Applied Microbiology and Biotechnology (2019) 103:6867–6883
https://doi.org/10.1007/s00253-019-10002-1

MINI-REVIEW

Factors influencing the membrane fluidity and the impact on production of lactic acid bacteria starters

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Received: 19 April 2019 / Revised: 25 June 2019 / Accepted: 27 June 2019 / Published online: 12 July 2019

Abstract

Production of lactic acid bacteria starters for manufacturing food, probiotic, and chemical products requires the application of successive steps: fermentation, concentration, stabilization, and storage. Despite process optimization, losses of bacterial viability and functional activities are observed after stabilization and storage steps due to cell exposure to environmental stresses (thermal, osmotic, mechanical, and oxidative). Bacterial membrane is the primary target for injury and its damage is highly dependent on its physical properties and lipid organization. Membrane fluidity is a key property for maintaining cell functionality, and depends on lipid composition and cell environment. Extensive evidence has been reported on changes in membrane fatty acyl chains when modifying fermentation conditions. However, a deep characterization of membrane physical properties and their evolution following production processes is scarcely reported. Therefore, the aims of this mini-review are (i) to define the membrane fluidity and the methods used to assess it and (ii) to summarize the effect of environmental conditions on membrane fluidity and the resulting impact on the resistance of lactic acid bacteria to the stabilization processes. This will make it possible to highlight existing gaps of knowledge and opens up novel approaches for future investigations.

Keywords Fluorescence anisotropy · Lipid phase transition · Preservation processes · Environmental stress

Introduction

Lactic acid bacteria (LAB) are of great importance for the food industry because of their role in the manufacture of fermented meat, vegetables, fruit, and dairy products. The market of concentrated LAB cultures (starters) is continuously growing due to the development of health benefit products and green chemistry applications. However, the most promising bacteria will have no commercial value if the long-term stability of the target functional properties (acidifying activity, production of aroma compounds and texturizing agents, probiotic activity,...) is not ensured up to their final use (fermentation of food, direct ingestion of probiotics). The commercialization of LAB requires the application of successive processes including fermentation, concentration, stabilization, and storage, for delivering LAB under the form of ready-to-use, highly concentrated, and stable starters to food companies or to consumers. Stabilization strategies are based on the decrease of water activity to inhibit or strongly slow down degradation reactions. Freezing and freeze-drying are the stabilization processes most commonly used since they allow to maximize the technological properties and shelf-life of LAB cells (Fonseca et al. 2015; Béal and Fonseca 2015).

During the stabilization process, bacteria are exposed to several environmental changes, such as change in temperature, solute concentration, and hydration level (Fonseca et al. 2006; Santivarangkna et al. 2008; Fonseca et al. 2015). Cells will thus exhibit passive responses to these environmental changes and membrane fluidity will play a key role in the cellular response (Beney and Gervais 2001; Santivarangkna et al. 2008). Membranes of LAB are mainly composed of phospholipids forming a lamellar lipid bilayer with embedded proteins. Membrane fluidity characterizes the dynamics of lipids within the bilayer, and is thus the inverse of membrane viscosity

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(Denich et al. 2003). To maintain cell integrity while ensuring the main functions of cell membrane (regulating the transport and diffusion of biological substances), an optimal value of cell membrane viscosity of 0.1 Pa.s (100 times higher than water viscosity) is reported in literature. This value corresponds to a 90% glycerol solution (Schechter 2004). Membrane fluidity is thus dependent on temperature; decreasing temperature results in increasing viscosity and in a more rigid membrane. During freezing, bacterial cells are exposed to cold and osmotic stresses, resulting in membrane stiffening, and cell dehydration and volume reduction, due to the cryoconcentration of the extracellular medium (Dumont et al. 2004; Gautier et al. 2013; Fonseca et al. 2016). These events can induce membrane damage, such as membrane leakage or loss of integrity, whose degree will be dependent on membrane fluidity. The fermentation conditions and the resulting membrane lipid composition are commonly related to the resistance of LAB to stabilization processes. Even it is well admitted that membrane fluidity is governed by the fatty acid composition of the membrane, few works report direct assessment of membrane fluidity after fermentation (Velly et al. 2015; Bouix and Ghorbal 2017) and following stabilization process (Schwab et al. 2007; Passot et al. 2014; Meneghel et al. 2017b).

This review is structured in four sections aiming at (i) summarizing the lipid membrane composition of LAB and its contribution to membrane fluidity; (ii) reviewing the progress on methods for characterizing membrane fluidity; (iii) overviewing the influence of environmental conditions during production and stabilization processes on membrane fluidity and the consequences on LAB resistance; (iv) sharing future prospects for LAB research.

Contribution of lipid composition to LAB's membrane organization and fluidity

The principal types of lipids involved in LAB membrane are polar phospholipids, although other polar glycolipids and neutral lipids can contribute to membrane organization (Drucker et al. 1995; Gómez-Zavaglia et al. 2000). Phospholipids (PLs) contain both a hydrophilic region including a phosphate group, and a hydrophobic region including two acyl chains. The acyl chains of fatty acids are mainly composed of an even number of carbons, from 12 to 22, involving no, one, or two unsaturations (C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C20:0, C22:0). Fatty acids (FA) of 16 and 18 carbons account for more than 60% of total FA of LAB membranes (Johnsson et al. 1995; Gómez-Zavaglia et al. 2000; Wang et al. 2005; Li et al. 2009a; Broadbent et al. 2010; Gautier et al. 2013; Velly et al. 2015). Furthermore, cyclic fatty acids (CFA, such as cycC19:0) are widely found in LAB and are formed by the addition of a methylene group to the carbon-carbon double bond of unsaturated fatty acids (UFA, mainly C18:1).

Under physiological conditions, membrane lipid bilayers are in a lamellar liquid crystalline state (Lα), as illustrated in Fig. 1 a. In this state, lipids have considerable motional freedom (rotation, rocking, and lateral diffusion) that allows the embedded proteins to work properly and ensures the main functions of bacterial membrane (fluid-mosaic model). Upon decreasing temperature (or removing water), the fatty acid chains and head-groups (or the PLs) start packing into a crystal-like structure, forming a more stable and ordered gel phase (Lβ), where lipid movements are highly reduced.

The fatty acyl chain structure and geometry govern the lipid's shape, the degree of lipid packing within the bilayer (Fig. 1b). Any conformation of the acyl chain that will make it more difficult to pack densely and regularly the chains will contribute to increase membrane fluidity. For instance, compared with straight chain of saturated fatty acid, the presence of unsaturation in *cis* conformation within the chain will clearly limit the chain packing (Loffhagen et al. 2001). As a consequence, the unsaturated/saturated fatty acids' ratio (UFA/SFA) is widely related to bacterial membrane fluidity (Denich et al. 2003). Furthermore, short length chains and

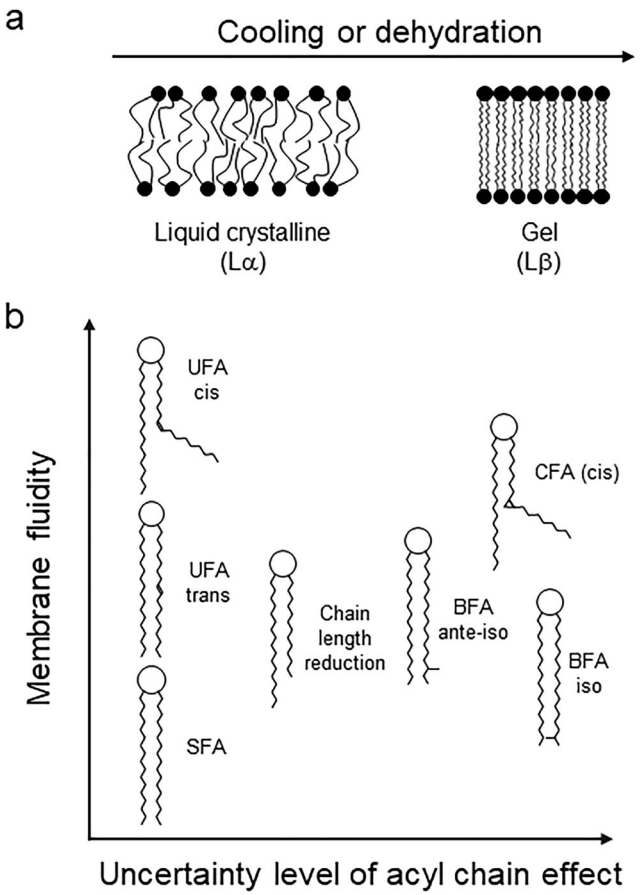

Fig. 1 Lipid phase transition between a lamellar liquid crystalline structure (Lα) and a gel state (Lβ) during cooling or dehydration (**a**). Schema of the membrane fluidity changes expected according to acyl chains structure and geometry (**b**). FA, fatty acids; UFA, unsaturated FA; SFA, saturated FA; BFA, branched FA; CFA, cyclic FA (cycC19:0)

ante-iso-branched chains of fatty acids have also been reported to increase membrane fluidity (Kaneda 1991; Denich et al. 2003; Wang et al. 2011).

The presence of cyclic fatty acids (CFA) also modulates membrane fluidity but their effect remains unclear and contradictory. For some authors, CFAs should have the same physical properties as UFA and would contribute to increase membrane fluidity (Machado et al. 2004; Zhang and Rock 2008). Other researchers reported a decrease of membrane fluidity with increasing content of CFA (Li et al. 2009a; Velly et al. 2015), or no modification of membrane fluidity (To et al. 2011).

The fatty acid composition of LAB membrane determines the temperature of phase transition from the liquid crystalline (Lα) to the gel (Lβ) phases. The longer and more saturated the chains of FA are, the higher the transition temperature is. For instance, for a diacylphosphatidylethanolamine bilayer, the temperature of lipid phase transition (Tm) changes from 30 to 90 °C when increasing the number of carbons of the acyl chains from 12 to 22 (Koynova and Tenchov 2013). When considering lipids with unsaturated chains, the position and type of the double bond substantially modulate the membrane lipid phase transition. For a dioctadecenoyl phosphatidylcholine bilayer, the Tm vary from 20, − 20, and 0 °C when the position of the double bond changes from 4, 9, or 13 position in carbon chains (Koynova and Tenchov 2013).

Although it is generally accepted that modification of FA composition is an effective way for modulating membrane fluidity and phase transition (Denich et al. 2003), little is known about the effect of polar head-group composition on membrane properties. Phospholipid heads are composed of phosphate groups covalently bound to glycerol, leading to phosphatidylglycerol (PG), or diphosphatydilglycerol (DPG) also called cardiolipin (CL) (Gómez-Zavaglia et al. 2000; Machado et al. 2004; Tymczyszyn et al. 2007), or lysylphosphatidylglycerol (LPG) (Russell et al. 1995; Machado et al. 2004), all anionic phospholipids. LAB phospholipid head can also be bounded to ethanolamine (Teixeira et al. 2002), thus leading to phosphatidylethanolamine (PE), a zwitterionic PL. Some phospholipids located at the outer surfaces of the LAB membranes are associated with a sugar group (mono and oligosaccharides) attached by a glycosidic bond to the polar head-group (glycolipids). Each type of polar head is associated to a given number of water molecules, tightly bound through hydrogen bonds (Luzardo et al. 2000), determining the radius of the polar head and the geometry of the lipid. PLs thus present different sizes and shapes that will affect the extent of interfacial area between head-groups as well as the PL distribution and packing in the membrane, eventually modulating membrane fluidity.

Membrane fluidity appears thus a complex property that depends on several factors, membrane FA composition, organization, and temperature. A reliable measurement of this property is thus mandatory in order to well understand the relationship between membrane fluidity and resistance of LAB to production processes.

Methods for evaluating membrane fluidity

Three main ways for assessing membrane fluidity of LAB are commonly reported:

- the measurement of the *UFA/SFA ratio* by identifying and quantifying the fatty acid methyl esters (FAME) by gaseous chromatography coupled to mass spectrometry (GC-MS);
- the direct measurement of membrane fluidity by quantifying the *fluorescence anisotropy* of a probe inserted in the lipid bilayer;
- the characterization of *membrane lipid phase transition* from the liquid crystalline to the gel phases (Lα <=> Lβ) by FTIR (Fourier transform infrared) spectroscopy, fluorescence spectroscopy using Laurdan probe, or differential scanning calorimetry (DSC).

Assessment of membrane fluidity and lipid phase transition can also be measured by nuclear magnetic resonance, electron spin resonance, and X-ray diffraction (Denich et al. 2003; Da Silveira et al. 2003; Mykytczuk et al. 2007).

Table 1 summarizes the works characterizing the membrane physical properties of LAB. Some details concerning the sample preparation, the conditions of measurement are also reported.

Fluorescence anisotropy

This is the most common method applied to measure the relative changes in fluidity of bacterial membranes under environmental conditions. Fluorescent lipid soluble membrane probes are used as biomarkers of membrane lipid structure and motion. The degree of polarization of the fluorescent probe is generally characterized by the anisotropy (r), which decreases when cell membrane fluidity increases. The most reported works concern steady-state anisotropy measurements by using spectrofluorometer with probes exhibiting fluorescence lifetimes (of 10^{-8} and 10^{-9} s) corresponding to the rate of lipid movement. In the last 15 years, this technique has been increasingly employed to study intact membranes of the whole LAB to better understand the role of membrane fluidity on the physiological responses to different environmental conditions (Table 1). The most commonly used probe is 1,6-diphenyl-1,3,5-hexatriene (DPH), an extremely hydrophobic and symmetrical probe that penetrates into the hydrophobic core orientating itself parallel to the fatty acid side chains. Another DPH analogue (1-[4 (trimethylamino)phenyl]-6-

Table 1 Reported work on the characterization of LAB cells and LAB lipids, and the associated methods

Method (scale)	Micro-organism	Sample type	Measurement conditions	Membrane physical properties	References
Fluorescence anisotropy (*r*)					
Fluorescence spectroscopy (population scale)	*O. oeni* Lo84.13	FCP	At 42 °C following heat; at 30 °C following acid and ethanol shocks	*r*-DPH	Tourdot-Maréchal et al. (2000)
	Lb. casei ATCC 393	Liposomes	From 10 to 55 °C, at 5 °C intervals, hyperosmotic conditions		Machado et al. (2004)
	O. oeni ATCC BAA-1163	FCP	At 30 °C, following cold, acid, and ethanol shocks		Chu-Ky et al. (2005)
	Lb. bulgaricus CIDCA 333	FCP, liposomes	From 15 to 50 °C, at 5 °C intervals, following osmotic stress		Tymczyszyn et al. (2005)
	Lb. bulgaricus L2	FCP	At 30 °C, following various fermentation pH and temperatures		Li et al. (2009a)
	Lc. cremoris MG1363	FCP	At 30 °C, following ethanol stress or acid shock		To et al. (2011)
	Lb. casei Zhang and acid-resistant mutant Lbz-2	FCP	At 37 °C, following lactic acid and gastric juice stress in chemostat		Wu et al. (2012)
	Lb. buchneri R1102, *B. longum* R0175	FCP	From 37 to 0 °C and back 0 to 37 °C, following harvest at exponential and stationary growth phases		Louesdon et al. (2015)
	Lc. lactis ML3	PLs	At 25 °C	*r*-DPH and *r*-TMA-DPH	In't Veld et al. (1992)
	Lb. bulgaricus CFL1	FCP	At 0 °C and 25 °C, following osmotic stress (sucrose)		Meneghel et al. (2017a)
Flow cytometry (cellular scale)	*Lb. bulgaricus* CFL1	Fresh cells	At 42, 25, and 5 °C following growth in MRS and whey medium	*r*-TMA-DPH	Passot et al. (2014)
	Lc. lactis TOMSC161		At 20 °C, fermentation (22 °C, 30 °C), different growth phases	*r*-DPH	Velly et al. (2015)
	Lb. bulgaricus CFL1 *S. thermophilus* CFS2		At 42 °C, following growth in (MRS or whey) and harvested at different growth phases	*r*-DPH and *r*-TMA-DPH	Bouix and Ghorbal (2017)
Fluorescence microscopy (subcellular scale)	*Lb. bulgaricus*CFL1	Fresh single cell	At 0 °C and 25 °C, following osmotic stress (sucrose)	*r*-TMA-DPH	Meneghel et al. (2017a)
			From 0 to 37 °C at 5 °C intervals, following growth in MRS and whey medium		Passot et al. (2014)
Membrane lipid phase transition					
FTIR spectroscopy (population scale)	*Lb. plantarum P743*	FCP, liposomes	FT − 50 °C/+ 80 °C; protectants: sorbitol, maltose, trehalose	Tm, PLs head-groups	Linders et al. (1997)
	Lb. bulgaricus CFL1	FCP, dried cells	FT − 50 °C/+ 80 °C; protectants: sucrose, maltodextrin, skim milk	Tm	Oldenhof et al. (2005)
		FCP	FT − 50 °C/+ 80 °C, MRS and whey growth media	Ts, Tm	Gautier et al. (2013)
			FT − 50 °C/+ 80 °C; protectants: glycerol, DMSO, sucrose	Ts, Tm	Fonseca et al. (2016)
			FT − 50 °C/+ 80 °C; osmotic stress (sucrose)	Ts,, Tm, PLs head-groups	Meneghel et al. (2017b)
	Lc. lactis TOMSC161	FCP	FT − 50 °C/+ 80 °C; following fermentation (22 °C, 30 °C), different growth phases	Ts and Tm	Velly et al. (2015)
	Lc. cremoris MG1363	FCP	Heating 0 to 40 °C; addition of sucrose, NaCl, pressure	Tm	Molina-Höppner et al. (2004)
Fluorescence spectroscopy (population scale)	*Lc. cremoris* MG1363	FCP	Heating 0 to 40 °C; addition of sucrose, NaCl, pressure	Laurdan	Molina-Höppner et al. (2004)
	Lb. acidophilus CRL 640	Liposomes	At 5, 15, 25, 37, 50 °C, following salt and bile stress	Tm	Fernández Murga et al. (1999)
	Lb. casei ATCC 393	Liposomes	At 20 °C and 37 °C, following hyperosmotic conditions		Machado et al. (2004)
	Lb. reuteri TMW1.106	FCP	From 20 to 50 °C at 10 °C intervals; protectants: Inulin, FOS, IMO, sucrose, skim milk		Schwab et al. (2007)

r, anisotropy; FCP, fresh cell pellet; *FT*, freeze-thawing; *PLs*, phospholipids; *FOS*, fructo-oligosaccharides; *IMO*, isomalto-oligosaccharides; *DPH*, 1,6-diphenyl-1,3,5-hexatriene/hydrophobic probe located at the hydrophobic core of lipid bilayer; *TMA-DPH*, 1-[4 (trimethylamino) phenyl]-6-phenyl-1,3,5-hexatriene/amphiphilic probe located at the aqueous interface between lipid bilayer and extracellular environment (PLs head-groups); *Laurdan*, 6-Dodecanoyl-N,N-dimethyl-2-naphthylamine/amphiphilic fluorescence probe located at the hydrophobic core of lipid bilayer; *Ts*, temperature of lipid phase transition during cooling (s, solidification); *Tm*, temperature of lipid phase transition during heating (m, melting); *PLs head-groups*, PO_2^- band by 1220 cm^{-1} associated to PLs head-group hydration

phenyl-1,3,5-hexatriene (TMA-DPH)) has also been used on LAB. TMA-DPH anchors at the aqueous membrane interface because the side chains contribute to amphipathic behavior (Trevors 2003).

Recently, fluorescence anisotropy measurements were performed on LAB using flow cytometry. A three co-staining method, involving DPH, propidium iodide (PI), and carboxyfluorescein diacetate (cFDA), was developed to assess membrane fluidity of viable, injured, and dead cells of *Lb. bulgaricus* and *S. thermophilus* (Bouix and Ghorbal 2017).

By replacing fluorescence spectroscopy with fluorescence microscopy to measure the emitted fluorescence of the probe, it is possible to obtain subcellular mapping of membrane fluidity and to detect any membrane heterogeneity. For instance, Passot et al. (2014) evidenced the formation of rigid domains within the membrane of *Lb. bulgaricus* cells when submitting to cold stress. Meneghel et al. (2017a, b) investigated the evolution of membrane fluidity of two strains of *Lb. bulgaricus* (ATCC11842, resistant/CFL1, sensitive) submitted to cold and osmotic stresses. The measurements of membrane fluidity were carried out at population (Fig. 2a) and subcellular scales (Fig. 2b). Similar values of fluorescence anisotropy were observed at the population level, regardless of the stress conditions applied, whereas significant differences were observed at the cellular level when quantifying the number of rigid domains within the bacterial membrane.

The main advantage of fluorescence anisotropy measurements is that it directly measures membrane fluidity through the mobility of a fluorescent probe within the membrane, while requiring low volumes of dyes. Adequate co-staining makes the membrane fluidity quantification of subpopulations (viable, injured, and dead cells) or subcellular mapping if coupled to flow cytometry or fluorescence microscopy possible, respectively. The main disadvantage is that cells must be in suspension and it is not possible to study the membrane fluidity in dried matrices. Moreover, as each fluorescent probe has a well-defined target (i.e., interface-carbonyls-, polar heads-phosphates-, hydrophobic region-acyl chains-), to have a full landscape of lipid membranes, different probes have to be used.

Membrane lipid phase transition (Lα ⇔ Lβ)

FTIR spectroscopy is a non-invasive technique for studying in situ the membrane lipid phase transition of LAB, the change from liquid crystalline to gel phases when decreasing temperature (Table 1). It is a particularly useful technique because of its large flexibility, making the study of both liquid and dried samples with no need of reagents possible. Despite that the FTIR spectroscopy does not provide a direct measurement of membrane fluidity, the spectra can provide complete information about the different membrane regions, namely the interface, the polar heads, and the hydrophobic region, which is of great advantage over other techniques used to this aim (i.e., fluorescence anisotropy, DSC, X-ray diffraction, electron spin resonance spectroscopy (ESR)). Furthermore, FTIR spectroscopy can be used to characterize the membrane lipid behavior during stabilization processes (i.e., freezing, drying).

Membrane phase behavior is commonly monitored by observing the evolution of the position of the symmetric CH_2 stretching band at approximately 2850 cm^{-1} (νCH_2 symmetric) with cooling and subsequent heating (Fig. 3) (Crowe et al. 1989). Figure 3 shows the membrane phase behavior for two population of *Lb. bulgaricus* CFL1 exhibiting different cryotolerance (black diamond symbols: bacteria resistant to freezing when cultured in MRS medium/gray triangle symbols: bacteria sensitive to freezing when cultured in whey medium). A shift in νCH_2 symmetric from 2854 cm^{-1} to lower wavenumbers (2851 cm^{-1} for the resistant condition and 2850 cm^{-1} for the sensitive one) was observed with decreasing temperature. Resistant bacteria were characterized by a lower lipid phase transition (− 8 °C) than sensitive bacteria (22 °C)

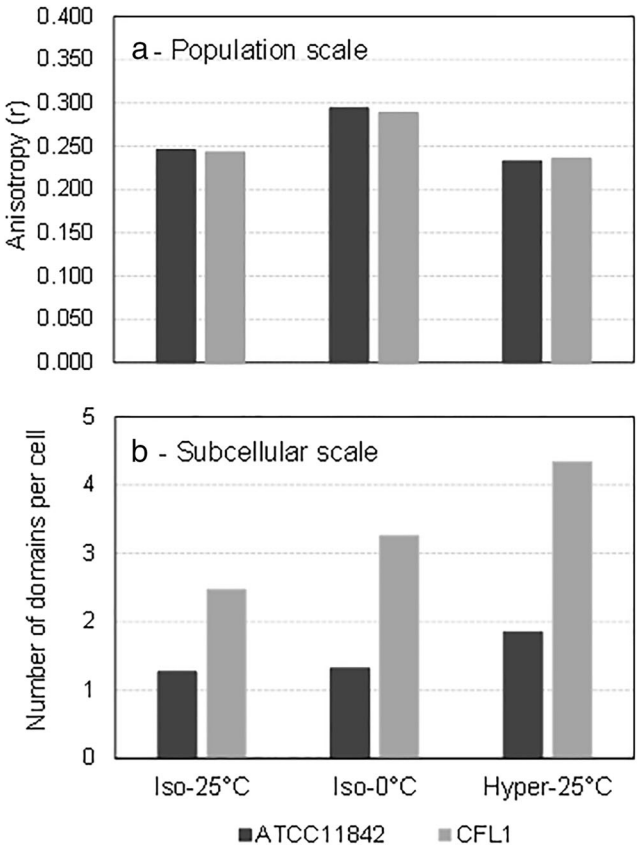

Fig. 2 Membrane fluidity characterization of two strains of *Lb. bulgaricus* (ATCC11842, freeze-resistant and CFL1, freeze-sensitive) submitted to cold and osmotic stress. At the population level, **a** anisotropy values correspond to cell suspensions, while at a subcellular level, **b** values of the number of rigid lipid domains observed on single cells are reported. TMA-DPH was the fluorescence probe used in both data sets. Stress conditions 300 mOsm sucrose solution at 25 °C (Iso-25 °C) and 0 °C (Iso-0 °C) and 1800 mOsm sucrose solution at 25 °C (Hyper-25 °C) (adapted from Meneghel et al. 2017a and b)

during cooling. The evolution of fluorescence anisotropy with temperature was also reported in Fig. 3 for both culture conditions (black circles for the freeze-resistant bacteria and gray circles for the freeze-sensitive bacteria). When considering the sensitive condition, the shift of νCH_2 symmetric to lower wavenumbers is associated with an increase of fluorescence anisotropy (i.e., a decrease in membrane fluidity).

A native membrane has different types of lipids (acyl chains and head-groups) with different melting temperatures and capacities to bind water. As a result, during freezing or drying, the various phospholipids enter their respective gel phases at different temperatures, and the gel and liquid-crystal phases transiently coexist. The gel-phase domains would exclude more fluid domains, and in such two-phase systems, membranes are expected to leak during thawing or rehydration with potentially negative consequences for cell survival (Crowe et al. 1989). Consequently, in addition to the determination of the lipid phase transition temperatures (Ts (following cooling) and Tm (following heating)), other useful parameters can be obtained from the membrane lipid transition curves: (i) the broadness of the transition indicates lipid heterogeneity (Oldenhof et al. 2005; Gautier et al. 2013) and possible phase separation due to the coexistence of rigid and fluid domains (Hazel and Williams 1990; Hazel 1995); (ii) the wavenumber increase at high and/or low temperatures denotes high disorder and fluidity (Gautier et al. 2013); and (iii) hysteresis between cooling and heating has been ascribed to irreversible phenomena occurring during freezing, probably lateral phase separation (Gautier et al. 2013).

Besides, the phosphate symmetric stretching vibration band (νPO_2^-asym, around 1220 cm^{-1}) has been employed for studying the interaction between phospholipid head-groups and the extracellular environment of LAB cells (Meneghel et al. 2017b).

The liquid crystalline to gel phase transition in LAB membrane was also detected (Table 1) by using Laurdan (an amphiphilic fluorescence probe) fluorescence spectroscopy (Harris et al. 2002). Molina-Höppner et al. (2004) thus investigated the effect of milk buffer with 0.5 M sucrose and milk buffer with 4 M sodium chloride on the membrane phase behavior of *Lc. lactis* by applying FTIR spectroscopy and Laurdan fluorescence spectroscopy. Although only FTIR lipid phase transition is presented, the authors declared a similar temperature-dependent phase behavior with Laurdan approach.

Membrane-fluidity-related responses to environmental conditions occurring during the production process of LAB concentrates and their impact on bacterial resistance

Changes of environmental conditions that take place during the production process of LAB starters generate stresses that induce, in turn, different LAB responses. Stresses are mainly caused by modifications in temperature, pH, medium composition, solute concentration, water activity, atmosphere

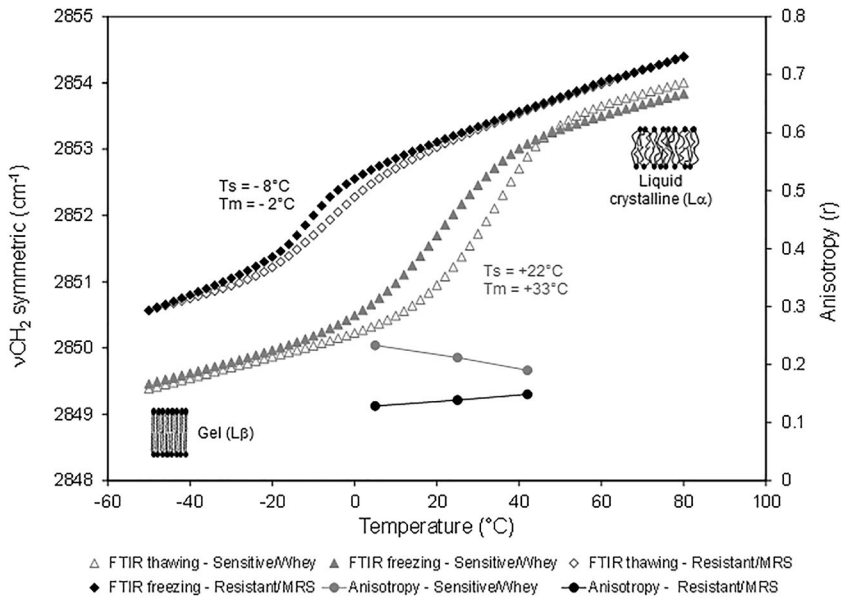

Fig. 3 Membrane lipid phase behavior of two populations of *Lb. bulgaricus* CFL1 exhibiting different resistance to freeze-thawing. Resistant (black diamonds) and sensitive (gray triangles) cells' lipid transitions (liquid-crystalline ↔ gel phase) were obtained by FTIR following cooling (close symbols) and heating (open symbols) by FTIR spectroscopy. Lipid transition temperatures during cooling (Ts, solidification) and during heating (Tm, melting) are indicated. Anisotropy values are also presented at three temperatures for both resistant (black circles) and sensitive cells (gray circles). Resistant and sensitive cells were obtained when culturing in MRS broth and mild whey-based culture medium, respectively (adapted from Gautier et al. 2013)

composition, etc. According to the process step (fermentation, cooling, concentration, stabilization, and storage), the kinetics of the stressful event (sudden or gradual), its intensity, and duration can vary. Consequently, different cellular responses can be promoted:

- The active responses that generally help bacteria to withstand the stress taking place during first steps of production (fermentation and cooling). They lead to modifications of membrane lipid composition, changes in protein contents, and regulation of some genes' expression. They could also result in cell adaptation to the stresses taking place during the stabilization processes.
- The passive responses that are related to cell's physicochemical modifications taking place during downstream processes of stabilization (membrane phase change, lipid oxidation, protein denaturation, modification of ionic force, and viscosity). They are influenced by the cellular active responses and lead to various degrees of deterioration of cell biological properties and functionalities.

Table 2 summarizes the studies reported on the impact of environmental factors on LAB resistance to the stabilization processes (freezing, freeze-drying, and drying) that have reported either modification of lipid composition and/or quantification of membrane physical properties (lipid phase transition or direct assessment of membrane fluidity by fluorescence anisotropy).

Changes in fatty acyl chain composition are more largely investigated and quantified than changes in phospholipid classes, e.g., head-group composition. Moreover, research works on changes of membrane physical properties due to process conditions are also scarcely reported. To our knowledge, only two works have investigated simultaneously fatty acid composition, membrane phase transition, and fluidity in relation with the LAB resistance to process (Velly et al. 2015; Meneghel et al. 2017b).

Fermentation (LAB active responses)

Temperature

Applying temperature lower than the optimal one for cell's growth improved the resistance of LAB to freezing (Fernández-Murga et al. 2000), to frozen storage (Wang et al. 2005), and to freeze-drying (Li et al. 2009a). Velly et al. (2015) reported no improvement of freeze-drying resistance of *Lc. lactis*. Cold adaptation resulted in a modification of fatty acid membrane with an increase of the UFA/SFA ratio, which could be related to an increase of membrane fluidity. Only Velly et al. (2015) characterized the physical properties of *Lc. lactis* and reported an increase of membrane fluidity and a decrease of the lipid phase transition temperature when cells were grown at low temperature. When cells are grown at non-optimal temperatures, they adapt their membrane fatty acid composition in order to maintain membrane fluidity (i.e., liquid crystalline phase) (Hazel and Williams 1990; Hazel 1995).

Modulation of cyclic fatty acid (cycC19:0) membrane content following cold treatment remains unclear and varies according to the LAB strains. When decreasing culture temperature, the cycC19:0 content decreased for *Lb. acidophilus* CRL 640 (Fernández-Murga et al. 2000), *Lactobacillus delbrueckii* ssp. *lactis* (Veerkamp 1971), and *Lactobacillus plantarum* (Russell et al. 1995), or increased for *Lb. acidophilus* RD758 (Wang et al. 2005) and *Lc. Lactis* ssp. *cremoris* (Guillot et al. 2000), whereas a bell shape dependence with temperature was observed for *Lb. fermentum* (Suutari and Laakso 1992).

Furthermore, when increasing the growth temperature (from 42 to 60 °C), a decrease of phospholipid content compared with proteins was reported for *O. oeni* (Garbay and Lonvaud-Funel 1996).

The application of a moderate cold stress after cell growth at optimal temperature could afford resistance to upcoming stabilization process. The production of cold shock proteins and the modulation of membrane FA composition are proposed as the main adaptive responses to suboptimal temperatures (Papadimitriou et al. 2016). Wang et al. (2005) and Schoug et al. (2008) reported increase in the UFA/SFA ratio, similarly to the low temperature effect observed during fermentation, but leading to opposite effects on the resistance to stabilization process. The better resistance to frozen storage of "cold adapted" *Lb. acidophilus* cells was related to the increase in cycC19:0 membrane fatty acid content (Wang et al. 2005). In turn, the cold step induced a lower resistance of *Lb. coryniformis* to freeze-drying accompanied of a decrease in the content of cycC19:0 when compared with optimal growth conditions (Schoug et al. 2008).

pH

Exposing LAB to pH values lower than the optimal one during growth resulted in improvement of cell survival either after the stabilization processes (freezing or freeze-drying) or the application of acidic treatment. Only Gilliland and Speck (1974) and Li et al. (2009a) observed a decrease of survival of *Lc lactis* and *Lb. bulgaricus* with decreasing pH when bacteria were grown at uncontrolled pH and 39 °C, respectively. However, opposite results were obtained when growth of *Lb. bulgaricus* was carried out at 30 °C, suggesting that these two factors, pH and temperature, have important and combined impact on LAB survival after stabilization processes. The effect of low pH on the modulation of membrane fatty acid composition appeared to be dependent on the LAB considered: 60% of the works reported a decrease of the UFA/SFA ratio, whereas 66% of the studies observed an increase of

Table 2 Impact of environmental factors on lipid composition, membrane physical properties, and LAB's resistance to stabilization processes (freezing, freeze-drying, and drying)

Factor	LAB strain	Stress conditions	Lipid composition	Membrane physical properties	Resistance to stabilization process	References
Fermentation (LAB active responses)						
Temperature	*Lb. acidophilus* RD758	Cold: growth at 30 °C	**UFA/SFA** (+), **CFA** (+) C18:0 (−), C16:0 (+)	NR	(+) **to FT and FS at − 20 °C**	Wang et al. (2005)
	Lb. acidophilus CRL 640	Cold: growth at 25, 30 °C	***UFA/SFA*** (~), *CFA* (−) *MCL* (−) C18:2 (+), GLs/PLs (+)	NR	(+) **to FT**	Fernández-Murga et al. (2000)
	Lc. lactis TOMSC161	Cold: growth at 22 °C	**UFA/SFA** (+), ***CFA*** (~)	Tm (−), **fluidity** (+) at 22 °C	(~) to FD and storage at 25 °C	Velly et al. (2015)
	Lb. bulgaricus L2	Cold: growth at 30 °C, 35 °C, 37 °C, 39 °C (at pH 5)	**UFA/SFA** (+), ***CFA*** (~)	NR	(+) **to FD**	Li et al. (2009a)
	Lb. acidophilus RD 758	Cold step after culture (8 h at 15 °C)	**UFA/SFA** (+), **CFA** (+)	NR	(+) **to FS**	Wang et al. (2005)
	Lb. coryniformis	Cold step after culture (26 °C, 6-8 h)	**UFA/SFA** (+), *CFA* (−)	NR	(−) *to FD*	Schoug et al. (2008)
pH	*Lc. lactis* (AC1, AC11, E8, ML1)	Acid: uncontrolled pH or pH 6	*UFA/SFA* (−), **CFA** (+) at uncontrolled pH	NR	(−) *to FT at − 17 °C and FS if uncontrolled pH*	Gilliland and Speck (1974)
	O. oeni SD-2a	Uncontrolled pH Initial pH (4.8; 4.0; 3.5) ATB medium	*UFA/SFA* (−), **CFA** (+)	NR	(+) **to FD at low pH (3.5)**	Li et al. (2009b)
	S. thermophilus CFS2	Acid, controlled pH 5.5, 6, and 6.5	**UFA/SFA** (+), **CFA** (+), C18:1 (9c) (+), C20:1 (+) at low pH (5.5)	NR	(+) **to FS at − 20 °C at low pH**	Béal et al. (2001)
	Lb. acidophilus RD758	Acid, controlled pH 4.5, 5, and 6	**UFA/SFA** (+), **CFA** (+) C16:0 (+), C18:0 (−) at pH 5	NR	(+) **to FT and FS at − 20 °C at low pH (5)**	Wang et al. (2005)
	Lb. bulgaricus L2	Controlled pH from 5 to 6.5 (at 30 °C)	***UFA/SFA*** (~), ***CFA*** (~)	NR	(+) **to FD at low pH**	Li et al. (2009a)
		Controlled pH from 5 to 6.5 (at 39 °C)	*UFA/SFA* (−), *CFA* (−) at low pH	NR	(−) *to FD at low pH*	
	Lb. bulgaricus CFL1	Acid shock (pH 5.25 at end of fermentation) of cells from controlled pH 6 culture	*UFA/SFA* (−), *CFA* (−) and ***MCL*** (~)	NR	(+) *to FT and FS at − 20 °C*	Streit et al. (2008)
	S. gordonii DL1, *S. salivarius* 57.I, *Lb. casei* 4646	Chemostat at controlled pH (7, 6, and 5) and uncontrolled pH	**UFA/SFA** (+), **MCL** (+) at low pH and uncontrolled pH	NR	NR after stabilization (+) Survival at low pH after fermentation	Fozo et al. (2004)
	Lc. cremoris MG1363	Acid, uncontrolled pH Initial pH 7 or 5 (adaptation), shock at pH 3	*UFA/SFA* (−), **CFA** (+) at low pH (5)	*Fluidity* (−) *at low pH*	NR after stabilization (+) Survival of acid adapted cells after acid shock	To et al. (2011)
	Lb. casei ATCC 334	Acid shock, initial pH 4.5 and 2, on cells harvested at stationary phase after culture at controlled pH (6)	*UFA/SFA* (−), **CFA** (+) *MCL* (−)	NR	NR after stabilization (+) Survival of acid adapted cells (pH 4.5) after fermentation	Broadbent et al. (2010)
	Lb. casei Zhang, and acid-resistant mutant Lbz-2	Acid shock, initial pH 5.0, 3.5 of steady state chemostat cells (pH 6.5)	**UFA/SFA** (+), **CFA** (+), **MCL** (+) specially for the mutant	*Fluidity* (−) *if low pH*; less decrease of fluidity for the mutant	NR after stabilization (+) Survival of mutant after fermentation	Wu et al. (2012)
	O. oeni ATCC BAA-1163	Acid shock at pH 5.0 and 3.0, on cells harvested at stationary phase after uncontrolled pH culture	NR	*Fluidity* (−) specially at pH 3.0	NR after stabilization No effect on survival after fermentation	Chu-Ky et al. (2005)
Growth phase	*O. oeni* SD-2a	Uncontrolled pH ATB medium Mid-exponential and early stationary	***UFA/SFA*** (~), **CFA** (+) in stationary growth phase corresponding to lowest pH (3.6)	NR	(+) **to FD at stationary**	Li et al. (2009b)
	Lb. acidophilus RD758	Lactate, lactose depletion (starvation)	**UFA/SFA** (+), **CFA** (+), **MCL** (+), BFA (+), of starved cells (stationary)	NR	(+) **to FT of starved cells (stationary)**	Wang et al. (2011)
	Lb. buchneri R1102	Lactate (controlled pH)	*UFA/SFA* (−), **CFA** (+) C16:0 (+), in stationary growth phase	*Fluidity* (−)	(−) *to FT if stationary phase*	Louesdon et al. (2015)
	Bifidobacterium longum R0175	Lactate (controlled pH)	*UFA/SFA* (−), **CFA** (+) C16:0 (+), in stationary growth phase	*Fluidity* (−)	(+) **to FT if stationary phase**	Louesdon et al. (2015) Velly et al. (2015)
	Lc. lactis TOMSC161	Lactate (controlled pH)	*UFA/SFA* (−), **CFA** (+) at late stationary	Tm (+), *fluidity* (−) with growth	(+) **to FD and storage if stationary phase**	

Table 2 (continued)

Factor	LAB strain	Stress conditions	Lipid composition	Membrane physical properties	Resistance to stabilization process	References
Medium composition	*Lb. bulgaricus* NSC1 to NSC4	Addition of sodium oleate	**UFA/SFA** (+), **CFA** (+) **MCL** (+) C18:1 (+),	NR	(+) **to FT in liquid N_2**	Smittle et al. (1974)
	Lb. sp. A-12, *Lc. lactis*	Addition of Tween 80	**UFA/SFA** (+), **CFA** (+) **MCL** (+) C18:1 (+),	NR	(+) **to FT at − 17 °C**	Goldberg and Eschar (1977)
	S. thermophilus CFS2	Addition of Tween 80	**UFA/SFA** (+) C18:1 (+), C20:1 (+),	NR	(+) **to FT at − 20 °C and FS**	Béal et al. (2001)
	Lb. bulgaricus CFL1	MRS and Whey	**UFA/SFA** (+), **CFA** (+), **MCL** (+) with MRS	Ts/Tm (−) if MRS	(+) **to FT at − 80 °C if MRS**	Gautier et al. (2013)
		MRS and Whey	**UFA/SFA** (+), **CFA** (+), **MCL** (+) with MRS	**Fluidity** (+) **if MRS**		Passot et al. (2014)
	O. oeni SD-2a	Uncontrolled pH (initial pH 4.8) ATB, FMATB, MATB medium (Malate/sucrose ratio)	**UFA/SFA (+) CFA** (+) when glucose proportion increase	NR	(+) **to FD when glucose proportion increase**	Li et al. (2009b)
Hyperosmotic condition	*Lb. bulgaricus* CIDCA 33	Addition of PEG in MRS	*UFA/SFA* (−), *CFA* (−) GLs/PLs (+)	*Fluidity* (−) at presence of PEG	NR	Tymczyszyn et al. (2005)
	Lb. casei ATCC 393	Addition of NaCl in MRS	*UFA/SFA* (−), **CFA** (+) Glycolipid/phospholipid (~) (+) **LPG**, **CL** (−) *PG*	Fluidity (~) (+) lateral lipid packing and proton permeability	NR	Machado et al. (2004)
	Lb. plantarum	Addition of KCl	(+) **PG**, **CL** (−) *LPG*	NR	NR	Russell et al. (1995)
		Addition of KCl and betaine	***UFA/SFA*** (~), **CFA** (~) (+) **LPG** (−) *PG*, *CL*	NR	NR	
After fermentation/stabilization processes (LAB passive responses)						
Freezing	*O. oeni* ATCC BAA-1163	Cold stress cold shocks: 5 and 30 min, at 8 °C or 14 °C	NR	*Fluidity* (−) at 8 °C	NR after stabilization No effect on survival	Chu-Ky et al. (2005)
	Lb. bulgaricus CFL1	Culture medium (MRS vs. Whey) Cooling from 42 °C to 0 °C	UFA/SFA (−), CFA (−), MCL (−) with whey	Ts/Tm (+) in whey *Fluidity* (−) and lipid rigid domains at low T° *in whey*	(−) *FT* when LAB grown in *whey* medium	Gautier et al. (2013) Passot et al. (2014)
	Lb. bulgaricus CFL1 and ATCC 11842	Exposure to cold (5 °C) and/or osmotic stress (50% of sucrose) Culture in whey medium	UFA/SFA (−), MCL (−) for CFL1	Ts (+), *Fluidity* (−) PO_2^- (−), in *CFL1* Broad rigid lipid domains in *CFL1*	(−) *to FT for CFL1*	Meneghel et al. (2017b) Meneghel et al. (2017a)
	Lb. plantarum P743	Air drying Protective solutes: Maltose, **sorbitol**, trehalose	NR	Tm (+) dried, Tm (~) with protectants PO_2^- (−) with sorbitol	(+) **to AD with sorbitol**	Linders et al. (1997)
Drying	*Lb. reuteri* TMW1.106	Freeze-drying Protective solutes: **FOS**, sucrose, inulin, IMO	NR	**Fluidity** (+) with FOS	(+) **to FD with FOS**	Schwab et al. (2007)
	Lb. bulgaricus CFL1	Air drying Protective solutes: sucrose (S), maltodextrin (MD), S/MD mixture	NR	Tm (+) when drying Tm (~) with protectants	(~) *AD*	Oldenhof et al. (2005)
	Lb. plantarum CWBI-B534, *L. mesenteroïdes* Kenya MRog2	Freeze-drying Protective solute: maltodextrin + glycerol Storage conditions	C18:2/C16:0 (−) and C18:3/C16:0 (−) following storage in presence of air, at high moisture content and high temperature	NR	(−) *Storage in presence of air*, at high temperature and moisture content	Coulibaly et al. (2009)
	Lb. acidophilus W	Freeze-drying (FD) Vacuum drying (VD)	***UFA/SFA*** (~) *CFA* (−) for VD	NR	(~) FD, VD	Brennan et al. (1986)
	Lb. bulgaricus NCFB 1489	Spray-drying	*UFA/SFA* (−), *CFA* (−) after spray-drying and storage	NR	(−) *SD and storage*	Teixeira et al. (1996)

The modifications on membrane fatty acid composition, fluidity as well as resistance to stabilization processes are indicated as follows: increase (bold and "+"), decrease (italic and "−"), and not significant variation (bold italic and "~")

NR, not reported; *FOS*, fructo-oligosaccharides; *IMO*, isomalto-oligosaccharides; *PLs*, phospholipids; *GLs*, glycolipids; *PG*, phosphatidylglycerol; *CL*, cardiolipin; *LPG*, lysylphosphatidylglycerol; *PGL*, phosphoglycolipid; *FA*, fatty acids; *UFA*, unsaturated FA; *SFA*, saturated FA; *CFA*, cyclic FA (cycC19:0); *BFA*, branched FA; *MCL*, mean acyl chain length; *Ts*, lipid transition during cooling (s, solidification); *Tm*, lipid transition during heating (m, melting); PO_2^-, band by 1220 cm^{-1} associated to PL head-group hydration; *FT*, freeze-thawing; *FS*, freezing storage; *FD*, freeze-drying; *AD*, air drying; *VD*, vacuum drying; *SD*, spray-drying

the CFA content. The scarce works performing direct measurements of membrane fluidity evidenced a membrane stiffness when bacterial growth was carried out at low pH.

This might be ascribed either to a decrease of UFA/SFA combined with an increase of CFA, or to the increase of CFA and long chain FA (higher mean acyl chain length, MCL) content. The increase in the proportion of long length chain FA is often associated to an increase in UFA/SFA, thus probably counterbalancing the effect of FA unsaturation on membrane fluidity.

Growth phase (or age of the culture)

During growth, LAB encounter various environmental changes: decrease of pH (for uncontrolled pH culture condition), production of metabolites (lactate for controlled pH condition, reactive oxygen species), and eventual nutrient depletion.

An increasing content of CFA was systematically evidenced with increasing culture time associated with a decrease of the UFA/SFA ratio for 60% of them. This modulation of membrane fatty acid composition during growth seems to result in a decrease of membrane fluidity (similar to the pH effect) and for most of the reported cases, in an improvement of LAB resistance to stabilization processes. Velly et al. (2015) correlated the membrane stiffness to an increase if the CFA/UFA ratio by means of C18:1 cyclopropanation.

When examining the effect of pH and growth phase parameters, LAB growth at uncontrolled pH conditions up to low pH values or at controlled pH up to stationary phase induces similar effects on membrane FA composition (increase CFA, decrease UFA/SFA) and membrane stiffness when measured.

Cultures performed at controlled and low pH values (acid adaptation) and at standard pH but followed by acid shock/challenge induce similar bacterial responses than growth under lactose depletion (Wang et al. 2011): increase CFA, UFA/SFA, and MCL, and decrease of membrane fluidity.

Composition of culture medium and growth in hyperosmotic condition

Modifying the composition of the culture medium is often an efficient way for modulating membrane fatty acid profile (Table 2). Culture medium promoting the synthesis of UFA (increase UFA/SFA ratio), CFA, and long chain FAs made it possible to improve the LAB resistance to freezing and freeze-drying processes. These membrane composition modifications seem to be correlated with an increase of membrane fluidity, in particular at low temperatures (Passot et al. 2014; Gautier et al. 2013). An increase of membrane fluidity can be expected when increasing CFA content due to its decreasing effect on the lipid phase transition temperature (Perly et al. 1985) and from recent molecular dynamic simulations of model membranes containing CFA (Poger and Mark 2015). However, the reported studies on LAB did not make it possible to clarify the effect of CFA on membrane fluidity. Addition of lipids with high content of UFA, such as oleic derivatives (Tween 80, sodium oleate) or using MRS (containing Tween 80) medium, appears as an efficient way to increase membrane content of UFA and CFA. By varying pH and culture age in MRS medium, accumulation levels of CFA up to 33% and 42% were reported for *Lb. fermentum* and *Lb. buchneri*, respectively (Nikkilä et al. 1996).

Some authors have investigated the effect of the osmolarity of the culture medium (addition of salts and solute to decrease water activity) on the membrane properties. Unfortunately, no data on LAB survival after stabilization processes were reported. Increasing osmolarity (decreasing water activity) resulted in modification not only of the FA composition but also on the lipids polar head-groups. A decrease of the UFA/SFA ratio and an increase of CFA were evidenced by several authors for *Lb. pentosus* (Gilarová et al. 1994), *Lc. lactis* ssp. *cremoris* (Guillot et al. 2000), *Lb. helveticus* (Guerzoni et al. 2001), and *Lb. casei* (Machado et al. 2004). However, other LAB exhibited different behaviors: increase of UFA/SFA ratio and decrease of CFA in *Lb. acidophilus* (Fernández Murga et al. 1999), decrease of UFA/SFA ratio and CFA in *Lb. bulgaricus* (Tymczyszyn et al. 2005), and no modification *Lb. plantarum* (Russell et al. 1995).

Furthermore, the effect of hyperosmotic condition on the composition of the membrane phospholipid head-groups remains difficult to generalize and appears to be dependent on the bacteria and on the solutes. Increasing proportions of anionic PLs (PG, CL, and LPG) were reported in *Lb. plantarum* (Russell et al. 1995) and *Lb. casei* (Machado et al. 2004) when subjected to KCl 0.8 M and NaCl 1M respectively. Osmotic stress adaptation conducts to a high overall negative charge of bacterial membrane lipids for acting as a binding site for cations (Mykytczuk et al. 2007). The relative proportions of anionic phospholipids, however, varied according to microorganisms.

After fermentation/stabilization processes (LAB passive responses)

The main strategy to stabilize and increase the shelf life of LAB starters is to reduce the availability of water by freezing or drying. Due to the heat sensitivity of LAB, freeze-drying is often the drying method of choice. However, stabilization processes generate some undesirable side effects that induce decreased cell activity and death. Freezing process induces mainly cold and osmotic stresses, whereas mechanical, osmotic, oxidative, and heat stresses characterize the drying processes. Two main kinds of cellular damage are reported: (i) changes in the physical state of cytoplasmic membrane, resulting in loss of membrane integrity (Linders et al. 1997; Schwab et al.

2007); and (ii) modifications in the secondary structure of proteins (Carpenter and Crowe 1988; Oldenhof et al. 2005).

Modification of membrane properties following stabilization processes

Few studies have investigated the modification of membrane physical properties following stabilization processes. Following cooling, LAB membrane fluidity decreases with the transition from the disordered liquid crystalline (Lα) to the ordered gel phase (Lβ) of the lipid bilayer. Gautier et al. (2013) and Passot et al. (2014) studied the evolution of membrane properties of two populations of *Lb. bulgaricus* CFL1 exhibiting different freezing resistance following cooling by FTIR spectroscopy and anisotropy of fluorescence (Fig. 3). Freeze-resistant cells exhibited a lower lipid phase transition (Ts) during freezing (Ts = − 8 °C) and a higher membrane fluidity ($r = 0.240$) at the ice nucleation temperature range, than the freeze-sensitive cells (Ts = + 22 °C and $r = 0.388$, respectively). A sub-zero value of lipid phase transition, associated to high membrane fluidity, allowed the maintenance of the cell membrane in a relatively fluid state during freezing. Therefore, water flux from the cell and the concomitant volume reduction following ice formation in the extracellular medium (and associated solute cryoconcentration) was facilitated.

During drying processes, removal of unfrozen water results in profound changes in the physical properties of biomolecules, particularly phospholipids and proteins (Crowe et al. 1989). A decrease of the lateral spacing of the polar head-groups and the subsequent packing of the hydrocarbon chains lead to a considerable increase of the membrane lipid phase transition after drying (Potts 1994). In LAB, the membrane lipid phase transition (Tm) of *Lb. plantarum* was reported to shift from 4 °C in hydrated cells to 20 °C in dried cells (Linders et al. 1997) and from 35 °C to 40 °C in *Lb. bulgaricus* (Oldenhof et al. 2005).

No significant modification of fatty acid composition was observed upon freeze-drying of *Lb. acidophilus* while a decrease of CFA was observed following vacuum drying (Brennan et al. 1986). However, the fatty acid composition of freeze-dried and spray-dried *Lb. bulgaricus* was reported to evolve upon storage (Castro et al. 1995; Teixeira et al. 1996). Teixeira et al. (1996) showed that UFA/SFA ratio was stable within 49 days of storage and then decreased, while CFA content decreased from 32 days of storage after spray drying. Similarly, the low survival of *Lb. plantarum* and *Leuconostoc mesenteroïdes* to 90 days storage at 20 °C was associated to the decrease in C18:2/C16:0 and C18:3/C16:0 ratios (Coulibaly et al. 2009). These decreases were ascribed to the oxidation of UFA and CFA that are sensitive to oxygen (Castro et al. 1996) and accentuated by an increase in the residual relative humidity that probably activates the oxidation processes (Castro et al. 1995). Damage through reactive oxygen species is indeed recognized as one of the main stress that face micro-organisms during dehydration process (Potts 1994). Oxidation of cell components upon drying and storage has been confirmed by the improvement of survival when adding antioxidants to starters before stabilization (Andersen et al. 1999; Kurtmann et al. 2009) and by storage in nitrogen atmosphere (Castro et al. 1995; Andersen et al. 1999).

Furthermore, no direct measurements of membrane fluidity are reported upon drying and rehydration of LAB.

Interaction of protective molecules with membrane and effect on LAB resistance

The production and cellular accumulation of sugars (i.e., trehalose, sucrose, fructo-oligosaccharides (FOS)) is one of the most studied phenomena in organisms resistant to anhydrobiosis-involving processes (García 2011). In order to mimic the processes naturally occurring in cells, protective molecules such as sugars, amino acids, polyols, polysaccharides, and antioxidants are currently added, after fermentation, to cell concentrates (Santivarangkna et al. 2008). The protective mechanisms of these molecules, in particular their potential interaction with membranes, are still controversial and have been studied mainly on model lipid systems (liposomes, monolayers) (Crowe 2015).

The interaction of the polar head-groups of cell membranes with water molecules present in the environment is crucial to maintain membrane in functional state. During dehydration, sugars like trehalose or sucrose are reported to directly interact with the polar head-groups by establishing hydrogen bonds, and replacing water molecules. The consequence is the decrease of the lipid membrane phase transition temperature after dehydration. Membranes dehydrated in the presence of sugars remain in the liquid crystalline phase as if they were hydrated, thus preserving their biological function (Crowe et al. 1988; Milhaud 2004).

Works on LAB have not confirmed Tm depression of whole cells of model micro-organisms stabilized with sugars (Leslie et al. 1995). No significant effect of maltose, trehalose, and sorbitol was observed on Tm of *Lb. plantarum* dried cells (Linders et al. 1997). These results were explained by an already low modification of Tm on drying without protectant and the authors ascribed the protective effect of carbohydrates to their free radical scavenging activity and not to the direct interaction with the polar lipid head-groups. Similarly, sucrose, maltodextrin, and skim milk had also minor effects on membrane phase behavior and the overall protein secondary structure of *Lb. bulgaricus*-dried cells (Oldenhof et al. 2005). Furthermore, an increased stability upon freeze-drying of stationary phase cells of *Lb. reuteri* in the presence of FOS was ascribed to direct interaction of FOS with membranes (Schwab et al. 2007), but no lipid phase transition was

assessed. In the presence of FOS, the authors evidenced a decreased generalized polarization that they interpreted as an increased membrane fluidity of *Lb. reuteri*. However, Molina-Höppner et al. (2004) reported a decrease of Tm of *Lc. lactis* suspended in milk buffer in the presence of sucrose or NaCl, from 21.4 to 16.8 °C or 16.6 °C, respectively. In this study, accumulation of sugars within the intracellular medium was observed.

By describing the cooling process as a combination of cold and osmotic stresses, Meneghel et al. (2017a, b) proposed a complete characterization of the membrane physical behavior following freezing in presence of sucrose, using FTIR spectroscopy and fluorescence of anisotropy at the subcellular level. The organization of membrane phospholipid head-groups and its modification with osmotic stress (the most cell-damaging stress of freezing process) monitored by FTIR spectroscopy (PO_2^- (+)) suggested preferential exclusion of sucrose from the LAB membrane as the preservation mechanism of the freeze-resistant cell. When considering freeze-sensitive cells, direct interaction between sucrose and membrane was proposed to explain loss of biological activity following freezing. Furthermore, occurrence of rigid domains within the membrane was more important in the freeze-sensitive bacterial population following cold and/or osmotic stresses. The broadening of existing highly rigid lipid domains in freeze-sensitive cells when applying osmotic stress is proposed to be caused by the interaction of sucrose with membrane phospholipids, leading to membrane disorganization and cell degradation. The visual observation of rigid lipid domains within the membrane of LAB and the identification of FTIR markers of phospholipid organization requires further investigation, in particular to identify the precise composition of lipid domains and their mechanisms of formation in LAB.

Future prospects for research on LAB membranes

Existence of lipid domains within bacterial membrane

Domains of specific lipid composition have recently been evidenced within bacterial membranes and the characterization of mechanisms underlying the local enrichment of PLs has become an active research area (Romantsov et al. 2009; Passot et al. 2014; Lin and Weibel 2016). Membrane poles and septa of bacilli including Gram-positive bacteria were reported to be enriched in anionic phospholipids, especially cardiolipin (CL) (Kawai et al. 2004; López 2006; Bernal et al. 2007; Seydlová et al. 2013). CL content varies from 5 to 30% in bacteria, and has been associated to the membrane rigidification of *B. subtilis* (Seydlová et al. 2013) and *P. aeruginosa* (El Khoury et al. 2017). A spiral-shaped phosphatidylglycerol (PG) domain that extends along the long axis of *B. subtilis* was also evidenced (Barák et al. 2008; Hachmann et al. 2009; Muchová et al. 2010). Moreover, the existence of domains similar to lipid rafts in eukaryotic cells was suggested (Donovan and Bramkamp 2009; López and Kolter 2010). Lipid domains, lipid rafts, and membrane physical properties have been mainly studied in model organisms (*B. subtilis and E. coli*) and evidenced their relevance for several important cellular processes (Barák and Muchová 2013). Many interesting findings are thus expected to come to shed light on other bacteria, with new markers and imaging technologies continuously been developed. The recent visualization of lipid domains of high rigidity within the membrane of freeze-sensitive *Lb. bulgaricus* (Meneghel et al. 2017a) raises scientific questions concerning the composition of LAB lipid domains (head-groups and acyl chains), their mechanisms of formation, and their role on LAB resistance to stress.

Lipid-protein interactions and the modulation of membrane fluidity by proteins

Bacterial membrane proteins, accounting for about 20–30% of the cell proteome, are inserted in the membrane lipid bilayer through protein-aqueous channels. The lipid composition of the membrane can affect the biogenesis, activity, and function of integral membrane proteins (Lee 2004; Schneiter and Toulmay 2007).

The coordination of lipid and protein position in the membrane is however still poorly understood.

Lipid-protein interactions are controlled by several factors: the thickness, curvature, and fluidity of membrane; composition of lipid head-group (charge, size, hydration); and fatty acid (chain length, transition temperature) (Denich et al. 2003; Lee 2004). For example, non-bilayer forming lipid assemblies will occupy spaces in the protein surface to ensure a good contact between the protein and the lipid bilayer. Proteins also contribute to the stability of the membrane by limiting the flexibility of acyl chains, and decreasing their motion (Heipieper et al. 1994; Epand 1998), particularly in the stationary growth phase (Souzu 1986). The insertion of large proteins in the lipid bilayers results in ordering the lipid acyl chains, which in turn causes an increase of the membrane lipid phase transition temperature.

Some proteins can modify the membrane fluidity when cells are exposed to short-term changes in environmental conditions, such as heat shock. In this case, the bacterial metabolic pathways would not make a rapid change in membrane lipid composition possible. Heat shock proteins (such as GroEL, a soluble chaperonin from *E. coli*) can associate with lipids, leading to an increasing molecular order in the lipid bilayer, thus counterbalancing the increased membrane fluidity induced by high temperature (Torok et al. 1997).

Furthermore, the membrane lipid phase transition from the liquid crystalline to the gel phases occurring following a

decrease of temperature or hydration level can induce protein segregation and thus the formation of concentrated domains of lipids within bacterial membranes (Letellier et al. 1977; Sperotto et al. 1989). This phenomenon called lateral phase separation can perturb the membrane biological function, inactivating some proteins. Better knowledge about lipid-protein interactions and lipid domain formation in LAB would help to improve the maintenance of membrane properties following stabilization processes.

The "sensing" role of membrane fluidity

Membrane fluidity modifications also contribute to the perception of environmental changes (cold, hyperosmotic, etc.) by bacterial cells and to the subsequent expression of genes that ensures acclimation to a new set of environmental conditions. Putative sensors that perceive changes in membrane fluidity have been reviewed for bacteria and plants (Los and Murata 2004). A cytosolic thermosensor governs the temperature-dependent adjustment of membrane fluidity (associated to production of unsaturated fatty acid) in *E. coli*, while in *B. subtilis* and cyanobacteria, this control is exerted by a membrane-associated thermosensor (Mansilla et al. 2004). Membrane-integrated osmosensors have also been proposed for *E. coli* and *Lc. lactis* and it has been suggested that the changes in fluidity and in physical state of membrane lipids regulate the activity of osmosensors (Los and Murata 2004). The identification of lipids or lipid domains that interact with these environmental sensors can provide clues for understanding how bacteria and in particular LAB respond to environmental stresses.

LAB biomimetic membranes as research tools

The complexity of bacterial membrane composition and organization, as well as the asymmetric characteristic of lipid bilayers, makes it highly difficult to identify key components that govern the membrane properties and bacterial resistance to environmental changes induced by stabilization processes. The use of model membranes with well-defined lipid composition is a promising way to circumvent this high complexity and to identify the role of specific lipids (polar head-groups as well as fatty acyl chains). To our knowledge, no work has been reported on the development of model membranes that are good representations of LAB. Most of the reported studies have focused on single-component model lipid membrane. Considerable work is needed to generate data on more complex model lipid membranes and real biological membranes.

Methodological breakthroughs

Complementary physical methods are continuously improved to investigate interactions between molecules and lipid bilayers (Deleu et al. 2014; Kent et al. 2015).

As mentioned before in this mini-review, FTIR spectroscopy is a powerful tool for studying the structure and organization of membrane lipid bilayers (whole cells or vesicles) in physiological conditions and following exposure to environmental stresses without introducing extrinsic probes. However, since the lipid phase transition does not give a direct measurement of membrane fluidity, it still needs to be combined to fluorescence anisotropy. Besides, FTIR spectroscopy remains underexploited and could provide relevant information on lipid domain formation (Mendelsohn and Moore 1998), lipid-protein interactions in model membranes (Silvestro and Axelsen 1998), and interactions between lipid polar head-groups (Lewis and McElhaney 2013). The maximal extraction of structural information encoded in the IR spectra will certainly require input from complementary approaches (isotopic labelling, recently developed sub-micron IR micro-spectroscopy, fluorescence microscopy, light scattering, neutron scattering). Polarized FTIR ATR measurements can also deliver valuable information on the preferential orientation of functional groups on the membrane's surface (Silvestro and Axelsen 1998; Hutter et al. 2003).

A summary of the main factors identified as having an influence on membrane fluidity is proposed in Fig. 4. The factors relate to membrane composition and process parameters, and are classified according to their ability to induce increasing, decreasing, or uncertain effect on membrane fluidity.

	Membrane fluidity		
	Increasing	**Decreasing**	**Uncertain effect**
Membrane composition	⇧ UFA/SFA	⇧ FA chain length	CFA Head-groups Lipid domains Proteins
Fermentation parameters	⇩ Growth temperature ⇧ Oleic derivatives in culture medium	⇧ Growth time (stationary phase) ⇧ Medium osmolarity	pH Combined factors
Stabilisation-related parameters		⇩ Temperature ⇧ Solute concentration	Process kinetics Oxidation level Hydration level Interaction with protective molecules

Fig. 4 Summary of main factors affecting LAB membrane fluidity. FA, fatty acids; UFA, unsaturated FA; SFA, saturated FA; CFA, cyclic FA (cycC19:0)

Conclusion

The prediction of membrane fluidity from the membrane fatty acid composition has shown strong limitations. Despite all reported work on LAB lipid membranes, research is still needed to understand lipid organization and their interaction with proteins, to elucidate the factors governing membrane fluidity.

The systematic assessment of complete lipid composition (e.g., lipid classes, fatty acids, and head-group quantification) and physical properties of LAB membranes (membrane fluidity and lipid phase transition) becomes essential for understanding the role of different fatty acids (i.e., CFA), lipid classes (phospholipids and glycolipids), and lipid head-groups (i.e., PG, PE, etc.) in membrane fluidity.

For fully understanding the causes of bacterial membrane modulation and injury by environmental stress, it appears mandatory to assess membrane physical properties, through going back and forth real cells and model membranes, and to combine complementary methods: covering different observation scales (from cell population to molecules), involving in real-time measurements, close to environmental conditions: if possible, in situ or mimicking temperature and water activity process changes.

The systematic deep characterization of lipid composition and physical properties of membranes at different steps of the production of stabilized LAB would make it possible to relate process environmental conditions, membrane fluidity, and LAB active or passive responses. This knowledge is of paramount importance for the optimization of industrial fermentations and stabilization processes of LAB starters. It will also allow by reverse engineering, to select, produce, and deliver populations of LAB with preferred characteristics in terms of membrane fluidity and physiological state.

Funding This work has received funding from the European Union's Horizon 2020 Marie Skłodowska-Curie research and innovation program under grant agreement no. 777657.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

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