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ORIGINAL ARTICLE

Conjugated linoleic acid conversion by dairy bacteria cultured in MRS broth and buffalo milk

C.P. Van Nieuwenhove, R. Oliszewski, S.N. González and A.B. Pérez Chaia

Centro de Referencia para Lactobacilos (CERELA-CONICET), Tucumán, Argentina
Universidad Nacional de Tucumán, Tucumán, Argentina

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Correspondence
Adriana B. Pérez Chaia, Universidad Nacional de Tucumán, Chacabuco 145- 4000- S.M. de Tucumán, Argentina.
E-mail: apchaia@cerela.org.ar

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Abstract

Aims: To evaluate strains of Lactobacilli, Bifidobacteria and Streptococci for their ability to produce conjugated linoleic acid (CLA) from free linoleic acid (LA).

Methods and Results: Eight dairy bacteria tolerant to LA were grown in MRS broth containing LA (200 μg ml^{-1}) and CLA was assessed. Seven bacteria were able to form CLA after 24 h of incubation, varying percentage conversion between 17% and 36%. *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Streptococcus thermophilus* showed the highest LA conversion and were inoculated into buffalo milk supplemented with different concentration of LA. The production of CLA at 200 μg ml^{-1} of LA was two- or threefold in milk than MRS broth. All evaluated strains were able to produce CLA from high LA levels (1000 μg ml^{-1}).

Conclusions: The most tolerant strain to LA was *Lact. casei*. *Lacttobacillus rhamnosus* produced the maximum level of CLA at high LA concentrations (800 μg ml^{-1}). The selected bacteria may be considered as adjunct cultures to be included on dairy fermented products manufacture. Low concentration of LA must be added to the medium to enhance CLA formation.

Significance and Impact of the Study: The production of CLA by strains using milks from regional farms as medium offer a possible mechanism to enhance this beneficial compound in dairy products and those the possibility to develop functional foods.

Introduction

Conjugated linoleic acid (CLA) is a term that refers to a mixture of positional and geometric isomers of linoleic acid (LA) in which double bonds are conjugated. It has received great attention for their beneficial health properties. CLA has been reported to prevent carcinogenesis (Ha *et al*. 1990; Ip *et al*. 1991) and atherosclerosis (Lee *et al*. 1994; Nicolosi *et al*. 1997), modulate immune response (Hayek *et al*. 1999) and reduce body fat (Park *et al*. 1997).

At least 16 CLA isomers are known, some of them produced by alkaline isomerization of LA (Sehat *et al*. 1998). The main isomer (*cis*9, *trans*11) occurs in dairy products, which are considered the most important natural source of CLA. Moreover, this isomer has been considered to have the main biological activities.

CLA is formed during rumen biohydrogenation, being *Butyrivibrio fibrisolvens* (Kepler and Tove 1967) the first discovered bacteria with this function. The ability of many bacteria to produce CLA has been probed in last years, among them some lactic acid bacteria (Jiang *et al*. 1998; Lin 2000). Moreover, many bacteria of different origin were able to form CLA like strains isolated from human intestines (Kamlage *et al*. 2000; Alonso *et al*. 2003; Coakley *et al*. 2003), cow's rumen (Kim *et al*. 2002), intestine of dogs and cats (Fukuda *et al*. 2002) and intestinal microflora of rats (Chin *et al*. 1994). Rainio *et al*. (2001) reported about CLA production by strains of *Propionibacterium freudenrehichii*, usually present in Swiss

cheese. In addition, Lin *et al.* (2003) isolated and studied enzimatic extracts of *Lact. acidophilus* and *Propionibacterium freudenrehichii* sp *shermanii* and found high CLA production rate in both.

Human production of CLA does not appear to occur to great levels. Therefore, many researchers are looking for the incorporation of bacterial strains able to form CLA into processed foods in order to increase its concentration in the human diet.

Previous studies demonstrated that CLA content in cheese varies according to strain (Lin *et al.* 1999) and ripening time (Shanta *et al.* 1995). Therefore, it seems to be appropriate to study particular behaviour of bacteria in the media that they will be used. Mozzarella cheese is the main product manufactured with buffalo milk.

The aim of this study was to evaluate the ability of some dairy bacteria to produce CLA in MRS broth and in buffalo milk supplemented with free LA.

Materials and methods

Bacterial strains, growth media and conditions

Eight lactic acid bacteria obtained from Centro de Referencia para Lactobacilos (CERELA) collection (Table 1) were used in the study. Cultures were activated by successive subculturing into MRS broth (De Man *et al.* 1960) or pasteurized buffalo milk at their optimal growth conditions. *Lactobacilli* and *Bifidobacterium* strains were grown anaerobically at 37°C and *Streptococcus thermophilus* at 42°C, during 24 h. MRS agar and LA (99% pure, Sigma) were used to asses the tolerance of active cultures of each strain to this fatty acid. The ability of some strains to growth and produce CLA was evaluated in MRS broth and in buffalo milk, both supplemented with LA. Buffalo milk was obtained from regional farms. It was refrigerated before transporting to the laboratory where was pasteurized (62°C, 30 min.) before use.

Table 1 Conversion rate of linoleic acid into CLA in MRS broth

Strain	CLA production (%)*
Streptreptococcus thermophilus CRL728	33·9
Lactactobacillus casei CRL431	35·9
Lact. acidophilus CRL730	23·8
Lact. casei CRL87	17·0
Lact. rhamnosus C14	34·5
Lact. acidophilus Q42	20·0
Bifidobacterium bifidum CRL1399	24·8
Lact. bulgaricus CRL423	ND

*Mean of triplicate analysis. CLA percentages were determined using the following equation: % CLA = CLA/(CLA + LA) × 100.
ND, not detected.

Growth in media with linoleic acid

A stock solution of LA (30 mg ml^{-1}) was prepared in 1% (v/v) Tween 80 (polyoxyethylene sorbitan monooleate) (Merck, Darmstadt, Germany) to improve its solubility. To evaluate the ability of bacteria to grow in presence of LA, an aliquot of an overnight culture of each strain was spread on MRS agar plates supplemented with 0, 50, 75 or 100 μg ml^{-1} of LA and incubated during 48 h at 37°C or 42°C under anaerobic conditions depending on strain (see above). After initial screening in presence of low fatty acid concentration, strains able to grow under this condition were assayed for CLA production in MRS broth. Inocula of 1% of each strain were added to MRS broth containing 200 μg ml^{-1} of LA and CLA production was determined after incubation at the appropriated temperature for each strain during 24 h. CLA content in the cultures was expressed as % of LA added. Growth and CLA production was also assayed in buffalo milk. Inocula of 1% of each strain were added to pasteurized buffalo milk supplemented or not (control) with 200 μg ml^{-1} of LA. Viable cells number, pH and CLA production were determined throughout time during 24 h of incubation at the condition above described.

Pasteurized buffalo milk supplemented with 200–1000 μg ml^{-1} of LA was used to asses the LA concentration effect on the maximal CLA production in 24 h. Viable cell number was determined by plating serial dilutions on MRS agar incubated at the conditions previously described for each strain.

Fatty acid analysis

Samples from cultures and sterile media were taken to determine fatty acid profile by gaseous chromatography (GC). Lipids were extracted by using chloroform/methanol (2:1, v/v) solution according to Folch procedure (1957) and derivatized to methyl ester (FAME) according to Chin *et al.* (1992). Pentadecanoic acid (C15:0) was used as internal standard. Fatty acid methyl esters were analyzed by GC (Agilent Technologies, series 6890N) equipped with a flame ionization detector and an automatic injector (Model 7683).

One micro litre of fatty acid methyl esters was injected to a HP-5 capillary column 30 m × 0·32 mm i.d. × 0·25 μm of thickness). GC conditions were: injector and detector temperature 250°C; initial oven temperature 50°C was increased to 150°C at 20°C/min (50 min), then increased to 225°C at 10 °C/min (20 min). Nitrogen was used as carrier gas with a pressure of 37·8 psi. Fatty acids were identified by comparison with the retention times of methylated standards (99%, Sigma, St Louis, MO, USA). *Cis*9, *trans*11

Table 2 Long-chain fatty acids composition of buffalo milk

Fatty acid (mg g^{-1} of fat)	Buffalo milk
C14 : 0	72·6 ± 6·6
C16 : 0	302·4 ± 22·7
C18 : 0	126·9 ± 9·9
C18 : 1 *trans*11	48·8 ± 7·2
C18 : 1 *cis*9	275·3 ± 15·1
C18 : 2	13·1 ± 3·1
C18 : 3	5·6 ± 1·3
*c*9, *t*11-CLA	4·7 ± 0·5
*t*10, *c*12-CLA	0·2 ± 0·1
SFA (%)	58·5
MUFA (%)	37·8
PUFA (%)	3·7

was used as CLA standard. Fatty acids were expressed as mg g^{-1} of fat in and CLA production as μg ml^{-1}.

When milk was used as culturing medium, total fatty acids were analyzed and CLA production was calculated by subtracting natural CLA content (0 h). Buffalo milk composition is shown in Table 2.

Statistical analysis

Samples were carried out by triplicate, expressing results as mean ± standard deviation (SD). Data were statistically evaluated by one-way ANOVA test (Minitab® Release 14 Statistical Software, 2003 Minitab Inc.). Significance level of 0·05 was used.

Results

Tolerance to linoleic acid and CLA production in MRS broth

The inhibitory effect of LA on bacterial growth has been reported by many authors (Jiang *et al.* 1998; Coakley *et al.* 2003), which demonstrated that there is a different tolerance according to strain. In the present study, the LA tolerance was evaluated by addition of increasing fatty acid concentrations (0, 50, 75 and 100 μg ml^{-1}) in MRS agar plates where bacteria were spread. Although previous studies (Jiang *et al.* 1998) reported bacterial growth inhibition using lower fatty acid level (25 μg ml^{-1}), in the present study all strains were able to grow in presence of higher LA concentrations (100 μg ml^{-1}).

Overnight inocula of strains were cultured into MRS broth containing LA at 200 μg ml^{-1}. After 24 h of incubation, lipids were extracted and analyzed (Fig. 1). CLA production was detected in seven bacteria, varying percentage conversion between 17% and 36% (Table 1). Percentages conversion were calculated by using CLA/(CLA + LA) × 100 equation. In the present study,

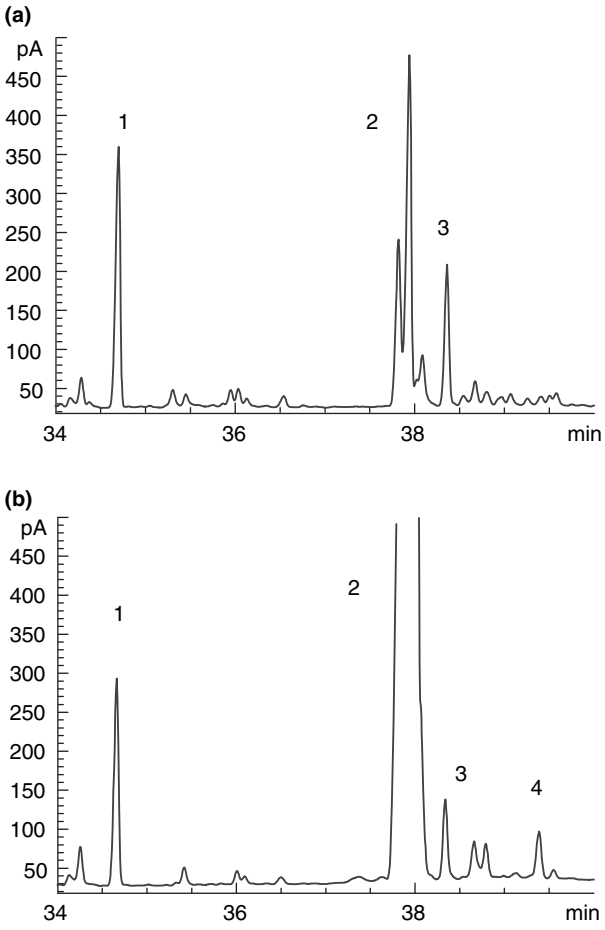

Figure 1 GC chromatogram of MRS broth before and after 24 h of incubation GC chromatogram of the fatty acid composition of the media supplemented with 200 μg ml^{-1} of LA and bacteria inoculum at 0 h (a) and 24 h (b) of incubation. (1): internal standard, (2): oleic acid, (3): linoleic acid, (4): conjugated linoleic acid (CLA).

Lact. casei CRL431 showed the highest CLA production in MRS broth (35·9%), followed by *Lact. rhamnosus* C14 (34·5%) and *Strep. thermophilus* CRL728 (33·9%). Jiang *et al.* (1998) did not found CLA production by lactobacilli, lactococci or streptococci strains. Coakley *et al.* (2003) determined CLA production only by *Bifidobacterium* strains cultured in MRS broth with 550 μg ml^{-1} of free LA. In the present study, *Bif. bifidum* CRL 1399 showed a LA percentage conversion value of 24·8%. *Lact. bulgaricus* CRL87 was the only strain unable to form CLA in the assayed conditions.

The four strains with the highest LA conversion were selected to study CLA production in buffalo milk.

Growth and CLA production in buffalo milk

The selected bacteria were cultured in buffalo milk supplemented with 200 μg ml^{-1} of LA. During 24 h of

incubation, samples were taken at regular intervals (0, 4, 8, 15 and 24 h) to determine pH, number of viable cells and fatty acid profile. Bacterial growth (expressed as log CFU ml^{-1}) and pH are shown in Fig. 2. At different intervals, pH of fermented buffalo milk was similar either in presence or absence of LA for each bacterium, suggesting that the bacterial metabolism is not affected by low concentration of fatty acid in the medium. Cell growth was not significantly affected by LA presence, except in *Lact. rhamnosus* where high viable count in absence of fatty acid ($P < 0{\cdot}05$) was observed after 24 h of incubation.

CLA content in buffalo milk was previously reported (Van Nieuwenhove *et al.* 2004). In our study, we determined total CLA in milk before and after bacterial fermentation.

Milk CLA content increased as fermentation progress. LA isomerization initiated after the first 4–7 h of incubation (Fig. 3). After 24 h of incubation, milk inoculated with *Lact. rhamnosus* C14 showed the highest CLA level, increasing from 5 to 6 mg g^{-1} of fat with respect to the initial CLA content. *Lactobacillus casei* CRL431 and *Strep. thermophilus* CRL728 also showed high CLA production (3·9 and 3·5 mg g^{-1} of fat, respectively). The lowest LA conjugation was determined in milk containing *Bif. bifidum* CRL1399, increasing CLA value 3 mg g^{-1} of fat with respect to the initial level (0 h).

An inverse relationship between CLA and LA concentration in milk as fermentation progressed was observed (Fig. 3).

To compare CLA production, results were expressed as μg ml^{-1} (Table 3). CLA production varied from 78 to 190·2 μg ml^{-1}, corresponding to percentage of conversion from 39% to 95%. In all evaluated strain higher LA conversion was determined in milk than MRS broth. This fact was previously reported by other authors (Rainio *et al.* 2001), which found an increase of twofold of CLA production in Propinoibacteria incubated in milk.

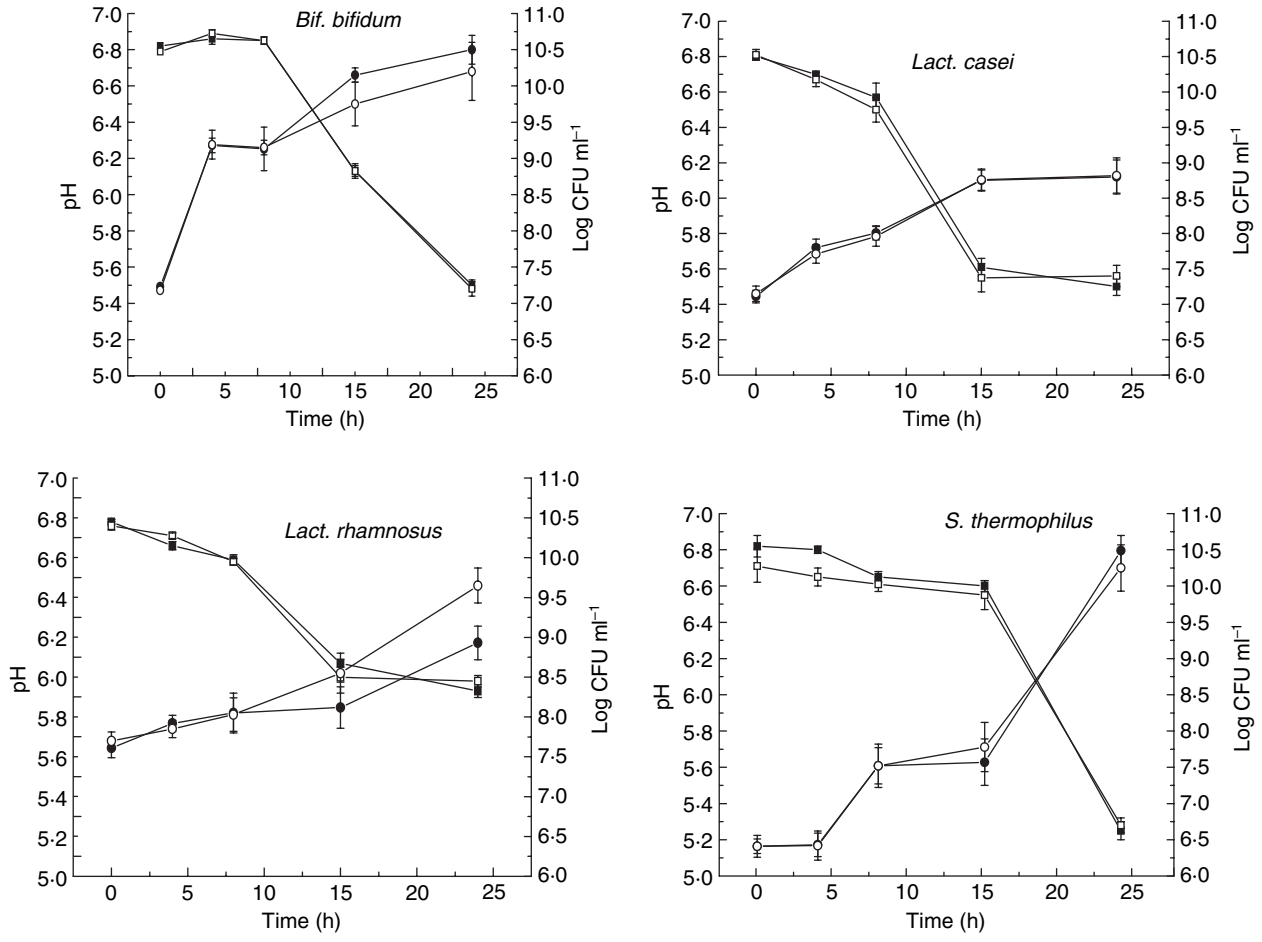

Figure 2 Growth of bacteria in buffalo milk containing free linoleic acid Bacteria were grown in buffalo milk supplemented with 200 μg ml^{-1} of linoleic acid. (■) pH with linoleic acid; (□) pH without linoleic acid; (•) Log CFU ml^{-1} with linoleic acid; (○) Log CFU ml^{-1} without linoleic acid.

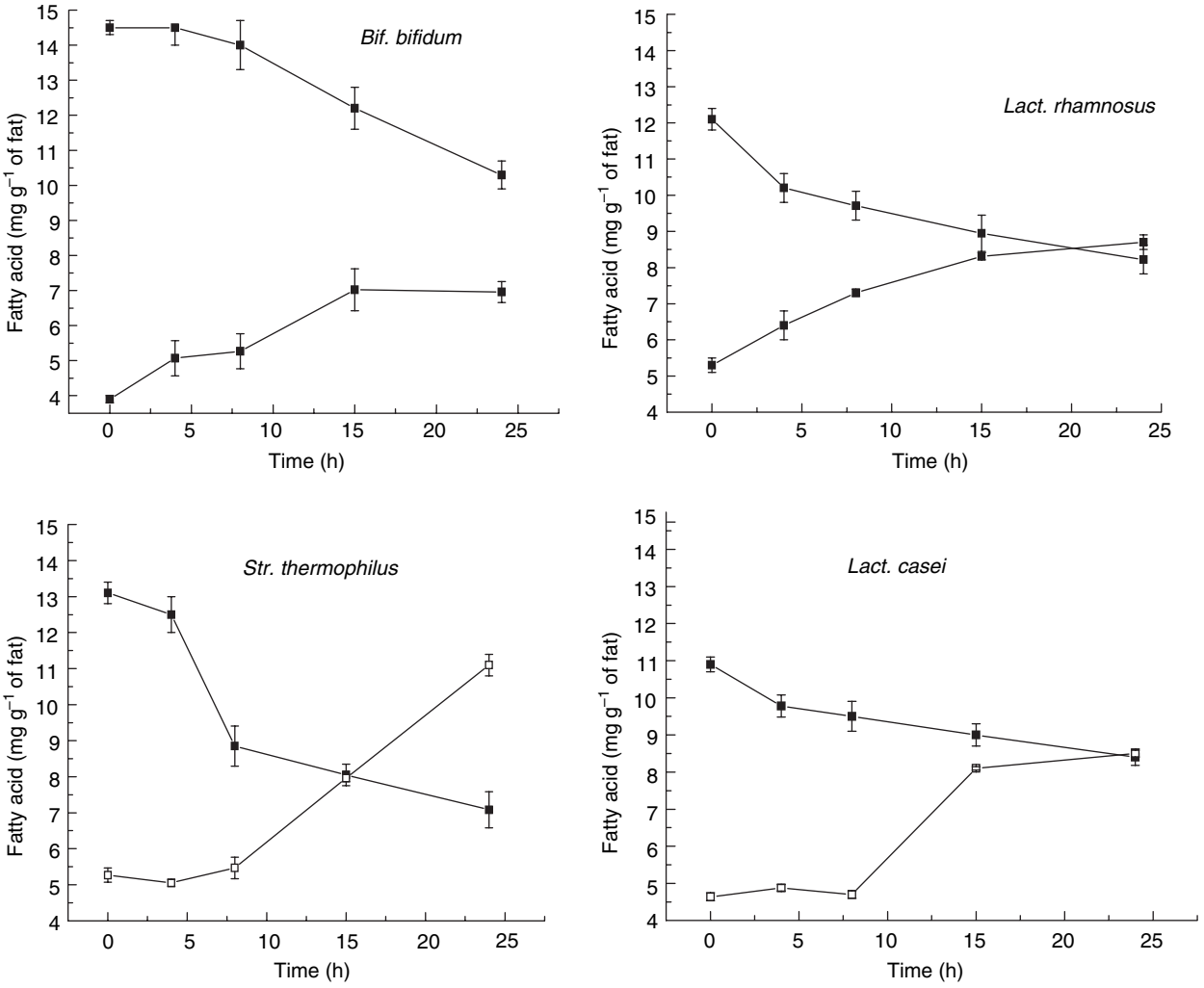

Figure 3 Linoleic acid and CLA concentration in buffalo milk during 24 h of incubation (■) Linoleic acid; (□) CLA.

Table 3 CLA production in buffalo milk at different linoleic acid levels

Linoleic acid (μg ml^{-1})	CLA production (μg ml^{-1})			% conversion				
Lactactobacillus casei	*Lactactobacillus rhamnosus*	*Bifidobacterium bifidum*	*Streptreptococcus Thermophilus*	*Lact. casei*	*Lact. rhamnosus*	*Bif bifidum*	*Strep. thermophilus*	
200	115·8	190·2	78	105	57·9	95·1	39	52·5
400	175·2	36·3	90	101·4	43·8	9·1	22·5	25·4
800	25·5	79·8	30·3	198·6	3·19	10	3·8	24·8
1000	46·2	18·3	20·4	96·9	4·6	1·8	2·0	9·7

Lactobacillus rhamnosus was the most efficient converser strain in presence of 200 μg ml^{-1} of LA (95%) and *Bif. Bifidum* was the lowest (39%).

All strains showed the highest CLA production near stationary phase. Therefore, following experiences were carried out after 24 h of fermentation to ensure the higher CLA production.

Tolerance to increasing levels of linoleic acid in milk

Increasing concentrations of LA (400–1000 μg ml^{-1}) were added into milk to evaluate the fatty acid tolerance and CLA production by strains. Cultures were incubated during 24 h, determining pH, viable cells count and lipid profile. Results about pH changes and bacterial growth are shown in Fig. 4.

Lactobacillus rhamnosus and *Bif. bifidum* evidenced similar final pH values in presence or absence of fatty acid. Both showed a growth inhibition at high LA doses. *Streptococcus thermophilus* was significantly inhibited from 800 μg ml^{-1} of LA, observing an increase on final pH. *Lactobacillus casei* was able to grow even at the highest LA levels probed, observing an increase on final pH too. The higher viable cell count could be due to the addition of Tween 80 which enhance the growth of many lactobacilli and to high LA tolerance of this strain. Citrate utilization by lactobacilli led to a lower pH decrease. This phenomenon can be attributed to the formation of carbon dioxide from sodium citrate present in milk. Carbon dioxide in the form of sodium acid carbonate could neutralize the acidity generated by the acid products formed (De Figueroa *et al.* 1996; Rea and Cogan 2003).

Conjugated linoleic acid production

Samples were collected to determine lipid profile and CLA production in presence of different concentrations of LA in milk.

CLA production at different LA levels was strain dependent (Table 3). *Lactbacillus casei* showed the higher CLA production at 400 μg ml^{-1} of LA levels while in *Lact. rhamnosus* was at 200 μg ml^{-1}. *Bifidobacterium Bifidum* again was the worst CLA producer, varying conversion percentage from 39% to 2% (200 and 1000 μg ml^{-1}, respectively). *Streptococcus thermophilus* evidenced the high CLA production at 800 μg ml^{-1} of LA. In all selected strain the higher percentage of LA conversion was determined at low LA levels.

Discussion

LA isomerase is present in many bacteria. So that, CLA production was reported in lactobacilli, bifidobacteria, propionibacteria and others (Lin 2000; Coakley *et al.* 2003; Lin *et al.* 2003; Lee *et al.* 2006). CLA production is influenced on many factors such as pH, media and phase of growth.

In our study, eight dairy bacteria were evaluated for CLA production in MRS broth. Seven of them were able

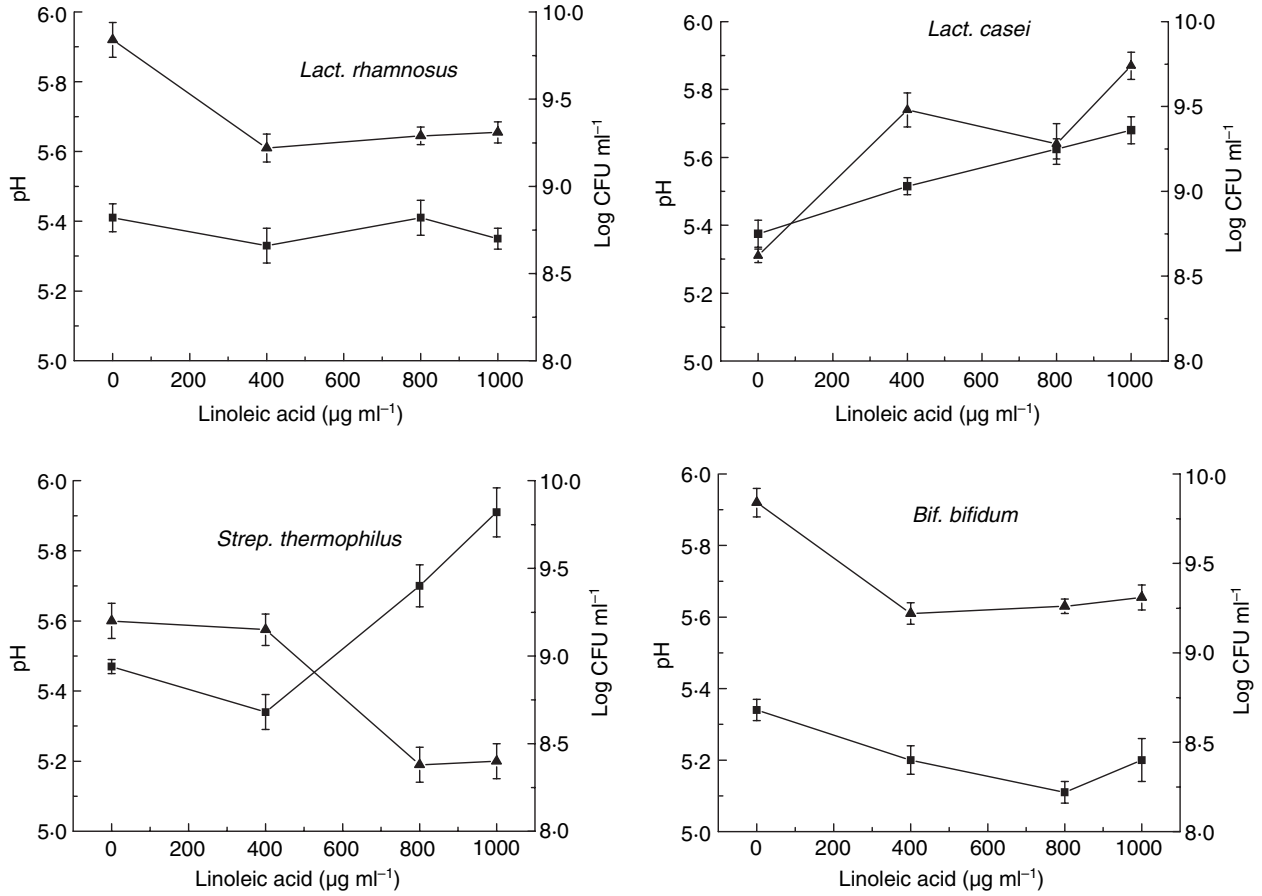

Figure 4 Growth and final pH in buffalo milk at different concentrations of linoleic acid Bacteria were cultured during 24 h in buffalo milk supplemented with free linoleic acid at different concentrations (400–1000 μg ml^{-1}). (▲) Log CFU ml^{-1}; (■) pH.

to generate CLA and four were selected according to the higher CLA production. These strain efficiently converted LA to CLA in MRS broth, but the percentage of CLA conversion was higher in milk than MRS broth.

All selected strains were able to grow in buffalo milk, showing the highest CLA formation near stationary phase. CLA production varied among strain, but all showed the higher percentage of conversion at low LA concentration.

Previous studies reported that many milk compounds, like proteins, could neutralize the negative effects of fatty acids on bacterial metabolism (Boyaval *et al.* 1995; Kim and Liu 2002). This process could explain bacterial growth in buffalo milk even at high concentrations of LA and the higher CLA production.

Our results are according to other authors which reported CLA production from 60% to 93% in milk (Jiang *et al.* 1998).

In our study, *Bif. bifidum* showed higher CLA production to results reported by Coakley *et al.* (2003). This bacterium is known for their several health and nutritional benefits (Boyaval *et al.* 1995).

Streptococcus thermophilus is commonly used as starter culture for fermented dairy products and *Lact. casei* and *Lact. rhamnosus* are usually included as adjunct cultures. Recently was reported that *Lact. rhamnosus* could produce CLA in rats and may be used as probiotic strain (Lee *et al.* 2006).

The reason why bacteria would convert LA to CLA is unclear. Jiang *et al.* (1998) proposed that conversion of LA into CLA may be a detoxification mechanism to avoid growth inhibitory effect of fatty acid.

The effect of bacteria on CLA production during cheese manufacture is in progress in our laboratory. Addition of strains with selective lipases is being studied too to liberate natural LA of milk and this way to serve as substrate for CLA production.

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