EFSA, 2019). All these data indicate that, in many countries and poultry settings, these emergent serovars have already supplanted *S.* Typhimurium and *S.* Enteritidis as the most relevant serovars associated with poultry production, at least form a food production and animal health perspective. The potential causes underlying these population shifts have been discussed in detail by Foley et al. (2011).

Nevertheless, the data accumulated to date demonstrate that higher on-farm prevalence does not always imply a higher incidence of disease in humans, as these *Salmonella* serovars (*e.g.* Mbandaka and Livingstone) are associated with a low incidence in humans (Foley et al., 2008). Causes of this phenomenon have been partially explored although not completely identified. These studies have focused on the host specificity of the different strains and the mechanisms of egg contamination. These two aspects would explain, for example, the causes of the high incidence in humans of *S.* Enteritidis, which is not host-specific and, moreover, can be transmitted to the egg by transovarian route (Martelli and Davies, 2012). However, none of these reasons could explain why some serovars that are frequently isolated in chickens, such as *S.* Mbandaka or *S.* Livingstone (EFSA, 2019) have such a low incidence in humans, despite not being poultry specific serovars. Unluckily, in spite of their increasing relevance, information on these serovars is still scarce, especially regarding their stress resistance.

Therefore, the aim of this work was to determine the resistance to several stressing agents and food preservation technologies, in laboratory media and in egg products, of 4 strains belonging to emerging *Salmonella* serovars associated to poultry and poultry products and to compare it with that of *S.* Enteritidis strains.

2. Material and methods

2.1. Bacterial strains

To carry out this investigation, 8 strains belonging to *Salmonella enterica* subsp. *enterica* were selected, 4 of them corresponding to *S.* Enteritidis. The strains of *S.* Enteritidis (STCC 4155, STCC 4396, STCC 7160 and STCC 7236) were supplied by the Spanish Type Culture Collection. *S.* Heidelberg DMS 9379 was supplied by the German Collection of Microorganisms. *S.* Kentucky NCTC 5799, *S.* Mbandaka NCTC 7892 and *S.* Livingstone NCTC 9125 were supplied by Public Health England. All strains were maintained frozen at −80 °C in cryovials for long-term preservation.

2.2. Growth conditions

Cultures were grown in 96 wells microtiter plates (Thermo Scientific, Roskilde, Denmark). They were prepared by inoculating 100 µL of tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (Oxoid; TSB-YE) with a single colony previously isolated on a plate of tryptone soy agar supplemented with 0.6% w/v yeast extract (Oxoid; TSA-YE). Microtiter plates were sealed with a polyester impermeable film (VWR International, Leuven, Belgium) and incubated overnight at 37 °C under static conditions. One µL of these pre-cultures was inoculated into 100 µL of fresh TSB-YE and incubated for 24 h under the same conditions to obtain the stationary growth phase cultures that were used for stress resistance determinations. Preliminary studies showed that growth fitness and stress resistance of *Salmonella* cells grown following this methodology was comparable to that of cell suspensions obtained in conventional 250 mL flask under agitation (150 r.p.m.). For some experiments, strains were also grown in commercial pasteurized liquid whole egg (Pascual, Aranda de Duero, Spain).

2.3. Inoculation of poultry products and poultry manure

Medium-sized eggs (53–63 g) were purchased from a local supermarket. The eggshells were thoroughly washed with 70% ethanol, allowed to air dry, and held at room temperature for at least 1 h before each experiment. Eggshell inoculation procedure was similar to that described by Keklik et al. (2010). Ten µL of inoculum solution was spread on the top surface in an area of 2 × 1 cm rendering 7 to 8 Log_{10} CFU/cm^2 on the inoculated egg surface, approximately. To enhance the fixation of the cells, samples were kept under laminar flow in a biological hood for 30 min before the treatments.

Commercial pasteurized liquid whole egg (Pascual, Aranda de Duero, Spain) was inoculated at an initial concentration of 10^7 CFU/mL. The pasteurized liquid whole egg was characterized by measuring its pH, water activity and electrical conductivity. The pH was measured using a pHmeter BASIC 20 (Crison Instrument, Barcelona, Spain), water activity was measured at room temperature with a dew point instrument (Water Activity System mod. CX-1, Decagon Devices, Pullman, WA, USA) and electrical conductivity was measured with a FYA641LFP1 conductivity probe (Ahlaborn, Holzkirchen, Germany) connected to an Almemo 2590 data logger (Ahlaborn, Holzkirchen, Germany).

Commercial mayonnaise (1 g; Hellmann's Mayonesa Ecológica, Univeler España, Viladecans, Spain) was inoculated at an initial concentration of 10^7 CFU/g. This mayonnaise is mainly composed of oil (78%) and egg yolk (7.4%), is acidified to with vinegar and lemon juice and has no other preservative added. Its pH and a_w were measured as described above.

The poultry manure was collected from a *Salmonella* free breeding flock, at the Faculty of Veterinary of the University of Zaragoza (Spain). Its pH and a_w were also measured as described previously. For the inoculation of poultry manure, 5 g of it were inoculated with 20 µL of the inoculum and mixed homogeneously (manually shaking within a Petri dish for 1 min), giving an initial concentration of 10^7 CFU/g.

2.4. Acid, hydrogen peroxide, and sodium chloride and desiccation resistance determinations

Resistance to chemical agents in laboratory media was carried out as described in Guillén et al. (2020). The treatment medium for acid-resistance determinations was citrate-phosphate McIlvaine buffer adjusted to pH 2.5 (Dawson et al., 1974). Hydrogen peroxide resistance was evaluated in 100 mM Tris–HCl buffer (pH 7.0) with hydrogen peroxide added at final concentration of 30 mM (Sigma, St Louis, USA). Resistance to osmotic medium was evaluated in TBS-YE supplemented with 30% w/v of sodium chloride (a_w = 0.786 ± 0.01) (VWR International; NaCl). In all cases, treatments were performed on microtiter plates, and cells were added to the treatment medium to an initial concentration of 10^7 CFU/mL. After inoculation, the suspensions were incubated at a constant temperature of 25 °C throughout the treatment, except for the NaCl determinations, which were carried out at 37 °C due to the low lethality of this agent at room temperature (25 °C). After the selected contact time, up to 50 min, 100 min and 32 h for acid, hydrogen peroxide and sodium chloride determinations, respectively, 20 µL samples were withdrawn at preset intervals and transferred into 180 µL of buffered peptone water (Oxoid; BPW). Subsequent serial dilutions were prepared and pour-plated for survival counts as described below.

Decontamination (washing) experiments in eggshells were carried out following the protocol of Cox et al. (2002), slightly adapted for our purposes. Briefly, treatments consisted in the application of 1.5 mL per

egg of a solution of 2% citric acid or 0.15% hydrogen peroxide to the inoculated eggs with a fine mist sprayer. In parallel, the decontamination efficacy of washing with 1.5 mL of sterile distilled water was also tested, as a control. Treated eggs were air-dried for 1 h before microbiological sampling. For microbial recovery, eggs were gently broken, discarding the contents, and each eggshell was deposited in a sterile stomacher bag (VWR) containing 50 mL of BPW and homogenized for 30 s at 230 r.p.m. in a stomacher laboratory blender (model 400, Seward, West Sussex, UK).

Salmonella resistance to acid conditions was also determined in acidified mayonnaise. After its inoculation, the mayonnaise samples were preserved for up to 12 h at room temperature. Microbial recovery was carried out as described for chemical agents using the laboratory media indicated above.

Resistance to desiccation was determined in two conditions by testing the viability of *Salmonella* strains after its inoculation in eggshells and in poultry manure. The inoculated eggshells and poultry manure were maintained at room temperature and under normal room atmosphere (RH = 69–75%) for up to 24 and 72 h, respectively. Microbial recovery was carried out as described above. In the case of the poultry manure, 5 g were diluted in 45 mL of BPW.

2.5. Heat treatments

Heat treatments were carried out in a Mastia thermoresistometer (Conesa et al., 2009). Briefly, this instrument consists in a 400 mL vessel provided with an electrical heater for thermostation, a cooling system, an agitation device to ensure inoculum distribution and temperature homogeneity, and ports for the injection of microbial suspension and for the extraction of samples. The thermoresistometer was programmed to perform a linear temperature profile from 25 to 58 or 60 ± 0.1 °C at a rate of 2 °C/min. Once treatment temperature had attained stability, 100 µL of the microbial cell suspension was injected into the main chamber containing the treatment media, tryptic soy broth or pasteurized liquid whole egg. After inoculation, samples were collected at different heating times, up to 5 min, and immediately pour plated and incubated for survival counting.

2.6. High hydrostatic pressure (HHP) treatments

HHP treatments were carried out in a Stansted Fluid Power S-FL-085-09-W (Harlow, London, England) apparatus (Ramos et al., 2015). The pressure-transmitting fluid was a mixture of propylene glycol and distilled water (50/50, v/v). An automatic device was employed to set and/or record pressure and time during the pressurization cycle. Cell suspensions were diluted to a cell concentration of 10^7 CFU/mL in citrate-phosphate McIlvaine buffer of pH 7.0 or commercial pasteurized liquid whole egg. Samples were packed in plastic bags, which were sealed without headspace and introduced in the treatment chamber. Treatments were applied at 300 MPa for different treatment times up to 30 min, and temperature never exceeded 40 °C.

2.7. Pulsed electric field (PEF) treatments

The PEF equipment used in this investigation was supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The equipment and treatment chamber have been previously described by Saldaña et al. (2009). Prior to PEF treatments, 100 µL of the microbial cell suspension were dissolved in citrate-phosphate McIlvaine buffer (pH 7.0 and 1 mS/cm of electrical conductivity) or commercial pasteurized liquid whole egg (pH 7.5 ± 0.3 and a conductivity of 6.7 ± 0.3 mS/cm) at a concentration of approximately 10^7 CFU/mL. Samples were placed with a sterile syringe in the treatment chamber. Two different treatment chambers were used, one with a gap of 0.25 cm and an area of 2.0 cm^2 for treatments carried out in McIlvaine buffer and another with a gap of 0.4 cm and an area of 0.79 cm^2 for treatments in liquid whole egg. Treatments were based on square pulses with a width of 3 µs, applied at a frequency of 1 Hz for buffer treatments and at 0.5 Hz for egg treatments. Electric field strengths were set at 25 and 23 kV/cm. Under these experimental conditions, the energy per pulse was 1.88 and 5.63 kJ/kg for buffer and egg treatments, respectively. Treatments of up to 50 pulses (150 µs) were applied. Under these conditions, the final temperature of the treatment media was always below 35 °C.

2.8. Ultraviolet C light (UV-C) treatments

UV-C treatments were carried out in a microtiter plate under static conditions. Microtiter plates were coated with 1 layer of a microplate sealing film (BREATHseal, Greiner bio-one, Frickenhausen, Germany) and located at a distance of 22.50 cm from a 32 W UV-C lamp (VL-208G, Vilber, Germany). Radiation intensity was measured by means of a UVX radiometer (UVP, LLC, Upland, CA). Under these experimental conditions, an intensity of 0.47 ± 0.2 mW/cm^2 was attained. The treatment medium was citrate-phosphate McIlvaine buffer of pH 7.0, and the initial concentration was of 10^7 CFU/mL approximately. Treatment times of up to 120 s were applied and temperature never exceeded 30 °C. The surface-inoculated eggs were exposed to 6.36 ± 0.2 mW/cm^2 up to 15 s, giving a fluence of 0.10 J/cm^2. After its exposure to UV-C light, microbial recovery was carried out as indicated above.

2.9. Recovery after different treatments and survival counting

After treatments, all samples were adequately diluted in BPW and plated in the recovery medium, which was TSA-YE for all the samples but for those of poultry manure that were plated in in Xylose Lysine Desoxycholate Agar (Oxoid; XLD). Plates were incubated for 24 h (48 for XLD agar) at 37 °C, after which the number of colony forming units (CFU) per plate was counted.

2.10. Curve fitting and statistical analysis

All the determinations were carried out by triplicate in different working days. Survival curves (including at least 5 data points) were obtained by plotting the logarithm of the survival fraction (Log_{10} N/N_0) *versus* treatment time (hours for NaCl determinations; minutes for acid, heat, HHP, and peroxide treatments; seconds for UV treatments, and microseconds for PEF treatments). Since deviations from linearity were observed in survival curves to the majority of agents/technologies, the Geeraerd inactivation model-fitting tool from GInaFiT 1.7 (KU Leuven, Belgium), was used to fit survival curves and calculate resistance parameters. Eq. (1) is used to describe survival curves with shoulder and Eq. (2) for those with tail.

$$N_t = N_0 \cdot \exp^{-K_{max} \cdot t} \cdot \left[\frac{\exp^{K_{max} \cdot S_l}}{1 + (\exp^{K_{max} \cdot S_l} - 1) \cdot \exp^{-K_{max} \cdot t}} \right] \quad (1)$$

$$N_t = (N_0 - N_{res}) \cdot \exp^{-K_{max} \cdot t} + N_{res} \quad (2)$$

In these equations, N_t represents the number of survivors, N_0 the initial count, and t the treatment time.

This model describes the survival curves by means of three parameters: shoulder length (S_l), defined as the time before exponential inactivation begins; inactivation rate (K_{max}), defined as the slope of the exponential portion of the survival curve; and N_{res} which describes residual population density (tail). Therefore, the traditional decimal reduction time value (D-value) can be calculated from the K_{max} parameter using Eq. (3).

$$D - \text{value} = 2.303/K_{max} \quad (3)$$

Goodness of the fits of Eqs. (1) and (2) were estimated through R^2 and RMSE calculated with Excel software. Standard deviations (SD) and

Table 1
Resistance (K_{max}, S_l and N_{res}) and goodness of the fit (R^2, *RMSE*) parameters calculated after fitting the survival curves to the 7 agents investigated of the 8 *Salmonella* strains studied to the Geeraerd's model.

	pH						H_2O_2					
	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/mL)	*2D*-value (min)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/mL)	*2D*-value (min)	R^2	*RMSE*
S. Enteritidis 4155	0.208 (0.012)[a]	1.087 (1.883)	–	23.29 (0.639)	0.98–0.99	0.222–0.303	0.098 (0.002)	12.343 (3.729)	–	59.53 (3.041)	0.99–0.99	0.145–0.1-90
S. Enteritidis 4396	0.128 (0.016)	–	–	36.29 (4.255)	0.97–0.99	0.146–0.228	0.111 (0.007)	19.097 (4.507)	–	60.61 (3.775)	0.97–0.98	0.237–0.3-53
S. Enteritidis 7160	0.100 (0.003)	–	–	45.88 (1.240)	0.91–0.98	0.155–0.321	0.121 (0.005)	15.943 (3.566)	–	53.92 (4.384)	0.97–0.98	0.353–0.4-02
S. Enteritidis 7236	0.113 (0.006)			40.72 (2.06)	0.86–0.88	0.428–0.508	0.142 (0.014)	24.560 (2.520)		57.27 (4.318)	0.99–1.00	0.118–0.1-97
S. Heidelberg	0.174 (0.012)	1.055 (1.827)	–	27.69 (3.790)	0.99–1.00	0.084–0.234	0.135 (0.069)	17.953 (7.463)	–	57.72 (8.794)	0.98–1.00	0.134–0.2-95
S. Kentucky	0.320 (0.029)	3.072 (4.995)		17.53 (4.050)	0.99–1.00	0.117–0.344	0.191 (0.059)	25.820 (12.920)		51.93 (3.369)	0.98–1.00	0.097–0.3-23
S. Mbandaka	0.265 (0.078)	6.712 (5.913)	–	25.30 (0.344)	0.99–1.00	0.095–0.188	0.132 (0.025)	21.413 (10.249)	–	57.19 (6.144)	0.96–1.00	0.075–0.3-36
S. Livingstone	0.195 (0.023)	2.148 (3.720)	–	26.04 (1.046)	0.98–0.99	0.181–0.253	0.103 (0.020)	15.238 (8.584)	–	61.17 (4.113)	0.98–1.00	0.091–0.2-13

	NaCl						Heat					
	K_{max} (h^{-1})	S_l (h)	N_{res} (CFU/mL)	*2D*-value (hour)	R^2	*RMSE*	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/mL)	*2D*-value (min)	R^2	*RMSE*
S. Enteritidis 4155	0.520 (0.048)	–	4.374 (0.034)	9.32 (0.944)	0.98–0.99	0.104–0.-195	3.303 (0.398)	0.222 (0.197)	–	1.63 (0.02)	0.99–1.00	0.118—0.319
S. Enteritidis 4396	0.512 (0.154)	–	3.719 (0.202)	8.22 (1.703)	0.95–0.96	0.291–0.-302	2.951 (0.499)	0.053 (0.091)	–	1.64 (0.194)	0.95–1.00	0.120—0.557
S. Enteritidis 7160	0.539 (0.033)		4.338 (0.036)	9.00 (0.645)	0.97–0.99	0.141–0.-205	2.730 (0.292)	0.010 (0.012)		1.71 (0.181)	0.98–1.00	0.01–0.-330
S. Enteritidis 7236	0.547 (0.061)	–	4.556 (0.120)	9.18 (1.112)	0.95–0.99	0.106–0.-260	2.093 (0.279)	0.040 (0.069)	–	2.26 (0.209)	0.96–1.00	0.124—0.472
S. Heidelberg	1.306 (0.268)	–	3.204 (0.681)	3.66 (0.810)	0.98–1.00	0.090–0.-360	1.874 (0.120)	0.237 (0.411)	–	2.70 (0.570)	0.97–0.99	0.171—0.335
S. Kentucky	0.642 (0.093)	–	2.578 (0.390)	7.28 (0.988)	0.98–1.00	0.019–0.-275	1.614 (0.646)	0.995 (1.416)	–	4.21 (0.865)	0.93–0.99	0.076—0.329
S. Mbandaka	0.540 (0.084)	–	2.955 (0.383)	8.69 (1.197)	0.95–1.00	0.083–0.-441	1.472 (0.280)	–	–	3.21 (0.659)	0.88–0.98	0.142—0.618
S. Livingstone	0.539 (0.042)		3.832 (0.424)	8.72 (0.744)	0.98–0.99	0.167–0.-208	1.231 (0.307)			3.89 (0.870)	0.90–0.97	0.252—0.322

	HHP						PEF						UV-C					
	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/mL)	*2D*-value (min)	R^2	*RMSE*	K_{max} (μs^{-1})	S_l (μs)	N_{res} (CFU/mL)	*2D*-value (μs)	R^2	*RMSE*	K_{max} (s^{-1})	S_l (s)	N_{res} (CFU/mL)	*2D*-value (s)	R^2	*RMSE*
S. Enteritidis 4155	1.032 (0.060)	–	3.165 (0.075)	4.48 (0.261)	0.98–1.00	0.019–0.158	0.085 (0.004)	–	4.309 (0.048)	56.29 (2.343)	0.97–0.99	0.168–0.366	0.0581 (0.011)	7.727 (9.170)	–	65.39 (5.896)	0.98–1.00	0.009–0.337
S. Enteritidis 4396		–			0.98–0.99	0.405–0.523		–			0.97–1.00	0.104–0.404			–		0.98–0.99	0.160–0.320

(*continued on next page*)

Table 1 (*continued*)

	HHP					PEF					UV-C				
	K_{max} (min^{-1})	S_l (min)	N_{res} (CFU/mL)	2D-value (min)	R^2	*RMSE*	K_{max} (μs^{-1})	S_l (μs)	N_{res} (CFU/mL)	2D-value (μs)	R^2	*RMSE*	K_{max} (s^{-1})	S_l (s)	N_{res} (CFU/mL)	2D-value (s)	R^2	*RMSE*
	1.617 (0.027)		2.159 (0.158)	2.85 (0.047)			0.172 (0.005)		4.151 (0.086)	27.35 (0.910)			0.092 (0.006)	3.766 (3.454)		54.13 (4.082)		
S. Enteritidis 7160	0.501 (0.003)	–	–	9.19 (0.048)	0.90–0.91	0.475–0.517	0.087 (0.004)	–	4.356 (0.087)	55.61 (2.028)	0.97–0.99	0.237–0.366	0.074 (0.003)	5.986 (5.297)	–	68.63 (7.837)	0.93–0.99	0.179–0.655
S. Enteritidis 7236	0.488 (0.010)	–	–	9.44 (0.196)	0.92–0.94	0.382–0.460	0.078 (0.001)	–	4.419 (0.120)	52.25 (1.276)	0.99–1.00	0.108–0.163	0.052 (0.001)	12.357 (0.835)	–	78.25 (0.876)	0.98–1.00	0.046–0.329
S. Heidelberg	0.569 (0.052)	–	–	8.13 (0.724)	0.95–1.00	0.082–0.443	0.180 (0.035)	–	3.977 (0.626)	26.63 (5.101)	0.99–1.00	0.070–0.161	0.078 (0.011)	8.820 (12.473)	–	68.64 (4.045)	0.99–0.98	0.180–0.292
S. Kentucky	0.837 (0.077)	0.493 (0.405)	–	5.86 (0.784)	0.96–1.00	0.225–0.408	0.185 (0.022)	–	4.133 (0.295)	25.78 (3.458)	0.99–1.00	0.018–0.189	0.073 (0.005)	13.560 (19.177)	–	76.44 (14.849)	0.99–1.00	0.091–0.165
S. Mbandaka	1.178	–	3.632	4.02	0.93–1.00	0.089–0.543	0.178	–	4.032	26.60	0.95–0.99	0.203–0.600	0.084	32.180	–	87.93	0.99–1.00	0.098–0.176
	(0.185)		(0.079)	(0.655)			(0.030)		(0.171)	(4.142)			(0.016)	(9.164)		(1.202)		
S. Livingstone	0.692	–	3.733	6.83	0.96–1.00	0.036–0.375	0.095	–	4.456	51.34	0.99–1.00	0.117–0.195	0.068	17.925	–	89.48	0.99–1.00	0.052–0.247
	(0.077)		(0.176)	(0.810)			(0.006)		(0.215)	(2.644)			(0.023)	(12.482)		(11.730)		

[a] Values in parentheses represent the SD of the means.

Pearson's and Spearman correlation coefficient were calculated using GraphPad PRISM® statistical software (GraphPad Prism version 7.00 for Windows, GraphPad Software, San Diego, California, USA). The same software was used to carry out the Iterative Grubbs' test (Alpha = 0.05) and the statistical analyses (Welch's *t*-test, student t-test and ANOVA; *p*-value < 0.05).

3. Results and discussion

The resistance against seven different preservation technologies and environmental stresses of 4 strains belonging to emerging *Salmonella* serovars associated with poultry and poultry products has been evaluated in this study and subsequently compared with that of 4 strains of *S.* Enteritidis. The adequacy of the methodology used has already been discussed in Guillén et al. (2020). It was previously checked that the resistance of *Salmonella* cells to all the studied agents was similar regardless if they were grown in pasteurized liquid whole egg or in TSB-YE (data not shown). Therefore, for methodological reasons, mainly because liquid whole egg could not be sterilized, all the experiments were carried out with cells grown in TSB-YE. Survival curves to the 7 agents were obtained by plotting the logarithm of the survival fraction *vs* the treatment time, displaying different profiles. These profiles showed deviations from linearity, as an example, the survival curves for hydrogen peroxide and UV showed shoulders, while those for NaCl and PEF displayed tails. Therefore, in order to accurately describe them, the non-linear Geeraerd model (Geeraerd et al., 2000), was used to calculate the corresponding resistance parameters (N_0; S_l; K_{max}, N_{res}). The mean values obtained for these parameters and their standard deviation, along with the goodness-of-fit parameters, are included in Table 1. The traditional decimal reduction time value (*D*) of each survival curve was calculated from its corresponding K_{max} (Eq. (3)). It was decided to use the *2D*-value parameter (time required to inactivate the first 2-Log_{10} cycles) in order to establish meaningful comparisons among strains and/or agents as described in Guillén et al. (2020). Since the *2D*-values obtained for each agent/technology cannot be directly compared because of the different time scale of survival curves, for comparison purposes the calculated resistance parameters were normalized by dividing them by the average *2D*-value of the resistance of all the *Salmonella* Enteritidis strains here studied.

3.1. Resistance to stressing agents and food preservation technologies in laboratory media

Table 1 includes the resistance parameters (S_l; K_{max}, N_{res} and *2D*-values) to the 7 different agents/technologies studied of the 8 strains (4 strains of *S.* Enteritidis, *S.* Heidelberg, *S.* Kentucky, *S.* Mbandaka and *S.* Livingstone) studied. As a way of example the *2D*-values to acid pH (2.5) for the 8 strains of *Salmonella* varied from 17.53 to 45.88 min, being *S.* Kentucky the most sensitive, and *S.* Enteritidis 7160 the most resistant one. In fact, other studies have shown that *S.* Kentucky was more sensitive to acid stress than the serovars Enteritidis and Mbandaka when exposed to the media at a pH of 2.5 (Joerger et al., 2009). Results obtained for all the agents studied are in the range of those previously obtained following the same methodology (Guillén et al., 2020). Only some particular behaviors are worth being noted, such as the low resistance to NaCl observed for *S.* Heidelberg, the barosensitivity of two strains of *S.* Enteritidis, 4155 and 4396, and of *S.* Mbandaka, the high thermotolerance of *S.* Kentucky and *S.* Livingstone and the high UV-C resistance of *S.* Mbandaka and *S.* Livingstone, as compared with previous studies (Guillén et al., 2020).

Variability in resistance among the 8 strains varied depending on the technology investigated. As can be deduced from Table 1 and also from Fig. 1 (see below) the lower variability in resistance was found for H_2O_2 resistance and the highest for HHP resistance. Thus, up to a 3.3-fold difference in resistance to HHP (*2D* values) between the most and least resistant strains was observed. By contrast the *2D*-value of the

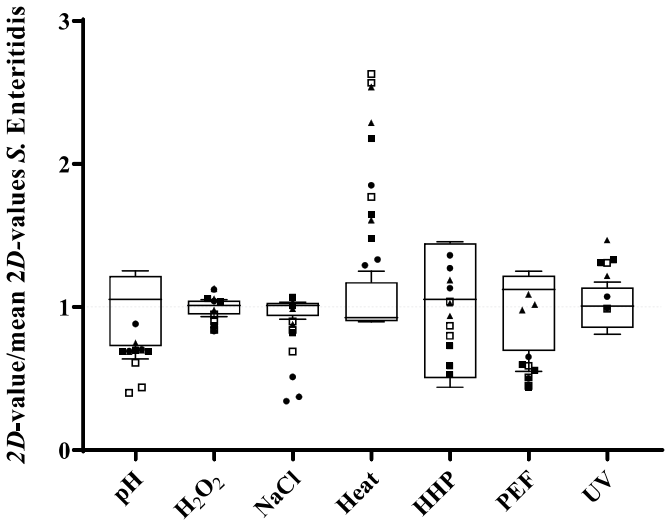

Fig. 1. Variability in resistance (expressed as the ratio between the *2D*-value calculated for each strain and biological replicate and the mean *2D*-value of all *S.* Enteritidis strains) to different environmental stresses and non-thermal food preservation technologies among the *Salmonella* strains studied. The boxes depict the variability among the 4 *S.* Enteritidis strains tested and the points (each one corresponding to a biological replicate) correspond to the values calculated for each emerging poultry-associated *Salmonella* serovar: *S.* Heidelberg ●, *S.* Kentucky □, *S.* Mbandaka ■ and *S.* Livingstone ▲.

most H_2O_2 resistant strain was only 1.2-fold higher than that of the most sensitive one. These ranges are similar to those reported for *Salmonellae* in Guillén et al. (2020), and also to those reported for heat resistance in the meta-analysis carried out by van Asselt and Zwietering (2006) and in the study of Lianou and Koutsoumanis (2013), but lower than those reported by the later authors for acid resistance (up to 6-fold).

To determine whether any positive or negative association could be found among *Salmonella* resistance to the different stresses, Pearson's and Spearman's correlation tests were conducted (Table 2). Before, the iterative Grubbs's test was carried out to detect possible outliers. Grubbs's test identified a unique outlier: the *2D*-value to osmotic medium of *S.* Heidelberg. Therefore, this value was excluded from further analysis.

For this set of strains, a positive correlation was found between resistance to PEF and osmotic stress (Pearson $r = 0.792$, p-value = 0.034; Spearman $r_s = 0.929$, p-value = 0.007) and between UV and heat resistance (Pearson $r = 0.737$, p-value = 0.037; Spearman $r_s = 0.786$, p-value = 0.028). In the previous study these positive correlations were not observed (Guillén et al., 2020). On the other hand, correlations between UV and PEF resistance and between NaCl and H_2O_2 resistance observed in our previous work (Guillén et al., 2020) were not observed for the set of strains used in the present study. These differences might be attributed to different factors, most probably the different number of strains tested and the particular characteristics of the strains included in each set. Thus, in order to obtain a wider and more robust view, the same statistical analysis was carried out including all the strains (15 from the previous study + 8 included in the present study). Results obtained indicate that if the 23 strains are included in the comparison, a positive correlation between PEF and NaCl (Pearson $r = 0.507$, p-value = 0.016; Spearman $r_s = 0.625$, p-value = 0.002) and between H_2O_2 and NaCl (Pearson $r = 0.629$, p-value = 0.002; Spearman $r_s = 0.559$, p-value = 0.008) resistance would exist, but the relationship between PEF and UV and between UV and heat resistance turned to be non-significant. In any case, all these conclusions should be taken with care given the relative low number of strains studied. Potential explanations for the existence or absence of these correlations have been given elsewhere (Guillén et al., 2020).

Fig. 1 illustrates the differences in resistance between the emerging *Salmonella* serovars and *S.* Enteritidis. The normalized resistance values to each agent/technology were calculated as described in materials and methods. In this figure, the resistance of *S.* Enteritidis is depicted in a box and whiskers format, whereas the resistance of the other serovars is included as data points. As can be observed in the figure, emerging serovars (as a cluster) tended to be less acid, NaCl and PEF resistant and more heat resistant than *S.* Enteritidis strains. However, clear deviations from these general trends were observed, such as the PEF resistance of *S.* Livingstone, which was comparable to that of *S.* Enteritidis strains. Further comparison (Welch test) of the resistance parameters calculated for each of the emerging serovars *vs* that of *S.* Enteritidis (the 4 strains considered together) revealed that the pH resistance of the 4 emerging serovars and the PEF resistance of 3 strains (Heidelberg, Kentucky and Mbandaka) was significantly lower ($p < 0.05$) than that of *S.* Enteritidis, in line with the results indicated above. In addition, *S.* Kentucky and *S.* Livingstone strains were found to be significantly more heat resistant, *S.* Heidelberg significantly less NaCl-resistant and *S.* Mbandaka significantly less HHP-resistant and more UV-resistant than *S.* Enteritidis strains.

Altogether, results obtained in laboratory media indicate that, in general terms, the strains of the emerging serovars would display a lower acid and NaCl resistance, a higher heat resistance and similar oxidative HHP, PEF and UV resistance than *S.* Enteritidis cells. However, given the fact that only one strain of each serovar was studied, what it is not representative of the whole serovar and also results in comparisons – *vs S.* Enteritidis – with a different number of samples/replicates (3 *vs* 12), these conclusions should be taken with care. Further work will be required in order to validate these conclusions but, if these results are verified, they offer a potential explanation for the low incidence in humans of these emerging serovars. Thus, in spite of their high on-farm prevalence, their lower resistance to osmotic stress (commonly encountered in surfaces such as the eggshell) and, especially acid stress (which they will face in the stomach) would limit the number of cells reaching the gut and, therefore, the risk of illness. Nevertheless, it is also plausible these emerging serovars might be lacking some virulence gene/s that would play a role in human diseases but that are not necessary to colonize chickens or that they would have a lower ability to use some metabolites, thus making them unable to overcome the microbiota present in the intestine of mammals. In fact,

Table 2
Pearson correlation coefficient values calculated for the *2D* resistance values of the 8 *Salmonella* strains to the different environmental stresses and non-thermal food preservation technologies studied. Values in parentheses correspond to the p-value ($p = 0.05$).

	pH	H_2O_2	NaCl	Heat	HHP	PEF	UV
pH		0.013 (0.976)	0.480 (0.276)	−0.661 (0.075)	0.481 (0.228)	0.476 (0.233)	−0.314 (0.449)
H_2O_2	0.013 (0.976)		0.452 (0.309)	−0.264 (0.528)	−0.363 (0.377)	0.154 (0.716)	−0.089 (0.834)
NaCl	0.480 (0.276)	0.452 (0.309)		−0.606 (0.149)	0.325 (0.477)	0.792 (0.034)	0.028 (0.952)
Heat	−0.661 (0.075)	−0.264 (0.528)	−0.606 (0.149)		0.010 (0.981)	−0.374 (0.361)	0.737 (0.037)
HHP	0.481 (0.228)	−0.363 (0.377)	0.325 (0.477)	0.010 (0.981)		0.526 (0.181)	0.234 (0.577)
PEF	0.476 (0.233)	0.154 (0.716)	0.792 (0.034)	−0.374 (0.361)	0.526 (0.181)		0.113 (0.789)
UV	−0.314 (0.449)	−0.089 (0.834)	0.028 (0.952)	0.737 (0.037)	0.234 (0.577)	0.113 (0.789)	

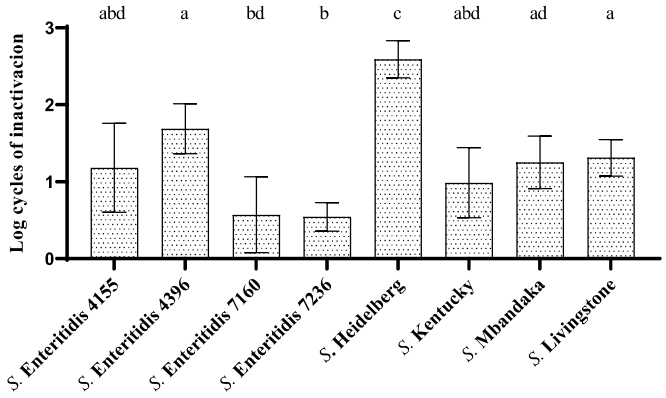

Fig. 2. Log_{10} cycles of inactivation after 2 days of incubation in poultry manure (25 °C; pH 8.42; aw = 0.857) of 8 the *Salmonella* strains studied. Different letters indicate statistically significant differences between strains. Error bars correspond to the standard deviation of the means.

Dhanani et al., 2015, observed that the four *S.* Kentucky strains they studied lacked several SPI2-associated genes, and suggested that this might explain in part their inability to induce diseases in humans. Furthermore, as observed by Shah (2014) it is also plausible that these stress sensitive strains would also display an impaired expression of virulence genes. Future studies examining the genetic and metabolic differences between serovars isolated in chickens and humans are needed in order to elucidate why certain serovars are associated with different hosts.

Given the relevance of these results, in the second part of this investigation the resistance to the different agents/technologies here studied of these strains belonging to emerging *Salmonella* serovars was compared to that of *S.* Enteritidis in eggs and egg products. Poultry manure was also included as it is a very relevant source of contamination and infection of eggs and laying hens, respectively.

3.2. Survival in poultry manure

Fig. 2 includes the Log_{10} cycles of inactivation of the 8 strains after a fixed incubation time (2 days) in poultry manure (pH = 8.42 ± 0.06 and a_w = 0.857 ± 0.02). Survival varied widely depending on the strain (between 0.57 and 2.59 Log_{10} cycles of inactivation) (Fig. 2). *S.* Heidelberg was the most sensitive, and *S.* Enteritidis 4155, *S.* Enteritidis 7160, *S.* Enteritidis 7236 and *S.* Kentucky (no statistically significant differences among these four; $p > 0.05$) the most resistant strains. Thus, a variation of more than 4-fold between the most and least resistant strains was observed (Fig. 2). In general terms, it can be concluded that Enteritidis strains survived better than emerging serovars in manure.

In order to compare these data with those obtained in laboratory media, the following calculations were done. *S. S.* Enteritidis STCC 4155 inactivation after 2 days in poultry manure was taken as the reference value (1.18 Log_{10} cycles). With this value and the inactivation parameters previously determined for this strain in NaCl-added laboratory media (Table 1) the time required to achieve the same level of inactivation (1.18 Log_{10} cycles) for this strain in NaCl-added media was calculated. Then, this time (5.27 h) was used to calculate the Log_{10} cycles of inactivation attained for each of the other 7 *Salmonella* strains studied. These calculations enabled us to make direct comparisons (Log_{10} cycles of inactivation in NaCl-added media *vs* Log_{10} cycles of inactivation in poultry manure) between treatments of a similar lethality. The same procedure was followed to establish the comparisons between laboratory and food products for all the other agents/technologies here studied.

A strong correspondence between these data and those obtained in laboratory media was observed (Pearson $r = 0.859$, p-value = 0.006). This correlation could be explained by the fact that in both cases the water activity (0.786 for NaCl-added media and 0.857 for poultry manure) was well below the *Salmonella* growth boundaries. Regarding poultry manure, the water activity was not so low (0.857) but the pH, among other factors, might also be contributing to *Salmonella* inactivation. The results obtained by Himathongkham et al. (2000) were comparable with those obtained in this study. A lower survival capacity in manure could explain the lower incidence of emerging strains in humans, while the higher survival capacity of *S.* Enteritidis would be a hazard as a source of contamination of eggs and chickens.

3.3. Survival in eggshells and resistance to eggshell decontamination processes

The differences in resistance (Log_{10} cycles of inactivation) in eggshells and to eggshell decontamination processes such as acid and hydrogen peroxide washing and UV-light, of the 8 *Salmonella* strains studied is shown in Fig. 3. As can be observed in Fig. 3A, the number of Log_{10} cycles of *Salmonella* inactivated 24 h after their inoculation in the surface of eggshells varied from 0.71 to 2.88 depending on the strain/serovar studied. As in poultry manure *S.* Heidelberg was among the most sensitive strains being its resistance significantly lower than that of *S.* Enteritidis 7160, *S.* Enteritidis 7236 and *S.* Livingstone. The variability in resistance was also similar to that observed in manure (approx. 4-fold), and a very good correlation between survival in eggshell and NaCl resistance ($r = 0.867$, p-value = 0.005) was found.

Eggshell decontamination, especially for hatching eggs, is critical to the poultry industry in terms of reducing the horizontal transmission of *Salmonella* in the laying house (Cox et al., 2000). Acid and hydrogen peroxide washing and UV-light have been widely demonstrated to be effective methods to eggshell decontamination. Furthermore, UV-light has even been proposed as an alternative to chemical agents given its minimal negative impact on hatchability and cuticle (Al-Ajeeli et al., 2016; Cox et al., 2007; Melo et al., 2019).

Reductions in the number of viable *Salmonella* cells attached to the eggshell between 0.66 and 1.98 Log_{10} cycles for citric acid washings and between 0.55 and 2.13 Log_{10} cycles for peroxide washings were observed depending on the strain/serovar (Fig. 3B and C). A similar result was obtained by Melo et al. (2019), who reported a reduction of 0.84 logarithmic cycles in the number of total microorganisms present in the eggshell after the application of a hydrogen peroxide solution, using a protocol similar to ours.

No correlation was found between the data obtained in laboratory media and washing experiments. In addition, whereas the variability is H_2O_2 resistance among *Salmonella* strains was very low, that to H_2O_2 washings was almost comparable to that of acid washings. These differences might be attributed to different factors/phenomena. For instance, it should be reminded that whereas freshly grown cells were used in the inactivation experiments carried out in laboratory media, in eggshell washing experiments the cells were previously inoculated and dried in the surface of the eggshell, imposing a desiccation stress to *Salmonella* cells that, as described above, would affect them to a different extent depending on the strain.

On the other hand, the number of Log_{10} cycles of inactivation attained after an UV treatment, 0.10 J/cm^2, varied between 1.38 and 3.16, for *S.* Mbandaka and *S.* Enteritidis 4396, respectively (Fig. 3D). These results on eggs are in agreement with several previous reports, in which reductions from 0.60 to 3.24 were observed after the application of UV at a fluence of 0.10 J/cm^2 (Chavez et al., 2002; Holck et al., 2018). It should be noted that in spite of the facts that *Salmonella* cells were also exposed to a desiccation stress and that the intensity applied in eggshell experiments was more than 10-fold higher than in buffer experiments (6.36 *vs* 0.47 mW/cm^2), a good correspondence was found between the results obtained in buffer and in eggshell decontamination experiments ($r = 0.953$, p-value = 0.0002). Despite this strong correlation, the variability in resistance among the strains on the eggshell surface was

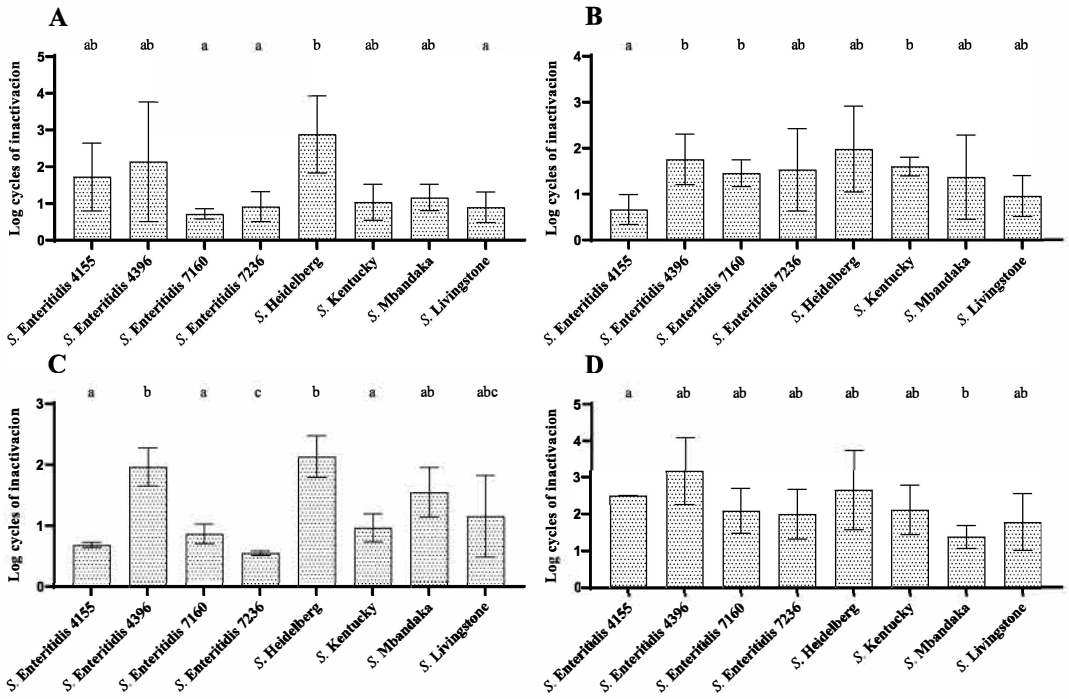

Fig. 3. Survival in eggshells and resistance to eggshell decontamination processes of the 8 *Salmonella* strains studied. A) Log_{10} cycles of inactivation in eggshell after 24 h (25 °C, RH = 69–75%). B) Log_{10} cycles of inactivation after citric acid (2%) washing. C) Log_{10} cycles of inactivation after hydrogen peroxide (0.15%) washings. D) Log_{10} cycles of inactivation after UV treatments (6.36 mW/cm^2). Different letters indicate statistically significant differences between strains. Error bars correspond to the standard deviation of the means.

nearly 2-fold higher than that observed in liquid medium (Table 1).

3.4. Survival in mayonnaise

Acidity is probably the most important intrinsic factor determining *Salmonella* survival in mayonnaise. In this case, the pH of the mayonnaise was pH 3.81 ± 0.3 (and its a_w = 0.937 ± 0.01) and the acidulants included were acetic and citric acid. As can be observed in Fig. 4 *Salmonella* counts after 12 h of incubation (25 °C) in mayonnaise decreased from 1.33 to 2.20 Log_{10} cycles. Variability between strains/serovars was 1.65-fold, with *S.* Enteritidis 4396, *S.* Enteritidis 7169 and *S.* Enteritidis 7236 showing the highest tolerances and *S.* Livingstone the lowest. *S.* Enteritidis strains tended to display a higher resistance in mayonnaise than the emerging serovars, similarly to that observed in acid buffer (Table 1). A strong correlation was obtained between the

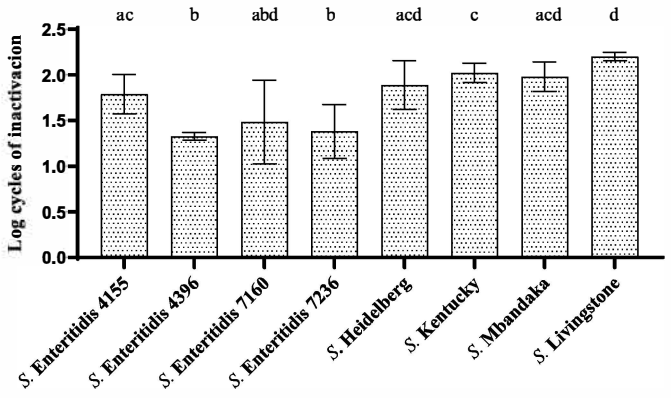

Fig. 4. Log_{10} cycles of inactivation after 12 h of incubation in acidified mayonnaise (25 °C; pH 3.81) of 8 the *Salmonella* strains studied. Different letters indicate statistically significant differences between strains. Error bars correspond to the standard deviation of the means.

results obtained in mayonnaise and those in acidic buffer (r = 0.724, p-value = 0.042). Similar results were found by Zhu and coworkers, who observed that a mixture of *S.* Enteritidis strains was significantly more resistant than a mixture of strains belonging to different serovars of *Salmonella*, including Heidelberg, in acidified mayonnaise with citric acid and acetic acid (Zhu et al., 2012).

3.5. Resistance to liquid egg decontamination/pasteurization technologies

A part of the egg production is intended for the manufacture of liquid egg. In the egg industry, the microbiological safety of liquid products is ensured mainly by heat pasteurization (Lechevalier et al., 2017; Silva and Gibbs, 2012) but other alternatives are being considered, such as PEF or HHP treatments (Monfort et al., 2010, 2012). Thus, in the final part of this work the resistance of the 8 *Salmonella* strains to these 3 technologies (heat, PEF and HHP) when treated in liquid whole egg was determined and compared. The pH and a_w of this liquid whole egg were 7.5 ± 0.3 and 0.996 ± 0.01 respectively, and its electrical conductivity was 6.7 ± 0.3 mS/cm.

First, it should be noted that the resistance of all the *Salmonella* strains studied to these three technologies was higher in liquid egg that in buffer, indicating that the complexity of the composition of liquid whole egg would exert a protective effect, as already described elsewhere (Cebrián et al., 2016). Furthermore, our results indicate that this protective effect would outbalance the sensitization effect that some antimicrobial egg components, such as lysozyme, might induce on *Salmonella* cells (Liang et al., 2002; Masschalck et al., 2001; Smith et al., 2002).

Fig. 5A shows the number of Log_{10} cycles inactivated after 2 min at 60 °C in liquid whole egg. As can be observed, up to 2.71 Log_{10} cycles of inactivation were attained, for *S.* Enteritidis 4155. Our results are similar to those obtained by Gurtler et al. (2015) in liquid egg. Thus, in both studies *S.* Heidelberg was the most heat-sensitive strain, followed by *S.* Mbandaka. Enteritidis strains showed a heterogeneous profile. When comparing data obtained in buffer and in liquid whole egg, a

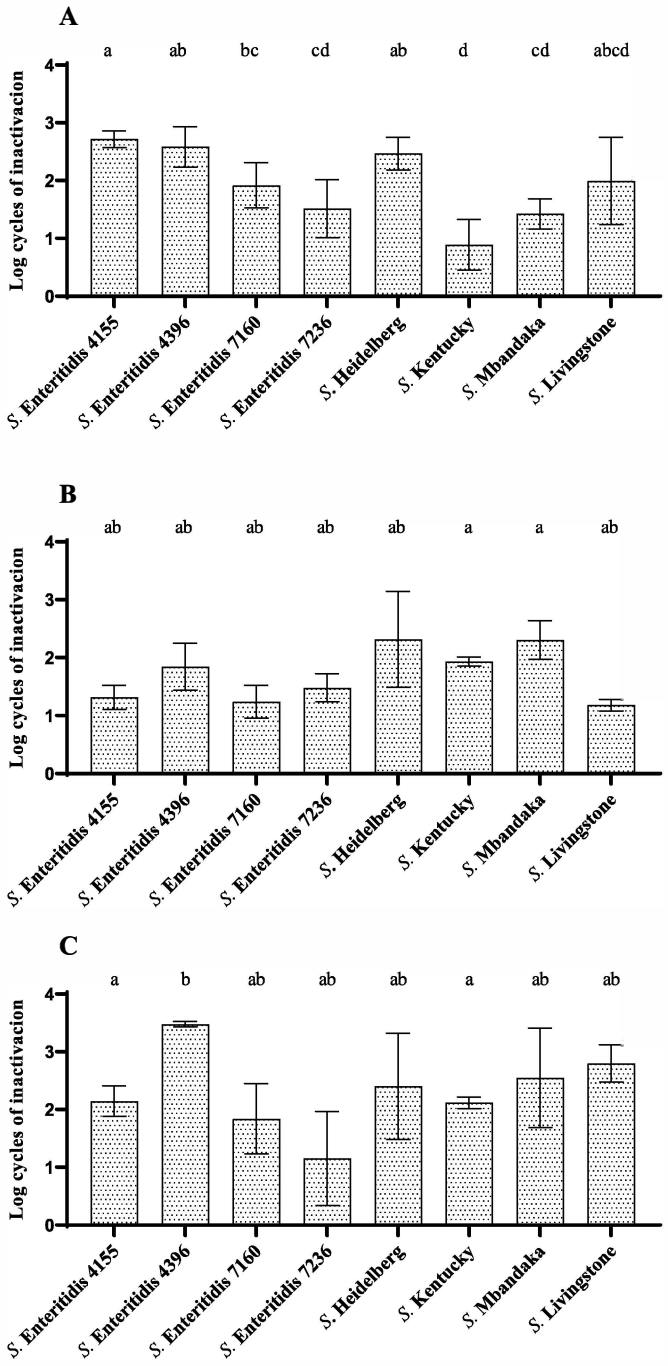

Fig. 5. Resistance (Log_{10} cycles of inactivation) to different liquid egg decontamination/pasteurization technologies of 8 the *Salmonella* strains studied A) Heat treatment (2 min at 60 °C). B) PEF treatment (60 μs 23 kV/cm). C) HHP treatment (20 min at 300 MPa). Different letters indicate statistically significant differences between strains. Error bars correspond to the standard deviation of the means.

Pearson correlation coefficient of 0.701 (p-value = 0.053) and a Spearman correlation coefficient of 0.738 (p-value = 0.046) were obtained. This indicates that, at least, a trend towards an association between both parameters (heat resistance in buffer and in liquid whole egg) would exist. In any case, these results also suggest that the protective effect exerted by liquid whole egg might be different depending on the strain/serovar studied. In any case, given the low number of strains studied and limited treatment conditions explored (a single temperature in each medium) these conclusions should be taken with care and further work will be required in order to fully elucidate if trends observed in buffer can be extrapolated to more complex media such as liquid whole egg.

Regarding PEF, *Salmonella* inactivation in liquid whole egg after 60 μs at 23 kV/cm ranged from 1.18 to 2.32 Log_{10} cycles, these values correspond to *S.* Heidelberg and *S.* Livingstone respectively (Fig. 5B). The variability in resistance among the studied *Salmonella* strains was very low, less than 2-fold, similarly to that observed when they were treated in laboratory media (2.1-fold). These results are similar to those obtained by Monfort et al. (2010) who reported reductions around 2 Log_{10} cycles, after treatments at 20 and 25 kV/cm in liquid whole egg for *S.* Typhimurium, but higher than those observed by Hermawan et al. (2004). As for osmotic and acid stresses and UV treatments, a significant correlation was found between resistance to PEF in McIlvaine and in liquid whole egg (r = 0.914, p-value = 0.002). Thus, in general, in both media, *S.* Enteritidis strains showed a slightly higher resistance than the emerging strains.

The lethality of HHP treatments of 20 min at 300 MPa varied between 1.15 and 3.48 Log_{10} cycles of inactivation, for *S.* Enteritidis 4396 and *S.* Enteritidis 7236, respectively, as depicted in Fig. 5C. Thus, as described for the treatments in buffer, differences in HHP resistance among the 8 strains in liquid whole egg varied approximately 3-fold. Furthermore, a significant correlation was also found between the baroresistance of *Salmonella* in buffer and in liquid whole egg (r = 0.762, p-value = 0.028).

4. Conclusions

From the first part of the study, it can be concluded that variability in resistance among the eight strains studied varied depending on the technology investigated. However, differences in resistance (*2D*-values) were always lower than 3.3-fold. Our results indicate that the strains of the emerging serovars studied would display a lower acid and NaCl resistance, a higher heat resistance and similar oxidative, HHP, PEF and UV resistances than *S.* Enteritidis.

For some agents, including osmotic stresses, UV and PEF, there was a very good correspondence between the results obtained in laboratory media and in real food matrices and/or poultry manure ($r > 0.85$; $p < 0.01$). A significant relationship was also found for acid and HHP resistance ($p < 0.05$) and a trend for heat ($p < 0.10$). Therefore, in general terms, conclusions drawn from the study carried out in laboratory media -regarding intraspecific variability and the relative resistance of the different strains- might be extrapolated, although with caution, to real food scenarios. Further work would be required in order to fully elucidate if this is also true for heat treatments.

Results obtained in this investigation would help to better understand the physiology and ecology of *Salmonella* and to design better egg preservation strategies. It is noteworthy that the lower acid and osmotic stress resistance of these emergent serovars might explain their relatively low incidence in humans. However, this hypothesis is based in the results obtained only with a single strain of each serovar and further work will be required to validate it.

CRediT authorship contribution statement

Silvia Guillén: Investigation, Methodology, Formal analysis, Writing-original draft preparation. **María Marcén**: Investigation, Writing - review & editing. **Ignacio Álvarez**: Methodology, Writing - review & editing. **Pilar Mañas**: Conceptualization, Writing - review & editing. **Guillermo Cebrián**: Conceptualization, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Manuscrito X/Manuscript X

Del laboratorio a la mesa: ejemplo de la aplicación práctica de los datos obtenidos en esta tesis doctoral a la evaluación cuantitativa del riesgo de *Salmonella* en huevos y ovoproductos

Silvia Guillén, Ignacio Álvarez, Pilar Mañas y Guillermo Cebrián

Del laboratorio a la mesa: ejemplo de la aplicación práctica de los datos obtenidos en esta tesis doctoral a la evaluación cuantitativa del riesgo de *Salmonella* en huevos y ovoproductos

Silvia Guillén[1], Ignacio Álvarez[1], Pilar Mañas[1] and Guillermo Cebrián[1]*

[1] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón—IA2—(Universidad de Zaragoza-CITA), 50013, Zaragoza, Spain;
* Correspondence: guiceb@unizar.es (G.C); Tel.: +34-876554127

1. Antecedentes

Tanto en la introducción como en los anteriores capítulos/manuscritos se ha descrito detalladamente la situación actual de la salmonelosis como enfermedad de transmisión alimentaria en la Unión Europea, así como la relevancia de los huevos y ovoproductos como principal grupo de alimentos involucrados en su transmisión. Asimismo, se han indicado algunas de las lagunas de conocimiento que todavía existen a día de hoy como el hecho de que mientras que se han asociado con la enterocolitis aproximadamente unas 2.000 serovariedades de *Salmonella*, es un conjunto más pequeño, de aproximadamente 10-20 serovariedades, el responsable de más del 80% de las toxiinfecciones; destacando *S*. Typhimurium y *S*. Enteritidis (CDC, 2018; EFSA, 2021).

De la misma forma hay muchas serovariedades específicas de determinados grupos de animales, por ejemplo, de los reptiles y así, se calcula que tan solo un 10% de todos las serovariedades se suelen asociar a los huevos y ovoproductos (Gast, 2007). En este sentido cabe destacar que las serovariedades que más han preocupado (por diferentes razones) a la industria avícola han ido variando a lo largo de los últimos 100-150 años. A principios del siglo XX las serovariedades Gallinarum y Pullorum estaban ampliamente distribuidos en las explotaciones avícolas, causando numerosas bajas en las mismas (Shivaprasad and Barrow, 2008). Por ello se pusieron en marcha campañas de saneamiento con objeto de erradicar estas dos serovariedades, lo que condujo a su casi completa desaparición hacia los años 60-70 (Bäumler et al., 2000). A día de hoy se cree que fue este fenómeno el que condujo a la emergencia de *S*. Enteritidis como principal serovariedad asociado a los huevos y ovoproductos, si bien las causas no están aún del todo claras. Una de las teorías más interesantes para explicar este fenómeno postula que esto podría haber sido debido a que inmediatamente tras la erradicación de las serovariedades Gallinarum y Pullorum se produjo un descenso en el nivel de inmunidad de grupo, escenario que aprovechó *S*. Enteritidis (que en un principio se asociaba principalmente a los roedores) para ocupar este nicho ecológico que había quedado vacío (Foley et al., 2011). A esto hay que añadir que estudios realizados mediante el uso de modelos matemáticos, sugieren que la presencia de *S*. Gallinarum sería capaz de excluir por competición a *S*. Enteritidis de las aves (Bäumler et al., 2000). Finalmente, varios autores también han indicado que los cambios en los sistemas productivos que ocurrieron a lo largo de las últimas décadas del siglo XX, como el aumento en la densidad de cría o la integración vertical, podrían haber contribuido también a la diseminación de *S*. Enteritidis (Velge et al., 2005). En cualquier caso, este episodio constituye el primer caso documentado de sustitución de serovariedad/es dominantes de *Salmonella* en el sector aviar y una clara evidencia de que las intervenciones humanas tienen consecuencias que pueden no resultar beneficiosas en todos los sentidos, ya que *S*. Enteritidis representa un peligro para la salud humana mayor que Gallinarum o Pullorum.

De forma análoga a lo descrito anteriormente las serovariedades responsables de la de la aparición de toxiinfecciones alimentarias en humanos por consumo de huevos y ovoproductos no siempre han sido las mismas ni han tenido la misma importancia relativa. No obstante, los datos acumulados hasta

la fecha demuestran que una mayor prevalencia en granja no siempre implica una mayor incidencia de enfermedades en humanos (EFSA, 2021). Las causas de este fenómeno han sido parcialmente exploradas, aunque no del todo identificadas. Así, la mayor parte de los esfuerzos de la comunidad científica para explicarlo se han centrado en el estudio de la especificidad de hospedador de las diferentes cepas y en el de los mecanismos de contaminación del huevo (Foley et al., 2013; Martelli and Davies, 2012; Sabbagh et al., 2010). Estos dos aspectos permitirían explicar, por ejemplo, las causas de la elevada incidencia en humanos de *S*. Enteritidis, ya que es serovariedad de las más prevalentes en los animales/huevos, que no es específicas de hospedador y, además, se puede transmitir al huevo por vía transovárica (Martelli and Davies, 2012). Sin embargo, ninguna de estas razones podría explicar por qué algunas serovariedades que se aíslan con frecuencia en gallinas y pollos, como *S*. Mbandaka o *S*. Livingstone (Bellido-Blasco et al., 2006; EFSA, 2021) tan apenas tienen incidencia en humanos, pese a no ser serovariedades específicas de aves.

Esto podría ser debido, como ya se ha sugerido con anterioridad, a que hay aspectos que todavía no han sido estudiados en profundidad como las diferencias en resistencia al estrés (condiciones medioambientales o tratamientos posteriores) entre las diferentes serovariedades o su capacidad de crecimiento/competición. En este sentido es necesario señalar que, si bien es cierto que existen numerosos estudios acerca de la resistencia de *Salmonella* a diferentes agentes de inactivación como el calor, el medio ácido o el estrés osmótico (como el de Sherry et al. (2004)), las diferentes condiciones de ensayo, o diseños experimentales no adecuados para este fin (por ejemplo, por una inadecuada o insuficiente elección de serovariedades) no permiten sacar conclusiones sólidas a este respecto.

En los últimos años estamos asistiendo a un cambio en las serovariedades de *Salmonella* con una mayor prevalencia tanto en las instalaciones avícolas como en los huevos y ovoproductos. Así, en Estados Unidos *S*. Heidelberg reemplazó a *S*. Enteritidis como las serovariedad más frecuentemente aislada de pollos desde 1996 hasta 2006 y desde 2007 esta ha sido reemplazada por *S*. Kentucky (Foley et al., 2011). De forma similar en Europa *S.* Infantis, *S.* Mbandaka y *S.* Livingstone ya superan a *S.* Enteritidis en frecuencia de aislamiento en pollos de engorde y ponedoras (EFSA, 2021). Esta es una tendencia que es previsible que se consolide a tenor de los planes específicos para el control de determinadas serovariedades, como Enteritidis que se están estableciendo tanto en Europa como Estados Unidos.

Así, en el caso de Europa y en base a estudios de prevalencia de *Salmonella* en distintas especies de consumo realizados en los primeros años de este siglo, la Comisión Europea estableció unos objetivos comunitarios de reducción de la prevalencia de *Salmonella* en los alimentos (de origen aviar). Estos quedan reflejados en el Reglamento (CE) N° 2160/2003, que establece la obligatoriedad de adoptar medidas apropiadas y eficaces para detectar y controlar la presencia de *Salmonella* en todas las etapas de la producción. Basándose en los objetivos marcados en el Reglamento, cada Estado miembro elaboró un programa de control, que en el caso de España se denominaron Programas Nacionales de Control de *Salmonella*.

Los Programas tienen una duración de tres años y afectan aquellas poblaciones animales de producción primaria que tienen riesgo potencial de transmisión de *Salmonella* como zoonosis. Actualmente las especies que son objeto de los Programas son las aves de corral (reproductoras, gallinas ponedoras, pollos de engorde y pavos) y cerdos. Las serovariedades que se controlan dependen de la especie y del tipo de explotación, como se recoge en la siguiente Tabla 1.

Así, en todas las categorías se controlan las serovariedades Enteritidis y Typhimurium, y además en las reproductoras también se controlan las serovariedades Hadar, Infantis, y Virchow.

Estos Programas Nacionales de Control aplican medidas de control en todas las etapas de la cadena alimentaria, desde la producción primaria hasta que los alimentos llegan a los consumidores, lo que, aunque lentamente, está conduciendo a un descenso en la prevalencia de estas serovariedades. Sin embargo, como se ha indicado anteriormente, y también indica EFSA en su informe de 2019 (EFSA BIOHAZ PANEL et al., 2019) esto puede conducir a simples fenómenos de sustitución, con consecuencias difíciles de prever a día de hoy.

Tabla 1. Objetivos de prevalencia *Salmonella* (MAPA, 2021)

Agente	Población animal	Fase de la cadena alimentaria	Fecha de fijación del objetivo
5 serovariedades (SE, ST, SH, SI, SV)	Gallinas reproductoras líneas pesadas (carne) y líneas ligeras (huevos)	Explotaciones de selección, multiplicación y recría de reproductoras	Máximo 1% prevalencia Reglamento (CE) 1003/2005
2 serovariedades (SE, ST)	Gallinas ponedoras	Explotaciones de producción de huevos (recría y puesta)	Máximo 2% de prevalencia Aprobado 7/06/06
2 serovariedades (SE, ST)	Pollos de engorde	Explotación de producción de pollos de carne	Máximo 1% R.584/2008
2 serovariedades (SE, ST)	Pavos	Explotaciones de reproductoras, explotaciones producción de pavos para carne	Objetivo 1% R.584/2008

SE: *S*. Enteritidis; ST: *S*. Typhimurium; SH: *S*. Hadar; SI: *S*. Infantis; SV: *S*. Virchow.

Dado que en esta Tesis Doctoral, y dentro del marco del proyecto "Caracterización de la resistencia al estrés y a los tratamientos tecnológicos, de la capacidad de crecimiento y del potencial patógeno de *Salmonella* Heidelberg, *Salmonella* Kentucky, *Salmonella* Livingstone y *Salmonella* a Mbandaka" que fue galardonado con el Premio a la Investigación del Instituto del Huevo en el año 2018, se abordó la caracterización fenotípica de una cepa de cada una de las serovariedades arriba indicadas y también la de 5 cepas de *S*. Enteritidis, en este capítulo se presenta una aproximación básica a la evaluación cuantitativa del riesgo, en este caso en términos relativos, que supondrían estas serovariedades utilizando como base los datos obtenidos en dicha caracterización.

En este punto es necesario señalar que, como se discutirá más adelante, esta no deja de ser una aproximación extremadamente simplista al problema, dada, entre otras cuestiones, la limitada cantidad de datos existentes acerca de determinados aspectos entre los que destacan el desconocimiento acerca de la variabilidad en resistencia, capacidad de crecimiento y patogenicidad dentro de las diferentes serovariedades estudiadas (salvo *S*. Enteritidis) y, la falta de datos acerca de determinados aspectos extremadamente importantes en las rutas "de contaminación e infección" estudiadas como, por ejemplo, las vías de transmisión al huevo (específicamente si se pueden transmitir por vía vertical) de estas serovariedades emergentes, sus concentraciones en huevo y ovoproductos, y/o sus temperaturas mínimas de crecimiento.

2. Consideraciones previas

En relación a las cadenas alimentarias (o rutas "de contaminación e infección") objeto de estudio se seleccionaron 3 por su relevancia (todas ellas centradas en el estudio de huevo cáscara transformado y consumido en el hogar): mayonesa acidificada, mayonesa sin acidificar y huevo tratado térmicamente (como una tortilla, por ejemplo) aunque una de ellas no se pudo abordar (mayonesa sin acidificar).

A continuación, se describirá en detalle el diseño experimental y consideraciones tenidas en cuenta para la estimación del riesgo en mayonesa acidificada.

En primer lugar, es necesario indicar que el objetivo de este estudio no es hacer una cuantificación absoluta del riesgo que suponen estas serovariedades sino relativa, en comparación con *S.* Enteritidis. Dicho de otra forma, lo que se pretende determinar es si el hecho de que estas serovariedades acaben por sustituir completamente a *S.* Enteritidis de los entornos productivos y cadenas de distribución y transformación de huevos y ovoproductos significaría un incremento en el riesgo (número de casos) de salmonelosis en humanos. Como se ha detallado antes, para poder hacer una evaluación completa serían necesarios datos más detallados acerca de la prevalencia en granjas de cada una de las serovariedades una vez no estuviera presente en ellas *S.* Enteritidis, así como una estimación del nivel de contaminación del huevo que causarían. Asimismo, harían falta nuevos datos acerca de las fuentes posibles de contaminación posterior, y frecuencia y dosis de contaminación de las mismas. Por ello, además de evaluar sólo el riesgo relativo se ha investigado únicamente un supuesto en el que el nivel de contaminación de los huevos era fijo e igual para todas las serovariedades.

En segundo lugar, que, dado que las rutas de contaminación del huevo varían entre serovariedades, y aunque la ruta vertical no es exclusiva de *S.* Enteritidis, tampoco existen datos suficientes en la literatura (al menos que tengamos conocimiento) para poder estimar la proporción de huevos que se contaminaría por cada ruta y para cada serovariedad ni el nivel de contaminación. Por ello el modelo de estudio se restringió al de huevos contaminados externamente (en su cáscara).

En tercer lugar, que, a falta de un modelo de infección mejor, el riesgo de contraer salmonelosis se estimará a partir de los ensayos de adhesión e invasión de células Caco-2. Así, en este caso se interpretará que un mayor número de células capaces de invadir las células epiteliales será indicativo de un mayor riesgo, sin tener en cuenta las posibles diferencias en gravedad de la enfermedad que causarían ni la capacidad de crecimiento y competición en el intestino humano.

Y, en cuarto lugar, que, aunque de forma habitual en los análisis del riesgo estructurados en módulos no se tiene en cuenta posibles respuestas de desarrollo de resistencia ni lo contrario, no es menos cierto que esto puede conducir a una infra- o sobre-estimación notable del riesgo. Por ello de forma previa se realizaron ensayos para determinar si existían estos fenómenos. Así se determinó si la exposición previa al pH ácido de la mayonesa conducía a un cambio en la resistencia al fluido gástrico y si la exposición al fluido gástrico conducía a cambios en el porcentaje de células capaces de infectar los cultivos celulares, no observándose cambios estadísticamente significativos en ninguno de estos dos parámetros. Asimismo, también se observó que en número inicial de células no afectaba significativamente a la resistencia al medio ácido ni al calor ni al fluido gástrico simulado y se asumió que tampoco lo haría a la capacidad de invasión, aunque las concentraciones testadas en laboratorio fueron muy superiores a las utilizadas en la simulación (datos no mostrados).

Así pues, lo que se evaluó en último término (lo que se determinó como "riesgo relativo") fue el número de células capaces de invadir un cultivo de células Caco-2 (o Caco-2/HT29) partiendo de un supuesto en el que las cáscaras de huevo estuvieran contaminadas con 100 células de *Salmonella* y tras ser expuestas a las diferentes condiciones características y representativas de cada una de las cadenas/rutas estudiadas.

3. Metodología

Para hacer las simulaciones, que se llevaron a cabo utilizando el programa @risk (@risk for Excel v. 8.0, Palisade, Ithaca, USA), se seleccionaron las siguientes funciones de probabilidad para cada uno de los tiempos y fases.

En el caso del tiempo de exposición de las células de *Salmonella* al pH ácido de la mayonesa se seleccionó una función log-logística con máximo a las 1,5 horas y valores limitados entre las 0 y 24 horas de exposición, y en el caso de la temperatura de refrigeración de dicha mayonesa una función normal con los valores descritos en (Jofré et al., 2019).

En el caso de los tratamientos térmicos se seleccionó una función normal para la temperatura (Media = 65 °C; desviación estándar = 2 °C) y para el tiempo de tratamiento (Media = 0,5 minutos; desviación estándar = 0,1 °C).

Por su parte para el tiempo de exposición al pH ácido del estómago se escogió una función tipo beta, en base a los modelos de vaciado del estómago descritos por varios autores (Bürmen et al., 2014; Elashoff et al., 1982; Yokrattanasak et al., 2016).

Por otra parte, los parámetros de resistencia (cinéticos) y de capacidad de crecimiento e invasión se obtuvieron a partir de nuestros estudios previos (Guillén et al., 2020b, 2020a, 2021, 2022). Adicionalmente se tuvo que determinar la supervivencia al Fluido Gástrico Simulado formulado según Minekus et al. (2014) de las 8 cepas investigadas. Como puede observarse en la figura 1, las cepas de las serovariedades emergentes estudiadas mostraron una resistencia al mismo comparable a la de las cepas de *S.* Enteritidis. Así, *S.* Mbandaka y *S.* Livingstone mostraron una resistencia similar a la de las cepas más resistentes y *S.* Heidelberg y *S.* Kentucky a la de las más sensibles.

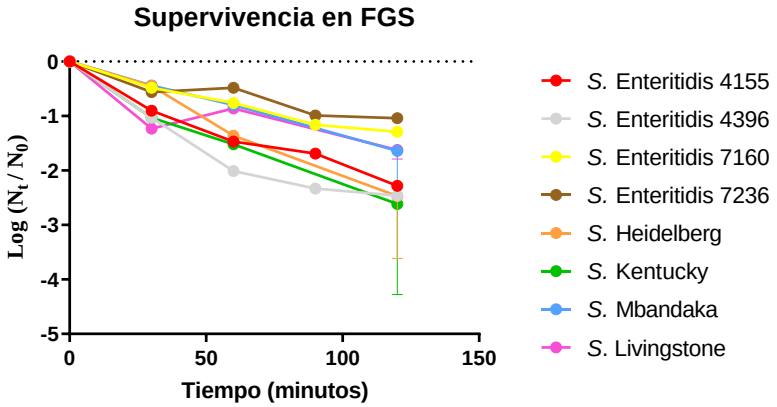

Figura 1: Gráficas de supervivencia frente al Fluido Gástrico Simulado (FGS) de las 8 serovariedades estudiadas. Las barras de error indican la desviación estándar.

4. Resultados y discusión

4.1 Mayonesa acidificada

Como se ha indicado anteriormente en todos los escenarios estudiados el riesgo relativo se determinó a partir del número de células capaces de invadir un cultivo de células Caco-2 (o Caco-2/HT29) partiendo de un supuesto en el que las cáscaras de huevo estuvieran contaminadas con 100 células de *Salmonella*. En este caso, estas células contaminarían una mayonesa acidificada a pH 3,8 que se conservaría entre 0 y 24 horas en la nevera y que posteriormente se ingeriría, estando las células de las diferentes cepas de *Salmonella* expuestas a un pH de 3,0 en el estómago durante un periodo de tiempo variable de entre 0 y 360 minutos, para, finalmente estas células acceder al intestino donde invadirían las células epiteliales en una proporción previamente estimada y característica para cada cepa. La figura 2 ilustra el modelo diseñado para establecer las comparaciones.

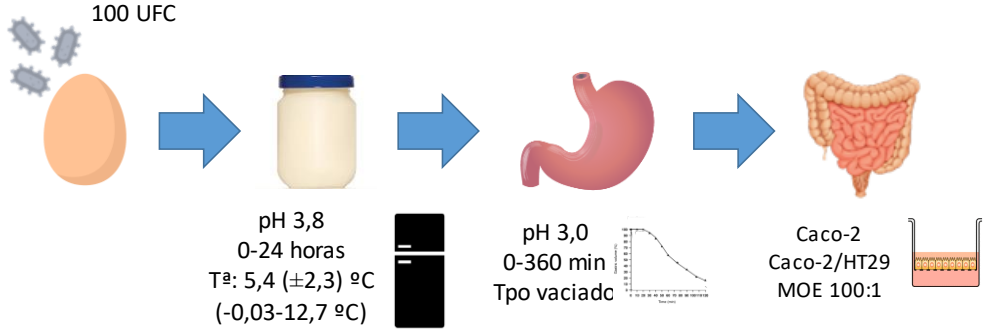

Figura 2: Esquema del modelo de cadena alimentaria (mayonesa acidificada) estudiada.

Los resultados de las simulaciones se ilustran en las figuras 3A y 3B. En las misma se incluyen las gráficas de probabilidad acumulada en función del número de células de *Salmonella* capaces de infectar el cultivo celular. La figura A incluye los datos obtenidos para las 4 cepas de *S*. Enteritidis estudiadas mientras que en la segunda se compraran las dos cepas de *S*. Enteritidis con comportamientos más extremos (las que representarían un mayor y menor riesgo) junto con las 4 cepas de las serovariedades emergentes estudiadas. En dichas figuras aquellas gráficas que aparecen más desplazadas a la derecha son las que se asociarían a cepas/serovariedades de mayor riesgo, ya que esto indicaría que la probabilidad de que el número de células de *Salmonella* que finalmente fueran capaces de infectar el cultivo será mayor.

Como puede observarse en la figura A existieron grandes diferencias entre las 4 cepas de *Salmonella* Enteritidis estudiadas siendo la cepa CECT 7236 la que representaría un mayor riesgo y 4396 la que supondría uno menor (siempre hablando en términos de capacidad de invasión). Al comparar estas dos cepas con las cepas de las otras 4 serovariedades (figura 2B) se puede observar que estas últimas supondrían un riesgo menor que las cepas de la serovariedad CECT 7236. Si bien esto indica que hay cepas de Enteritidis que representarían un menor riesgo no es menos cierto que la existencia de cepas de Enteritidis poco patógenas en vivo, un hecho asociado a una baja resistencia al estrés, ya ha sido documentado por (Shah, 2014).

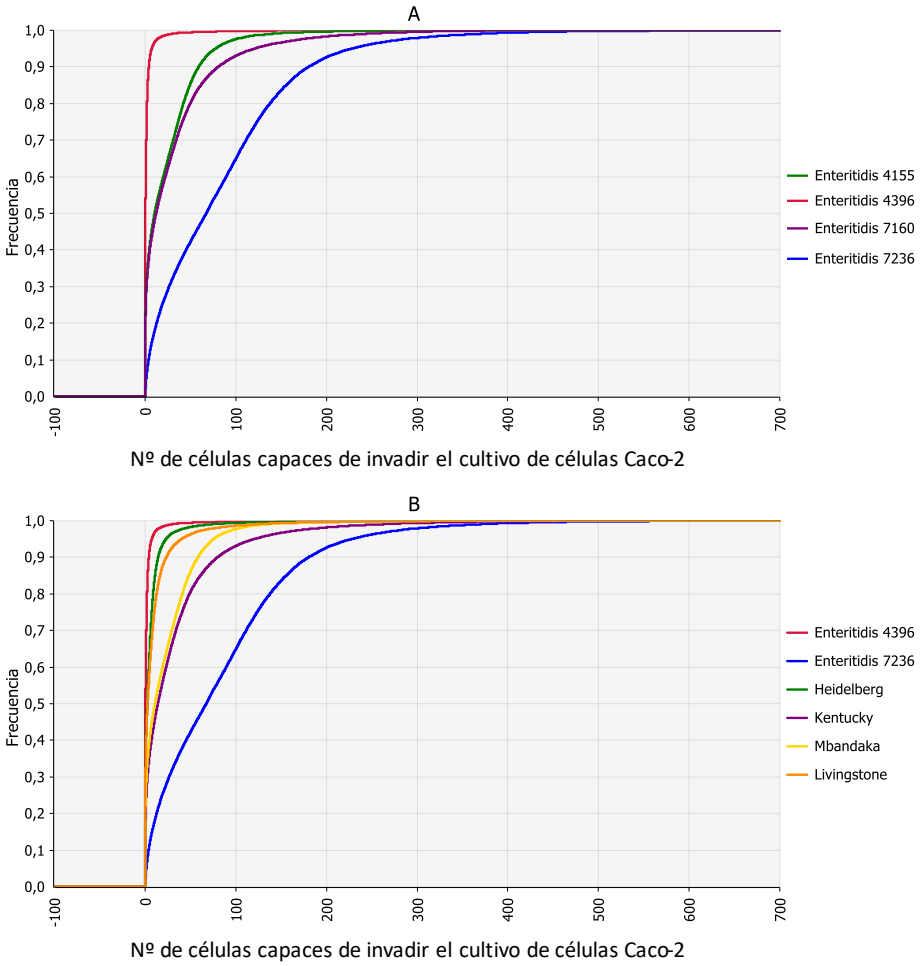

Figura 3: Riesgo relativo de causar enfermedad por consumo de mayonesa acidificada (ver figura 2) de cada una de las 8 serovariedades estudiadas (expresado como distribución de frecuencias del número de células de *Salmonella* capaces de invadir un cultivo de células Caco-2). A: Cepas de *S*. Enteritidis. B: *S*. Enteritidis *vs* serovariedades emergentes.

Adicionalmente también se exploró si estas conclusiones eran extrapolables a una mayonesa acidificada conservada a temperatura ambiente (25 °C). Como puede observarse en la figura 4, los resultados fueron similares a los obtenidos para la mayonesa conservada en refrigeración.

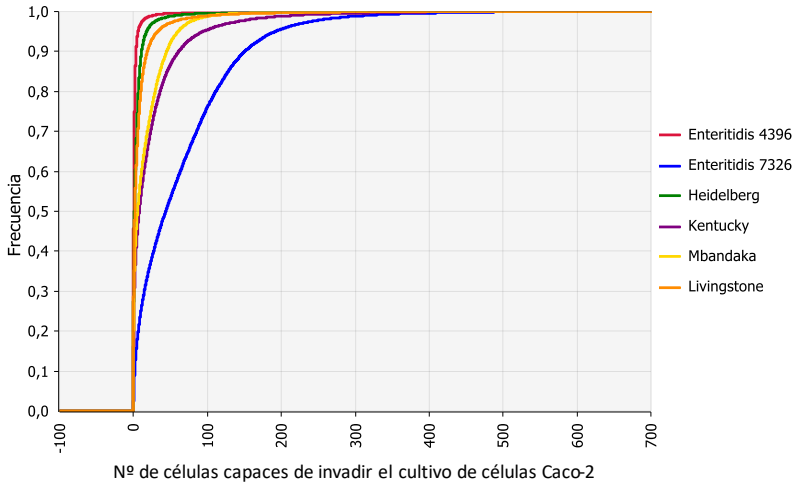

Figura 4: Riesgo relativo de causar enfermedad por consumo de mayonesa acidificada conservada a temperatura ambiente de cada una de las cepas de las serovariedades emergentes en comparación de las cepas de *S*. Enteritidis con mayor y menor riesgo de causarlo (expresado como distribución de frecuencias del número de células de *Salmonella* capaces de invadir un cultivo de células Caco-2).

4.2 Mayonesa sin acidificar

En el caso de la mayonesa no acidificada los resultados obtenidos en este proyecto no resultan suficientes para hacer una adecuada comparación del riesgo relativo. En este sentido resulta imprescindible caracterizar con precisión la temperatura mínima de crecimiento de las diferentes cepas en mayonesa, así como su velocidad de crecimiento a temperaturas permisivas y como esta temperatura modifica la velocidad de crecimiento.

Si, como se deduce de los datos obtenidos en TSB-YE, medios con que simulan las condiciones intestinales y huevo y sus fracciones la velocidad de crecimiento de las serovariedades emergentes en mayonesa es comparable a la de *S*. Enteritidis se puede asumir que el crecimiento post-contaminación en la mayonesa ocurrirá al mismo ritmo para todas las serovariedades por lo que el riesgo relativo dependerá, esencialmente, de la resistencia al fluido gástrico y capacidad de invasión de cada una de las cepas. Partiendo de este supuesto se han hecho también simulación con resultados similares (en términos de riesgo relativo) a los descritos para la mayonesa acidificada. En la figura 5 se muestran los resultados que se obtendrían para la mayonesa recién contaminada. Asumiendo iguales velocidades y temperaturas mínimas de crecimiento y en caso de que la mayonesa fuera conservada en condiciones que permitan el crecimiento todas las gráficas se irían desplazando progresivamente hacia la derecha, pero manteniéndose estables las diferencias entre ellas. En este punto es importante señalar que la resistencia al medio ácido es menor para células en fase exponencial de crecimiento por lo que, paradójicamente, podría darse el caso de, durante la primera parte del almacenamiento, el riesgo de enfermedad sea menor que en el momento de la contaminación a pesar de que el número de células sea mayor. En cualquier caso, este es un hecho que también debe ser estudiado en más profundidad.

Es importante señalar también que en este segundo escenario no testado en el que se produce un crecimiento de *Salmonella* tras la contaminación de la mayonesa, y a falta de nuevos datos, habría también que verificar si el número inicial también afecta a la velocidad de crecimiento en huevo y ovoproductos de estas serovariedades (Guillén et al., 2021) y si los datos obtenidos en huevo líquido son extrapolables a la mayonesa. De hecho, es más que probable que dado que *S*. Enteritidis parece estar muy adaptada a los componentes antimicrobianos y limitación de nutrientes del huevo a estas cepas no

sólo les afecte este fenómeno, sino que lo haga más, aunque, por otra parte, también se ha observado que las diferencias entre cepas y dosis se minimizan en aquellos casos en los que la clara está mezclada con la yema, como sería el caso.

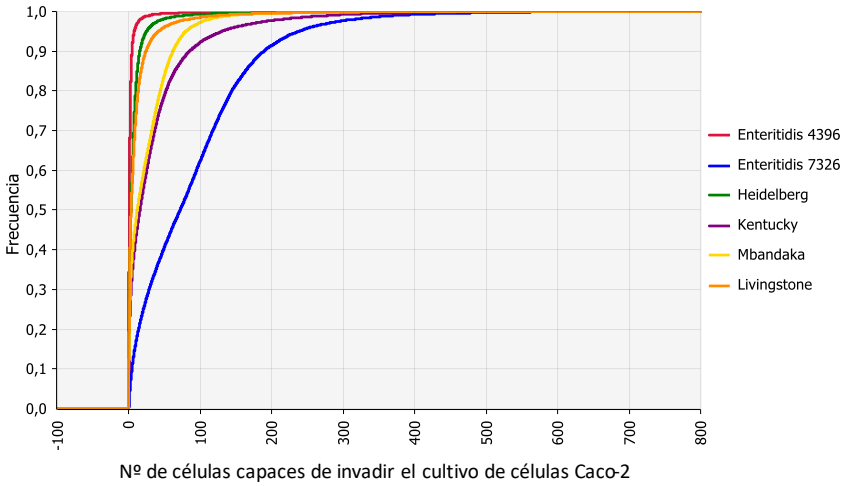

Figura 5: Riesgo relativo de causar enfermedad por consumo de mayonesa (independientemente de su pH) en el momento de su contaminación (dosis=100 células) de cada una de cepas de las serovariedades emergentes en comparación de las cepas de *S*. Enteritidis con mayor y menor riesgo de causarlo (expresado como distribución de frecuencias del número de células de *Salmonella* capaces de invadir un cultivo de células Caco-2).

4.3 Huevo tratado térmicamente

Por último, también se estudió el riesgo asociado al consumo de productos tratados térmicamente como podría ser el caso de una tortilla. Si bien, como en el caso de la mayonesa no acidificada no poseemos datos acerca de los límites inferiores de crecimiento para cada una de las cepas sí se pueden simular diferentes escenarios a partir de los resultados experimentales obtenidos que se presentan en la siguiente figura.

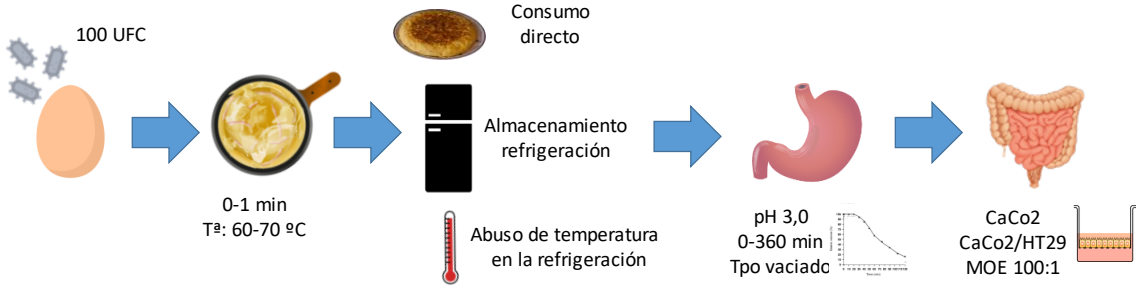

Figura 6: Esquema del modelo de cadena alimentaria (tortilla o producto tratado térmicamente) estudiada.

En relación al primero de los escenarios en el que se haría un consumo directo justo después de la preparación del producto la figura 7 muestra los resultados obtenidos de la simulación. Como puede observarse en este caso los resultados apuntan a que en este caso la cepa estudiada de *S*. Kentucky podría representar un riesgo superior que las cepas de *S*. Enteritidis y de las otras 3 serovariedades estudiadas.

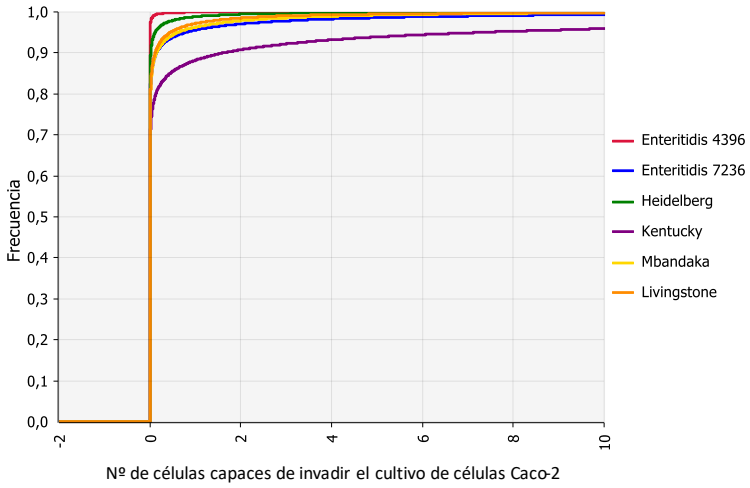

Figura 7: Riesgo relativo de causar enfermedad por consumo de huevo tratado térmicamente (ver figura 6) y consumido inmediatamente después de su preparación de cada una de las cepas de las serovariedades en comparación de las cepas de *S*. Enteritidis con mayor y menor riesgo de causarlo (expresado como distribución de frecuencias del número de células de *Salmonella* capaces de invadir un cultivo de células Caco-2).

No obstante, hay que señalar que, en comparación con los datos obtenidos para la mayonesa, y como se puede observar al comparar las figuras 8A y 8B, este mayor riesgo ocurriría sólo cuando los tratamientos aplicados fueran más elevados lo que, por su parte, corresponde con los escenarios en los que el riesgo de salmonelosis sería más bajo.

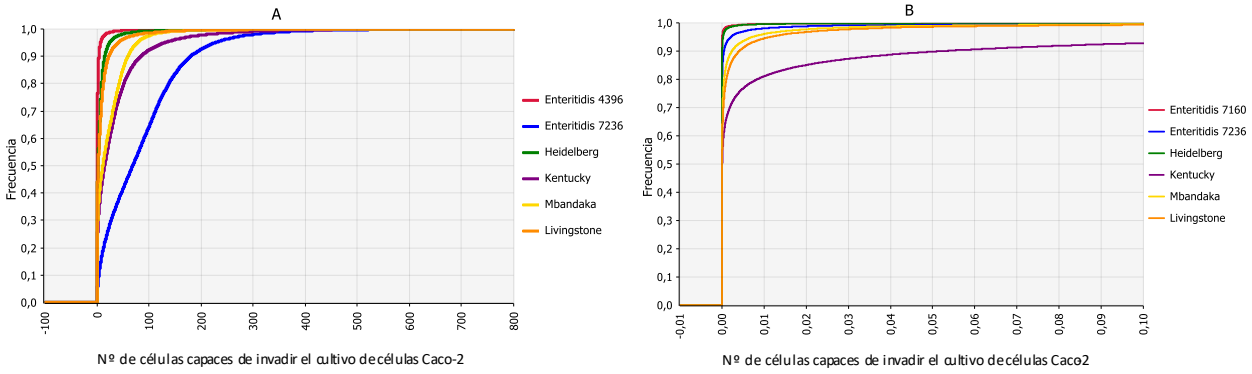

Figura 8: Riesgo relativo de causar enfermedad por consumo de huevo tratado térmicamente (A: 50 °C; B: 65 °C) y consumido inmediatamente después de su preparación de cada una de las cepas de las serovariedades en comparación de las cepas de *S*. Enteritidis con mayor y menor riesgo de causarlo (expresado como distribución de frecuencias del número de células de *Salmonella* capaces de invadir un cultivo de células Caco-2).

Como en el caso de la mayonesa sin acidificar la falta de datos acerca de la temperatura mínima de crecimiento y velocidad de crecimiento a bajas temperaturas impide hacer simulaciones en los escenarios más relevantes, como temperaturas justo por encima de las de refrigeración (7-15 °C). En cualquier caso, usando datos obtenidos para una temperatura de crecimiento de 25 °C para las diferentes cepas -obtenidos en huevo liquido pasteurizado y con un número inicial=10^6 UFC/ml (datos no mostrados)- y aplicando una función log-logística (máximo a 1,5 horas y valores limitados entre las 0 y 24 horas de crecimiento/incubación) para el tiempo de incubación a esta temperatura se puede observar que la inclusión de esta etapa no sólo resultaría en un aumento del riesgo por el crecimiento de los supervivientes sino que también conduciría a un cambio en el riesgo relativo ya que en este caso, por ejemplo, el riesgo relativo de *S*. Kentucky y *S*. Enteritidis CECT 7236 sería muy similar (figura 7 *vs* figura 9).

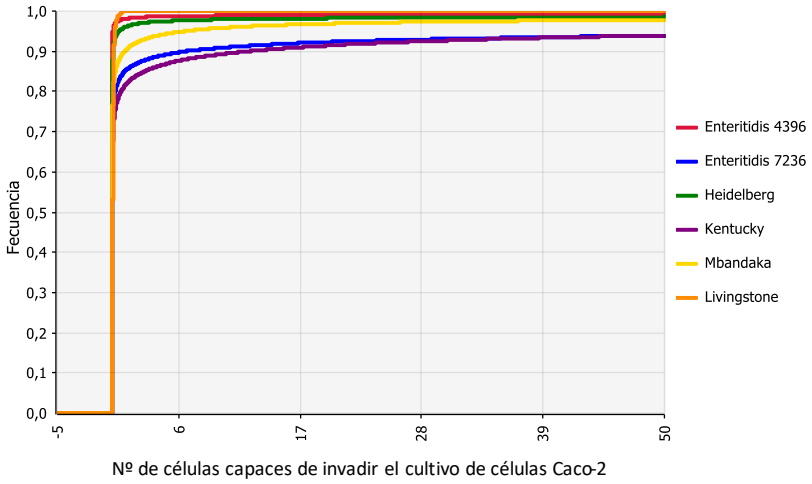

Figura 9: Riesgo relativo de causar enfermedad por consumo de huevo tratado térmicamente (ver figura 6) almacenado a 25 °C durante diferentes periodos de tiempo de cada una de las cepas de las serovariedades en comparación de las cepas de *S*. Enteritidis con mayor y menor riesgo de causarlo (expresado como distribución de frecuencias del número de células de *Salmonella* capaces de invadir un cultivo de células Caco-2).

En este caso, además, y como se ha descrito para los productos con huevo consumidos inmediatamente tras su tratamiento, conforme menor fue la intensidad del tratamiento térmico, menor fue el riesgo relativo de *S*. Kentucky en comparación al de *S*. Enteritidis.

5. Conclusiones

Los datos obtenidos a partir de estas simulaciones sugerirían que, en los escenarios estudiados, el riesgo asociado al consumo de mayonesa acidificada contaminada a partir de células de *Salmonella* de estas 4 serovariedades emergentes presentes en la cáscara no sería superior al que representaría Enteritidis, al menos no el de las cepas de Enteritidis que supondrían un mayor riesgo. Por su parte, son necesarios más estudios para confirmar si los resultados obtenidos para la mayonesa sin acidificar son extrapolables otras condiciones más representativas de las que realmente se dan en los hogares y establecimientos de restauración colectiva, pero también apuntan en el mismo sentido. Por último, tan sólo la cepa de *S.* Kentucky estudiada supondría un riesgo relativo de causar salmonelosis mayor al de las cepas de *S.* Enteritidis estudiadas en productos de huevo tratado térmicamente pero este escenario sólo se daría en los productos sometidos a los tratamientos térmicos más severos que, a su vez, son los de menor riesgo asociado.

Además, es necesario señalar que el hecho de que la resistencia al medio hiperosmótico y en la cáscara de huevo de estas serovariedades también, al menos en las condiciones ensayadas, sea inferior a la de Enteritidis resultaría en un menor número de células en el momento del cascado de los mismos y, por lo tanto, en un menor riesgo. A todo ello hay que añadir que también es más que probable que la transmisión vertical de estas serovariedades sea muy inferior a la de *S*. Enteritidis, lo que todavía refuerza más la hipótesis de que el riesgo que suponen estas serovariedades (o al menos las cepas estudiadas de las mimas) para el ser humano sería igual o menor que el de *S*. Enteritidis.

En cualquier caso, y como es evidente, estas conclusiones deben tomarse con extremada cautela dado lo limitado de los escenarios estudiados, la simplicidad del enfoque aplicado y la falta de datos para describir adecuadamente alguno de los módulos/procesos.

Finalmente, los resultados obtenidos de estas simulaciones también revelan la enorme variabilidad existente entre las cepas de *S*. Enteritidis, que es incluso superior a la variabilidad existente entre cepas de diferentes serovariedades (como las otras cuatro aquí investigadas). Esto evidencia que para hacer una estimación precisa del riesgo asociado a estas serovariedades emergentes también sería necesario analizar varias cepas de las mismas. De hecho, si bien se ha concluido que estas serovariedades/cepas emergentes no supondrían mayor riesgo que las cepas de mayor riesgo de *S.* Enteritidis, sí que tendrían

un mayor riesgo asociado que algunas cepas de esta última serovariedad. En este sentido, una posible explicación a por qué a pesar de la creciente frecuencia con la que estas serovariedades se aíslan de huevo y ovoproductos el número de casos que causan en humanos sigue siendo tan bajo podría basarse en los hallazgos de Shah (2014) que observó al menos dos grupos de cepas dentro de la serovariedad Enteritidis, con grandes diferencias en potencial patógeno. Así, la mayoría de los casos de *Salmonella* Enteritidis sería debidos a tan sólo un subgrupo de cepas y, por lo tanto, para estimar el riesgo asociado a la sustitución de esta serovariedad por otras en los entornos productivos, serían estas últimas las más relevantes, lo que implicaría que las comparaciones, tal y como se han establecido aquí, sí que podrían ser significativas y, sobre todo, representativas.

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Discusión general

Aunque los resultados de esta Tesis Doctoral ya han sido discutidos individualmente en los manuscritos adjuntos en la sección de resultados, el objetivo de esta sección es presentar una discusión global de los resultados con el fin de ofrecer una visión conjunta de los distintos capítulos previamente presentados.

A pesar de la relevancia del género *Salmonella* en la salud y la economía global, todavía se desconocen muchos aspectos de su fisiología, que serían de importancia para su control y prevención. El número tan elevado de serovariedades que pertenecen a este género, más de 2500 serovariedades identificadas hasta la fecha, hace aún más complejo el estudio de este microorganismo. Además, también es bien conocido que las cepas de una misma serovariedad pueden mostrar características fenotípicas muy diferentes (Shah, 2014). No obstante, esto también hace de *Salmonella* un modelo único para el estudio de determinados aspectos como el estudio de la magnitud de la variabilidad en la resistencia microbiana al estrés, la capacidad de crecimiento y la virulencia a diferentes niveles (intra-poblacional, intra-serovariedad e

intra-especie) y también para el estudio de las relaciones existentes entre estas características fenotípicas. Además, el hecho de que semejante diversidad fenotípica se pueda dar entre microorganismos tan próximos genéticamente (de la misma subespecie en el caso de las *Salmonellas* responsables de salmonelosis en humanos) constituye una enorme ventaja para abordar el estudio de las fuentes (mecanismos) de esta variabilidad, o, dicho de otra forma, para el estudio de los mecanismos de resistencia, crecimiento y virulencia.

El **primer bloque** de esta Tesis aborda precisamente el estudio de la resistencia al estrés, capacidad de crecimiento y virulencia de diferentes serovariedades, de las relaciones existentes entre estas tres características fenotípicas y, por último, el de los mecanismos responsables de las diferencias existentes entre las diferentes cepas/serovariedades (con especial atención a la resistencia).

En el estudio de la **resistencia a los diferentes agentes** ambientales o tecnologías de procesado de alimentos utilizados en esta Tesis, abordado en los manuscritos II y IX, se encontró una baja variabilidad de la resistencia entre las 23 cepas de *Salmonella* en medios de laboratorio. Dado que estas 23 cepas pertenecían a 15 serovariedades diferentes se podría haber esperado una elevada variabilidad entre las respuestas a los diferentes agentes. Sin embargo, si se excluye del análisis la elevada termorresistencia de *S*. Senftenberg 775W que es un caso particular ya conocido (Ng et al., 1969) y que se discutirá más adelante, la tecnología en la que se encontró la variabilidad en resistencia más elevada fue frente a las altas presiones hidrostáticas, observándose en todo caso diferencias de menos de 3,3 veces entre el valor *2D* de la cepa más resistente y la cepa más sensible, y los agentes con menor variabilidad fueron la resistencia al medio oxidativo y al UV-C. Esta variabilidad encontrada concordaría, en cuanto a su magnitud, con estudios previamente publicados (van Asselt y Zwietering, 2006; Cebrián et al., 2016b; Sherry et al., 2004). Además, en el estudio realizado por Cebrián et al. (2016b), donde se incluían los datos de 5 cepas de *Salmonella*, 4 de ellas pertenecientes a la serovariedad Typhimurium, se observó, como en esta Tesis Doctoral que las diferencias en resistencia entre las cepas de *Salmonella* eran menores frente a la radiación UV-C que frente a las APH o los PEAV. También cabe destacar que, de acuerdo a dicho estudio, la variabilidad en resistencia frente a diferentes agentes entre las cepas de *Salmonella* fue

menor, en términos generales, que la observada dentro de otras especies microbianas. Si bien el número de cepas de aquel estudio era muy reducido (5) el hecho de que en esta Tesis Doctoral se haya observado una variabilidad de la misma magnitud utilizando muchas más cepas (23) indica que las conclusiones de aquel estudio eran válidas a pesar del reducido número de cepas y que, por lo tanto, y en lo que respecta a los mecanismos de resistencia microbiana , la proximidad genética entre las diferentes cepas de *Salmonella*, sí que se correspondía con una gran homogeneidad en esta característica fenotípica (resistencia al estrés). Por último, el caso de *Salmonella* Senftenberg 775W es muy particular, y su estudio presenta un gran interés desde el punto de vista científico y también práctico. Pese a los esfuerzos realizados, todavía no se ha logrado esclarecer las causas de la mayor resistencia al calor de esta cepa y sería necesario continuar estudiando, habiéndose sugerido que esto podría estar relacionado con las características de sus envolturas y/o con la generación de un menor nivel de especies reactivas del oxígeno durante el calentamiento (Mañas et al., 2001; Serrano, 2015).

En paralelo el diseño experimental de esta Tesis también nos permitió estudiar la variabilidad dentro de una misma serovariedad (intra-serovariedad) y compararla con la variabilidad existente dentro de la subespecie *enterica* (inter-serovariedad), para ello se utilizaron cinco cepas de Enteritidis y 5 cepas de Typhimurium. En el caso de la resistencia al estrés, la variabilidad intra-serovariedad fue comparable o mayor que la variabilidad entre las diferentes serovariedades (inter-serovariedad). Esto podría explicarse porque a pesar de que, en un principio, se asume que la proximidad genética entre las cepas de una misma serovariedad sería mayor que con cepas de otras serovariedades, ya se ha descrito el desarrollo de estrategias de generación de diversidad fenotípica dentro una misma serovariedad, que podría conducir a que cepas de diferentes serovariedades acabaran siendo geno- y feno-típicamente más parecidas entre sí que con otras cepas de sus mismas serovariedades (Gupta et al., 2019; Ko y Choi, 2021; Zheng et al., 2014). A su vez, el hecho de que esta variabilidad fenotípica intra-serovariedad fuera comparable a la inter-serovariedad, explicarían por qué no hay diferencias entre los resultados de este estudio y la comparativa de Cebrián et al. (2016b), en la que se incluían mayoritariamente (4 de 5) cepas de la serovariedad Typhimurium.

Por otra parte, si bien existen mecanismos de desarrollo de resistencia al estrés inespecíficos o generales (como se discutirá en más profundidad más adelante) y se han documentado numerosos casos en los que las cepas de *Salmonella* más resistentes a un estrés también lo son a otros, como en el caso de la resistencia al calor y al pH ácido (Álvarez-Ordóñez et al., 2008; Humphrey et al., 1995) las cepas de *Salmonella* estudiadas en esta Tesis Doctoral mostraron diferentes perfiles de resistencia frente a cada uno de los estreses estudiados, y las cepas más resistentes a un determinado estrés no fueron las más resistentes a otros tipos de estrés. Esto se explicaría ya que los diferentes modos de acción y las dianas celulares no son los mismos para cada agente estresante o tecnología (Cebrián et al., 2016b; Sherry et al., 2004), y a su vez, sería debido a que las respuestas de desarrollo de resistencia de *Salmonella* también varían notablemente en función del estrés que las induce (Arsène et al., 2000; Mackey, 2000). No obstante, se observó una correlación positiva entre la resistencia de las cepas de *Salmonella* al estrés osmótico y a la resistencia a los PEAV. Además, los resultados del manuscrito II sugieren una correlación positiva entre la resistencia a los PEAV y al UV-C. En este punto es conveniente indicar que el mecanismo de acción de cada uno de estos agentes es muy diferente (revisados en Guillén et al. (2020)). En general, el estrés osmótico inactiva las células debido a la perturbación el equilibrio hidrofóbico-electrostático que mantiene la estructura de las proteínas estables/funcionales y, en algunos casos también está asociado al efecto tóxicos específico del soluto utilizado para generar dicho estrés osmótico, como es el caso de la capacidad del ión Na^+ de inhibir la actividad de ciertas enzimas y de los canales iónicos de la célula bacteriana (Murguía et al., 1996; Stewart et al., 2005), mientras que la principal diana de los PEAV son las envolturas celulares (Mañas y Pagán, 2005) y la inactivación microbiana por UV-C se debe principalmente a su efecto sobre el material genético de los microorganismos (Gayán et al., 2014), aunque otros componentes celulares como las proteínas también pueden sufrir daños. En cuanto a esta segunda asociación (PEAV - UV-C) hay que señalar que la fluidez de la membrana se ha propuesto como un factor que interviene en la resistencia microbiana a los UV-C (Gayán et al., 2013), de tal manera que una membrana más fluida haría una célula más sensible a los UV-C. Sin embargo, el papel de la fluidez de la membrana en la resistencia a la PEAV, aunque ha sido ampliamente discutido, aún está por aclarar (Cebrián, Condón, et al., 2016a). Por consiguiente, esta diversidad en los mecanismos

de acción de estos tres agentes, sugiere la existencia de un sistema de respuesta al estrés común, y que, además, debería jugar un papel capital/importante en la resistencia a los tres estreses.

Para mantener la homeostasis celular en una variedad de entornos de estrés, las bacterias pueden utilizar factores sigma alternativos de la ARN polimerasa para así expresar selectivamente subconjuntos discretos de genes. *Salmonella* tiene dos familias de factores sigma, la familia σ^{54} (RpoN) y la familia σ^{70} (RpoD), y dentro de esta última familia se incluye los siguientes factores sigma alternativos: $\sigma^{70/D}$ (rpoD), $\sigma^{24/E}$ (rpoE), $\sigma^{32/H}$ (rpoH), $\sigma^{38/S}$ (rpoS) y σ^{28} (fliA) (Bang et al., 2005; Hartman et al., 2016). El factor sigma alternativo σ^{S} (RpoS) de la ARN polimerasa controla una respuesta adaptativa global, y se ha demostrado que está fuertemente inducido bajo diversas condiciones de estrés, como la inanición, la hiperosmolaridad, la disminución del pH o las temperaturas altas o bajas (Battesti et al., 2011; Hengge-Aronis, 1996). Asimismo, se ha sugerido que RpoS desempeña también un papel en la resistencia a los PEAV y los UV-C en *Salmonella* (Child et al., 2002; Sagarzazu et al., 2013). Precisamente, tres de las cinco variantes resistentes aisladas/investigadas en esta Tesis mostraron una mutación en un gen represor de RpoS, en *hnr*. En *Salmonella*, *hnr* funciona favoreciendo la degradación de RpoS y también controla que ARN mensajero se destruye estimulando la poliadenilación (Carabetta et al., 2009; Zhou y Gottesman, 1998). Así, cualquier cambio en *hnr* tendría el potencial de afectar la actividad RpoS y, en consecuencia, la resistencia al estrés de las células.

Los resultados obtenidos demostraron que las tres mutaciones identificadas condujeron a una mayor actividad RpoS y que, por lo tanto no sólo la mayor resistencia de estos clones era debido a una mayor actividad RpoS (ver manuscrito V) sino también sugería que RpoS tendría un papel muy relevante frente a estos tres agentes (algo que debería verificarse utilizando, por ejemplo, mutantes knock-out en este gen) y que, como ya se ha sugerido previamente, una diferencia en actividad RpoS podría ser una de las causas en diferencia al estrés observadas entre las cepas investigadas. No obstante, no se observó ninguna correlación significativa entre la actividad RpoS y la resistencia a ningún estrés, o, lo que es lo mismo, los resultados indicaron que la actividad RpoS, no explicaría, al menos por sí sola, las diferencias

en resistencia mostradas entre las cepas estudiadas. En este punto es conveniente indicar que tal vez el método utilizado (qRT-PCR) no sea el método más preciso para determinar la actividad RpoS y poder realizar comparaciones entre cepas o que tal vez sería conveniente estudiar la actividad de RpoS en otros puntos de la curva de crecimiento, ya que se ha visto que RpoS se induce en el inicio de la fase estacionaria, durante la transición de la fase exponencial a la fase estacionaria (Lange y Hengge-Aronis, 1991), ya que en este caso el nivel de expresión de RpoS se midió en cultivos de 24 horas, es decir en una fase estacionaria avanzada. Siendo todo esto cierto también es necesario señalar que las alternativas (para la cuantificación de la actividad RpoS) son muchísimo más costosas experimentalmente (como por ejemplo a través del uso de proteínas fluorescentes como GFP con un promotor controlado por RpoS) dado que habría que introducir el inserto en 23 cepas y que estas técnicas no están exentas de problemas metodológicos. Además, también es importante recordar que la variabilidad en resistencia observada fue muy baja para todos los agentes (lo que indicaría pocas diferencias en la expresión de mecanismos de resistencia) y que para cada agente el comportamiento de las cepas fue diverso, mientras que, de ser RpoS la principal fuente de variabilidad y dado que se ha demostrado su relevancia en la resistencia a varios de los estreses aquí estudiados, cabría esperar que para varios de ellos la respuesta de las cepas (la resistencia relativa) fuera similar.

Teniendo en cuenta todo lo arriba indicado es más que razonable concluir que, probablemente, los resultados obtenidos sí que estén reflejando de forma bastante precisa la realidad y por ello se podría afirmar que, en un principio, la actividad RpoS no sería la principal fuente de variabilidad en resistencia al estrés entre las cepas de *Salmonella*. Esto no implica que esta proteína reguladora no sea relevante en la resistencia al estrés de este microorganismo. De hecho, lo más probable es que mientras que la expresión de RpoS sea una condición necesaria para el desarrollo de resistencia frente a al menos algunos estreses, lo que sucedería es que existirían otros factores específicos que tendrían una mayor relevancia en la resistencia de *Salmonella* frente a cada uno de los agentes/tecnologías.

En el primer bloque de esta tesis también se pretendía verificar si, como han demostrado diferentes autores para otros microorganismos o condiciones experimentales, el **desarrollo**

de respuestas al estrés (o adquisición de resistencia a ciertos antibióticos) tendría un coste en términos de capacidad de crecimiento en condiciones adversas y de competición frente a otros grupos microbianos en el caso del género *Salmonella* (Andersson y Hughes, 2010; Zambrano et al., 1993). Por ello se estudiaron las posibles relaciones entre la resistencia al estrés, la capacidad de crecimiento o virulencia, y otras características fenotípicas como fueron la formación de biofilms o la resistencia a antibióticos.

Si bien los datos obtenidos con las variantes resistentes indican que, efectivamente, algunas mutaciones puntuales que pueden conducir a un incremento en resistencia tienen un coste en términos de capacidad de crecimiento (especialmente en los medios más limitantes) no se encontró ninguna correlación (en un principio debería ser inversa) entre la resistencia al estrés y la capacidad de crecimiento de las 23 cepas de *Salmonella* estudiadas. Este último hecho podría ser debido a diferentes causas, como por ejemplo que el coste en términos de capacidad de crecimiento se ha demostrado que depende de la naturaleza del agente estresante/respuesta microbiana desencadenada (Karatzas, Hocking, et al., 2008; Karatzas, Randall, et al., 2008; Urdaneta et al., 2019). Otra posible explicación sería que a lo largo de su proceso evolutivo estas cepas (o las cepas de *Salmonella* en general) habrían adquirido mutaciones compensatorias que les hicieran recuperar la capacidad de crecimiento que perderían cuando aparecieran mutaciones que les confirieran resistencia al estrés. Esta parece una posibilidad razonable dado que, salvo ciertas excepciones, las células de las diferentes cepas de *Salmonella* se ven expuestas a condiciones medioambientales muy cambiantes, lo que hace que estén sometidas a diferentes presiones selectivas, que pueden ser agentes inactivantes en unos casos, pero en otras la necesidad de competir por nutrientes. Además es importante matizar que si bien es posible que se produzca una reversión de la mutación, se ha demostrado que es más frecuente encontrar mutaciones compensatorias que reversión de la mutación (Andersson y Hughes, 2010; Guillén et al., 2021; Levin et al., 2000)

Por lo que respecta a la relación entre resistencia al estrés y virulencia se puede concluir que, en general, y tal y como se ha indicado para la relación entre resistencia y capacidad de crecimiento no se observó ninguna correlación, con algunas y muy particulares excepciones como la particular correlación entre resistencia al medio ácido y adhesión e invasión o las

correlaciones positivas entre la resistencia a los PEAV y al UV-C y la capacidad de invasión. El modelo *in vitro* de adhesión e invasión seleccionado para establecer las comparaciones fue el constituido únicamente por células epiteliales de Caco-2, ya que, como se indica en el Anexo I, se obtuvo una muy buena correlación entre los resultados de adhesión e invasión obtenidos en este modelo y los obtenidos en modelos más complejos basados en el uso de co-cultivos de células Caco-2 y HT29-MTX con o sin la adición de una microbiota sintética (viva o inactivada), lo que indicaría que la virulencia relativa de las diferentes cepas podía estimarse en este modelo más sencillo. Todas las matizaciones de tipo general arriba descritas para los resultados obtenidos acerca del coste del desarrollo de respuestas de resistencia en términos de capacidad de crecimiento son aplicables para la relación de las primeras con la virulencia, pero, además, hay que tener en cuenta que en el caso de las expresión de factores de virulencia es un proceso muy complejo y estrechamente regulado de múltiples pasos en el que la adhesión y la invasión de las células epiteliales es tan importante como la supervivencia al ácido, la bilis, el estrés oxidativo y otros mecanismos de defensa del huésped, y tanto como la proliferación intra y extracelular. Es más, desde la ingestión del patógeno hasta el establecimiento de la enfermedad, y dependiendo de la zona de tracto digestivo y/o condiciones reinantes los factores de virulencia que *Salmonella* debe expresar son completamente diferentes y, en algunos casos hasta opuestos. Un ejemplo paradigmático de la complejidad de este proceso y la dificultad de establecer correlaciones entre virulencia y otros caracteres geno- o fenotípicos es el caso de RpoS, regulador que se sabe que juega un papel fundamental en varias etapas de este proceso (Kim et al., 2021; Nickerson y Curtiss, 1997), pero para el que se han publicado observaciones contradictorias (Krogfelt et al., 2000; Methner et al., 2004), lo que sugiere que RpoS podría estar desempeñando diferentes funciones en cada uno de estos pasos.

En cualquier caso, es evidente que son necesarios más estudios, no sólo para dilucidar completamente el papel de RpoS en la patogénesis de *Salmonella*, y en el caso que nos ocupa, sino también, si esto podría explicar la correlación positiva observada entre invasión y la resistencia a la radiación UV-C y los PEAV (que parecen estar especialmente influenciadas por dicha actividad RpoS).

Y finalmente, en lo que respecta a la relación entre resistencia al estrés y el resto de características fenotípicas estudiadas indicar que, más allá del estrés ácido, no se observó ninguna correlación entre ninguno de los agentes estresantes estudiados o la capacidad de crecimiento y la formación de biofilms. Al igual que no se encontró ninguna correlación entre la resistencia a los estreses y a los antibióticos estudiados.

A la vista de todo lo arriba indicado y si bien hay que tener en cuenta que los resultados obtenidos aquí -con sólo 23 cepas- no pueden extrapolarse (o esto debe hacerse con cuidado) directamente a toda la subespecie *enterica*, la combinación de la baja variabilidad en resistencia y capacidad de crecimiento existente con la incertidumbre asociada a este tipo de determinaciones podría estar dificultando la identificación de correlaciones o llevando a la aparición de relaciones casuales, pero no causales, y que, por último, mutaciones puntuales, como las observadas en las variantes resistentes aisladas en este estudio, sí que implicaron un coste en la capacidad de crecimiento de las células, los resultados obtenidos en esta Tesis Doctoral indican que, al menos a nivel de subespecie, la hipótesis de que "el desarrollo de respuestas de resistencia al estrés conlleva un coste en términos de capacidad de crecimiento o virulencia" no se cumpliría a nivel de la subespecie *enterica* de *Salmonella enterica*.

Por otra parte, verificar esta hipótesis también implicó cuantificar la variabilidad en la capacidad de crecimiento de *Salmonella*, en la virulencia y el resto de características fenotípicas estudiadas.

La **variabilidad en la capacidad de crecimiento** de las 23 cepas de *Salmonella* fue comparable (en órdenes de magnitud) a la variabilidad en resistencia al estrés determinada. La mayor variabilidad en la velocidad máxima de crecimiento (μ_{max}) y las mayores μ_{max} se encontraron en TSB-EL, diferencia inferior a 3 veces entre la cepa con mayor y menor μ_{max}. A pesar de ser un medio nutritivo, sin ninguna limitación de nutrientes, las cepas mostraron similar variabilidad que en un medio con concentraciones limitadas de hierro (LB-DYP) o un medio mínimo con gluconato como fuente exclusiva de carbono. En estudios previos se sugirió que la variabilidad de las cepas de *S. enterica* y *Listeria monocytogenes* era mayor cuando las condiciones de crecimiento se volvían desfavorables, o dicho de otra forma, cuanto más próximo a los límites de crecimiento en pH y concentración de NaCl (Aryani et al., 2015;

Godínez-Oviedo et al., 2021; Lianou y Koutsoumanis, 2011) pero hay que señalar que esto sería un reflejo no de la capacidad de crecimiento sino, probablemente de la diferencia en capacidad de resistencia al estrés de las diferentes cepas, aunque en condiciones subletales. Son varias las hipótesis que pueden plantearse para explicar las diferencias entre nuestros resultados y los obtenidos por estos autores. Por una parte, está el hecho de que, como se ha indicado previamente, la capacidad de crecimiento en condiciones subóptimas estaría determinada por una combinación entre capacidad de crecimiento, adquisición y utilización de nutrientes y resistencia al estrés. Siendo en nuestro caso las variabilidades en capacidad de crecimiento (en medio óptimo: TSB-EL) y resistencia al estrés bajas y muy parecidas (aproximadamente 3x) y teniendo las células microbianas unos recursos genéticos y energéticos limitados sería razonable asumir que cerca de los límites de crecimiento la variabilidad sería similar ya que aquellas cepas con mayor capacidad de crecimiento probablemente no fueran las que mayor resistencia tuvieran (aunque los resultados arriba indicados parecen refutar esta hipótesis). Otra explicación podría ser que mientas en los estudios de Aryani, Godínez-Oviedo y Lianou las condiciones subóptimas de crecimiento se generaban aplicando un estrés físico-químico, en esta Tesis se trataría de un estrés "nutricional" por lo que las conclusiones de dichos autores podrían no ser extrapolables a los datos aquí presentados. No obstante, también hay que señalar que al estudiar la velocidad de crecimiento de cuatro cepas de *S.* Enteritidis en huevo y ovo-productos (resultados que se discutirán con más detalle más adelante) se observó que la variabilidad en velocidad de crecimiento aumentaba sustancialmente al disminuir la dosis de inóculo, lo que correspondía con una menor biodisponibilidad de hierro, e indicaría que la variabilidad en capacidad de crecimiento sí podría ser mayor en condiciones más limitantes. En cualquier caso, estos resultados corresponden a tan sólo cuatro cepas y será necesario determinar si los mismo son extrapolables al resto de cepas aquí estudiadas.

Por el contrario, la **variabilidad en el resto de características fenotípicas** estudiadas fue mucho mayor. Como ya se ha comentado anteriormente la complejidad de los factores de virulencia y su regulación es muy elevada (Velge et al., 2012; Winstanley y Hart, 2001) y se ha sugerido que los mecanismos de adhesión e invasión podrían ser dependientes de la serovariedad (Gagnon et al., 2013). De acuerdo a nuestros datos, la serovariedad Enteritidis,

en promedio, mostró la mayor capacidad tanto de adhesión como de invasión en las condiciones estudiadas, pero, además, en invasión demostró ser el grupo más heterogéneo de cepas. Esta heterogeneidad ya ha sido descrita por Shah (2014), que observó al menos dos subgrupos de cepas con diferente virulencia dentro de la serovariedad Enteritidis y explicaría a su vez los resultados obtenidos en las simulaciones presentadas en el capítulo 5. Este hecho es extremadamente importante pues indicaría que, probablemente, la dominancia de *S*. Enteritidis como agente causal de salmonelosis sería atribuible no a todas las cepas de esta serovariedad, sino a un subgrupo más específico, que debería estudiarse en mayor profundidad y, probablemente clasificarse, de forma separada (de hecho ya se ha sugerido en varias ocasiones que la clasificación por serotipos debería ser sustituida por otro tipo de clasificaciones (EFSA BIOHAZ PANEL, 2019).

Por otra parte, todas las *Salmonellas* incluidas en el estudio fueron capaces de formar biofilms, en mayor o en menor grado, pero esta característica fue, junto con la resistencia a los antibióticos, en la que las diferentes cepas de *Salmonellas* presentaron una mayor variabilidad. Las causas de esta gran variabilidad han sido intensamente estudiadas aunque no del todo aclaradas (Berger et al., 2009; Klerks et al., 2007; Patel y Sharma, 2010). Si bien la resistencia a los antibióticos es un tema de mayor relevancia, este queda fuera del ámbito de esta investigación y por ello no se discutirá. Mientras tanto, y en relación a la formación de biofilms, cabe destacar que se ha sugerido que existe una conexión entre una mayor adaptación del huésped (cepas específicas *vs* inespecíficas de hospedador) y la capacidad de formar biofilms (MacKenzie et al., 2017). Así, la formación de biofilms está muy conservada en las cepas de *Salmonella* asociadas a la gastroenteritis, pero esta capacidad se habría perdido en las cepas de *Salmonella* responsables de enfermedades invasivas o adaptadas a un huésped concreto, como sería el caso de la serovariedad Typhi (MacKenzie et al., 2017; Römling et al., 2003). Nuestros resultados no contribuyen a aclarar la veracidad de esta afirmación ya que, salvo Gallinarum, las serovariedades estudiadas no son específicas de hospedador, aunque es cierto que esta cepa fue una de las que poseyó una menor capacidad de formarlos.

En el **segundo bloque** de esta Tesis Doctoral se pretendía validar los resultados más relevantes de entre los obtenidos en la primera parte en huevo y ovoproductos. Esto es debido a que, aunque, en general, los resultados obtenidos en medios de laboratorio pueden ofrecer predicciones precisas de la resistencia o el crecimiento microbiano en los alimentos, es posible que no tengan en cuenta factores importantes como la estructura del alimento, la competencia/interacción con otros microorganismos o el estado fisiológico de las células microbianas, entre otros, y por tanto serían necesarios estudios para verificar estos resultados en condiciones reales.

En los primeros ensayos de validación del crecimiento en huevo y ovoproductos se observaron diferencias en la capacidad de crecimiento de las *Salmonellas* dependiendo de la dosis inicial de inóculo, y se vio que la dosis inicial de inóculo parecía afectar a la velocidad de crecimiento microbiana, un fenómeno extremadamente curioso que no había sido descrito hasta la fecha y que además aparecía o no en función de la fracción de huevo estudiada y su historia térmica. Así, los resultados revelaron que las curvas de crecimiento iniciadas a dosis más bajas condujeron a fases de latencia o adaptación más largas, y a tasas de crecimiento específicas máximas más bajas *(μ_{max})* en el huevo entero líquido crudo.

Aunque la influencia del número de bacterias presentes en un cultivo en la duración de la fase de latencia ya había sido previamente observada en medios de laboratorio (Bertrand, 2019), nadie ha documentado previamente que esto pueda influir en la velocidad máxima de crecimiento. Estos resultados no sólo resultaban curiosos, sino que parecían contradecir la asunción (generalmente aceptada) de que la máxima velocidad de crecimiento es específica para cada microorganismo y medio de crecimiento pero que no se ve afectada por otros factores como la dosis. Sin embargo, el estudio más detallado de los mecanismos responsables del fenómeno proporcionó la clave para explicarlo. Así, esta invariabilidad de la velocidad máxima se basa en el principio de que la máxima velocidad de crecimiento en un determinado medio viene determinada por las condiciones del mismo y, en último lugar, por el factor más limitante para el crecimiento en él, ya sea un parámetro físico-químico, un nutriente o una combinación de los dos. Así, nuestros resultados se explicarían por el hecho de que el nutriente más limitante en huevo entero crudo y clara (cruda y/o pasteurizada) es el hierro,

cuya biodisponibilidad crecería conforme aumentaba la dosis inicial/número de células en el medio, lo que haría que cuanto más altas sean estas últimas mayor sea la velocidad de crecimiento de *Salmonella* en estos productos.

Igualmente es relevante el hecho de que nuestros resultados también demuestran que la biodisponibilidad del hierro es mayor en productos pasteurizados y que, por lo tanto, la velocidad de crecimiento en estos productos de *Salmonella* sería mayor. Y lo es porque el riesgo de sufrir salmonelosis está relacionado con uno o varios de los siguientes escenarios: la existencia de una población muy elevada de *Salmonella* en el huevo crudo antes de la pasteurización, la aplicación de un tratamiento inadecuado/insuficiente, o la contaminación posterior a la pasteurización. Según los datos mostrados en esta Tesis, la recontaminación (en cualquier otro escenario el problema sería mayor en huevo crudo) posterior a la pasteurización del huevo entero líquido o la clara de huevo representaría un riesgo mayor para los consumidores que el de los productos no pasteurizados. Esto es de gran relevancia, ya que los productos de huevo comercialmente pasteurizados se utilizan a menudo como ingredientes en los alimentos sin ningún tratamiento térmico adicional durante la preparación de los mismos.

Como se ha descrito en el manuscrito VII, tanto el efecto de la fracción, del tratamiento térmico como el de la dosis en la velocidad de crecimiento de *Salmonella* en huevo y ovoproductos tendrían en común la causa subyacente: la biodisponibilidad de hierro. Así, de acuerdo a nuestro modelo, las máximas velocidades de crecimiento se alcanzarían en la yema, una fracción en la que no hay limitación de hierro (ni en concentración ni por la presencia de proteínas que lo secuestren). Al estar el hierro ya suficientemente biodisponible en la yema cruda en este producto no se observó efecto del tratamiento térmico ni de la dosis. En el otro extremo se encuentra la clara, en el que la biodisponibilidad del hierro es muy limitada, especialmente debido a la presencia de proteínas como la ovotransferrina y Ex-FABP. En este medio las células de *Salmonella* requieren el uso de sideróforos (Enterobactinas y Salmochelinas, especialmente estas últimas) para la captación de hierro y para alcanzar su máximo potencial de crecimiento (tasa). Cuando las células de *Salmonella* están presentes a baja densidad celular en el huevo entero crudo, los sideróforos tienen un efecto privativo (sólo

haciendo el hierro accesible a la propia célula), y el crecimiento sería lento. Sin embargo, si están presentes/inoculadas a una densidad suficientemente alta, los sideróforos se liberan al medio; los sistemas antimicrobianos (limitantes del hierro) presentes en la clara serían parcial o totalmente superados, y las células de *Salmonella* serían capaces de crecer (a una velocidad más alta conforme mayor es la concentración de sideróforos en el medio). Estos dos fenómenos: baja biodisponibilidad de base y la dosis-dependencia de la liberación de sideróforos explicarían por qué la dosis determina la velocidad de crecimiento en la clara. La aplicación de tratamientos térmicos de intensidad creciente provocaría una desnaturalización progresiva de esas proteínas limitadoras del hierro (principalmente de la ovotransferrina), lo que permitiría a las células de *Salmonella* captar hierro liberado por esta proteína por otros sistemas, lo que resultaría en una mayor velocidad de crecimiento de *Salmonella* y, eventualmente, la desaparición de la dependencia inicial de la dosis en la tasa de crecimiento de *Salmonella*. No obstante, nuestros resultados indican que esto último no llega a ocurrir en la clara, probablemente por la baja intensidad de los tratamientos térmicos que se le pueden aplicar. Finalmente, el huevo líquido entero se encuentra en una situación intermedia ya que resulta de la mezcla de las dos fracciones. Eso explicaría que estando crudo la velocidad de crecimiento de *Salmonella* en él sea dosis dependiente pero que una vez tratado térmicamente desaparezca completamente el efecto dosis (la concentración de proteínas con efecto antimicrobiano sería menor y los tratamientos que se pueden aplicar también más intensos).

Todo lo arriba indicado también explicaría el efecto que estos tres factores: fracción, historia térmica y dosis tienen en la temperatura mínima de crecimiento de *Salmonella* en huevo y ovoproductos que es, probablemente, un parámetro incluso más relevante desde el punto de vista de la gestión y prevención de riesgos que la velocidad máxima de crecimiento. Además, hay que señalar que, en este caso, y como ya se ha comentado cuando se han discutido los resultados obtenidos acerca de la capacidad de crecimiento de *Salmonella* en medios de laboratorio, la variabilidad en la capacidad de crecimiento entre las cepas de *S.* Enteritidis fue mayor conforme menor era la biodisponibilidad del hierro, lo que sugería que podría estar asociada a la capacidad de producción de sideróforos. En cualquier caso, los resultados obtenidos en esta Tesis Doctoral a este respecto no son concluyentes y sería necesaria una

investigación más profunda para determinar si esta es la última o mayor causa, de las diferencias en temperatura mínima observada.

Por último, como ya se ha descrito en la introducción y en los manuscritos IX y en el ejemplo de aplicación práctica (manuscrito X), las serovariedades que más han preocupado a la industria avícola han ido variando a lo largo de los últimos 100-150 años debido a la intervención humana en la cadena agroalimentaria. En los últimos años, la prevalencia de serovariedades como Kentucky, Heidelberg, Livingstone o Mbandaka está aumentando en ganado aviar e incluso reemplazando a *S*. Enteritidis en pollos de engorde o gallinas ponedoras (EFSA, 2021; Kaldhone et al., 2017). En cambio, una mayor prevalencia en granja no siempre implica una mayor incidencia de enfermedades en humanos (EFSA, 2021). Dada la relevancia de este hecho y con objeto de verificar otra de las hipótesis planteadas en esta Tesis Doctoral "las diferencias en resistencia al estrés y capacidad de crecimiento entre las diferentes serovariedades de *Salmonella* podrían explicar, o contribuir a explicar, la diferente frecuencia con la que dichas serovariedades causan gastroenteritis en humanos" y de tratar de determinar, aunque de forma aproximada, el riesgo que supondría para la salud humana la completa sustitución de *S*. Enteritidis por otras serovariedades en las gallinas ponedoras. Dicho de otra forma, lo que se pretendía determinar es si el hecho de que estas serovariedades acaben por sustituir completamente a *S*. Enteritidis de los entornos productivos y cadenas de distribución y transformación de huevos y ovoproductos significaría un incremento en el riesgo (número de casos) de salmonelosis en humanos.

Por ello en primer lugar se estudió la resistencia a las mismas frente a diferentes agentes/tecnologías y se comparó con la de 4 cepas de Enteritidis, la principal serovariedad asociada a huevos y ovoproductos durante los últimos años (Foley et al., 2011). Estos datos, que demuestran que existe una buena correlación entre la resistencia determinada en medios de laboratorio y en matrices reales (lo que implicaba que los datos de resistencia relativa e influencia de los parámetros de tratamiento determinados en medios de laboratorio podrían extrapolarse a escenarios alimentarios reales), junto a los de velocidad de crecimiento y capacidad de invasión permitieron además hacer una serie de simulaciones acerca de diferentes escenarios que sugieren que estas serovariedades emergentes no supondrían un riesgo para la salud mayor que, al menos, las cepas más "peligrosas" de *S*. Enteritidis. Las

limitaciones del enfoque aplicado han sido comentadas y discutidas extensamente, pero demuestran al menos a modo de ejemplo la utilidad de los datos obtenidos en esta Tesis para mejorar las evaluaciones cuantitativas del riesgo que supone *Salmonella* en huevo y ovoproductos.

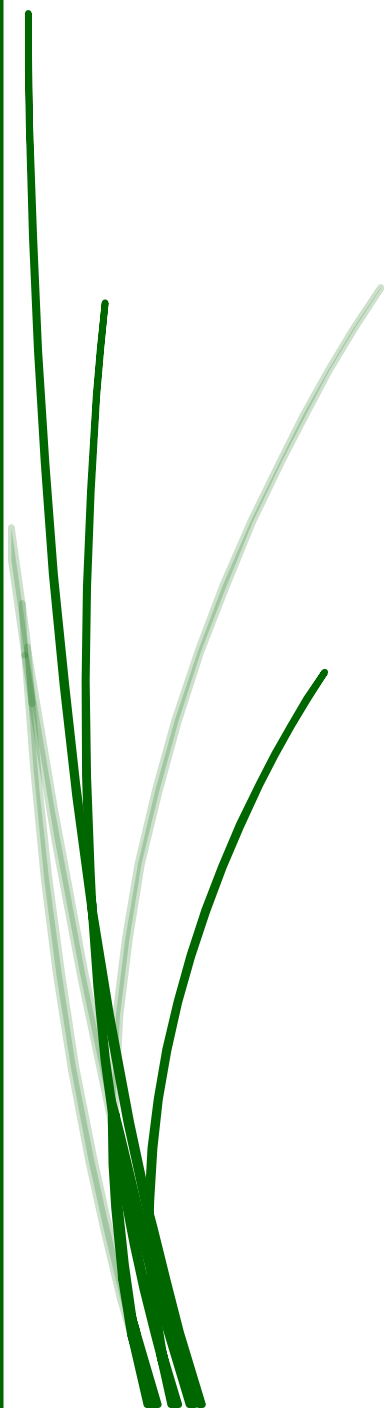
Pese a que el crecimiento y supervivencia de *Salmonella* en huevos y ovoproductos es probablemente uno de los temas más estudiados en microbiología alimentaria y predictiva, resulta evidente que todavía quedan muchos aspectos por investigar para así poder desarrollar medidas de control más eficaces que las actuales. Así, a la vista de la complejidad de las relaciones entre resistencia, crecimiento y patogenicidad microbianas y dado que en muchos casos, las células de *Salmonella* (y otros patógenos alimentarios) se ven sometidas a múltiples estreses pero también pasan por fases en las que se pueden multiplicar en los alimentos, parece claro que los enfoques determinísticos (criterios de proceso) deberían ser progresivamente combinados y/o sustituidos por enfoques probabilísticos (evaluaciones del riesgo) que proporcionarían información más realista acerca del riesgo asociado a los diferentes productos y agentes patógenos. Esto permitiría desarrollar herramientas predictivas más precisas que, por ejemplo, podrían ayudar a predecir el impacto que podrían tener las serovariedades o microrganismos emergentes incluso antes de que hayan sustituido a los ya dominantes, y con ello implementar las medidas adecuadas para controlar definitivamente a este patógeno alimentario.

Una de estas nuevas herramientas que están ganando interés en los últimos años, a la vista de estas diferencias en la variabilidad dentro de una misma serovariedad, son los modelos "multinivel", los cuales posibilitan cuantificar (e integrar en los modelos) la variabilidad y la incertidumbre de los datos. Estos modelos ayudarían a distinguir entre la incertidumbre (fuentes de variación que no se consideran en el sistema, como errores de medición o errores de especificación del modelo) y la variabilidad biológica (diferencias en la respuesta de células individuales). Los modelos disponibles son todavía escasos, probablemente porque para una mejor aproximación requiere la adquisición de nuevos datos experimentales para diferenciar y cuantificar adecuadamente las fuentes de variabilidad. De hecho, actualmente con los datos obtenidos en esta Tesis se está desarrollando un modelo multinivel, de base probabilística, que integre la incertidumbre del ensayo y la variabilidad entre réplicas y entre cepas y que

permita determinar con mayor precisión el crecimiento de *S*. Enteritidis en huevo y ovoproductos en función de la cepa, la temperatura de almacenamiento, la historia térmica del huevo y el número inicial con la que el producto resulte contaminado (Anexo II).

En resumen, los resultados obtenidos en esta Tesis Doctoral contribuyen a comprender mejor la fisiología y la ecología de Salmonella, siendo especialmente útiles para definir procesos de conservación de alimentos seguros y para mejorar las evaluaciones cuantitativas del riesgo microbiológico. No sólo proporcionan una estimación de la variabilidad intra e inter-serovariedad en la resistencia al estrés, el crecimiento y la virulencia dentro de las Salmonella no tifoideas, grupo al que corresponden todas las cepas estudiadas en esta Tesis Doctoral, sino que también ayudan a identificar las cepas que podrían suponer un mayor riesgo para la seguridad alimentaria debido a su mayor capacidad de crecimiento, resistencia al estrés o virulencia. Particularmente relevantes son los resultados obtenidos en relación al crecimiento e inactivación de Salmonella en huevos y ovoproductos, ya que estos datos que pueden resultar muy útiles para desarrollar evaluaciones de riesgo cuantitativas de Salmonella más precisas, mejorar sus procesos de producción, almacenamiento y distribución y/o para, en base a ellos, revisar o perfeccionar las directrices actuales para la gestión de estos productos, tanto dentro de la industria agroalimentaria como para los consumidores.

Conclusiones/Conclusions



CONCLUSIONES

1 La variabilidad en resistencia al estrés encontrada entre las 23 cepas de *Salmonella* estudiadas fue baja, con una diferencia de menos de 3,3 veces en el valor *2D* para todos los agentes estudiados (si se excluye del análisis la resistencia de *S.* Senftenberg 775W frente al calor). La variabilidad intra-serovariedad fue similar o mayor que la variabilidad interserovar, a pesar de la mayor proximidad genética existente entre las cepas pertenecientes a una misma serovariedad.

2 Las cepas de *Salmonella* que mostraron la mayor resistencia frente a un agente/estrés no lo fueron frente a otros agentes/estreses. No obstante, se encontró una correlación entre la resistencia de las cepas de *Salmonella* frente al estrés osmótico y a los PEAV.

3 La variabilidad en capacidad de crecimiento entre las diferentes cepas de *Salmonella* fue similar (en magnitud) a la variabilidad en resistencia al estrés, con una diferencia de menos de 3 veces entre cepas independientemente del medio de crecimiento estudiado. Las cepas que mostraron una mayor velocidad de crecimiento en medios ricos en nutrientes (no selectivos) también la mostraron en medios deficientes en hierro o que contenían gluconato como única fuente de carbono.

4 La capacidad de invasión y adhesión dependió de la serovariedad estudiada, con *S.* Enteritidis mostrando la mayor capacidad de invasión de las investigadas. Las cepas con una mayor capacidad de adhesión no fueron siempre las más invasivas.

5 Las características fenotípicas en las que se observe la mayor variabilidad fueron la capacidad de formar biofilms (hasta 30 veces) y la resistencia a los antibióticos que, dependiendo del antibiótico investigado varió hasta en 64 veces entre las cepas.

6 En términos generales, una mayor resistencia al estrés no supuso un coste en términos de capacidad de crecimiento para las cepas de *Salmonella* estudiadas.

7 RpoS parece desempeñar un papel esencial en la resistencia de *Salmonella* al estrés osmótico, los PEAV y la luz UV-C. No obstante, las diferencias en actividad RpoS existentes entre cepas no pueden explicar, al menos por sí solas, las diferencias en resistencia (frente a ningún agente) observadas entre las cepas de *Salmonella* aquí estudiadas.

8 Tres de las cinco variantes resistentes aisladas/investigadas mostraron mutaciones en el gen *hnr*, lo que sugiere que esto podría constituir una estrategia evolutiva conservada (entre el género *Salmonella*) de adquisición de resistencia. En este caso, este desarrollo de resistencia sí que tuvo un coste en términos de velocidad de crecimiento.

9 La dosis inicial y la historia térmica del huevo líquido entero y la clara determinan la velocidad de crecimiento de *Salmonella* en estos ovoproductos. Sin embargo, esto no ocurre en la yema de huevo. Este fenómeno parece estar relacionado con la biodisponibiidad del hierro que sería mayor en productos pasteurizados y cuanto mayor fuera el número inicial de células.

10 De la misma forma, la temperatura mínima de crecimiento de *S.* Enteritidis también dependería de la dosis inicial y la historia térmica del huevo entero líquido y la clara, pero no en yema de huevo.

11 Para la mayoría de los agentes estudiados se observó una correspondencia significativa entre la resistencia determinada en medios de laboratorio y en matrices reales (alimentos y gallinaza).

12 Los resultados obtenidos sugieren que las serovariedades emergentes en ganado aviar no supondrían un riesgo para la salud humana superior a *S*. Enteritidis, al menos en los escenarios estudiados/simulados en esta Tesis Doctoral.

CONCLUSIONS

1 Low variability (less than 3.3-fold change in 2D-values the for all agents studied) in stress resistance was found among the 23 *Salmonella* strains studied (if S. Senftenberg 775W heat resistance is excluded from the analysis). Intra-serovar variability was comparable or higher than inter-serovar variability, despite the similar genetic backgrounds of strains belonging to the same serovar.

2 *Salmonella* strains that were the most resistant to a given stress were not more resistant to other types of stress. Nevertheless, a positive correlation was observed between the resistance of *Salmonella* strains to osmotic stress and to PEF.

3 The variability in growth capacity was similar (in magnitude) to the variability in resistance, with less than a 3-fold change in μ_{max} values regardless of the growth media analysed. Strains that showed a higher growth rate under non-limiting conditions also showed a higher growth rate on media with reduced amounts of Fe, or with gluconate as the sole carbon source.

4 The invasion and adhesion capacity was serovar dependent, with serovar Enteritidis displaying the highest invasion ability under the conditions studied. Strains with a high adhesion ability were not always the most invasive ones.

5 The phenotypic aspects in which the highest variability was found were the ability to form biofilms, with differences of more than 30-fold, and antibiotic tolerance, with differences of up to 64-fold between strains depending on the antibiotic.

6 In general terms, the higher stress resistance of some strains/serovars did not impose a fitness cost to them.

7 RpoS seems to play a crucial role in *Salmonella* resistance to osmotic stress, PEF and UV-C light. However, RpoS activity alone would not explain the differences in resistance observed among *Salmonella* strains to any of the stressing agents/food preservation technologies here studied.

8 Three out of the five resistant variants isolated displayed mutations in *hnr*, suggesting that this could constitute a conserved strategy (within *Salmonellae*) for the acquisition of stress resistance. It was observed that, in this case, this increase in stress resistance had a fitness cost.

CONCLUSIONS

9 The initial dose and thermal history of liquid whole egg and egg white determine the growth fitness of *S.* Enteritidis cells in these products, whereas this does not occur in egg yolk. This phenomenon seems to be related to iron bioavailability, which would be higher in pasteurized products and when *Salmonella* cells were inoculated at a high initial dose.

10 Likewise the minimum growth temperature of *S.* Enteritidis cells would depend on the initial dose and product's thermal history in whole liquid egg and in egg white, but not in egg yolk.

11 For most of the agents studied there was a significant correspondence between the resistance determined in laboratory media and real food matrices and/or poultry manure.

12 Results obtained suggest that emerging poultry serovars/strains would not pose a higher risk for human health than Enteritidis serovar, at least in the scenarios studied/simulated within this PhD thesis.

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Anexos

Anexo I/Annex I

Estudio comparativo de la capacidad de adhesión e invasión de diferentes cepas de *Salmonella* en diferentes modelos *in vitro*

Estudio comparativo de la capacidad de adhesión e invasión de diferentes cepas de *Salmonella* en diferentes modelos in vitro

1. Objetivo

El objetivo de este trabajo fue estudiar la capacidad de *Salmonella* de adherirse e invadir cultivos celulares en un modelo más complejo que los que existen actualmente. Para ello se determinó y comparó la capacidad de adhesión e invasión de diferentes serovares de *Salmonella* en 2 modelos in vitro diferentes (células Caco-2 y co-cultivos Caco-2-HT29-MTX) y el efecto de la adición de una microbiota sintética (viva o inactivada) a estos procesos.

2. Metodología

2.1. Microorganismos y condiciones de cultivo

2.1.1. Cepas de *Salmonella*

Las cepas de *Salmonella enterica* subsp. *enterica* utilizadas en este estudio se enumeran en la Tabla 1. Los cultivos se realizaron en caldo de tripticasa-soja (Oxoid, Basingstoke, Reino Unido) suplementado con 0,6% p/v de extracto de levadura (Oxoid, TSB-YE) en placas microtiter de 96 pocillos, y se incubaron a 37 °C en condiciones estáticas como se describe en Guillén et al. (2020).

Tabla 1. Cepas utilizadas en este estudio y su procedencia

Cepa	Colección	Cepa	Colección
S. Typhimurium SL1344	IFR (Norwich)	*S*. Derby 4397	CECT
S. Typhimurium 443	CECT	*S*. Hadar 13033	NCTC
S. Typhimurium 4594	CECT	*S*. Heidelberg 9379	DMS
S. Typhimurium 7162	CECT	*S*. Infantis 4373	CECT
S. Typhimurium 722	CECT	*S*. Kentucky 5799	NCTC
S. Enteritidis 4155	CECT	*S*. Livingstone 9125	NCTC
S. Enteritidis 4300	CECT	*S*. Mbandaka 7892	NCTC
S. Enteritidis 4396	CECT	*S*. Senftenberg 775W 4565	CECT
S. Enteritidis 7160	CECT	*S*. Stanley 4141	CECT
S. Enteritidis 7236	CECT	*S*. Virchow 4154	CECT

*CECT: Colección Española de Cultivos Tipo; NCTC: Colección Nacional de Cultivos Tipo del Reino Unido; DMS: Colección Alemana de Microorganismos; IFR: Institute of Food Research, Norwich.

2.1.2. Modelo de microbiota intestinal humana

El modelo de microbiota humana consistió en una mezcla de 17 cepas de colección suministradas por Microviable Therapeutics (MT; Gijón, Asturias, España) (Tabla 2). Esta mezcla se diseñó para intentar asemejarse a la composición de los principales grupos microbianos (Firmicutes, Bacteroidetes y Actinobacteria-Protebacteria-Verrumicrobia) identificados tanto cualitativa como cuantitativamente en el íleon y en el colon humano. Las diferentes especies se cultivaron por separado en frascos de 200 ml durante 3 días a 37 °C en condiciones anaeróbicas en una cámara de anaerobiosis (MACS VA500,

Don Whitley Scientific Limited, Shipley, Reino Unido), con una composición de gases de 0% de oxígeno, 5% de hidrógeno, 5% de dióxido de carbono y 90% de nitrógeno, además de un catalizador de paladio, para crear una atmósfera anaeróbica y utilizando los medios indicados en la Tabla 2.

Tabla 2. Cepas que componían la microbiota humana y su medio de cultivo

Cepa	Medio de cultivo
Firmicutes	
Eubacterium cylindroides	GAM + cys
Ruminococcus faecis	GAM + cys
Ruminococcus bicirculans	GAM + cys
Faecalibacterium prausnitzii	BHI + YE + cys + celobiosa + maltosa
Blautia sp.	BHI + YE + cys + celobiosa + maltosa
Blautia coccoides	BHI + YE + cys + celobiosa + maltosa
Dorea longicatena	BHI + YE + cys + celobiosa + maltosa
Lactobacillus delbruekii	MRS
Lactobacillus amylovorus	MRS
Enterococcus faecalis	BHI
Enterococcus faecium	BHI
Bacteroidetes	
Bacteroides uniformis	GAM + cys
Bacteroides vulgatus	GAM + cys
Bacteroides dorei	GAM + cys
Bacteroides coprophilus	GAM + cys
Actinobacterias-Protebacterias-Verrumicrobia	
Escherichia coli	BHI
Collinsella aerofaciens	BHI + YE + cys + celobiosa + maltosa
Bifidobacterium longum	GAM + cys

*BHI: Brain Heart Infusion (Oxoid);
BHI + YE + cys + celobiosa + maltosa: 37 g/l BHI (Oxoid) + 5 g/l extracto de levadura (Oxoid) + 0,05% cisteína (Sigma, St. Louis, USA) + 0,1% celobiosa (Sigma) + 0,1% maltosa (Sigma);
GAM + cys: 59 g/l GAM (Gifu Anaerobic Medium Broth (Nissui, Uffing, Alemania) + 0,25% cisteína (Sigma);
MRS: Man, Rogosa y Sharpe (Merck, Darmstadt, Alemania)

Se prepararon tres pre-mezclas separadas, correspondientes a cada uno de los tres grupos microbianos indicados anteriormente, una incluyendo las especies del filo Firmicutes, otra las pertenecientes a Bacteroidetes y otra con las cepas pertenecientes a los grupos Actinobacteria + Proteobacteria + Verrumicrrobia (Tabla 2). Cada una de estas pre-mezclas se obtuvieron mezclando volúmenes iguales de las diferentes especies que lo integraban. Una vez preparadas estas suspensiones mixtas o pre-mezclas se hizo un recuento microscópico de cada una de ellas y se mezclaron para obtener la microbiota modelo completa. Esta microbiota modelo se construyó mezclando diferentes volúmenes de cada una de las 3 pre-mezclas, de manera que la proporción final fue 55% Firmicutes/35%

Bacteroidetes/10% Actinobacterias-Protebacterias-Verrumicrobia. A continuación, se centrifugó toda la mezcla (12.000xg 20 min a 4 °C) y se resuspendió en el mismo medio de crecimiento de los cultivos celulares (Dulbecco's Modified Eagle's Medium + Gluta-MAX™ (DMEM, Invitrogen, France)) a las diferentes concentraciones ensayadas (10^9-10^{11} UFC/ml).

Para obtener una microbiota modelo inactivada, la mezcla completa se resuspendió en PBS (Solución salina tamponada con fosfato, Oxoid), se colocó en una placa de Petri de 150 mm con agitación magnética y se expuso a la luz UV-C, se situó a una distancia de 22,50 cm de una lámpara UV-C de 32 W (VL-208G, Vilber, Germany) durante 30 minutos. Tras este tratamiento fueron inactivados al menos 7 ciclos logarítmicos de la mezcla y el número de células de *E. coli* fue inferior a 30 UFC/ml. A continuación, se centrifugaron las células y se resuspendieron en DMEM hasta alcanzar las diferentes concentraciones ensayadas (10^9-10^{11} UFC/ml).

En los ensayos se trabajó con una concentración de esta microbiota de aproximadamente 10^9 UFC/ml (mezcla original). Se evaluó el uso de concentraciones superiores. Así, la adición de la microbiota a una concentración de 10^{10} UFC/ml ya condicionó mucho el momento en el que podían ser añadida al cultivo celular (como mucho media hora antes de inocular las *Salmonellas*) mientras que el uso de 10^{11} UFC/ml (similar a las concentraciones en el colon) resultó inviable ya que afectaba inmediatamente a la viabilidad y adhesión de las células Caco-2 y HT29-MTX a la placa multipocillo.

2.2. Ensayos de adhesión e invasión

El protocolo para la obtención de los cultivos de las células Caco-2 y HT29-MTX se detalla en Guillén et al. (2022). Para los ensayos con co-cultivos, Caco-2-HT29-MTX, las células fueron resuspendidas en una ratio de 90:10, se sembraron a una densidad de aproximadamente 15.000 células por pocillo en placas de cultivo de 96 pocillos y fueron cultivadas durante 16 días a 37 °C 5% CO_2 (Dostal et al., 2014). Los protocolos de adhesión e invasión fueron descritos en Guillén et al. (2022). En los ensayos con la microbiota intestinal humana, tanto inactivada como no, esta se añadía 30 minutos antes sobre los co-cultivos de Caco-2-HT29-MTX. Todos los ensayos se hicieron por triplicado con cultivos obtenidos en diferentes días (tanto de *Salmonella* spp. como de la microbiota).

2.3. Caracterización de la microbiota sintética

Se utilizó un kit de extracción de DNA Maxwell 16 Lev Blood (Promega, Madison, EE.UU.) para purificar el DNA bacteriano de 0,5 ml del mix de la microbiota siguiendo las instrucciones del fabricante. A continuación, se llevó a cabo el análisis de secuenciación de alto rendimiento del 16S rRNA. Se prepararon bibliotecas dirigidas a las regiones hipervariables V3-V4 del 16S rRNA (Klindworth et al., 2013) utilizando el protocolo de preparación de bibliotecas de secuenciación metagenómica 16S de Illumina. Las bibliotecas de DNA generadas se secuenciaron con el kit MiSeq Reagent Kit v3 en la plataforma MiSeq de Illumina, utilizando lecturas de secuenciación de 300 pb por pares. Las muestras secuenciadas se procesaron con QIIME2 v2018.6.0 (Caporaso et al., 2010) y el plug-in DADA2 (Callahan et al., 2016). Las secuencias resultantes se agruparon en variantes de secuencias de amplicones (ASV) y luego se clasificaron por taxones utilizando un clasificador ajustado. Se utilizó el clasificador scikit-learn para clasificar las secuencias utilizando la base de datos SILVA (versión 132 QIIME), con un umbral de agrupación del 97% de similitud. Para la clasificación, sólo se consideraron significativas las ASV que contenían al menos 10 lecturas de secuencia.

2.4. Análisis estadístico

Todas las determinaciones se realizaron por triplicado en diferentes días de trabajo. Las desviaciones estándar (SD) y los análisis estadísticos (coeficientes de correlación de Pearson, ANOVA y la prueba de Tukey; valor p <0,05) se calcularon utilizando el software estadístico GraphPad PRISM® (GraphPad Prism versión 8.00 para Windows, GraphPad Software, San Diego, California, EE.UU.).

3. Resultados

3.1. Caracterización de la microbiota

Como se puede observar en la Figura 1 el porcentaje del grupo de Actinobacteria + Proteobacteria + Verrumicrrobia se ajustó bastante al deseado (12,43 vs 10%). Por el contrario, el porcentaje de Bacteroides fue alrededor de un 10 % superior al deseado (46,09 vs 35%) y el de Firmicutes un 15% inferior (40,46 vs 55%). Por otra parte, dentro de cada grupo el porcentaje de cada especie tampoco fue igual. Así, dentro de Firmicutes destacan las bajas concentraciones de *Dorea*, *Blautia* y, especialmente *Faecalibacterium*, las que, además, serán probablemente, la causa de las principales desviaciones entre el % de Firmicutes objetivo y el real. Por otra parte, no se puede olvidar que esta técnica de cuantificación también tiene sus deficiencias, como que no permite determinar qué porcentaje de las células es viable y cual no.

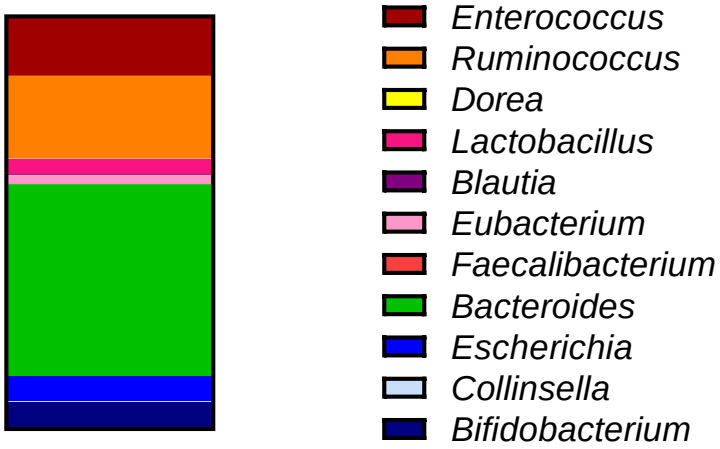

Figura 1. Composición promedio de la microbiota utilizada en los ensayos (los grupos indicados representan el 98,98% de los identificados por secuenciación del 16S rRNA).

3.1. Estudio comparativo de las diferentes cepas y comparación entre modelos in vitro

En estudios previos se probaron diferentes MOEs (desde 1:1 a 1000:1), ratio entre células de *Salmonella* y Caco-2. La MOE de 100:1 resultó la más operativa ya que ratios más bajas nos llevaban muchas veces por debajo del límite de detección y la superior sólo significaba tener que hacer una dilución más. También se hicieron ensayos para comparar la variabilidad existente entre réplicas técnicas y los resultados indican que esta metodología tiene una repetitividad "limitada" con SDs de hasta el 50% (algo menos en adhesión), siendo estos valores comparables a los observados entre réplicas biológicas.

Con el modelo de co-cultivo in vitro de células epiteliales intestinales (Caco-2), células secretoras de mucosa (HT29-MTX) y la adición de flora sintética se quería desarrollar un modelo más complejo que imitara el intestino de una persona sana. Los resultados obtenidos en los diferentes modelos in vitro se ilustran en Figura 2. Como se puede observar, en general en el co-cultivo (Caco-2-HT29-MTX) las células de *Salmonella* mostraron un aumento en la capacidad de adhesión e invasión, encontrándose diferencia estadísticamente significativa (p <0,05) entre estos dos modelos y en ambos procesos. Además de los resultados se puede deducir que la presencia de microbiota reduce ambos fenómenos (adhesión e invasión), no encontrándose diferencias en adhesión entre la adición de flora viva o sintética (p >0,05).

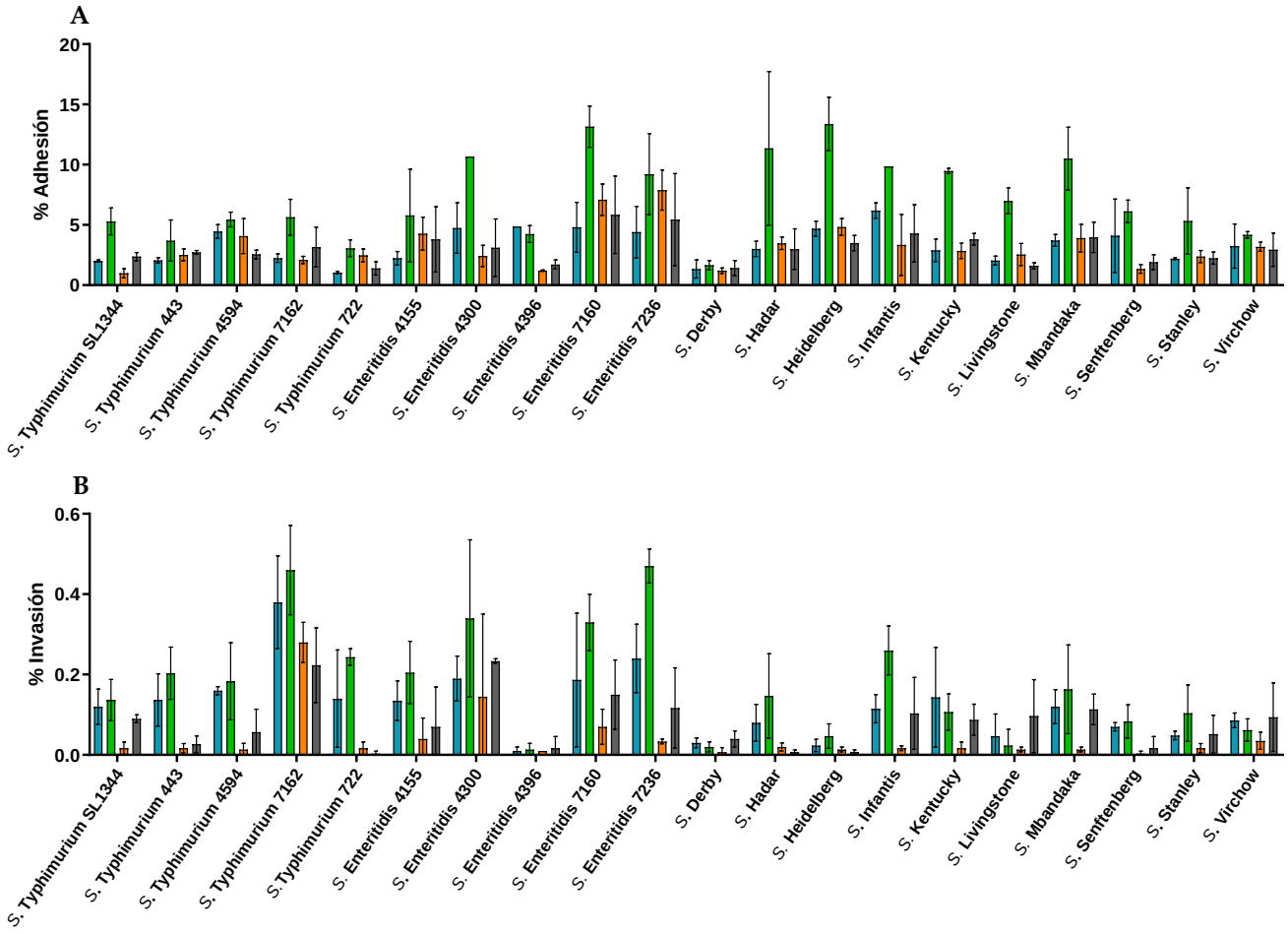

Figura 2. Capacidad de adhesión (A) e invasión (B) de las diferentes cepas de *Salmonella* a los diferentes modelos in vitro: células de Caco-2 (azul), co-cultivos de Caco-2-HT29-MTX (verde), co-cultivos Caco-2-HT29-MTX + flora (naranja) y co-cultivos Caco-2-HT29-MTX + flora inactivada (gris)

Para determinar si existía una correlación entre los diferentes modelos in vitro se realizó el test de correlación de Pearson. Los resultados de este test para los valores de adhesión se muestran en la Tabla 3 y los valores de invasión en la Tabla 4. Se observaron correlaciones positivas entre todos los modelos in vitro (p <0,05), excepto entre los valores de adhesión en Caco-2 y co-cultivo con flora que se observó una tendencia (p <0,10).

Tabla 3. Valores del coeficiente de correlación de Pearson calculados para los valores de adhesión de las cepas de *Salmonella* en los diferentes modelos de in vitro. Los valores entre paréntesis corresponden al p-valor.

	Caco-2	Co-cultivo	Co-cultivo + Flora	Co-cultivo + Flora Inactivada
Caco-2		**0,614 (0,004)**	0,417 (0,067)	**0,527 (0,017)**
Co-cultivo	**0,614 (0,004)**		**0,608 (0,004)**	**0,709 (0,000)**
Co-cultivo + Flora	0,417 (0,067)	**0,608 (0,004)**		**0,843 (0.000)**
Co-cultivo + Flora Inactivada	**0,527 (0,017)**	**0,709 (0,000)**	**0,843 (0.000)**	

Tabla 4. Valores del coeficiente de correlación de Pearson calculados para los valores de invasión de las cepas de *Salmonella* en los diferentes modelos de in vitro. Los valores entre paréntesis corresponden al *p*-valor.

	Caco-2	Co-cultivo	Co-cultivo + Flora	Co-cultivo + Flora Inactivada
Caco-2		**0,905 (0,000)**	**0,797 (0,000)**	**0,721 (0,000)**
Co-cultivo	**0,905 (0,000)**		**0,657 (0,002)**	**0,657 (0,002)**
Co-cultivo + Flora	**0,797 (0,000)**	**0,657 (0,002)**		**0,779 (0,000)**
Co-cultivo + Flora Inactivada	**0,721 (0,000)**	**0,657 (0,002)**	**0,779 (0,000)**	

De este análisis estadístico, y en concreto, en lo que respecta a la adición de la microbiota sobre los co-cultivos, se deduce que la adición de la microbiota sintética tiene un efecto estadísticamente significativo en la adhesión e invasión y, lo más sorprendente es que, en el caso de la invasión, hay diferencias entre añadir la microbiota viva o inactivada. Esto era poco esperable dado el poco tiempo de contacto, y teniendo en cuenta las limitaciones del modelo (aerobiosis, medio nada similar al que se encontrarían en el intestino) y tal vez sería objeto de estudio en un futuro.

Y en lo que respecta al objetivo inicial del estudio, el análisis estadístico indica que, además de obtenerse una correlación positiva, los coeficientes de correlación de Pearson entre los % de adhesión e invasión de las diferentes cepas en cada uno de los modelos estudiados son elevados. Esto nos estaría indicando, que incluso el modelo más sencillo podría valer para hacer estimaciones relativas de la capacidad de invasión de las diferentes cepas/serovariedades de *Salmonella*, aunque no puede descartarse que en condiciones que se parezcan más al intestino humano esto deje de cumplirse, a pesar de que ninguno de los modelos sirviera para hacer estimaciones cuantitativas absolutas del riesgo. Por ello, teniendo en cuenta los resultados de este estudio y dada esta buena correlación entre los diferentes modelos in vitro, sobre todo el obtenido entre las células Caco-2 y el co-cultivo, para el estudio de la variabilidad de las diferentes cepas/serovares de *Salmonella* spp. se seleccionó este modelo más sencillo, cultivos únicamente con células de Caco-2, para realizar los ensayos dentro del marco de esta tesis doctoral. Además, esta simplificación condujo a una disminución en la variabilidad en los resultados.

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Anexo II/Annex II

Development of a predictive growth model of *Salmonella* Enteritidis in whole liquid egg using multilevel modelling: a preliminary step

Development of a predictive growth model of S*almonella* Enteritidis in whole liquid egg using multilevel modelling: a preliminary step

1. Introduction

Risk assessment emerged already a few decades ago as an extremely efficient methodology to assess a wide variety of environmental risks, including those of foodborne pathogenic microorganisms, and as a basis for a food safety approach (WHO/FAO, 2007). Originally, most of these risk assessments were qualitative or semi-quantitative (e.g., high or low probability of causing illness). However, as scientific data have accumulated, and given the greater flexibility of quantitative models, the current trend is to work with the latter type of models. In turn, these models can be deterministic, a single value for the variables of the model, or probabilistic, through the use of probability distributions that describe the probability associated with each value. The main problem for the development of these quantitative models is that it is necessary to fill some knowledge gaps that still exist today. Model parameters may depend on a wide range of implicit (e.g. bacterial strain), intrinsic (e.g. media type) and extrinsic (e.g. temperature) factors in a way that is not yet fully understood. Therefore, they must be estimated using experimental data and, since experimental error is unavoidable, it would be relevant to include variability and uncertainty parameters in the analysis since their values cannot be known with absolute certainty. In the context of food safety, variability includes inherent sources of variation (e.g., differences in individual cell response or food composition) and uncertainty would encompass those sources of variation that are not considered in the system (e.g., measurement errors or model misspecification). For these reasons, quantitative models have certain limitations, and given current efforts to quantify variability and uncertainty in microbial responses, multilevel models are likely to gain popularity in the coming years and replace the single-level models that are now so relevant to predictive microbiology.

The relevance of salmonellosis as an agent responsible for foodborne toxi-infections, especially associated to egg and egg products, is well known (EFSA, 2021) and has been widely discussed in this thesis. Predictive models are a particularly useful tool for developing strategies to combat this disease, however, nearly all growth and survival models currently available for this microorganism are deterministic based (kinetic models). Although these models have certain advantages, such as the fact that they can be provided with a mechanistic basis (although this is not usually the case) and lead to obtaining parameters of (relatively) easy interpretation, they do not integrate within them neither the uncertainty nor the variability inherent to different processes/aspects such as data collection, their modelling or the existing biological variability between strains or even within the same microbial population. Including the variability and uncertainty of these variables would improve the current predictive models that estimate the growth or inactivation of *Salmonella* in eggs so that better strategies could be designed to ensure the safety of these products.

In view of the above, our final goal is to develop a probabilistic multilevel model that integrates the uncertainty of the assay and the variability between replicates and between strains and that allows determining -with higher precision than the current ones- the growth of *S*. Enteritidis in whole liquid egg as a function of the strain, the storage temperature, the thermal history of the egg and the initial number with which the product is contaminated.

As a preliminary step, this work has been focused on studying the parameter variance, which can give very useful information, as already described above, for predictive microbiology. This is a parameter associated with a biological characteristic of the microorganism (variability) and that can be as relevant as other parameters, such as growth rates for the case here studied, especially for quantitative microbial risk assessments (QMRA). In this sense, multilevel models are a good framework for the analysis of variation because they incorporate variability and uncertainty in the first steps of the modelling process. In this approach, model assumptions are not limited to model kinetics, but an error

model must be defined to describe variability and uncertainty using statistical assumptions that can later be refuted by other experts or compared using model selection techniques (Garre et al., 2020).

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Five strains belonging to *Salmonella enterica* serovar Enteritidis were used in this study. The strains of *S*. Enteritidis (STCC 4155, STCC 4300, STCC 4396, STCC 7160 and STCC 7236) were supplied by the Spanish Type Culture Collection (STCC). Strains were maintained frozen at −80 °C in cryovials for long-term preservation. Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (Oxoid, TSB-YE) in 96 wells microtiter plates and incubated at 37 °C under static conditions as described in Guillén et al. (2020).

2.2. Growth media and growth curves

Growth experiments were carried out in raw liquid whole egg obtained from medium-sized raw eggs (53-63 grams) purchased from a local supermarket and in commercial pasteurized liquid whole (Pascual, Aranda de Duero, Spain). The egg products were inoculated with different initial *Salmonella* doses, 10^2 (low dose) and 10^6 (high dose) CFU/mL, and were then incubated at 10, 20, 30, 37 and 42 °C. Samples were taken at preset time intervals, from 0 to 480, 60 or 30 h for the 10, 20 or 30-37-42 °C curves, respectively, adequately diluted in buffered peptone water (Oxoid, BPW), and plated on Xylose Lysine Deoxycholate agar (Oxoid, XLD), the recovery medium. XLD plates were incubated for 48 h at 37 °C, and the number of colony forming units (CFU) per plate was counted.

2.5. Primary growth model

Growth curves were constructed by plotting the decimal logarithm of the number of *Salmonella* versus time under the different conditions assayed. Each point in the growth curve corresponds to the average value of all samples analyzed (at least three replicates). The curves obtained were fitted with the Baranyi and Roberts model (Baranyi and Roberts, 1994):

$$Log_{10}\,N = Log_{10}\,N_{max} + Log_{10}\left(\frac{1+e^{(\ln(10)\cdot\ \mu_{max}\cdot(t-\lambda))}-e^{(-\ln(10)\cdot\mu_{max}\cdot\lambda)}}{e^{(\ln(10)\cdot\mu_{max}\cdot(t-\lambda))}-e^{(-\ln(10)\cdot\mu_{max}\cdot\lambda)}+10^{(Log_{10}N_{max}-Log_{10}N_0)}}\right) \quad \text{Eq. 1}$$

where $Log_{10}N$ is the Log_{10} of cell concentration at time t (CFU/mL); $Log_{10}N_0$ is the Log_{10} of the initial cell concentration (CFU/mL); $Log_{10}N_{max}$ is the Log_{10} of maximum cell concentration (CFU/mL); μ_{max} is the maximum growth rate (Log_{10}/h); λ is the lag phase (h).

2.4. A multilevel model including variability and uncertainty

A multilevel model provides a framework to include in the model stochastic hypotheses regarding the sources of variability, in this case the initial dose and the variability between Enteritidis strains. The variability between strains describes the fact that some strains have more capacity to growth than others, and the initial dose describes the influence on growth parameters when the curves are initiated at different cell concentrations. Therefore, the hypotheses of the multilevel model are not limited to how the growth rate varies (i.e. the Baranyi and Roberts model in the single-level approach), but also include hypotheses regarding how different sources of variation affect the variation of the model variables (Garre et al., 2020). The hypotheses of multilevel models and Bayesian parameter estimation were similar to those defined by Garre et al. (2020).

The parameters of the multilevel model have been estimated using Stan (Carpenter et al., 2017), through the interface provided in the rethinking R package (McElreath, 2016). The convergence of the Markov chain was assessed according to typical guidelines (Brooks et al., 2011; McElreath, 2016). Trace and pair plots were visually inspected to ensure appropriate mixing and convergence of the chain. Furthermore, the number of iterations was increased until the parameter $\hat{R}$ was lower than 1.1,

following usual guidelines for Bayesian modelling (McElreath, 2016). Accordingly, 4000 iterations after 1000 warmup iterations were required to fulfil these requirements.

3. Results

First, growth curves starting at different concentrations between 10^2 (low dose) and 10^6 (high dose) CFU/mL were obtained in raw and pasteurized liquid whole egg at different temperatures, between 10 and 42 °C. As an example, the curves obtained for the Enteritidis STCC 4300 strain at the highest and lowest doses in raw and pasteurized liquid whole eggs are shown in Figure 1.

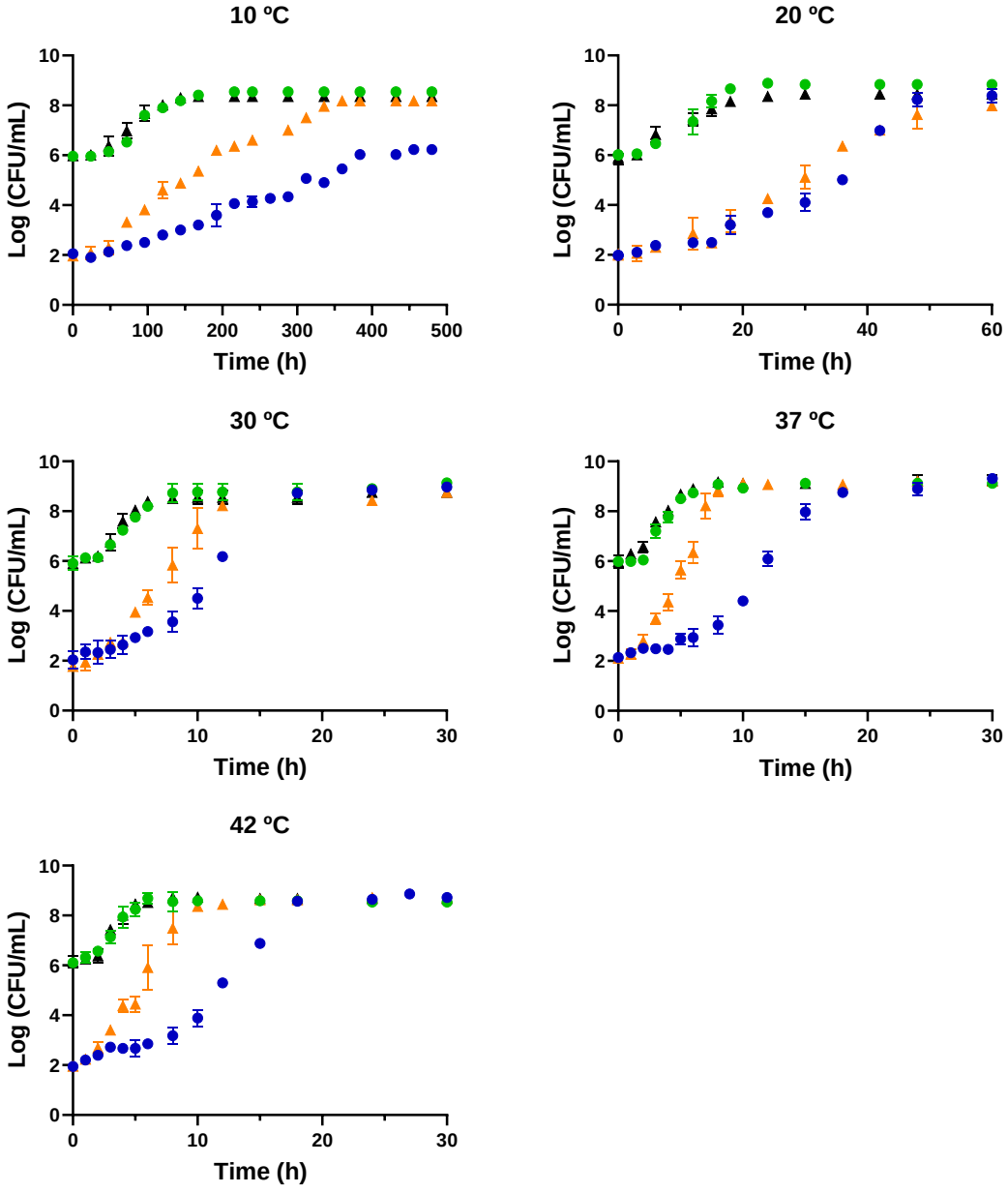

Figure 1. Growth curves of *Salmonella* Enteritidis STCC 4300 at different temperatures: 10, 20, 30, 37, 42 °C in raw liquid whole egg starting at different concentrations 10^2 (●) and 10^6 CFU/mL (●) and pasteurized liquid whole egg at 10^2 (▲) and 10^6 CFU/mL (▲). Error bars represent the standard deviation.

Then, in order to get further insight into the study of variance and, more specifically, on unexplained variance, the sources of variability, in this case the initial dose and the variability between Enteritidis strains, were introduced at the different levels of the model. The estimation of this parameter was used to quantify and compare different sources of variability. Figure 2 includes an explanatory diagram of how the sources of variability in the model parameters were included in the study, leading to the generation of the different models.

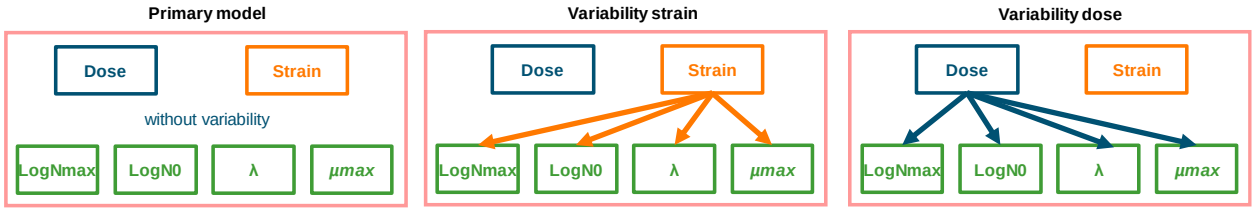

Figure 2. Explanatory diagram depicting how the different models (a single source of variability) were constructed.

In order to facilitate comparisons between the models obtained in raw and/or pasteurized liquid whole egg, the graphs included in Figure 3 were constructed. This figure shows, as a way of example the evolution of the variance throughout the models obtained for raw or pasteurized liquid whole egg incubated at 37 °C. Based on the existing literature, we established a lower limit of unexplained variance, 0.25, whereby lower variance values would indicate an overestimation of the model.

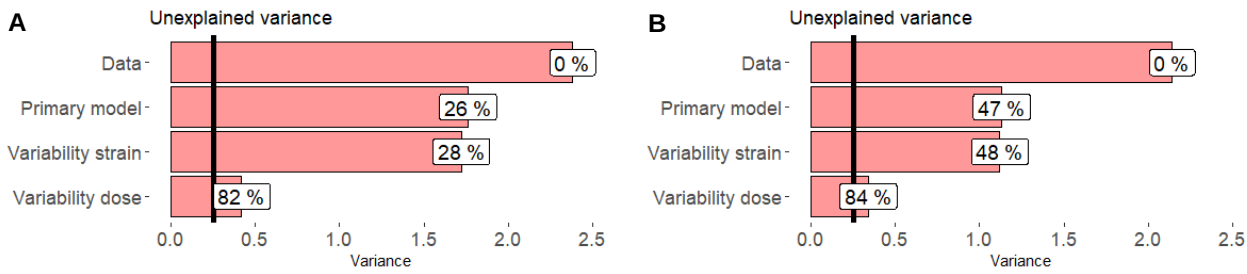

Figure 3. Evolution of the unexplained variance parameter in the models in raw whole egg (A) and pasteurized whole egg (B) at 37 °C. Percentages indicate the part of the variance that was explained by including the initial dose or strain variability in the model parameters. The black line indicates the lower limit of estimated variance that could be reached without overestimating the model.

The first bar (Data) depicts the variability between the microbial counts of the curves obtained. After modelling with the Baranyi and Roberts primary model (primary model), this variance was reduced by 26 and 47% in raw and pasteurized liquid whole egg, respectively. Including the variability among strains in the different parameters of the model, $Log_{10} N_0$, $Log_{10} N_{max}$, μ_{max} and λ, did not resulted in any significant improvement in the model (Variability strain); however, when the variability of the initial dose was included (Variability dose), a high reduction in variance was obtained. Therefore, from these data it can be concluded that the variability of the initial dose was more relevant than the variability of the strain, at least under the conditions here studied.

In these early trials, only a single source of variability had been included in the models, either the initial dose or the variability between strains, but multilevel models allow the incorporation of both, including correlations between parameters that allow explaining/minimising the source of uncertainty. In the successive models, both sources of variability were included in the model parameters in a summative way and in the following order: first, the variability of both sources in the $Log_{10} N_0$ parameter was added (Variability in $Log_{10} N_0$), then, and maintaining this variability in $Log_{10} N_0$, both sources of variability in λ (+ λ), μ_{max} (+ μ_{max}) and $Log_{10} N_{max}$ (+ $Log_{10} N_{max}$) were incorporated to the model; finally,

the correlation between the parameters of the parameters of μ_{max} and λ (+ correlation μ_{max}_ λ) was also integrated. Figure 4 includes diagram illustrating how these two sources of variability were sequentially incorporated to the different kinetic parameters, leading to the development of increasingly complex models.

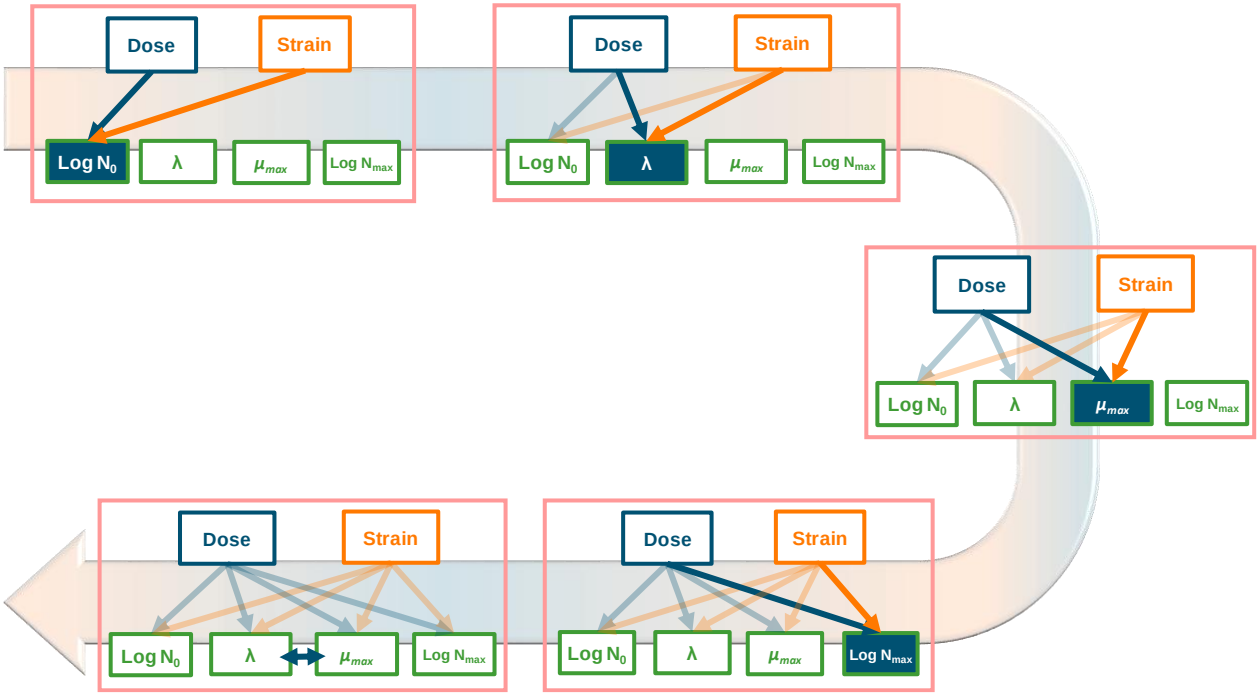

Figure 4. Diagram illustrating the how the sources of variability (strain and initial dose) were sequentially incorporated to the model.

As can be deduced from Figure 5, incorporating the two sources of variability studied (initial dose and strain) to the different kinetic parameters did not improved the model for pasteurized liquid whole egg (Figure 5B) but for the $Log_{10} N_0$ parameter. At this point it should be noted that, as already discussed and depicted in Figure 3, this improvement was almost only due to the variability in the initial dose. Thus, when the variability of the initial dose was included in the $Log_{10} N_0$ parameter, the maximum reduction in unexplained variance was already achieved. In addition, it should also be noted no influence of this source of variability on the other model parameters was observed. This result can be considered obvious, at least to some extent, because the growth curves were obtained in a wide range of initial concentrations between 2 and 6 Log_{10} CFU/mL. However, what it is more remarkable is that it did have an impact on various parameters of the model developed for raw liquid whole egg (Figure 5A). In this case, and in contrast to what was observed for the pasteurized liquid whole egg, the variability in the $Log_{10} N_0$ parameter alone did not explain the uncertainty of the data obtained in raw liquid whole egg. Thus, the variability of the initial dose also had an impact on both the lag phase parameter (λ) and the maximum growth rate (μ_{max}). Therefore, it can be concluded that the initial dose had a greater influence on the raw liquid whole egg than on the pasteurized liquid whole egg. These results confirmed the results previously published in Guillén et al. (2021), where an association was observed between these same parameters. In that work we observed that growth curves starting from lower initial counts led to longer lag phases (λ) and lower growth rates (μ_{max}) in raw liquid whole egg, but this phenomenon was not observed in pasteurized liquid whole egg; i.e. no influence of initial concentration on *Salmonella* growth parameters was observed in pasteurized whole egg.

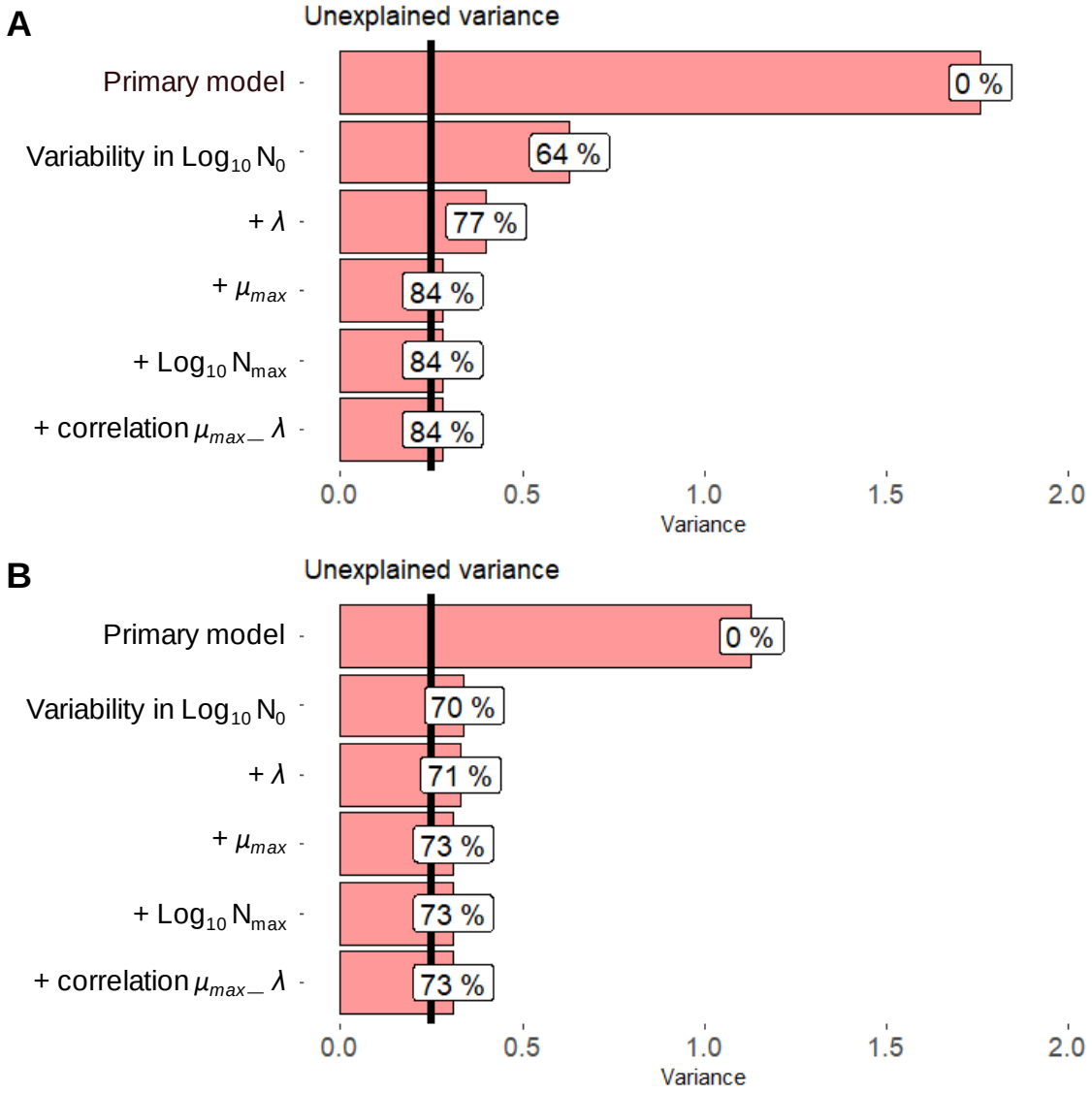

Figure 5. Evolution of the unexplained variance parameter in the models in raw whole egg (A) and pasteurized whole egg (B) at 37 °C. Sequential effect of each parameter in variability (Log_{10} N_0, λ, μ_{max} and Log_{10} N_{max},). Percentages indicate the part of the variance that was explained by including the initial dose or strain variability in the model parameters. The black line indicates the lower limit of estimated variance that could be reached without overestimating the model.

The same approach was applied to the growth curves obtained at the other growth temperatures studied. When comparing results obtained at low growth temperatures, i.e. 10 °C, to those obtained at 37 °C it should be noted that, at low incubation temperatures the influence of strain variability was greater than at high temperatures, 37 °C, and that, conversely, the influence of dose variability was greater the higher the growth temperature (Figure 6). These results suggest that this higher variability between strains at lower temperature could be related to the minimum growth temperature of the different strains, which would be consistent with previously reported results on raw whole liquid egg, where it was found that the minimum growth temperature of *Salmonella* Enteritidis cells varied among the different strains, differences of up to 0.6 °C, and furthermore this was dependent on the initial dose (Guillén et al., to be submitted for publication).

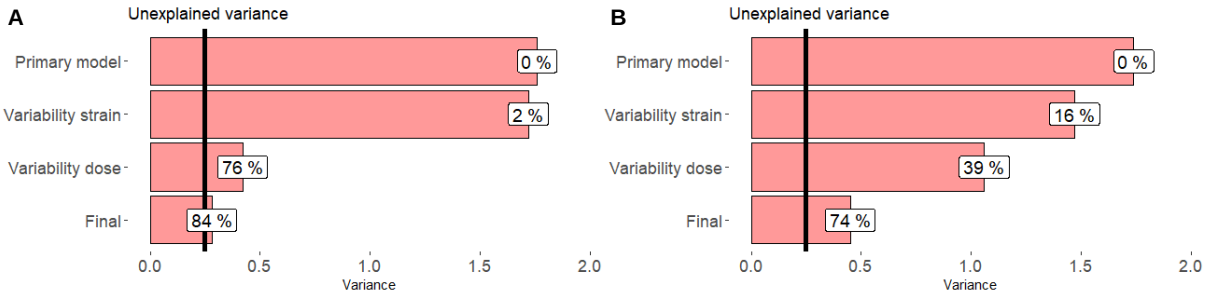

Figure 6. Comparison of evolution of the unexplained variance parameter in the models in raw whole egg at 37 °C (A) and at 10 °C (B). Percentages indicate the part of the variance that was explained by including dose or strain variability in the model parameters. The black line indicates the lower limit of estimated variance that could be reached without overestimating the model.

It should also be noted that at low temperatures with these two sources of variability, not all the variance would be explained, but this may be due to experimental errors since it would be closer to the lower growth limit and fluctuations in temperature would have a higher effect in the growth.

In summary, the results obtained clearly indicate that thanks to the use of multilevel models the main sources of variability within the growth curves of *Salmonella* in whole liquid egg were identified, being the initial dose (almost the only one for high growth temperatures) and the differences in the growth capacity of the different strains (this one only at low temperatures). In addition, this approach also allowed us to determine whether or not these sources of variability were affected by the thermal history of the liquid whole egg and/or the growth temperature.

We are currently further working - in collaboration with the Wageningen University- on the application of these multilevel models for the development of a tertiary model enabling the prediction of *Salmonella* growth in whole liquid egg, egg white and egg yolk at different temperatures, between 10 and 42 °C, as a function of the strain, the thermal history of the egg and cell concentration and also incorporating the sources of variability and uncertainty within it. This will help to improve *Salmonella* growth prediction models in egg and egg products and, therefore, to the quantitative risk assessment of *Salmonella* in these products.

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Anexo III/Annex III

Factor de impacto de las revistas y áreas temáticas

Factor de Impacto y quartil en el que se sitúan, dentro de cada área temática, las revistas en las que se han publicado los artículos de esta Tesis Doctoral. El Factor de Impacto hace referencia al del año de publicación o al último disponible. Todos los parámetros se han obtenido del Journal Citation Reports® disponible en la página web de ISI Web of Knowledge. Además, se incluye la justificación de la contribución del doctorando en cada trabajo.

Publicación I

Guillén, S., Nadal, L., Álvarez, I., Mañas, P., Cebrián, G., 2021. Impact of the resistance responses to stress conditions encountered in food and food processing environments on the virulence and growth fitness of non-typhoidal *Salmonellae*. Foods 10, 617. https://doi.org/10.3390/foods10030617

Revista: Foods

Año: 2021

Área temática: Food science & technology

Factor de impacto: 4.350 (Q2, posición 37/143)

Contribución doctorando: Writing-original draft preparation, Writing-review and Editing

Publicación II:

Guillén, S., Marcén, M., Mañas, P., Cebrián, G., 2020. Differences in resistance to different environmental stresses and non-thermal food preservation technologies among *Salmonella enterica subsp. enterica* strains. Food Res. Int. 132, 109042. https://doi.org/10.1016/j.foodres.2020.109042

Revista: Food Research International

Año: 2020

Área temática: Food science & technology

Factor de impacto: 6.475 (Q1, posición 9/143)

Contribución doctorando: Investigation, Methodology, Formal analysis and Writing- original draft.

Publicación III:

Guillén, S., Marcén, M., Fau, E., Mañas, P., Cebrián, G., 2022. Relationship between growth ability, virulence, and resistance to food-processing related stresses in non-typhoidal *Salmonellae*. Int. J. Food Microbiol. 361, 109462. https://doi.org/10.1016/j.ijfoodmicro.2021.109462

Revista: International Journal of Food Microbiology

Año: 2022

Área temática: Food science & technology

Factor de impacto: 5.277 (Q1, posición 25/143)

Contribución doctorando: Investigation, Methodology, Formal Analysis and Writing-Original draft preparation.

Publicación IV:

Guillén, S., Marcén, M., Álvarez, I., Mañas, P., Cebrián, G., 2021. Influence of the initial cell number on the growth fitness of *Salmonella* Enteritidis in raw and pasteurized liquid whole egg, egg white, and egg yolk. Foods 10, 1621. https://doi.org/10.3390/foods10071621

Revista: Foods

Año: 2021

Área temática: Food science & technology

Factor de impacto: 4.350 (Q2, posición 37/143)

Contribución doctorando: Conceptualization, Methodology, Formal Analysis, Investigation and Writing—original draft preparation

Publicación V:

Guillén, S., Marcén, M., Álvarez, I., Mañas, P., Cebrián, G., 2020. Stress resistance of emerging poultry-associated *Salmonella* serovars. Int. J. Food Microbiol. 335, 108884. https://doi.org/10.1016/j.ijfoodmicro.2020.108884

Revista: International Journal of Food Microbiology

Año: 2022

Área temática: Food science & technology

Factor de impacto: 5.277 (Q1, posición 25/143)

Contribución doctorando: Investigation, Methodology, Formal analysis and Writing-original draft preparation.