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Isolation and characterization of resistant variants of *Salmonella* Typhimurium after sequential exposure to plasma activated water (PAW)

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ABSTRACT

Plasma-activated water (PAW) is emerging as a promising disinfectant. However, its use can inadvertently contribute to the isolation of bacterial resistant variants (RVs). In this study, four distinct populations of *Salmonella enterica* serovar Typhimurium were subjected to 30 cycles consisting of lethal treatments with PAW followed by recovery of survivors. One RV from each population was isolated for genotypic and phenotypic characterization. Globally, genomic analysis of four isolated RVs (SePAW1–4) uncovered mutations affecting proteins directly or indirectly related to oxidative stress response (QseC in SePAW1; ArcB in SePAW2; and ZntB, and CsrA in SePAW3); to cell envelopes (MreB in SePAW1; and MpeM, and WbaV in SePAW2); or to transcriptional factors (RpoD in SePAW4). Significantly, these genetic alterations were found to underlie the observed cross-tolerance of each RV to one or more disinfectants: a chlorine-based solution (sodium hypochlorite), an oxidative compound (peracetic acid), or an individual compound from essential oils (carvacrol).
Industrial relevance: The research findings provide crucial insights into the adaptability of *Salmonella* Typhimurium to plasma activated water (PAW) and its implications for industrial disinfection practices. Understanding the genetic mechanisms underlying bacterial resistance to PAW offers valuable guidance for the development of targeted disinfection strategies in the food industry. The study's emphasis on exploiting compromised cell envelopes highlights the potential for enhancing the effectiveness of PAW-based disinfection technologies in industrial settings, thus contributing to improved food safety standards.

1. Introduction

Foodborne diseases are a global concern, and ensuring food safety during production and processing is of utmost importance. The presence of harmful microorganisms in the food chain can come from the raw material as well as cross-contamination from contact surfaces where *Salmonella* spp. can survive among other microorganisms (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). Therefore, disinfection plays a vital role to control such contaminants. Chlorine-based solutions, such as sodium hypochlorite, have long been favoured in the food industry due to their effectiveness, wide availability, and affordability (Drogui & Daghrir, 2015). However, it is essential to consider the potential risks associated with chlorine-based water treatment, as it can result in disinfection by-products such as trihalomethanes, haloacetic acids, haloacetonitriles, and haloketones, which have been associated with cancer and reproductive outcomes (Wawryk, Wu, Zhou, Moe, & Li, 2020).

To address this concern, alternative disinfectants have been explored such as acetic acid and peracetic acid (PAA) (Ölmez & Kretzschmar, 2009) which are residue-free green disinfectants. However, their strong corrosive nature on treated surfaces represents a challenge (Horn & Niemeyer, 2022). Another alternative are the plant-based extracts such as the essential oils (EOs) and their individual constituents, e.g. carvacrol (Falco, Verdegue, Aznar, Sanchez, & Randazzo, 2019).

Abbreviations: PAW, Plasma activated water; RV, Resistant variants; PAA, Peracetic acid; BS, Bile salts.

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Nevertheless, their use is compromised due to their lipophilic character and their susceptibility to external factors (such as oxygen, light) that can affect their antimicrobial capacity. Additionally, the exposure to certain biocides and disinfection agents can exert selective pressure and potentially promote the emergence of bacterial resistant variants (RVs) (Berdejo et al., 2022; Cadena, Kelman, Marco, & Pitesky, 2019; Mavri & Smole Možina, 2013; Rodríguez-Melcón, Alonso-Calleja, & Capita, 2023). One experimental approach for understanding the adaptive response of microorganisms after their exposure to particular environments is the implementation of adaptive laboratory evolution (ALE), where the microorganisms are cultured under defined conditions for an extended period of time.

The food industry has shown significant interest in new environmentally friendly sterilization technologies. These technologies include ozone, acidic electrolyzed water, ultrasound technology, pulsed electric field, and plasma-activated water (PAW) (Knorr et al., 2011; Roobab et al., 2023). PAW generated by non-thermal plasma has emerged as a novel and highly efficient disinfectant for fresh produce, such as strawberries (Ma et al., 2015), button mushrooms (Xu, Tian, Ma, Liu, & Zhang, 2016), kale, and spinach (Perinban, Orsat, Lyew, & Raghavan, 2022), as well as lentil sprouts (Medvecka et al., 2022). Remarkably, the use of PAW does not significantly affect the colour, firmness, pH, or antioxidant properties of the treated products. Numerous studies have investigated the antimicrobial properties of PAW and explored its generation, reactive species origin, and biological applications (Roobab et al., 2023; Thirumdas et al., 2018; Zhao, Patange, Sun, & Tiwari, 2020; Zhou et al., 2020). The composition of the reactive oxygen and nitrogen species (RONS) in PAW depends on the working gas and the system's operating conditions (Bradu, Kutasi, Magureanu, Puac, & Zivkovic, 2020). Typically these species include long-lived species such as nitrates (NO_3^-), nitrites (NO_2^-), hydrogen peroxide (H_2O_2), and ozone (O_3), as well as relatively short-lived ones like hydroxyl radicals ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$), superoxide (O_2^-), peroxynitrate ($OONO_2^-$), and peroxynitrites ($ONOO^-$) (Ma, Zhang, Lv, & Sun, 2020; Qi et al., 2018; Rathore, Patel, Butani, & Nema, 2021; Volkov et al., 2021). These RONS have been shown to significantly contribute to bacterial inactivation by creating a high oxidation-reduction potential. However, the mechanisms underlying bacterial tolerance and inactivation by PAW are not yet fully understood.

Consequently, the objective of this study is to isolate *Salmonella* Typhimurium RVs during ALE assays using PAW and characterize them phenotypically. Additionally, we aim to identify the genetic modifications associated with the increased tolerance of these RVs and assess their cross-tolerance to other disinfectants. This research aims to enhance our understanding of the mechanisms underlying bacterial tolerance against PAW and improve disinfection processes.

2. Material and methods

2.1. Microorganisms, growth conditions, and reagents

Salmonella enterica subsp. *enterica* serovar Typhimurium LT2 (SeWT) was provided by the Spanish Type Culture Collection (CECT 722; ATC700720). Strains were maintained in cryovials at −80 °C with glycerol (20% *v*/v), from which plates of tryptone soya agar (Oxoid, Basingstoke, England) with 0.6% yeast extract (Oxoid; TSAYE) were prepared on a weekly basis. To prepare the overnight preculture, one colony was inoculated in a tube containing 5 mL of fresh tryptone soya broth (Oxoid) with 0.6% yeast extract (TSBYE) and was incubated aerobically in an orbital shaker (130 rpm; Heidolph Vibramax 100, Schwaback, Germany) for 12 h at 37 °C (Incubig, Selecta, Barcelona, Spain). Subsequently, working bacterial cultures were prepared in flasks containing 10 mL of TSBYE, which were inoculated with an initial concentration of 10^6 colony forming units per mL (CFU/mL) from the preculture and were incubated for 24 h/37 °C/130 rpm until the stationary growth phase (approximately 3×10^9 CFU/mL was reached). The same protocol was applied to obtain the working bacterial cultures of the RVs resulting from the ALE experiments with PAW.

2.2. PAW set-up

The PAW set-up was based on that of Darmanin et al. (2020) with some modifications. In summary, the set-up consisted of an electrochemical cell (with a volume of 50 mL). High-purity water was used to facilitate the precise determination of PAW's mechanism of action, eliminating interference from other compounds in the system. Thus, the electrochemical cell was filled with 20 mL of high-purity water and connected to a reservoir through a peristaltic pump, containing 240 mL of water. This circulation of water in the electrochemical cells ensured a steady volume of 20 mL within the cell during treatment. The cell had a copper electrode (0.5 mm in diameter; AlfaAesar, 99.999% purity) positioned approximately 4 mm above the water surface and connected to a self-regulated HV-DC power supply with a pulsating frequency of constant polarity of around 37.35 kHz. The voltage and electric current were measured using a UNI-T, UTD2102CEX 100 MHz Oscilloscope (UNI-T, Dongguan City, China) equipped with a Victor BK81560 High Voltage Probe (Bokles, Taiwan) and a UT-P04 100 MHz Passive Probe (UNI-T). The current measurement was conducted through 100 Ω resistor connected in series to the circuit. During the plasma discharge, filtered air was passed over the copper electrode with a flow rate ranging between 125 and 150 L/h. The produced PAW had a pH of 2.35 ± 0.03, a conductivity of 1830 ± 50 μS with a composition of 300 ppm NO_3 and 15 ppm H_2O_2. This PAW was used for the lethal treatments and all the evolution essays (Sections 2.3, 2.4).

2.3. Direct-tolerance to PAW

1 mL of a culture at stationary growth phase was centrifuged for 2 min at 10,000 ×g (Heraeus Megafuge 1.0R, Langenselbold, Germany), and resuspended in PAW. Then, it was diluted 1:10 in 10 mL of fresh PAW (initial concentration: 3×10^8 CFU/mL). After 45 min of PAW treatment at room temperature, samples were taken and adequately diluted in phosphate-buffered saline (Sigma-Aldrich; PBS) and spread on TSAYE plates. Plates were counted after incubation (24 h/37 °C).

2.4. PAW adaptive laboratory evolution assays

The ALE assays were based on the isolation of strains by recovering surviving cells after lethal treatments with PAW. This methodology was adapted from Berdejo, Merino, Pagán, García-Gonzalo, and Pagán (2020), and four lineages were carried out in parallel L(1–4). For this, 1 mL from stationary growth phase of four cultures of SeWT was centrifuged for 2 min at 10,000 ×g, and resuspended in PAW. Then, it was diluted 1:10 in 10 mL of fresh PAW (initial concentration: 3×10^8 CFU/mL) for 45 min at room temperature. Subsequently, treated cells were centrifuged for 20 min at 4000 ×*g* and washed twice with TSBYE to stop the PAW-reaction with the microorganisms. Then, 1 mL from each lineage was diluted 1:10 in TSBYE (10 mL) and incubated for 24 h at 37 °C. This procedure was repeated 30 times. After the 30th step, an aliquot from each lineage was diluted in PBS and spread on TSAYE plates from which 5 colonies were randomly selected for phenotypic and genotypic characterization.

2.5. Whole genome sequencing (WGS) and identification of mutations

Total genomic DNA (gDNA) from the SeWT and the most tolerant strains was extracted using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and sequenced by the Novogene firm (UK) on an Illumina Hiseq 4000 platform, using 150 bp paired-end reads. The original sequencing data acquired in image files were first transformed into sequence reads by base calling with the CASAVA software.

The effective sequencing data were aligned with the reference sequences through BWA software. For the current 4,951,383 bp reference genome, the mapping rate of each sample ranges from 99.86% to 99.91%. GATK was used to detect single nucleotide variants (SNVs), and they were then annotated using ANNOVAR. The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (BioProject ID: PRJNA1062270). The accession numbers of the samples are SAMN39289816 (SePAW1), SAMN39289817 (SePAW2), SAMN39289818 (SePAW3), SAMN39289819 (SePAW4).

2.6. Lethal treatment with sodium hypochlorite, peracetic acid (PAA), and carvacrol

Prior to the treatments, stationary growth phase cultures of SeWT and the RVs were centrifuged for 3 min at 10,000 ×g and washed in PBS. Then cultures were centrifuged and resuspended in sterile distilled water. Lethal treatments were carried out in 10 mL of sterile distilled water to which sodium hypochlorite (2 mg/L; pH 6.0), PAA (2.5 mg/L; pH 4.6), or carvacrol (250 μL/L) were added before being inoculated at a final concentration of 2×10^8 CFU/mL. After 45 min, samples were taken, diluted in PBS and spread on TSAYE plates. After plates' incubation (24 h/37 °C), colonies were counted by an automatic plate counter (Analytical Measuring Systems, Protos, Cambridge, United Kingdom).

2.7. Maximum non-inhibitory concentration of sodium chloride (NaCl) and bile salts (BS)

The determination of the maximum non-inhibitory concentration (MNIC) allows detection of damages to the cytoplasmic membrane and the outer membrane through the use of selective agents such as NaCl or BS, respectively (Pagán, Berdejo, Merino, García-Gonzalo, & Pagán, 2021).

This technique highlights the loss of both the integrity and functionality of the membrane. First, selective media were prepared with TSAYE and with increasing concentrations of each solute (NaCl or BS); in the case of NaCl, from 2.5% to 6.0% (*w/v*) at intervals of 0.5%; and from 0.05% to 0.30% (w/v) at intervals of 0.05% for BS. Then, aliquots of native cells from a stationary growth phase culture were plated onto the non-selective medium (TSAYE), as a control, and on the selective media at different NaCl or BS concentrations, and subsequently incubated for 24 h and 48 h at 37 °C, respectively. After incubation, colony-forming units (CFU) were counted. The MNIC is defined as the highest concentration that inhibits the growth of <20% of the cells initially inoculated in the selective medium compared to those grown in the non-selective medium used as a control.

2.8. Statistical analysis

Results from phenotypic characterization were obtained from at least 3 independent experiments carried out on different working days with different bacterial cultures. Data were compared using unpaired *t*-tests with Prism 4.03 software (GraphPad Software, San Diego, CA, USA), and differences were considered significant if $p \leq 0.05$.

3. Results

3.1. ALE assay under selective exposure to PAW

Four independent cultures of *S.* Typhimurium were adaptively evolved under selective exposure to PAW. After 30 days, 5 colonies evolved from each lineage L(1–4) were randomly selected to carry out phenotypic characterization. PAW treatments against SeWT and evolved strains were performed to evaluate whether the colonies showed an increased tolerance to PAW (Fig. 1).

All the strains from all the lineages showed a significant increase in tolerance compared to SeWT ($p \leq 0.05$), with the exception of C1 and C10. The tolerance exhibited by the five evolved strains within each lineage was similar ($p > 0.05$); however, C5 demonstrated the highest average level of tolerance among them.

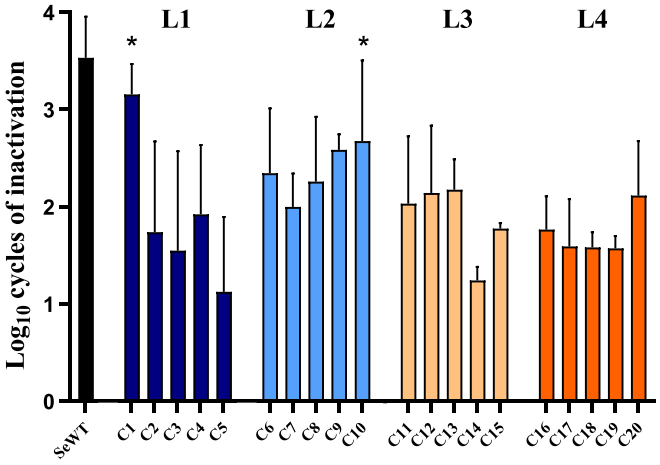

Fig. 1. Direct-tolerance to plasma activated water. Log_{10} cycles of inactivation of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (■; SeWT) and its evolved strains C(1−20) from the four lineages: L1 (■), L2 (■), L3 (■), L4 (■), after 45 min of plasma activated water treatment. Data are means ± standard deviations (error bars) obtained from at least three independent experiments. *: Indicates non-significant differences ($p > 0.05$) with SeWT.

Therefore, within each lineage we selected the evolved strain with the highest average PAW tolerance, namely C5, C7, C14, and C19, for further genotypic and phenotypic characterization. Those RVs were subsequently designated as SePAW1, SePAW2, SePAW3, and SePAW4, respectively.

3.2. Whole-genome sequencing of PAW RVs

The identification of mutations of the RVs was carried out by comparing their genomes against SeWT (Table 1) to identify the cause of the increased tolerance to PAW. For this purpose, WGS was conducted and the reads were mapped onto the reference genome: *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 (NCBI accession: NC_003197.2). Mutations in our SeWT were identified with regard to the reference strain (Berdejo et al., 2021) to discard them. The following single nucleotide variants (SNVs) were found in RVs:

a) In **SePAW1:** a silent mutation in *narZ* which codifies a nitrate reductase subunit alpha; a replacement of threonine by serine at position 477 of the transcriptional regulator CadC; an amino acid substitution of methionine by isoleucine at position 363 of the cadaverine antiporter CadB; a change of cytosine by an adenosine caused a stop gain at position 136 in the histidine kinase QseC; and a replacement of glutamic acid by glutamine in position 142 of rod shape determining protein MreB.
b) In **SePAW2:** a substitution of glycine by valine at position 386 of murein DD-endopeptidase MepM; a change from proline to leucine at position 72 of abequosyltranferase; an amino acid replacement of isoleucine by threonine at position 450 of the two-component sensor histidine ArcB; and a substitution of an alanine by serine in position 304 of cytochrome *c* biogenesis protein CcmH.
c) In **SePAW3:** a replacement of glycine by alanine at position 293 of zinc transporter ZntB; two synonymous mutations were detected in Fels-2 prophage proteins at positions 70 and 313; and a change of valine by glycine in CrsA, a carbon storage regulator at position 25.
d) Finally, two SNVs were detected in **SePAW4** related to RNA polymerase: a glycine replacement by arginine at position 42 of the sigma

Table 1
Mutations of resistant variants (RVs) isolated by cyclic exposure to short lethal treatments of plasma activated water in comparison with SeWT (*Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type).

RVs	Genome position	Genes	Locus tag	Mutation	Change	Information
SePAW1	1,663,776	*narZ*	STM1577	G3096T	L1032	Nitrate reductase subunit alpha
	2,698,061	*cadC*	STM2557	C1430G	T477S	Transcripcional regulator CadC
	2,699,625	*cadB*	STM2558	G1089A	M363I	Lysine cadaverine antiporter
	3,341,867	*qseC*	STM3178	C407A	S136X (stop gain)	Two component system sensor histidine kinase QseC
	3,544,143	*mreB*	STM3374	G424C	E142Q	Rod shape determining protein MreB
SePAW2	1,985,240	*mepM*	STM1890	G1157T	G386V	Murein DD-endopeptidase MepM
	2,168,403	*wbaV*	STM2087	C215T	P72L	Abequosyltranferase
	3,491,487	*arcB*	STM3328	T1349C	I450T	Two-component sensor histidine ArcB
	4,012,467	*ccmH*	STM3812	G910T	A304S	cytochrome *c* biogenesis protein CcmH
SePAW3	1,750,248	*zntB*	STM1656	G878C	G293A	Zinc transporter ZntB
	2,852,124	STM2703	STM2703	C210T	N70N	Fels-2 prophage protein
	2,854,635	STM2706	STM2706	T939C	N313N	Fels-2 prophage protein
SePAW4	2,971,067	*csrA*	STM2826	T74G	V25G	Carbon storage regulator
	3,376,396	*rpoD*	STM3211	G124A	G42R	RNA polymerase sigma factor RpoD
	3,583,388	*rpoA*	STM3415	A910G	K304E	DNA-directed RNA polymerase subunit alpha

factor RpoD; and lysine replacement by glutamic acid at position 304 of the subunit alpha RpoA.

3.3. Cross-tolerance to sodium hypochlorite, peracetic acid (PAA), and carvacrol of PAW RVs

Cross-tolerance of RVs was evaluated against three different disinfectants: chlorine-based (sodium hypochlorite), oxidative compound (PAA), and an individual compound from EOs (carvacrol). Fig. 2 shows the cycles of inactivation of SeWT and SePAW1, SePAW2, SePAW3, SePAW4 after 45 min-treatment with sodium hypochlorite (Fig. 2A), PAA (Fig. 2B), or carvacrol (Fig. 2C) in sterile distilled water. After 45 min of sodium hypochlorite treatment, inactivation of initial populations of SePAW1, SePAW3, and SePAW4 reached around 3.5 cycles, similar to that of SeWT ($p > 0.05$). However, SePAW2 demonstrated significantly higher tolerance compared to the other strains, with <1 cycle of inactivation ($p \leq 0.05$).

In contrast, all RVs showed higher tolerance to PAA (Fig. 2B) than SeWT, with varying degrees of cross-protection. While inactivation of SeWT exceeded the limit of detection (6.7 log cycles), inactivation levels of SePAW1 and SePAW3 were 5.5 and 4.0 log cycles respectively. SePAW2 and SePAW4 showed the highest levels of tolerance as the PAA treatment only resulted in a 0.5-log cycle reduction of the initial populations of those RVs.

Finally, after the 45-min treatment with 250 μL/L carvacrol (Fig. 2C), the inactivation of SePAW1 was similar to that of SeWT ($p > 0.05$), exhibiting a reduction of approximately 5 log cycles. In contrast, SePAW2, SePAW3, and SePAW4 demonstrated higher tolerance to carvacrol, with SePAW4 showing an almost two-fold increase in tolerance to carvacrol compared to SeWT ($p \leq 0.05$).

3.4. Resistance to sodium chloride and bile salts

As shown in Table 2, resistance to NaCl in the growth medium of SePAW4 was similar to that of SeWT (MNIC of NaCl in selective medium of 5.0%). However, SePAW1 and SePAW3 showed higher sensitivity to NaCl (MNIC of 3.0%) followed by SePAW2 (4.5%).

On the other hand, all the RVs showed a lower resistance to BS in the growth medium than SeWT (MNIC of 0.20%). Specifically, SePAW1 and SePAW4 were the most sensitive RVs (0.05%), followed by SePAW2 (0.10%) and SePAW3 (0.15%) (Table 2).

4. Discussion

The association between extended exposure to oxidative stress and the development of resistance/tolerance has been extensively documented, akin to the phenomena observed with antibiotics and various other antibacterial agents. It has been shown that acquired resistance can lead to reductions in susceptibility to different disinfectants or antimicrobial compounds, which could lead to food safety concerns. In order to assess whether sequential exposure to PAW could lead to the emergence of RVs, in this study, 4 lineages L(1–4) of *S.* Typhimurium were exposed to PAW for 30 days. As shown in Fig. 1, this study has demonstrated for the first time that sequential exposure to PAW also favors the isolation of RVs. The most tolerant strain within each lineage (SePAW1, SePAW2, SePAW3, and SePAW4) was selected for further phenotypic and genomic analysis (Table 1), in order to identify the possible genes causing the increase in tolerance.

The mutations identified in the four RVs (SePAW1–4) encompass a variety of genes involved in key cellular processes. These mutations are associated with alterations in protein functionality, collectively contributing to the distinctive cross-resistance phenotypes observed. In this context, the intricate details of these mutations and their potential implications are explored:

a) SePAW1 harbors interesting mutations in *cadC*, *cadB*, *mreB*, and *qseC* genes. The mutations in *cadC* and *cadB* are linked to acid tolerance mechanisms (Lee & Kim, 2017), while the mutation in *mreB* might impact cell wall synthesis. We should highlight the relevance of the SNV in *qseC*, part of the *qseBC* operon. The insertion of a stop codon caused the loss of functionality of QseC. Bearson, Bearson, Lee, and Brunelle (2010) demonstrated that its loss of functionality in *S.* Typhimurium decreased bacterial motility and colonization fitness. In this sense, the loss of motility has already been related by Lyu, Yang, Villanueva, Singh, & Ling (2021) with increased resistance to oxidative stress since flagellar motility uses cellular energy stored in the form of proton motive force and makes cells less efficient in pumping out toxic molecules such as antibiotics or H_2O_2 (Karash, Liyanage, Qassab, Lay, & Kwon, 2017).

Therefore, these mutations related to acid tolerance, cell envelopes and oxidative stress could explain the increased tolerance of SePAW1 against PAW (Fig. 1) given its extremely acidic pH, as well as its cross-tolerance to PAA at pH 4.6 (Fig. 2B). As observed in Table 2 the integrity and/or functionality of both the cytoplasmic and outer membranes in SePAW1 was affected since its resistance to NaCl and BS was lowered as compared to that of SeWT. However, this membrane alteration did not present an advantage in the inactivation caused by sodium hypochlorite, and carvacrol (Fig. 2).

b) SePAW2 exhibits mutations in genes related to peptidoglycan synthesis (*mepM*) (Kim et al., 2021), lipopolysaccharide biosynthesis (*wbaV*) (Hernández et al., 2015), oxygen sensing (*arcB*), and cytochrome *c* maturation (*ccmH*) (Kagan et al., 2009). Of particular

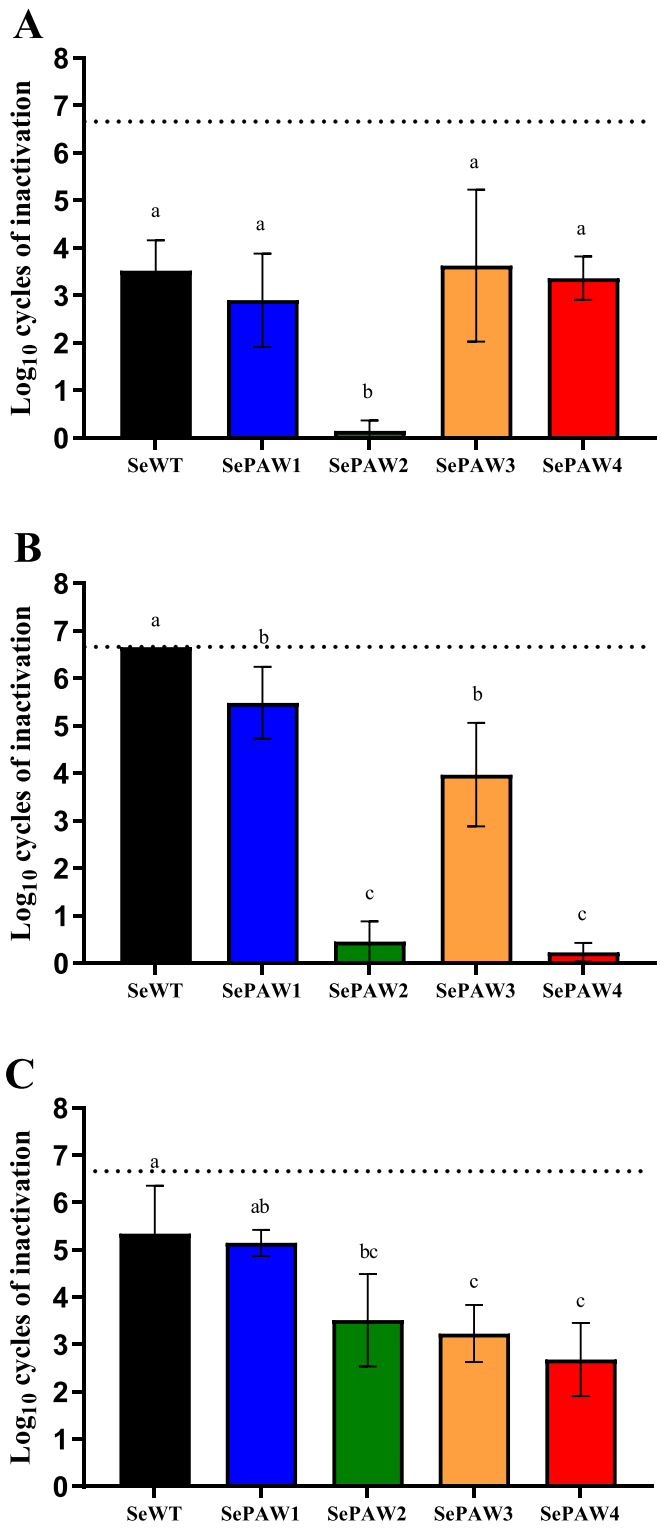

Fig. 2. Cross-tolerance to disinfectants. Log_{10} cycles of inactivation of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (■; SeWT) and its resistant variants: SePAW1 (■), SePAW2 (■), SePAW3 (■), SePAW4 (■), after 45 min of sodium hypochlorite (A; 2 mg/L; pH 6.0), peracetic acid (B; 2.5 mg/L; pH 4.6), and carvacrol (C; 250 μL/L; pH 5.8) in distilled water. Data are means ± standard deviations (error bars) obtained from at least three independent experiments. Same letters in each graph indicate non-significant differences among mean values ($p > 0.05$). The dashed line represents the detection limit (− 6.7 log_{10} cycles of inactivation).

Table 2
Maximum non-inhibitory concentration (MNIC) of sodium chloride (%NaCl, w/v) and bile salts (%BS, w/v) for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and its resistant variants SePAW1, SePAW2, SePAW3, and SePAW4. Each value represents the result of at least 3 different experiments carried out with different bacterial cultures and on different working days.

Strain	NaCl (%)	BS (%)
SeWT	5.0	0.20
SePAW1	3.0	0.05
SePAW2	4.5	0.10
SePAW3	3.0	0.15
SePAW4	5.0	0.05

importance is the mutation in *arcB*, leading to an amino-acid change in histidine kinase domain of ArcB which belongs to the anoxic redox control (Arc) system, also known as aerobic respiration control. Arc system has been related to response against nitrosative and oxidative stress in *S.* Typhimurium and *S. e*Enteritidis (Cabezas et al., 2021; Lu, Killoran, Fang, & Riley, 2002).

Although resistance of SePAW2 against NaCl and BS was slightly decreased as compared to SeWT (Table 2), it was the only RV that showed cross-tolerance against all the tested disinfectants (sodium hypochlorite, PAA and carvacrol) (Fig. 2). Thus, collectively, these mutations enhanced tolerance of SePAW2 to oxidative stress-inducing agents.

c) SePAW3 displays mutations in genes associated with zinc homeostasis (*zntB*), which is related to nitrosative stress (Frawley et al., 2018); prophage proteins (STM2703, STM2706) which may contribute to enhancing bacterial fitness and survival in specific environments or conditions (Wahl, Battesti, & Ansaldi, 2019); and global gene regulation (*csrA*), integral to the bacterium's ability to adapt to dynamic environmental changes, responding to factors like oxidative stress, nutrient availability, and temperature shifts (Potts, Guo, Ahmer, & Romeo, 2019; Pourciau et al., 2019).

These mutations, especially in *zntB* and *csrA*, might lead to enhanced tolerance of SePAW3 to nitrosative and oxidative stress-inducing agents, such as PAW (Fig. 1), PAA and carvacrol treatments (Fig. 2B, C).

d) SePAW4, on the other hand, shows mutations in RNA polymerase genes (*rpoA*, *rpoD*). On the one hand, mutation in RpoA is located in the alpha C-terminal domain, involved in interaction with transcriptional regulators and with upstream promoter elements. On the other hand, *rpoD* codes for the housekeeping sigma factor $\sigma^{70/D}$, responsible for the transcription of constitutive genes. Specifically, more of one-third of genes have been identified to RpoD sigmulon (Lee et al., 2024). Therefore, although *rpoA* and *rpoD* are not directly involved in resistance to oxidative stress, mutations in these genes have been associated with multi-drug resistance (Piovesan Pereira, Wang, & Tagkopoulos, 2021; Webber et al., 2015) and tolerance to heat and acid stress (Harden Mark et al., 2015; Tenaillon et al., 2012).

Therefore, mutations of *rpoA* and especially *rpoD*, might explain tolerance of SePAW4 against oxidative stress caused by PAW (Fig. 1), PAA and carvacrol (Fig. 2).

In summary, the mutations identified in SePAW1–4 underscore the intricate nature of bacterial adaptation to PAW. In this regard, mutations were detected in proteins directly or indirectly related to oxidative stress response in strains SePAW1 (*qseC*), SePAW2 (*arcB*), and SePAW3 (*zntB*, *csrA*); whereas mutations have been found in proteins related to cell envelopes in strains SePAW1 (*mreB*), and SePAW2 (*mpeM* and *wbaV*). Although strain SePAW4 does not exhibit mutations directly related to oxidative stress or cell envelopes, particularly the mutation detected in

rpoD may have indirect effects on the regulation of genes related to stress resistance and/or cell envelopes. These mutations may alter protein functionality, leading to enhanced resistance to oxidative stress and disinfectants. However, further research is needed to fully elucidate the precise mechanisms underlying these observed phenotypes.

In order to provide a comprehensive overview of these genetic adaptations, Fig. 3 was generated, which illustrates the biological processes as outlined in the Gene Ontology of all the mutated proteins (Liebermeister et al., 2014). The analysis reveals that the genomic alterations observed in RVs significantly impact genes involved in regulatory mechanisms responding to external fluctuations in pH, oxygen levels, or transcriptional factors. Additionally, these mutations influence metabolic processes linked to the synthesis of cell envelopes. As a result, it becomes evident that these regulatory mechanisms and the composition of cell envelopes play a pivotal role in the mechanism of inactivation by PAW and other disinfectants, including sodium hypochlorite, PAA, and carvacrol. It is important to emphasize that the purpose of this figure is to delineate a generalized pattern in the mutations detected in independent RVs isolated after ALE with PAW, without implying uniform relevance across all mutations.

Intriguingly, despite the noticeable reduction in the functionality and/or integrity of the cell envelopes observed in all the RVs, as evidenced by their heightened sensitivity to NaCl and/or BS, these RVs paradoxically exhibited an increased tolerance to PAW and other disinfectants. This observation opens an exciting avenue for further investigation: the possibility of identifying a disinfectant that can exploit this compromised cell envelope to gain access to the bacterial cytoplasm and effectively inactivate *S.* Typhimurium cells. Such a targeted approach could potentially lead to the development of more efficient and selective disinfection strategies.

Of particular interest in our study is the effectiveness of sodium hypochlorite as a disinfectant against the RVs that emerged in response to sequential PAW exposure. Sodium hypochlorite stands out as a promising disinfectant option due to its ability to effectively inactivate these RVs. Notably, all RVs, except SePAW2, exhibited similar tolerance to sodium hypochlorite compared to the wild-type strain (SeWT). This observation suggests that sodium hypochlorite could serve as a potent disinfectant to counteract the tolerance developed by these RVs. Nevertheless, despite its strong disinfectant capabilities, sodium hypochlorite use warrants caution due to potential health risks (Wawryk et al., 2020). In contrast, similar tolerance levels against PAA of SePAW1, SePAW3, and SePAW4 in comparison to SeWT would suggest that the mechanism of tolerance against PAA is independent of the mutations found in these strains. This finding underscores the complexity of resistance/tolerance mechanisms and the need for a multifaceted approach to disinfection. It suggests that sodium hypochlorite, while effective against certain disinfectant-tolerant strains, may not be equally potent against all RVs, which might require alternative strategies. The distinctive tolerance profiles observed among RVs emphasize the importance of tailored disinfection strategies and the potential benefits of combining PAW with appropriate disinfectants to ensure effective disinfection in various contexts, including food safety and preservation. This combinatorial approach can help address the diverse challenges posed by disinfectant-tolerant strains, offering a comprehensive solution for maintaining food safety. Consequently, this calls for careful consideration when selecting disinfectants for specific applications, particularly in scenarios where various RVs might be encountered.

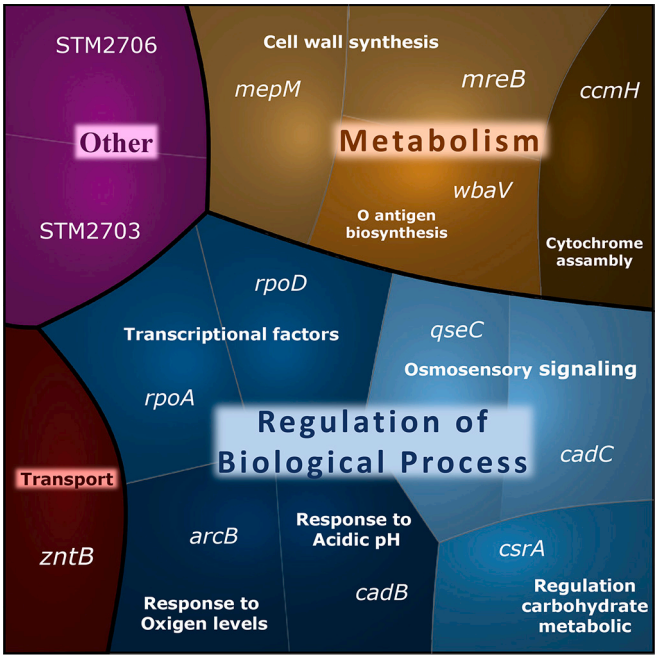

Fig. 3. Voronoi diagram. Every tile (small polygon) represents each of the mutated genes identified in the four evolved resistant strains in comparison with *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type. Tiles are arranged and colored according to the biological processes annotated in Gene Ontology.

In summary, this study focused on investigating the emergence of RVs of *Salmonella* Typhimurium under sequential exposure to PAW and their subsequent tolerance to various disinfectants. We identified specific mutations in four different strains (SePAW1, SePAW2, SePAW3, and SePAW4) and assessed their impact on tolerance phenotypes. Those mutations collectively underscore the complex genetic mechanisms that contribute to the enhanced tolerance of these RVs to oxidative stress and various disinfectants. Understanding these genetic adaptations is valuable for selecting appropriate disinfectants in food industry settings based on strain genotypes.

5. Conclusions

This study is the first to demonstrate that sequential exposure to PAW can lead to the emergence of RVs exhibiting direct-tolerance to PAW. While the RVs were isolated under stringent conditions, further investigations are necessary to comprehensively understand the conditions conducive to RV emergence. This understanding is crucial for the effective utilization of PAW as a sanitizer, whether in farm or food industry settings. Recent studies have proposed the use of PAW as a disinfectant, highlighting its brief contact in some applications (Bradu et al., 2020) or more prolonged exposure, such as in PAW ice (Katsaros, Koseki, Ding, & Valdramidis, 2021; Liao et al., 2018). These findings underscore the importance of preventing the emergence of tolerant mutants within the existing microbiota, as the isolated mutants have shown cross-tolerance not only to other disinfectants and natural compounds but also to various stresses encountered in the food chain, including preservation processes.

In conclusion, our study offers valuable insights into the genetic adaptations that underlie resistance to PAW and other disinfectants, emphasizing the need for careful consideration when implementing PAW as a sanitizer in different applications within the food industry. Preventing the emergence of resistant mutants is imperative to maintain the effectiveness of disinfection processes and ensure food safety.

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CRediT authorship contribution statement

Elisa Pagán: Conceptualization, Methodology, Investigation, Writing – original draft. **Foteini Pavli:** Methodology, Investigation. **Sarah Happiette:** Investigation. **Daniel Berdejo:** Methodology, Formal analysis. **Ruben Gatt:** Supervision. **Rafael Pagán:** Supervision, Conceptualization, Methodology, Writing – original draft, Project administration, Funding acquisition. **Vasilis Valdramidis:** Writing – review & editing, Funding acquisition. **Diego García-Gonzalo:** Supervision, Conceptualization, Methodology, Writing – original draft, Project administration, Funding acquisition.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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