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Research Article

Fermented Milk Manufacture Conditions Affect *Bifidobacterium animalis subsp. lactis* BB12 Survival as a Result of Membrane Fatty Acid Composition

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Abstract

The effects of the kind of milk (organic and conventional), fermentation temperature (37°C and 42°C), final pH (4.8 and 4.4), intermediate cooling temperature (22°C, 25°C and 28°C) and duration (4, 8 and 12 hours) were investigated on acidification activity, bacterial survival and membrane fatty acids composition of *Bifidobacterium animalis* subsp. *lactis* BB12 after fermentation and storage at 4°C. Acidification time was reduced when fermentation was performed at 42°C until pH 4.4. Superior cell counts were achieved in organic fermented milks at the end of fermentation and after storage, independently of process conditions. Cultivability was higher when the cells were fermented at 42°C until pH 4.4 or maintained at 28°C for 12h before being cooled to 4°C. These conditions led to modifications of membrane fatty acid composition, thus resulting in changes of membrane fluidity. However, if the unsaturated fatty acid percentage was higher in organic fermented milks, it was lower when fermentation was conducted at 42°C until pH 4.4 and after cooling at 28°C for 12h. Finally, this study suggests that other biological mechanisms are involved in the bacterial responses to environmental conditions.

Keywords: *Bifidobacteria; Fermented Milk; Membrane Fatty Acids; Organic Milk; Survival; Adaptation*

Introduction

Interest in bifidobacteria is growing significantly due to their potential health-promoting effects on the host [1,2], including stimulation of immune system, reduced risk of gastrointestinal disorders such as antibiotic associated diarrhea [3]. In the last decade, considerable attention has been paid to the incorporation of these bacteria into functional products, including food-based products, feed supplements and pharmaceutical preparations [4,5]. By considering fermented milk products, bifidobacteria are involved in their transformation process by fermentation, generally in mixed cultures with other lactic acid bacteria [5]. In these products, viability of bifidobacteria should be at least 10^{6-7} cfu per g of product until consumption, since this concentration is necessary to determine their effectiveness [4,6].

However, bifidobacteria included in fermented milk products generally display low survival, as a result of low temperature of storage, acidic pH of the product [7] and high sensibility to oxidative stress [8]. Microbial antagonism with other bacteria is another cause of limited survival [9]. Nevertheless, during fermented milk production, acidification activity and survival through chilled storage of bifi-

dobacteria depend on the operating conditions that include the matrix composition, the fermentation temperature and final pH [10,11]. Moreover, cooling conditions also act on bacterial survival during refrigerated storage [12].

The adaptive ability of bifidobacteria to withstand acidic stress and cold temperature appears to be highly important for technological applications [13,14]. Some authors demonstrated that ensuring suitable incubation temperature and final pH [15,16] as well as cooling conditions [17,18], made it possible to obtain high survival rates at the end of the fermentation process and during storage. According to Shafiee G., *et al*. [19], the viability of probiotic microorganisms depends simultaneously on the incubation temperature and final fermentation pH. In addition, probiotic bacteria submitted to a sub-lethal thermal and acid stress increase their resistance to subsequent stress [15,20]. Cooling the cells with an intermediate step at 26°C for 8h allow lactobacilli to better resist the cold stress endured during freezing [17]. Piazentin ACM., *et al*. [12] showed that implementing a cooling process in two phases (25°C for 8h, then storage at 4°C) was responsible for an enhancement of *Lactobacillus paracasei* viability and increased concentrations of lactic acid and linoleic acid in soybean fermented milks. From this information, enhancement of cold and acid tolerance of bifidobacteria in refrigerated dairy products could be achieved after exposure of the fermented products to sub-lethal stress during manufacture.

In addition to these environmental factors, milk composition also affect the bacterial growth and survival. More specifically, organic milk was shown to be appropriate for fermented milk production, since bacterial concentrations were increased in this food matrix as compared to conventional milk [21,22]. The difference observed with conventional milk was ascribed to the specific fatty acid composition of organic milk that was characterized by higher concentrations of bioactive unsaturated fatty acids [23]. In addition, a relationship between milk fatty acid composition and bacterial membrane fatty acid content has been demonstrated and linked to bacterial viability [24].

The mechanisms underlying cold and acid tolerance include changes in cell membrane composition and properties, as well as alterations in metabolic pathways [14]. Bacterial membrane is the first target to stress conditions [25]. Thus, under moderate stress conditions, probiotic bacteria are able to adapt their fatty acids composition in order to modulate their membrane permeability and fluidity, which help them to maintain their viability under stronger stress conditions [15,26]. From these authors, this ability of the cells to modify their membrane properties following a moderate stress is a key factor to increase their survival. The modification of the synthesis of some specific proteins, which has been observed under various stress conditions, may also occur [15,27].

In this context, this work intends to investigate the physiological alterations of *Bifidobacterium animalis* subsp. *lactis* BB12 induced by the kind of milk and the operating conditions during fermentation and cooling. Their effect on acidification activity and survival during chilled storage will be related to bacterial membrane fatty acids composition.

Materials and Methods

Milks

Commercial organic and conventional UHT semi-skimmed milks (Auchan, France) were purchased from a local supermarket. In order to avoid any effect of milk composition, the same batches of milk were used in all independent experiments. UHT milks were stored at 4°C before manufacture of fermented milks.

Bacterial strain and culture conditions

Frozen cells of *Bifidobacterium animalis* subsp*. lactis* BB12 were kindly donated by Chr. Hansen, Arpajon, France. They were sub-cultured in Reinforced Clostridial Medium (RCM, Oxoid, Basingstoke, UK), supplemented with 15% (v/v) glycerol as a cryoprotectant and stored at -80°C in cryotubes. Inocula were prepared from two sub-cultured on RCM for 24h at 37°C before milk inoculation.

Manufacture of fermented milks

Organic and conventional UHT semi-skimmed milks were poured into 250 mL Erlenmeyer flasks, tempered at desired temperature and inoculated with *Bifidobacterium animalis* subsp. *lactis* BB12 at an initial concentration of 3.0×10^6 ($\pm 7.0 \times 10^5$) cfu/mL. Milk samples were incubated in a thermostatically controlled water bath until achievement of the final pH. The Cinac system (Ysebaert, Frépillon, France) was used to assess acidification activity and pH reduction during milk fermentation. The pH was continuously recorded and led to the determination of the acidification rate (Vm, in upH/min), the time at which the maximum acidification rate was achieved (tm, in min) and the time necessary to reach the desired pH (tpHfinal, in min). The higher tm and tpH final, the lower the acidification activity was [28].

Measurement of cultivability

Cultivability measurements were carried out before and after (day 1) fermentation and after 21 days storage at 4°C. Cell populations were measured by pour plate technique using Reinforced Clostridial Agar (RCA, Oxoid, Basingstoke, UK. Agar plates were incubated at 37°C for 72h under anaerobic conditions (Genbox anaer, Bio-Mérieux, Marcy l'Etoile, France). Each result was the mean of at least 12 counts and was expressed in CFU/mL.

Measurement of post-acidification

Post-acidification was determined by the difference between the pH at the end of the fermentation and the pH measured after 21 days of storage at 4°C, by using a pH-meter.

Bacterial membrane fatty acids analysis

The membrane fatty acid composition of the bacteria was determined as described by Béal C., *et al.* [29] and adapted by Florence ACR., *et al* [24].

The analyses were performed on a gas chromatograph (HP 6890, Hewlett Packard, Avondale, PA) equipped with a mass selective detector (Agilent 5973, Hewlett Packard). A capillary column packed with 70% cyanopropyl polysilphenylene-siloxane (BPX 70, 60m 0.25 mm, SGE, Victoria, Australia) was employed. Helium was used as the carrier gas (1.2 mL/min) and injection volume was 1.5 µL. Injection was done splitless for 2 minutes. Oven temperature was raised from 65 to 230°C at 5°C/min and held 10 minutes at 230°C. Injection and detection temperatures were 230°C.

Methylated fatty acid esters were identified by comparing their retention times with those of two standards: BAME and FAME (Supelco, Bellefonte, USA). The use of a mass-selective detector allowed confirming identification. Each mass spectrum was compared with data banks (NBS75K and WILEY 275.L, Mass Spectral Library, Hewlett Packard) to determine the exact carbon number. Results were expressed as relative percentages of each fatty acid, which were calculated as the ratio of the surface area of the considered peak to the total area of all peaks.

Experimental designs and statistical analyses

Two experimental designs were conducted in order to evaluate the physiological changes of *Bifidobacterium lactis* BB12 in various operating conditions during milk fermentation and to quantify its survival during cold storage (Figure 1). The first one allowed quantifying the effect of three fermentation conditions, including the type of milk (conventional and organic milks), the growth temperature that was set at 37°C or 42°C and the final pH that was fixed at pH 4.4 or pH 4.8. The second experimental design was conceived to test different cooling conditions after fermentation at 37° until pH 4.4 in the two types of milks. Fermented milks were submitted to an intermediate

cooling step, at various temperatures (22°C, 25°C and 28°C) for 4h, 8h or 12h, before final cooling in an ice bath until 4°C. Samples were stored for 21 days at 4°C. All conditions were quadruplicated.

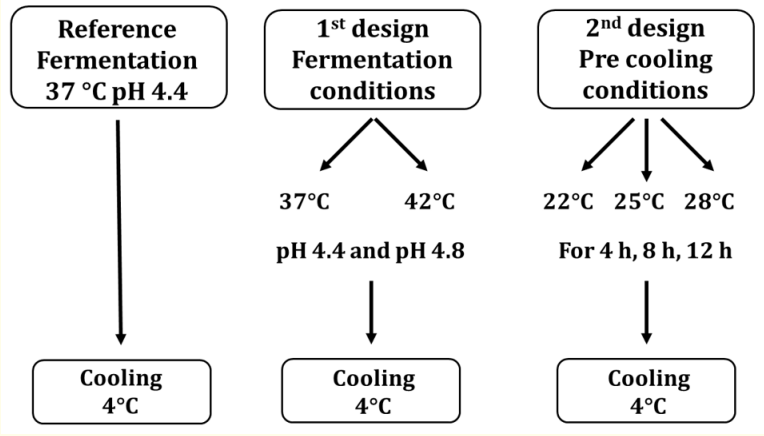


Figure 1: *Experimental designs used in the study.*

Multifactor analyses of variance and multiple comparison tests were done using Statistica 6.0 (Statsoft, Tulsa, USA) in order to determine statistical significance of differences among samples. Mean values were compared using the Newman Keuls test at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Results and Discussion

Influence of fermentation conditions on acidification activity of *B. lactis* BB12

Acidification activity of *Bifidobacterium animalis* subsp*. lactis* BB12 was affected by the type of milk and the fermentation conditions ($P < 0.001$). As expected, the highest rate of acidification (Vm) was observed when fermentation temperature was established at 42°C (5.9 ± 0.2 10^{-3} upH/min) instead of 37°C (5.2 ± 0.2 10^{-3} upH/min). These data agree with those obtained by Garro MS., *et al.* [10] with other *Bifidobacterium* strains. In addition, increasing fermentation temperature to 42°C allowed shortening the fermentation time by 2 h, in accordance with previous reports [30]. Moreover, Vm was higher ($P < 0.01$) when the fermentation was conducted in organic milk (5.6 ± 0.2 10^{-3} upH/min) as compared to conventional milk (5.1 ± 0.2 10^{-3} upH/min), thus indicating a positive effect of organic milk on the fermentation performance.

These characteristics directly influenced the time at which fermentation stopped, in addition to the final pH to be reached. From figure 2, fermentation time was firstly influenced by the final fermentation pH, as it was 2.9 ± 0.2h longer ($P < 0.05$) to attempt pH 4.4 instead of pH 4.8, in both milks, thus validating previous works [16,30]. This observation was expected as it is the direct consequence of the acidification process. Secondly, incubation temperature of 42°C allowed reducing by 15% the time to reach the final pH, by comparison with 37°C. This observation is in agreement with previous works that recommended temperatures ranging from 41 to 43°C for optimal growth of *Bifidobacterium* species of animal origin [31]. Conversely, other studies revealed that 37°C was the optimum temperature for growth of different bifidobacteria species [10,11], thus pointing out that this behavior was strain dependent. Finally, cells grown in organic milk showed significantly ($P < 0.05$) shorter fermentation times as compared to conventional milk, independently of the incubation

temperature and final pH. Metabolic activity of *B. lactis* BB12 was improved in organic milk for all the tested conditions, in accordance with the conclusions of Florence ACR., *et al.* [22,24,32] who related this difference to the different fatty acid composition of organic and conventional milks.

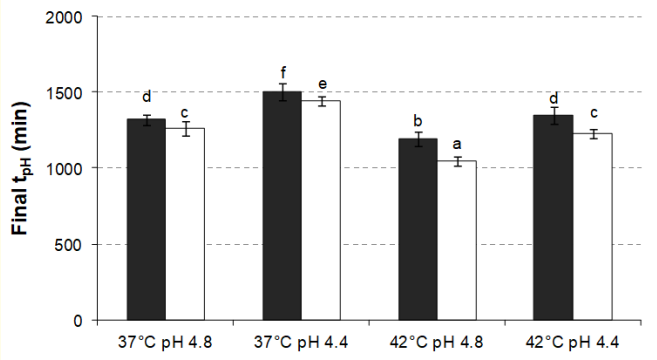


Figure 2: *Effect of fermentation temperature (37°C or 42°C) and type of milk, conventional (■) or organic (□) on the time necessary to reach final fermentation pH (Final tpH4.4 or tpH4.8, in min).*

Mean values ± standard deviation (n = 12) with different letters are significantly different; P ≤ 0.05.

Influence of fermented milk manufacture conditions on post-acidification during cold storage

Post-acidification occurred during storage of fermented milks at 4°C for 21 days. From the first experimental design, fermentations stopped at pH 4.8 and pH 4.4 showed, respectively, final pH equal to 4.65 ± 0.02 and 4.28 ± 0.04 at the end of chilled storage, thus showing a significant effect of the final fermentation pH. The post-acidification was however similar in the two conditions, with a pH variation that was equal to 0.14 ± 0.04. No significant difference ($P > 0.18$) in final pH values was observed by considering the other fermented milk manufacture conditions (kind of milk and growth temperature).

From the second experimental design, cooling conditions did not affect the pH values at the end of the storage (21 days at 4°C) that remained around 4.28 ± 0.03. This result is in agreement with the previous results obtained by Piazentin ACM., *et al*. [12] with *Lactobacillus paracasei* and by Barona M., *et al.* [33] with *Bifidobacterium breve*, *Bifidobacterium longum* and *Bifidobacterium bifidum*. Conversely, Jayamanne and Adams [4] displayed no significant post-acidification in products recovered at different final pH (4.0, 4.25 and 4.5), after fermentation by *Bifidobacterium longum* NCTC11818 and *Bifidobacterium animalis* ssp. *lactis* USCC50051. According to Garro MS., *et al.* [34], the pH of fermented products with *Bifidobacterium longum* CRL 849 decreased by one unit of pH or remained unchanged after 28 days of storage. These contradictory observations indicated that the bacterial species strongly affected the post-acidification level. However, it can be considered that *B. lactis* BB12 displayed a weak post-acidification activity of during storage at low temperature, as reported by Barona M., *et al* [33].

Influence of fermented milk manufacture conditions on cultivability of *B. lactis* BB12 after fermentation and during cold storage

During fermentation, *B. lactis* BB12 grew appreciably, from 3.0×10^6 (± 7.0×10^5) cfu/mL to 4.6×10^9 (± 1.2×10^9) cfu/mL. The final bacterial concentration was however influenced ($P < 0.01$) by the kind of milk, the fermentation temperature, the final pH and the cooling conditions (Figure 3 and 4). Final concentrations at day 1 were comprised between 3.9 ± 0.2 and 7.7 ± 0.2 10^9 cfu/mL, which was consis-

tent with previous results [21,32]. In addition, and as expected, a significant reduction ($P < 0.001$) in *B. lactis* BB12 cultivability occurred during cold storage for 21 days, independently of fermentation temperature and final pH (Figure 3B) or cooling conditions (Figure 4B). This decrease in cell concentrations occurred as a result of pH reduction, low temperature and possible oxidative stress during storage, these factors being considered as crucial for bifidobacteria survival [7,13].

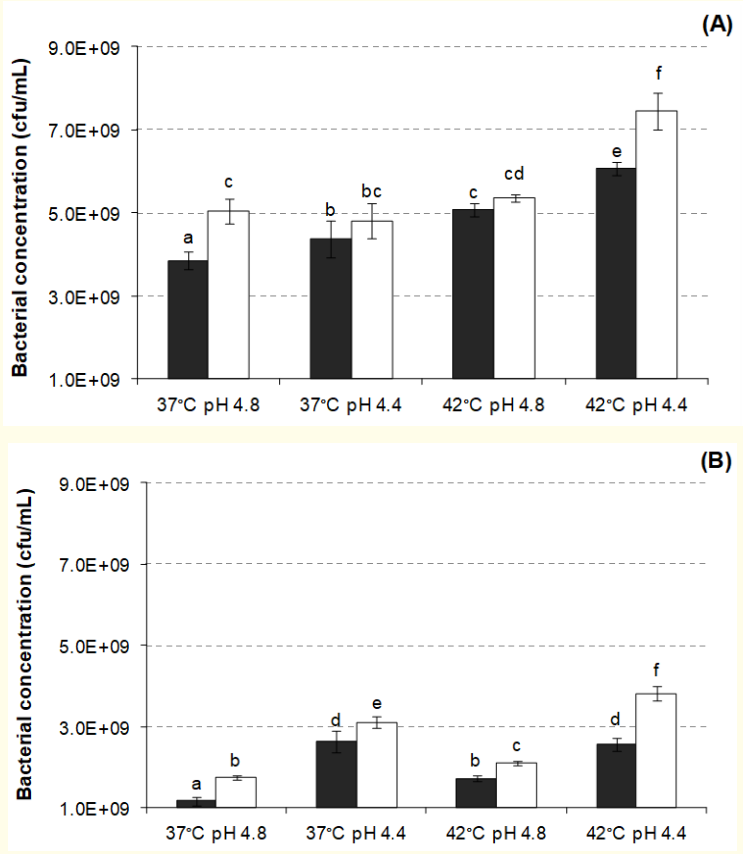


Figure 3: *Bacterial concentrations of Bifidobacterium animalis subsp. lactis BB12 in conventional (■) and organic (□) probiotic fermented milks grown at 37°C or 42°C until pH 4.8 or 4.4, after 1 day (A) and 21 days (B) of storage at 4°C. Mean values ± standard deviation (n = 12) with different letters are significantly different; P ≤ 0.05.*

Regarding the influence of kind of milk, cultivability was always higher using organic milk ($P < 0.001$), independently of fermented milk process conditions or storage time, as compared to conventional milk (Figure 3 and 4). A positive effect of organic milk was demonstrated, as bacterial concentrations at day 1 were 1.2 times higher as compared to conventional milk, whatever the culture and cooling conditions employed (Figure 3 and 4). This result agrees with previous ones [21,22,24] who showed that *B. lactis* BB12 showed significant higher counts in organic milk as compared to conventional milk.

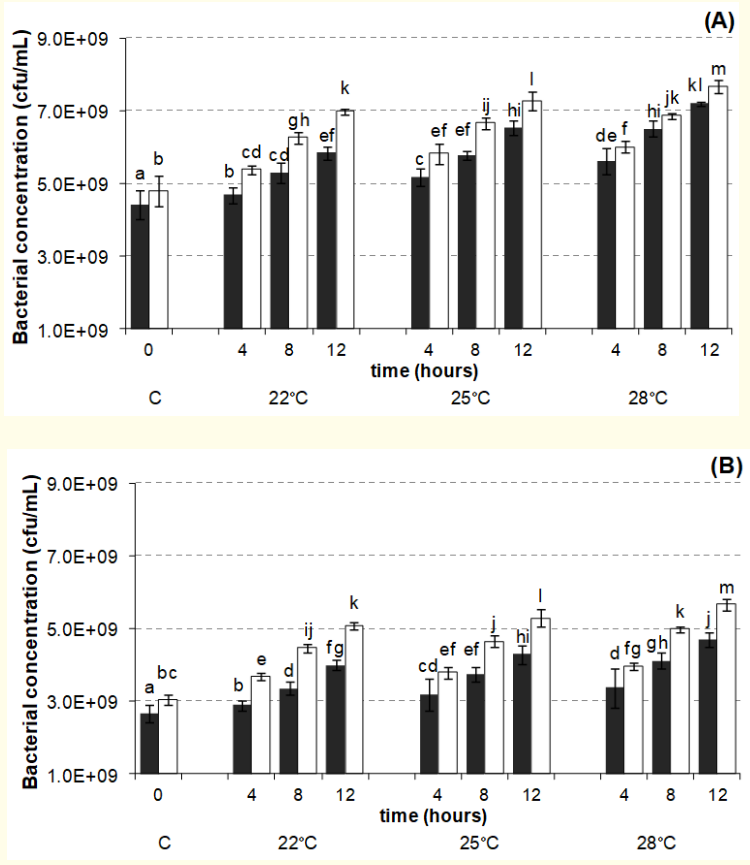


Figure 4: *Bacterial concentration of Bifidobacterium animalis subsp. lactis BB12 in conventional (■) and organic (□) probiotic fermented milks cooled directly to 4°C (control condition, C) or with a step at an intermediate temperature at 22°C, 25°C or 28°C for 4h, 8h or 12h, after 1 day (A) and 21 days (B) of storage at 4°C.*

Mean values ± standard deviation (n = 12) with different letters are significantly different; P ≤ 0.05.

Independently of fermented milk manufacture conditions, the reduction in cultivability during cold storage was less pronounced when using organic milk as raw material instead of conventional milk ($P < 0.001$). Ratios (in %) between cultivable cells concentrations measured after 21 days of storage at 4°C and concentrations measured after 1 day were calculated to quantify the cell survival. They were significantly improved ($P < 0.05$) using organic instead of conventional milk, whatever the fermentation temperature and the final pH. Survival was equal to 58.3 ± 1.7% in organic milk as compared to 53.1 ± 1.9% in conventional fermented milks. Under the different cooling conditions, survival was higher (68.5 ± 5.4%) using organic milk instead of conventional milk (62.8 ± 5.3%). This result confirms and broadens precedent results obtained by Florence, ACR., *et al*. [22,24] after at least 21 days of storage, as it proved that organic milk was more favorable to bifidobacteria survival at the end of shelf-life of the fermented products.

Fermentation temperature strongly acted on bifidobacteria cultivability (Figure 3). At day 1, bacterial concentrations were improved when the cultures were conducted at 42°C, thus indicating that this temperature was preferred by this strain. This observation is in agreement with the results obtained from acidification activity measurements, as well as with previous studies [34]. At 42°C, cell concentra-

tions were higher ($P < 0.05$) than at 37°C, with a multiplying factor of 1.6 times at pH 4.4 and 1.2 times at pH 4.8, independently of the type of milk (Figure 3A). These data agree with those of Garro MS., *et al.* [10], but they differ from those reported by Abe F., *et al.* [11] and Mortazavian A.M., *et al*. [16], who noticed lower counts of *B. longum* and *B. lactis* BB12 at 41°C instead of 37°C. After 21 days of storage at 4°C, differences triggered by fermentation temperature were reduced, even if bacterial concentrations remained higher after growth at 42°C (Figure 3B). Survival rates were improved ($P < 0.05$) when fermentation was done at 37°C (59.1 ± 1.6%) instead of 42°C (41.9 ± 6.5%). These results agree with those of Mortazavian A.M., *et al*. [16] who observed lower losses in bifidobacteria cells counts when cultivated at 37°C instead of 40°C or 44°C.

In addition to fermentation temperature, final pH also acted on bacterial concentrations (Figure 3). Cultivability at day 1 was higher when the fermentations were stopped at pH 4.4 instead of 4.8. By considering that fermentation duration was longer 2.9h at pH 4.4, this result indicated that *B. lactis* BB12 was able to grow at low pH, e.g. between 4.8 and 4.4. Previously, Béal C., *et al.* [30] also reported higher concentrations of *L. bulgaricus* when grown until pH 4.4 instead of pH 4.8. According to Ruiz L., *et al.* [35], the optimal growth pH of *Bifidobacterium* strains is around 6.7, but *B. animalis* subsp. *lactis* is considered as acid tolerant and survives well at pH 3.5 - 4.0.

After 21 days of storage at 4°C, higher cultivability was achieved when the cultures were performed until pH 4.4, as compared to pH 4.8 (Figure 3). Moreover, survival rates were significantly lower ($P < 0.05$) at pH 4.8 (34.7 ± 3.4%) as compared to pH 4.4 (60.9 ± 6.4%). These results indicate that the acid stress engendered at lower final pH, resulted in higher resistance of bifidobacteria to the stress suffered during post acidification. This interpretation is supported by previous studies [13,36] who conclude that bifidobacteria may become more tolerant during cold storage after exposition to low pH in stationary growth phase.

By considering the effect of cooling conditions (Figure 4), bacterial concentrations were increased after fermentation (day 1) and storage (day 21), when a temperature step (22, 25 or 28°C) was carried out for 4, 8 or 12h, before final cooling to 4°C. Moreover, an increase in cooling temperature and duration progressively improved cultivability during storage, whatever the kind of milk used. By comparison with the control condition (C) achieved without any temperature step, an increase of at a least 20% in cell concentration was noted after one day of storage at 4°C when an intermediate cooling step at a moderate temperature was used (Figure 4A). This positive effect was slightly more pronounced when the intermediate temperature value was enhanced ($P < 0.01$) or when the duration of this step was longer ($P < 0.03$). The higher cultivability was then achieved when cells were first cooled at 28°C for 12h, before chilling until 4°C. After 21 days of storage at 4°C, cultivability was progressively improved with the increase in temperature ($P < 0,05$) and duration ($P < 0.05$) of the cooling step, independently of the type of milk (Figure 3B). Cell concentrations at day 21 were significantly higher when the bacteria were kept at 28°C before being cooled to 4°C. In addition, cells cooled for 12h before being chilled until 4°C showed superior concentrations after 21 days of storage as compared to other conditions. Although cultivability was affected by the cooling temperature, survival during storage was not influenced by this factor ($P \geq 0.05$). The ratio between cell concentrations at day 21 and day 1 was equal to 67.2 ± 6.3% when specific cooling conditions were applied, instead of 61.9 ± 3.3% in control conditions. Cooling duration, in contrast, displayed significant positive effect on survival rates, as they were equal to 69.9 ± 3.5% when cells were maintained 12h at an intermediate temperature, instead of 61.9 ± 3.3% in control conditions. These findings are in accordance with those of Panoff JM., *et al.* [37] and Wang Y., *et al*. [17] with *L. bulgaricus* CIP 101027T and *L. acidophilus* RD758, respectively. These authors reported that cells better resisted to frozen storage when a temperature step was carried out before freezing. Recently, Piazentin ACM., *et al*. [12] demonstrated higher survival *of L. paracasei* when the cells were submitted to an intermediate cooling at 25°C for 8h. However, as this procedure was never performed by considering chilled storage with bifidobacteria, no comparison may be done with previous data. This result may thus be of great interest for further industrial applications.

Finally, some interactions occurred between the different factors (type of milk, fermentation temperature, final pH, cooling conditions), at a significant level of 0.001%. From these interactions, higher cultivability after 21 days of storage when fermentation was conducted at 42°C until pH 4.4, whereas better survival was observed when fermentations were carried out at 37°C until pH 4.4. The com-

bination of an intermediate cooling temperature (28°C) and long duration (12h) also improved *B. lactis* BB12 concentration and survival during three weeks of cold storage. Finally, cells grown in organic milk were always more tolerant to chilled storage, whatever the other operating conditions.

Impact of fermented milk manufacture conditions on membrane fatty acid composition of *B. lactis BB12* after fermentation and during cold storage

A total of 18 fatty acids were identified in the membrane of *B. lactis* BB12 under different fermented milk manufacture conditions, in organic and conventional milks. Ten of them accounted for more than 90% of membrane fatty acid composition and were identified as decanoic (capric) acid C10:0, dodecanoic (lauric) acid C12:0, tetradecanoic (myristic) acid (C14:0), pentadecanoic acid (C15:0), hexadecanoic (palmitic) acid (C16:0), hexadecenoic (palmitoleic) acid (C16:1), octadecanoic (stearic) acid (C18:0), octadecenoic (oleic) acid (C18:1), octadecadienoic (linoleic) acid (C18:2) and octadecatrienoic (linolenic) acid (C18:3). Some branched chain fatty acids, mainly represented by iso and ante-iso C15:0 and as well as 2-hydroxy decanoic (2-OH C10:0), 3-hydroxy dodecanoic (3-OH C12:0), tridecanoic (C13:0), 3-hydroxyl tetradecanoic (3-OH C14:0) and heptadecanoic acid (C17:0) were also identified. Cyclic fatty acids are basically composed of cyclopropaneoctanoic acid (cyc C19:0). These fatty acids have been previously identified in *B. animalis* subsp. *lactis* BB12 [24], in *B. longum* NCC2705 [38], *B. animalis* subsp. *lactis* IPLA 4549 and 4549dOx [39] and in other commercial strains of *B. longum* [40]. They also fitted with the reports of Denich TJ., *et al*. [41] who observed that gram-positive bacteria contained large proportions of saturated (SFA) and branched chain (BFA) fatty acids.

The membrane fatty acid compositions of *B. lactis* BB12 cells obtained under different operating conditions, including kind of milk, incubation temperature, final fermentation pH, cooling temperature and duration and storage time, are summarized in table 1 and 2.

Fatty acids	Storage time (days)		Type of milk		Incubation temperature		Final fermentation pH	
(%)	1	21	Conventional	Organic	37°C	42°C	4.8	4.4
C10:0	25.9^{d}	23.4^{c}	25.5^{d}	24.0^{c}	23.9^{c}	25.5^{d}	24.5^{ns}	24.9^{ns}
C12:0	18.7^{d}	18.3^{c}	18.9^{d}	18.2^{c}	18.0^{c}	19.0^{d}	18.6^{ns}	18.5^{ns}
C14:0	21.5^{c}	22.6^{d}	21.7^{c}	22.4^{d}	22.3^{d}	21.7^{c}	22.2^{b}	21.9^{a}
C15:0	1.0^{a}	1.2^{b}	1.0^{c}	1.2^{d}	1.2^{d}	1.0^{c}	1.1^{ns}	1.1^{ns}
C16:0	17.1^{c}	19.6^{d}	17.7^{c}	18.9^{d}	19.2^{d}	17.4^{c}	18.2^{ns}	18.4^{ns}
C16:1	1.4^{ns}	1.4^{ns}	1.4^{ns}	1.3^{ns}	1.4^{ns}	1.3^{ns}	1.4^{ns}	1.3^{ns}
C18:0	0.7^{ns}	0.7^{ns}	0.8^{ns}	0.7^{ns}	0.8^{ns}	0.7^{ns}	0.7^{ns}	0.7^{ns}
ΣC18:1	4.8^{c}	5.2^{d}	4.9^{c}	5.1^{d}	5.2^{d}	4.9^{c}	5.1^{d}	4.9^{c}
C18:2	0.5^{ns}	0.5^{ns}	0.5^{d}	0.4^{c}	0.4^{c}	0.5^{d}	0.5^{ns}	0.5^{ns}
C18:3	0.2^{ns}	0.2^{ns}	0.1^{c}	0.3^{d}	0.2^{ns}	0.2^{ns}	0.2^{ns}	0.2^{ns}
SFA	85.4^{c}	86.1^{d}	85.9^{b}	85.6^{a}	85.7^{ns}	85.8^{ns}	85.8^{ns}	85.7^{ns}
UFA	6.9^{c}	7.2^{d}	6.8^{c}	7.3^{d}	7.2^{b}	6.8^{a}	7.1^{b}	6.9^{a}
CFA	0.4^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}
BFA	7.3^{d}	6.4^{c}	6.8^{ns}	6.9^{ns}	6.8^{ns}	6.9^{ns}	6.9^{ns}	6.8^{ns}

Table 1: *Means and Newman Keuls multiple comparison tests for the main bacterial membrane fatty acids of B. lactis BB12 grown in organic and conventional fermented milks under different fermentation conditions.*

ΣC18:1: Sum of C18:1 Isomers; C18:2: Linoleic Acid Isomer Cis 9,12; SFA: Total Saturated Fatty Acids Concentration; UFA: Total Unsaturated Fatty Acids Concentration; CFA: Total Cyclic Fatty Acids Concentration; BFA: Total Branched Fatty Acids Concentration.

Different letters account for means significantly different at P < 0.01 (a, b); P < 0.001 (c, d); ns: no significant difference at P ≥ 0.05.

Fatty acids	Storage time (days)		Type of milk		Cooling temperature				Cooling duration			
(%)	1	21	Conventional	Organic	4°C	22°C	25°C	28°C	0h	4h	8h	12h
C10:0	26.8^{f}	22.7^{e}	26.0^{f}	23.6^{e}	23.5^{e}	26.0^{h}	24.4^{f}	25.2^{g}	23.6^{e}	25.2^{g}	25.5^{g}	24.8^{fg}
C12:0	18.9^{b}	17.4^{a}	18.7^{f}	17.7^{e}	17.9^{ns}	18.7^{ns}	18.0^{ns}	18.2^{ns}	17.9^{a}	18.2^{b}	18.6^{c}	18.1^{b}
C14:0	21.6^{a}	23.0^{b}	21.9^{e}	22.7^{f}	22.3^{ns}	22.4^{ns}	22.0^{ns}	22.5^{ns}	22.3^{ns}	22.3^{ns}	22.1^{ns}	22.5^{ns}
C15:0	1.0^{ns}	1.1^{ns}	0.9^{e}	1.2^{f}	1.2^{ns}	1.0^{ns}	1.0^{ns}	0.9^{ns}	1.2^{ns}	0.9^{ns}	1.0^{ns}	1.0^{ns}
C16:0	17.1^{e}	21.9^{f}	19.0^{e}	20.0^{f}	19.1^{a}	18.9^{a}	20.2^{b}	19.7^{b}	19.3^{ns}	19.6^{ns}	19.3^{ns}	19.7^{ns}
C16:1	1.2^{ns}	1.4^{ns}	1.2^{ns}	1.3^{ns}	1.5^{h}	1.1^{e}	1.3^{g}	1.2^{f}	1.5^{ns}	1.2^{ns}	1.2^{ns}	1.3^{ns}
C18:0	0.6^{ns}	0.8^{ns}	0.7^{ns}	0.7^{ns}	0.8^{ns}	0.6^{ns}	0.8^{ns}	0.6^{ns}	0.8^{ns}	0.6^{ns}	0.6^{ns}	0.7^{ns}
C18:1	4.4^{e}	5.4^{f}	4.6^{e}	5.2^{f}	5.4^{h}	4.4^{e}	5.1^{g}	4.7^{f}	5.3^{h}	4.8^{f}	4.6^{e}	4.9^{g}
C18:2	0.4^{ns}	0.4^{ns}	0.5^{f}	0.4^{e}	0.5^{ns}	0.3^{ns}	0.5^{ns}	0.4^{ns}	0.5^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}
C18:3	0.1^{ns}	0.1^{ns}	0.0^{e}	0.2^{f}	0.2^{ns}	0.1^{ns}	0.1^{ns}	0.1^{ns}	0.2^{ns}	0.1^{ns}	0.1^{ns}	0.1^{ns}
SFA	86.3^{e}	87.1^{f}	87.3^{f}	86.1^{e}	85.2^{e}	87.6^{g}	86.7^{f}	87.3^{g}	85.3^{e}	87.6^{g}	87.4^{g}	86.9^{f}
UFA	6.2^{e}	7.3^{f}	6.3^{e}	7.2^{f}	7.6^{f}	5.9^{e}	7.1^{f}	6.3^{e}	7.5^{g}	6.4^{e}	6.3^{e}	6.7^{f}
CFA	0.5^{b}	0.3^{a}	0.4^{ns}	0.3^{ns}	0.4^{ns}	0.3^{ns}	0.3^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}	0.4^{ns}
BFA	7.0^{f}	5.4^{e}	6.0^{e}	6.4^{f}	6.8^{f}	6.1^{e}	5.9^{e}	6.0^{e}	6.7^{f}	6.0^{e}	5.9^{e}	6.0^{e}

Table 2: *Means and Newman Keuls multiple comparison test for the main bacterial membrane fatty acids of B. lactis BB12 grown in organic and conventional fermented milks and cooled under different conditions.*

ΣC18:1: Sum of C18:1 isomers; C18:2: Linoleic Acid Isomer Cis 9,12; SFA: Total Saturated Fatty Acids Concentration; UFA: Total Unsaturated Fatty Acids Concentration; CFA: Total Cyclic Fatty Acids Concentration; BFA: Total Branched Fatty Acids Concentration.

Different letters account for means significantly different at P < 0.01 (a, b, c, d); P < 0.001 (e, f, g, h) and ns: no significant difference at P ≥ 0.05.

By considering the effect of storage time, the percentages of C10:0, C12:0 and total BFA and CFA significantly decreased (P < 0.001) in cellular membranes of *B. lactis* BB12, while the relative contents of C14:0, C15:0, C16:0, C18:1 and total SFA and unsaturated fatty acids (UFA) increased, during three weeks of storage at 4°C (Table 1 and 2). These changes corresponded to a decrease in medium chain fatty acids (C10:0 to C12:0) relative contents and a rise in the proportion of long chain unsaturated fatty acids (UFA), mainly represented by C18:1. This increase in C18:1 concentration as a response to stress conditions agrees with the results reported by Russell NJ., *et al.* [42] with *L. plantarum* in response to low temperature, by Béal C., *et al*., [29] with *Streptococcus thermophilus* submitted to acidic conditions and by Montanari C., *et al.* [43] with *Lactobacillus helveticus* cells after cold and acid stress. The increase in the relative levels of the straight chain fatty acids C14:0 and C16:0 was also associated to acid adaptation of lactic acid bacteria [36].

The use of the two different types of milk, organic or conventional, induced some variations on membrane fatty acid composition of *B. lactis* BB12 (Table 1 and 2). Whatever the fermentation and cooling conditions used, cellular fatty acids showed similar trend distribution. *B. lactis* BB12 cells cultured in organic milk were characterized by significant (P < 0.001) higher relative levels of C14:0, C15:0, C16:0, C18:1 and C18:3 fatty acids, to the detriment of C10:0, C12:0 and C18:2 in their membranes. The saturation degree of membrane fatty acids of *B. lactis* BB12 was lower after growth in organic milk, which allowed unsaturated and branched fatty acids to be relatively more abundant. In addition, the differences between fatty acids contents among the two types of milks were more pronounced after the different cooling treatments, as compared to the various fermentation conditions. These differences in the membrane fatty acid composition as a function of kind of milk have never been observed earlier. They may be related to the initial fatty acid composition of raw organic milk that differs from that of conventional milk, as previously stated by Florence ACF., *et al* [24]. More specifically, higher percentages of

bioactive unsaturated fatty acids, such as *trans*-vaccenic (TVA), conjugated linoleic acid (CLA) and alpha-linolenic acid (ALA) were demonstrated in organic milks as compared to conventional milks [21,22,24,32,44]. These characteristics may be exploited in the context of fermented milks production, as these fatty acids are recognized as giving health benefits to the host such as anti-arrhythmic effects in the heart, positive impact on neurological function and reduction of the incidence of some cancers [45].

As expected, growth of *B. lactis* BB12 at various fermentation temperatures resulted in different membrane fatty acid compositions (Table 1). Higher relative contents in C10:0, C12:0 and C18:2 fatty acids were found when cells were grown at 42°C. In contrast, percentages in C14:0, C15:0, C16:0 and C18:1 were lower, as compared to 37°C, whereas no significant changes were detected on C16:1, C18:0 and C18:3 fatty acids. These results agree with previous ones, as C16:0 relative content in *L. acidophilus* CRL640 increased at low temperature [46] and as C18:1 relative concentration in *L. plantarum* was enhanced by lowering the temperature [42]. Nevertheless, they differed from those obtained with *L. acidophilus* RD758, as lower relative levels of these two fatty acids were found at 37°C, as compared to 42°C [18]. These different results may be ascribed to the different bacterial species tested. The chain length was also affected by fermentation temperature, as cells of *B. lactis* BB12 showed an increase of 3 % in the chain length of the fatty acids at 37°C as compared to 42°C. This observation was in agreement with that reported by Veerkamp JH. [50] with *Bifidobacterium bifidum* subsp. *pennsylvanicus*. Despite the differences observed in individual fatty acids distribution, the total percentages of SFA, CFA and BFA were not significantly influenced by the fermentation temperature ($P > 0.07$). On the contrary, the relative content of UFA slightly decreased by increasing growth temperature (Table 1), in accordance with previous reports [46,47].

The final fermentation pH appeared to have minor impact on membrane fatty acid composition of *B. lactis* BB12, as previously reported by Veerkamp JH. [47] with *B. bifidum.* When final fermentation pH was set at pH 4.8, slightly higher percentages of C14:0 and C18:1 were found in bacterial membranes, as compared to pH 4.4. A reduction in C14:0 and C18:1 relative contents after acidification was also observed in the membranes of *L. acidophilus*, *L. plantarum*, *L. lactis* and *L. sanfranciscensis* [43]. Moreover, the C18:1 percentage was shown to decrease with decreasing fermentation pH in *S. thermophilus* [29] and *L. acidophilus* [15].

When different cooling temperatures were applied, the fatty acids distribution in *B. lactis* membranes only slightly differed as compared to control conditions. For most of the fatty acids, difference was not significant ($P \geq 0.05$). However, significant higher contents in C10:0 and C16:0, but lower concentration in C16:1 were pointed out, when a specific cooling procedure was performed, as compared to a rapid cooling until 4°C. These results are in agreement with the findings of Wang, Y., *et al.* [17], who demonstrated that low growth temperatures led to a larger synthesis of unsaturated fatty acids in *L. acidophilus* membranes, as compared to high temperatures. These findings led to an increase of SFA and a decrease of BFA when a temperature step was applied before cooling at 4°C. A decrease in branched fatty acids concentration was also induced by a temperature increase in *Pediococcus* sp. [48].

Finally, the analysis of variance showed slight differences in the fatty acid content as a function of cooling duration. The C10:0 and C12:0 relative contents were increased after at least 4h of cooling, whereas the C18:1 content decreased when the cooling duration increased. The other cellular fatty acids of *B. lactis* BB12 were not significantly affected ($P \geq 0.05$). As a consequence of these changes, the SFA content was enhanced when the UFA and BFA contents were reduced. These modifications were already observed after a 4h cooling step, with only small changes after 8h and 12h. They indicated that the membrane modifications occurred rapidly after cooling and that the maintenance of the cells at an intermediate temperature between fermentation and storage temperature for a longer time only slightly modified more their fatty acid content. As this experimental approach has never been implemented earlier, these results are new and cannot be compared with previous ones.

Finally, these results indicated that, if the main changes in the membrane composition occurred during the fermentation step and the beginning of the cooling step, they also happened during the storage, despite the fact that the cells were not growing. As the cell cultivability decreased during storage, a relationship might be established between these characteristics.

Relationship between fermented milk manufacture conditions, membrane fatty acid composition and *B. lactis* BB12 survival during cold storage

Survival of *B. lactis* BB12 during chilled storage in fermented milks was affected by milk composition (conventional or organic), fermentation conditions (temperature and pH) and cooling conditions (step at an intermediate temperature and duration). It was improved by using organic milk, by performing the milk acidification at 42°C until pH 4.4 and by precooling the fermented product with an intermediate step at 28°C for 12h before cooling at 4°C. In the same time, membrane fatty acids composition of *B. lactis* BB12 changed, as a result of these environmental conditions.

By considering the effects of environmental fermentation and cooling conditions, higher cellular concentration and better resistance during storage at 4°C of *B. lactis* BB12 were linked to a low unsaturated fatty acid content that was mainly explained by a low C18:1 relative concentration and high C10:0 and C12:0 contents. These characteristics were achieved at high fermentation temperature (42°C), low fermentation pH (pH 4.4) and by applying an intermediate cooling temperature for a few hours. However, by considering the type of milk, the modifications induced in the cellular membrane acted differently on cell survival. The better cultivability and survival rates that were found in cells grown in organic milk as compared to conventional milk, independently of process conditions, were related to a lower saturated fatty acids content, but higher unsaturated and branched fatty acid relative concentrations. These observations seemed to be contradictory as high survival of *B. lactis* BB12 was associated to low unsaturated fatty acid level when considering the effects of fermentation and cooling conditions, but to high unsaturated fatty acid content with respect to milk composition. This apparent discrepancy indicated that other biological mechanisms may be involved in the responses of *B. lactis* BB12 to the different conditions encountered within this study. Among them, the expression level of some genes and the differential synthesis of specific intracellular proteins are two other physiological responses to environmental and stress conditions that may be taken into account [49]. These biological responses can be identified through transcriptomic analyses and proteomic approaches, respectively, as suggested by Streit F., *et al.* [27] and Zomer A., *et al* [49].

Conclusion

This work demonstrated that acidification activity, cultivability and membrane fatty acid composition of *Bifidobacterium lactis* BB12 after growth and during storage at 4°C in fermented milks were affected by the kind of milk and the operating conditions during fermented milk manufacture and cooling. Fermentation time was lower when temperature was set at 42°C instead of 37°C and when final pH was higher (pH 4.8 vs. 4.4). The highest cultivability after fermentation and at the end of cold storage was obtained when the cells were cultured in organic milk at 42°C until pH 4.4 and cooled with an intermediate step at 28°C for 12 h before reaching 4°C. In addition, whatever the conditions used, handling of organic milk instead of conventional milk always led to better survival of the *Bifidobacteria*.

The survival of *B. lactis* BB12 was related to its membrane fatty acid composition that varied according to the operating conditions and to the kind of milk. Surprisingly, modifications of membrane composition differed according to the considered factor, as unsaturated fatty acid relative content was higher in organic fermented milk products, but lower after using optimized operating conditions. These results indicated that other physiological modifications affected the bacterial survival. In the future, the characterization of the transcriptome and the proteome of *B. lactis* BB12 may be done to achieve a more in-depth understanding of the biological responses of this species to environmental changes. In addition, as bifidobacteria may display health-promoting effects on the host, the effects of the studied operating on these specific properties may also be characterized.

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Conflict of Interest

None declared.

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