International Journal of Food Sciences and Nutrition

ISSN: 0963-7486 (Print) 1465-3478 (Online) Journal homepage: http://www.tandfonline.com/loi/iijf20

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To cite this article: Nadia Manzo, Fabiana Pizzolongo, Immacolata Montefusco, Maria Aponte, Giuseppe Blaiotta & Raffaele Romano (2015) The effects of probiotics and prebiotics on the fatty acid profile and conjugated linoleic acid content of fermented cow milk, International Journal of Food Sciences and Nutrition, 66:3, 254-259, DOI: 10.3109/09637486.2014.992005

To link to this article: http://dx.doi.org/10.3109/09637486.2014.992005

Published online: 06 Feb 2015.

INTERNATIONAL JOURNAL OF FOOD SCIENCES and NUTRITION

http://informahealthcare.com/ijf
ISSN: 0963-7486 (print), 1465-3478 (electronic)

Int J Food Sci Nutr, 2015; 66(3): 254–259
 DOI: 10.3109/09637486.2014.992005

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FOOD COMPOSITION AND ANALYSIS

The effects of probiotics and prebiotics on the fatty acid profile and conjugated linoleic acid content of fermented cow milk

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Abstract

The ability of probiotic bacteria (*Lactobacillus acidophilus* La5 and *Bifidobacterium animalis* Bb12), to produce conjugated linoleic acid (CLA) in association with *Streptococcus thermophilus* and *Lb. bulgaricus* during milk fermentation has been evaluated in this study. Pasteurized cow milk and infant formula were used. Infant formula was selected for its high linoleic acid content, for being a source of CLA and for its prebiotic compounds, e.g. galacto-oligosaccharides. The microorganisms were not able to increase the CLA content of the fermented products under the given experimental conditions. No statistically significant differences ($p > 0.05$) occurred between the CLA content in milk and the fermented samples. The CLA contents of 10 commercial fermented milk products were determined. The highest CLA content was observed in fermented milk containing only *Str. thermophilus* and *Lb. bulgaricus*.

Keywords

Bifidobacterium animalis, CLA, dairy products, *Lactobacillus acidophilus*, yoghurt

History

Received 3 June 2014
Revised 4 November 2014
Accepted 14 November 2014
Published online 6 February 2015

Introduction

There is a growing interest in using conjugated fatty acids as novel types of beneficial and functional lipids. The term CLA refers to a mixture of positional and geometric isomers of octadecadienoic acid (C18:2) with conjugated double bonds, among which, the cis-9,trans-11-octadecadienoic acid (18:2) is about the 80% of all possible CLA isomers (Pariza et al., 2001). These compounds are formed in the rumen by anaerobic bacteria (*Butyrivibrio fibrisolvens*) as an intermediate in the biohydrogenation of linoleic acid and from desaturation of vaccenic acid (trans-11 octadecenoic acid) in the mammary gland via Δ9-desaturase (Griinari & Bauman, 1999). Some authors report potential beneficial effects of CLA, like reduction of carcinogenesis (Kelley et al., 2007), tumorigenesis, atherosclerosis, and body fat content (Park et al., 2007), improving of hyperinsulinemia and enhancing of the immune system (Field & Schley, 2004). In considering the possible use of CLA for medicinal and nutraceutical purposes, an emphasis on increasing the CLA content of foods has grown in recent years. According to literature reported data, several strains of *Lactobacillus* (*Lb.*), *Propionibacterium*, *Bifidobacterium* (*B.*) and *Enterococcus* are able to form CLA from linoleic acid and could be used to increase the CLA level in fermented dairy products, such as yoghurt and cheese. However, the available literature is inconclusive regarding whether the addition of probiotic bacterial strains can increase the CLA content of dairy products (Kishino et al., 2002). The possibility of increasing CLA using different microbial cultures as starters in fermented dairy foods has not been completely explored. Shantha et al. (1995) reported an increased CLA content from 4.4 mg/g milk fat to 5.3 mg/g in skimmed yoghurt, but these findings were not the same as those found under our experimental conditions. A recent study reported CLA production by *Streptococcus* (*Str.*) *thermophilus* and *B. animalis* Bb12 during the fermentation of milk from cows that consumed organic feed, and no CLA increase was observed in milk from cows that consumed conventional feed (Rodrigues Florence et al., 2009).

Similarly, Akalin et al. (2007) observed an increase in the CLA content from 2 to 6 mg of CLA/g in total fat from yoghurt containing starter and *Lb. acidophilus* La5/*B. animalis* Bb12 with 2% fructo-oligosaccharides (FOS). A significant increase in the c9-t11CLA content was found in skimmed yoghurt in which *Lb. acidophilus* was used as a starter, added with 0.1% linoleic acid and 5% FOS (Lin et al., 2003). The use of maltodextrin can also induce an increased CLA concentration relative to that of the control; there was a 21% increase when using *Str. thermophilus* and *Lb. bulgaricus*, and no less than 38% was reported with *Str. thermophilus* and *Lb. acidophilus* (Oliveira et al., 2009).

Bifidobacteria and lactic acid bacteria are commonly used as probiotic in production of fermented dairy products and some of these strains (*B. animalis* Bb12) are able to produce CLA after incubation in skim milk (Rodríguez-Alcalá et al., 2011).

Fermented derivatives have been prepared from pasteurized cow milk and infant formula to study the CLA production capability of probiotic strains (*Lb. acidophilus* La5 and *B. animalis* Bb12). The use of infant formula has been prompted by its polyunsaturated fatty acids (PUFAs), particularly linoleic acid, which is a source of CLA (Rodríguez-Alcalá et al., 2013), and for its prebiotic compounds, galacto-oligosaccharides (GOS) and dextrins. These substances, so-called prebiotics, are considered the excellent substrates to improve milk fermentation, promoting bifidobacteria and lactobacilli growth and/or activity (Gibson et al., 2004).

A secondary objective of this work was to determine the CLA content of commercial fermented milk, and to evaluate whether

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different probiotic and prebiotic matrices could increase the CLA content.

Materials and methods

Starter cultures and fermented milk

Fermented products were prepared from pasteurized cow milk and infant formula, both of which were purchased from the local market. Infant formula was reconstituted with water before use as recommended by the producer (4.6 g of powder in 30 mL of water).

A commercial yoghurt starter culture containing *Str. thermophilus* and *Lb. bulgaricus* (YC-350), and both probiotic bacteria cultures (*Lb. acidophilus* La5 and *B. animalis* Bb12) were obtained from Chr. Hansen (Chr. Hansen A/S, Hørsholm, Denmark). Commercially available FOS extracted from chicory roots with a degree of polymerization from 2 to 30 (average value 9) were obtained from Cosucra S.A. (Fontenoy, Belgium). Maltodextrins (MD), with a dextrose equivalent (DE) of 19 (Sigma-Aldrich, St. Louis, MO), have been used. All reagent and organic solvents used were of analytical grade.

To evaluate the CLA content of commercial products, five yoghurt (samples FM 1–FM 5) and five fermented milk (samples FM 6–FM 10) samples were purchased from the local Italian market. Yoghurt is milk fermented only by the starter microorganisms *Str. thermophilus* and *Lb. bulgaricus*, and fermented milks are fermented by different microorganisms that are also in co-culture with the starters. Table 1 shows the fat percentage, microorganisms and prebiotic content reported on the commercial product label.

Fermented milk preparation

A cow milk sample (1350 ml) was divided into three aliquots; the first was not mixed with FOS or MD, the second aliquot was fortified with 2% FOS and the third was mixed with 2% MD. Each batch was heated to 85 °C for 30 min, cooled to 43 °C and mixed with yoghurt starter culture. Each batch was divided into three batches again. *Lb. acidophilus* La5 (1.2×10^7 CFU/mL) was added to the second batch, and *B. animalis* Bb12 (1.2×10^7 CFU/mL) was added to the third batch. Glass jars with a 150 mL capacity were filled with the mixtures and incubated at 40 °C until a pH of 4.7 was reached. The same procedure was repeated starting from infant formula (Figure 1).

Samples analysis

Determination of microbial counts, total titratable acidity and pH

The drop method (Collins et al., 1989) was used to determine the population level reached by each tested microorganism. Briefly, samples (1 mL) were diluted (10^{-1} to 10^{-9}) with sterile quarter strenght Ringer's solution (Oxoid, Basingstoke, UK) and aliquots of 12 μL were dropped onto agar plates of the proper media using a calibrated 20 μL micropipette. The plates were incubated at 37 ± 1 °C for 48 h under anaerobic conditions and those showing individual colonies in the drop areas were counted. Viable cell counts were calculated as colony-forming units per mL and the results expressed as Log_{10} values. Each experiment was carried on in triplicate.

Titratable acidity was determined using the official AOAC method Nr. 92307 (AOAC, 1999). During the fermentation process, pH was measured every 30 min.

Lipid extraction

Extraction of fat from the samples was carried out according to the Official Method of Cheese Analysis (DM, 1986, International Norm FIL-IDF 5A, 1969), and the Schmid–Bondzynski–Ratzlaff SBR method of lipid extraction (International Standard 5B, 1986) with some modifications. Samples (5 g) were homogenized with ethanol (6.7 mL) and mixed using a Vortex mixer for 60 s. Then, a diethyl ether–heptane mixture (10 mL, 2:1 v/v) was added and mixed by vortexing for 60 s. Samples were then centrifuged at 3000*g* for 10 min. The diethyl ether phase containing the extracted lipids was transferred and the residue was extracted using the same procedure three more times. The combined filtrates were concentrated in a rotary evaporator at 36 °C. Then, extracted lipid phase was dissolved in hexane and purified using sodium chloride saturated solution (3 mL). The hexane phase containing purified lipids was dried over anhydrous sodium sulfate and under nitrogen. The total lipid obtained was determined gravimetrically using the AOAC method (1999).

Fatty acid analysis

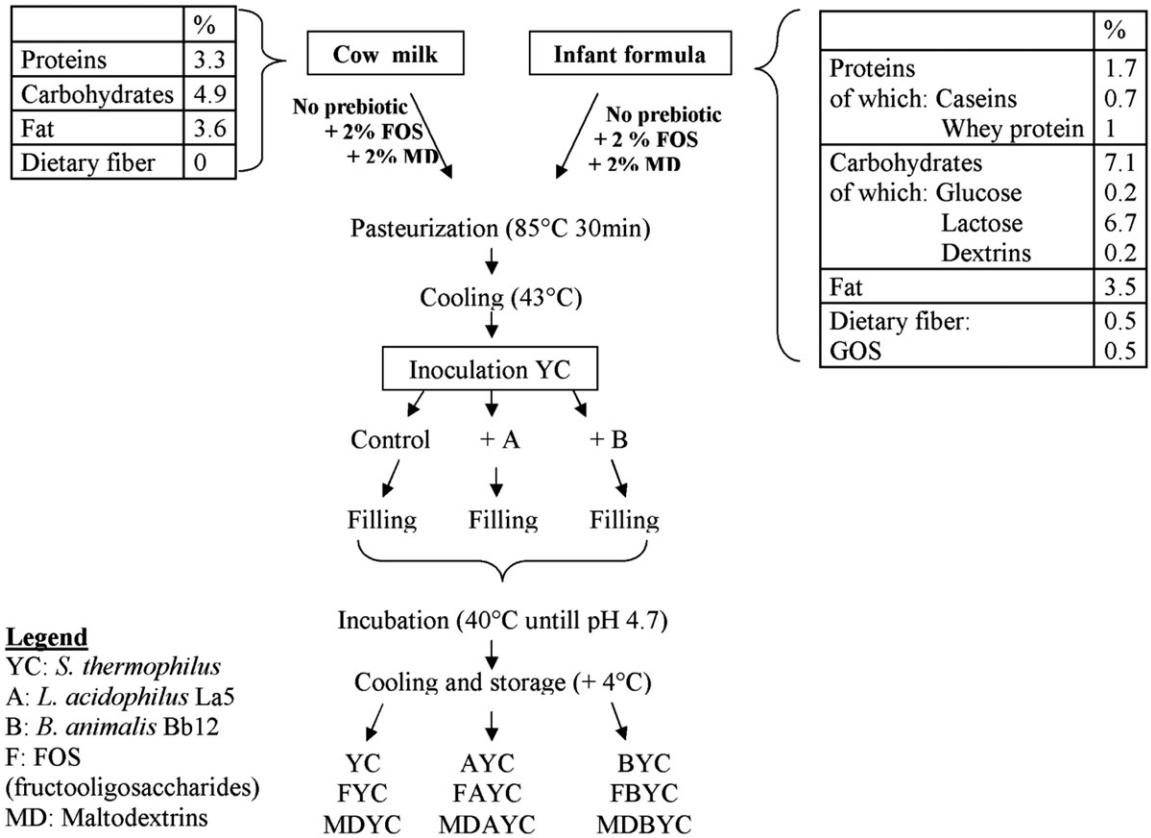
Fatty acids (FA) composition was obtained by gas chromatography (GC) after derivatization to fatty acid methyl esters (FAME) using diazomethane (Kuhnel et al., 2007) and 2 N potassium hydroxide in methanol (Romano et al., 2011).

An Agilent Technologies 6850 Series II model gas chromatograph (Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Milano, Italy) equipped with a programmed temperature vaporizer (PVT), a flame ionization detector (FID), and a fused silica capillary column, 100 m × 0.25 mm i.d.; 0.20 μm film thickness (Supelco Bellefonte, PA), was used. The identification and the quantification of the obtained peaks was performed using the Supelco 37 Component FAME MIX (Supelco Bellefonte, PA), a CLA isomers mixture (Nu-Chek Prep., Inc. Elysian, MN) as external standards and GC retention time data available in the literature. FA concentration was calculated through response factors to convert peak areas into weight percentages.

Table 1. Total fat content and bacteria strains used in commercial fermented milk (information reported on the product label).

Sample	Total fat (%, w/w)	Bacteria	Prebiotic
FM1	3.6	*S. thermophilus* and *L. bulgaricus*	
FM2	3.8	*S. thermophilus* and *L. bulgaricus*	
FM3	4.2	*S. thermophilus* and *L. bulgaricus*	
FM4	4.0	*S. thermophilus* and *L. bulgaricus*	
FM5	4.5	*S. thermophilus* and *L. bulgaricus*	
FM6	3.5	*S. thermophilus*, *L. bulgaricus* and *L. acidophilus*	
FM7	0.9	*S. thermophilus*, *L. bulgaricus* and *L. jonsonii* La1	
FM8	3.5	*L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. lactis* and *Acetobacter aceti*	Inulin, Fructooligosaccharides
FM9	3.5	*S. thermophilus*, *L. bulgaricus* and *L. paracasei*	
FM10	1.7	*S. thermophilus*, *L. bulgaricus*	Fructooligosaccharides

Prebiotic components when present are indicated.



	%
Proteins	3.3
Carbohydrates	4.9
Fat	3.6
Dietary fiber	0

	%
Proteins	1.7
of which: Caseins	0.7
Whey protein	1
Carbohydrates	7.1
of which: Glucose	0.2
Lactose	6.7
Dextrins	0.2
Fat	3.5
Dietary fiber:	0.5
GOS	0.5

Figure 1. Processing procedure for the laboratory prepared fermented milks.

Statistical analysis of results

All determinations and experiments were performed in triplicate and results reported as the average values of three determinations. Analysis of variance (ANOVA) was carried out using the software XLSTAT 2012 (Addinsoft, Paris, France) to compare data obtained for different fermentation processes. The differences with $p \leq 0.05$ were considered significant.

Results and discussion

Basic parameters: total microbial count, pH and titratable acidity

The microbial count showed a regular increase during fermentation. At beginning of fermentation, as expected, the total count was about 10^7 CFU/mL (7.14 ± 0.24 and 7.05 ± 0.28 Log CFU/ml in cow milk and infant formula, respectively). At the end of the process, microbial loads increased more than one order of magnitude reaching 8.66 ± 0.28 Log CFU/ml in fermented cow milk and 8.61 ± 0.45 Log CFU/ml in fermented infant formula.

Figure 2 shows the pH variation during the fermentation process. A regular pH decrease occurred from the initial pH value of 6.7. Products obtained from cow milk reached a pH value of 4.7 after 180 min of fermentation. Products obtained from infant formula reached a pH value of 4.7 after 120 min of fermentation. Fermented cow milk showed the highest values for titratable acidity (expressed in % w/w of lactic acid), within a range from 0.72 ± 0.02 to 1.03 ± 0.01, with respect to fermented products obtained from infant formula, with acidity values ranging from 0.35 ± 0.02 to 0.40 ± 0.01. This difference could be explained by considering the lower titratable acidity of infant formula (0.14 ± 0.01) with respect to that of cow milk (0.26 ± 0.01%). The titratable acidity also depends on other different factors, e.g. casein content (Alais, 2000). It is also notable that infant formula

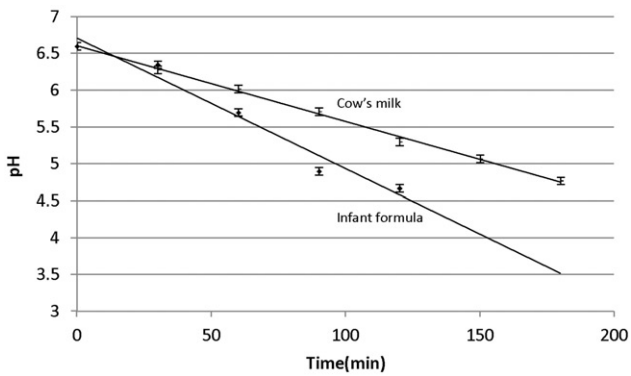


Figure 2. pH variation during fermentation process for cow milk and infant formula.

contains lower protein contents, with 1.7 ± 0.01 (% w/w) in comparison with the 3.3 ± 0.02 (% w/w) of cow milk. The pH of the products from the local market ranged between 3.77 and 4.55, and the titratable acidity ranged between 0.8 and 1.0%.

Fatty acid composition and CLA content

The fatty acid profile showed some significant differences between fermented cow milk (Figure 3a) and fermented infant formula (Figure 3b). Cow milk was richer in short (SCFA) and middle chain fatty acids (MCFA), with values of 8.7% and 51.9%, respectively. The long-chain fatty acids (LCFA) made up 37.5% of the profile. By contrast, the infant formula contained 1.4% SCFA, 32.8% MCFA and 65.4% LCFA (data not shown).

The saturated fatty acid (SFA) concentration of cow milk was 68%, and the same fraction in infant formula accounted for 38%. The monounsaturated fractions were 27% and 43% in cow milk

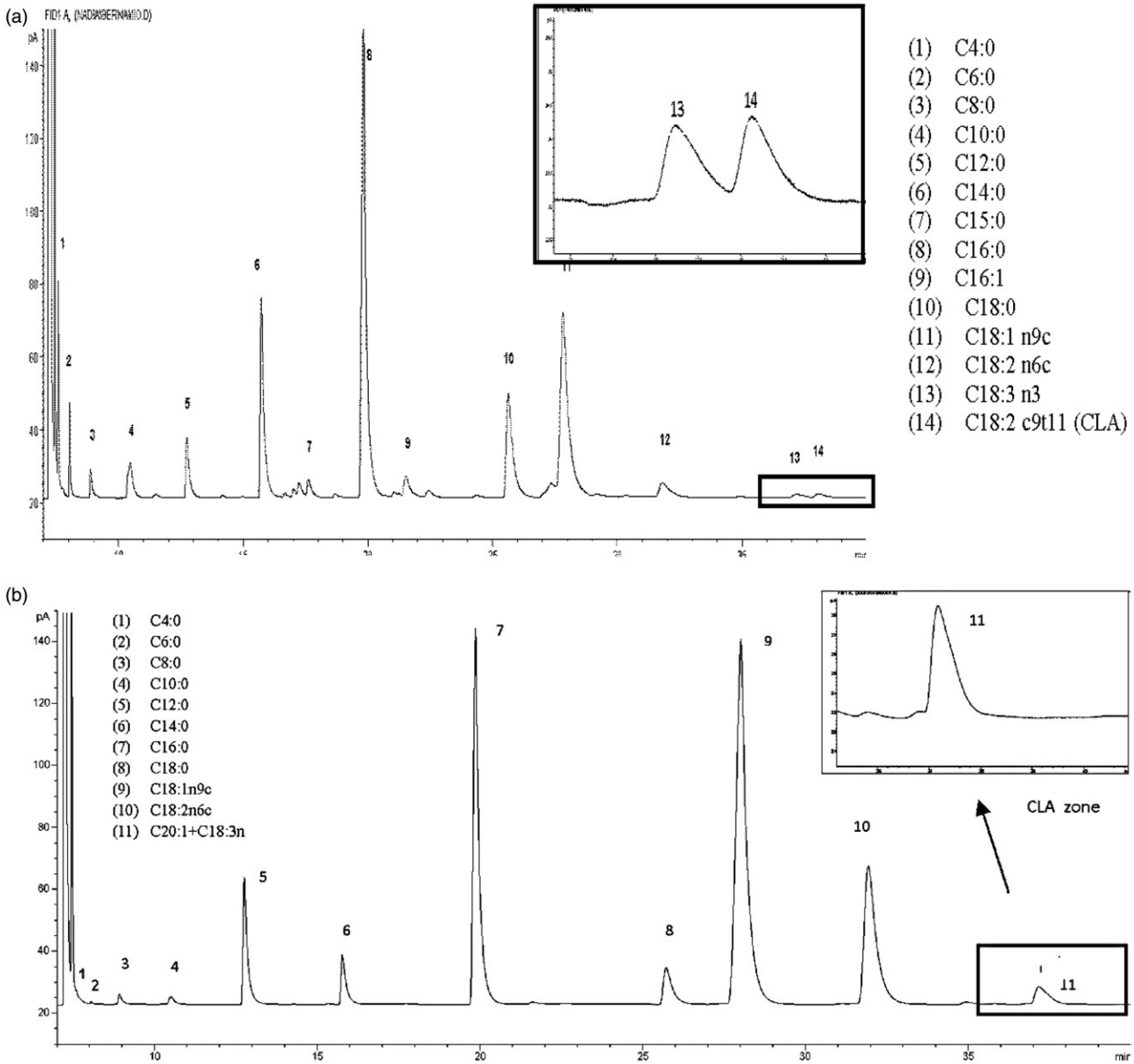


Figure 3. (a) Fermented cow milk gas chromatogram showing characteristic peaks. (b) Fermented infant formula milk gas chromatogram showing characteristic peaks.

and infant formula, respectively. Infant formula was richer in PUFA with 18.8% relative to the 3.4% PUFA in cow milk because of the higher presence of linoleic acid in the infant formula. Only c9-t11CLA was identified among all the CLA isomers in cow milk and its fermented products.

Table 2 shows the linoleic acid, c9-t11CLA, SFA, monounsaturated fatty acid (MUFA) and PUFA contents of cow milk and its fermented products. A statistical analysis showed no significant differences ($p > 0.05$) between cow milk and fermented milk in the presence and absence of probiotic microorganisms. The same result was obtained in the presence or absence of MD and FOS in a different manner from that of Akalin et al. (2007) which indicated that *Lb. acidophilus* La5 and *B. animalis* Bb12 are able to produce CLA during milk's transformation into fermented milk. Our results seem to suggest that the fermentation process did not consistently affect the fatty acid profile.

Table 3 shows linoleic acid, c9-t11CLA, SFA, MUFA and PUFA in infant formula and its fermented products. No CLA isomer was detected in infant formula or its fermented products, despite the high linoleic acid concentration (17.8%). No significant difference was observed in the SFA, MUFA and PUFA contents between milk and its fermented products.

Table 4 reports the linoleic acid, c9-t11CLA, SFA, MUFA and PUFA content of commercially available fermented products. Since all of them were derived from cow milk, their fatty acid profiles were similar to that of cow milk with significant differences among different products, because the fatty acid profile can be influenced by genetic factors, the environment, the stage of lactation, animal welfare and the nature of feeding (Chin et al., 1992). FM3 contained the highest amount of SFA. FM6 contained the highest amount of PUFA (4.38%). C18:2 was the primary fatty acid within the PUFA fraction, and it was present in the highest amount in sample FM6 (2.89%).

The amounts of c9-t11CLA found in commercial samples are shown in Figure 4. An average of 0.67% was detected. Three samples (FM1, FM2 and FM3) with values higher than the average (dot line) were produced by using only starter culture (*Str. thermophilus* and *Lb. bulgaricus*). FM6 contained the well-known

Table 2. Fatty acid composition (% w/w on total) of cow milk and of derived fermented products; average values (of three determination and experimentation) and standard deviation are reported.

Fatty acid	Milk	BeYC	BeFYC	BeAYC	BeFAYC	BeBYC	BeFBYC	MDB	MDBA	MDBB
C18:2n6c	2.25 ± 0.09	2.35 ± 0.08	2.29 ± 0.07	2.15 ± 0.02	2.26 ± 0.01	2.08 ± 0.13	2.34 ± 0.02	2.34 ± 0.06	2.28 ± 0.13	2.35 ± 0.05
CLA (9-c, 11-t)	0.55 ± 0.01	0.56 ± 0.01	0.57 ± 0.03	0.55 ± 0.01	0.54 ± 0.03	0.56 ± 0.05	0.56 ± 0.01	0.48 ± 0.05	0.54 ± 0.01	0.47 ± 0.01
∑SFA	68.10	67.84	67.90	68.08	68.00	68.07	67.86	67.49	67.13	68.10
∑MUFA	26.64	26.80	26.78	26.92	26.76	26.90	26.64	27.31	27.48	26.69
∑PUFA	3.40	3.50	3.44	3.27	3.39	3.23	3.55	3.42	3.45	3.45

c, cis; t, trans; CLA, conjugated linoleic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; BeYC, cow's milk fermented with starter; BeFYC, cow's milk fermented with starter + 2% FOS; BeAYC, cow's milk fermented with starter + *L. acidophilus* La5; BeFAYC, cow's milk fermented with starter + *L. acidophilus* La5 + 2% FOS; BeBYC, cow's milk fermented with starter + *B. animalis* Bb12; BeFBYC, cow's milk fermented with starter + *B. animalis* Bb12 + 2% FOS; MDB, cow's milk fermented with starter + 2% maltodextrin; MDBA, cow's milk fermented with starter + *L. acidophilus* La5 + 2% maltodextrin; MDBB, cow's milk fermented with starter + *B. animalis* Bb12 + 2% maltodextrin.

Table 3. Fatty acid composition (% w/w on total) of infant formula and of derived fermented products; average values (of three determination and experimentation) and standard deviation are reported.

Fatty acid	Infant formula	YC	YC + A	YC + B	FYC	FYCA	FYCB	MDH	MDHA	MDHB
C18:2n6c	17.80 ± 0.02	17.85 ± 0.06	17.88 ± 0.02	17.78 ± 0.19	17.67 ± 0.01	17.88 ± 0.01	17.75 ± 0.05	17.92 ± 0.26	17.78 ± 0.09	17.74 ± 0.14
CLA (9-c, 11-t)	–	–	–	–	–	–	–	–	–	–
∑SFA	38.18	38.35	38.23	38.14	38.03	38.05	38.07	37.75	38.20	37.91
∑MUFA	42.69	42.43	42.53	42.78	42.52	42.25	42.47	42.79	42.23	42.50
∑PUFA	18.88	18.93	18.95	18.85	18.74	18.96	18.95	18.96	18.84	18.84

c, cis; t, trans; CLA, conjugated linoleic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; YC, infant formula fermented with starter; YC + A, infant formula fermented with starter + *L. acidophilus* La5; YC + B, infant formula fermented with starter + *B. animalis* Bb12; FYC, infant formula fermented with starter + 2 % FOS; FYCA, infant formula fermented with starter + *L. acidophilus* La5 + 2% FOS; FYCB, infant formula fermented with starter + *B. animalis* Bb12 + 2% FOS; MDH, infant formula fermented with starter + 2% maltodextrin; MDHA, infant formula fermented with starter + *L. acidophilus* La5 + 2% maltodextrin; MDHB, infant formula fermented with starter + *B. animalis* Bb12 + 2% maltodextrin.

Table 4. Fatty acid composition (% w/w on total) of fermented products available from local market. Average values (of three determination and experimentation) and standard deviation are reported.

Fatty acid	FM1	FM2	FM3	FM4	FM5	FM6	FM7	FM8	FM9	FM10
C18:2n6c	$1.55^{c} \pm 0.06$	$1.70^{c} \pm 0.02$	$1.70^{c} \pm 0.04$	$2.46^{b} \pm 0.18$	$2.40^{b} \pm 0.01$	$2.89^{a} \pm 0.04$	$2.32^{b} \pm 0.01$	$1.65^{c} \pm 0.02$	$1.64^{c} \pm 0.02$	$2.37^{b} \pm 0.04$
CLA (9-c, 11-t)	$0.73^{bcd} \pm 0.03$	$0.91^{a} \pm 0.04$	$0.78^{bc} \pm 0.00$	$0.48^{g} \pm 0.05$	$0.57^{efg} \pm 0.02$	$0.64^{def} \pm 0.01$	$0.54^{fg} \pm 0.00$	$0.82^{ab} \pm 0.01$	$0.67^{cde} \pm 0.02$	$0.53^{g} \pm 0.03$
∑SFA	68.43^{ab}	67.09^{bc}	69.45^{a}	67.42^{bc}	66.79^{bc}	62.98^{d}	66.58^{c}	66.45^{c}	69.60^{a}	67.02^{bc}
∑MUFA	25.81^{d}	26.15^{cd}	24.40^{e}	26.80^{bcd}	27.26^{bc}	30.42^{a}	27.62^{b}	27.27^{bc}	24.28^{e}	27.12^{bc}
∑PUFA	3.14^{d}	3.66^{bc}	3.42^{cd}	3.67^{bc}	3.80^{b}	4.38^{a}	3.76^{bc}	3.43^{bcd}	3.26^{d}	3.72^{bc}

Different letters in the same line correspond to statistically significant differences ($p < 0.05$). c, cis; t, trans; CLA, conjugated linoleic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

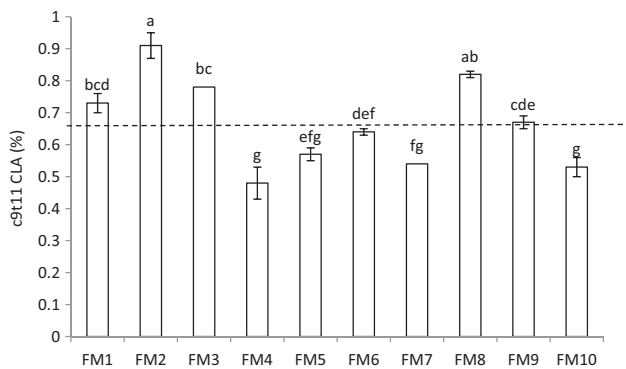


Figure 4. c9-t11CLA content in commercial yoghurt and fermented milk. Mean values and standard deviation. Different letters correspond to statistically significant differences ($p < 0.05$).

potential CLA producers *Lb. acidophilus*, *Lb. rhamnosus* (Sa et al., 2006), *Lb. casei* (Yadav et al., 2007) and *Lb. lactis* (Kim & Liu, 2002) and exhibited the highest percentage of PUFA, but a lower concentration of c9-t11CLA was observed. Sample FM2 was produced without probiotic microorganisms, and it contained the highest amount of CLA (0.91%).

Conclusion

Our results seem to suggest that the tested microorganisms were not able to produce linoleic acid isomers during the milk fermentation process, even if prebiotic matrices were added. Notwithstanding the high concentration of linoleic acid, which is primary source of CLA, fermentation process did not increase CLA levels in fermented products obtained from infant formula.

High CLA content values were expected for commercial products fermented by probiotics; however, FM2 had the highest c9-t11CLA content, and it contained only starter microorganisms. These results may confirm the hypothesis that the amount of CLA found in the fermented products is already present in the cow milk. This finding could suggest that the CLA quantity is not related to probiotic fermentation. This hypothesis is enforced by the results; the added microorganisms did not lead to statistically significant changes in the CLA concentration. The conjugated linoleic acids present in fermented milk were already there; these

CLAs are produced in the rumen and they are therefore maintained during the fermentation process.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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