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Abstract

Listeria monocytogenes is a psychrotrophic food-borne pathogen mostly associated with consumption of ready-to eat foods. Due to its high prevalence in raw materials, it is fundamental to control its growth at low temperature. In lipid-rich products, fatty acids can be heterogeneously distributed in the food matrix and can be present in the environment immediately surrounding the pathogen.. In this study, we sought to understand the impact of exogenous fatty acids on the growth and membrane physiology of *L. monocytogenes* according to the temperature and strain. We demonstrate that exogenous unsaturated fatty acids promote the growth of *L. monocytogenes* at 5°C but not at 37°C. The level of growth modifications is dependent upon the strain. At 5°C, there is high incorporation of unsaturated fatty acids, which decreases the weighted-average melting temperature of membrane fatty acids allowing *L. monocytogenes* to compensate for the decrease in fluidity caused by the temperature, thus leading to increased growth. In contrast, the incorporation of saturated fatty acids decreases membrane fluidity and prevents growth at 5°C. This study underlines the absolute necessity to understand better the cold adaptation of *L. monocytogenes* in lipid-rich foods in order to adjust their shelf-life and guarantee their microbiological safety.

Keywords

Exogenous fatty acids, growth promotion, cold adaptation, fatty acid incorporation, shelf-life compromising

Abbreviations

FA fatty acids

#FA exogenous fatty acids

FAME fatty acid methyl esters

SFA saturated fatty acids

i-BFA iso-branched fatty acids

ai-BFA anteiso-branched fatty acids

UFA unsaturated fatty acids

SCFA short-chain fatty acids

GC-MS gas chromatography coupled with mass spectrometry

RTE ready-to-eat

WAMT weighted-average melting temperature

1. Introduction

Infectious foodborne diseases remain a real public health issue worldwide. In 2019, more than 320,000 human cases due to zoonoses were reported in Europe (EFSA and CDC, 2021). Among them, *L. monocytogenes* caused 2,621 invasive human cases which confirms a stable trend since 2015 after a long period of increasing trend. Invasive listeriosis causes the highest rate of case fatality (17.6%) compared to other zoonoses (EFSA and CDC, 2021). The ubiquitous nature of *L. monocytogenes* leads to its high prevalence in many raw materials and its persistence in the whole food chain. Consequently, its prevalence in ready-to-eat (RTE) foods is also high at the processing stage, in particular for fish production (5.8%) (EFSA and CDC, 2021). Ready-to-eat (RTE) foods are highly concerned because they are typically stored at low temperature which allows the growth of *L. monocytogenes* and they don't receive any subsequent treatment that could inactivate it before consumption. Regulation (EC) No. 2073/2005 defines a limit at the retail stage of 100 CFU/g (n=5, c=0) for RTE foods that are able to support the growth of *L. monocytogenes*. Nevertheless, more than 90% of listeriosis cases were caused by ingestion of RTE foods containing more than 2,000 CFU/g, which is significantly above this regulation limit (Ricci et al., 2018).

Therefore, control of product safety regarding listeriosis risk mainly lies in control of pathogen growth at low temperature. When microorganisms are exposed to temperatures out of their "comfort zone" they have to adapt to counteract the effects of temperature on their cellular components (Booth, 2002). The cold adaptation response is a universal bacterial mechanism, which has been particularly studied in psychrotrophic bacteria such as *B. cereus* and *L. monocytogenes* (Alvarez-Ordóñez et al., 2015). Loss of membrane fluidity is the first consequence faced by cells when the temperature decreases. At a given temperature, membrane fluidity and permeability depend on lipid acyl chain composition (Parsons and Rock, 2013). To sustain optimum membrane fluidity and survive in harsh environments such

as sub-optimal temperatures or in the presence of toxic compounds, bacterial cells can alter the acyl chain structure of membrane glycerophospholipids depending on their biosynthesis pathway systems. Several adaptations can be made, such as changing the ratios of: 1) saturated (SFA) versus unsaturated fatty acids (UFA), 2) *cis* to *trans* UFA, 3) branched (BFA) versus non-branched FA, changing 4) acyl chain length, and 5) synthesizing cyclopropane FA from UFA (Denich et al., 2003). At low temperature, *L. monocytogenes* adapts its membrane composition by increasing the ratio of *anteiso*/*iso* branched chain fatty acids (*ai*-BFA / *i*-BFA) and/or by shortening the FA chain length, both of which counterbalance the physical loss of fluidity (Julotok et al., 2010; Mastronicolis et al., 2005, 1998; Neunlist et al., 2005).

Beside this intrinsic bacterial ability to adapt to harsh environments, several compounds in the environment could help bacteria to overcome stressful conditions. For instance, osmo-protective compounds present in food products, namely betaine in vegetables and carnitine in meats, are taken up by *L. monocytogenes* and favor its growth at 4°C (Tasara and Stephan, 2006). Moreover, several studies have shown that numerous bacteria could use exogenous FA from the environment to adapt their membrane composition (Ando et al., 1992; Brinster et al., 2010, 2009). In particular, *B. cereus* was shown to insert fatty acids from spinach in its membrane at low temperature under anaerobiosis and thus to recover the same maximal population density as at optimal temperature (de Sarrau et al., 2013). Here, we studied the impact of different types of FA (UFA, SFA) available in the environment on the growth of *L. monocytogenes* and on its membrane physiology according to temperature and strain.

2. Materials and methods

2.1. Chemicals

Myristic acid (C14), palmitic acid (C16), oleic acid (C18:1cis9), linoleic acid (C18:2w6) and linolenic acid (C18:3w6) were from Larodan Fine Chemicals (Malmö, Sweden). Solutions were obtained by dispersion of the specific FA in a bovine serum albumin (BSA) solution in phosphate buffer pH 8. The final concentrations in the culture medium were 0.045 mM FA in 0.05% BSA.

2.2. Bacterial strains and storage

The strains of *Listeria monocytogenes* used in this study were EGD-e (Murray et al., 1926), CNL895805 (Van Langendonck et al., 1998) and seven other strains isolated from foods (Table 1). The full names of the strains (e.g. Lm_b3d_208) were shorten in the text (e.g. Lm208) to simplify the reading. Stock cultures of *L. monocytogenes* were kept in Tryptone Soya Broth (TSB) (BioMérieux, Marcy l'Etoile, France) supplemented with 20% (v/v) glycerol at −80 °C.

Table 1: *L. monocytogenes* strains used in this study

Strain code	Origin	Serotype	CC
EGDe	Guinea pig	1/2a	CC9
Lm_ b3d_208 (CNL 895805)	Sheep's brain	1/2a	CC7
Lm_ b3d_226	Milk	4	CC1
Lm_ b3d_540	Ham sandwich	1/2a	-
Lm_ b3d_541	Eggs & tuna sandwich	1/2a	CC121
Lm_ b3d_543	Raw salmon	1/2a	CC121
Lm_ b3d_551	Duck foie gras	1/2c	CC9
Lm_ b3d_553	Smoked salmon	3a	CC121
Lm_ b3d_554	Slices of bacon	3a	CC121
Lm_ b3d_562	Raw sheep's milk	-	CC19

2.3. Culture conditions

The strains were inoculated in TSB at 1% v/v with a standardized inoculum (~10^8 CFU.mL^{-1}) obtained after two subcultures at 30°C in the same broth. Cultures were then grown in 100-well microplates (Bioscreen C, Labsystems, Helsinki, Finland) or 20 mL flasks. When indicated, the medium was supplemented by a specific exogenous FA (#FA) solution. Bacterial growth was followed for 48 h at 37°C, 6 days at 8°C and 15 days at 5°C by measuring the optical density (OD) at 600 nm in microplates in an automatic spectrophotometer (Bioscreen C, Labsystems, Helsinki, Finland) or discontinuously in 20 mL flasks (spectrophotometer Genesis 30, Thermo Fisher Scientific, Waltham, USA). The addition of 0.05% BSA in TSB was shown to have no significant effect on the growth rate of *L. monocytogenes* at 5°C (0.045±0.004 h^{-1} versus 0.042±0.01 h^{-1}).

2.4. Determination of the growth rate

Maximum specific growth rates (μ_{max}) of *L. monocytogenes* were estimated from the OD growth kinetics by fitting the modified Gompertz model (Guillier et al., 2007; Pernin et al., 2018) using the complementary macro SolverAid (de Levie, 2012).

2.5. Shelf-life alteration in the presence of exogenous FA

Growth simulations were performed to evaluate the impact of the presence of exogenous FA on shelf-life, which is here considered to be the time when the population reaches 100 *L. monocytogenes*/g. Five thousand values of growth rates were calculated with the statistical function NORMINV(RAND(),M,SD) which was used to return the inverse of the normal distribution from the experimentally determined mean (M) and standard deviation (SD) of μ_{max} for each condition (see 2.4). The lag time was calculated from each of the 5,000 growth

rate values with the following relation by assuming that the physiological state (K) is at the average value of 2.5 (Augustin et al., 2011):

$K = \mu_{max} . lag$ (1)

Five thousand simulations of growth curves were then performed for each condition using the Gompertz growth model with the following parameters: the growth rate (μ_{max}), the lag time (*lag*), the initial contamination (N_0) and the final population (N_f). The initial and final populations were respectively set at 1 and 10^9 CFU/g. The mean and standard deviation of the shelf-life were calculated from the 5,000 growth simulations in each condition.

2.6. Membrane fatty acid analysis

Bacterial cultures grown as described above were harvested by centrifugation (7,000 g, 20°C) in the early stationary phase according to OD growth curves. Pellets were washed twice with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) (Brinster et al, 2009). Extraction and methylation of FA were carried out directly on bacterial pellets as previously described (Dubois-Brissonnet et al., 2016; Méchin et al., 1999). FA of whole cells were first saponified and esterified by methanolic NaOH and methanolic HCl (1^{st} step: 1 mL NaOH 3.75 M in 50% v/v methanol solution for 30 min at 100°C; 2^{nd} step addition of 2 mL HCl 3.25 M in 45% v/v methanol solution for 10 min at 80°C). Fatty acid methyl esters (FAME) were extracted with a diethyl ether / cyclohexane solution (1:1 v/v). The organic phase was then washed with a dilute base (NaOH 0.3 M). Analytical gas chromatography of FAME was carried out on a GC-MS Trace 1300 / ISQ 7000 (Thermo Fisher Scientific, Waltham, USA) equipped with a BPX70 capillary column (25m, 0.22 mm id) (SGE, Victoria, Australia). Column temperature was set at 100°C for 1 min and then increased to 170°C at the rate of 2°C/min.

FAME were expressed as a percentage of the total area and grouped in classes: saturated fatty acids (SFA), unsaturated fatty acids (UFA), *iso* and *anteiso* branched-chain fatty acids (*i*-BFA

and *ai*-BFA). Additional indicators of membrane adaptation were also calculated, namely short-chain fatty acid (< C15) contents (SCFA) and weighted-average melting temperature (WAMT). The latter is calculated from the melting temperature of each individual FA and its ratio in the membrane composition (Seel et al., 2018).

$$\text{WAMT (°C)} = \sum_{n}^{1} T_i . FA\ (\%)_i \quad (2)$$

where T_i (°C) is the melting temperature of FA_i, $FA(\%)_i$ is the percentage of FA_i, and FA_1 to FA_n are each of the FA present in the membrane composition. Melting temperatures of FA (°C) were obtained from CAS Common Chemistry (https://commonchemistry.cas.org/).

2.7. Statistics

The number of replicates is specified in the legend of each figure. All experiments were conducted at least in triplicate with independent subcultures. Statistical analyses were performed using GraphPad Software 9.2.0 (Prism, USA). Two-tailed paired Student's t-tests were performed between #C18:1 and control for each strain in Figure 1. One-way ANOVA with Dunnett's multiple comparison tests (95% confidence interval) were performed between the different conditions and the control for each strain and temperature in Figures 2, 4, 5 and 6. According to the *p*-value, results are reported as non-significant (ns) or significantly different * if $p<0.0332$; ** if $p<0.0021$; *** if $p<0.0002$; **** if $p<0.0001$.

3. Results

3.1. The medium supplementation with oleic acid promotes the growth of several strains of L. monocytogenes at 8°C but not at the same level

We first aimed to study the growth of nine different strains of *L. monocytogenes* at 8°C in TSB supplemented or not with oleic acid (#C18:1). The growth rates at 8°C without supplementation (control) varied among the different strains, ranging from 0.060±0.03 h^{-1} to 0.088±0.005 h^{-1}. Except for EGD-e, the growth rate of *L. monocytogenes* in #C18:1 medium was always significantly higher than that of the control. The ratio μ_{max} (#C18:1) / μ_{max} (control) was ranging from 1.1± 0.09 h^{-1} for EGD-e to 1.3±0.29 h^{-1} for Lm208 (Figure 1). EGD-e and Lm208, which respectively showed the lowest and highest increase of growth rate in #C18:1 medium, were chosen for further experiments.

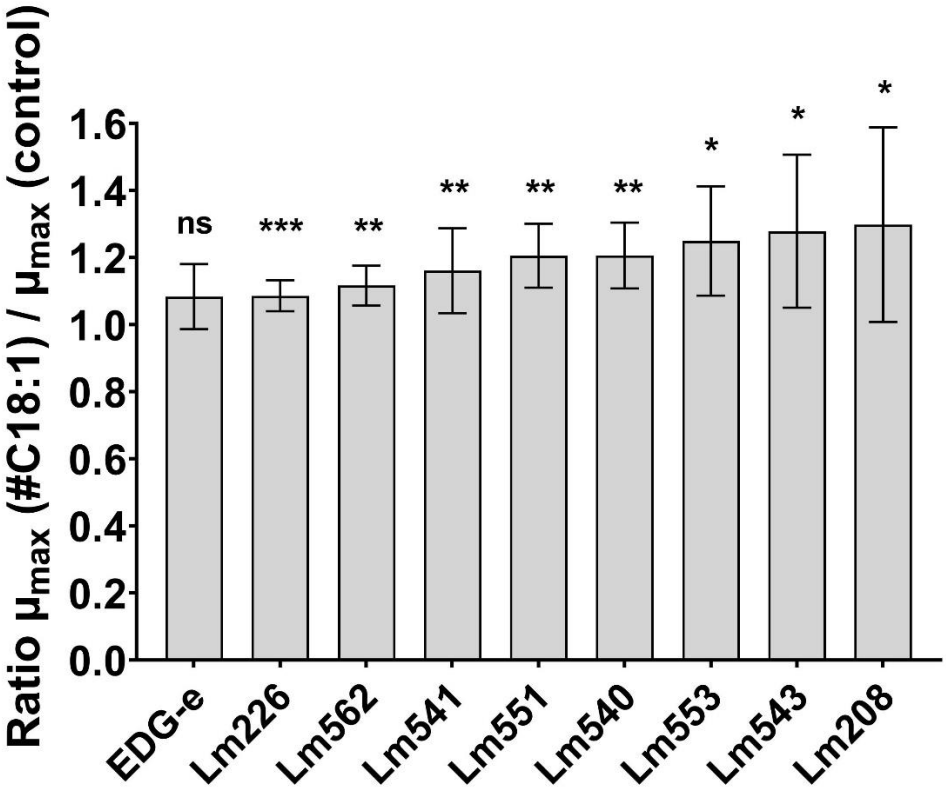

Figure 1: Ratios of growth rates at 8°C of nine different strains of *L. monocytogenes* in TSB with C18:1 supplementation or without [Ratio = μmax (#C18:1) / μmax (control)]. Mean and standard deviation (n=6) are represented. Growth rates in both conditions were compared for each strain by two-tailed paired Student's t-tests (95% confidence interval).

3.2. The level of growth promotion depends on the type of exogenous fatty acid and on temperature

The growth of strains EGD-e and Lm208 was followed at 5°C or at 37°C in medium supplemented with different SFA, namely myristic acid (#C14) or palmitic acid (#C16) or different UFA, namely oleic acid (#C18:1), linoleic acid (#C18:2) or linolenic acid (#C18:3). Stearic acid (C18) was not used in this study because of the lack of efficiency in dispersing this FA in BSA solution. At 5°C, the growth rates in control conditions for EGD-e and Lm208 were similar, being respectively 0.044±0.013 h^{-1} and 0.044±0.008 h^{-1} (Figures 2A and 2C). With UFA supplementation, the growth rate of Lm208 significantly increased and the ratio μ_{max} (#UFA) / μ_{max} (control) was above 1.4 (Figure 2A). Supplementation with #C18:1 increased EGD-e growth rate slightly, but not significantly, as previously observed at 8°C (see 3.1). Nevertheless, #C18:2 or #C18:3 supplementations led to a significant increase of EGD-e growth rate at 5°C (Figure 2C).

Supplementation with SFA led to a significant decrease of the growth rate at 5°C for Lm208 and the ratio μ_{max} (#UFA) / μ_{max} (control) was 0.7 for #C14 and 0.6 for #C16 (Figure 2A). A slight but non-significant decrease was also observed in EGD-e growth rate with these supplementations (Figure 2C).

We analyzed the impact of temperature by following the growth of both strains in the same supplemented media at 37°C. The growth rates in control conditions for Lm208 and EGD-e were respectively 0.961±0.091 h^{-1} and 1.015±0.016 h^{-1} (Figures 2B and 2D). Whatever the supplementation and strain, no significant differences were recorded in growth rates at 37°C, except for a significant decrease observed for the #C16 EGD-e culture (Figure 2D).

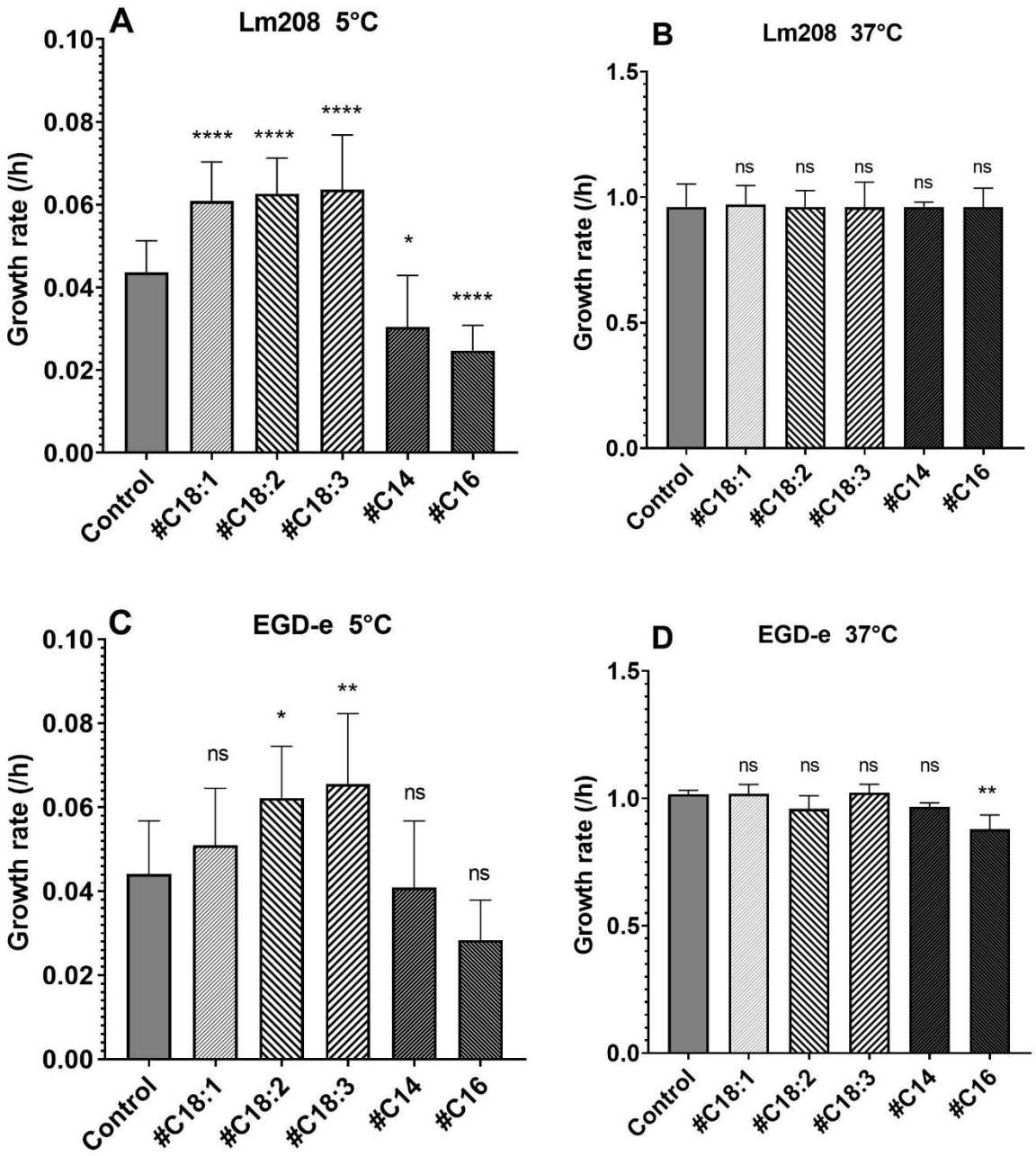

Figure 2: Growth rates at 5°C (A and C) and 37°C (B and D) of *L. monocytogenes* Lm208 (A and B) and EGD-e (C and D) with # C18:1, #C18:2, #C18:3, #C14, or #C16 supplementation or without (control). Mean and standard deviation (n=6 to 18) are represented. Growth rates were analyzed in comparison with the control by Dunnett's one-way ANOVA (95% confidence interval).

3.*3. Shelf-life predictions under FA supplementations*

The mean of the 5,000 growth simulations performed from the experimental values of growth rates in cultures without supplementation (Control) or with supplementations are plotted in Figure 3A. The mean and standard deviation of the shelf-life, considered as the time when the population reaches 100 *L. monocytogenes*/g, is represented in Figure 3B. Without supplementation, the population reached 100 CFU/g in 6.9 ± 1.3 days. The shelf-life is significantly lower in #UFA (4.9 ± 0.8 days in #C18:1, 4.8 ± 0.7 days #C18:2 and #C18:3) and higher in #SFA (12.3 ± 8.2 days in #C14 and 12.7 ± 4.1 days in #C16) than in control cultures (Figure 3B).

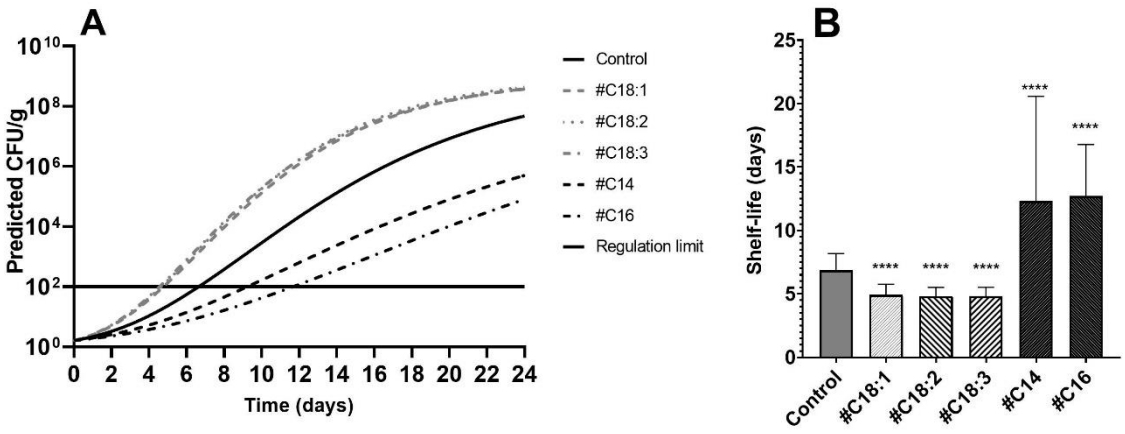

Figure 3: Growth simulations of CFU/g over time (A) and shelf-life (B) in TSB at 5°C with supplementations or without (Control). Mean of growth curves and mean and standard deviation of shelf-life are presented. 5,000 simulations were processed considering that N_0 was 1 CFU/g, K was 2.5 and the growth rate follows a normal distribution around the experimentally determined mean and standard deviation of μ_{max} for each condition. Shelf-lives were analyzed in comparison with the control by Dunnett's one-way ANOVA (95% confidence interval).

3.4. *The level of incorporation of exogenous FA by L. monocytogenes depends upon the strain, temperature and type of FA*

The FA composition of *L. monocytogenes* mainly consists of BFA with *i*-BFA (*i*-C14, *i*-C15, *i*-C16, *i*-C17) and *ai*-BFA (*ai*-C13, *ai*-C15, *ai*-C17) (Figure 4). The three most abundant BFA in both strains at both temperatures are *ai*-C15, *i*-C15 and *ai*-C17. At 37°C in control conditions, BFA represent 84% of the membrane FA of Lm208, SFA (C12, C14, C15, C16, C18) represent 13% of the composition and UFA (*cis9* C18:1) 3% (Figure 4B). At 5°C, total BFA and SFA respectively decreased to 78.1 % and 12.6% and UFA increased to 9.3% (Figure 4A).

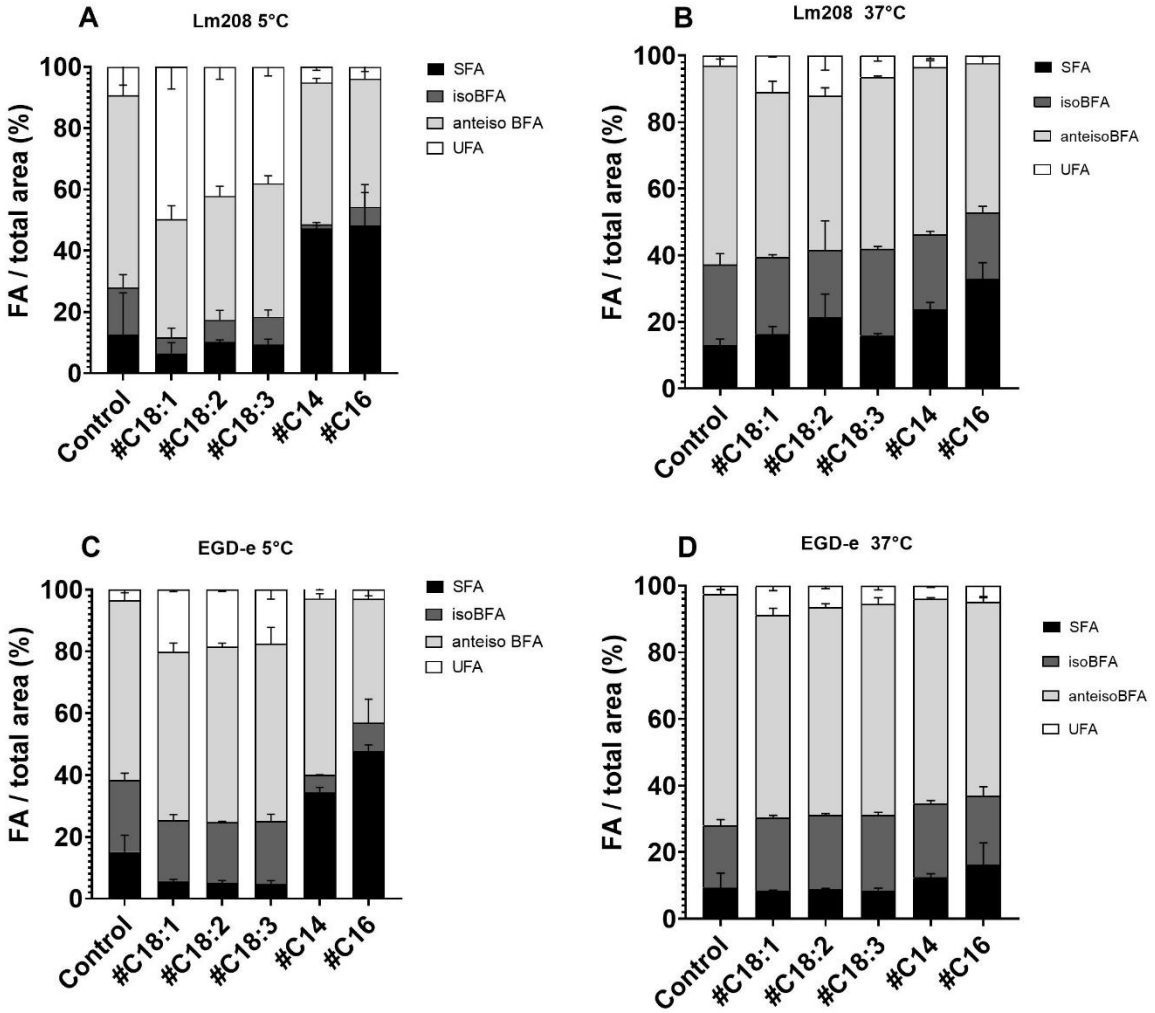

Figure 4: Fatty acid composition expressed in 4 FA categories (SFA, *i*-BFA, *ai*-BFA and UFA) of *L. monocytogenes* Lm208 (A and C) and EGD-e (B and D) at 5°C (A and B) and 37°C (C and D) with # C18:1, #C18:2, #C18:3, #C14, or #C16 supplementation or without (control). Mean and standard deviation (n=3 to 6) are represented.

Medium supplementation with exogenous FA induced modifications in membrane FA composition, but differently according to the strain, temperature and type of FA. In the case of #UFA supplementations at 37°C, UFA in Lm208 membrane composition was respectively 11%, 12% and 10.8% for #C18:1, #C18:2 and #C18:3 in comparison with 3% in the control (Figure 4B). At 5°C, levels of UFA were much higher in the membrane, reaching respectively 49.7%, 42.1% and 38.1% for #C18:1, #C18:2 and #C18:3 in comparison with 9% in the control (Figure 4A). Moreover, the supplementation with C18:2 or C18:3 induced a significant increase in C18:1 together with the supplemented FA at 5°C (Figure 5A). At both temperatures, Lm208 membrane composition in SFA was not significantly different between #UFA cultures and control (Figure 4 A and B). With #SFA supplementation, the level of SFA in Lm208 membrane significantly increased. In #C14, the membrane contained 17.1% of C14 versus 4.6% in the control at 37°C and 44% versus 1% at 5°C (Figure 4A et 4B). Similarly, in #C16 cultures, it contained 30.3% of C16 versus 6.2% in the control at 37°C and 45.9% versus 2.1% at 5°C. In contrast, UFA were not significantly different from the control in #SFA cultures (Figure 5A). Short-chain FA (below C15) of Lm208 were also impacted by these FA supplementations (Figure 5B). In the control, SCFA increased at 5°C compared to 37°C. At 5°C in UFA and C16 supplemented cultures, SCFA significantly decreased in comparison to the control. C14 supplementation induced no significant change even though C14 belongs to SCFA, which suggests a decrease in de *novo* synthesized SCFA (Figure 5B). The WAMT of Lm208 membrane FA was lower at 5°C than at 37°C in control cultures (Figure 6A). The WAMT in #UFA cultures was significantly lower to that in the control at

5°C, whereas it was similar at 37°C. It was significantly higher in #SFA cultures at both temperatures (Figure 6A).

The EGD-e strain behaved the same way as Lm208 even though #UFA incorporation was less effective at both temperatures, but especially at 5°C (Figures 4 C and D). The same trends for WAMT adaptation were observed with EGD-e as with Lm208, but to a lesser extent (Figure 6B).

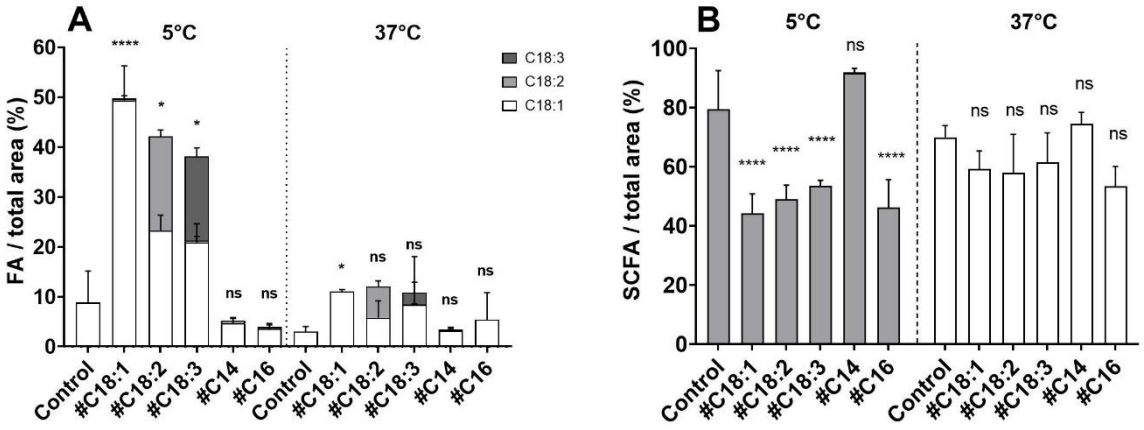

Figure 5: Fatty acid composition in unsaturated FA (C18:1, C18:2 and C18:3) (A) and in short-chain FA (C<15) (B) at 5°C and 37°C of *L. monocytogenes* Lm208 with # C18:1, #C18:2, #C18:3, #C14, or #C16 supplementation or without (control). Mean and standard deviation (n=3 to 6) are represented. FA were analyzed in comparison with the control by Dunnett's one-way ANOVA (95% confidence interval).

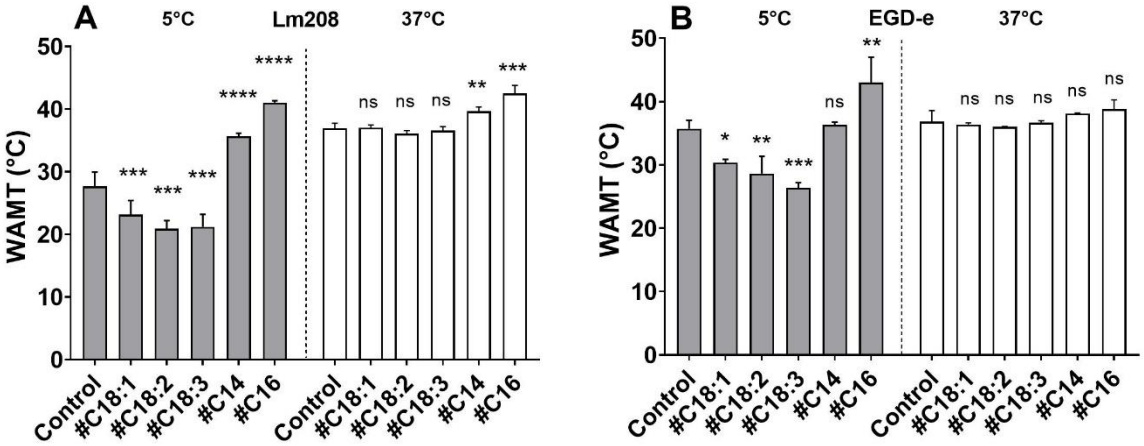

Figure 6: Weighted-average melting temperature of the membrane fatty acids of *L. monocytogenes* Lm208 (A) and EGD-e (B) at 5°C and 37°C with # C18:1, #C18:2, #C18:3, #C14, or #C16 supplementation or without (control). Mean and standard deviation (n=3 to 6) are represented. WATM were analyzed in comparison with the control by Dunnett's one-way ANOVA (95% confidence interval).

4. Discussion

In RTE foods, food safety is mainly controlled by preventing *L. monocytogenes* from growing at low temperature and from reaching 100 CFU/g at the end of the shelf-life. Food matrices are heterogeneous systems where microorganisms encounter several types of microstructures and gradients of available resources or inhibitors (Brocklehurst et al., 1995; Malakar et al., 2000). In lipid-rich foods, in particular, available FA could be heterogeneously distributed and unequally available for bacterial contaminants. Thus, we explored in this study the impact of different types of exogenous FA on *L. monocytogenes* growth and membrane adaptation at low temperature.

First, nine strains isolated from different food sources were cultivated in the presence or absence of oleic acid at 8°C. This UFA is in high proportion in many foods products including vegetable oils, such as olive (76% of total FA), peanut (48.7% of total FA) and sunflower (15.3% of total FA) oils, which are commonly used in salad sauces or other RTE preparations (Zambiazi et al., 2007). Compared to the other strains, Lm540, Lm553 and Lm208 showed the greatest increase of growth rate in the presence of oleic acid. These strains were respectively isolated from raw or smoked salmons and from sheep brain. We hypothesize that these three strains are habituated to lipid-rich media containing UFA,

318 respectively salmon (about 30.5% of UFA) (Horn et al., 2018) and sheep brain phospholipids
319 (more than 37% UFA) (Palmer et al., 1985).
320 Thereafter, we aimed to evaluate the impact of several UFA and SFA on the growth of
321 Lm208 and EGD-e at 5°C or 37°C. We demonstrated that UFA supplementations generally
322 induced a significant increase of growth rate at 5°C in contrast to SFA supplementations. Free
323 SFA or UFA are both generally known as antimicrobial agents. Straight-chain FA inhibit the
324 growth of *S. aureus* and other Gram-positive bacteria, lauric acid being the most active FA
325 (Kabara et al., 1972; Kelsey et al., 2006). UFA, including C16:1, C18:1, C18: and C18:3, also
326 show antibacterial activity (Kabara et al., 1972), which could be attributed to the ability of
327 these molecules to interact with the membrane, to create pores and to affect the production of
328 energy (Desbois and Smith, 2010). Nevertheless, scarce studies have described a positive
329 impact of FA on bacterial growth, in particular at low temperature. The addition of the apolar
330 phase of spinach to *B. cereus* cultures allows bacterial growth at low temperature without
331 oxygen (de Sarrau et al., 2013). Addition of Tween 80 (containing esterified oleic acid) to a
332 growth medium composed of cheddar cheese extract results in a significant increase in the
333 final cell density of *Lactobacillus casei* at 8°C (Tan et al., 2012). Moreover, a very recent
334 study shows that, similarly to our results, C18:1 and C18 supplementations, by Polysorbate 80
335 and Polysorbate 60, respectively increase and decrease the growth of *L. monocytogenes* at low
336 temperature (6°C) (Flegler et al., 2022). Unlike what occurred at low temperature, we found
337 no significant modifications of bacterial growth rate at 37°C, whatever the supplementation
338 (UFA or SFA). Similarly, growth patterns of *E. coli* in minimal media supplemented with
339 exogenous UFA at 37°C are similar to the control patterns (Herndon et al., 2020). In contrast,
340 supplementation with Polysorbate 80 or Polysorbate 60 results in little increase in the growth
341 rate of *L. monocytogenes* at 37°C (Flegler et al., 2022).

342 The increased growth rate we have demonstrated with UFA supplementation can compromise
343 shelf-life. According to growth simulations, two days of shelf-life are lost when UFA are
344 available in the growth environment. UFA are present in numerous food matrices, in
345 particular fish, such as salmon, and sauces containing vegetable oils. Moreover, the FA profile
346 of food products tends to be modified nowadays in favor of UFA at the expense of SFA, so as
347 to respect nutritional recommendations (INRA and Anses, 2013). FA in complex foods
348 generally occur in esterified forms such as surfactants (Polysorbates), phospholipids, or
349 triglycerides but plant, animal or bacterial lipases can partially break them down into free FA.
350 Polysorbates, phospholipids or free FA can be dispersed in the aqueous phase, but not
351 triglycerides which are trapped in emulsions. According to their localization and to their
352 ability to get in contact with bacteria, lipids and FA can potentially have different levels of
353 impact on *L. monocytogenes* growth. Studies investigating the influence of lipid content on
354 the growth parameters of *L. monocytogenes* in complex foods are scarce. Most have been
355 conducted in model emulsified systems, in which oil droplets structure the medium from
356 liquid to solid according to the percentage of fat and where most of the lipids are inside
357 droplets and thus are not in contact with the bacteria. In this type of emulsion, the presence of
358 fat droplets was shown to increase the μ_{max} of *L. monocytogenes* at 4°C, though the authors
359 could not explain why (Verheyen et al., 2018). We can hypothesize that some free UFA could
360 be dispersed in the aqueous phase during the emulsifying process and could impact the
361 growth of *L. monocytogenes*.
362 In order to further understand the impact of UFA on the growth of *L. monocytogenes*, we
363 explored bacterial membrane FA composition. The membrane of the two strains of
364 *L. monocytogenes* is composed of SFA, BFA and UFA, as already shown in several studies
365 (Annous et al., 1997; Rogiers et al., 2017). Despite UFA synthesis is not described in
366 *L. monocytogenes*, many studies found them in small proportions in its membrane when

grown without UFA supplementation (Bisbiroulas et al., 2011; Hingston et al., 2017; Mastronicolis et al., 2010; Püttmann et al., 1993). We could hypothesize that C18:1 could be incorporated from trace C18:1 present in the culture medium, even though we could not detect any UFA in the medium alone (data not shown). Another hypothesis is that the membrane UFA could be converted from corresponding SFA by a desaturase. *B. cereus* produces two acyl-lipid desaturases, DesA and DesB, creating double bonds in the FA chain in positions 5 and 10, respectively (Alvarez-Ordóñez et al., 2015). Hingston et al. (2017) suggest that a desaturate-like system exists in *L. monocytogenes* that could convert C16:0 to C16:1 and C18:0 to C18:1. They made this assertion based on the fact that UFA levels in the membrane decreased when *L. monocytogenes* was treated with a desaturase inhibitor (Vadyvaloo et al., 2002).

Low temperature induces modifications in FA composition of *L. monocytogenes* such as the shortening of FA length, the switching of branching from *i*- to *ai*-BFA and the increase in UFA. These well-known membrane modifications tend to counterbalance the physical reduction of membrane fluidity due to low temperature by synthesizing FA with lower melting point temperatures (Annous et al., 1997; Hingston et al., 2017).

When both studied strains of *L. monocytogenes* were cultivated in FA-supplemented medium, their membrane composition was impacted by the incorporation of exogenous FA. Consistent with our study, it was shown that exogenous FA are incorporated in the membrane phospholipids of *L. monocytogenes* (Flegler et al., 2022). This FA incorporation is concomitant with a decrease of SFA and BFA synthesis which could save bacterial energy. FA incorporation was here shown to be strain- and temperature-dependent (higher in Lm208 than in EGD-e and higher at 5°C than at 37°C), but not selective regarding the type of FA. Indeed, every exogenous FA that we made available in the medium was incorporated. Nevertheless, the consequences of this incorporation are different according to the type of FA.

When UFA are available in the medium at low temperature, their great uptake dramatically decreases the WAMT of the membrane FA. Although the melting temperature of the cytoplasmic membrane also depends on the total polar lipid structure, this parameter can be considered as an indicator for membrane fluidity (Seel et al., 2018). The decrease of WAMT due to UFA incorporation probably allows *L. monocytogenes* to preserve its membrane fluidity despite the low temperature and thus to grow faster than the control. Similarly, it was hypothesized that the incorporation of environmental FA into the membrane of *L. casei* favors bacterial growth by optimizing membrane fluidity while saving energy (Tan et al., 2012). Due to their high melting temperature, exogenous SFA increase the WAMT of the membrane FA when incorporated. Despite the energy saving of FA synthesis, the higher WAMT of the membrane leads to a more rigid membrane which prevents an optimal exchange of metabolites and thus decreases the growth rate. The mechanisms of FA incorporation in the membrane phospholipids of *L. monocytogenes* should be explored. In Gram-positive bacteria, exogenous FA are bound by the FA binding protein, FakB, phosphorylated by the FA kinase, FakA, and the resulting acyl-phosphate is converted into acyl-ACP by an acyltransferase PlsX (Yao and Rock, 2017). Further investigation at genomic and transcriptomic levels is needed to understand why EGD-e have lower rates of incorporation of UFA which are concomitant with lower growth increase compared to Lm208, and to decipher the whole mechanisms of FA incorporation in *L. monocytogenes* at a molecular level.

5. Conclusion

In this study, we show that various FA are highly incorporated by the membrane of *L. monocytogenes* at low temperature, probably because this is a way to save energy used in FA synthesis. Nevertheless, UFA and SFA incorporations have opposite effects on

L. monocytogenes growth at low temperature. UFA supplementation induces a decrease in phospholipid melting point temperature and promotes growth of the pathogen. In contrast, SFA supplementation inhibits bacterial growth. These behaviors could have fundamental consequences in terms of food safety and shelf-life definition. In lipid-rich food products, where the distribution of FA is not homogeneous, the composition of the microenvironment immediately surrounding the bacteria should be considered to predict pathogen growth. More generally, models of predictive microbiology should consider the heterogeneity of bacterial behavior in relationship to food composition heterogeneity.

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