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Growth fitness, virulence, and heat tolerance of *Salmonella* Typhimurium variants resistant to food preservation methods

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ABSTRACT

To study potential ramifications of antimicrobial resistance, we carried out adaptive laboratory evolution assays (ALE) to isolate three resistant variants (RVs) of *Salmonella enterica* Typhimurium, employing three different types of food preservation methods: 1) an emergent technology, plasma-activated water (PAW), leading to variant RV-PAW; a traditional method, heat, leading to variant RV-HT, and a natural antimicrobial compound, carvacrol, leading to variant RV-CAR. The variant resistant to plasma-activated water, RV-PAW, had mutations in *rpoA* and *rpoD*; it showed increased tolerance to heat in orange juice but ultimately did not pose a significant threat, as it exhibited a fitness cost at refrigeration temperature (8 °C), whereas its virulence against *Caenorhabditis elegans* decreased. The variant resistant to heat, RV-HT, had mutations in *flhC*, *dnaJ*: it exhibited a fitness cost at high growth temperatures (43 °C) and induced morphofunctional alterations in *C. elegans*. The variant resistant to carvacrol, RV-CAR, had mutations in *sseG*, *flhA*, *wbaV*, *lon*; this variant not only exhibited significantly higher thermotolerance in both laboratory media and food models but also effectively increased its growth fitness at refrigeration temperatures while retaining its virulence, evidenced by the highest percentage of Smurf phenotype in *C. elegans*.

To address these challenges, we applied a process combining thermal treatment with citral, with the aim of leveraging the sublethal damage caused in RVs by heat treatments in orange juice. This approach achieves enhanced microbial inactivation without having to escalate the intensity of the thermal treatment. The result was particularly encouraging in the case of RV-CAR, the most challenging strain, for which we improved lethality by up to 3 $\log_{10}$ inactivation cycles.

1. Introduction

In 2021, non-typhoidal *Salmonella* serovars were the principal cause of foodborne outbreaks in the EU; most *Salmonella* infections result from the ingestion of contaminated foods, such as poultry meat, pork, beef, eggs, milk, seafood, and fresh produce (EFSA and ECDC, 2022). Moreover, Van Boxstael et al. (2012) have shown that most isolated strains are resistant to at least one antimicrobial, and that multi-resistance is ubiquitous in these bacterial populations.

Moreover, foodborne pathogens can develop resistance mechanisms to withstand the stressful environmental and processing conditions they face along the food chain. Exposure to certain biocides and disinfecting agents can exert selective pressure and potentially promote the emergence of resistant variants (RVs) of these pathogenic bacteria, which, apart from exhibiting direct resistance to the selective agent, can also develop cross-resistance to antibiotics or food preservation methods (Berdejo et al., 2022; Mavri and Smole Možina, 2013; Rodríguez-Melcón et al., 2023). The food industry thus also contributes to the emergence of bacterial resistance. This not only compromises food safety but also gives rise to antimicrobial resistance (AMR) microorganisms, which, in

Abbreviations: AMR, antimicrobial resistance; ALE, adaptive laboratory evolution; EOs, essential oils; TSAYE, tryptone soya agar supplemented with yeast extract; CFU, colony-forming units; PBS, phosphate-buffered saline; FUDR, fluorodeoxyuridine; HT, heat treatment; PAW, plasma-activated water; RV-CAR, resistant variant to carvacrol; RV-HT, resistant variant to heat treatment; RV-PAW, resistant variant to plasma-activated water.

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Table 1
Resistant variants of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 evolved by cyclic exposure to prolonged sublethal doses of carvacrol: RV-CAR, by cyclic exposure to short lethal heat treatments: RV-HT and by cyclic exposure to short lethal plasma activated water treatments: RV-PAW.

Reference	Treatment	Antimicrobial agent/technology	Description	Name
SeCarA (Pagán et al., 2024a)	Sublethal	Carvacrol	*sseG* 113C; *flhA* Pro306Gl; *wbaV* Tyr194Ser; *lon* Leu241Pro	RV-CAR
MT4 (Berdejo et al., 2024)	Lethal	Heat	*dnaJ* Frameshift variant (Lys62) and *flhC* Thr148Pro	RV-HT
SePAW4 (Pagán et al., 2024b)	Lethal	Plasma activated water	*rpoD* Gly42Arg; *rpoA* Lys304Glu	RV-PAW

turn, reach consumers through the food chain (Oniciuc et al., 2019; Samtiya et al., 2022; Verraes et al., 2013).

In recent years, our laboratory has isolated RVs of *Salmonella* Typhimurium through adaptive laboratory evolution (ALE) assays by subjecting this bacterial strain to sublethal doses of carvacrol, an individual constituent found in numerous essential oils (Berdejo et al., 2020; Merino et al., 2023; Pagán et al., 2024a). Through a series of lethal treatment cycles, we have also isolated RVs of the same bacterial strain against traditional microbial inactivation technologies, such as heat treatments (Berdejo et al., 2024), and emerging technologies, including plasma-activated water (PAW) (Pagán et al., 2024b). Numerous resulting RVs have shown increased resistance against the agent applied for their isolation and cross-resistance to further antimicrobials, including antibiotics, as well as to other food preservation methods. It is nevertheless unknown whether these increases in resistance come at a fitness cost (a decreased growth rate and an increased lag time) and/or a virulence cost, as has been suggested by previous reports on microorganisms subjected to stress adaptation (Beceiro et al., 2013; Melnyk et al., 2015). Neither do we yet know whether a correlation can be observed between these different types of resistance (Cepas and Soto, 2020; Guillén et al., 2021). To evaluate microbial virulence, the use of *C. elegans* was proposed (Sanz-Puig et al., 2019). This animal model has been successfully applied in microbial virulence studies (Burkhardt et al., 2023; Labrousse et al., 2000; Silva et al., 2015) as it is well characterized, easy, and inexpensive to maintain due to the worm's short lifespan.

Given that RVs of *S.* Typhimurium can emerge in the food chain as a consequence of the use of antimicrobials and/or food preservation methods (Berdejo et al., 2024; Pagán et al., 2024a; Pagán et al., 2024b), our study's objective was threefold: 1) to evaluate whether the mutations identified in ALE-obtained variants resistant to CAR, HT and PAW offer any advantage or entail a fitness cost when growing at different temperatures, or cause any changes in these RVs' degree of virulence; 2) to evaluate whether these RVs, suspended in laboratory media at two different pH levels (7.0 and 4.0), show enhanced tolerance to heat, which is the inactivation technology most commonly employed to preserve food; 3) whether the RVs, when suspended in a liquid matrix (in this case, orange juice), show enhanced resistance to heat and to the simultaneous application of heat and citral, a combined process with an effective synergism that has been previously described (Espina et al., 2017; Somolinos et al., 2010). Research results thereby obtained should enable scientists to achieve greater precision in defining the extent and consequences of the occurrence of these RVs within the food chain; moreover, such knowledge should facilitate the development of improved food preservation strategies designed to control them.

2. Materials and methods

2.1. Microorganisms, growth conditions, and reagents

Salmonella enterica subsp. *enterica* serovar Typhimurium str. LT2 (SeWT) was provided by the Spanish Type Culture Collection (CECT 722). RVs had been previously obtained either by cyclic exposure to prolonged sublethal doses of carvacrol (100 μL/L of carvacrol for 24 h at 37 °C and pH 7.0: designated hereafter as RV-CAR), by cyclic exposure to short lethal heat treatments (30 cycles 55 °C/20 min, designated hereafter as RV-HT), or by exposure to brief lethal PAW treatments (pH 2.3/45 min: designated hereafter as RV-PAW). Whole-genome sequencing was carried out to identify genetic variations (Table 1). *Escherichia coli* OP50 (OP50) was provided by the Caenorhabditis Genetics Center (CGC) of the University of Minnesota.

All strains featured in this study were stored in cryovials containing glycerol (20 % v/v) at a temperature of −80 °C. On a weekly basis, plates of the strains were prepared in tryptone soya agar (Oxoid, Basingstoke, United Kingdom) supplemented with 0.6 % yeast extract (Oxoid; TSAYE). We prepared the working bacterial cultures by inoculating a single colony into test tubes containing 5 mL of tryptone soya broth supplemented with 0.6 % yeast extract (Oxoid; TSBYE). The test tubes were then incubated at 37 °C/16 h/130 rpm. We inoculated the resulting subcultures in flasks containing 10 mL of fresh TSBYE until an initial concentration of 10^6 colony-forming units per mL (CFU/mL) was reached. The flasks were incubated at 37 °C for 24 h and 130 rpm up to stationary growth phase (approximately 3×10^9 CFU/mL). We verified the bacterial concentration in the cultures by diluting them in phosphate-buffered saline (Sigma-Aldrich, Steinheim, Germany; PBS); plates were subsequently counted on TSAYE.

2.2. Fitness cost evaluation: growth curves at different temperatures

Growth kinetics were evaluated in tubes containing 5 mL of TSBYE at 37 °C, 43 °C, and 8 °C, at an initial concentration (N_0) of 3×10^7 CFU/mL and 3×10^6 CFU/mL. Every 15 min, optical density (OD_{595}) of the test tubes was measured at 595 nm wavelength by a microplate reader (100 μL/well), as well as in two-day intervals when they were incubated at 8 °C. OD_{595} values at time 0 corresponding to absorbance caused by the growth medium were subtracted. We graphically displayed the bacterial growth curves based on OD_{595} and modeled them using the following modified Gompertz equation:

$$y = A\,exp\{-exp[\,(\mu_m\, e/A\,)(\lambda - t) + 1\,]\} \quad (1)$$

where y: OD_{595}; *t*: time (h); *A*: maximum value reached (OD_{595} max); μ_m: maximum growth rate (1/h); λ: lag time/time to detection (h).

To build the model and obtain *A*, μ_m, and λ values, a least-squares adjustment using the GraphPrism® program (GraphPad Software, Inc., San Diego, USA) was applied. We evaluated the adjustment's goodness of fit by applying standard error (R^2), R^2 adjusted values, and RMSE (the root mean square error).

2.3. Evaluation of virulence potential: viability of Caenorhabditis elegans

C. elegans strain N2 provided by CGC were maintained and propagated on nematode growth medium (NGM) plates according to standard techniques described in Stiernagle (2006). Worm eggs were recovered from adult worms after exposure to a sodium hypochlorite/sodium hydroxide solution, as described in Porta-de-la-Riva et al. (2012). Egg suspensions were incubated overnight at 20 °C to allow hatching. Synchronized L1 worms were transferred onto fresh NGM plates covered with OP50 for the different assays.

2.3.1. Longevity assay

L1 nematodes were seeded on NGM plates ad libitum with OP50 and maintained at 20 °C for 52 h until they reached L4 stage. The nematodes were then transferred to fluorodeoxyuridine-containing 6-well plates (FUDR) with a maximum of 12 synchronized nematodes per well. The

nematodes were fed with either OP50, SeWT, RV-CAR, RV-HT, or RV-PAW. Three times per week for 21 days, we assessed the number of dead and alive nematodes with a Nikon stereoscopic microscope (Nikon SMZ745T, Tokyo, Japan) equipped with a camera. A worm was considered dead if it did not move and did not respond to stimulation by gentle prodding with a platinum pick. Three independent assays were performed with approximately 60 worms per assay.

2.3.2. Dye leakage assays

We studied morphofunctional alterations of the intestinal barrier using dye leakage assays (known as the "Smurf" assay), following the methodology described by Kissoyan et al. (2019), with certain modifications. Nematodes collected on days 1, 4, and 7 were transferred to FUDR plates, collected in S-buffer (consisting of 100 mM NaCl, 6.5 mM K_2HPO_4, and 43.5 mM KH_2PO_4), and washed twice. Subsequently, 1 mL of 5 % w/v erioglaucine disodium salt solution (Sigma-Aldrich) was added to 1 mL of washed nematodes and incubated for 3 h in rotation. Worms were washed in S-buffer five times until the supernatant appeared clear. The worms were then anesthetized with 20 μM levamisole (Sigma-Aldrich), mounted on microscope slides, and observed under a Nikon Ti-2 U microscope. Nematodes were quantified as "Smurf" type if blue dye could be observed outside the intestinal lumen or throughout the body cavity. Results are shown as the percentage of Smurf-type worms per condition. At least 30 worms were analyzed per condition and experiment.

2.4. Cross-resistance to heat treatments and combined processes

To evaluate the heat tolerance of SeWT and its RVs, we applied lethal heat treatments in "McIlvaine buffer" (citrate-phosphate buffer), elaborated from citric acid monohydrate (PanReac-AppliChem, Darmstadt, Germany) and disodium hydrogen phosphate (PanReac) and adjusted to pH 7.0 and pH 4.0. Polystyrene tubes containing 990 μL of treatment medium were heated to 53 °C (for pH 7.0) and to 50 °C (for pH 4.0) in a thermoblock incubator (FX Incubator, Zeulab, Zaragoza, Spain). Once the targeted temperature was reached, the tubes housed in the incubator were inoculated to achieve an initial concentration of 10^7 CFU/mL, thereby launching the treatment phase. Aliquots were taken after 40 and 20 min for pH 7.0 and pH 4.0, respectively. Aliquots were then diluted in PBS, pour-plated in TSAYE, and incubated at 37 °C for 24 h. Microbial counts were taken in an automatic plate counter via image analysis (Analytical Measuring Systems, Protos, Cambridge, United Kingdom).

Following the same methodology, we validated the heat tolerance of the WT and the RVs in commercial sterilized orange juice (pH 3.6) (Don Simón, Madrid, Spain). For this purpose, a heat treatment at 50 °C was applied for 10 min. Similarly, we assessed the cross-tolerance of the WT and the RVs to a combined process based on the simultaneous application of heat (50 °C/10 min) and citral (200 μL/L) (Sigma-Aldrich) in orange juice. Antimicrobials were added once the treatment medium was thermostated, prior to microbial inoculation.

2.4.1. Detection of sublethal injury

To assess the degree of sublethal injury, we measured maximum non-inhibitory concentrations (MNIC) of sodium chloride (SC) (data not shown), following a procedure previously described by Pagán et al. (2021). After heat treatment, samples were plated onto non-selective medium (TSAYE) and onto the following selective media: TSAYE with sodium chloride (TSAYE-SC) at 5.0 % for SeWT, RV-CAR, and RV-PAW; 4.5 % for RV-HT. Plates with TSAYE were incubated for 24 h, and those with TSAYE-SC for 48 h. The percentage of sublethally injured cells was expressed as the difference between the counts (CFU/mL) on TSAYE and on TSAYE-SC after treatments.

2.5. Statistical analysis

Results were obtained from at least 3 independent experiments conducted on different bacterial cultures on different working days. Lethal treatment graphics are displayed as the mean ± S.D. of the replicates. Data were analyzed and subjected to comparison of averages using analysis of variance (ANOVA), followed by *post-hoc* Tukey test using GraphPad Prism® program (GraphPad Software, San Diego, CA, USA). Data shown in graphs from the *C. elegans* experiments represent mean values ± S.D. Normality of the data was evaluated using the Kolmogorov-Smirnov Test and the Shapiro-Wild Test for Normality. Nonparametric sets of data were analyzed by Kruskal Wallis. Differences were considered significant if $p \leq 0.05$.

3. Results

3.1. Growth fitness of S. Typhimurium RVs to carvacrol, heat, and plasma-activated water

Fig. 1 displays the growth curves of SeWT, RV-CAR, RV-HT, and RV-PAW, obtained at three different temperatures: 37 °C (A, B), 43 °C (C, D), and 8 °C (E, F), with an initial concentration of 3×10^7 CFU/mL (A, C, E) and 3×10^6 CFU/mL (B, D, F), after modeling by modified Gompertz equation (Eq. (1)). The values from the model are represented in Table 2 and the goodness of fit in Table S1.

As shown in Fig. 1A, no significant differences ($p > 0.05$) in the maximum absorbance value were observed at 37 °C or in the lag time (1.5 h) for the four strains. However, once this had occurred, the RV-CAR strain demonstrated the slowest growth rate (0.158 ± 0.019 OD_{595}/h) compared to SeWT (0.220 ± 0.027 OD_{595}/h) ($p \leq 0.05$).

When we increased the temperature to 43 °C (Fig. 1C), a generalized decrease in the maximum absorbance value could be observed in all four strains compared to 37 °C. The RV-HT strain reached a lower *A* value and growth rate compared to the other strains ($p \leq 0.05$), which exhibited behavior of similar proportions among themselves. The SeWT strain experienced a 30 % reduction of lag time with the temperature rise, in contrast to the evolved strains, whose time to detection remained consistent across both temperatures.

With a decrease in temperature to 8 °C (Fig. 1E), the maximum absorbance values and growth rates were similar between SeWT and RV-HT, differing from those of RV-CAR and RV-PAW strains, which exhibited significant increases along both parameters. Notably, the lag time of the RV-PAW was approximately six times longer than the time to detection of the other strains.

Once the N_0 had been reduced by a factor of 10, the maximum absorbance values and growth rates were similar to those previously described at 37 °C (Fig. 1B) and 43 °C (Fig. 1D). However, as expected, the time to detection was more extended: twice as long in some cases. Nevertheless, the most remarkable results were obtained at 8 °C, as neither SeWT, RV-HT, nor RV-PAW were able to grow after 33 days, unlike RV-CAR, which initiated its growth after 9 days. Therefore, RV-CAR was the only RV with a better growth fitness than SeWT, specifically under refrigeration temperatures (8 °C), given its shorter lag time and higher growth rate.

3.2. Virulence potential of S. Typhimurium variants resistant to carvacrol, heat, and plasma-activated water

As a first approach to assess the virulence of SeWT and its RVs, we tested whether the lifespan of *Salmonella*-fed *C. elegans* worms was affected in comparison to that of the control, OP50. Thus, as shown in Fig. 2, nematodes exposed to SeWT, RV-CAR, RV-HT, and RV-PAW had a shorter overall life span than those exposed to OP50, being superior to 5 % at 21 days for OP50, 20 days for PAW, 19 days for RV-HT, 17 days for RV-CAR and 15 days for SeWT.

Moreover, the nematodes' decrease of survival rate during their lifespan was significantly different ($p > 0.05$) depending on which *Salmonella* strain was applied. Fig. 3 shows the values of time to death of 50 % (TD50) of the population. SeWT was the most virulent strain,

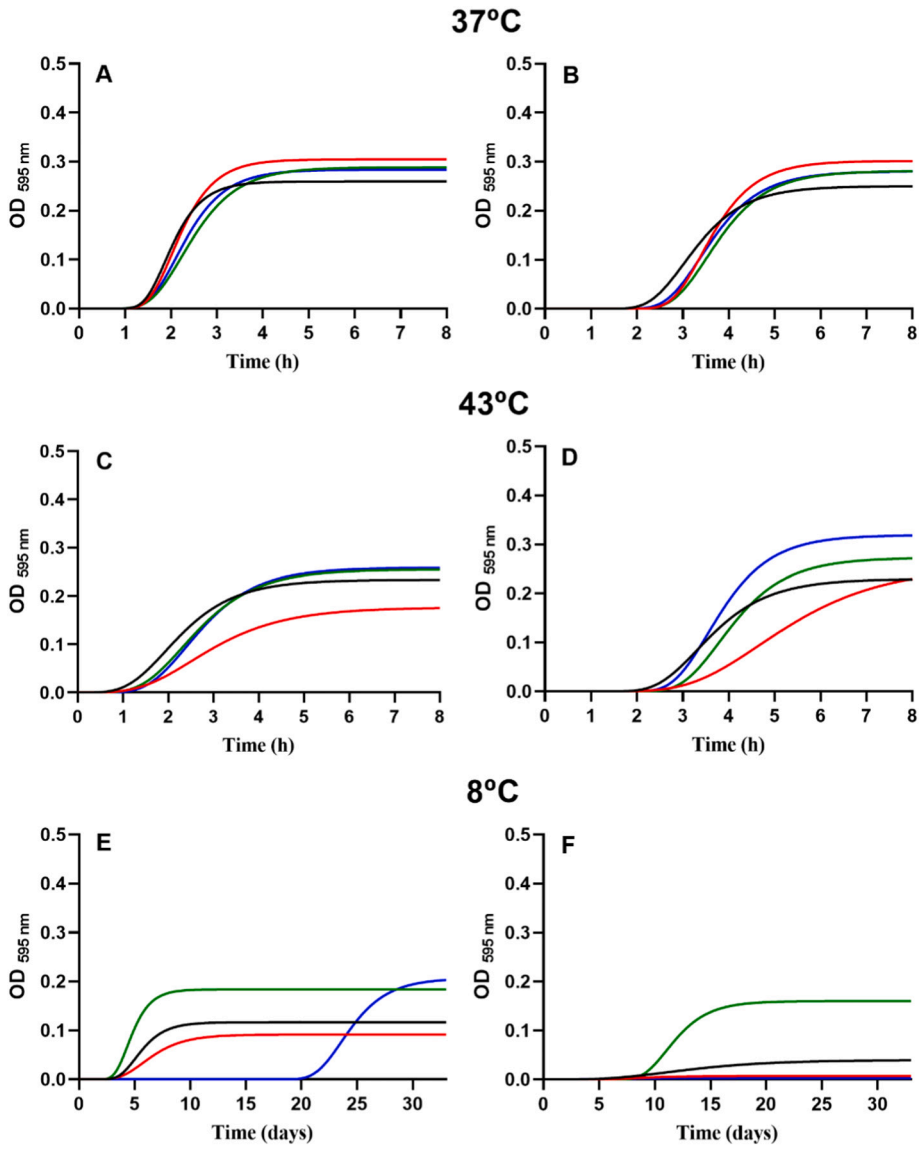

Fig. 1. Modeled growth curves of *Salmonella enterica* Typhimurium LT2 (SeWT; —) and resistant variants: RV-CAR (—), RV-HT (—) and RV-PAW (—) in TSBYE at 37 °C (A, B), 43 °C (C, D) and 8 °C (E, F) with an initial concentration of 3×10^7 CFU/mL (A, C, E) and 3×10^6 CFU/mL (B, D, F).

decreasing TD50 from 12.7 to 7 days compared to OP50 ($p \leq 0.05$). There were no significant differences ($p > 0.05$) among the TD50 values for RVs compared to SeWT (7 days), although RV-PAW had a lower degree of virulence (a TD50 of 11 days), quite similar to that of the OP50 control (a TD50 of 12.7 days).

To assess severity of damage due to infection, the intestinal barrier function of *C. elegans* was evaluated in this study by applying the "Smurf assay", i.e., exposing the worms to blue dye (Fig. 4). The blue dye remained inside the worms' intestine when intestinal integrity was intact; however, dye leaked into the body cavity whenever the intestinal barrier function was disrupted. In control worms (OP50), we noted that the blue dye remained in the infected intestine (Fig. 4B). On the other hand, in the worms infected with the SeWT strain, the blue dye leaked into the body cavity (Fig. 4A). The latter type of cases is colloquially designated as "Smurf phenotype".

As can be observed in Fig. 5, the percentage of Smurf phenotype for SeWT, RV-CAR, and RV-HT was similar: around 80 % ($p > 0.05$), which was 40 % higher than the OP50 ($p \leq 0.05$). Notably, RV-PAW demonstrated loss of virulence in contrast to RV-CAR and RV-HT, which induced disruption of intestinal barrier function of the infected nematodes, similarly to the effects caused by SeWT ($p > 0.05$). Therefore, virulence assays in *C. elegans* nematodes showed that mutations affecting RV-CAR and RV-HT did not result in a substantial loss of pathogenicity; in contrast, mutations affecting RV-PAW significantly reduced its virulence potential.

3.3. Heat tolerance in laboratory media of S. Typhimurium variants resistant to carvacrol, heat, and plasma-activated water

To further characterize the heat tolerance of the RVs we had obtained, we carried out heat treatments in laboratory media under neutral and acid pH conditions. The results obtained after 40 min at 53 °C in pH 7.0 (Fig. 6A) and after 20 min at 50 °C in pH 4.0 (Fig. 6B) were similar: the three RVs showed a significant increase in heat tolerance ($p \leq 0.05$) in comparison to SeWT. The degree of inactivation of the RVs was reduced by 2–3 $\log_{10}$ cycles, regardless of the pH of the treatment medium. These results also demonstrate the existence of cross-resistance between the antimicrobial agent employed to obtain the RV (carvacrol and PAW) and lethal heat treatment.

Table 2
A (maximum OD_{595}), μ_m (maximum growth rate) and λ (lag time) parameters of the modified Gompertz model obtained from growth curves in TSBYE of *Salmonella enterica* Typhimurium LT2 (SeWT) and resistant variants (RV-CAR, RV-HT, RV-PAW) at 37, 43 and 8 °C with different initial concentration (A; 3×10^7 CFU/mL) and (B; 3×10^6 CFU/mL). Each value represents the mean ± standard deviation from 3 independent experiments.

A) Initial concentration: 3×10^7 CFU/mL

Temperature (°C)	Parameter	Strains			
		SeWT	RV-CAR	RV-HT	RV-PAW
37	*A* (OD_{595})	0.259 ± 0.027^{a}	0.289 ± 0.008^{a}	0.305 ± 0.029^{a}	0.283 ± 0.021^{a}
	μ_m (OD_{595}/h)	0.220 ± 0.027^{a}	0.158 ± 0.019^{b}	0.227 ± 0.014^{a}	0.184 ± 0.029^{ab}
	λ (h)	1.427 ± 0.149^{a}	1.557 ± 0.062^{a}	1.530 ± 0.076^{a}	1.545 ± 0.108^{a}
43	*A* (OD_{595})	0.234 ± 0.010^{a}	0.256 ± 0.026^{a}	0.176 ± 0.011^{b}	0.259 ± 0.007^{a}
	μ_m (OD_{595}/h)	0.102 ± 0.002^{a}	0.105 ± 0.008^{a}	0.059 ± 0.005^{b}	0.116 ± 0.005^{a}
	λ (h)	1.096 ± 0.079^{a}	1.406 ± 0.128^{ab}	1.410 ± 0.196^{ab}	1.584 ± 0.192^{b}
8	*A* (OD_{595})	0.117 ± 0.005^{a}	0.184 ± 0.021^{b}	0.092 ± 0.019^{a}	0.203 ± 0.017^{b}
	μ_m (OD_{595}/days)	0.031 ± 0.005^{a}	0.057 ± 0.010^{b}	0.018 ± 0.001^{a}	0.050 ± 0.007^{b}
	λ (days)	3.735 ± 0.372^{a}	3.113 ± 0.422^{a}	3.811 ± 0.571^{a}	22.49 ± 2.169^{b}

B) Initial concentration: 3×10^6 CFU/mL

Temperature (°C)	Parameter	Strains			
		SeWT	RV-CAR	RV-HT	RV-PAW
37	*A* (OD_{595})	0.250 ± 0.009^{a}	0.281 ± 0.007^{bc}	0.302 ± 0.008^{b}	0.280 ± 0.008^{c}
	μ_m (OD_{595}/h)	0.132 ± 0.017^{a}	0.142 ± 0.003^{a}	0.177 ± 0.015^{b}	0.146 ± 0.007^{ab}
	λ (h)	2.326 ± 0.137^{a}	2.786 ± 0.114^{b}	2.790 ± 0.177^{b}	2.673 ± 0.149^{b}
43	*A* (OD_{595})	0.230 ± 0.015^{a}	0.273 ± 0.035^{ab}	0.257 ± 0.018^{a}	0.319 ± 0.008^{b}
	μ_m (OD_{595}/h)	0.098 ± 0.008^{a}	0.125 ± 0.013^{c}	0.064 ± 0.011^{b}	0.158 ± 0.002^{d}
	λ (h)	2.442 ± 0.098^{a}	2.984 ± 0.044^{bc}	3.182 ± 0.150^{b}	2.803 ± 0.145^{c}
8	*A* (OD_{595})	–	0.160 ± 0.017	–	–
	μ_m (OD_{595}/days)	–	0.028 ± 0.002	–	–
	λ (days)	–	8.987 ± 0.342	–	–

Different superscript letters represent statistically significant differences ($p \leq 0.05$) among the means of the same row.

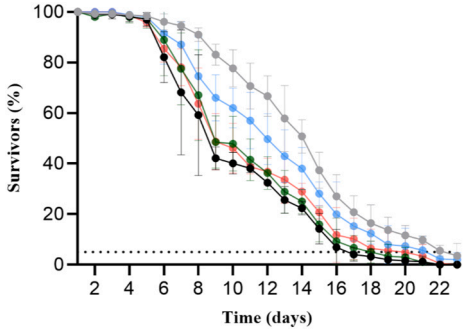

Fig. 2. Survival curves of *Caenorhabditis elegans* worms fed with apathogenic *Escherichia coli* OP50 (●; control), *Salmonella enterica* Typhimurium LT2 (●; SeWT) and resistant variants: RV-CAR (●), RV-HT (●) and RV-PAW (●). Dashed line represents 0.05 % of survivors.

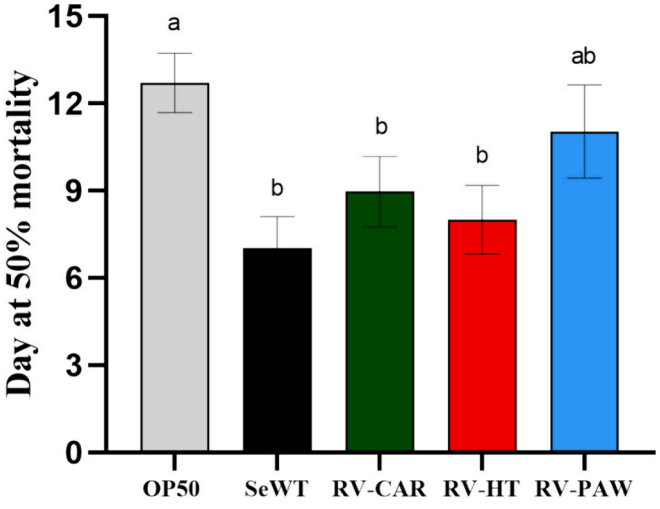

Fig. 3. TD50 of *Caenorhabditis elegans* worms fed with apathogenic *Escherichia coli* OP50 (OP50; ■), *Salmonella enterica* Typhimurium LT2 (SeWT, ■), and resistant variants: RV-CAR (■), RV-HT (■) and RV-PAW (■). TD50: time required for 50 % of the nematodes to die. Data are displayed as mean ± SD and were obtained from three independent experiments. Different superscript letters represent statistically significant differences ($p \leq 0.05$).

3.4. Evaluation of RVs' heat tolerance in orange juice and exploration of strategies to improve their inactivation

We tested the heat tolerance of SeWT and its RVs in orange juice, conducting the evaluation at a lower temperature due to that medium's higher acidity (pH 3.6). A higher heat tolerance of RVs ($p \leq 0.05$) was again observed in comparison to that of SeWT, especially in the case of RV-PAW (Fig. 7).

To enhance the efficacy of the thermal treatment against the RVs, we started by assessing the occurrence of sublethal damages to the cytoplasmic membrane, as such damages have been shown to be crucial in the design of efficient combined processes (Arroyo et al., 2010; Espina et al., 2010; Pagán et al., 2018). For this purpose, survivors to the heat treatment were plated on two different recovery media: TSAYE, which allows for a maximum proportion of survivors, and TSAYE-SC, which impairs the repair of cells that accumulate damages in the cytoplasmic membrane. A comparison among the tested strains of the proportion of cells whose cytoplasmic membrane was sublethally injured (as depicted in Fig. 7) shows that WT and RV-CAR have the highest proportion of sublethally injured cells (>90 %), whereas the proportion is slightly lower in RV-HT (85 %) and significantly lower in RV-PAW (approximately 50 %).

Since the application of HT in presence of natural antimicrobials has been shown to exert a synergistic lethal effect through the inactivation

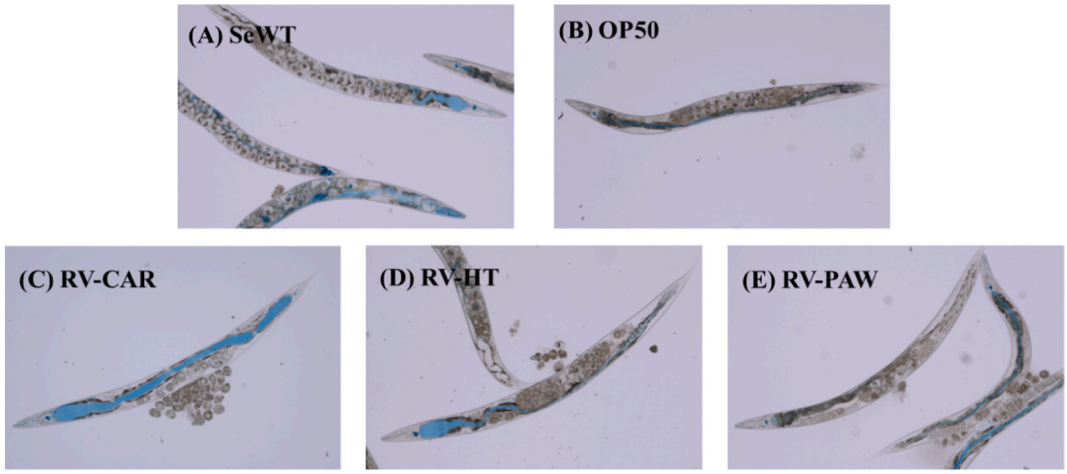

Fig. 4. Representative images of *Caenorhabditis elegans* worms fed with *Salmonella enterica* Typhimurium LT2 (A), apathogenic *Escherichia coli* OP50 (B) and resistant variants: RV-CAR (C), RV-HT (D) and RV-PAW (E). Nematodes were exposed to erioglaucine for evaluating the permeability of the gastrointestinal barrier. Photographs were taken with a Ti-u2 Nikon microscope and a 4× Apochromat objective.

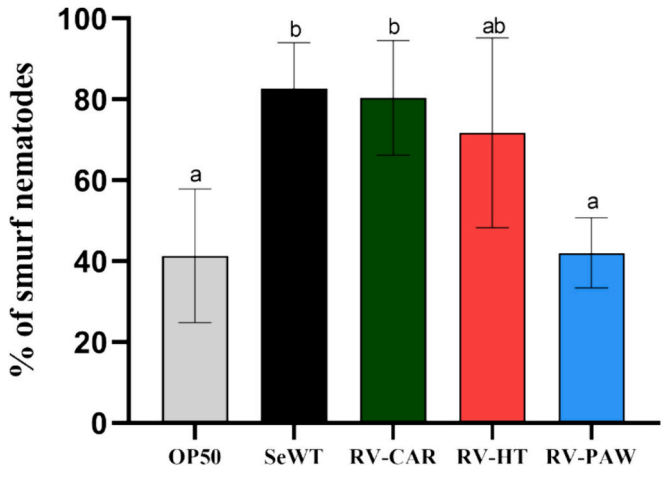

Fig. 5. Percentage (%) Smurf phenotype of apathogenic *Escherichia coli* OP50 (■), *Salmonella enterica* Typhimurium LT2 (■; SeWT) and resistant variants: RV-CAR (■), RV-HT (■) and RV-PAW (■) after 7 days of incubation. Data are displayed as mean ± SD and were obtained from eight independent experiments. Different superscript letters represent statistically significant differences ($p \leq 0.05$).

of damaged bacteria, we explored a process combining HT (50 °C/10 min) with citral (200 μL/mL), a natural antimicrobial present in EOs. We started by assessing the lethal efficacy of 200 μL/mL of citral against the four strains for 20 min at room temperature. No significant inactivation was attained (data not shown). As a result of the combined treatment, we were able to observe a synergistic lethal effect in the four strains, as the total achieved inactivation was greater than the sum of the inactivations caused by heat and citral applied individually. The highest synergism was observed in SeWT, as >4 extra $\log_{10}$ cycles of inactivation were obtained with the combined process. A similar synergism also stood out with RV-CAR and RV-HT, reaching almost 3 and 1.5 extra $\log_{10}$ cycles of inactivation, respectively. However, the presence of citral scarcely improved the heat treatment's effectiveness when it was applied to RV-PAW, given the latter variant's greater degree of heat resistance compared to the other strains, and in view of the fact that the cytoplasmic membrane of RV-PAW accumulated less overall damage.

4. Discussion

AMR is a major current threat already affecting human and animal health on a widespread global scale. The application of antibiotics and other antimicrobials can exert selective pressure after long-term sublethal exposure and favors the emergence of specific RVs. In previous studies, we isolated RVs in ALE assays with subinhibitory concentrations of carvacrol (RV-CAR (Pagán et al., 2024a)), lethal heat (RV-HT (Berdejo et al., 2024)), and plasma-activated water treatments (RV-PAW (Pagán et al., 2024b)). In those three studies, RVs showed direct resistance to the treatment for which they were isolated, and cross-resistance to heat and antibiotics (chloramphenicol) in the case of RV-CAR. However, further important aspects, such as their growth fitness, virulence, and heat tolerance when contaminating a food matrix, are still unknown. More in-depth knowledge of those factors could inform us about the relevance of the emergence of these RVs in case they reach the food chain, compete with SeWT, display virulence, and survive heat treatments.

Under optimal growth conditions (37 °C), the kinetics of the RVs compared with SeWT demonstrate that mutations occurring in RVs entail a slight fitness cost (a decreased growth rate and an increased lag time), especially in RV-CAR (Fig. 1A), as similarly occurs with *L. monocytogenes* resistant to TCO (Berdejo et al., 2021). Karatzas et al. (2008) observed that the acquisition of acid resistance also had a fitness cost for *Salmonella* cells (at neutral pH). The fitness cost is accentuated when the increase in temperature acts as a stress factor: we observe that RV-HT was the most affected strain. RV-HT has two mutations: an SNV in *flhC* and a frameshift in *dnaJ*. The latter caused the loss of its function: specifically, the production of a heat shock protein that acts as a molecular co-chaperone of DnaK and enables its role in protein folding and disaggregation. This result is in accordance with Takaya et al. (2004), who found that a DnaK/DnaJ-depleted *Salmonella* mutant was temperature-sensitive for growth, i.e., not viable above 39 °C. It is also in accordance with Sell et al. (1990), who found that *dnaJ* is essential for the growth of *E. coli* at 43 °C.

Conversely, when growth temperature was reduced to refrigeration conditions (8 °C), the mutations occurring in RV-CAR and RV-PAW caused the growth rate to be doubled compared with SeWT and RV-HT. This improvement in the growth rate of RV-CAR at low temperatures could be due to a mutation in the *lon* gene, since the Lon protease plays a vital role in protein quality control by eliminating faulty proteins during stress responses and activating pathways to prevent any further damage to the bacterial cell that could be caused by low temperatures (Kirthika et al., 2020). Lastly, although RV-PAW has a high growth rate,

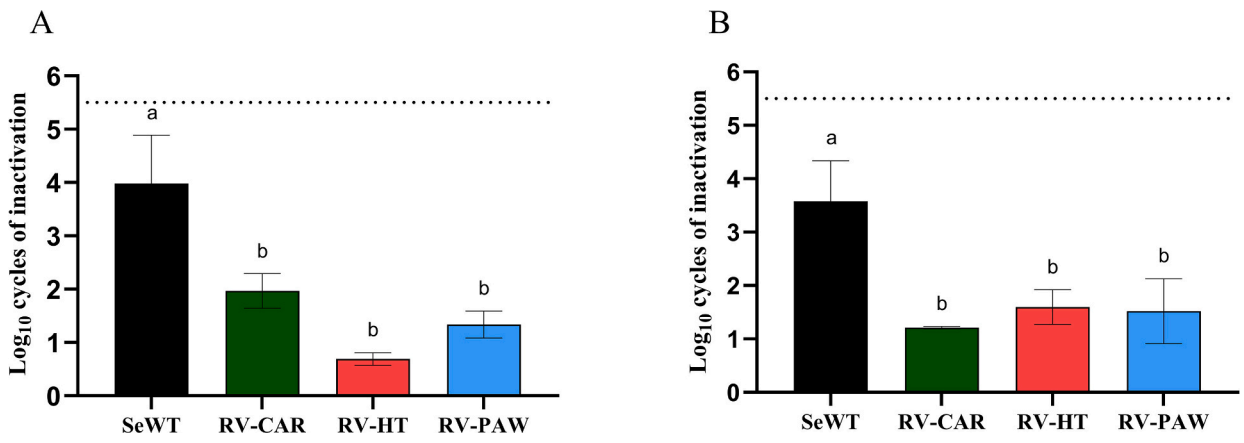

Fig. 6. Log_{10} cycles of inactivation of *Salmonella enterica* Typhimurium LT2 (■; SeWT) and resistant variants: RV-CAR (■), RV-HT (■) and RV-PAW (■) after heat treatments: (A) 53 °C/40 min in pH 7.0; (B) 50 °C/20 min in pH 4.0. Data are means ± standard deviations (error bars) obtained from at least three independent experiments. Different superscript letters represent statistically significant differences ($p \leq 0.05$). The dashed line represents the detection limit (−5.5 log_{10} N_t/N_0).

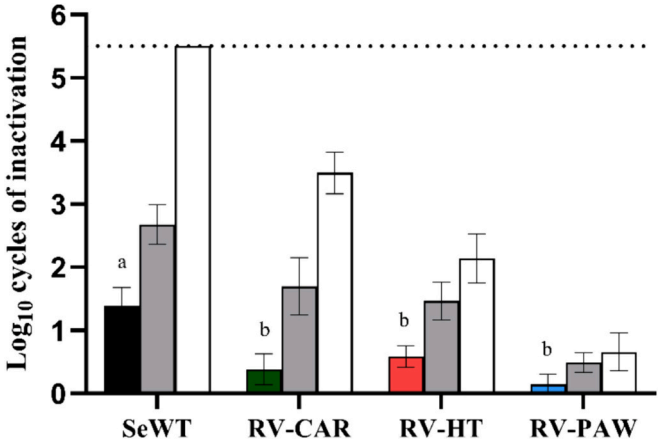

Fig. 7. Log_{10} cycles of inactivation of *Salmonella* Typhimurium LT2 (■; SeWT) and resistant variants: RV-CAR (■) RV-HT (■) and RV-PAW (■) after 50 °C/10 min heat treatment in orange juice recovered in TSA (■; and combined treatments), TSA-SC ■) and combined with 200 μL/L of citral (□). Data are means ± standard deviations (error bars) obtained from at least three independent experiments. Different superscript letters represent statistically significant differences ($p \leq 0.05$). The dashed line represents the detection limit (−5.5 log_{10} N_t/N_0).

its long lag time or adaptation to the environment could be due to the mutations found in the RNA polymerase subunit (*rpoA*) and the transcriptional factor $\sigma^D/^{70}$ (encoded by *rpoD*), which is responsible for expression of most housekeeping genes required for normal cellular metabolism during exponential growth (Bang et al., 2005). Thus, although RV-PAW has a growth rate similar to RV-CAR at cold temperatures, its highly extended lag time entails that RV-HT reaches the stationary phase much earlier, despite being the RV with the lowest growth rate. Therefore, if any of these three RVs were selected during processing or reached the food chain through cross-contamination, the one that would pose the most significant hazard would be RV-CAR, especially in processed foods kept under refrigeration.

To test whether the mutations conferring resistance might affect the RVs' virulence, we used *C. elegans* as a test organism in a pathogenicity assay. *S.* Typhimurium has been previously shown to shorten the lifespan of *C. elegans* by colonizing its intestinal epithelium and pharynx (Burkhardt et al., 2023; Labrousse et al., 2000). The reduced lifespan of the SeWT-fed nematodes compared to *E. coli* OP50-fed ones reflected the virulence of SeWT. Bearing in mind that RV-PAW was isolated by selective pressure from plasma-activated water, which is a highly acidic medium (pH 2.3), this RV can also be regarded as an acid-resistant variant. According to Berk et al. (2005), acid-resistant strains can enjoy enhanced chances of survival in the stomach, which, in turn, increases their numbers in the intestinal epithelium and allows them to establish an infection. Our results do not confirm that assumption, as the TD50 of RV-PAW was slightly higher than that of SeWT (Fig. 3), a finding further reinforced if we take the percentage of Smurf phenotype into account (Fig. 5). As shown in Fig. 4E, *C. elegans* fed with RV-PAW maintained their intestinal barrier at a rate similar to that of OP50-fed nematodes (Fig. 4B). These results are in agreement with those of Karatzas et al. (2008), who reported that an increased acid resistance can lead to a lower ability of *S. enterica* to establish a systemic infection in mice.

Secondly, RV-HT maintained the same virulence as SeWT, as demonstrated by its TD50 rate and the abnormal and enlarged intestinal tube we were able to observe (Fig. 4D). Similar results were obtained by Berdejo et al. (2024), who showed that a *Salmonella* Δ*dnaJ* mutant was capable of colonizing the large intestine of antibiotic-pretreated mice. However, they further explored the virulence factor and found that the Δ*dnaJ* mutant neither infected the mesenteric lymph nodes nor triggered acute inflammation. Thus, loss of DnaJ severely attenuated the virulence of *Salmonella*. This fact is also in line with a previous observation that *dnaK* deletion (encoding a co-chaperone for DnaJ) causes reduced virulence of *S.* Typhimurium in mice (Takaya et al., 2004).

Finally, RV-CAR was as virulent as SeWT; thus, its mutations that had increased in terms of resistance and tolerance did not suffer any setback in terms of virulence (Kirthika et al., 2020; Takaya et al., 2003). These results are in agreement with those of Yuan and Yuk (2019), who found that *E. coli* O157:H7 adapted to thymol, carvacrol, and trans-Cinnamaldehyde did not become more virulent (in terms of adhesion and invasion) than SeWT. We conclude that RV-CAR is an especially alarming RV, given that it practically maintains its virulence and growth fitness, or even improves them at refrigeration temperature.

The next step consisted in exploring whether these RVs showed cross-resistance to thermal treatments under acidic conditions: specifically, in orange juice, which we chose as a model for acid foods. The three RVs not only showed increased tolerance to heat treatment at pH 7.0, as described in previous studies (Berdejo et al., 2024; Pagán et al., 2024a), but also to laboratory media at acid pH (Fig. 6). The mechanisms responsible for this behavior varied widely among the three RVs. The *flhA* gene, involved in flagellar biosynthesis, was implicated in the resistance displayed by RV-CAR, as described in a previous study (Pagán et al., 2024a). Other studies featuring *E. coli* have also underscored the relationship between flagellar synthesis and tolerance to HHP (Gayán et al., 2019). The thermal resistance of RV-HT was described by Berdejo

et al. (2024), who confirmed that loss of DnaJ functionality was, indeed, causative and in itself sufficient to explain RV-HT's increased heat resistance. As previously mentioned, RV-PAW is indirectly resistant to acid; thus, the mutations observed in the *rpoA* and *rpoD* genes could be related to membrane fatty acid composition, that is, to lower membrane fluidity, which, in turn, is associated with heat resistance (Álvarez-Ordóñez et al., 2008).

Finally, we were also able to observe a higher tolerance of all three RVs in orange juice in comparison to the SeWT (Fig. 7). Thus, in order to achieve a greater degree of microbial inactivation, we chose to assay a combined process based on the simultaneous application of heat and citral (Chueca et al., 2015). Our objective was to inactivate the RVs without increasing heat treatment intensity, thereby avoiding the adverse effects of heat on sensory and nutritional food properties while simultaneously maintaining the level of energy consumption. As a previous step, we tested for the existence of sublethal damage, which we were able to confirm in SeWT, RV-CAR, and RV-HT, thereby pointing to a likely synergism between heat and citral (Espina et al., 2010; Targino de Souza Pedrosa et al., 2021). In this sense, Somolinos et al. (2010) demonstrated a strongly synergistic lethal effect of a mild heat treatment combined with citral on *E. coli*, associated with the damage caused to cell envelopes by heat. As a result, while maintaining the heat treatment's intensity, the degree of inactivation we were able to observe after applying the combined process for the RV-CAR and RV-HT strains was higher than the degree of inactivation of SeWT obtained after applying the heat treatment alone. These results show that the design of appropriate combined processes could serve as a relevant strategy to mitigate the consequences of the occurrence of variants with specific mutations that cause increased heat tolerance in food. For instance, we observed a significant increase in the intrinsic resistance displayed by RV-PAW in orange juice, as shown by the slight differences detected among the number of survivors in non-selective and selective media. We observed no synergism when we applied the combined treatment under these circumstances. Nevertheless, RV-PAW had suffered a significant fitness and virulence cost; thus, its higher survival rate in heat treatments does not need to be regarded as a relevant risk. On the contrary, the combined process of HT and citral succeeded in improving the extent of microbial inactivation, at least of RV-CAR, which was the strain presenting the greatest hazard due to its virulence and its ability to improve its growth fitness at refrigeration temperature.

5. Conclusions

The question of whether the development of a stress resistance response can lead to an increase or decrease in the risk of salmonellosis is not only defined by the impact on the expression of virulence factors but also by the degree of RVs' resistance to food stressors and their degree of growth fitness. Our research in this study further characterizes RVs that emerged after ALE assays with three different food preservation methods: a traditional technology (thermal treatments: RV-HT), an emerging technology (PAW: RV-PAW), and a natural antimicrobial (carvacrol: RV-CAR). While RV-PAW showed an enhanced tolerance to heat in food, this did not pose a significant threat, as RV-PAW displayed reduced growth fitness compared to the parent strain and lost its degree of virulence. However, another RV, namely RV-CAR, displayed not only a significantly higher thermotolerance but also excellent growth fitness compared to SeWT while retaining its virulence. Our study thus underscores the importance of developing combined processes capable of leveraging sublethal damage caused by heat treatments. Finally, we observed a notable synergism between heat and citral for purposes of microbial inactivation. This, in turn, allowed us to limit the enhanced tolerance we observed in RV-CAR suspended in orange juice.

These results show that, in order to improve the inactivation of RVs that could be present in the food chain, such as those featured in this study, it would be necessary to increase the intensity of heat treatments or to develop combined processes that synergize with heat, e.g., essential oils or their main antimicrobial constituents, such as citral.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110810.

Compliance with ethical standards

This research complies with ethical standards.

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Ethical approval

This article does not contain any studies performed by any of the authors on human participants or animals.

CRediT authorship contribution statement

Elisa Pagán: Writing – original draft, Methodology, Investigation, Conceptualization. **Noelia López:** Investigation, Formal analysis. **Ana Sánchez:** Investigation, Formal analysis. **Raúl Campillo:** Investigation. **Daniel Berdejo:** Writing – review & editing, Methodology, Conceptualization. **Diego García-Gonzalo:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Rafael Pagán:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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