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Emerging mutant populations of *Listeria monocytogenes* EGD-e under selective pressure of *Thymbra capitata* essential oil question its use in food preservation

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ABSTRACT

Due to their excellent antimicrobial properties, essential oils (EO) have been proposed as potential preservatives for certain kinds of foods, such as dairy products. However, the occurrence of pathogenic populations that are resistant to EOs could pose a health risk. This report seeks to assess the emergence of resistant populations in *Listeria monocytogenes* EGD-e growth at 37 °C under selective pressure of *Thymbra capitata* EO (TCO), to characterise their resistance in laboratory media, and to identify their genotypic changes, as well as to evaluate the resistance in skimmed milk. TCO cyclic treatment allowed the isolation of two *L. monocytogenes* EGD-e resistant strains against the EO: LmSTCO by sublethal doses (75 μL/L TCO) and LmLTCO by lethal doses (300 μL/L TCO) after 20 and 30 cycles, respectively. Both strains displayed an increase of the minimum inhibitory and bactericidal concentration against TCO and a higher survival rate after lethal treatments than the wild-type strain (LmWT). Growth kinetics revealed a better adaptation of LmSTCO in presence of TCO, while LmLTCO grew more slowly compared to LmWT, even in the absence of the antimicrobial. Moreover, a slight increase in cross-resistance to antibiotics was observed: LmSTCO to β-lactams and LmLTCO to a series of broad-spectrum antibiotics. The genomic study revealed one sole nucleotide change in LmSTCO located in *plsC* gene codifying an enzyme involved in the production of phosphatidic acid, a precursor in cell membrane synthesis. Five genetic variations were found in LmLTCO: among them, the deletion of an ATP-synthesis system involved in slowing bacterial growth. Inhibition and inactivation assays in skimmed milk confirmed the increased resistance of both strains, thereby indicating a safety risk in case these strains emerge in the food chain. These results strongly suggest that the occurrence of such resistances should be taken into account in order to ensure the efficacy of natural antimicrobials in the design of food preservation strategies.

1. Introduction

Natural antimicrobials are emerging as an alternative to chemically synthesized food preservatives, which are subject to greater restrictions and are increasingly rejected by consumers (Carocho, Barreiro, Morales, & Ferreira, 2014). Essential oils (EOs) and their individual constituents (ICs) have demonstrated excellent antimicrobial and antioxidant properties (Faleiro & Miguel, 2020) that point toward their potential use in the food industry (Quinto et al., 2019). Their current use in food preservation is nevertheless limited by some drawbacks. Due to the strong organoleptic properties of EOs and ICs, the current required doses lead to an undesirable change of taste and smell in most foods, leading to their rejection by the consumer (Espina, García-Gonzalo, & Pagán, 2014). Many studies thus focus on understanding their antimicrobial activity in order to improve it and thereby reduce the doses required in food preservation (Falleh, Ben Jemaa, Saada, & Ksouri, 2020). On the other hand, the increase in antimicrobial resistance (AMR), mainly against antibiotics (Peterson & Kaur, 2018), has called into question the long-term effectiveness of EOs and ICs, and raised the question whether resistance to natural antimicrobials could also appear. The antioxidant activity of EOs and ICs at low doses (Hashemi, Khorram, & Sohrabi, 2017) has been associated with a low mutagenic rate in bacteria (Chueca, Berdejo, Gomes-Neto, Pagán, & García-Gonzalo, 2016; Hammer, Carson, & Riley, 2008) through the neutralisation of reactive

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oxygen species (ROS), one of the main causes of DNA damage (Sakai, Nakanishi, Yoshiyama & Maki, 2006). This, in turn, suggested that the occurrence of mutations caused by the application of EOs and ICs would be unlikely (Leite de Souza, 2016). Recent studies have shown, however, that IC treatments can lead to the emergence of resistant and tolerant strains. Prolonged evolution assays at sublethal and lethal doses demonstrated the emergence of resistant and tolerant strains against ICs, such as carvacrol, citral and limonene oxide, in food pathogens: *Escherichia coli* (Chueca et al., 2016), *Salmonella enterica* (Berdejo, Merino, Pagán, García-Gonzalo, & Pagán, 2020) and *Staphylococcus aureus* (Berdejo et al., 2019). It should be noted that "resistance" is the ability of bacteria to replicate and not just survive in the presence of a drug, whereas "tolerance" is the general ability of a population to survive longer treatments (Balaban et al., 2019).

The development of resistance against EOs had been discarded in view of their great complexity and compositional variety and, therefore, to the multitude of antimicrobial action mechanisms that their ICs can exert on bacteria (Lingan, 2018). For instance, *Thymbra capitata* EO is one of the EOs with the best antimicrobial properties and is composed of more than 28 different ICs (Candela, Maggi, Lazzara, Rosselli, & Bruno, 2019). Nevertheless, the development of resistance and tolerance against a complex EO, *Citrus sinensis,* has recently been observed in *S. aureus* (Berdejo, Pagán, Merino, Pagán, & García-Gonzalo, 2020), and even some of these evolved strains not only showed resistance to natural antimicrobials, but also developed cross-resistance to a wide range of antibiotics (Berdejo, Merino, et al., 2020; Chueca et al., 2018). These results would support the assumption that the mutations selected by the selective pressure exerted by EOs and ICs may also be associated with resistance to antibiotics.

The specific conditions under which resistance to natural antimicrobials can occur are unknown, as well as whether it may also occur in other foodborne pathogens, such as *Listeria monocytogenes*. However, elucidating which mutations occur and which stress response or metabolic pathways are affected would allow us to gain a better understanding of the mechanisms of cellular response to natural antimicrobials, thereby revealing their main modes of action and leading to safer, more efficient food preservation strategies.

We therefore carried out this study with the following goals: a) to assess whether the use of *Thymbra capitata* EO applied at sublethal or lethal doses could lead to the emergence of resistant or tolerant strains in *Listeria monocytogenes* EGD-e, b) to describe their direct resistance and tolerance to TCO, as well as cross-resistance to antibiotics, c) to identify their genetic changes in comparison to wild-type and d) to assess the magnitude of the increased resistance and tolerance in skimmed milk.

2. Materials and methods

2.1. Microorganisms, growth conditions, and reagents

We selected *L. monocytogenes* EGD-e for our study of genetic evolution because this strain has been completely sequenced and characterized in detail (Glaser et al., 2001; Toledo-Arana et al., 2009). *L. monocytogenes* EGD-e was kindly provided by Prof. Chakraborty (Institute for Medical Microbiology, Giessen, Germany). Throughout this investigation, the strain was kept 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 working bacterial cultures, test tubes containing 5 mL of tryptone soya broth (Oxoid) with 0.6% yeast extract (TSBYE) were inoculated with one colony and then incubated aerobically overnight on 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 stationary growth phase was reached (2×10^9 CFU/mL approximately). We applied the same protocol to obtain the working bacterial cultures of the isolated strains that resulted from the evolution assays with *T. capitata* essential oil (TCO).

TCO was kindly provided by the TELIC Group (Barcelona, Spain). This EO was kept in the dark at 4 °C 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.

UHT skimmed milk (Central Lechera Asturiana, Asturias, Spain) was purchased in supermarket; to ensure sterility, a new bottle was opened before each experiment.

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

MIC determination was performed according to CLSI (2015) with some modifications due to hydrophobicity of the EO. From the bacterial cultures, test tubes with 5 mL of cation-adjusted mueller hinton broth (MHB; Sigma-Aldrich) were inoculated in presence of different concentrations of TCO: from 0 to 500 μL/L, based on previous experiments (results not shown). Following the method described by Friedman, Henika & Mandrell (2002), a vigorous shaking by vortex was used to prepare TCO dispersions in MHB, avoiding the use of solvents for their possible detriment in the antibacterial activity. Once tubes were incubated for 24 h, MIC was determined as the lowest concentration of the antimicrobial compound that was capable to avoid bacterial growth. To determine objectively bacterial growth, the optical density was read at 595 nm (OD_{595}) using a microplate reader (Genios, Tecan, Männedorf, Switzerland). 10% of the OD_{595} measure of the positive control was established as the lower limit to consider that bacterial strain was grown (Kohanski, DePristo, and Collins, 2010). The minimum bactericidal concentration (MBC) of TCO was evaluated in parallel to MIC test. From the test tubes employed in the MIC determination after incubation, 100 μL aliquot of each tube was spread onto mueller hinton agar cation adjusted (Sigma-Aldrich; MHA) plates and incubated at 37 °C for 48 h. Colonies were counted and the lowest concentration of TCO that killed $\geq$ 99.9% of the initial bacterial concentration (5×10^5 CFU/mL) was defined as the MBC end point

Additionally, MIC and MBC determinations were conducted in a food model, skimmed milk, following the same protocols applying a concentration range from 600 to 1,500 μL/L TCO. Since milk turbidity hinders a correct OD_{595} measurement, MIC was assessed by cell counting of the samples. An increase of 50% of the initial bacterial population (CFU/mL), i.e. one generation in bacterial growth, was established as the minimum to take bacterial growth into consideration. MBC determination in skimmed milk was conducted following the same protocol as in TSBYE.

2.3. TCO evolution assays

The use of EOs in food preservation can lead either to the inhibition of bacterial growth or to bacterial inactivation, depending on the concentration. To obtain mutant *L. monocytogenes* EGD-e strains against TCO, we followed two different protocols in order to simulate bacteriostatic and bactericidal conditions: a) cyclic exposure to prolonged sublethal treatments, and b) cyclic exposure to short lethal treatments.

The first protocol was based on the isolation of strains by prolonged exposure to a sub-inhibitory concentration of TCO during growth phase, applying a methodology adapted from Kohanski, DePristo, and Collins (2010) and Andersson and Hughes (2014). *L. monocytogenes* EGD-e wild-type strain (LmWT) was grown on TSAYE plates for 48 h at 37 °C. A single colony was inoculated in 5 mL TSBYE and incubated under agitation for 16 h at 37 °C. This preculture was diluted 1:1000 into 50 mL TSBYE and incubated for 6 h to obtain an exponential phase culture. From that culture, 5 mL TSBYE were inoculated at an initial bacterial

concentration of 10^6 CFU/mL in the presence of 75 μL/L of TCO (1/2x MIC for LmWT). This bacterial suspension was incubated 24 h/37 °C/130 rpm and, once the stationary phase was reached, the same step was repeated: the previous culture was diluted (10^6 CFU/mL) in 5 mL TSBYE with 75 μL/L TCO and incubated 24 h/37 °C/130 rpm. This procedure was repeated 20 times. An aliquot was then diluted in phosphate buffered saline (Sigma-Aldrich, Steinheim, Westphalia, Germany; PBS) and spread on TSAYE plates (without TCO). After the incubation period, 5 colonies ($LmSTCO_{1\text{-}5}$) were randomly selected to carry out phenotypic and genotypic characterization.

The second protocol was based on the isolation of strains by recovering survivors after lethal TCO treatments. This methodology was adapted from Levin-Reisman et al. (2017). A stationary phase culture of LmWT was diluted 1:100 in 50 mL fresh TSBYE with 300 μL/L of TCO (2x MIC for LmWT) for 4.50 h at 37 °C. Treated cells were centrifuged for 20 min at 15,000 relative centrifuge force (RCF), washed twice with TSBYE, resuspended in 1 mL TSBYE, and incubated overnight at 37 °C. This procedure was repeated 30 times. An aliquot was then diluted in PBS and spread on TSAYE plates (without TCO), from which 5 strains ($LmLTCO_{1\text{-}5}$) were randomly selected to carry out phenotypic and genotypic characterization.

Once the 5 strains had been isolated by each evolution assay, $LmSTCO_{1\text{-}5}$ and $LmLTCO_{1\text{-}5}$, were obtained; the first approach to evaluate their resistance and tolerance was to determine the MIC and the MBC of TCO and to compare it with those of LmWT.

2.4. Growth curves in presence of TCO

In order to study the behaviour of the isolated strains against TCO, the growth kinetics of LmWT and of evolved strains were evaluated in TSBYE in the presence of different concentrations of TCO following the protocol described by Berdejo, Pagán, et al. (2020): from 0 up to 300 μL/L TCO.

Bacterial growth curves based on OD_{595} of LmWT, LmSTCO, and LmLTCO were graphically displayed and modelled by a modified Gompertz equation (Zwietering, Jongenburger, Rombouts, & van 't Riet, 1990):

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

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

A least-squares adjustment was carried out to build the model and obtain A, μm, and λ values using the Prism program (GraphPad Software, Inc., San Diego, USA). The experiment was prolonged for more than 24 h at high TCO concentrations until reaching stationary phase to allow an optimal adjustment to 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).

2.5. Survival curves in the presence of TCO

The tolerance of LmWT and of the evolved strains against TCO was evaluated by lethal treatments following the protocol described by Berdejo, Pagán, et al. (2020). Treatments were performed in citrate–phosphate buffer, also called "McIlvaine buffer", at 25 °C with 150 μL/L TCO, at pH 4,0, and 200 μL/L TCO at pH 7,0. Those pH treatments were chosen as representative of neutral and acid conditions within the usual pH range of food. Treated samples were diluted in PBS and subsequently spread on TSAYE plates, which were incubated for 48 h at 37 °C. Once survival curves of LmWT and evolved strains were obtained, inactivation kinetics were compared in order to evaluate the increase in tolerance of LmSTCO and LmLTCO against TCO. Next, following the same protocol, lethal treatments were performed in skimmed milk, at 1600 μL/L TCO for 60 min, to assess the relevance of the increased tolerance of evolved strains in a food model.

2.6. Antibiotic susceptibility test

Agar disk diffusion assay was conducted to test antimicrobial susceptibility according to CLSI (CLSI, 2012, 2014). Following the suggestions for fastidious bacteria (CLSI, 2010), bacterial cultures were grown in MHB supplemented with 2.5% lysed horse blood (Sigma-Aldrich). Bacterial suspensions were then spread on MHA plates supplemented with 2.5% lysed horse blood and, after 5 min at room temperature, blank disks (Ø: 6.0 mm) (Thermo Scientific™ Oxoid™ Antimicrobial Susceptibility Disk Dispenser, ST6090, Waltham, MA, USA) were placed on the surface of plates and individually impregnated with the antibiotics: 30 μg kanamycin sulphate, 30 μg tetracycline, 30 μg chloramphenicol, 400 μg nalidixic acid sodium, 5 μg rifampicin, 30 μg norfloxacin, 150 μg novobiocin sodium, 10 μg trimethoprim, 10 μg ampicillin, and 150 μg cephalexin (Sigma-Aldrich). These plates were incubated at 37 °C for 24 h, after which the diameters of the resulting inhibition zones were measured (paper disks included).

2.7. Statistical analysis

Each phenotypic characterization result was obtained from at least 3 independent experiments carried out on different working days with different bacterial cultures. MIC and MBC data correspond to the results obtained from 5 different assays. Growth curve parameters, lethal treatment graphics, and antibiotic susceptibility tests are displayed as the mean ± standard deviation, using the Prism program (GraphPad 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

From bacterial culture of LmWT and isolated strains: LmSTCO and LmTCO, genomic DNA (gDNA) was extracted using gDNA extraction and purification columns, following the protocol provided in the kit (GeneJET Genomic DNA, Thermo Scientific, Waltham, MA, USA). Illumina technology was used to carry out whole genome sequencing (WGS) of LmWT, LmSTCO, and LmLTCO, on NextSeq equipment at mid output flow, with a total of 2 × 150 cycles (Illumina; Fasteris, SA, Geneva, Switzerland). Quality control and genetic study was carried out as described by Berdejo, Pagán, et al. (2020). The quality-control-filtered paired-end reads were mapped on the reference genome sequence (National Center for Biotechnology Information; NCBI accession: NC_003210.1): *Listeria monocytogenes* EGD-e (Toledo-Arana et al., 2009). A total of 3.66, 4.31 and 4.55 million of 150 bp-reads were mapped for LmWT, LmSCar and LmLCar, respectively with an average Phred quality score of 33.07, 33.05 and 33.01. Single nucleotide variants (SNVs), short insertion (Ins), deletions (Del), and structural variations (SVs) were identified between LmWT 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: PRJNA669703). The accession numbers of the samples are SAMN16457448 (LmWT), SAMN16457449 (LmSTCO), SAMN16457450 (LmLTCO). Finally, specific primers (Table S1) were designed to carry out PCR amplifications, as well as Sanger sequencings to verify the mutations detected in the WGS.

3. Results and discussion

3.1. Emergence of resistant and tolerant strains by evolution assay with TCO

After carrying out the TCO evolution assays with *L. monocytogenes* EGD-e (LmWT) with two different protocols: cyclic exposure to a)

Table 1
Minimum inhibitory concentration (MIC; μL/L) and minimum bactericidal concentration (MBC; μL/L) of *Thymbra capitata* essential oil (TCO) for *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: $LmSTCO_{1\text{-}5}$ (5 strains selected by cyclic exposure to prolonged sublethal doses) and $LmLTCO_{1\text{-}5}$ (5 strains selected by cyclic exposure to short lethal treatments). 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)
LmWT	150	200
$LmSTCO_{1\text{-}5}$	200	250
$LmLTCO_{1\text{-}5}$	300	400

prolonged sublethal doses, and b) short lethal treatments, we selected 5 colonies at random from each lineage, called $LmSTCO_{1\text{-}5}$ and $LmLTCO_{1\text{-}5}$ respectively. The evolved strains were kept and re-cultivated in absence of the EO to avoid a phenotypic adaption, and hence their behaviour can be associated with genotypic changes. The first assay with the purpose of assessing the emergence of resistant and tolerant strains was the phenotypical characterisation of $LmSTCO_{1\text{-}5}$ and $LmLTCO_{1\text{-}5}$ against TCO by MIC and MBC determinations, and their comparison with LmWT (Table 1). MIC and MBC results of the 5 colonies selected from the evolution assays were grouped in the same cell because they showed the same values for both parameters.

Comparing evolved strains with LmWT, we observe that both strains exhibited higher MIC and MBC values against TCO. On the one hand, all $LmSTCO_{1\text{-}5}$ revealed an increase of 33% in MIC, from 150 to 200 μL/L, and of 25% in MBC, from 200 to 250 μL/L. On the other hand, $LmLTCO_{1\text{-}5}$ showed even higher resistance and tolerance than $LmSTCO_{1\text{-}5}$, reaching a MIC of 300 μL/L and a MBC of 400 μL/L, i.e., a 100% increase for both values compared to LmWT.

We thus observe that *L. monocytogenes* EGD-e evolved during cyclic exposure, both under sub-inhibitory doses and under lethal concentrations of TCO, resulting in an increase in resistance and tolerance against TCO. Previous studies of evolution assays did not evidence any change in bacterial susceptibility in *L. monocytogenes* against ICs such as eugenol and citral (Apolónio, Faleiro, Miguel, & Neto, 2014), or against EOs such as *Rosmanirus officinalis* (Gomes Neto, Luz, Honório, Tavares, & de Souza, 2012) or *Origanum vulgare* (Luz, Neto, Tavares, Magnani, & de Souza, 2012). To the best of our knowledge, this is the first report to demonstrate that *L. monocytogenes* can evolve under EO treatments to the point of developing resistant strains. This is the first time that resistant and tolerant strains have been isolated under the application of sublethal and lethal treatments of a complex EO. Until now, increased resistance and tolerance to a complex EO had only been observed in *Staphylococcus aureus*, and only when the antimicrobial was applied at subinhibitory concentrations (Berdejo, Pagán, et al., 2020). These findings indicate that the use of TCO as a food preservative can permit the emergence of mutant subpopulations with either increased resistance and/or tolerance to EOs.

In addition, it is likely that the bacterial populations after the two evolution assays were homogeneous, since the five isolated colonies showed the same increase in MIC and MBC against TCO. These results would suggest that the mutations occurred during the evolution assays and were maintained due to selective TCO pressure before finally becoming fixed in the bacterial population. For this reason, we carried out phenotypic and genotypic characterization on only one of the 5 strains from each lineage, from here onward referred to as LmSTCO and LmLTCO. It should be noted that the temperatures employed during bacterial growth in both evolution assays may influence the mutagenic rate. In this regard, higher temperatures during incubation could favour the emergence of resistant genetic variants (Chu et al, 2018).

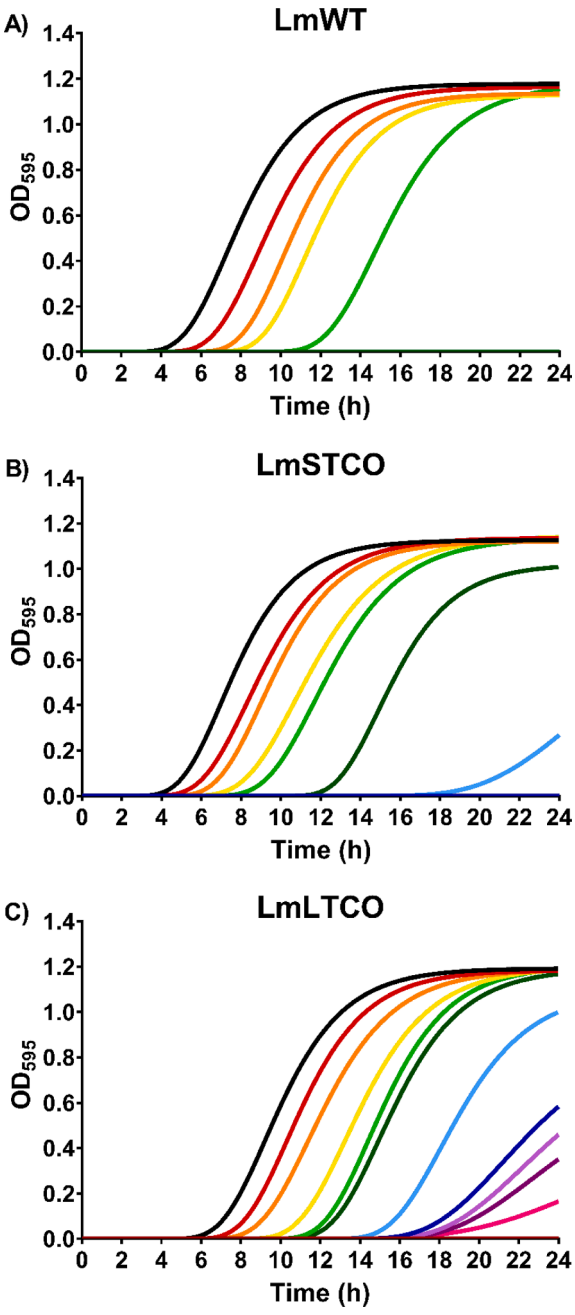

Fig. 1. Growth curves of *Listeria monocytogenes* EGD-e wild type (A; LmWT) and evolved strains: LmSTCO (B; by cyclic exposure to prolonged sublethal doses) and LmLTCO (C; by cyclic exposure to short lethal treatments), in the absence (-) and presence of 50 (), 75 (), 100 (), 125 (), 150 (), 175 (), 200 (), 225 (), 250 (), 275 () or 300 () of *Thymbra capitata* essential oil (TCO). Growth curves were modelled using the modified Gompertz equation (Eq. (1)). Concentrations above the MIC were tested but they are not displayed to facilitate the visualization of the data.

3.2. Better adaptation and growth of LmSTCO than LmWT 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. By least-squares adjustment model, growth curves were obtained with excellent goodness of fit (Table S2). Fig. 1 displays the growth curves modelled by Gompertz modified equation obtained from LmWT, LmSTCO, and LmLTCO in presence of varying concentrations of TCO: from 0 to 300 μL/L. As can be seen in Fig. 1A, the presence of TCO in the growth

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 of *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: LmSTCO (by cyclic exposure to prolonged sublethal doses) and LmLTCO (by cyclic exposure to short lethal treatments), at 37 °C in TSBYE under continuous agitation (130 rpm) with different concentrations of *Thymbra capitata* essential oil (TCO).

A (OD_{595})		Strains		
		LmWT	**LmSTCO**	**LmLTCO**
TCO (μL/L)	**0**	1.178 ± 0.035^{a}	1.126 ± 0.031^{a}	1.192 ± 0.039^{a}
	50	1.165 ± 0.029^{a}	1.136 ± 0.030^{a}	1.184 ± 0.040^{a}
	75	1.136 ± 0.031^{a}	1.122 ± 0.030^{a}	1.189 ± 0.032^{a}
	100	1.130 ± 0.033^{a}	1.145 ± 0.039^{a}	1.198 ± 0.043^{a}
	125	1.180 ± 0.040^{a}	1.137 ± 0.031^{a}	1.205 ± 0.045^{a}
	150		1.021 ± 0.034^{b}	1.192 ± 0.038^{a}†
	175		0.996 ± 0.021^{b}	1.099 ± 0.023^{a}†
	200			0.920 ± 0.029^{b}
	225			0.851 ± 0.036^{b}
	250			0.724 ± 0.039^{c}
	275			0.715 ± 0.055^{c}
μ_m (OD_{595}/h)		Strains		
		LmWT	**LmSTCO**	**LmLTCO**
TCO (μL/L)	**0**	0.203 ± 0.017^{a}	0.204 ± 0.013^{a}	0.199 ± 0.017^{a}
	50	0.194 ± 0.008^{a}	0.182 ± 0.009^{ab}	0.197 ± 0.015^{a}
	75	0.201 ± 0.010^{a}	0.189 ± 0.010^{ab}	0.183 ± 0.007^{ab}
	100	0.198 ± 0.010^{a}	0.167 ± 0.011^{b}	0.186 ± 0.010^{a}
	125	0.180 ± 0.007^{a}	0.172 ± 0.006^{b}	0.198 ± 0.011^{a}
	150		0.181 ± 0.007^{a}	0.190 ± 0.007^{a}
	175		0.075 ± 0.003^{c}	0.162 ± 0.007^{b}†
	200			0.095 ± 0.002^{c}
	225			0.083 ± 0.004^{cd}
	250			0.066 ± 0.009^{de}
	275			0.040 ± 0.004^{e}
λ (h)		Strains		
		LmWT	**LmSTCO**	**LmLTCO**
TCO (μL/L)	**0**	5.149 ± 0.261^{a}	4.984 ± 0.199^{a}	7.047 ± 0.298^{a}†
	50	6.624 ± 0.139^{b}	6.048 ± 0.177^{b}*	8.121 ± 0.262^{b}*†
	75	8.039 ± 0.165^{c}	6.832 ± 0.180^{c}*	9.020 ± 0.130^{c}*†
	100	9.110 ± 0.161^{d}	8.198 ± 0.244^{d}*	10.910 ± 0.182^{d}*†
	125	12.280 ± 0.124^{e}	9.266 ± 0.112^{e}*	12.220 ± 0.162^{e}†
	150		12.820 ± 0.107^{f}	12.652 ± 0.105^{e}
	175		20.430 ± 0.162^{g}	15.590 ± 0.133^{f}†
	200			17.648 ± 0.058^{g}
	225			18.392 ± 0.172^{gh}
	250			18.686 ± 0.481^{h}
	275			19.873 ± 0.513^{i}

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 LmWT ($p < 0.05$). †Significantly different from LmSTCO ($p < 0.05$).

medium mainly affected the lag phase, and, to a lesser extent, the growth rate of LmWT, as well as that of the evolved strains. Moreover, concentrations higher than 150 μL/L caused a decrease of the maximum bacteria concentrations achieved in the stationary phase of LmSTCO and LmLTCO.

Table 2 displays the values of the parameters *A* (maximum OD_{595}), μm (maximum specific growth rate) and λ (lag phase), obtained from the models of the three strains at all the tested concentrations. The *A* parameter revealed that there are no significant differences ($p > 0.05$) in the bacterial concentration reached in the stationary phase for LmWT, LmSTCO, and LmLTCO when grown in the absence or in the presence of TCO up to a concentration of 125 μL/L. Nevertheless, there was a significant decrease ($p < 0.05$) at concentrations above 125 μL/L: from 1.126 to 0.996 (OD_{595}) for LmSTCO at 175 μL/L TCO, and from 1.192 to 0.715 (OD_{595}) for LmLTCO at 275 μL/L TCO. Similar results were obtained for the μm parameter: LmWT showed no variation based on TCO concentration, and only the evolved strains showed a decrease in growth rate as EO concentration increased. The greatest differences were

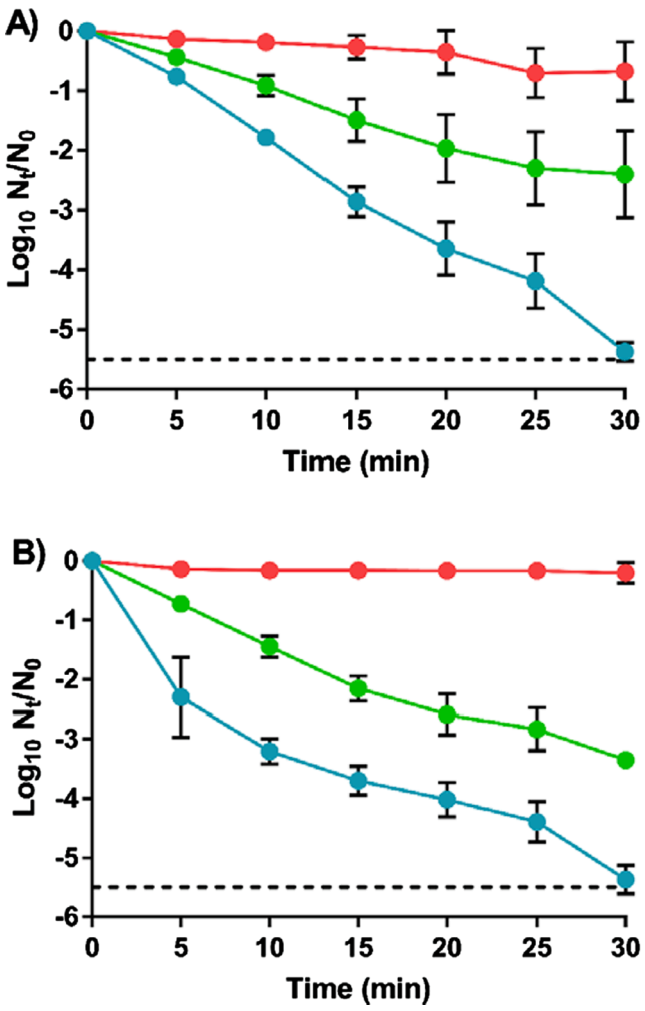

Fig. 2. Survival curves of *Listeria monocytogenes* EGD-e wild type (●; LmWT) and evolved strains: LmSTCO (●; by cyclic exposure to prolonged sublethal doses) and LmLTCO (●; by cyclic exposure to short lethal treatments), after lethal treatment of *Thymbra capitata* essential oil (TCO) in citrate–phosphate buffer at pH 4.0 (150 μL/L TCO; A) and at pH 7.0 (200 μL/L TCO; B). 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/N0).

observed in the λ parameter both among the concentrations applied and between the evolved strains and LmWT. The three strains showed remarkable growth delay ($p < 0.05$) as the TCO concentration increased. For instance, LmWT increased its lag phase from 5.149 h to 12.280 h when TCO was added at a concentration of 125 μL/L. Comparing the evolved strains with LmWT, significant differences were observed in the lag phase at different concentrations of TCO ($p < 0.05$). On the one hand, LmSTCO exhibited a growth behaviour similar to LmWT in absence of TCO; however, when TCO was added to medium, the lag phase of LmSTCO was shorter than that of LmWT (from 50 to 125 μL/L). On the other hand, the growth behaviour of LmLTCO was completely different from the other evolved strain. LmLTCO displayed a growth delay in presence of TCO compared to LmWT, but also in absence of the EO.

Previous studies on *L. monocytogenes* have also shown a slower growth rate and a higher lag phase as the concentration of thyme EO in the medium increased (Braschi et al., 2018). The EOs caused an alteration of cell membrane integrity and increased the membrane permeability of bacteria, thus prolonging adaptation and lag phase time (Bouyahya, Abrini, Dakka, & Bakri, 2019). *S. aureus* resistant to orange EO also exhibited a decrease in lag phase time in comparison with the

Table 3
Zones of growth inhibition for agar disk diffusion assays of *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: LmSTCO (by cyclic exposure to prolonged sublethal doses) and LmLTCO (by cyclic exposure to short lethal treatments) against antibiotics: 30 μg kanamycin sulfate, 30 μg tetracycline, 30 μg chloramphenicol, 400 μg nalidixic acid sodium, 5 μg rifampicin, 30 μg norfloxacin, 150 μg novobiocin sodium, 10 μg trimethoprim, 10 μg ampicillin, and 150 μg cephalexin. Each value represents the mean diameter of the inhibition halo ± standard deviation (mm) from three independent experiments.

Antibiotics	Strains		
	LmWT	**LmSTCO**	**LmLTCO**
Kanamycin	22.86 ± 0.99	22.20 ± 0.97	19.27 ± 0.75*
Tetracycline	35.43 ± 0.76	35.70 ± 0.63	31.03 ± 0.83*
Chloramphenicol	24.28 ± 0.92	25.02 ± 0.67	22.40 ± 1.27
Nalidixic acid	21.62 ± 1.22	18.09 ± 2.34	19.88 ± 1.14
Rifampicin	33.91 ± 0.97	32.19 ± 1.64	32.36 ± 0.96
Norfloxacin	22.03 ± 1.16	24.74 ± 2.50	19.98 ± 1.21
Novobiocin	31.18 ± 0.41	33.20 ± 1.40	29.43 ± 0.41*
Trimethoprim	35.30 ± 1.05	33.56 ± 1.61	31.47 ± 1.68*
Ampicillin	20.28 ± 0.14	16.65 ± 0.44*	18.21 ± 0.61*
Cephalexin	21.97 ± 1.32	16.89 ± 0.86*	20.41 ± 0.47

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

wild-type strain (Berdejo, Pagán, et al., 2020), similarly to what we have observed regarding LmSTCO (Fig. 1B).

These results would explain how LmSTCO, the strain evolved by sub-inhibitory doses, could emerge during the evolution assays against LmWT. At the concentration used in the evolution cycles, 75 μL/L TCO, LmSTCO had a lag phase 1.2 h shorter than LmWT. This sub-population might therefore grow better than LmWT, to the point of taking over the culture. In regard to LmLTCO, the kinetics study revealed that its mutations have a fitness cost, and thereby lead to a growth delay in absence of TCO, even though this strain was more resistant and tolerant than LmWT in MIC and MBC determination. No previous reports have shown that evolved strains with increased resistance and tolerance to natural antimicrobials grow slower than wild-type strain in the presence, and also in the absence, of EO as observed in LmLTCO. This phenomenon is probably caused by the evolution assay protocol we applied to select and isolate LmLTCO. These findings support the assumption that mutations fixed after the two evolution assays would be different and would behave differently.

3.3. No inactivation of LmLTCO at lethal TCO doses for LmWT

To further evaluate and compare the tolerance of the evolved strains with LmWT, lethal TCO treatments were carried out in citrate–phosphate buffer at pH 4.0 and 7.0. Fig. 2 shows survival curves of LmWT, LmSTCO, and LmLTCO, after treatments with 150 μL/L TCO at pH 4.0 and 200 μL/L TCO at pH 7.0 for 30 min. Both treatments managed to inactivate more than 5 $\log_{10}$ cycles of LmWT, but not of the evolved strains. As can be seen in Fig. 2A, only 2.5 and 0.5 $\log_{10}$ cycles of reduction were observed after 30 min of treatment for LmSTCO and LmTCO, respectively, at pH 4.0. Similar increased survival was noted in both evolved strains at neutral conditions. At pH 7.0, LmTCO was the most tolerant: no inactivation was observed in LmLTCO during the experiment, whereas LmSTCO reached a reduction of 3.3 $\log_{10}$ cycles after 30 min. Other studies also revealed an increased tolerance in *E. coli* (Chueca et al., 2016), *S. enterica* (Berdejo, Merino, et al., 2020), and *S. aureus* (Berdejo et al., 2019; Berdejo, Pagán, et al., 2020), after evolution assays with natural antimicrobials.

These data explain how LmLTCO could emerge more favourably along the evolution cycles than LmWT. The increased tolerance of the evolved strain allowed it to better survive lethal treatments than LmWT. It can thus be assumed that LmWT concentration would progressively decrease during the evolution assay until genetic variations of LmLTCO became fixed in the bacterial population.

Table 4
Minimum inhibitory concentration (MIC; μL/L) and minimum bactericidal concentration (MBC; μL/L) of *Thymbra capitata* essential oil (TCO) for *Listeria monocytogenes* EGD-e (LmWT) and evolved strains: LmSTCO (by cyclic exposure to prolonged sublethal doses) and LmLTCO (by cyclic exposure to short lethal treatments) in skimmed milk. 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)
LmWT	900	900
LmSTCO	1000	1200
LmLTCO	1200	1300

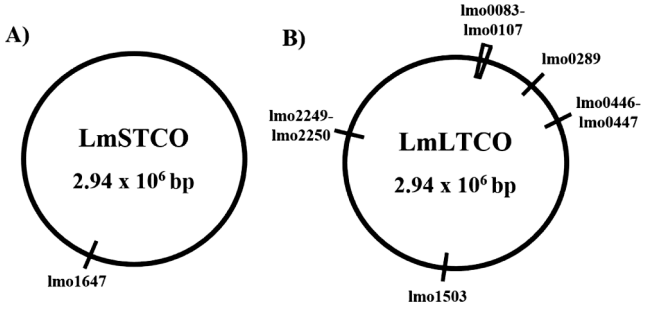

Fig. 3. Genomic maps of the *Listeria monocytogenes* EGD-e evolved strains by cyclic exposure to prolonged sublethal doses (LmSTCO; A) and to short lethal treatments (LmLTCO; B) of *Thymbra capitata* essential oil (TCO).

3.4. Cross-resistance of evolved strains against antibiotics

We determined the susceptibility of LmWT and evolved strains to antibiotics to assess whether cross-resistance to other types of antimicrobials could also occur. Table 3 displays the inhibition halos of LmWT, LmSTCO and LmLTCO against several antibiotics: kanamycin, tetracycline, chloramphenicol, nalidixic acid, rifampicin, norfloxacin, novobiocin, trimethoprim, ampicillin, and cephalexin. Limited information is provided in CLSI documents (CLSI, 2010, 2012) for testing *Listeria* strains, so concentrations were chosen and adjusted according to Yehia, Elkhadragy, Aljahani, and Alarjani (2020) and previous experiments (data not shown) to achieve inhibition halos higher than 20.0 mm of LmWT, and thus to enhance analysis sensitivity in the study of increased antibiotic resistance in the evolved strains.

LmSTCO only exhibited a significant ($p < 0.05$) decrease of inhibition halos in comparison with LmWT for two antibiotics: ampicillin and cephalexin. Both antibiotics belong to β-lactams, whose main target is the inhibition of cell wall biosynthesis. These results suggest that the key to increased resistance and tolerance to the EO in LmSTCO lies in the cell envelope structure. On the other hand, in LmLTCO, an increase in resistance was observed for several kinds of antibiotics. Kanamycin, tetracycline, novobiocin, trimethoprim and ampicillin caused smaller inhibition halos in LmTCO than in LmWT. This decrease in antibiotic susceptibility could be associated with a general mechanism of defence against antimicrobial compounds. According to Pontes and Groisman (2019), a slow growth rate could lead to antibiotic resistance. It is likely that the delay of LmLTCO growth, previously observed in the absence of TCO, might lead to an increased resistance to a wide range of antimicrobials.

The antibiotic susceptibility tests evidence that strains resistant or tolerant to TCO could also develop a slight increase in cross-resistance to antibiotics. These results indicate that certain of the mechanisms of resistance to EOs could be involved in the response to antibiotics, and that cross-resistance between both types of antimicrobials may occur.

Table 5
Mutations of LmSTCO (strain evolved by cyclic exposure to prolonged sublethal doses of *Thymbra capitata* essential oil) in comparison with *Listeria monocytogenes* EGD-e (LmWT), verified by Sanger sequencing. Single nucleotide variation (SNV).

Genome position	Locus tag	Mutation	Change	Information
1,696,296	lmo1647	SNV: C17A*	Ala6Glu	1-acylglycerol-3-phosphate O acyltransferase (Involved on the membrane phospholipid synthesis in Gram+)

* Position respect to the start of the coding region.

Table 6
Mutations of LmLTCO (strain evolved by cyclic exposure to short lethal treatments of *Thymbra capitata* essential oil) in comparison with *Listeria monocytogenes* EGD-e (LmWT), verified by Sanger sequencing. Single nucleotide variation (SNV), insertion (Ins) and deletion (Del).

Genome position	Locus tag	Mutation	Change	Information
86,323–114,201	lmo0083-lmo0107	Large deletion	25 genes deleted	Table S4
313,664	lmo0289	Del: -A13*	Thr5* Frame shift	Hypothetical protein
479,717	lmo0446-lmo0447	SNV: C by T	Intergenic region	Non-coding DNA / No regulatory region
1,532,296	lmo1503	Ins: +A105*	Gly36* Frame shift	Hypothetical protein
2,339,533	lmo2249-lmo2250	SNV: T by C	Intergenic region	Non-coding DNA / No regulatory region

* Position respect to the start of the coding region.

3.5. Genetic variations responsible for the increased resistance and tolerance to TCO

The identification of mutations of the evolved strains was carried out by comparing the genomes between LmWT (origin strain) LmSTCO, and LmLTCO (Fig. 3) in order to identify the cause of the increased resistance and tolerance to TCO, as well as to antibiotics. For this purpose, WGS was conducted and the reads were mapped onto reference genome: *L. monocytogenes* EGD-e (NCBI accession: NC_003210.1) (Toledo-Arana et al., 2009). Mutations in our LmWT were then identified with regard to the reference strain (Table S3) in order to discard them as the cause of increased resistance and tolerance, since they would also be found in the evolved strains. WGS revealed 1 SNV in LmSTCO (Table 5) and 5 genetic variations in LmLTCO: 2 SNVs, 1 Ins, 1 Del and 1 large deletion (Table 6). All mutations observed in WGS were verified by Sanger sequencing.

The only LmSTCO mutation was found in *plsC* gene (lmo1647), a replacement of a cytosine by an alanine at position 17. This SNV led to a change from alanine to glutamic acid in the enzyme 1-acylglycerol-3-phosphate O acyltransferase, in the amino acid 6. This enzyme, PlsC, is located in the cell membrane and is involved in the biosynthesis of phosphatidic acid, the central precursor of membrane phospholipids, from acyl-ACP (Yao & Rock, 2013). For this reason, PlsC plays an essential role in the synthesis and repair of cell envelopes in Gram-positive bacteria (Geiger, Sohlenkamp, & López-Lara, 2010). Although there are no data on this gene in relation to resistance to natural antimicrobials, several authors have observed its involvement in antibiotic resistance. According to Sutterlin, Zhang, and Silhavy (2014), the overexpression of *plsC* increases phosphatidic acid, and consequently leads to a protective effect against vancomycin. In another study, Li et al. (2015) isolated *Acinetobacter baumanii* strains from evolution assay with tigecycline, and observed that the cause of increased resistance was a mutation in *plsC*. In both studies, the increased resistance occurred against antibiotics targeting the bacterial membrane, as we observed in our study (ampicillin and cephalexin, table 3). Thus, it can be seen that the mutation of the *plsC* gene is responsible for the selection of LmSTCO and highlights the capital importance of the cell envelope in the bacterial defence response to natural antimicrobials.

Regarding LmLTCO, the 2 SNVs we found were located in two intergenic areas: between lmo0446 and lmo0447, and between lmo2249 and lmo2250, respectively. Neither of the two mutations was present in either coding or regulatory zones; thus, their involvement in the increased resistance of LmLTCO strain to TCO can be discarded. WGS also detected 1 insertion of an alanine in lmo0289 and 1 deletion of an alanine in lmo1503. These two genes code hypothetical proteins whose functions have not been evidenced *in vivo*. Further studies would be necessary to describe their function in *L. monocytogenes* EGD-e, and to ascertain their potential role in bacterial defence against TCO and antibiotics. Finally, 1 large deletion was detected in LmLTCO removing an amount of 25 genes from lmo0083 to lmo0107, coding of MerR family transcriptional regulator, oxidoreductase, 6 subunits of ATP synthases, 3 subunits of mannose transporter, 2 transcriptional regulators, NADH oxidase, chitinase B and ABC transporter (Table S4). In this regard, it was observed that most of the deleted genes have a function in carbohydrate and sugar catabolism, as well as in the synthesis of ATP and energy production. Their loss could explain the results obtained in the phenotypic characterization of LmLTCO. The reduction in energy production would explain the growth delay observed in the growth curves both in the absence and in the presence of varying TCO concentrations, as well as the higher generic resistance and tolerance to antibiotics (Græsbøll, Nielsen, Toft, & Christiansen, 2014; Pontes & Groisman, 2019), and probably to TCO. It should be noted that the deletion of the rest of the genes, such as the MerR family regulator related to the response to heavy metals, oxidative stress and antibiotics, or other transcriptional regulators, as well as the other mutations detected, could also mean a variation in the resistance and tolerance of LmLTCO. Moreover, although our evolution assays have resulted in the selection of these resistant strains, it does not mean that every evolution assay will lead to the isolation of the same genetic variants.

3.6. Evolved strains show increased resistance and tolerance to TCO in skimmed milk

A phenotypic characterization of LmSTCO and LmLTCO in a food model was carried out to assess whether these strains could pose a microbiological risk in case they reach the food chain. Their resistance and tolerance of the evolved strain to TCO was determined in skimmed milk as a food matrix because EOs and ICs have been proposed as food preservatives for dairy products due to their antimicrobial, anti-oxidant, and functional properties (Mishra et al., 2020). In this regard, *T. capitata* is one of the most widely studied EOs in milk preservation due to its excellent antimicrobial properties (Ben Jemaa et al., 2017, 2018). Moreover, we chose skimmed milk to avoid the effect of fat on the antimicrobial activity of TCO and, thus, to achieve the greatest sensitivity in our experiment and to ease the observation of the differences in resistance and tolerance between evolved strain and LmWT.

Table 4 shows the MICs and MBCs of TCO for LmWT, LmSTCO, and LmLTCO in skimmed milk. The concentration required to inhibit and to inactivate the growth of the three tested strains was around 4 or 5 times higher than in TSBYE. As in TSBYE, LmWT was the most susceptible strain in skimmed milk, MIC and MBC of 900 μL/L TCO. LmLTCO exhibited the highest MIC and MBC values of TCO: 1200 μL/L and 1300 μL/L, respectively, followed by LmSTCO: 1000 μL/L and 1200 μL/L. Nevertheless, the increase in resistance and tolerance in skimmed milk against TCO was proportionally lower than in TSBYE. It is therefore likely that certain milk components interfere with the antimicrobial activity of EO and/or modify the resistance of LmWT and evolved strains. For the inactivation treatments, the concentration was increased to 1600 μL/L TCO because the concentration previously used in

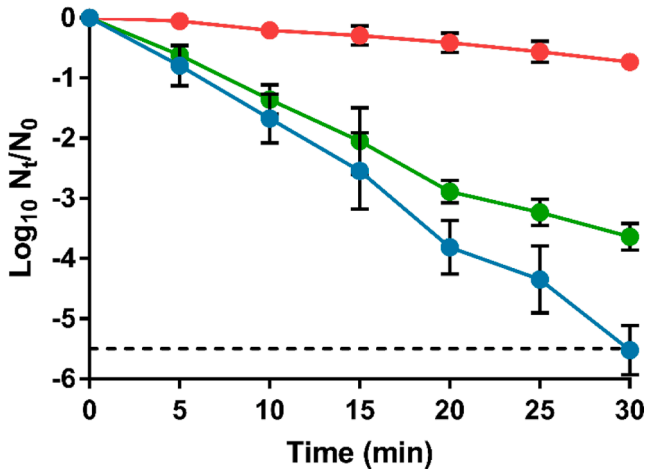

Fig. 4. Survival curves of *Listeria monocytogenes* EGD-e wild type (●; LmWT) and evolved strains: LmSTCO (●; by cyclic exposure to prolonged sublethal doses) and LmLTCO (●; by cyclic exposure to short lethal treatments), after lethal treatment of 1,600 μL/L *Thymbra capitata* essential oil (TCO) in skimmed milk at 25 °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).

McIlvaine buffer was not capable of inactivating any of the 3 strains. As mentioned above, this is due to the protective effect that skimmed milk components may exert. Similarly to lethal treatments in McIlvaine buffer, Fig. 4 revealed a higher survival of evolved strains to TCO inactivation treatments compared to LmWT. LmLTCO showed the highest tolerance: less than 1 $\log_{10}$ cycles of inactivation were achieved after 30 min treatment, whereas LmSTCO and LmWT reached a bacterial reduction of 3.6 and 5.5 $\log_{10}$ cycles, respectively. Several authors highlight natural antimicrobials as an effective preservation method for dairy products; no previous studies have assessed the emergence of resistant bacterial population in dairy food, or evaluated the food safety risk if mutant strains appear. Our results suggest, however, that the contamination of the food chain with these strains could pose a microbiological risk, since the increases in resistance and tolerance to TCO were also observed in food matrices such as skimmed milk.

4. Conclusions

This study evidences the emergence of resistant (LmSTCO) and tolerant (LmLTCO) strains of *L. monocytogenes* EGD-e against a complex essential oil: specifically, *Thymus capitata* (TCO). LmSTCO showed a better adaptation to TCO by decreasing its lag phase when growing in the presence of the antimicrobial. The increased tolerance of LmLTCO allowed it to emerge against wild-type strain LmWT, despite the fact that this had a fitness cost in the presence as well as in the absence of TCO. In addition, both strains developed a slight increased cross-resistance to antibiotics: LmSTCO to β-lactams antibiotics, and LmLTCO to a wide range of broad-spectrum antibiotics. These findings support the relevance of knowing the mechanisms of action of natural antimicrobials as alternative or in combination with antibiotics to combat the multi-drug resistant bacteria.

In LmSTCO, the genomic study identified the mutation in the *plsC* gene, coding an enzyme involved in the biosynthesis of phosphatidic acid, which was responsible for increasing resistance and tolerance to TCO, as well as to β-lactams. A total of 5 genetic changes were found in LmLTCO; among them, the deletion of an ATP synthesis system and energy production which produced a fitness cost and slowed down bacteria growth. This study adds to the knowledge about the mechanism of action of natural antimicrobials; however, it is still necessary to conduct further research with the aim of designing more efficient and safe food preservation strategies.

Our study has likewise shown a large increase in resistance of the evolved strains against TCO compared to the wild-type strain in a food matrix: skimmed milk.

Overall, these results indicate that it would be necessary to consider the emergence of mutant sub-populations in the design of food preservation strategies, since they could represent a microbiological risk due to their ability to grow and survive under conditions established for their corresponding wild-type strains. Further studies are required to understand how resistant strains could appear in the food chain, and to ascertain the real risk to food safety they might represent.

CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110403.

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