Quantitative and qualitative determination of CLA produced by Bifidobacterium and lactic acid bacteria by combining spectrophotometric and Ag^+-HPLC techniques

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Keywords:
CLA production
Probiotic bacteria
UV spectrophotometry
Ag^+-HPLC

A B S T R A C T

Bifidobacterium and lactic acid bacteria (LAB), especially from the genera *Lactobacillus* and *Lactococcus*, are commonly used in the production of fermented dairy products due to their potential probiotic characteristics. Moreover, some strains of these microorganisms also have the ability to produce conjugated linoleic acid (CLA) from linoleic acid (LA), which has attracted much attention as a novel type of beneficial functional fermented milk. In the present work 22 probiotic bacteria were tested for the production of CLA, using a UV screening method and HPLC techniques. Five microorganisms, two strains of the genera *Bifidobacterium*, two *Lactobacillus* and one *Lactococcus* were selected for their ability to produce CLA after incubation in skim milk with free LA as a substrate. It was possible to quantify the production of CLA (in the range of 40–50 μg CLA/ml) and identify the CLA isomers produced as C18:2 *cis* 9, *trans* 11 (60–65%), C18:2 *trans* 10, *cis* 12 (30–32%), C18:2 *trans* 9, *trans* 11 and C18:2 *trans* 10, *trans* 12 (2–5%).

1. Introduction

Conjugated linoleic acid (CLA) is a mixture of positional and geometric conjugated isomers of the essential fatty acid linoleic acid (LA) with conjugated double bonds at carbon positions from 6–8 to 13–15. CLA isomers (C18:2 *cis* 9, *trans* 11 and C18:2 *trans* 10, *cis* 12) confer a number of beneficial biological effects that have been identified in a range of animal models and include anti-carcinogenesis, immuno-modulation, anti-atherosclerosis and reduction of whole body fat (Hur, Park, & Joo, 2007; Lin, Lin, & Lee, 1999; Park & Pariza, 2007; Tanaka, 2005).

These compounds occur naturally in a variety of foods, including ruminant products such as milk-fat and meat which have been found to contain relatively large amounts of CLA. Dairy products from ruminants are very rich sources of CLA, among which 18:2 *cis* 9, *trans* 11, is the main isomer (Chin, Liu, Storkson, Ha, & Pariza, 1992). The presence of these compounds in dairy products is partly due to the isomerization and biohydrogenation of linoleic and linolenic acids that take place in the rumen; these processes are performed by ruminal bacteria, such as *Butyrivibrio fibrisolvens* and *Megasphaera elsdenni* (Bauman & Griinari, 2003; Jouany, Lassalas, Doreau, & Glasser, 2007; Sieber, Collomb, Aeschlimann, Jelen, & Eyer, 2004). Such observation has raised the hypothesis that other microorganisms may also be able to produce CLA. This hypothesis and the fact that several fermented dairy products contain higher levels of CLA than non-fermented counterparts, created the possibility of producing fermented dairy products with high levels of CLA. Lactic acid bacteria (LAB), especially from the genera *Lactobacillus*, *Bifidobacterium* and *Lactococcus*, are commonly used, due to their potential probiotic characteristics, to produce fermented dairy products (Almeida et al., 2008; Antunes et al., 2009; Parvez, Malik, Kang, & Kim, 2006). The identification of LAB able to produce CLA from a source of LA is of great importance since their use in the production of fermented dairy products will be of interest for human consumption as a probiotic dairy product with high CLA content.

Gas chromatography systems fitted with polar capillary columns and FID detectors are widely used in the fatty acid routine analysis (Jensen, 2002), as well as in the identification and quantification of minor compounds. However, when several isomers are presented, a combination of methodologies is needed. In analysis of CLA, GC has to be combined with Ag^+-HPLC, in order to obtain a full resolution of all the CLA isomers in the sample (Bondia-Pons, Molto-Puigmarti, Castellote, & Lopez-Sabater, 2007; Luna, Fontecha, Juarez, & de la Fuente, 2005; Sehat et al., 1998). Furthermore HPLC and GC are time consuming but due to conjugated double bonds can be detected using a 233 nm wavelength; in this case, UV spectrophotometers are able to perform a simple and rapid measurement in the high CLA producer LAB screening assays (Barrett, Ross, Fitzgerald, & Stanton, 2007).

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The aim of the present research work is to select CLA-producing bacteria in skim milk from a pool of potential probiotic LAB, by using a UV screening method to measure the CLA concentration followed by HPLC analytical techniques that are able to detect and identify the CLA isomers, toward their future application in the manufacture of fermented products.

2. Material and methods

2.1. Analytical reagents

All reagents used in the lab procedures were HPLC grade: hexane and isopropanol were obtained from Labscan (Dublin, Ireland), CLA isomers (C18:2 *cis* 9, *trans* 11 (rumenic acid) and C18:2 *trans* 10, *cis* 12) and linoleic acid (C18:2 *cis* 9, *cis* 12 from Sigma–Aldrich (St. Louis, MO, USA) and high CLA content oil (Tonalin®) from Cognis (Illertissen, Germany).

2.2. Bacterial strains

Twenty-two potentially probiotic strains were selected for this study; these included 16 strains of *Lactobacillus*, five strains of *Bifidobacterium* and one strain of *Lactococcus lactis* (Table 1). Among these, nine strains were isolated from commercial fermented milks on selective media, purified and identified in the Escola Superior de Biotecnologia (ESB), according to their morphological and physiological characteristics.

2.3. Screening for the bacteria producers of CLA

A first screening method to select high CLA-producing bacteria was performed in culture medium (Fig. 1). The bacterial strains were activated overnight at 37 °C in M17 broth for *L. lactis* (Oxoid, Hampshire, UK) or in MRS broth for the remaining strains (Pronadisa, Madrid, Spain). The media used to grow the *Bifidobacterium* and *Lactobacillus acidophilus* strains were supplemented with 0.05% (w/v) cysteine-HCl (Sigma) to lower the redox potential and incubated under anaerobic conditions. These activated cultures were transferred at 5% (v/v) to the appropriate culture medium (10 ml) containing 1% (w/v) Tween-80 (Scharlau, Sentmenat, Barcelona, Spain) and 1 mg/ml of linoleic acid (Sigma) and incubated 24 h.

Only the strains showing ability to produce CLA were subsequently tested in skim milk. In order to do so, the selected bacteria were activated in M17 or MRS broth as previously indicated. Ten milliliter of skim milk (Scharlau, Sentmenat, Barcelona, Spain; 0.5 g fat/l, 10% (w/v) were inoculated (5% v/v and incubated under appropriate conditions for 24 h. Thereafter, 2% (v/v) were transferred to 10 ml skim milk (10% w/v) containing 1% (w/v) Tween-80 and 1 mg/ml linoleic acid or 1 mg/ml safflower oil and incubated at 37 °C, these were monitored at 24 h and 48 h to know the optimal conditions for CLA production. Use of two linoleic acid sources (free fatty acids and safflower oil) allowed the authors to understand whether substrate affects CLA production. All experiences were carried out at least in duplicate.

2.4. Lipid extraction

Lipid isolation from culture media was carried out as a variation of the method described by Alonso, Cuesta, and Gilliland (2003). Briefly: 10 ml of culture media were centrifuged at 7500 rpm, 5 min, 4 °C. Three ml from the resulting supernatant were added with 6 ml of isopropanol and vortexed for 1 min. Addition of hexane (5 ml) followed which were vortex for 1 min and finally centrifuged at 2000 rpm, 5 min, 4 °C.

2.5. Quantification of CLA production by UV spectroscopy

Total CLA determination was carried out at a wavelength of 233 nm in a Perkin-Elmer spectrophotometer (Lambda650 model, Beaconsfield, UK) with a scan program (190–350 nm). Measurements were obtained in triplicate from 2 ml of the lipid extract in hexane placed into quartz cuvettes.

In order to verify the suitability of this method, a standard curve was constructed for the absorbance at 233 nm versus CLA (C18:2 c9, t11) concentration (0–30 μg/ml),

Table 1
Strains selected for this study, CLA production and source.

Strain	μg CLA/ml MRS[a]	Source
Lactobacillus brevis LMG 6906	–	Laboratorium voor Microbiologie en Microbiele Genetica–Rijksuniversiteit (Gent, Belgium)
Lactococcus lactis LMG S 19870	6.73 (4.53)	
Lactobacillus plantarum LMG S 19557	–	
Bifidobacterium animalis Bb12-1	7.12 (5.46)	Christian Hansen (Hoersholm, Denmark)
Bifidobacterium animalis Bb12-2		
Lactobacillus acidophilus La5	–	
Lactobacillus acidophilus LAC1	3.89 (9.81)	DELVO-PRO® DSM (Moorebank, Australia)
Bifidobacterium animalis BLC-1	–	
Lactobacillus paracasei ssp. *paracasei* LCS1	–	
Lactobacillus casei 01	–	
Lactobacillus acidophilus Ki	8.57 (10.23)	CSK–frozen concentrates (Leeuwarden, The Netherlands)
Bifidobacterium animalis Bo	–	
Lactobacillus acidophilus ATCC 4356	–	American Type Culture Collection (USA)
Lactobacillus acidophilus 1	–	Commercial fermented milks
Lactobacillus casei 1	–	
Lactobacillus casei 2	–	
Lactobacillus casei 3	–	
Lactobacillus rhamnosus 1	–	
Lactobacillus rhamnosus 2	–	
Lactobacillus plantarum-2	6.77 (2.52)	
Lactobacillus casei LCS	–	
Bifidobacterium animalis BLC	6.93 (7.85)	

CLA concentration in μg/ml of MRS medium with free LA (1 mg/ml) for 24 h and calculated spectrophotometrically at wavelength of 233 nm from the linear trend of the standard curve.

[a] Results expressed as mean values of triplicate determination (coefficient of variation, RSD).

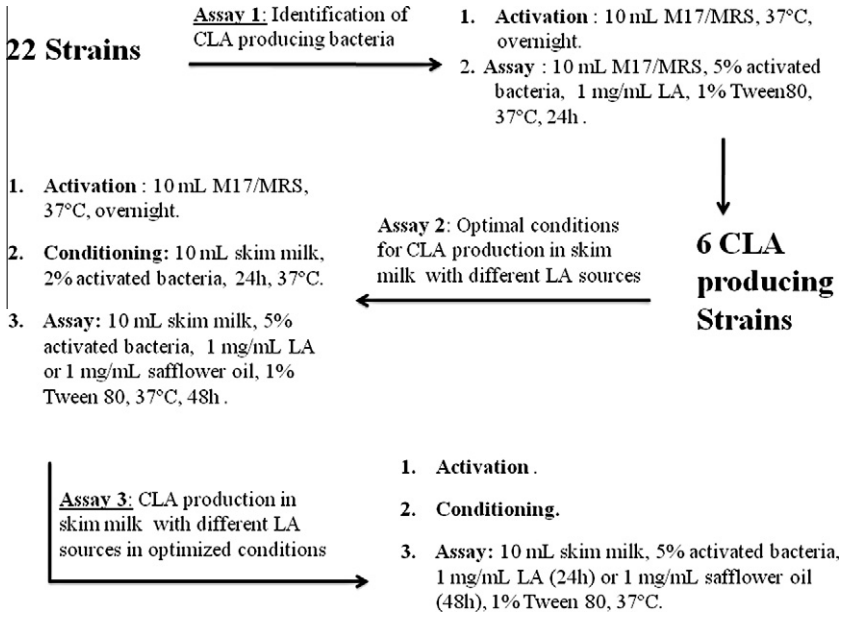

Fig. 1. Scheme of the sequential experiments performed to identify the probiotic bacteria capable of producing CLA and test this ability in skim milk using free linoleic acid or safflower oil as precursor substrates.

2.6. CLA isomers determination by Ag^+-HPLC

CLA isomers profile produced by LAB in skim milk was determined by Ag^+-HPLC. Previously, 4 ml of extract were evaporated, and methylated using sulphuric acid in mild conditions according to Aldai, Murray, Nájera, Troy and Osoro (2005) to obtain the FAME extract.

Separation of CLA methyl esters was carried out using a HPLC system (Shimadzu Vp Series, Duisburg, FR, Germany) equipped with UV detector operated at 233 nm. FAMEs were separated using three ChromSpher five lipid analytical column (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size; Varian, Palo Alto, CA, USA). The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 ml/min. The flow was initiated 0.5 h prior to the sample injection (10 μl).

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was carried out using SPSS v. 11.5 (SPSS, Chicago, IL, USA) to quantify the effects of the parameters bacterial strain and substrate on the CLA producing capacity. Tukey's HSD test at the 5% significance level was applied to all experimental data, to assess statistically significant differences among all possible combinations of parameters. All statistical analyses were performed using Statistica 6.1 (Statsoft, Tulsa, OK, USA).

3. Results

According to the spectrophotometric determination of CLA, the graph obtained from the standard curve demonstrated that an increase in the CLA concentration (from 0 to 0.30 μg/ml) coincided with a linear increase in absorbance ($R^2 = 0.993$; $y = 0.073x$) for the C18:2 *cis*-9, *trans* 11 CLA isomer up to an absorbance of 2.2.

Nevertheless, in order to confirm the results, a second calibration curve was arranged using Tonalin® (CLA-TG80 oil; 80% CLA) in hexane solution applying the RA regression curve equation and obtaining also a good linearity ($R^2 = 0.997$). These results agree with previous studies that demonstrated no differences among pure CLA standards and high CLA containing oils (Barrett et al., 2007).

Therefore the CLA concentration in the lipid extract of the culture supernatants could be calculated from the linear trend of the standard curve. The lipid extracts, obtained from the 22 bacteria assayed in this work, were screened spectrophotometrically, at wavelength of 233 nm, for CLA production after growth in the presence of linoleic acid. With this approach, a total of six of the 22 bacteria assayed in this work were identified with the ability to transform Linoleic Acid (LA) into CLA (Table 1).

Once identified, each of the six CLA producing strains were subsequently assayed for their ability to transform the substrate (LA or Safflower oil) and the optimum incubation time 24 h and 48 h was simultaneously assessed (Table 2). The concentration of CLA produced by each bacteria under each specific condition was calculated using the previous standard curve equation but taking into account that the extraction recovery percentage of CLA in skim milk was of 82.3% (determined by using a blank of CLA *C18:2 cis*-9, *trans* 11 isomer without the presence of LAB, data not shown). Table 2 shows that the highest levels of CLA production occurred for *L. acidophilus* Lac1 and *B. animalis* BLC (41.6 and 36.3 μg/ml, respectively) at 24 h of incubation when safflower oil was added as a substrate to skim milk, although other strains were also able to increase the bioconversion of linoleic acid to CLA production at 48 h of incubation time. Nevertheless, when free LA was used as substrate the highest rate of bioconversion of CLA was obtained for *B. animalis* Bb12-1 (21.6 μg/ml) at 24 h of incubation time.

Finally, a third trial was carried out using the optimum conditions previously identified in order to test the growth and viability of producing a high CLA probiotic dairy product. *L. acidophilus* Ki was excluded from this final study due to the very low concentrations of CLA produced in skim milk. Therefore, the 5 strains selected for their high CLA production capacity were grown, in duplicate, in skim milk added with either free LA or safflower oil as substrates and incubated during 24 h or 48 h, respectively (Table 3). In what concerns bacteriological growth, all the strains tested were able to grow in the presence of both LA and safflower oil. The highest growth in the presence of LA was achieved by *L. lactis* and the same occurred when safflower oil was added to skim milk (Fig. 2). It is important to observe that the presence of

Table 2
CLA concentration (μg/ml) of the culture supernatant's obtained after incubation of the selected strains in Skim milk with free LA (1 mg/ml) and safflower oil (1 mg/ml) for 24 and 48 h.

Strain	μg CLA/ml skim milk							
	Linoleic acid (FFA)				Safflower			
	24 h		48 h		24 h		48 h	
	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
B. animalis Bb12-1	21.57[a]	8.95	10.17[b]	7.50	16.72[c]	3.59	26.06[d]	8.95
L. acidophilus Lac1	15.29[a]	6.69	0.89[b]	10.00	41.62[c]	5.76	16.27[a]	6.69
B. animalis BLC	20.46[a]	9.98	13.86[b]	10.23	36.33[c]	8.25	24.41[a]	9.98
L. lactis LMG	18.94[a]	4.28	13.35[b]	5.67	24.03[c]	5.48	32.80[d]	4.28
L. acidophilus Ki	2.31[a]	6.65	0.89[b]	7.58	7.08[c]	12.47	8.01[c]	6.65
L. plantarum-2	11.44[a]	8.59	17.54[b]	10.47	10.30[a]	4.58	18.82[b]	8.59

[a] Results expressed as mean values of triplicate determination (coefficient of variation, %RSD). Superscript letters in a row means significant differences among assayed conditions ($p < 0.05$).

Table 3
CLA concentration (μg/ml) of the culture supernatant's obtained after incubation of the selected strains in skim milk medium with free LA (1 mg/mL) during 24 h and safflower oil (1 mg/ ml) for 48 h.

Strain	μg CLA/ml skim milk				
	Linoleic acid		Safflower		
	Mean	%RSD	Mean	%RSD	*P*
L. lactis LMG	45.51	6.22	23.05	9.56	0.025
B. animalis BLC	48.25	5.40	18.41	8.87	0.010
B. animalis Bb12-1	42.21	10.19	22.29	9.97	0.010
L. acidophilus Lac1	40.94	7.03	4.68	5.76	0.006
L. plantarum-2	51.68	5.22	21.53	11.53	0.014

P, significant value of the *t* Student test ($P < 0.05$).

either of these substrates did not have any influence upon the bacteriological growth ($P > 0.05$) during incubation.

All of the CLA-producing bacteria displayed homologous values of bioconversion of LA and all were found to be around 40–50 μg/ml when incubated in skim milk with free LA during 24 h and around 20 μg/ml when incubated with safflower oil during 48 h (with the exception of *L. acidophilus* Lac1 with only 4.7 μg/ml) (Table 3).

Since spectrophotometric methods do not distinguish between CLA isomers as the determination is based on the measurement of the conjugated double bound in the fatty acid, a chromatographic analysis by Ag^+-HPLC was carried out. The chromatogram profile (Fig. 3) showed the presence of four major conjugated linoleic moieties confirmed by the second derivative of the spectra (Banni, Day, Evans, Corongiu, & Lombardi, 1995) and by the injection of pure CLA standards. As expected, the C18:2 *cis* 9, *trans* 11 (rumenic acid) was the predominant isomer generated with a 60–65% and

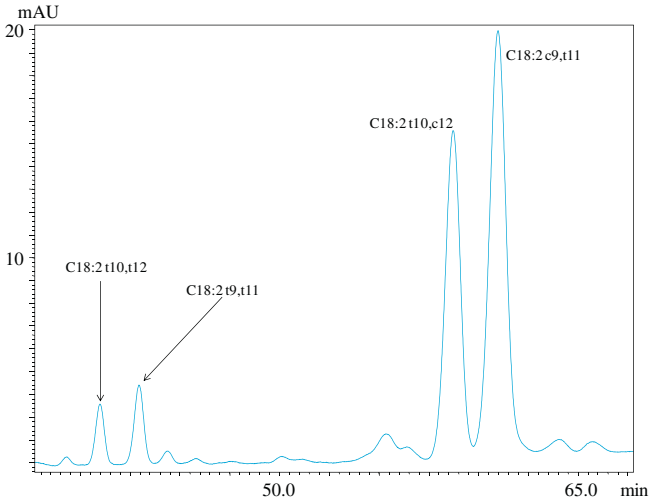

Fig. 3. Partial chromatogram of the CLA isomers profile assessed by Ag^+-HPLC of the culture supernatant obtained of *L. acidophilus* LAC1 in skim milk with free linoleic acid as precursor substrate.

also the C18:2 *trans* 10, *cis* 12 that accounted a 30–32%. The same fatty acids isomers but with *trans*, *trans* configurations (9, 11 and 10, 12) were also generated as minor compounds (2–5%). Similar profiles were obtained for all the five selected strains.

4. Discussion

In addition to the increased interest in the physiological effects conferred upon humans following CLA consumption, there has

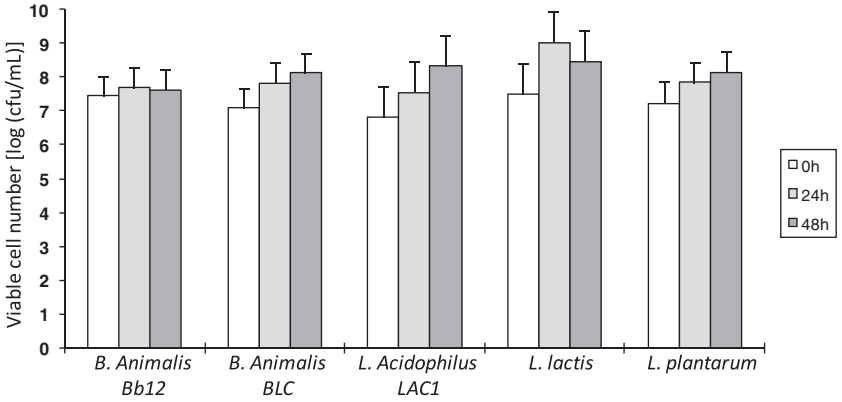

Fig. 2. Viable cell numbers of selected strains cultured in skim milk with free linoleic acid or safflower oil added, and incubated at 24 h and 48 h, respectively.

been concomitant interest in the isolation of bacterial strains (*Bifidobacterium* and LAB, especially from the genera *Lactobacillus* and *Lactococcus*) with the ability to produce CLA in milk or dairy products (Alonso et al., 2003; Bisig, Eberhard, Collomb, & Rehberger, 2007; Ogawa et al., 2005; Sieber et al., 2004). Furthermore, the combination of UV spectrophotometric and chromatographic techniques makes possible a rapid identification from a pool of microorganisms, of those able to produce a high quantity of CLA and at the same time quantify the amounts produced while GC and/or HPLC techniques allow the quantitative and qualitative analysis of the isomers (Barrett et al., 2007; Wang, Lv, Chu, Cui, & Ren, 2007).

Previous works have reported the ability of bifidobacteria and LAB to produce CLA in growth media with LA as substrate at concentrations that ranged from 3.5 to 350 μg/ml for *Bifidobacterium*, 60–1500 μg/ml for *Lactoccocus* and 20–4900 μg/ml for *Lactobacillus* strains (Ogawa et al., 2005; Sieber et al., 2004). In the present work when a screening procedure to identify possible CLA producers was performed, the amounts registered in the positive cases were within the results obtained by other authors as shown above. The differences found between our results and those obtained by other research groups may be due to intrinsic characteristics of the microorganisms and methodologies employed (concentration of LA, time and temperature of incubation) as well as the fact that LA may exert antimicrobial characteristics (Nieman, 1954; Wang et al., 2007), and that the isomerization of CLA has been proposed as a detoxifying mechanism that may even produce saturated fatty acids (Adamczak, Bornscheuer, & Bednarski, 2008).

When oils are used as LA sources, bacteria must have the ability to produce lipases and esterases to release the fatty acids from the triacyglycerides and it is well established that some strains are able to do it (Holland et al., 2005), which in fact represents an extra reaction step. Based on this fact, although our results showed a good CLA production during incubation of producing strains in skim milk with safflower oil during 24 h (Table 2), 48 h of reaction time was selected for the last experiment, since the CLA concentration seemed to increase for some of the selected strains. However, CLA production was in fact lower for those strains than before (Table 3). Decreases of CLA production due to oxidation reactions as well as oxidative metabolism of the microorganisms have been reported (Ogawa, Matsumura, Kishino, Omura, & Shimizu, 2001; Wang et al., 2007). This effect may explain the decrease of CLA production in skim milk using either LA or safflower as substrate when the incubation time was longer than 24 h.

Other authors (Xu, Boylston, & Glatz, 2004) reported no increments in the CLA concentration when the assay was carried out in skim milk with added milk-fat or soya oil (total fat content 1%), incubated for 24 h with *P. freudenreichii*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus, E. faecium*, *P. acidilactici,* and *B. bifidum*. However, when hydrolysed soya oil was added (total fat content 1%) CLA production was 0.63–2.21 mg CLA/g fat. Puniya, Chaitanya, Tyagi, De, and Singh (2008) found that *L. brevis* isolated from rumen fluid produced 10 mg CLA/g fat in skim milk using sunflower oil (0.25%) as a source of LA, while *L. lactis* with 1% sunflower oil was 9.22 mg CLA/g fat. The former research work supports the results presented in this study where concentrations of 2.72–3.44 mg CLA/g fat was obtained in skim milk after 24 h using LA as substrate while lower concentrations (0.31–1.53 mg CLA/g fat) were produced when safflower was added.

Several authors (Kishino, Ogawa, Ando, Omura, & Shimizu, 2002; Ogawa et al., 2005) have proposed pathways for CLA formation by LAB using LA as substrate. The fatty acid is converted into 10-hydroxy-12-*trans*-octadecadienoic and 10-hydroxy-12-*cis*-octadecadienoic and finally towards C18:2 *cis* 9, *trans* 11 (RA) and C18:2 *trans* 9, *trans* 11. It is generally accepted that LAB can transform polyunsaturated fatty acids to hydroxy fatty acids (Kim et al., 2003). Our results show that the selected probiotic strains produce CLA from LA transforming it mainly into C18:2 *cis* 9, *trans* 11 followed by C18:2 *trans* 10, *cis* 12 and small amounts of *trans*, *trans* isomers (9, 11 and 10, 12). Ogawa et al. (2005) supported all these results when he reviewed the CLA profile produced by *Bifidobacterium* and *Lactobacillus* strains when grown in the presence of LA.

In the present study *B. animalis* BLC produced the highest concentrations of CLA in skim milk with LA as substrate. Other studies elsewhere (Coakley et al., 2003; Hennessy, Ross, Devery, & Stanton, 2009; Park et al., 2009) reported that *Bifidobacterium* species were able to isomerize LA added as free fatty acid (0.35–0.65 mg/ml) or oil (sunflower, 0.25–1 mg/ml) to CLA ranging from 0.6–200 μg/ml in skim milk or in MRS giving an isomer profiles of C18:2 *cis* 9 *trans* 11, C18:2 *trans* 9, *trans* 11 and C18:2 *trans* 10, *cis* 12. According to these authors the reported presence of the C18:2 *trans* 10, *trans* 12 could be formed by the conversion of C18:2 *trans* 10, *cis* 12.

In this assay, the isomer composition produced by the probiotic bacterial strains assessed is similar to that found in synthetic mixtures (Ma, Wierzbicki, Field, & Clandinin, 1999) being a good alternative to avoid the chemical reagents if production is to be above the reported levels.

All the CLA producing strains studied in this work may be used in the dairy industry, namely in milk, in order to produce dairy products with increased CLA content, because substrates that can be used for that showed no inhibition. Future studies should be performed in order to optimise the CLA production and the probiotic growth in the presence of LA rich milk as substrates.

5. Conclusion

This study has concluded that it is possible to use LAB in milk as CLA producing microorganisms using different sources of Linoleic acid (free acid or oil). The working conditions and the substrate used to perform these assays is critical, hence due to the isomer profile similar to that of the synthetic preparations (constituting a good alternative) and the high interest of dairy products containing CLA, corroborated by previous reports where LAB have shown high potential to isomerize the substrate into CLA, future investigations should be carried out in order to improve and achieve the optimal production conditions.

Acknowledgements

This study was carried out with funds from the projects: CYTED-110AC0386, CM S-2009/AGR/1469, and CONSOLIDER-INGENIO CDS-2007-00063.

Financial support for author T.M. Braga was provided by a Ph.D. fellowship – BD/18667/2004 – issued by PRAXIS XXI (FCT, Portugal). The authors acknowledge Prof. Paula Teixeira and co-workers for the provision of probiotic isolates.

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