Fatty acid profile, trans-octadecenoic, α-linolenic and conjugated linoleic acid contents differing in certified organic and conventional probiotic fermented milks

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Fatty acid profile, *trans*-octadecenoic, α-linolenic and conjugated linoleic and acid contents differed in certified organic and conventional probiotic fermented milks

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Abstract

Development of dairy organic probiotic fermented products is of great interest as they associate ecological practices and benefits of probiotic bacteria. As organic management practices of cow milk production allow modifying the fatty acid composition of milk as compared to conventional milk, we intend to study the influence of the type of milk on some characteristics of fermented milks, such as acidification kinetic, bacterial counts and fatty acid content. Conventional and organic probiotic fermented milks were produced using *Bifidobacterium animalis* subsp. *lactis* HN019 in co-culture with *Streptococcus thermophilus* TA040 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340. The use of organic milk provided higher acidification rate and cultivability of *L. bulgaricus*. Fatty acids profile of organic fermented milks showed higher amounts of *trans*-octadecenoic acid C18:1 (1.6 times) and polyunsaturated fatty acids including *cis*-9 *trans*-11 C18:2 conjugated linoleic (CLA - 1.4 times), and α-linolenic acids (ALA - 1.6 times), as compared to conventional fermented milks. These higher levels were the result of both initial percentage in milk and increase during acidification, with no further modification during storage. Finally, using bifidobacteria slightly increased CLA relative content in the conventional fermented milks, after 7 days storage at 4°C, whereas no difference was pointed out in organic fermented milks.

Keywords:

Bifidobacterium, organic milk, fermented milk, *trans*-octadecenoic acid, conjugated linoleic acid, alpha-linolenic acid

1. Introduction

Organic methods of food production have gained increased public interest over the last couple decades, mainly in the western world. Organic and conventional dairy productions differ in feeding regimens, use of antibiotics and chemotherapeutic treatments, and handling of the animals (Collomb et al., 2008). Organic milk is produced in an agro-system under more constrained conditions in which the use of synthetic livestock additives or other artificial inputs, as well as genetically modified organisms are forbidden. This production relies on ecological practices that prohibit the use of antibiotics, hormones and any synthetic chemical fertilizers (Toledo, Andrén, & Bjorck 2002).

Milk is an excellent source of lactose, dairy proteins as caseins and whey proteins, calcium and it contains other minerals and trace elements. According to Ellis et al. (2006) there is little or no difference between organic and conventional milk samples by considering their carbohydrate, proteins and minerals contents. Conversely, significantly higher amounts of polyunsaturated fatty acids (PUFA), conjugated linoleic (CLA) and n-3 fatty acids are found in organic milk (Collomb et al., 2008). This is also confirmed by Butler, Stergiadis, Seal, Eyre, & Leifert (2011), who indicated that fatty acid profile and antioxidant content of milk are influenced by management (organic or conventional), season and brands. The distribution of these fatty acids in milk is important as it confers different characteristics to the milk (Ekinci, Okur, Ertekin, & Guzel-Seydim, 2008).

Among the unsaturated fatty acids, the relative concentration of three main long chain fatty acids (LCFA) differed according to the kind of milk. Conjugated linoleic acid, an isomer of linoleic acid (C18:2), has gained considerable attention due to its potentially beneficial biological effects (Gnädig, Xue, Berdeaux, Chardigny, & Sebedio, 2003), including anticarcinogenic, antiatherogenic, antidiabetic and immune stimulation. The *trans* fatty acids content in milk represents about 2% of total fatty acids, which can be increased to 4–10% of total fatty acids by enhancing dietary unsaturated oils content in the cow's diet. *Trans*-vaccenic acid, known as (*E*)-11-octadecenoic acid (C18:1 *trans*-11, or TVA), is the main *trans* fatty acid isomer found in the fat of ruminants and in dairy products such as milk and yogurts (Santora, Palmquist, & Roehrig, 2000). It participates in CLA production, through enzymatic action of Δ-9-desaturase in mammary glands (Gnädig et al., 2003), and contributes to supply the human body's in CLA (Butler et al., 2011). It is also an intermediate fatty acid of CLA biohydrogenation pathway (Bergamo, Fedeli, Iannibelli, & Marzillo, 2003). Finally, α-linolenic acid (ALA), the major omega-3 fatty acid in milk, has been related to the ability to exert anti-arrhythmic effect in the heart, to have a positive impact on neurological function by limiting central nervous system injury and to protect against coronary heart disease (Barceló-Coblijn & Murphy, 2009). It is also the dietary precursor for three long-chain omega-3 polyunsaturated fatty acids (LC-PUFA) synthesis: eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) (Brenna, Salem Jr, Sinclair, & Cunnane, 2009).

Production of fermented milks using bifidobacteria is a big challenge in dairy industry because milk, on the whole, is not a suitable matrix for the growth of lactic and probiotic bacteria since they lack essential proteolytic activity (Oliveira, Sodini, Remeuf, & Corrieu, 2001). Interest of bifidobacteria for human health is related to their survival through gut intestinal tract and to their role for stimulating the immune system and for preventing microbial gastroenteritis (Foligne et al., 2007; Hols et al., 2005). In addition, CLA production by bifidobacteria was shown to be a possible mechanism for their health enhancing properties (Oh et al., 2003).

Until now, few studies have explored the effect of organic milk on the growth of bifidobacteria and yogurt starters. From our knowledge, only the work of Florence et al. (2009) described the acidification profile, fatty acids contents, and chemical composition of organic and conventional milks fermented by bifidobacteria in co-culture with *Streptococcus thermophilus.* These authors detected higher protein and iron concentrations in organic fermented milks, although no difference was observed in the initial milk. In addition, they found higher relative concentrations of TVA and CLA in organic fermented milks. From this information, a better knowledge about acidification kinetics and milk composition of organic and conventional fermented milk products is needed. In this context, this study aimed at characterizing the behavior of bifidobacteria and yogurt starters during organic and conventional milk fermentation. Their impact on milk composition, in terms of overall fatty acid composition, and *trans*-octadecenoic, conjugated linoleic and α-linolenic acid relative contents, were determined and compared, during fermentation and cold storage of the fermented milks.

2. Material and methods

2.1. Milks

Commercial organic (Naturallis, São Paulo, Brazil) and conventional (Batavo, São Paulo, Brazil) UHT whole milks were purchased from local supermarket. They were heat treated at 85°C for 15 minutes in a water-bath (Lauda, Type A100, DR. R. Wobser GmbH & Co. KG, Germany), under constant stirring. They were cooled down to 10°C and stored overnight at 4 °C before manufacture of fermented milks.

Skimmed milk powder (Molico, Nestlé, São Paulo, Brazil) was reconstituted at 10% (w/w) and heat treated at 121 °C for 15 minutes. It was used for inoculum preparation.

2.2. Preparation of cultures

Three commercial freeze-dried strains of probiotic and yogurt cultures were employed: *Streptococcus thermophilus* TA040 (Danisco, Dangé-Saint-Romain, France), *Lactobacillus delbrueckii* subsp*. bulgaricus* LB340 (Danisco, Madison, WI) and *Bifidobacterium animalis* subsp*. lactis* HN019 (Danisco, Madison, USA).

Each lyophilized strain was weighted and rehydrated in 50 mL of sterilized skimmed milk at 42 °C for 15 min before use, as recommended by the manufacturer.

One mL of each rehydrated culture was inoculated in 500 mL of organic and conventional milk allowing initial counts of 6.0 $\log_{10}$ colony forming units (CFU)/mL.

2.3. Experimental procedure

Organic and conventional UHT heat treated milks were tempered at 42°C, divided into two batches, and inoculated with two combinations of starter cultures. Yogurt was achieved by inoculating both *S. thermophilus* TA040 (50%) and *L. bulgaricus* LB340 (50%) and probiotic fermented milk was prepared by inoculating these two strains (33% each) and *Bifidobacterium lactis* HN019 (33%). Inoculated milk samples were incubated at 42°C in a thermostatically controlled water bath until pH reached 4.5. The pH and the acidification rate (dpH/dt, in upH/min) of each microbial blend were monitored by using the Cinac system (Ysebaert, Frépillon, France). The time to reach pH 4.5 ($t_{pH4.5}$, in hours) was used to differentiate the mixed cultures. After achievement of pH 4.5, the fermentations were stopped by rapid cooling in an ice bath until 10 °C. The samples were dispensed into 50 mL polypropylene cups, thermally sealed using Selopar equipment (BrasHolanda, Pinhais, Brazil) and stored at 4 °C until required for analysis. The samples were prepared in duplicate, and the experiment was replicated twice on different days.

Before fermentation, at final fermentation time and after 7 days of storage at 4°C, the cultivability (CFU/mL) of yogurt and probiotic bacteria, the fatty acids profile of milk and fermented milks, including *trans*-octadecenoic acid, CLA and ALA relative contents were determined.

2.4. Chemical composition of milks

Fat, proteins, total solids content and density were determined with an ultrasonic milk analyzer Ekomilk (Eon Trading, Stara Zagora Bulgaria). Titratable acidity was analyzed as recommended by AOAC (1995) and lactose concentration was determined according to the Lane–Eynon method based on the reduction of copper (AOAC, 1995). A digital potentiometer (Mod.8603, Mettler-Toledo, Scherzenbach, Switzerland) was used for pH measurements. All analyses were duplicated.

2.5. Cultivability measurements

The CFU counts ($\log_{10}$ CFU/mL) were determined in triplicate. *S. thermophilus* and *L. bulgaricus* were respectively plated onto M17 lactose agar and MRS agar (Oxoid, Basingstoke, UK), previously acidified to pH 5.4 with acetic acid. *B. lactis* was enumerated in RCA (Oxoid, Basingstoke, UK) added with 2 μg/mL of dicloxacillin (pH 7.1) and 0.3 g/L aniline blue (InLab, São Paulo, Brazil). They were incubated at 37 °C for 48 h under anaerobic conditions (AnaeroGen, Oxoid, Basingstoke, UK). CFU were counted after anaerobic incubation at 37 °C for 72 h of at least four replicates.

2.6. Fatty acids extraction and analysis

The lipids were extracted from organic and conventional UHT milks, yogurts and probiotic fermented milks, according to Iso method 14156 (ISO, 2001), which is a dedicated method for extraction or separation of lipids and liposoluble compounds from milk and milk products. Fatty acids methyl esters (FAME) of milk lipids were prepared by transesterification according to Iso method 15884 (ISO, 2002), that consists in a base-catalyzed methanolysis of the glycerides, followed by a neutralization with crystalline sodium hydrogen sulfate to avoid saponification of esters.

Analyses of FAME were carried out in a gas chromatograph, model 3400CX (Varian, Walnut Creek, Ca., USA) equipped with a split-injection port, a flame-ionization detector and a software package for system control and data acquisition (model Star Chromatography Workstation version 5.5). Injections were performed in a 30 m long fused silica capillary column with 0.25 mm internal diameter, coated with 0.25 µm Chrompack CP-Wax 52CB (ChromTech, Apple Valley MN., USA). Helium was used as carrier gas at a flow rate of 1.5 $mL.min^{-1}$ and a split ratio of 1:50. The injector temperature was set at 250 °C and the detector at 280 °C. The oven temperature was initially set at 75 °C for 3 min, then programmed to increase to 150 °C at a rate of 37.5 °C min^{-1}, and then to 215 °C at a rate of 3 °C min^{-1} (Luna et al., 2004). Samples (1 µL) were injected manually after a dwell-time of *ca* 2s. Qualitative fatty acid composition of the samples was determined by comparing the retention times of the peaks with those of standards 05632 and 189-19 (Sigma, Chemical Co., St Louis, MO, USA). The relative content of each FAME was calculated from the area of each peak, and expressed as a percentage, according to the official method Ce 1-62 (AOCS, 1997). Results were grouped and expressed as percentages of short chain fatty acids (SCFA - C4:0 and C6:0), medium chain fatty acids (MCFA - C8:0 to C15:0), long chain fatty acids (LCFA - C16:0 to C18:3), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), according to Ackman (2007). All samples were analyzed in quadruplicate.

2.7. Statistical analysis

General Linear Models (GLM), multifactor analyses of variance (ANOVA) and multiple comparison tests were done using Statistica 8.0 (Statsoft, Tulsa, USA) in order to determine statistical significance of differences among samples. Mean values were compared using the Newman Keuls test at $P < 0.05$.

3. Results and discussion

3.1 Composition of organic and conventional milks

The chemical composition, which is expressed as percentage (%), was similar by considering conventional and organic milks. The contents in fat (3.0 ± 0.05%), total solids (11.7 ± 0.09%) and lactic acid (0.15 ± 0.01%) were similar in both milks, as measured before fermentation (day 0). Conversely, protein (2.4 ± 0.0%) and lactose (4.7 ± 0.1%) concentrations were significantly lower in organic milk than in

conventional milk (2.8 ± 0.1% and 4.9 ± 0.1%, respectively). The chemical composition of organic and conventional cow milks found in the present study was comparable to those reported by (Sola-Larrañaga & Navarro-Blasco, 2009). On the contrary, Toledo et al. (2002) reported similar levels of lactose but higher fat and protein concentrations. Differences in milk composition can be attributed to management system, season, and sampling periods in which the milk was purchased (Butler et al., 2011).

Table 1 summarizes the percentage of total identified fatty acid composition of the four kinds of fermented milks, before (0) and after fermentation (1) and after (7) days of storage at 4°C. The fatty acid composition of conventional and organic milks differed according to the kind of milk used for the fermentation. Their distribution according to chain length allowed separating short chain (SCFA), medium chain (MCFA) and long chain fatty acids (LCFA). The saturation degree allowed classifying the fatty acids into saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

The main fatty acids encountered in milk corresponded firstly to saturated fatty acids such as myristic acid (C14:0, 12.1-12.7%), palmitic acid (C16:0, 28.9-31.9%) and stearic acid (C18:0, 9.6-12.2 %). Monounsaturated fatty acids were also found. Among them, oleic acid (C18:1 cis-9, 21.3-21.8%), palmitoleic acid (C16:1 cis-9, 1.5-1.9%) and *trans*-octadecenoic acid (*trans*-C18:1, 2.1-3.3%) were the more abundant. Thirdly, polyunsaturated fatty acids were detected. The PUFA fraction was mostly composed by linoleic acid (cis-9 cis-12 C18:2, 1.6-1.9%), conjugated linolenic acid (*cis*-9 *trans*-11, CLA, 0.7-1.0%) and α-linolenic acid (cis-9 cis-12 cis-15 C18:3, ALA, 0.3-0.5%). PUFA and MUFA concentrations were, in this study, lower (2.5-3.5% and 27-28%, respectively) than those found by Rodríguez-Alcalá, Harte, & Fontecha (2009) in cow milk (5.7% for PUFA and 32.9% for MUFA). As a consequence, higher relative contents in SFA were found in the present study, 68-71% as compared to 60% obtained by Rodríguez-Alcalá et al. (2009).

From Table 1, the fatty acid composition results expressed as % of total fatty acids differed according to chain length in organic and conventional milks, as measured before fermentation. The relative content of short chain fatty acids (SCFA; C4:0 and C6:0) was lower in organic milk (5.6% instead of 6.4%) than in conventional milk. The medium chain fatty acid (MCFA; C8:0-C15:0) percentage was slightly lower in organic milk (difference of 0.6%). These data are in agreement with those reported by Collomb et al. (2008) that equally did not find significant difference according to the long chain length (LCFA; C16: -C18:3) in organic and conventional milks.

Table 1. Evolution of identified fatty acids methyl esters composition (%) in organic and conventional milks fermented by yogurt cultures or probiotic yogurt cultures

Kind of milk	Sample	Time (days)	SCFA	MCFA	LCFA	SFA	MUFA	PUFA
Organic	Y	0	5.51±0.18^{abc}	21.69±0.36^{a}	71.76±0.25^{abc}	68.86±0.20^{ab}	27.89±0.26^{ab}	3.36±0.04^{bc}
	Y	1	5.08±0.16^{a}	22.08±0.19^{ab}	72.82±0.312^{bc}	68.25±0.18^{a}	28.13±0.18^{b}	3.61±0.02^{cd}
	Y	7	5.29±0.14^{ab}	21.87±0.16^{ab}	72.84±0.30^{bc}	68.11±0.24^{a}	28.24±0.21^{b}	3.65±0.04^{d}
	PY	0	5.61±0.13^{abc}	22.69±0.15^{ab}	71.72±0.25^{abc}	68.89±0.15^{ab}	27.73±0.13^{ab}	3.39±0.01^{bc}
	PY	1	5.10±0.08^{a}	21.71±0.33^{a}	73.20±0.36^{c}	68.44±0.32^{a}	27.97±0.19^{ab}	3.60±0.05^{bcd}
	PY	7	5.48±0.22^{abc}	22.07±0.26^{a}	72.46±0.46^{abc}	68.48±0.13^{a}	27.92±0.13^{ab}	3.60±0.02^{bcd}
Conventional	Y	0	6.31±0.38^{bc}	23.97±0.84^{b}	70.00±0.97^{ab}	71.08±0.87^{c}	26.60±0.40^{a}	2.62±0.10^{a}
	Y	1	5.00±0.09^{a}	21.64±0.16^{a}	73.35±0.21^{c}	69.01±0.18^{ab}	28.19±0.16^{b}	2.58±0.08^{a}
	Y	7	5.56±0.25^{abc}	23.47±0.61^{ab}	70.97±0.85^{abc}	70.42±0.37^{bc}	26.96±0.32^{ab}	2.61±0.04^{a}
	PY	0	6.30±0.41^{bc}	23.99±0.89^{b}	70.08±1.01^{ab}	71.12±0.85^{c}	26.67±0.49^{a}	2.58±0.08^{a}
	PY	1	5.44±0.21^{abc}	22.79±0.60^{ab}	71.39±0.93^{abc}	69.67±0.50^{abc}	27.27±0.49^{ab}	2.69±0.14^{a}
	PY	7	4.89±0.01^{a}	21.66±0.04^{a}	73.44±0.04^{c}	69.18±0.01^{ab}	28.11±0.03^{b}	2.71±0.02^{a}

Abbreviations: Y = yogurt culture; PY = probiotic yogurt culture; Short Chain fatty acid (SCFA, C4:0 to C6:0); Medium Chain fatty acid (MCFA, C8:0 to C15:0); Long Chain fatty acid (LCFA, C16:0 to C18:3); SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid; 0 = days 0 (before fermentation), 1 (1 day after fermentation) and 7 = (7 days storage at 4°C). Mean values (N = 4), ± standard deviation with different letters in the same column are significantly different ($P \leq 0.05$).

The proportion in saturated fatty acids (SFA) was slightly higher in conventional milk (+2%). Conversely, for Collomb et al. (2008) and Ellis et al. (2006), organic and conventional milks did not significantly differ with respect to SFA. By regarding MUFA, their proportion was always lower in conventional fermented milks (-2%). Nevertheless, these results are contradictory with that obtained by Ellis et al. (2006) who found higher amounts of MUFA in conventional milks. More specifically, *trans*-C18:1 relative content was 1.6 times higher in organic products (Fig. 1A), in agreement with data reported by (Bergamo et al., 2003). After all, percentage of PUFA fraction was 1.3 times higher in organic products, when compared to conventional milks, as previously reported by (Ellis et al., 2006). Among these PUFA, the linoleic acid (LA - C18:2) was higher in organic milk, with 1.9 ± 0.02% instead of 1.6 ± 0.01% for conventional products. The initial relative contents of CLA (1.0 ± 0.01%) and ALA (0.5 ± 0.00%) were 1.4 and 1.6 times higher in organic milk (Fig. 1B and 1C). Even if Ellis et al. (2006) did not confirm that as a general rule, similar findings were reported by Bergamo et al. (2003) and Collomb et al. (2008).

Finally, the main difference observed in fatty acid composition of conventional and organic milks was related to the higher unsaturated fatty acid content in organic milk. It could be ascribed to the feeding regiment of the cows, as demonstrated by Bergamo et al. (2003), Butler et al. (2011) and Collomb et al. (2008).

3.2. Fermentation profile

The acidification profiles of yogurt made with *S. thermophilus* TA040 and *L. delbrueckii* subsp. *bulgaricus* LB340, and probiotic fermented milk containing the same yogurt culture plus *B. animalis* subsp. *lactis* HN019, in organic and conventional UHT milks, are shown on Fig. 2.

A similar acidification profile was observed for yogurt culture in both milks (Fig. 2A). Even if initial pH slightly differed (pH 6.54 ± 0.01 conventional milk, instead of pH 6.65 ± 0.01 in organic milk), the higher rate of acidification in organic milk (15.3 10^{-3} upH/min) than in conventional milk (11.7 10^{-3} upH/min) (Fig. 2B) allowed the final pH to be reached at the same time (tpH4.5 = 6.2 ± 0.3 h in both fermented milks). From Fig. 2B, two maximum acidification rates were observed whatever the kind of milk. This is explained by Pernoud, Fremaux, Sepulchre, Corrieu, & Monnet (2004), who demonstrated that *S. thermophilus* is an urease positive species, thus allowing urea conversion into ammonia and carbon dioxide. This transitory ammonia synthesis neutralized lactic acid, thus explaining the temporary pH stabilization, which resulted in these two peaks. This phenomenon has a direct impact on acidification profiles, due to natural variation of the urea level in milk (Hols et al., 2005). The previous phenomenon engendered by urease activity was not observed in the acidification profile of organic milk fermented with probiotic plus yogurt culture (Fig. 2C) that displayed a typical sigmoid behavior.

Fig. 1. Evolution of *trans*-octadecenoic acid (*trans*-C18:1, A), conjugated linoleic acid (CLA, B) and α-linolenic acid (ALA, C) relative contents in organic and conventional milks during fermentation by *Streptococcus thermophilus* TA040 and *Lactobacillus bulgaricus* LB340 (Y), and *Streptococcus thermophilus* TA040, *Lactobacillus bulgaricus* LB340 and *Bifidobacterium animalis* subsp. *lactis* HN019 (PY).
0: day 0 (before fermentation); 1: day 1 (1 day after fermentation); 7: day 7 (7 days storage at 4°C);
Means (n = 4) with different letters are significantly different; $P \leq 0.05$.

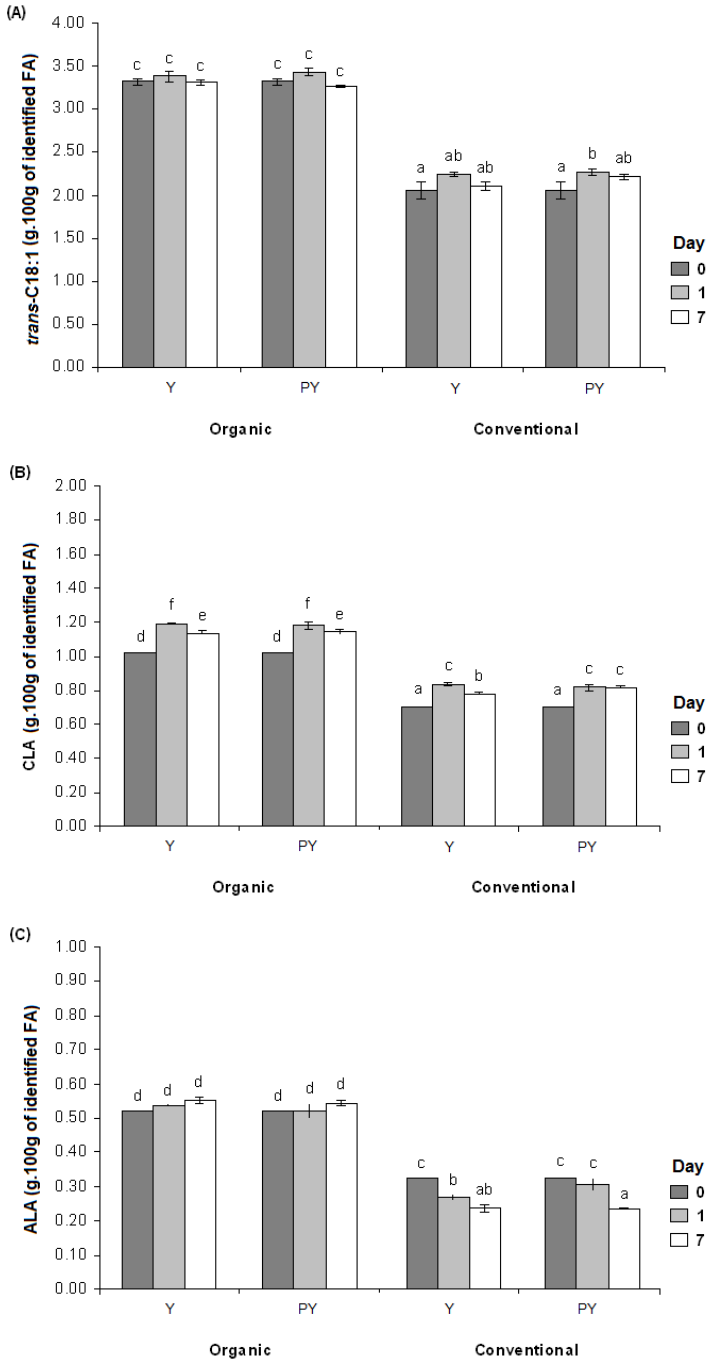
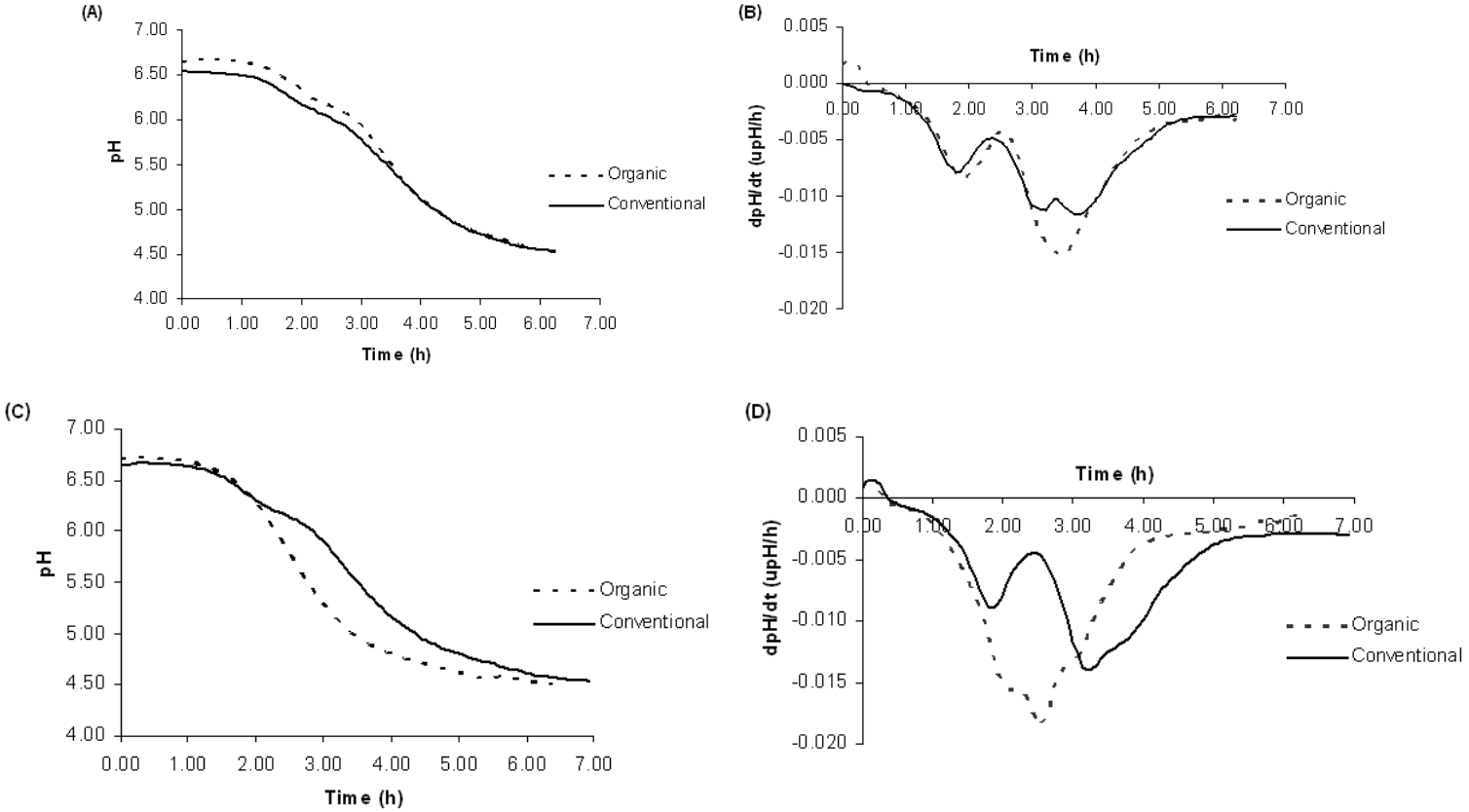


Fig. 2. Acidification kinetics in organic and conventional milks incubated at 42°C until pH 4.5, with (A and C) *Streptococcus thermophilus* TA040 and *Lactobacillus bulgaricus* LB340, and (B and D) *Streptococcus thermophilus* TA040, *Lactobacillus bulgaricus* LB340 and *Bifidobacterium animalis* subsp. *lacti*s HN019.

- - - - Organic milk

—— Conventional milk



This could be explained by the lower urea level in organic milk than in conventional milk, as previously reported by Toledo et al. (2002).

By considering the mixed culture including *B. lactis* HN019, the use of organic milk increased acidification rates as compared to conventional milk (Fig. 2B and 2D). This difference allowed the acidification of organic milk to be significantly more rapid (18.6 10^{-3} upH/min instead of 14.2 10^{-3} upH/min, $P < 0.05$) with bifidobacteria, lactobacilli and streptococci than with only yogurt bacteria. The time to reach pH 4.5 was equal to 6.2 ± 0.2 h in organic milk instead of 6.9 ± 0.1 h in conventional milk, which was significantly different ($P < 0.05$). This result is in agreement with those of Florence et al. (2009) that reported shorter fermentation time using binary cultures of *B. animalis* subsp. *lactis* and *S. thermophilus* in organic milks. It can be supposed that the strain *B. lactis* HN019 required specific nutriments that were found in organic milk, but not in conventional milk.

3.3. Bacterial concentrations during fermented milks production and storage

Bacterial growth differed by considering both type of milk and mixed culture composition. Indeed, microbial interactions can result either in stimulation, delay, inhibition, or the absence of effects, depending on bacterial species and strains (Vinderola, Costa, Regenhardt, & Reinheimer, 2002; Roy, 2005).

Growth of *S. thermophilus* TA040 occurred during the first two hours of fermentation, resulting from its rapid lactose assimilation, in agreement with earlier works of Beal & Corrieu (1994). Final concentrations of *S. thermophilus* achieved at the end of the fermentation ranged from 8.9 to 9.1 $\log_{10}$ CFU/mL, with no significant differences ($P > 0.05$) between the two different kinds of milk and types of cultures employed.

Growth of *L. bulgaricus* LB340 started after four hours of fermentation, in agreement with previous studies (Oliveira et al., 2009). Final concentrations were significantly higher ($P < 0.05$) in organic milk fermented by yogurt culture (8.1± 0.03 $\log_{10}$ CFU/mL) as compared to the other conditions (7.8 ± 0.03 $\log_{10}$ CFU/mL). A positive effect of organic milk was thus demonstrated on *L. bulgaricus* growth, which can be related to the higher poly-unsaturated fatty acid content (1.3 times higher) in this kind of milk as compared to conventional milks. Higher viability of lactic acid bacteria was previously achieved when the ratio between unsaturated and saturated fatty acids was increased (Castro et al., 1995; Béal et al., 2001). This could be explained by the stereochemistry of the double bounds of unsaturated fatty acids, which control membrane fluidity during exposure to the adverse environmental conditions found in fermented milks, such as low pH or low temperature. Moreover, the more rapid acidification observed in organic milk could be another factor of *L. bulgaricus* improvement.

No significant difference ($P > 0.05$) was noted for *B. lactis* HN019 growth in organic and conventional milk. Bacterial counts at the end of fermentation were equal to 7.9 ± 0.03 $\log_{10}$ CFU/mL and 8.1 ± 0.06 $\log_{10}$ CFU/mL for organic and conventional milk, respectively.

Final concentrations of *L. bulgaricus* and *S. thermophilus* at the end of the cultures were not significantly influenced by the presence of the probiotic culture *B. lactis* HN019 ($P > 0.05$). This result differs from those obtained by Vinderola et al. (2002) on the one hand and Donkor, Henriksson, Vasiljevic, & Shah (2006) on the other hand, who demonstrated that *L. bulgaricus* and *S. thermophilus* were either inhibited or stimulated by *Bifidobacterium* strains, respectively. This contradictory information indicates that the interactions between yogurt bacteria and *Bifidobacterium* are strongly strain dependent.

Growth of *B. lactis* HN019 in milk remained weak as final concentrations were around 8.1 ± 0.06 $\log_{10}$ CFU/mL. This result agreed with those reported by (Vinderola et al., 2002), who showed that addition of probiotic cultures to yogurt starters generally results in slower growth of the probiotic strains than if they were added alone in milk. This was explained firstly by the accumulation of lactic and acetic acids that affect the viability of bifidobacteria, and secondly by the low proteolytic activity of these bacteria (Roy, 2005).

Finally our results demonstrated that fermentation was mainly ascribed to *S. thermophilus*, which reached a final concentration 1 log higher than *L. bulgaricus* and *B. lactis*. Only a slight effect of the type of milk was noticed on the growth of *L. bulgaricus,* when associated with *S. thermophilus*, organic milk leading to a better growth of this species. The faster grow of starter cultures allowed rapid acidification which resulted in reduced availability of nutrients; thus, probiotic cultures do not have time to grow extensively (Roy, 2005).

By considering the bacterial concentrations measured after 7 days of storage at 4°C, the kind of milk did not affect the survival of the three bacterial species that was stable during cold storage. Concentrations were equal to 8.8 ± 0.2 $\log_{10}$ CFU/mL for *S. thermophilus* TA040, 7.6 ± 0.2 $\log_{10}$ CFU/mL for *L. bulgaricus* LB340 and 7.9 ± 0.1 $\log_{10}$ CFU/mL for *B. lactis* HN019*,* in both milks. Moreover, no significant difference ($P > 0.05$) was observed with the counts measured just after fermentation. This result differs from that obtained by Donkor et al. (2006), who indicated that the viability of *L. bulgaricus* Lb1466 was enhanced in the presence of probiotic organisms during cold storage. It was thus strain dependant.

3.4 Fatty acid profiles of milk during fermentation and storage

The fatty acid profiles varied during milk fermentation, as a result of the kind of milk and the type of starter culture. In contrast, no modification was observed during storage at 4 °C for 7 days.

The relative content of SCFA was slightly reduced during fermentation ($P < 0.05$), in both conventional and organic fermented products, independently of co-culture employed. During cold storage for 7 days, the SCFA of the fermented milks did not change anymore, whatever the type of milk. These data differ from those achieved by Ekinci et al. (2008), who observed higher amounts in short chain fatty acids in products fermented with other bacterial species. In conventional milks, independently of the co-culture used, the MCFA concentration decreased during

fermentation, whereas no significant difference was observed during 7 days storage at 4°C. In organic milk, the MCFA relative contents did not change during fermentation and after 7 days of cold storage. In addition, no significant difference ($P \geq 0.05$) was pointed out between organic and conventional milks. Nevertheless, relative concentrations of C14:1 and C15:0 were slightly higher ($P < 0.05$) in fermented conventional milks, which agrees with the study of Butler et al. (2011) who found higher concentration of MCFA in conventional milk. Finally, a significant increase in LCFA concentration was observed during fermentation (between 1 and 2%), but not during storage at 4°C, for both organic and conventional fermented milks. The relative contents of LCFA did not show significant difference ($P > 0.05$) between the two kinds of milks, in agreement with the findings of (Collomb et al., 2008; Ellis et al., 2006). Among these LCFA, higher relative contents of C16:0; C16:1 and C17:0 were found in conventional products, whereas relative amounts of C18:0 and C18:2 were higher in organic fermented milks.

In addition to these results that concerned the chain length of milk fatty acids, important changes were observed on the fatty acid saturation degree during fermentation ($P < 0.05$). In conventional milk, the proportion of saturated fatty acids (SFA) strongly decreased during fermentation (1-2%), whereas it diminished only slightly in organic milk (~0.4%). As a result of SFA level decrease during fermentation, the relative concentration of MUFA increased in conventional milk (1%) but not in organic milk (Table 1).

The levels of MUFA measured after fermentation were practically alike for both milks in our study. The percentage of PUFA increased during fermentation in organic milk (~0.2%) but remained stable in conventional milk. These results are in agreement with those obtained by Florence et al. (2009) with the cultures of *S. thermophilus* and four strains of *B. lactis*. They could be explained either by the different balance with MUFA or SFA, or by the synthesis of some polyunsaturated fatty acids by the bacteria (Oh et al., 2003).

The relative percentages of SFA, MUFA and PUFA at day 7 remained close to those measured at day 1 (Table 1). At 4°C, the metabolic activity of the bacteria was reduced as a consequence of the low temperature, and no more change occurred in the fatty acid content as a result of their metabolic activity. This result is in agreement with those reported by Rodriguez-Alcala & Fontecha (2007) with CLA fortified dairy products. They showed that the relative contents in SFA, MUFA and PUFA remained stable during storage. In contrast, Van de Guchte et al. (2006) observed that the total n-3 PUFA concentration slightly decreased during storage of conventional fermented milks. This difference can be ascribed to the different strains used.

Moreover, no significant effect of the type of starter culture was noticed on the chain length of milk fatty acids. The relative proportions of each group of fatty acids varied in the same way, whatever the probiotic culture was added to the yogurt culture or not. The same conclusion was achieved by comparing the fatty acid composition after 7 days of storage at 4°C, which was not affected by the starter and

remained stable. Finally, fermentation allowed increasing MUFA relative concentration in conventional milk, whereas organic fermented milks were characterized by an increase in PUFA relative contents. This indicates that the fatty acid composition of the fermented milk was the result of initial saturation degree as well as modification during fermentation. This result confirmed those obtained by Van de Guchte et al. (2006) with conventional fermented milks enriched or not with PUFA or whey proteins.

From these results, differences were observed according to fatty acid chain length and saturation degree by comparing organic and conventional fermented milks. We ascribed these differences to both initial milk composition and modification by fermentation. The initial fatty acid profile of milk was primarily determined by the balance of fatty acids in feeding regiment and the extent of rumen hydrogenation and mammary desaturase activity that differed in both systems of dairy production (Butler et al., 2011). Moreover, fatty acid composition of fermented milks was affected by growth and corresponding enzymatic activities of bacterial cells, which differed according to the milk, as a result of initial fatty acid profile (Kim & Liu, 2002; Ekinci et al., 2008).

In contrast, no differences were noted during cold storage of fermented milks. This fact may be due to the slower metabolic activity of bacteria at low temperature (Béal et al., 2001).

3.5. Evolution of trans-octadecenoic acid (*trans*-C*18:1), conjugated linoleic acid (CLA) and α-linoleic acid (ALA) relative contents during fermentation and cold storage*

During fermentation, *trans*-C18:1 relative concentration (Fig. 1A) showed a 20% increase in conventional fermented milks, with no significant difference among the starter cultures, whereas an enhancement of 8% was observed in organic milk. As the initial relative concentration in *trans*-C18:1 was 1.6 times higher in organic milk, the final *trans*-C18:1 percentage in the fermented milks was the result of both initial milk composition and modification during fermentation. It is interesting to maintain a high relative content of *trans*-C18:1 as it participates in CLA production in human's body (Gnädig et al., 2003; Butler et al., 2011) and acts as intermediate fatty acid in biohydrogenation pathway (Bergamo et al., 2003). During storage of the fermented products, the *trans*-C18:1 concentration remained stable, whatever the kind of milk and starters used. Finally, after 7 days storage at 4°C, it was higher in organic fermented milks (3.3±0.03%) than in conventional milks (2.2±0.03%).

During fermentation, CLA relative content significantly increased ($P < 0.05$), at different levels in organic (17%) and conventional (12%) milks (Fig. 1B). This was explained by Ekinci et al. (2008), who indicated that enzymatic reactions occurred on biohydrogenation pathway, thus increasing CLA level during the production of fermented products. Similar results were reported by Oliveira et al. (2009) in fermented milks, whereas no change was observed in probiotic fermented products made with conventional milk, as reported by Van de Guchte et al. (2006). As these authors used different strains, this behaviour was thus strain dependent. The

difference between conventional and organic fermented milks found in our study was considered as significant ($P < 0.05$). The CLA relative concentration was higher in organic fermented milks (1.2±0.01%) as compared to conventional fermented milks (0.8±0.01%) (Fig. 1B), in accord with previous results (Oliveira et al., 2009). This higher CLA relative content in organic fermented products was the result of both initial CLA percentage in milk and changes during fermentation. In addition to these results, CLA relative concentration did not significantly vary in fermented milks according to the co-cultures. This result indicates that *B. lactis* HN019 had no effect on CLA relative content, and that the variations observed during fermentation could be ascribed to *S. thermophilus* or *L. bulgaricus*, as suggested by Lin (2003). Finally, the CLA percentage slightly decreased during cold storage of three of the fermented milks ($P < 0.05$), that may be related to the activation of reduction steps in biohydrogenation pathway (Kim & Liu, 2002). However, by considering the conventional fermented milk with yogurt starters and bifidobacteria, a significant increase of relative CLA content was observed.

Fig. 1C shows that during fermentation ALA level did not vary significantly in organic milk (0.5±0.02%), for the two kinds of culture. In contrast, a significant decrease ($P < 0.05$) was noted during fermentation and storage of conventional milk products (from 0.38±0.02% to 0.30±0.02%). These results are not in agreement with those of Van de Guchte et al. (2006), who showed that the content of ALA was not affected during storage of conventional fermented milks at 4°C, which can be attributed to the different strains used. No significant difference was noticed between the two kinds of starters at the end of the fermentation. Finally, the ALA content in the fermented milks mainly resulted from its initial concentration in milk and from variation during fermentation and storage. During 7 days storage at 4°C, strong difference was observed between the two kinds of fermented milks. The ALA content remained high and stable in organic milk (0.54±0.02%), whereas it decreased from 0.30±0.02% to 0.24±0.01% in conventional milk. This decrease can be correlated to the increased levels of C18:0 and C18:1, independently of the co-culture used, as a result of modification of biohydrogenation and desaturation pathways (Destaillats, Trottier, Galvez, & Angers, 2005).

4. Conclusions

Our study demonstrated that the use of organic milk allowed more rapid acidification and provided higher amounts of PUFA content in the fermented milks that was related to an improvement of *L. bulgaricus* growth. In contrast, the growth of *S. thermophilus* and *B. lactis* HN019 was not affected by the type of milk. Bacterial concentrations remained stable after 7 days of storage at 4°C.

Acidification process also provided *trans*-C18:1 and CLA enhancement, together with ALA decrease, at different levels in conventional and organic milks. This result indicates that bacterial metabolism modified the relative fatty acid milk composition. By combining these differences with the initial fatty acid composition of organic and conventional milks, which depended on variations in dairy diet

manipulation, organic fermented milks showed higher relative amounts of *trans*-C18:1 (x 1.6), CLA (x 1.4) and ALA (x 1.6), as compared to conventional fermented milks at the end of fermentation and after storage at 4°C. Consequently, the fatty acid content of the fermented milks was the result of two factors: initial milk composition and modification during fermentation as a result of bacterial metabolic activities. The higher relative amounts of *trans*-C18:1, CLA and ALA in organic fermented milks and lower levels of SFA may be considered as desirable from a nutritional perspective.

In the future, it will be necessary to identify the specific role of each bacterial species, in pure cultures, in order to understand the biochemical mechanisms that support the changes in fatty acid composition in the fermented milks.

Conflict of interest statement:

None of the authors have any conflict of interest. ACRF collected data and wrote the manuscript; CB validated the data and revised the manuscript; RCS, CSBB, ALOPS, and LAG provided technical assistance; MNO designed the study, evaluated the data and revised the manuscript.

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References

Ackman, R. G. (2007). Application of gas liquid chromatography to lipid separation and analysis: qualitative and quantitative analysis. In: Chow, C.K. (Eds.), *Fatty acids in foods and their health implications* (pp. 47-62). Boca Raton: CRC Press USA.

AOAC, (1995). Fat, lactose, protein and solids in milk. Official methods of Analysis. In: AOAC Official Method 972.16. *Association of Official Analytical Chemists*, Washington, DC, USA.

AOCS. (1997). Official method Ce 1-62: fatty acid composition by gas chromatography. In: Official Methods and Recommended Practices of the AOCS. *American Oil Chemists Society*. Champaign, IL, USA.

Barceló-Coblijn, G., & Murphy, E. J. (2009). Alpha-linolenic acid and its conversion to longer chain n-3 fatty acids: Benefits for human health and a role in maintaining tissue n-3 fatty acid levels. *Progress in Lipid Research, 48,* 355-374.

Béal, C., & Corrieu, G. (1994). Viability and acidification activity of pure and mixed starters of *Streptococcus salivarius* ssp. *thermophilus* 404 and *Lactobacillus delbrueckii* ssp. *bulgaricus* 398 at the different steps of their production. *LWT – Food Science and Technology, 27* (Suppl.1), 86-92.

Béal, C., Fonseca, F. & Corrieu, G. (2001). Resistance to freezing and frozen storage of *Streptococcus thermophilus* is related to membrane fatty acid composition. *Journal of Dairy Science, 84,* 2347-2356.

Bergamo, P., Fedeli, E., Iannibelli, L., & Marzillo, G. (2003). Fat-soluble vitamin contents and fatty acid composition in organic and conventional Italian dairy products. *Food Chemistry, 82,* 625-631.

Brenna, J. T., Salem Jr, N., Sinclair, A. J., & Cunnane, S. C. (2009). Alpha-linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 80,* 85-91.

Butler, G., Stergiadis, S., Seal, C., Eyre, M., & Leifert, C. (2011). Fat composition of organic and conventional retail milk in northeast England. *Journal of Dairy Science, 94,* 24-36.

Castro, H. P., Teixeira, P. M. & Kirby, R. (1995). Storage of lyophilized cultures of *Lactobacillus bulgaricus* under different relative humidities and atmospheres. *Applied Microbiology Biotechnology, 44,* 172-176.

Collomb, M., Bisig, W., Bütikofer, U., Sieber, R., Bregy, M., & Etter, L. (2008). Fatty acid composition of mountain milk from Switzerland: Comparison of organic and integrated farming systems. *International Dairy Journal, 18* (Suppl. 10-11), 976-982.

Destaillats, F., Trottier, J. P., Galvez, J. M. G., & Angers, P. (2005). Analysis of α-Linolenic acid biohydrogenation intermediates in milk fat with emphasis on Conjugated Linolenic Acids. *Journal of Dairy Science, 88,* 3231-3239.

Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2006). Effect of acidification on the activity of probiotics in yoghurt during cold storage. *International Dairy Journal, 16,* 1181-1189.

Ekinci, F. Y., Okur, O. D., Ertekin, B., & Guzel-Seydim, Z. (2008). Effects of probiotic bacteria and oils on fatty acid profiles of cultured cream. *Journal of Lipid Science and Technology, 110,* 216-224.

Ellis, K. A., Innocent, G., Grove-White, D., Cripps, P., Mc Lean, W. G., Howard, C. V., et al. (2006). Comparing the fatty acid composition of organic and conventional milk. *Journal of Dairy Science, 89* (Suppl. 6), 1938-1950.

Florence, A. C. R., da Silva, R. C., do Espirito Santo, A. P., Gioielli, L. A., Tamime, A. Y., & de Oliveira, M. N. (2009). Increased CLA content in organic milk fermented by bifidobacteria or yoghurt cultures. *Dairy Science and Technology, 89,* 541-553.

Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., et al. (2007). Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. *World Journal of Gastroenterology, 13,* 236-243.

Gnädig, S., Xue, Y., Berdeaux, O., Chardigny, J. M., & Sebedio, J. L. (2003). Conjugated linoleic acid (CLA) as a functional ingredient. In: Mattila-Sandholm, T., & Saarela, M. (Eds.), *Functional dairy products* (pp. 263-298). Boca Raton: CRC Press USA.

Hols, P., Hancy, F., Fontaine, L., Grossiord, B., Prozzi, D., Leblond-Bourget, N., et al. (2005). New insights in the molecular biology and physiology of *Streptococcus*

thermophilus revealed by comparative genomics. *FEMS Microbiology Reviews, 29* (Suppl. 3), 435-463.

ISO. (2001). International Organization for Standarzation. Milk and milk products - Extraction methods for lipids and liposoluble compounds. *International Standard ISO 14156-IDF 172:2001*. 1 ed., 01-06, 2001.

ISO. (2002). International Organization for Standarzation. Milk fat - Preparation of fatty acid methyl esters. *International Standard ISO 15884-IDF 182:2002*. 1ed, 01-06, 2002.

Kim, Y. J. & Liu, R. H. (2002). Increase of conjugated linoleic acid content in milk by fermentation with lactic acid bacteria. *Journal of Food Science, 67* (Suppl. 5), 1731-1737.

Lin, T. Y. (2003). Influence of lactic cultures, linoleic acid and fructo-oligosaccharides on conjugated linoleic acid concentration in non-fat set yogurt. *Australian Journal of Dairy Technology, 58,* 11-14.

Luna, P., Martín-Diana, A. B., Alonso, L., Fontecha, J., de la Fuente, M. A., Requena, T. & Juárez, M. (2004). Effects of milk fat replacement by PUFA enriched fats on n-3 fatty acids, conjugated dienes and volatile compounds of fermented milks. *European Journal of Lipid Science and Technology, 106,* 417-423.

Oh, D. K., Hong, G. H., Lee, Y., Min, S. G., Sin, H. S., & Cho, S. K. (2003). Production of conjugated linoleic acid by isolated *Bifidobacterium* strains. World *Journal of Microbiology and Biotechnology, 19,* 907-912.

Oliveira, M. N., Sodini, I., Remeuf, F., & Corrieu, G. (2001). Effect of milk supplementation and culture composition on acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. *International Dairy Journal, 11* (Suppl. 11-12), 935-942.

Oliveira, R. P. S., Florence, A. C. R., Silva, R. C., Perego, P., Converti, A., Gioielli, L. A., et al. (2009). Effect of different prebiotics on the fermentation kinetics, probiotic survival and fatty acids profiles in nonfat symbiotic fermented milk. *International Journal of Food Microbiology, 128* (Suppl. 3), 467-472.

Pernoud, S., Fremaux, C., Sepulchre, A., Corrieu, G., & Monnet, C. (2004). Effect of the Metabolism of Urea on the Acidifying Activity of *Streptococcus thermophilus*. *Journal of Dairy Science, 87* (Suppl. 3), 550-555.

Roy, D. (2005). Technological aspects related to the use of bifidobacteria in dairy products. *Lait, 85,* 39–56.

Rodríguez-Alcalá, L. M., & Fontecha, J. (2007). Fatty acid and conjugated linoleic acid (CLA) isomer composition of commercial CLA-fortified dairy products: evaluation after processing and storage. *Journal of Dairy Science, 90* (Suppl. 5), 2083-2090.

Rodríguez-Alcalá, L. M., Harte, F., & Fontecha, J. (2009). Fatty acid profile and CLA isomers content of cow, ewe and goat milks processed by high pressure homogenization. *Innovative Food Science and Emerging Technologies, 10,* 32-36.

Santora, J. E., Palmquist, D. L., & Roehrig, K. L. (2000). *Trans*-Vaccenic Acid is desaturated to conjugated linoleic acid in mice. *The Journal of Nutrition, 130* (Suppl. 2), 208-215.

Sola-Larrañaga, C., & Navarro-Blasco, I. (2009). Chemometric analysis of minerals and trace elements in raw cow milk from the community of Navarra, Spain. *Food Chemistry, 112,* 189-196.

Toledo, P., Andrén, A., & Bjorck, L. (2002). Composition of raw milk from sustainable production systems. *International Dairy Journal 12,* 75-80.

Van de Guchte, M., Penaud, S., Grimaldi, C., Barbe, C., Bryson, K., Nicolas, P., et al. (2006). The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proceedings of the National Academy of Sciences of the United States of America, 103* (Suppl. 24), 9274-9279.

Vinderola, C. G., Costa, G. A., Regenhardt, S., & Reinheimer, J. A. (2002). Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *International Dairy Journal, 12* (Suppl. 7), 579-589.