International Journal of Food Microbiology 360 (2021) 109443

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Salmonella enterica serovar Typhimurium genetic variants isolated after lethal treatment with *Thymbra capitata* essential oil (TCO) showed increased resistance to TCO in milk

Daniel Berdejo, Elisa Pagán, Natalia Merino, Laura Botello-Morte, Rafael Pagán, Diego García-Gonzalo *

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

ARTICLE INFO

Keywords:
Salmonella Typhimurium
Thymbra capitata essential oil
Resistant genetic variants
Skimmed milk
Antibiotic resistance
soxR mutation

ABSTRACT

The high prevalence of *Salmonella enterica* in milk poses a risk of considerable concern in the preservation of certain dairy products, mainly those elaborated from raw milk. Essential oils (EOs) have been proposed as a promising food preservative for such products due to their strong antimicrobial properties. Additionally, these natural antimicrobials have been shown to be effective against multi-drug resistant strains. They can thus also be utilized to prevent the dissemination of antimicrobial resistances (AMR). However, recent evidence of the development of bacterial resistance under EO treatments may call their use into question. This study sought to assess the emergence of antimicrobial resistant genetic variants of *S. enterica* serovar Typhimurium from survivors after cyclic exposure to lethal doses (>5 $\log_{10}$ cycles of inactivation) of *Thymbra capitata* EO (TCO), in order to evaluate the impact that it could have on milk preservation, to ascertain whether cross-resistance to antibiotics occurs, and to identify the genomic changes responsible for their phenotype. Isolated strains by TCO (SeTCO) showed a two-fold increase in minimum inhibitory and bactericide concentrations (MIC and MBC) of TCO compared to *Salmonella enterica* serovar Typhimurium wild-type strain (SeWT) in laboratory growth medium, as well as a greater adaptation and growth rate in the presence of the EOs and a higher survival to TCO treatments in buffers of pH 4.0 and 7.0. The increased resistance of SeTCO was confirmed in skimmed milk: 300 μL/L TCO reduced only 1 $\log_{10}$ cycle of SeTCO population, whereas it inactivated more than 5 $\log_{10}$ cycles in SeWT. Moreover, SeTCO showed an increased cross-resistance against aminoglycosides, quinolones and tetracyclines. Whole genome sequencing revealed 5 mutations in SeTCO: 2 in genes involved in O-antigens synthesis (*rfbV* and *rfbX*), 2 in genes related to adaptation to the growing medium (*trkA* and *glpK*), and 1 in a redox-sensitive transcriptional regulator (*soxR*). The phenotypic characterization of a constructed SeWT strain with mutant $soxR_{SeTCO}$ demonstrated that the mutation of *soxR* was the main cause of the increased resistance and tolerance observed in SeTCO against TCO and antibiotics. The emergence of resistant strains against EOs might jeopardize their use as food preservatives. Further studies will thus be required to determine under which conditions such resistant strains might occur, and to assess the food risk they may pose, as well as to ascertain their impact on the spread of AMR.

1. Introduction

In spite of the continuous improvement of preservation methods for microbiological control, the dairy industry still faces important challenges in the ongoing prevention of food safety hazards. The presence and growth of pathogenic bacteria in raw milk and derived dairy products is one of the hazards that are the greatest cause for concern (Boor et al., 2017). Currently, *Salmonella* spp. stands out among key pathogenic bacteria, since it is highly prevalent in the food chain of milk production, as well as in the manufacturing of dairy products (Sonnier et al., 2018). Recent foodborne outbreaks caused by raw milk cheese consumption have been associated with the presence of *Salmonella* strains (Robinson et al., 2020; Ung et al., 2019).

In addition, the massive increase of multidrug resistant (MDR)

* Corresponding author at: Dpto. PACA, Facultad de Veterinaria, Universidad de Zaragoza, C/Miguel Servet, 177, 50013 Zaragoza, Spain.
E-mail address: Diego.Garcia@unizar.es (D. García-Gonzalo).

https://doi.org/10.1016/j.ijfoodmicro.2021.109443
Received 13 May 2021; Received in revised form 25 September 2021; Accepted 14 October 2021
Available online 19 October 2021

strains of *Salmonella* in the food industry is a serious worldwide public health issue in view of the spread of antimicrobial resistances (AMR) in agri-food environments (McMillan et al., 2019). Recent studies have reported the elevated occurrence of resistance genes, mainly in *S. enterica* strains, to a wide range of antibiotics along the milk production chain (Parry-Hanson Kunadu et al., 2018; Qamar et al., 2020). In fact, several serious foodborne outbreaks of MDR *Salmonella* strains associated with the consumption of milk and dairy products have been reported (Olsen et al., 2004; Plumb et al., 2019).

Essential oils (EOs) and their individual constituents (ICs) have been found to be promising agents in dairy products as food preservatives due to their bio-preservative, antioxidant, and antimicrobial activities (Mishra et al., 2020). These natural compounds have been studied extensively, and it has been shown that they have excellent antimicrobial properties against food-related pathogens (Calo et al., 2015; Pandey et al., 2017). Certain EOs, such as that derived from *Thymbra capitata*, have demonstrated high antimicrobial activity even against bacterial biofilms (Gagliano Candela et al., 2019), including those formed by *S. enterica* (Karampoula et al., 2016). Furthermore, EOs are also proposed as a possible alternative to antibiotics in the treatment of infectious diseases, with the purpose of combatting the generation and dissemination of resistance (Mittal et al., 2019; Yap et al., 2014). Therefore, their use could also be effective against MDR strains in food preservation.

Nevertheless, several studies support the assumption that the use of ICs can lead to the emergence of resistant and tolerant strains against natural antimicrobials, defining "resistance" as the ability of bacteria to grow in the presence of the antimicrobial, and "tolerance" as their ability to survive against lethal doses (Balaban et al., 2019). The application of cyclic treatments of ICs, such as carvacrol, citral or limonene oxide, has led to the selection of genetic variants in microbial populations of *Escherichia coli* (Chueca et al., 2016; Chueca et al., 2018), *Staphylococcus aureus* (Berdejo et al., 2019) and *Salmonella enterica* serovar Typhimurium (Berdejo et al., 2020a), with increased direct-resistance to the same ICs that were used for their selection. In addition, some of these strains also developed increased cross-resistance to a wide range of antibiotics, thereby supporting the assumption that those mutations contribute to non-specific bacterial resistance against antimicrobials (Berdejo et al., 2020a; Chueca et al., 2018). Due to the compositional complexity of EOs (each one composed of up to dozens of different ICs) and, consequently, the diverse mechanisms of antimicrobial action of their ICs (Lingan, 2018), EOs have been regarded as a safe alternative in the aim of preventing the emergence of antimicrobial resistance. However, a recent study also evidenced the emergence of resistant and tolerant strains of *S. aureus* against a complex *Citrus sinensis* EO after prolonged exposure to sub-inhibitory doses of EO (Berdejo et al., 2020b).

To the best of our knowledge, there is no evidence of the emergence of Gram-negative resistant strains through exposure to complex EOs. Moreover, the risk posed by the emergence of these strains in food preservation has not yet been assessed. For those reasons, this study seeks a) to assess the emergence of resistant mutants of *Salmonella enterica* serovar Typhimurium due to the use of a complex EO, *Thymbra capitata*; b) to evaluate the magnitude of the increased resistance in skimmed milk as a food model; c) to study the occurrence of cross-resistance to antibiotics in resistant mutants; and d) to identify the genetic modifications responsible for the increase in bacterial resistance.

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). The XTL298 strain, which contains *tetA-sacB* in the arabinose operon for strain construction, was kindly provided by Donald L. Court (National Cancer Institute at Frederick, USA). Throughout this study, the strains were kept in cryovials at −80 °C with glycerol (20% *v*/v), from which plates of tryptone soya agar (Oxoid, Basingstoke, United Kingdom) with 0.6% yeast extract (Oxoid; TSAYE) were inoculated on a weekly basis. To prepare the working bacterial cultures, test tubes containing 5 mL of tryptone soya broth (Oxoid) with 0.6% yeast extract (TSBYE) were inoculated with one colony, then incubated aerobically overnight in an orbital shaker (130 rpm; Heidolph Vibramax 100, Schwaback, Germany) at 37 °C (Incubig, Selecta, Barcelona, Spain). Subsequently, flasks containing 10 mL of fresh TSBYE were inoculated with the resulting subculture to achieve an initial concentration of 10^6 colony forming units per mL (CFU/mL), and incubated for 24 h at 37 °C and 130 rpm until the stationary growth phase was reached (5×10^9 CFU/mL approximately).

T. capitata essential oil (TCO) was kindly provided by the TELIC Group (Barcelona, Spain). This EO was kept in the dark and under refrigeration temperature in sealed glass bottles. The composition of this batch of TCO was previously analysed by Merino et al. (2019): 73.8% carvacrol, 9.2% p-cymene, 5.3% γ-terpinene, 2.0% (*E*)-caryophyllene and 9.7% other compounds. Sterile skimmed milk (Central Lechera Asturiana, Asturias, Spain) was purchased in a supermarket: prior to each experiment, a new bottle thereof was aseptically opened.

2.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC determination was performed according to Clinical and Laboratory Standards Institute (CLSI, 2015) with some modifications due to hydrophobicity of TCO. From an exponential culture (0.5 McFarland) of the bacterial strains, 5 mL of TSBYE were inoculated 1:100 achieving an initial concentration of 5×10^5 CFU/mL in the presence of different concentrations of TCO: 50–600 μL/L. Following the method described by Friedman et al. (2002), a vigorous shaking by vortex (Genius 3, Ika, Königswinter, Germany) was used to prepare TCO dispersions in TSBYE, avoiding the use of solvents due to their possible detrimental effect on antibacterial activity. Once the tubes were incubated at 37 °C for 24 h and 130 rpm, MIC was determined as the lowest concentration of the antimicrobial compound that was capable of preventing bacterial growth. To objectively determine bacterial growth, optical density was read at 595 nm (OD_{595}) using a microplate reader (Genios, Tecan, Männedorf, Switzerland). Under our working conditions, OD_{595} was linear in the range 0.0–1.4 (data not shown). A proportion of 10% of the OD_{595} measure of the positive control was established as the lower limit for considering that bacterial strain had grown (Kohanski et al., 2010). Positive control tubes with 5 mL TSBYE inoculated without TCO, and negative, non-inoculated control tubes with 5 mL TSBYE were also prepared in each experiment.

The minimum bactericidal concentration (MBC) of TCO was evaluated in parallel with the MIC test. From the test tubes employed in the MIC determination after incubation, 100 μL aliquot of each tube was spread onto TSAYE plates and incubated at 37 °C for 24 h. Colonies were counted and the lowest concentration of TCO that inactivated ≥99.9% of the initial bacterial concentration was defined as the MBC end point (Lallemand et al., 2016).

Additionally, MIC and MBC determinations were conducted in a food model, skimmed milk, following the same protocols, with a TCO concentration ranging from 200 to 1800 μL/L. It should be noted that in MIC determination, samples from each test tube were aliquoted and spread onto TSAYE plates to determine cell concentration, because the milk turbidity prevents a correct OD_{595} measurement. An increase of 50% of the initial bacterial population (CFU/mL) was established as the limit for considering that bacterial strain had grown.

2.3. TCO evolution assay

The evolution assay was based on the isolation of strains by

recovering surviving cells after lethal treatments with TCO. This methodology was adapted from Levin-Reisman et al. (2017). A stationary phase culture of SeWT was diluted 1:100 in 50 mL fresh TSBYE (initial concentration: 5×10^7 CFU/mL) with 500 μL/L of TCO (2× MIC for SeWT) for 4.50 h at 37 °C to achieve an inactivation greater than 5 $\log_{10}$ cycles. Inactivation was determined by plate count, as described in section 2.5. Subsequently, treated cells were centrifuged for 20 min at 15,000 × g (Heraeus Megafuge 1.0R, Thermo Electron LED GrnbH, Langenselbold, Germany), washed twice with TSBYE, resuspended in 1 mL TSBYE, and incubated overnight at 37 °C. This procedure was repeated 30 times. After the 30th step, an aliquot was diluted in phosphate buffer saline (PBS) and spread on TSAYE plates (without TCO), from which 5 colonies ($SeTCO_{1-5}$) were randomly selected to carry out phenotypic and genotypic characterization.

2.4. Growth curves in presence of TCO

First, TCO at different concentrations was added to tubes with 5 mL of TSBYE: from 0 up to 600 μL/L TCO. Test tubes were inoculated with the microbial culture at an initial concentration of 5×10^5 CFU/mL and incubated at 37 °C and 130 rpm for 24 h. Every hour, OD_{595} of the test tubes was measured by a microplate reader. A positive control (without antimicrobial added) and a negative control (without microbial culture added) were included in all the assays. The initial OD_{595} (at time 0) was subtracted from values of OD_{595} obtained during the experiment, corresponding to the absorbance caused by the growth medium and TCO presence. Bacterial growth curves based on OD_{595} of SeWT and SeTCO were graphically displayed and modelled by a modified Gompertz equation (Eq. 1) (Zwietering et al., 1990).

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

where y: OD_{595}; t: time (h); A: maximum value reached (OD_{595} max); μ_m: maximum specific growth rate (h^{-1}); λ: lag time (h).

A least-squares adjustment was carried out to build the model and obtain A, μ_m and λ values using Prism software (GraphPad Software, Inc., San Diego, USA). The experiment was prolonged for more than 24 h at high TCO concentrations until reaching the stationary phase to allow fitting of the growth curve. The adjustment's goodness of fit was evaluated using standard error, R^2 and R^2-adjusted values, and the root mean square error (*RMSE*). In addition, secondary models of Gompertz's parameters were built based on TCO concentration by least-squares adjustment; subsequently, tertiary growth models were obtained to estimate growth rate based on TCO concentration and incubation time.

2.5. Survival curves against TCO

The treatment medium used in the lethal treatments was citrate-phosphate buffer (McIlvaine buffer), prepared from citric acid monohydrate (Panreac) and disodium hydrogen phosphate (Panreac), adjusted to pH 4.0 and pH 7.0. These pH values were chosen as representative of neutral and acid conditions within the normal pH range of food. Stationary phase culture was centrifuged for 5 min at 6000 ×*g* in a microcentrifuge (Mini Spin, Eppendorf, Hamburg, Germany) and resuspended in the treatment medium. The treatment was carried out in 10 mL McIlvaine buffer previously tempered at 25 °C with 150 μL/L of TCO. Once TCO was dispersed, test tubes were inoculated at 10^7 CFU/mL. Aliquots were obtained every 5 min, subsequently diluted in PBS, and spread on TSAYE plates. After plate incubation (24 h/37 °C), colonies were counted in an automatic plate counter by image analysis (Analytical Measuring Systems, Protos, Cambridge, United Kingdom).

Following this same protocol, lethal treatments were performed on TSBYE and skimmed milk, with 300 μL/L and 1500 μL/L TCO respectively, for 30 min.

2.6. Antibiotic susceptibility test

The Kirby-Bauer disk diffusion test was conducted to test antimicrobial susceptibility according to CLSI (2012, 2014). Several antibiotics with different modes of action were chosen in order to cover different cellular targets that could be related to TCO resistance: 400 μg nalidixic acid sodium, 20 μg norfloxacin, 250 μg novobiocin sodium, 10 μg trimethoprim, 50 μg rifampicin, 30 μg chloramphenicol, 30 μg kanamycin sulphate, 30 μg tetracycline, 10 μg ampicillin, and 150 μg cephalexin (Sigma-Aldrich). First, stationary phase culture was spread on cation-adjusted Mueller Hinton agar plates (MHA; Sigma-Aldrich) and, after 5 min at room temperature, blank disks (Ø: 6.0 mm; Thermo Scientific™ blank anti-microbial susceptibility disk, Fisher Scientific, UK) were placed on the surface of the plates and individually impregnated with each antibiotic. These plates were incubated at 37 °C for 18–24 h, after which the diameters of the resulting inhibition zones were measured in horizontal plane using a digital calliper (paper disks included).

2.7. Statistical analysis

All phenotypic characterization results were obtained from at least 3 independent experiments carried out on different working days with different bacterial cultures. Growth curve parameters, lethal treatment graphics, and antibiotic susceptibility and motility tests are displayed as the mean ± standard deviation, using Prism software. Data were analysed and submitted to comparison of averages using analysis of variance (ANOVA), followed by post-hoc Tukey test and *t*-tests with Prism software, and differences were considered significant if $p \leq 0.05$.

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

Illumina technology was used to carry out WGS of SeWT and the genetic variant, on NextSeq equipment at mid-output flow, with a total of 2 × 150 cycles (Illumina; Fasteris, SA, Geneva, Switzerland). Quality control was then performed with FastQC software, evaluating reading quality (Q_{30}), sequence length, presence of adapters, and over-represented and duplicated sequences. A total of 3.65 and 3.77 million of 150 pb-reads were obtained for SeWT and SeTCO, which corresponds to a Phred quality score of 33.07 and 33.13, and 86.58% and 86.88% of reads above Q_{30}, respectively. The quality-control-filtered paired-end reads were mapped at 98.12% and 98.35% on the reference genome sequence (NCBI accession: NC_003197.2): *S. enterica* subsp. *enterica* serovar Typhimurium str. LT2, complete genome (McClelland et al., 2001), using a Burrows–Wheeler Alignment (BWA) Tool (Li and Durbin, 2010) and Samtools software (Li et al., 2009) (sources: http://bio-bwa.sourceforge.net/ and http://www.htslib.org/). A 150-fold coverage depth was achieved for both strains. Samtools was then applied to remove potential PCR duplicates according to reading positions on the reference genome; the resulting BAM files were then further processed using LoFreq-Star (source: http://csb5.github.io/lofreq/) to correct mapping errors and insert the quality values. Finally, single nucleotide variants (SNVs) and short insertion and deletions (InDels) were detected using LoFreq-Star, and toolbox snpEff (source: http;//snpeff.sourceforge.net/) was employed to identify involved genes and to predict functional effect variations (Cingolani et al., 2012). Coverage was further analysed with the Integrative Genomics Viewer (IGV; Broad Institute, source: https://software.broadinstitute.org/software/igv/) in order to find structural variations (SVs). Although mapping was carried out against the reference genome, SNVs, InDels, and SVs were identified between SeWT and isolated strains to ascertain the kind of mutations that had occurred during the evolution treatments. The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (BioProject ID: PRJNAPRJNA634825). The accession numbers of the samples are: SAMN15009803 (SeWT) and SAMN17313477 (SeTCO). Finally, specific primers (Table S1) were designed to carry out PCR amplifications, as

well as Sanger sequencing of PCR products to verify the mutations detected in the WGS.

2.9. Mutated gene replacement

In order to isolate the mutation detected in SeTCO, red recombinase technology was applied to perform the mutated gene replacement in SeWT. The *tetA-sacB* cassette (Li et al., 2013) was used as the PCR template to generate DNA fragments for primary chromosomal integration near to mutated gene (*soxR*$_{SeTCO}$) using specific primers containing 50 base pairs (bp) homologous sequences (Table S2). Plasmid pKD46, encoding the λ red recombinase genes behind the *araBAD* promoter, was used to enable the chromosomal integrations via double-crossover recombination, as previously described by Datsenko and Wanner (2000). To replace wild-type allele with gene variant in SeWT using intrachromosomal recombination, we first marked wild-type allele with *tetA-sacB* cassette and verified by colony PCR and Sanger sequencing. Then the *tetA–sacB* cassette of this strain was replaced intrachromosomally by gene variant with the aid of λ-red recombinase. For this purpose, 50 mL Luria-Bertani broth (LB; Oxoid) with 100 μg/mL ampicillin were inoculated from bacterial cultures and incubated at 30 °C with shaking at 130 rpm. After 2 h, arabinose was added at 4 g/L and cultures were re-incubated under the same conditions until the cultures' optical density at 590 nm (OD_{590}) reached 0.5. Cultures were subsequently centrifuged (5 min, 6000 ×*g*, 4 °C), the supernatant was discarded, and the remaining cell pellet was resuspended in 25 mL of 4 °C ultrapure water. This wash cycle was repeated three times and, finally, the supernatant was discarded and the cell pellet was resuspended in 500 μL ultrapure water to obtain electrocompetent cells. For electroporation, 40 μL of competent cells were mixed with 100–200 ng of DNA (0.2 cm/2.5 kV/5 ms). Following electroporation, cells were transferred to a sterile test tube containing 1 mL LB prewarmed and incubated at 30 °C for 3 h. Cells were then plated on LB agar containing tetracycline (20 μg/mL) to select clones by antibiotic resistance during primary integration, and on LB agar containing tetracycline (20 μg/mL) and sucrose (1.5% *w/v*), to isolate clones by loss of antibiotic resistance and sucrose insensitivity during secondary integration. PCR and Sanger sequencing were used to verify the insertion of genetic modification.

3. Results

3.1. Isolation of mutants with increased resistance and tolerance to TCO

Five colonies were randomly selected after TCO evolution assay in *Salmonella enterica* serovar Typhimurium: $SeTCO_{1–5}$. Then, MIC and MBC determinations of SeWT and evolved strains ($SeTCO_{1–5}$) against TCO were performed to compare their resistances and tolerances (Table 1). Evolved strains showed identical values among them, with a two-fold increase in resistance (as measured by MIC) and tolerance (as measured by MBC) against TCO after the evolution assay. While SeWT was inhibited at 250 μL/L TCO and inactivated at 300 μL/L TCO, $SeTCO_{1–5}$ showed MIC and MBC values of 600 μL/L TCO. Since the 5 strains isolated from the evolution lineage displayed the same degree of resistance and tolerance to TCO, we considered that the bacterial population was homogeneous and we pursued our research on only one of the 5 strains, henceforth referred to as SeTCO.

Table 1
Minimum inhibitory concentration (MIC; μL/L) and minimum bactericidal concentration (MBC; μL/L) of TCO for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strains ($SeTCO_{1–5}$). Each value represents the result of 5 different experiments carried out with different bacterial cultures and on different working days.

Strains	MIC (μL/L)	MBC (μL/L)
SeWT	250	300
$SeTCO_{1–5}$	600	600

3.2. Evolved strain grows faster than SeWT in the presence of TCO

Growth kinetics studies were carried out in the presence of TCO to characterise the adaptation of evolved strains to the EO, and subsequently modelled according to Gompertz equation (Eq. 1) by least-squares adjustment with excellent goodness of fit (Table S3).

Table 2 displays the values of the parameters *A* (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag phase), obtained from the models of SeWT and SeTCO at all the tested concentrations. Additionally, secondary models were obtained for parameters *A*, μ_m and λ based on TCO concentration in SeWT (Eqs. 2, 3, 4) and SeTCO (Eqs. 5, 6, 7).

$$A_{SeWT} = 1.19 - (C_{TCO}/240.68)\hat{}4.73 \quad (2)$$

$$\mu_{mSeWT} = 0.29 - 0.0038*C_{TCO} + 2.71*10^{-5}*C_{TCO}^2 - 6.54*10^{-8}*C_{TCO}^3 \quad (3)$$

$$\lambda_{SeWT} = 2.61\,exp(0.0081*C_{TCO}) \quad (4)$$

$$A_{SeTCO} = 1.17 - (C_{TCO}/586.42)\hat{}7.14 \quad (5)$$

Table 2
A (maximum OD_{595}), μ_m (maximum specific growth rate) and λ (lag time) parameters of the modified Gompertz model obtained from growth curves for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strain (SeTCO), at different concentrations of TCO.

		Strains	
		SeWT	SeTCO
A (OD_{595})			
TCO (μL/L)	**0**	1.159 ± 0.009^{a}	1.156 ± 0.006^{a}
	50	1.171 ± 0.014^{a}	1.156 ± 0.010^{a}
	100	1.162 ± 0.019^{a}	1.157 ± 0.025^{a}
	150	1.174 ± 0.073^{a}	1.168 ± 0.014^{a}
	200	0.716 ± 0.037^{b}	1.158 ± 0.035^{a}*
	250		1.146 ± 0.018^{a}
	300		1.161 ± 0.026^{a}
	350		1.160 ± 0.034^{a}
	400		1.101 ± 0.018^{a}
	450		1.101 ± 0.026^{a}
	500		0.792 ± 0.017^{b}
	550		0.518 ± 0.028^{c}
μ_m (OD_{595}/h)			
TCO (μL/L)	**0**	0.287 ± 0.021^{a}	0.288 ± 0.016^{a}
	50	0.152 ± 0.008^{b}	0.201 ± 0.011^{b}*
	100	0.114 ± 0.005^{c}	0.156 ± 0.016^{c}*
	150	0.091 ± 0.009^{cd}	0.156 ± 0.009^{c}*
	200	0.076 ± 0.003^{d}	0.147 ± 0.017^{c}*
	250		0.164 ± 0.009^{c}
	300		0.157 ± 0.010^{c}
	350		0.161 ± 0.011^{c}
	400		0.179 ± 0.007^{bc}
	450		0.178 ± 0.009^{bc}
	500		0.109 ± 0.005^{d}
	550		0.103 ± 0.014^{d}
λ (h)			
TCO (μL/L)	**0**	3.423 ± 0.170^{a}	3.391 ± 0.121^{a}
	50	4.641 ± 0.243^{b}	3.961 ± 0.185^{ab}*
	100	5.828 ± 0.245^{c}	4.581 ± 0.439^{bc}*
	150	7.001 ± 0.555^{d}	5.222 ± 0.241^{c}*
	200	14.160 ± 0.154^{e}	6.309 ± 0.518^{d}*
	250		9.190 ± 0.201^{e}
	300		10.158 ± 0.230^{f}
	350		12.561 ± 0.225^{g}
	400		13.650 ± 0.118^{h}
	450		14.767 ± 0.147^{i}
	500		17.961 ± 0.148^{j}
	550		18.352 ± 0.324^{j}

Each value represents the mean ± standard deviation from 3 independent experiments. Different superscript letters represent statistically significant differences ($p < 0.05$) among the means of the same column.
* Significantly different from SeWT ($p < 0.05$).

$$\mu_{mSeTCO} = 0.29 - 0.0017*C_{TCO} + 5.03*10^{-6}*C_{TCO}^2 - 5.51*10^{-9}*C_{TCO}^3 \quad (6)$$

$$\lambda_{SeTCO} = 3.35\,exp(0.0033*C_{TCO}) \quad (7)$$

where C_{TCO}: *Thymbra capitata* EO concentration; *A*: maximum value reached (OD_{595} max); μ_m: maximum specific growth rate (h^{-1}); λ: lag time (h).

Finally, growth models, integrating secondary models in Gompertz equation, were built for SeWT (Fig. 1A) and SeTCO (Fig. 1B) in order to estimate growth (OD_{595}) depending on TCO concentration and incubation time. The surface graph shows the independent variables "TCO concentration" (μL/L) and "incubation time" (h) on the x-axis and z-axis, respectively, and the OD_{595} of the culture as dependent variable on the y-axis.

On the one hand, as we can see in Fig. 1, the growth kinetics of both strains revealed that, as TCO concentration increased, *A* and μ_m parameters decreased considerably, while λ became longer. This means that the presence of TCO decreased the cell concentration in stationary phase, at high concentrations, decreased the cell growth rate and prolonged the lag phase. On the other hand, comparing SeTCO to SeWT, the behaviour of both strains was similar in the absence of TCO; however, when the EO was added, SeTCO exhibited a shorter lag phase and a higher growth rate than SeWT. In the presence of 200 μL/L TCO, the evolved strain showed 8 h shorter lag phase, and up to twice the growth rate of SeWT (Table 2).

3.3. TCO treatments lethal to SeWT are not effective against the evolved strain

The tolerance of SeTCO was further evaluated against lethal TCO treatments by comparing its inactivation kinetics with those of SeWT. Fig. 2 shows the survival curves of SeWT and SeTCO after TCO lethal treatments in phosphate-citrate buffer at pH 4.0 (Fig. 2A) and 7.0 (Fig. 2B), as well in TSBYE (Fig. 2C) which was the growth medium used to carry out the evolution assay. The evolved strain showed higher tolerance than SeWT in McIlvaine at both acid and neutral pH. As can be seen in Fig. 2A, inactivation of SeTCO at pH 4.0 reaches 5.5 log_{10} cycles after 30 min of treatment, i.e., 10 min later than SeWT. Similarly, the lethal treatment at pH 7.0 (Fig. 2B) resulted in more than 1 log_{10} cycle of inactivation in SeWT compared to the evolved strain after 25 min of treatment. Moreover, the lethal treatments in TSBYE revealed a large difference in tolerance between both strains (Fig. 2C): while SeWT showed an inactivation of more than 5 log_{10} cycles after 5 min of treatment, only 0.3 log_{10} cycles of population reduction were observed for SeTCO after 30 min.

3.4. Evolved strain as a risk in milk preservation by EOs

MIC and MBC were determined and lethal treatments were also performed in skimmed milk, to assess the microbiological risk of emergence of resistant strains in the use of natural antimicrobials as food

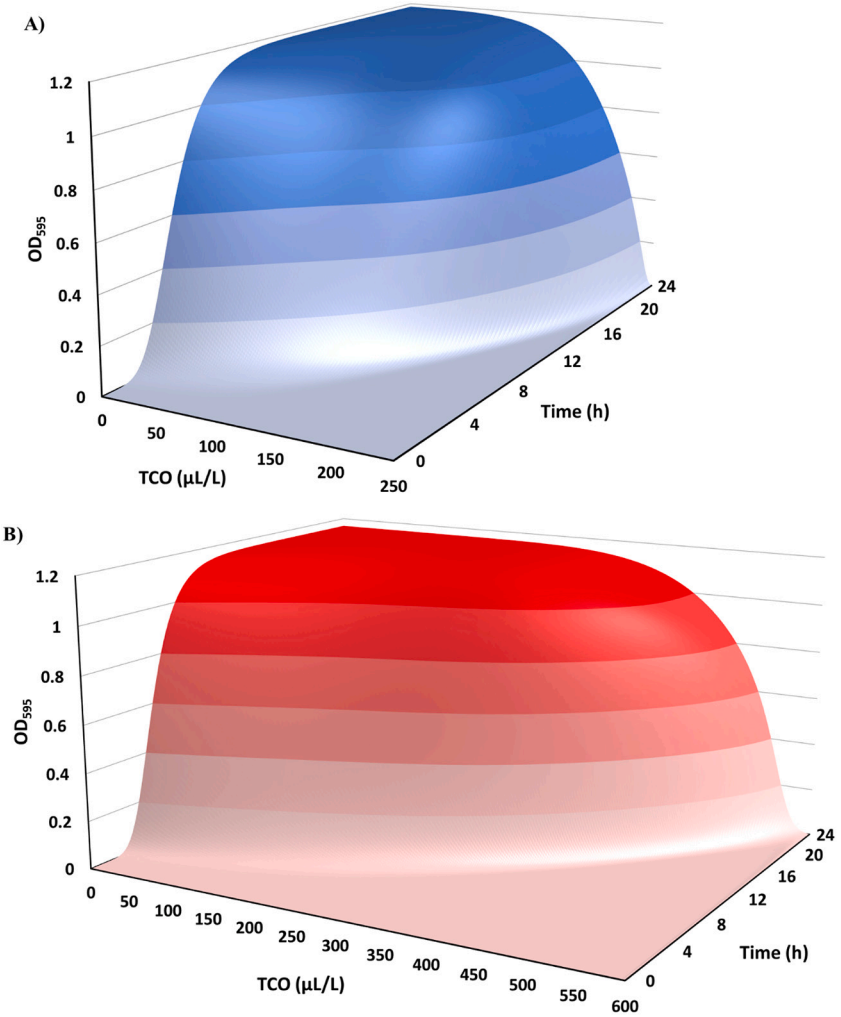

Fig. 1. Growth models of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT; A) and evolved strain (SeTCO; B) at difference concentrations of TCO, modelled using the modified Gompertz equation (Eq. 1).

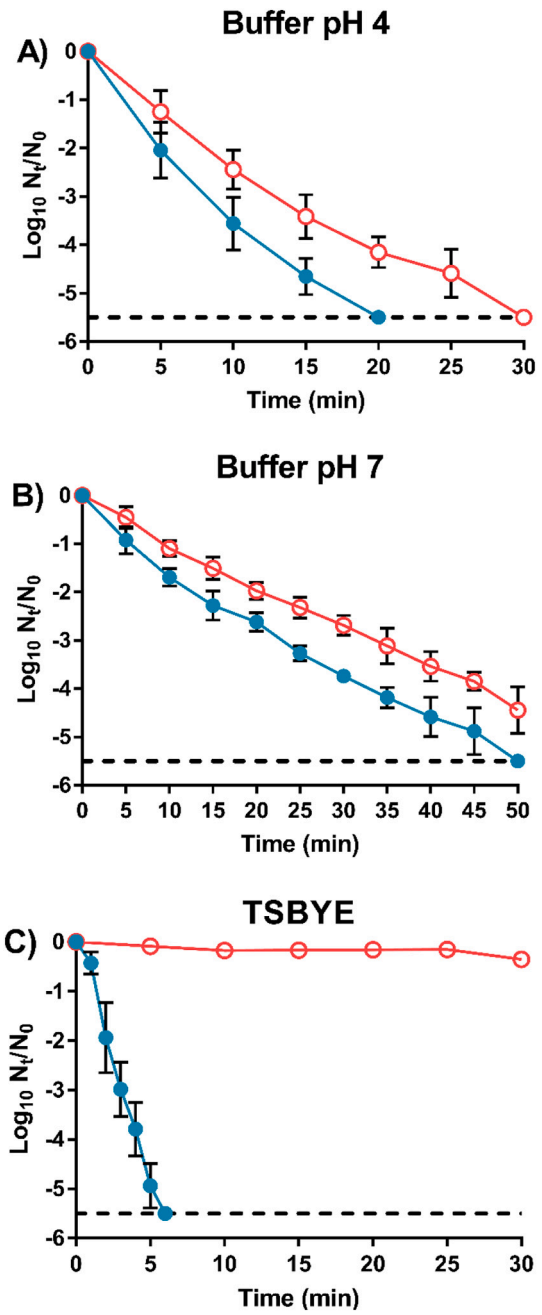

Fig. 2. Survival curves of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (●; SeWT) and evolved strain (○; SeTCO) after 150 μL/L TCO treatment in phosphate-citrate buffer at pH 4.0 (A) and pH 7.0 (B) or 300 μL/L TCO treatment in TSBYE (C). Data are means ± standard deviations (error bars) obtained from at least 3 independent experiments. The dashed line represents the detection limit (−5.5 $\log_{10} N_t/N_0$).

Table 3
Minimum inhibitory concentration (MIC; μL/L) and minimum bactericidal concentration (MBC; μL/L) of TCO for *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strain (SeTCO) in skimmed milk. Each value represents the result of 5 different experiments carried out on different bacterial cultures and on different working days.

Strains	MIC (μL/L)	MBC (μL/L)
SeWT	700	700
SeTCO	1600	1800

preservatives. Table 3 shows the MIC and MBC of TCO in skimmed milk for SeWT and SeTCO. The concentrations of TCO required to inhibit or inactivate both strains were more than double those observed in TSBYE. Likewise, SeTCO also showed an increased resistance and tolerance in skimmed milk in comparison with SeWT. MIC and MBC for SeTCO were 1600 and 1800 μL/L TCO, whereas SeWT was inhibited and inactivated with 700 μL/L.

Lethal treatments with 1500 μL/L TCO were also carried out to compare the tolerance of both strains (Fig. 3). As demonstrated in TSBYE, SeWT was more susceptible to TCO than SeTCO: after 25 min of treatment more than 5.5 $\log_{10}$ cycles of the initial population of SeWT were inactivated, whereas only 0.5 $\log_{10}$ cycles of SeTCO cells were reduced.

3.5. Emergence of TCO resistance leads to cross resistance to antibiotics

The Kirby-Bauer *disk diffusion test* was carried out to assess the cross-resistance of evolved strains against antibiotics. Table 4 shows the zones of growth inhibition (mm) of nalidixic acid, norfloxacin, novobiocin, trimethoprim, rifampicin, chloramphenicol, kanamycin, tetracycline, ampicillin and cephalexin against SeWT and SeTCO. Inhibition halos revealed cross-resistance against several antibiotics after the evolution assay. SeTCO exhibited zones of inhibition smaller than those of SeWT ($p \leq 0.05$) for tetracycline, chloramphenicol, both quinolone antibiotics (nalidixic acid and norfloxacin), novobiocin and trimethoprim. However, neither kanamycin nor the two β-lactams tested (ampicillin and cephalexin) showed significant differences ($p > 0.05$) in inhibition halos between the SeWT and the evolved strain.

3.6. Genetic variations in the resistant strain to antimicrobials

Genetic differences between the reference genome (NCBI accession: NC_003197.2) and our lab strain SeWT were identified to discard them as a consequence of the evolution assay, since they were already present at its onset (Table S4). The genomic comparison of SeWT and SeTCO allowed the identification of mutations fixed throughout the evolution assay with TCO (Fig. 4), causing increased resistance and tolerance against TCO, as well as cross-resistance to antibiotics. Sequencing data revealed 5 mutations in SeTCO: 1 deletion and 4 SNVs (Table 5). Sanger sequencing results verified the mutations in the 5 colonies isolated from the evolution assay, henceforth denoted as $SeTCO_{1–5}$, thereby demonstrating the homogeneity of the bacterial culture after the evolution assay.

WGS revealed the following genotypic changes in SeTCO:

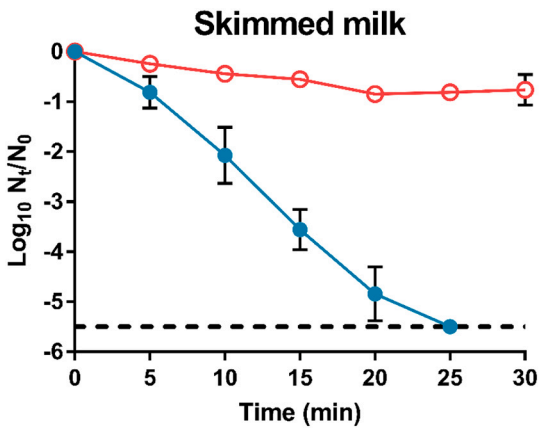

Fig. 3. Survival curves of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (●; SeWT) and evolved strain (○; SeTCO) after 1500 μL/L TCO in skimmed milk. Data are means ± standard deviations (error bars) obtained from at least 3 independent experiments. The dashed line represents the detection limit (−5.5 $\log_{10} N_t/N_0$).

Table 4
Zones of growth inhibition for agar disk diffusion assays of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 wild type (SeWT) and evolved strain (SeTCO) against antibiotics: 400 μg nalidixic acid sodium, 20 μg norfloxacin, 250 μg novobiocin sodium, 10 μg trimethoprim, 50 μg rifampicin, 30 μg chloramphenicol, 30 μg kanamycin sulfate, 30 μg tetracycline, 10 μg ampicillin, and 150 μg cephalexin. Each value represents the mean diameter of the inhibition halo ± standard deviation from three independent experiments.

Cell target	Antibiotic	Strains	
		SeWT	SeTCO
DNA replication	Nalidixic acid	30.08 ± 1.22	23.32 ± 0.76*
	Trimethoprim	27.82 ± 1.10	20.34 ± 0.43*
	Norfloxacin	26.43 ± 1.03	19.68 ± 0.23*
	Novobiocin	13.63 ± 0.40	8.21 ± 0.17*
RNA synthesis	Rifampicin	17.59 ± 0.23	15.34 ± 0.45*
Protein synthesis	Chloramphenicol	26.10 ± 1.37	22.17 ± 0.26*
	Tetracycline	25.80 ± 0.80	20.72 ± 1.80*
	Kanamycin	15.20 ± 1.40	17.62 ± 1.23
Cell wall synthesis	Cephalexin	22.36 ± 0.40	23.15 ± 0.69
	Ampicillin	14.12 ± 0.17	13.49 ± 0.39

* Significantly different from SeWT ($p < 0.05$).

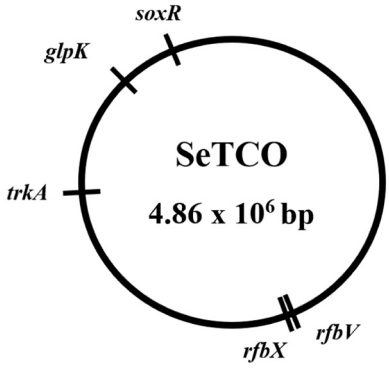

Fig. 4. Genomic map of SeTCO: *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 evolved strains by cyclic short lethal treatments of TCO in comparison with SeWT.

a) 2 mutations in the *rfb* O antigen gene cluster (Fitzgerald et al., 2003). On the one hand, a deletion of an adenine was observed in position 178 of *rfbV*, which encodes an abequosyltransferase. On the other hand, a SNV was detected in *rfbX*: a change of cytosine by guanine that led to a replacement of proline by alanine in the position 381 of the transmembrane protein.
b) 2 SNVs: a transversion from guanine to thymine was detected in position 619 of *trkA*, which encodes an essential subunit of the transmembrane protein of potassium transport systems (K^+); and a change of guanine by adenine located in *glpK* encoding a glycerol kinase.
c) a missense mutation located in *soxR* gene, a redox-sensitive transcriptional activator: a change of guanine by thymine, resulting in an amino acid substitution of arginine by leucine at position 20, specifically in the DNA-binding domain of the SoxR protein.

Once the genetic modifications in SeTCO were identified, based on the function of the mutated genes we decided to focus our study on the *soxR* mutation. Thus, we constructed $SeWT_{soxR}$ by replacing *soxR* in SeWT by the $soxR_{SeTCO}$ variant from SeTCO to determine the contribution of this unique mutation to the phenotype of the evolved strain. On the one hand, MIC and MBC results revealed a resistance and tolerance similar to SeTCO: MIC and MBC of 600 μL/L TCO. Similarly, MIC and MBC of $SeWT_{soxR}$ in skimmed milk showed the same values as SeTCO: 1600 and 1800 μL/L TCO, respectively. The antibiotic susceptibility test also showed that $SeWT_{soxR}$ developed cross-resistance to the same antibiotics as SeTCO (data not shown). No statistically significant differences ($p > 0.05$) were observed between the sizes of zones of inhibition obtained for SeTCO and for $SeWT_{soxR}$.

4. Discussion

Several studies have evidenced that the exposure to ICs such as carvacrol, citral, and limonene oxide could lead to the occurrence of resistant strains in food pathogen populations, such as *E. coli* (Chueca et al., 2016), *S. aureus* (Berdejo et al., 2019), and *Salmonella enterica* serovar Typhimurium (Berdejo et al., 2020a). Subsequently, despite the compositional complexity of EOs and their different mechanisms of action, Berdejo et al. (2020b) also observed the emergence of resistant strains in a Gram-positive bacterium, *S. aureus* against *Citrus sinensis* EO, thereby revealing the possibility that resistance to EOs may also occur.

Phenotypic characterization of SeTCO and SeWT against TCO by MIC and MBC determination, growth kinetics studies in the presence of the EO, and survival curves all evidenced a substantial, stable increase in resistance and tolerance of the evolved strain compared to the wild-type strain (origin of the evolution assay).

Previous studies featuring *S. enterica* did not observe an increase in resistance or tolerance after adaptation phases to natural antimicrobials of up to 72 h: both to ICs, such as cineole and carvacrol (Luz Ida et al., 2012), as also to EOs, such as *Rosmarinus officinalis* or *Origanum vulgare* (Gomes-Neto et al., 2014; Monte et al., 2014). This is thus the first study to have evidenced the emergence of evolved resistant and tolerant strains against a complex EO in a Gram-negative bacterium.

Mutations in the evolved strains responsible for improved adaptation (shorter lag phase and higher growth rate) to the natural antimicrobial do not come at a growth fitness cost in the absence of TCO (similar growth parameters to SeWT), as previously observed in *Salmonella enterica* serovar Typhimurium against carvacrol (Berdejo et al., 2020a). Growth kinetics in the presence of TCO would explain how the emergence of this genetic variant could pose a risk in food preservation when these natural antimicrobials are used as a bacteriostatic agent: the use of low doses to inhibit or slow down the growth of SeWT would be ineffective on genetic variants such as SeTCO.

Notably, the tolerance of SeTCO was higher in the nutrient-rich medium TSBYE than in citrate-phosphate buffers. The fact that the evolution assay was conducted in TSBYE probably led to the selection of other mutations that presented a higher tolerance in this medium. In addition, the increase in tolerance of SeTCO would explain its emergence during the evolution cycles: the increased survival of SeTCO after each lethal treatment allowed this genetic variant to become fixed in the microbial population, while SeWT concentration was progressively

Table 5
Mutations of SeTCO in comparison with SeWT verified by Sanger sequencing. Single nucleotide variation (SNV) and deletion (Del).

Genome position	Gene	Locus tag	Mutation*	Change	Information
2,168,086	*rfbV*	STM2087	Del: −A531	Frameshift (178)	Abequosyltransferase
2,168,774	*rfbX*	STM2088	SNV: C1141G	Pro381Ala	Putative O-antigen transferase
3,580,396	*trkA*	STM3409	SNV: G619T	Asp207Tyr	Trk system potassium transport protein TrkA
4,295,798	*glpK*	STM4086	SNV: G66A	Met22Ile	Glycerol kinase
4,504,454	*soxR*	STM4266	SNV: G59T	Arg20Leu	Redox sensitive transcriptional regulator SoxR

* Position with respect to the start of the coding region.

decreasing. These results also highlight the microbiological risk that the emergence of these strains would pose, since they would be capable of surviving lethal treatments.

EOs and ICs have been extensively studied in the preservation of dairy products due to their antimicrobial properties, among others (Mishra et al., 2020). TCO has demonstrated its excellent antimicrobial properties in milk: alone (Ben Jemaa et al., 2017), or combined with heat treatments (Ben Jemaa et al., 2018). However, no former study has assessed the food safety risk if a resistant bacterial population has emerged. In our study, MIC and MBC values for both strains were higher in skimmed milk than in growth media and treatment buffer, since milk carbohydrates and proteins can interact with the EO and reduce its antimicrobial properties (Mishra et al., 2020). As observed in TSBYE, the MIC and MBC values in milk also revealed twice the resistance and tolerance of SeTCO compared to SeWT (Table 3); the lethal treatments which inactivated more than 5 $\log_{10}$ cycles of SeWT population in milk were ineffective against the evolved strain (Fig. 3). Our results suggest that if these strains emerge in the food industry, they could grow and survive treatments designed for wild-type strains.

SeTCO also showed an increased cross-resistance against quinolones, tetracyclines, and aminoglycosides, with the exception of kanamycin (Table 4). However, both β-lactams tested displayed the same activity against SeWT and the evolved strain ($p > 0.05$). It is likely that the mutations selected by the evolutionary assay with TCO provide general antimicrobial resistance and are therefore also responsible for the development of cross-resistance to antibiotics. Berdejo et al. (2020a) reported that evolved strains of *Salmonella enterica* serovar Typhimurium in the presence of sub-inhibitory doses of carvacrol, the main IC of TCO, maintained the same susceptibility to antibiotics as wild-type strain, whereas evolution assays with lethal carvacrol treatments led to derivative strains with cross-resistance to a wide range of antibiotics, such as tetracycline or rifampicin. Regarding complex EOs, *S. aureus* evolved under sub-inhibitory orange EO doses (Berdejo et al., 2020b), and *Salmonella enterica* serovar Typhimurium adapted to *Origanum vulgare* EO did not show variations in antibiotic resistance.

Genetic analysis of $SeTCO_{1–5}$ strains revealed that mutations in *rfb* O antigen gene cluster are located in transferases involved in lipopolysaccharide O-antigen biosynthesis (Liu et al., 1996; Liu et al., 1995), which plays an important role in bacterial virulence (Liu et al., 2014). The close relationship between antibiotic resistance and virulence has been extensively studied; nevertheless, the correlation between them can be either positive or negative (Cepas and Soto, 2020). However, no data have been published about the relation between the resistances to natural antimicrobials and the virulence of the mutant strains.

According to Knöppel et al. (2018), the mutations in genes involved in metabolic pathways, *trkA* and *glpK*, usually occur as a consequence of adaptation to the growth medium. Indeed, Berdejo et al. (2020a) also observed a mutation in *trkA* after evolution assay with carvacrol in *Salmonella enterica* serovar Typhimurium. These mutations were probably selected because the nutrient medium TSBYE was employed in the evolution assay, and not due to the antimicrobial TCO.

The last SNV was detected in *soxR*, involved in the defence against redox-cycling drugs (Gu and Imlay, 2011). Its oxidized form activates the transcription of the *soxS* gene, which, in turn, modulates the expression of more than 100 genes of the *sox*RS regulon to provide cellular defence against oxidative stress (Pomposiello et al., 2001). The main role of the *sox*RS regulon is to minimize intracellular drug concentration through mechanisms that impede their entry, chemically modify them, or pump them out (Gu and Imlay, 2011). This bacterial response has been described against oxidizing agents and antibiotics; more recently, it has been linked to the bacterial response against ICs or EOs (Chueca et al., 2018; Sheng et al., 2016). Previous studies of resistant mutants of *E. coli* (Chueca et al., 2018) and *Salmonella enterica* serovar Typhimurium (Berdejo et al., 2020a) reported that *soxR* was mutated after cyclic treatments of carvacrol. In addition, a recent proteomic study of *S. enterica* observed a differential expression of stress-related proteins, such as superoxide dismutase, when the bacterium was exposed to *Origanum vulgare* EO, thereby supporting the assumption that oxidative stress would be related with the cell response to complex EOs (Barbosa et al., 2020).

Determination of MIC and MBC to TCO for $SeWT_{soxR}$ in TSBYE and skimmed milk revealed that *soxR* mutation was the main cause of the increased resistance and tolerance of SeTCO against TCO. Since $soxR\text{-}_{SeTCO}$ mutation (codon 20) is located in the DNA-binding domain of SoxR, it is likely that the regulation and the expression of the *sox*RS regulon may have been modified, leading to the development of resistance by an increase in efflux of intracellular antimicrobials. In addition, a genotypic study of a *Salmonella enterica* serovar Typhimurium variant with increased resistance against carvacrol isolated by Berdejo et al. (2020a), SeTCar, also revealed a SNV in *soxR*, on a different nucleotide but in the same codon as SeTCO: a replacement of arginine by cysteine. These findings highlight *soxR* as a key mechanism in the bacterial response to ICs and EOs, induced by the oxidative stress caused by these antimicrobial compounds (Chueca et al., 2014).

Furthermore, the increased cross-resistance to antibiotics of $SeWT_{soxR}$ demonstrates the important role of *soxR*, not only against natural antimicrobials, but also against antibiotics probably through the up-regulation of the multidrug efflux pumps (Du et al., 2018; Kumar et al., 2013). In fact, although SeTCar and SeTCO showed increased cross-resistance to most antibiotics, their resistance against cephalexin (β-lactam) was not modified. Thus, mutations in *soxR* may enhance the pump-out of drugs with intracellular targets, such as quinolones, tetracyclines and aminoglycosides. However, it is likely that the susceptibility to β-lactams is maintained, since these antibiotics act on the synthesis of the peptidoglycan wall in the periplasmic space. These results support the assumption that bacterial resistance mechanisms to EOs and antibiotics may be interrelated.

5. Conclusions

The efficacy of essential oils (EOs) as food preservatives might be called into question if resistant and tolerant strains emerge. Further studies are required to define under what conditions such resistant strains might appear, and to assess the food risk they may pose.

The fact that mechanisms of bacterial resistance to EOs and antibiotics may be interrelated supports the importance of attempting to gain a deeper understanding of how these resistances emerge under the application of natural antimicrobials when used as alternative to antibiotics against MDR strains.

soxR variation was the main contributor to the increased resistance and tolerance observed in SeTCO against TCO and antibiotics, probably enhancing the pump-out of antimicrobial compounds outside the cell and limiting the EO's antimicrobial effect. In this sense, our study provides relevant information regarding the mechanisms of action of EOs in the bacterial cell, supporting the assumption that these natural antimicrobials can also cause oxidative stress and severe intracellular damages.

Declaration of competing interest

None.

Acknowledgments

This study was financially supported by the European Social Fund and Agencia Estatal de Investigación (Spain) (Project No. PGC2018-0937389-BI00), and by the Aragon Agency for Research and Development (Project No. A03_20R). The Spanish Ministry of Sports, Culture and Education provided D. Berdejo and E. Pagán with a grant to carry out this investigation. The authors thank Elisa Gayan for her help on strain construction and S. Hanks for his collaboration on the English-language revision of this manuscript.

CRediT authorship contribution statement

Daniel Berdejo: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing. **Elisa Pagán**: Investigation, Validation. **Natalia Merino**: Investigation, Validation. **Laura Botello-Morte**: Investigation, Validation. **Rafael Pagán**: Conceptualization, Funding acquisition, Resources, Supervision, Writing. **Diego García-Gonzalo**: Conceptualization, Funding acquisition, Resources, Supervision, Writing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109443.

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