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ORIGINAL ARTICLE

Effect of a sublethal high-pressure homogenization treatment on the fatty acid membrane composition of probiotic lactobacilli

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Significance and Impact of the Study: This study contributed to understand the response mechanisms activated in cells exposed to pressure stress. It has been demonstrated that high-pressure homogenization (HPH) treatments, conducted at sublethal levels, could increase some important functional and technological characteristics of nonintestinal probiotic strains. The findings of this paper can contribute to elucidate the mechanisms through which these treatments can modify these strain probiotic properties that are related to outermost cell structures, also principal target of HPH.

Keywords
fatty acid membrane composition, high-pressure homogenization, *Lactobacillus acidophilus*, *Lactobacillus paracasei*, probiotics.

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Abstract

High-pressure homogenization (HPH) has been proposed to be applied directly to lactic acid bacterial cells at sublethal levels to enhance some functional properties. As the principal target of HPH are the cell surface envelope structures, the aim of this work was to study the effect of a HPH treatment, applied at 50 MPa, on cell membrane stress responses of already-known functional strains, isolated from Argentinean products. Specifically, the membrane fatty acid composition of cells before and after the sublethal treatment was investigated, and the results showed that plasma membranes, their level of unsaturation and their composition are involved in response mechanisms adopted by microbial cells when subjected to a sublethal HPH stress. In fact, the data obtained demonstrated that the treatment was able to modify the fatty acid profile of the different strains, although a uniform response was not observed. Further studies are necessary both to elucidate the role of each fatty acid in the cell response mechanisms and to clarify the changes in membrane compositions induced by HPH treatment also in relation to the applicative potential of this technique.

Introduction

High-pressure homogenization (HPH) has been proposed with several roles in the functional dairy sector, that is for the production of probiotic dairy products with improved sensorial or functional properties, such as strain viability over refrigerated storage and accelerated fermentation kinetics (Burns *et al.* 2008a; Patrignani *et al.* 2008a,b) with less environmental impact with respect to the traditional heat treatment, analogously, to PEF and high hydrostatic pressure (HHP) (Cruz *et al.* 2010; Tsevdou and Taoukis 2011).

When low pressure (50 MPa) was applied directly to probiotic bacteria cells, increased functional properties were reported *in vitro* and *in vivo* in mice without significant effects on cell viability (Tabanelli *et al.* 2012, 2013a, b). In particular, Tabanelli *et al.* (2013a,b) studied the effects of this HPH treatment on the *in vitro* functional and biological properties of some probiotic bacteria, demonstrating that HPH can modulate hydrophobicity and auto-aggregation, in a strain-dependent way. Moreover, this HPH treatment could increase the *in vitro* resistance to simulated gastric conditions and stomach–duodenum passage of some probiotic strains. Also, Muramalla and

Aryana (2011) published on the use of some low homogenization pressures (up to 13·80 MPa for 5 passes) to improve certain probiotic characteristics of yogurt bacteria and *Lactobacillus acidophilus* LA-K, demonstrating that treatments at 13·80 and 6·90 MPa, repeated for five times, improved strain acid and bile tolerance, respectively, without effects on protease activity and strain growth.

Additionally, these low homogenization pressure treatments, applied directly to probiotic strains, modified their interaction with the small intestines of BALB mice and induced a higher IgA response compared with untreated mice in a strain- and feeding period-dependent way (Tabanelli *et al.* 2012). This behaviour was attributed to the HPH-induced modification of the cell's outermost structures, involved in probiotic–immune cell interactions and representing the main target of the pressure stress (Fantin *et al.* 1996). The membrane systems were the most susceptible structures to the application of pressure (Kobori *et al.* 1995) because the modification of its lipidic composition permits to adapt to withstand the cell's exposure to a sublethal stress. This is fundamental to allow membrane functionality maintaining the state of fluidity and/or phase behaviour in the phospholipidic bilayer (Russell *et al.* 1995). In particular, the unsaturation level, the fatty acids (FA) length and the presence of branched chains or hydroxylic groups affect the compactness of the acyl chains (Guerzoni *et al.* 1997, 2001). The modulation of membrane FA composition in response to environmental conditions affects cell surface hydrophobicity, adhesion ability and, consequently, the functional features of the strains (Kankaanpää *et al.* 2004).

Despite its wide application potential, the literature on the effects of HPH on microbial membrane FA composition is scarce, even if its effects on *Saccharomyces cerevisiae* are known (Guerzoni *et al.* 1999). By contrast, it is reported that HHP modified the plasma membrane by increasing the packing density of the lipid molecules and inducing phase separation, owing to differences in compressibility between lipid species and between lipids and membrane proteins (de Freitas *et al.* 2012).

Pressure stress can reduce membrane fluidity by packing together more tightly the fatty acyl chains. An increase in the FA unsaturation can compensate for these effects and maintain the membrane in a functional liquid crystalline state (Fernandes 2008). Lanciotti *et al.* (1997) reported that sublethal hydrostatic pressure induced several physiological and biochemical modifications in *Yarrowia lipolytica*, increasing the polyunsaturated FAs (C18:2) and the odd unsaturated FAs (C17:1). These authors concluded that FA desaturase enzymes appeared to be involved in the active response to pressure and that these mechanisms are similar to those induced by thermal stresses (Somero 1992; Guerzoni *et al.* 1997).

With this perspective, the aim of this work was to study the effects of a low-pressure homogenization treatment (50 MPa) on the FA membrane composition of lactobacilli with already-known functional features (Burns *et al.* 2008b). These strains were chosen due to their ability to enhance certain functional properties in response to the treatment to contribute to the comprehension of their response mechanisms and to an understanding of the basis of these improved performances.

Results and discussion

Cell viability immediately after HPH treatment

HPH treatment, as performed at 50 MPa, did not affect the viability of cells suspended in MRS medium. In fact, the treatment reduced the strain cell loads by less than 0·2 Log CFU per ml (Fig. 1), which was not considered a significant result, confirming the tolerance of LAB to moderate pressure. On the other hand, the severity of HPH treatment was chosen on the basis of previous works demonstrating that pressure level did not affect the cell's viability but enhanced some probiotic and technological features (Lanciotti *et al.* 2007; Tabanelli *et al.* 2012, 2013a,b). The ability to maintain good cell viability is considered an indicator of probiotic capacity and is included in the selection criteria of innovative treatments that are performed to enhance the strain's probiotic properties (Vinderola and Reinheimer 2003; Granato *et al.* 2010).

Fatty acid (FA) analysis

The relative percentages of certain FAs are reported in Table 1. The presence of significant differences in the FA concentration was tested with ANOVA. The principal FAs found were palmitic (hexadecanoic acid), oleic [(*Z*)-9-octadecenoic acid], vaccenic [(*Z*)-11-octadecenoic acid] and cyclopropane fatty acids (CFAs). On the other hand, CFAs are regarded by many authors as the major fatty acids in LAB (Suutari and Laakso 1992; Dionisi *et al.* 1999). The branched and odd-numbered saturated FAs, together with octadecanoic acid, were present in small amounts in all the samples. However, wide differences were demonstrated in the FA percentages among the different strains that were independent of HPH treatment. In fact, each strain presented a peculiar fatty acid profile that differed from the others, mainly regarding cyclic fatty acids (CFAs) and oleic acid percentages. For example, *L. acidophilus* 08, *L. delbrueckii* subsp. *lactis* 200 and 200+ showed lower percentages of lactobacillic [(*Z*)-11,12-methyleneoctadecanoic acid] acid with respect to the other strains but had higher percentages of oleic acid.

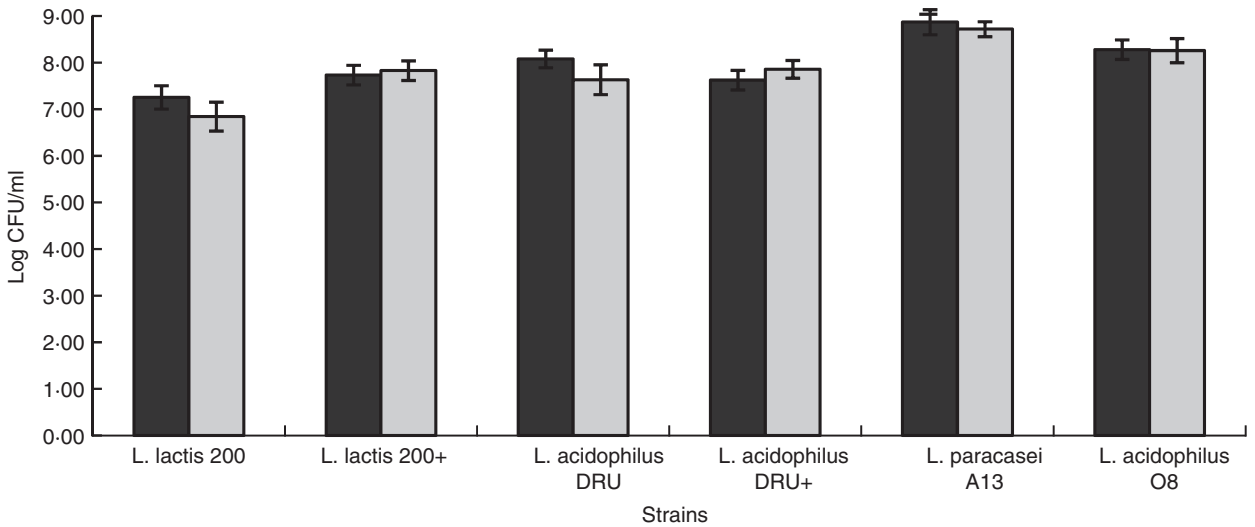

Figure 1 Cell counts of *Lactobacillus delbrueckii* subsp. *lactis* 200, 200+, *Lactobacillus acidophilus* DRU, DRU+, *Lactobacillus paracasei* A13 and *Lactobacillus acidophilus* 08 before (black bars) and after (grey bars) HPH treatment.

HPH treatment significantly ($P < 0{\cdot}05$) reduced dihydrosterculic [(*Z*)-9,10-methyleneoctadecanoic acid] and lactobacillic acids in *L. acidophilus* DRU and DRU+, *L. paracasei* A13 and *L. delbrueckii* subsp. *lactis* 200, while *L. acidophilus* 08 showed increases in the percentages of these FAs after the application of pressure. These cyclopropane FAs arise from the cyclization of oleic and (*Z*)-vaccenic acids, and they may play a role in bacterial adaptation to environmental stresses. In fact, Gómez Zavaglia *et al.* (2000) reported that these CFAs enhanced the stress tolerance of *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus* and *L. acidophilus*, as well as that the amount of CFAs in the membranes of numerous LAB increased under various stress situations (Guillot *et al.* 2000; Béal *et al.* 2001). However, the literature regarding the role of cyclic acids in membrane fluidity is quite contradictory. Grogan and Cronan (1997) attributed an increase in the structural stability and the dynamic properties of the biological membrane and a decrease in its fluidity to the presence of a cyclopropane ring among the membrane FAs. Cyclopropanation, likely the cis/trans isomerization of double bonds, was able to confer membrane chemical stability and protection against toxic compounds (including H^+) (Härtig *et al.* 2005). On the contrary, other authors asserted that CFAs, analogously to branched FAs, enhance the fluidity of the cell membrane, as they retain the ability to slide past each other and are unable to form a crystalline structure (Denich *et al.* 2003). Significant increases in palmitic and tetradecanoic acid were detected after HPH treatment in *L. paracasei* A13, *L. acidophilus* DRU and *L. delbrueckii* subsp. *lactis* 200, while *L. acidophilus 08* and *L. delbrueckii* subsp. *lactis* 200 + exhibited decreased percentages of these FAs. Vaccenic acid increased significantly in *L. paracasei* A13, *L. acidophilus* 08 and DRU+, but decreased in *L delbrueckii* subsp. *lactis* 200+. Moreover, an increase in oleic acid was detected among all of the HPH-treated strains, with the exception of *L. paracasei* A13, demonstrating the important role of specific unsaturated FAs. The crucial role of unsaturated FAs has been reported by several works and in response to several different stresses, including low or high growth temperatures, oxidative stress, acid stress and ethanol and salt addition stress (Chatterjee *et al.* 2000; Streit *et al.* 2008; Montanari *et al.* 2010; Wu *et al.* 2012). Additionally, Patrignani *et al.* (2008b) demonstrated the role of C18:1 and C18:2 in the resistance of some pathogenic species to antimicrobials, such as hexanal and (*E*)-2-hexenal.

In Table 2, the unsaturation degree, mean chain length and the ratio of CFA to saturated fatty acid (SFA) percentages are reported. The degree of FA unsaturation was significantly higher after HPH treatment for *L. acidophilus* DRU and DRU+ and *L. delbrueckii* subsp. *lactis* 200+, while no significant differences were observed for the other strains. Guerzoni *et al.* (2001) suggested that the increase in the unsaturation levels of FAs in cell membranes was a general response of certain thermotolerant strains or species when exposed to superoptimal temperatures and occurred in combination with other stresses, especially oxidative stress. In particular, oxygen-consuming desaturase activation or hyperinduction, with a consequent increase in FA desaturation, was observed as a response mechanism of *L. helveticus* to protect cells from toxic oxygen species and high temperatures. In fact, stress conditions often result in an oxidative stress for the cell due to an imbalance that occurs when the survival

Table 1 Membrane fatty acid compositions (expressed as percentages) of untreated controls (C) and treated (HPH) strains. The relative fatty acid percentages were calculated with respect to the total fatty acid area. The table only shows the compounds that reached a concentration of 0·6% in at least one sample; in particular, C12:0, C-anteiso-15:0, C15:0, C iso-17:0, C-anteiso-17:0 and C 17:0 are not reported. The results are the means of three independent experiments conducted on two different days ($N = 6$), and the standard deviation is reported within brackets

	C14:0	Ciso-15:0	C16:1Δ9	C16:1Δ11	C16:0	C18:2 (*Z,Z*)	C18:1Δ9	C18:1Δ11	C18:0	C18:2 (*Z,Z*)	C18:2 (*E,E*)	C19cyc9	C19cyc11
Lactobacillus paracasei A13 C	5·06 (±0·23)	0·12 (±0·07)	5·87 (±0·30)	1·59 (±0·16)	18·96 (±0·38)	0·79 (±0·06)	17·39 (±0·31)	12·75 (±0·30)	1·27 (±0·12)	1·42 (±0·23)	1·33 (±0·20)	20·16 (±0·11)	10·82 (±0·12)
L. paracasei A13 HPH	6·74 (±0·27)*	0·31 (±0·06)	7·24 (±0·26)*	2·13 (±0·18)*	20·28 (±0·27)*	0·40 (±0·05)*	14·97 (±0·28)*	14·16 (±0·24)*	1·06 (±0·28)	1·59 (±0·27)	1·29 (±0·17)	19·09 (±0·22)*	8·71 (±0·16)*
L. acidophilus Dru C	4·36 (±0·17)	0·07 (±0·02)	8·81 (±0·20)	0·10 (±0·06)	11·81 (±0·25)	0·39 (±0·08)	25·31 (±0·37)	18·58 (±0·25)	0·75 (±0·23)	0·50 (±0·20)	0·34 (±0·08)	19·93 (±0·31)	7·05 (±0·32)
L. acidophilus Dru HPH	5·05 (±0·18)*	0·64 (±0·04)*	9·09 (±0·21)	0·12 (±0·05)	12·79 (±0·20)*	0·42 (±0·07)	28·08 (±0·30)*	18·12 (±0·36)	0·77 (±0·24)	0·65 (±0·07)	0·49 (±0·05)	16·44 (±0·33)*	4·98 (±0·13)*
L. acidophilus Dru+ C	2·92 (±0·13)	0·07 (±0·03)	7·19 (±0·22)	–†	15·74 (±0·31)	–	12·08 (±0·23)	22·64 (±0·22)	0·63 (±0·21)	0·11 (±0·08)	0·11 (±0·03)	29·86 (±0·29)	7·47 (±0·20)
L. acidophilus Dru+ HPH	2·83 (±0·15)	0·16 (±0·07)	6·03 (±0·18)*	–	16·33 (±0·33)	–	16·54 (±0·25)*	23·97 (±0·28)*	0·60 (±0·29)	0·23 (±0·11)	0·23 (±0·06)	26·48 (±0·38)*	5·44 (±0·21)*
L. acidophilus 08 C	4·08 (±0·18)	0·29 (±0·04)	9·81 (±0·17)	0·44 (±0·12)	10·03 (±0·25)	1·00 (±0·13)	38·91 (±0·43)	1·18 (±0·16)	1·74 (±0·20)	1·55 (±0·14)	1·75 (±0·12)	25·31 (±0·30)	0·25 (±0·02)
L. acidophilus 08 HPH	3·25 (±0·17)*	0·24 (±0·07)	8·49 (±0·25)*	0·44 (±0·13)	7·71 (±0·32)*	0·89 (±0·06)	39·57 (±0·41)	2·17 (±0·15)*	0·97 (±0·13)*	1·83 (±0·18)	1·87 (±0·16)	28·25 (±0·25)*	0·30 (±0·04)
L. lactis 200 C	2·15 (±0·15)	0·23 (±0·05)	7·50 (±0·27)	0·41 (±0·14)	4·17 (±0·18)	1·75 (±0·14)	45·49 (±0·40)	2·00 (±0·17)	0·42 (±0·14)	2·94 (±0·21)	0·11 (±0·04)	28·80 (±0·21)	0·50 (±0·05)
L. lactis 200 HPH	3·22 (±0·17)*	0·26 (±0·06)	7·45 (±0·33)	0·41 (±0·09)	6·37 (±0·26)*	1·61 (±0·11)	45·94 (±0·32)	1·96 (±0·28)	1·06 (±0·18)*	2·09 (±0·25)*	0·12 (±0·03)	25·55 (±0·34)*	0·13 (±0·07)
L. lactis 200+ C	2·90 (±0·09)	0·12 (±0·04)	5·23 (±0·14)	0·21 (±0·08)	10·52 (±0·24)	0·72 (±0·07)	56·09 (±0·52)	5·55 (±0·24)	1·93 (±0·12)	1·20 (±0·20)	0·84 (±0·10)	12·02 (±0·21)	0·09 (±0·02)
L. lactis 200+ HPH	2·19 (±0·14)*	0·27 (±0·06)	5·98 (±0·20)*	0·26 (±0·11)	8·83 (±0·19)*	0·99 (±0·15)	59·32 (±0·55)*	4·07 (±0·21)*	1·02 (±0·04)*	1·97 (±0·17)	0·84 (±0·09)	12·62 (±0·23)	0·07 (±0·03)

*Significant differences ($P < 0·05$) in the fatty acid concentrations between treated and untreated strains, according to an ANOVA.
†Under the detection limit.

Table 2 The degree of unsaturation of membrane fatty acids, mean chain lengths and ratio of cyclic fatty acid (CFA) to saturated fatty acid (SFA) percentages of untreated controls (C) and treated (HPH) strains. The results are the means of three independent experiments conducted on two different days ($N = 6$), and the standard deviation is reported within brackets

	Unsaturation degree†	Mean chain length‡	CFAs/SFAs§
Lactobacillus paracasei A13 C	0·447 (±0·011)	17·293 (±0·010)	1·172 (±0·062)
L. paracasei A13 HPH	0·451 (±0·012)	17·216 (±0·013)*	0·943 (±0·064)*
L. acidophilus Dru C	0·553 (±0·012)	17·432 (±0·012)	1·522 (±0·090)
L. acidophilus Dru HPH	0·585 (±0·010)*	17·329 (±0·015)*	1·046 (±0·067)*
L. acidophilus Dru+ C	0·424 (±0·011)	17·639 (±0·014)	1·894 (±0·074)
L. acidophilus Dru+ HPH	0·475 (±0·016)*	17·625 (±0·010)	1·562 (±0·061)*
L. acidophilus 08 C	0·589 (±0·013)	17·330 (±0·013)	1·400 (±0·083)
L. acidophilus 08 HPH	0·599 (±0·017)	17·425 (±0·012)*	1·983 (±0·080)*
L. lactis 200 C	0·650 (±0·014)	17·558 (±0·011)	3·464 (±0·077)
L. lactis 200 HPH	0·634 (±0·020)	17·462 (±0·013)*	1.996 (±0·085)*
L. lactis 200 + C	0·726 (±0·015)	17·483 (±0·012)	0·707 (±0·051)
L. lactis 200 + HPH	0·772 (±0·012)*	17·685 (±0·014)*	0·920 (±0·060)

*Significant differences ($P < 0·05$) in the fatty acid concentration between treated and untreated strains, according to an ANOVA.
†Unsaturation level calculated as [percentage monoenes + 2(percentage dienes) + 3(percentage trienes)]/100.
‡Mean chain length calculated as (FAP*C)/100 (where FAP is the percentage of fatty acid and C the number of carbon atoms).
§Ratio of cyclic fatty acid (CFA) to saturated fatty acid (SFA) percentages.

mechanisms are unable to deal adequately with the reactive oxygen species (ROS) in the cells.

Also Lanciotti *et al.* (1997) concluded that fatty acid desaturase enzymes appeared to be involved in the active response of *Y. lipolytica* strains to pressure treatment through mechanisms similar to those induced by temperature stress (Somero 1992; Guerzoni *et al.* 1997). In fact, in addition to membrane fluidity, hydrostatic pressure also causes an increased production of ROS (Aertsen *et al.* 2005). Moreover, some authors reported an induction in the expression of OLE1, which encodes for a Δ9-desaturase in yeast, in *S. cerevisiae* subjected to HHP (Fernandes *et al.* 2004).

Data showed that HPH treatment decreased the fatty acid chain length in *L. paracasei* A13, *L. acidophilus* DRU and *L. delbrueckii* subsp. *lactis* 200 associated with an enhanced percentage of medium-chain fatty acid C14:0; similar results were found in the cellular FAs of lactobacilli, with proportions ranging from trace amounts to 21% (Behr *et al.* 2006; Streit *et al.* 2008). Montanari *et al.* (2010) suggested carbon chain shortening as the main strategy of *L. sanfranciscensis* to modulate the fluidity or physicochemical properties of the cytoplasmic membrane. By contrast, *L. delbrueckii* subsp. *lactis* 200+ had an increased fatty acid chain length after the treatment but associated with an increase in the ratio CFAs:SFAs.

Although the increase in unsaturation level after HPH treatment was observed in the major part of the strains considered, a uniform mechanism was not identified as response to the low pressure applied. Otherwise, despite changes in the ratio of saturation to unsaturation, cis to trans unsaturation, branched to unbranched structure and type of branching, acyl chain length and cyclization are reported as the main microbial cell responses to the physicochemical and environmental stresses (Fernández Murga *et al.* 2000; Montanari *et al.* 2010). Several authors have shown different modulation mechanisms in different strains that were also related to the cells' physiological states (Rock and Cronan 1996).

PCA

To better demonstrate the relationships between FA membrane composition and the HPH treatments, a principal component analysis (PCA) was carried out with the FA percentages detected in control and treated strains. On the other hand, the PCA is a very powerful technique, able to emphasize sample clusters in a two-dimensional space (Cruz *et al.* 2013; Tabanelli *et al.* 2013b).

Figure 2a reports the PCA loading plots for the two main factors explaining the variability of the FA profiles. Factor 1 accounted for 44·88% of the variability, and factor 2 for 18·19%. The samples can be grouped on the basis of the responses of the strain, independent of the pressure applied. Four clusters were present: cluster 1 included *L. acidophilus* DRU and its bile-resistant derivative DRU+; cluster two grouped *L. paracasei* A13; cluster three contained *L. acidophilus* 08 and cluster four grouped *L. delbrueckii* subsp. *lactis* 200 and 200+.

Figure 2b reports the variable factor coordinates for the first two factors, which represent the correlations of the single variable in each factor. Factor 1 was highly positively related with lactobacillic, vaccenic, palmitic and tetradecanoic acids and highly negatively related with

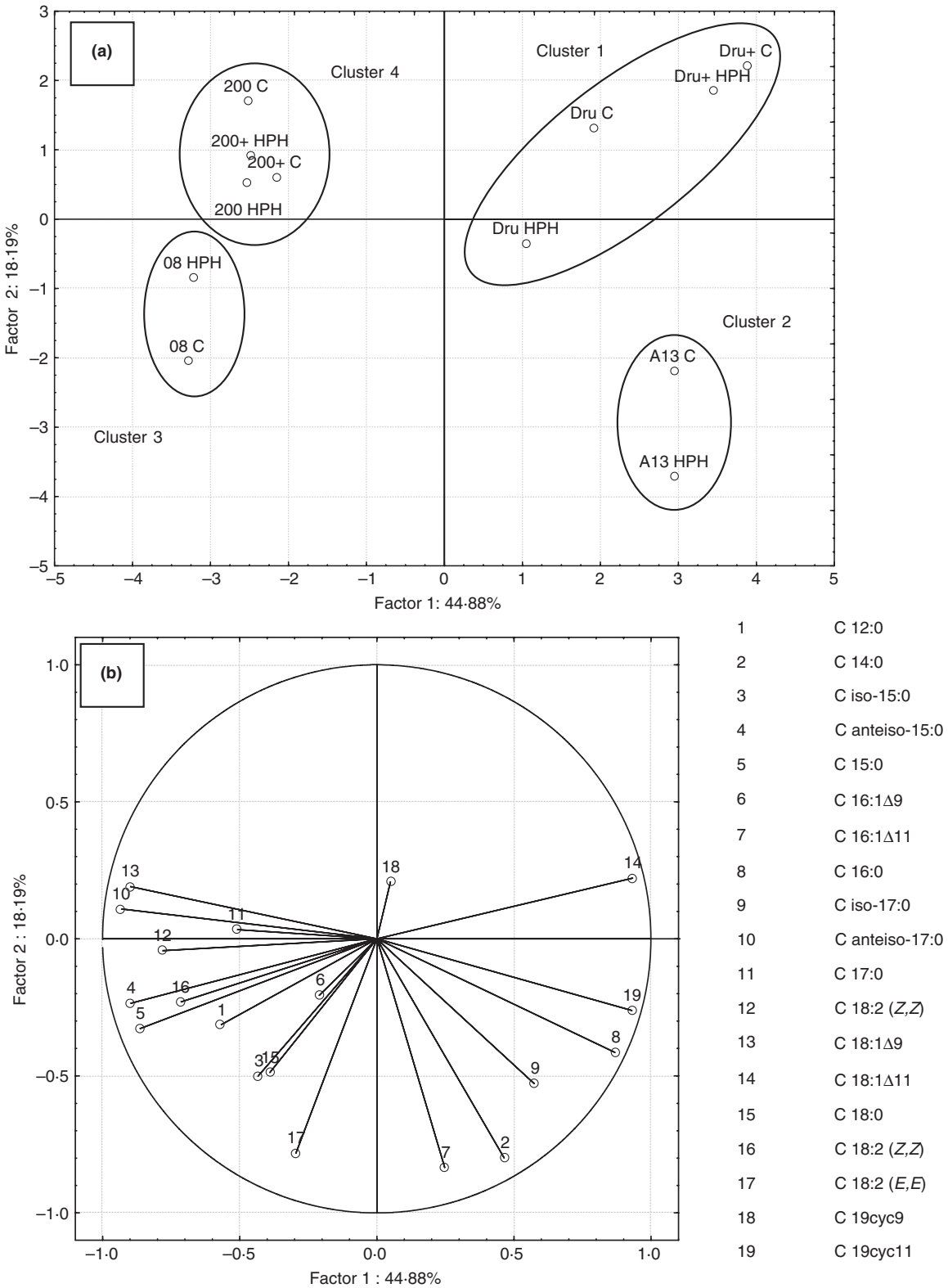

Figure 2 Principal component analysis (PCA) loading plots of the two-first factors relative to the fatty acid membrane composition of the different strains subjected or not subjected to HPH treatment (a) and variable factor coordinates for the two-first factors (b); the numbers correspond to the fatty acids reported on the right side of the figures.

polyunsaturated fatty acids, oleic acid, odd fatty acids (C15:0 and C17:0) and some branched fatty acids (C-anteiso 15:0 and C-anteiso 17:0).

Concerning factor 2, the main negative effects were determined by (*Z*)-11-hexadecenoic acid, (*E*,*E*) octadecadienoic acid and tetradecanoic acids, while the main positive effects were related with dihydrosterculic, vaccenic and oleic acids.

Lactobacillus acidophilus DRU and DRU+ are grouped in cluster 1, in which the effects of FAs such as dihydrosterculic and vaccenic acids prevailed. *L. paracasei* A13 samples (cluster 2) were grouped mainly for their high concentrations of tetradecanoic, palmitic, (*Z*)-11-hexadecenoic and lactobacillic acids. Moreover, treated *L. paracasei* A13 differed from the untreated controls regarding factor 2, which indicates an important effect of the treatment on FAs, such as lactobacillic and tetradecanoic acids. In the other clusters, the treated and untreated samples differed mainly on the basis of factor 2.

Lactobacillus acidophilus 08 and *L. delbrueckii* subsp. *lactis* 200 and 200+ were grouped in clusters 3 and 4 due to their higher percentages of oleic acid, *Z*-polyunsaturated FAs and odd FAs with respect to the other samples. Although the differences between treated and control cells were masked by the macroscopic differences among the different strains (which also belonged to different species), the PCA confirmed the lack of a uniform response for all of the strains to HPH treatment.

In conclusion, this work studied the changes in the fatty acid membrane composition in relation to the application of a low-pressure homogenization treatment, which was performed at 50 MPa. The results showed that plasma membrane composition and unsaturation are involved in the response mechanisms displayed by microbial cells when subjected to HPH stress. The PCA performed in this study allowed us to better highlight the differences in FA membrane composition before and after the treatment of each strain. It is well known that the composition of microbial FAs is the result of complex phenomena, which are implemented to maintain an optimal viability under various conditions in a different manner by different species and strains. However, further studies are necessary to elucidate the role of each FA in the cellular response mechanisms of the probiotic strains studied to further improve their technological and functional performances in functional foods.

Materials and methods

Strains

Lactobacillus paracasei A13, *Lactobacillus acidophilus* 08 and DRU, *Lactobacillus delbrueckii* subsp. *lactis* 200 and their bile-resistant derivatives *Lactobacillus acidophilus* DRU+ and *Lactobacillus delbrueckii* subsp. *lactis* 200+ were used. These strains, from the collection of the Instituto de Lactologia Industrial (INLAIN; UNL-CONICET, Santa Fe, Argentina), have been previously tested for their functional properties and are commonly used in commercial functional dairy products (Burns *et al.* 2008b). Bile-resistant derivatives were obtained as described by Burns *et al.* (2008b). The strains were maintained and cultured as reported by Tabanelli *et al.* 2013a,b.

High-pressure homogenization (HPH) treatment

Late exponential phase cells, grown in MRS broth for 18 h at 37°C up to a concentration of 9 Log CFU per ml, were subjected to a HPH treatment at a level of 50 MPa with a PANDA high pressure homogenizer (Niro Soavi, Parma, Italy) as described by Tabanelli *et al.* (2013a,b). As control samples, strain cultures were treated at a level of 0·1 MPa in the same homogenizer. The inlet temperature of samples was 37°C, and the increase rate of temperature (normally 3°C/10 MPa) was avoided using a thermal exchanger (Niro Soavi). Cell counts on MRS agar (37°C, 48 h, aerobic conditions) were performed before and after HPH treatment.

Fatty acid (FA) analysis

Control and treated cell cultures were harvested by centrifugation (6000 × ***g***, 15 min, 15°C) and washed twice with phosphate-buffered saline solution (PBS), pH 7·4. Lipid extraction was performed according to Suutari *et al.* (1990), while gas-chromatograph FA composition analyses were executed according to Montanari *et al.* (2010). FAs were identified by comparing their retention times and mass fragmentation profiles with those of the standards mix, BAME (Sigma-Aldrich, Milan, Italy). The results were the means of three independent experiments conducted on two different days ($N = 6$), and the data were expressed as a relative percentage of each FAs with respect to the total FA area.

Statistical analysis

The data were analysed by a one-way ANOVA procedure with Statistica 6.1 software (StatSoft Italy srl, Vigonza, Italy) and a principal component analysis (PCA) was applied (Statistica 6.1; StatSoft Italy srl). In the matrix used, rows represented the control and treated strains, while columns the fatty acid percentages reported in Table 2.

Conflict of interest

The Authors report no conflict of interest.

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