

Optical fibre-based methodology for screening the effect of probiotic bacteria on conjugated linoleic acid (CLA) in curdled milk

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A B S T R A C T

A methodology based on optical fibre (OF) detection was developed for screening the potential of CLA production by *Lactobacillus casei*-01, *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* B94 in probiotic curdled milk. The OF based methodology was validated by comparison with an analytical method based on gas chromatography–mass spectrometry (GC–MS) and it showed comparable linearity (between 5 and 130 µg), accuracy and detection limits, which ranged from 1.92 to 2.56 µg for CLA methyl ester and oleic acid methyl ester, respectively. Furthermore, the proposed OF based methodology was an advantageous analytical methodology for screening CLA production in probiotic curdled milk, due to its compact design and effective cost of analysis.

The application of the OF analytical method allowed to detect CLA in the probiotic curdled milk; the production of CLA by probiotic enzymes reached values of $297 \pm 19 \mu\text{g g}^{-1}$ by 60 days of ripening unfolding this potential functional food as very interesting from a technological point of view.

Keywords:

Optical fibre
GC–MS
Probiotic bacteria
CLA
Curdled milk

1. Introduction

The term conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exists as positional and geometric conjugated isomers of linoleic acid (LA, cis-9:cis-12–18:2n-6). These isomers have been found to have several beneficial physiological effects, demonstrated in animal models and cell cultures derived from humans/animals. CLA has been reported to have anti-carcinogenic, antiadipogenic, antiatherogenic, antidiabetogenic, immunomodulative and anti-inflammatory properties (Belury, 2002; Bhattacharya, Banu, Rahman, Causey, & Fernandes, 2006; Kelley, Hubbard, & Erickson, 2007; Wahle, Heys, & Rotondo, 2004). According to Ritzenthaler et al. (2001), an intake of cis-9:trans-11 intake must be 620 and 441 mg/d for men and women, respectively, to exhibit a cancer protective effect. Due to its potential health benefits, several CLA dietary supplements are currently commercialised, yet CLA occurs naturally in a variety of foods, especially in those derived from ruminant animals (Adamczak, Bornscheuer, & Bednarski, 2008; Fritsche et al., 1999). Dairy products are the most important source of CLA in the diet where cis-9:trans-11 isomer ((9Z,11E)-octadeca-9,11-dienoic acid) is the

principal form of CLA; its concentration depends on animal's diet, breed and age (Bisig, Eberhard, Collomb, & Rehberger, 2007).

Several microorganisms are able to synthesise CLA and therefore microbial conversion of LA to CLA by probiotic bacteria is desirable and adapts perfectly to the added-value of functional foods, a key research and innovation area in agro-research. Probiotics are viable microorganisms that can bring about health benefits to the host when administered in adequate amounts (Sanders, 2003). *Lactobacillus* and *Bifidobacterium* are among the common probiotic strains used in functional dairy products (Oliveira, Perego, Converti, & de Oliveira, 2009) and their ability to synthesise free CLA in cultured dairy products brings about additional health and nutritional benefits; studies focusing on CLA production in fermentation of milk by *Lactobacillus* and *Bifidobacterium* strains have shown this potential (Coakley et al., 2003; Lin, Lin, & Lee, 1999).

Due to the role of fatty acids in dairy products, fast and reliable methods for analysing CLA and other fatty acids are always desirable. Gas chromatography (GC) coupled to either a flame ionization detector (FID) or to a mass spectrometry detector (MS) are the most widely used methods for quantification of CLA (Rodríguez-Alcalá, Braga, Malcata, Gomes, & Fontecha, 2011).

The main objective of this research was to develop, validate, and apply a methodology based on optical fibre detection for screening the potential of CLA production by *Lactobacillus casei*-01, *Lactobacillus acidophilus* La-5 or *Bifidobacterium lactis* B94 in probiotic curdled milk matrices. To accomplish the objectives, three fatty acids

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were screened: oleic acid, LA and CLA (as the (9Z,11E)-octadeca-9,11-dienoic acid).

2. Experimental

2.1. Probiotic curdled milk

Probiotic curdled milk inoculated with either *L. casei*-01 (Chr. Hansen, Hørsholm, Denmark), *L. acidophilus* La-5 (Chr. Hansen, Hørsholm, Denmark) or *B. lactis* B94 (DSM) were produced according to [Rodrigues et al. \(2011\)](#). For each probiotic bacterium, 50-mL portions of cow milk previously sterilized (at 110–112 °C for 10 min) were prepared and divided into two groups, to obtain control and probiotic curdled matrices. For the control group and per liter of milk it was added 6 mL of three times diluted animal rennet (1:15,000) (Naturen[®], from Chr. Hansen, Hørsholm, Denmark), 6 mL of CaCl₂ (Panreac, Barcelona, Spain), and 10 g of NaCl (Panreac, Barcelona, Spain). Probiotic curdled milk shared the same composition, but were inoculated at 2% with *L. casei*-01, *L. acidophilus* La-5 or *B. lactis* B94, respectively. Milk coagulation was performed at 30 °C for 5 h. Upon coagulation, duplicates of each curdled milk matrix type were withdrawn and labelled as 0 d. The remaining samples were incubated at 12 °C throughout 60 days of ripening period; duplicate samples were withdrawn at 15, 30, 45 and 60 days.

2.2. Microbiological analyses

Viable cells of probiotic bacteria were obtained through 100 µL aliquots plated, in duplicate in MRS agar (Biokar Diagnostics, Beauvais, France) containing 0.5 g/L of L-cysteine-HCl (Panreac, Barcelona, Spain) after sequential dilutions which were made with sterile 0.1% (w/v) peptone water (Himedia, Mumbai, India) of curdled matrix homogenates ([Rodrigues et al., 2011](#)). Plating was followed by incubation at 37 °C for 48 h, under aerobic conditions for *L. casei*-01, and under anaerobic conditions for *L. acidophilus* La-5 and *B. lactis* B94. In order to check for contamination, microbiological counts were performed on plate count agar (Biokar Diagnostics, Beauvais, France) incubated aerobically at 37 °C for 48 h. No contamination was found over ripening period.

2.3. Chemical analyses

2.3.1. Extraction

Duplicated samples of each curdled milk (control or probiotic), were assessed for free fatty (FFA) acids and conjugated linoleic acids according to [Alonso, Cuesta, and Gilliland \(2003\)](#) with some modifications and [Rodrigues et al. \(2011\)](#) shaken vigorously. The mixture (sample and 2-propanol) was added to hexane (9 mL). The sample (6 mL) was added to 12 mL of 2-propanol (Fluka, Sintra, Portugal and Panreac, Barcelona, Spain) shaken for an extra 3 min and placed in an ultra-sound bath (Ultrasonik, Montreal, Canada) for 30 min. After settling, the upper layer was collected by aspiration and filtered with sodium sulphate anhydrous (Panreac, Barcelona, Spain). The sodium sulphate bed was washed with an additional 7 mL of hexane (added to the filtered mixture). The lipid fraction was collected in a 100 mL pear-shaped flask, and placed in a Rotavapor (Laborota 4000, from Heidolph, Schwabach, Germany) at 40 °C until dryness. The extract was redissolved in 500 µL of hexane, and transferred to a microtube. A methanolic solution (100 µL) was added to the tube containing the lipid fraction. The mixture was vortexed for 1 min, and held at 7 °C for 15 min in a sandy bath. Methylation was carried out with 200 µL of 14% (w/v) boron trifluoride in methanol (Sigma, Sintra, Portugal), at room temperature for 30 min and adding 0.2 mL of hexane

to the mixture. Extracts were stored at –20 °C until analysis. Oleic acid, linoleic acid and CLA were analysed as methyl esters by the developed optical fibre-based methodology after its validation.

2.3.2. GC–MS based methodology

GC–MS based methodology was ([Rodrigues et al., 2011](#)) used in order to test and compare the performance of the OF methodology. Standards of each fatty acid methyl ester for calibration or ten standard mixtures with different concentrations of each fatty acid methyl ester were injected (1 µL) in splitless mode, at 250 °C on a GC–MS (QP5000, Shimadzu, IZASA, Lisbon, Portugal) operated in SIM mode. The fatty acids chromatography took place in a DB5 capillary column (0.25 µm film × 0.25 mm × 30 m) (Sigma, Sintra, Portugal) using Helium as carrier gas (linear velocity of 35 cm/s). The temperature program started at 80 °C, and increased until 300 °C at a rate of 8 °C/min. The interface and MS detector temperatures were kept at 290 °C.

2.4. Optical fibre-based methodology

[Fig. 1a](#) shows the analytical apparatus of the optical fibre-based methodology (OF methodology) where the temperature of the injection cell (I) was controlled by a coiled tape heater from Cole Parmer, Montreal, Canada and a temperature sensor. The chromatographic column (C) contains a fused silica fibre coated with a film of phenyl methylpolysiloxane and was surrounded by another tape heater and a temperature sensor. The chromatographic column was connected to the flowmeter (F), which kept a constant flow of nitrogen, and to the analytical tube (AT). The AT design includes an internal narrowed region of 0.4 cm of diameter and 6.5 cm long. Inside the analytical tube was the optical fibre (O), which was previously uncladded and cleaved, with a Cleaver V6 (Future Instrument, Barcelona, Spain) precision fibre cleaver, to 15 mm of the optical path cord. In the cleaved fibre section a PDMS matrix was deposited resulting in the sensitive component of the OF methodology. The PDMS coating was deposited by dip-coating technique, dipping the optical fibre cleaved section into the PDMS solution. Besides optical fibre and the analytical tube the detection component of the OF methodology is also constituted by a laser diode optical source (L) with the working wavelength set at 1550 nm to generate the interrogating signal and a photodiode detector (P) to measure the intensity of the modulated signal which was connected to the optical coupler (OC). The optical component (O) of the detector is constituted by a monomode optical fibre pigtail, core and cladding diameters of 9 and 125 µm, respectively, integrated into a directional 50:50 Y optical coupler (OC). The optical coupler was also connected to the photodiode detector (P) which was connected to a laptop (PC) with home-made software.

Either individual standards of linoleic acid methyl ester, linoleic acid conjugated methyl ester and oleic acid methyl ester (Sigma, Sintra, Portugal), or ten different concentrations of standard mixtures, and samples (1 µL) were introduced by a gastight micro-syringe (Hamilton, Bonaduz, GR, Switzerland) at the top of the injector (I) kept at 290 °C. After separation on the column at 290 °C the analytes reached the analytical tube which contains the coated optical fibre (O) generating an analytical signal ([Fig. 1b](#)). The changes in the reflected optical power caused by variations in the refractive index of optical fibre have been previously discussed by [Silva, Rocha-Santos, and Duarte \(2008a, 2008b, 2008c, 2008d, 2009\)](#) and [Silva, M-Costa, Freitas, Rocha-Santos, and Duarte \(2009\)](#), and depend on both coating film (PDMS) and analytes (oleic acid methyl ester, linoleic acid methyl ester and CLA methyl ester) properties, as well as the chemical interactions which can take place between these two elements. The different sensitivities ([Fig. 1b](#)) obtained for the three compounds tested can be attributed

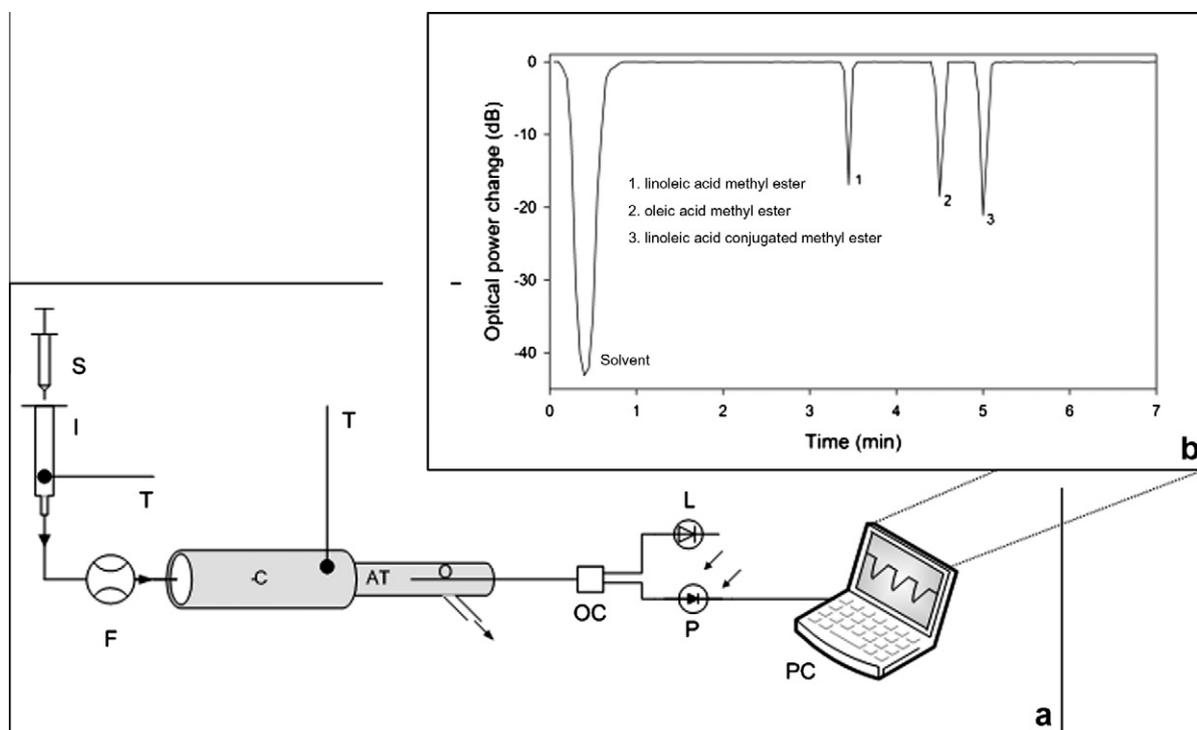


Fig. 1. (a) Experimental apparatus of optical fibre-based methodology (S: syringe, I: injection cell, T: temperature sensor, F: flowmeter, C: Chromatographic Column, AT: analytical tube, O: optical fibre, OC: optical coupler, L: laser diode source, P: photodiode detector, PC: laptop with home-made software); (b) analytical signal obtained by optical fibre-based methodology for a standard mixture 40.00 μg of oleic acid methyl ester, linoleic acid methyl ester and linoleic acid conjugated methyl ester.

to the different analyte properties, such as the boiling temperature, the molecular weight and the vapour pressure. In this case the sensitivity varies in the same way as the boiling temperature (linoleic acid methyl ester <oleic acid methyl ester <CLA methyl ester).

2.5. Quality assurance and quality control (QA/QC)

In order to assure representativeness and reliability of the results obtained, QA/QC procedures included blanks, duplicates and recovery assays. Replicate analysis of the curdled milk samples gave an uncertainty of <5% for the three fatty acids (oleic acid, linoleic acid and CLA). Recovery assays were carried out to ensure effectiveness of the extraction procedure of the three fatty acids from curdled milk samples and recoveries ranged from 95 to 101%. The calibration curves for both OF and GC-MS methodologies were obtained for five repeated measurements of five standard solutions containing a mixture of linoleic acid methyl ester, oleic acid methyl ester and CLA methyl ester in concentrations of 5.00, 10.00, 40.00, 70.00, 100.00 and 130.00 μg . The concentration of each compound was determined by direct interpolation in the standard curve within their linear dynamic range, and the

detection limits were calculated using $y = y_B + 3s_B$, where s_B is the standard deviation (SD) of the blank signal estimated as $s_{y/x}$, the residual SD taken from the calibration line and y_B is the blank signal estimated from the intercept taken also from the calibration line (Miller & Miller, 2005).

Ten different concentrations of standard mixtures (10.00, 30.00, 40.00, 50.00, 70.00, 80.00, 90.00, 100.00, 110.00, 120.00 μg) of each fatty acid methyl ester (linoleic acid methyl ester, oleic acid methyl ester and CLA methyl ester) were also obtained for five repeated measurements by both GC-MS and OF methodologies.

2.6. Statistical methods

All statistical analyses were performed using SigmaStat™ 3.0 (SigmaStat, 1994).

A two way repeated measures analysis of variance (ANOVA) and all pairwise multiple comparison procedures performed by Holm-Sidak method, were applied to the results obtained for the ten standard mixtures analysed by the GC-MS and the OF methodologies together with the expected value for each standard mixture.

Table 1
Analytical parameters obtained for linoleic acid methyl ester, oleic acid methyl ester and CLA methyl ester with GC-MS and OF based methodologies.

	Methodology	Linoleic acid methyl ester	Oleic acid methyl ester	CLA methyl ester
Retention time (s)	GC-MS	42.78	43.26	44.51
	OF	3.52	4.27	5.05
Linear range (μg)	GC-MS	5-130	5-130	5-130
	OF	5-130	5-130	5-130
Linear calibration	GC-MS	$y = 0.835x + 36.82$	$y = 0.835x + 40.59$	$y = 0.839x + 43.36$
	OF	$y = 0.108x + 12.56$	$y = 0.109x + 13.99$	$y = 0.135x + 15.53$
Squared coefficient of correlation (R^2)	GC-MS	0.9997 ($p < 3.22 \times 10^{-8}$)	0.9994 ($p < 2.52 \times 10^{-6}$)	0.9998 ($p < 2.08 \times 10^{-8}$)
	OF	0.9997 ($p < 3.39 \times 10^{-8}$)	0.9995 ($p < 1.02 \times 10^{-7}$)	0.9997 ($p < 3.21 \times 10^{-8}$)
Detection limit (μg)	GC-MS	1.92	2.57	1.72
	OF	1.94	2.56	1.92

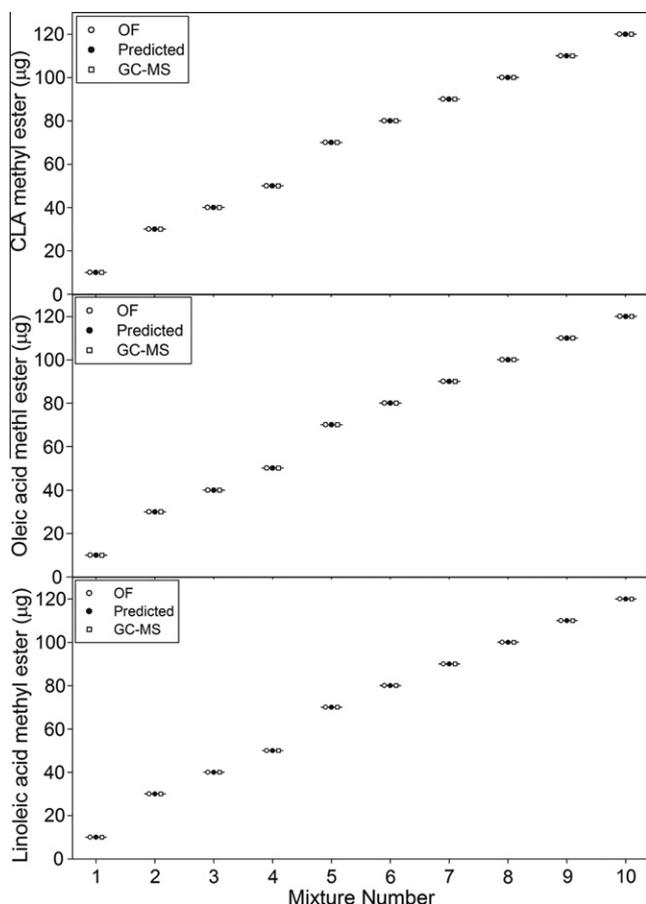


Fig. 2. Comparison of the results obtained with the OF and GC-MS methods for CLA methyl ester, oleic acid methyl ester, and linoleic acid methyl ester.

For control curdled milk as well as for each probiotic curdled milk, a one-way analysis of variance (ANOVA) was performed to analyse if the variation of oleic acid, linoleic acid, CLA and of viable cells of probiotic strain was significant throughout time at the 0.05 level of significance.

3. Results and discussion

The analytical time for the OF methodology (Fig. 1b) was 7 min against 46 min obtained for GC-MS due to different instrumentation and operational conditions of the analytical system, namely different chromatographic components, different temperature programs and different carrier gas flow rates. The retention times and other analytical parameters can be observed in Table 1. The detection limits, in µg, were in a range from 1.72 to 2.57, and from 1.92 to 2.56, for the GC-MS and OF methodologies, respectively. The linear range was 5–130 µg for both methodologies.

Table 2

Oleic acid, LA and CLA concentrations in control curdled milk throughout ripening time and variation of viable cells numbers in curdled milk inoculated with either probiotic strain.

Ripening time (d)	Control curdled milk (µg/g) ^a			Probiotic curdled milk (log cfu/g)		
	Oleic acid	LA	CLA	<i>B. lactis</i> B94	<i>L. casei</i> -01	<i>L. acidophilus</i> La-5
0	823.4 ± 3.0	87.68 ± 1.24	<1.92	8.1 ± 0.2	8.11 ± 0.05	8.26 ± 0.08
15	823.9 ± 1.7	87.60 ± 0.95	<1.92	9.6 ± 0.1	9.52 ± 0.01	8.3 ± 0.2
30	824.2 ± 2.5	87.67 ± 1.14	<1.92	9.525 ± 0.009	9.5 ± 0.1	8.2 ± 0.2
45	826.2 ± 0.4	87.76 ± 1.03	<1.92	8.8 ± 0.3	9.14 ± 0.02	8.5 ± 0.1
60	823.2 ± 0.9	87.42 ± 0.96	<1.92	7.66 ± 0.04	8.3 ± 0.2	8.52 ± 0.01

^a Mean + standard deviation values obtained from the three control curdled milk (one for each probiotic curdled milk) since no significant variation occurred ($p < 0.05$) for LA or oleic acid through ripening time.

At a 95% confidence level, the response of the OF methodology shows calibration sensitivities, measured as the slope of the calibration curve, which increases in the following order: linoleic acid methyl ester <oleic acid methyl ester <CLA methyl ester. The different sensitivity obtained for the three compounds tested was already discussed in the Section 2 and can be attributed to different analyte properties, such as the boiling temperature.

Fig. 2 shows the results (predicted value and obtained by OF and GC-MS methodologies) from the ten standard mixtures with different concentrations used to evaluate the performance of OF methodology. The ANOVA of these results allowed to conclude that there are no significant differences between the results obtained by the two methods ($p = 0.848, 0.706, 0.203$) for linoleic acid methyl ester, oleic acid methyl ester and CLA methyl ester, respectively). Although there is a statistical significance ($p < 0.001$) for the effects of different levels of the expected value, the results obtained by the two methods do not depend on what level the expected value is present for linoleic acid methyl ester ($p = 0.962$), oleic acid methyl ester ($p = 0.819$) and CLA methyl ester ($p = 0.992$).

The analytical error, measured as the residual standard deviation of both methods, was around 3×10^{-2} µg.

The OF methodology after preliminary tests, comparison and validation against GC-MS methodology, was applied to the analysis of oleic acid, linoleic acid (LA) and CLA on curdled milk matrices inoculated with either probiotic strain (*L. casei*-01, *L. acidophilus* La-5 or *B. lactis* B94) and ripened through a period of 60 d.

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO. Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Canada, & May 1, 2002; Sanders, 2003) and therefore the viability and metabolic activity of these microorganisms are key factors that must be controlled during processing operations and maturation period. A statistical significant increase of viable cells ($p < 0.05$) for both *B. lactis* B94 and *L. casei*-01 was observed in the first 30 days of ripening followed by a decrease until 60 days (Table 2). In turn, the number of viable cells of *L. acidophilus* La-5 remained approximately constant through all ripening period; the variation of viable cells of *L. acidophilus* La-5 through ripening time was not statistically significant ($p > 0.05$). Despite the different oscillations in viable cells observed for the three probiotic strains, the levels of each probiotic bacterium in curdled milk were around 10^8 cfu/g through the 60 days of ripening. Although it is still not possible to accurately generalise a minimum dose of probiotics that is needed for a beneficial effect in the gut, some studies revealed positive effects at levels of 10^8 cfu per day (Douglas & Sanders, 2008). Based on these guidelines, curdled milk revealed to be adequate food products to deliver probiotic concentrations capable of providing potential health.

No statistical significant variations of oleic or LA acid were observed through ripening period in the control curdled milk ($p > 0.05$); CLA concentrations found in those samples were below detection limit (Table 2).

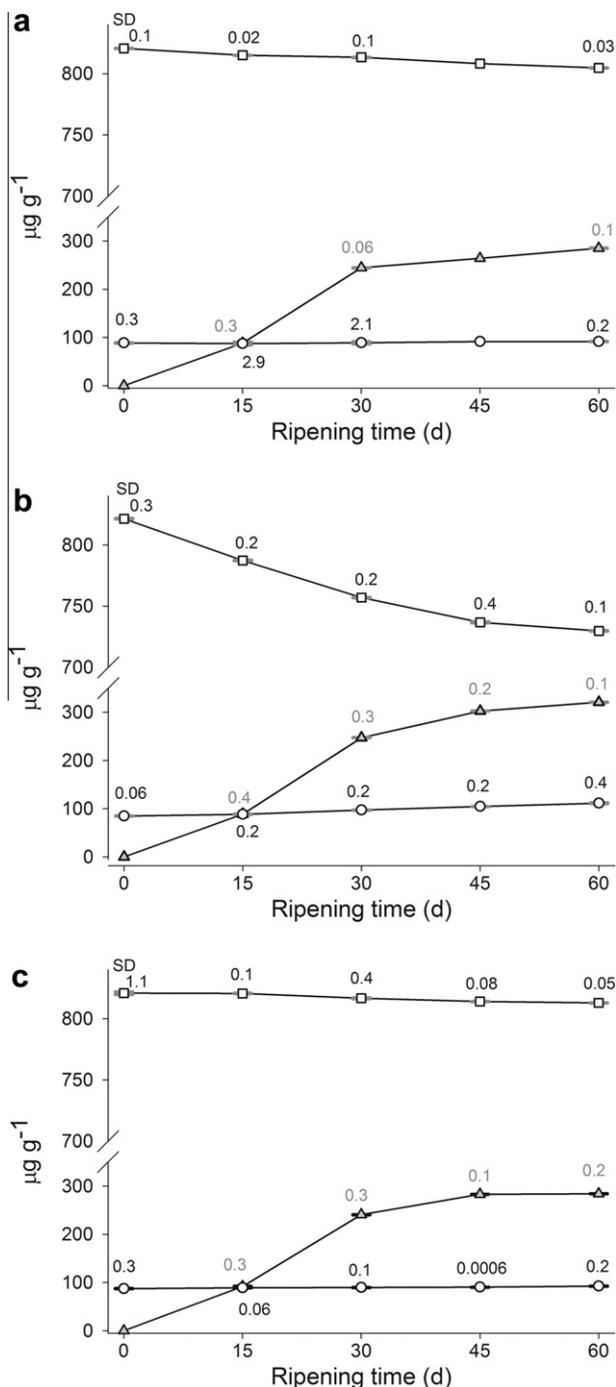


Fig. 3. Variation of concentrations of oleic acid (□), linoleic acid (○) or CLA (Δ) throughout ripening, in probiotic curdled milk inoculated with each probiotic strain: (a) *B. lactis* B94; (b) *L. casei*-01; (c) *L. acidophilus* La-5; SD - standard deviation.

In probiotic curdled milk inoculated with either *L. casei*-01, *B. lactis* B94 or *L. acidophilus* La-5 a statistical significant increase ($p < 0.05$) of CLA was observed reaching values from 284 to 321 $\mu\text{g/g}$ of curdled milk at 60 days of ripening (Fig. 3). Microbial enzymes are known to be major contributors to CLA production since linoleic acid isomerase activity can convert LA to CLA, which is probably responsible for the significant differences in CLA content found between inoculated curdled milk and their control counterparts. According to Kishino, Ogawa, Yokozeki, & Shimizu, 2009 and Ogawa, Matsumura, Kishino, Omura, & Shimizu, 2001, the isomerization of LA in CLA by lactic acid bacteria results from

two consecutive reactions: initially occurs hydration of LA forming 10-hydroxy-C18:1 which then undergoes isomerization by dehydration which is responsible to produce cis-9:trans-11, trans-9:cis-11 or trans-9:trans-11 C18:2 isomers. The LA concentration increased 3–5% in *B. lactis* B94 ($p > 0.05$) or *L. acidophilus* La-5 ($p > 0.05$) curdled milk over ripening period; in probiotic curdled milk inoculated with *L. casei*-01, the increase of LA was of 29% ($p < 0.05$) after 60 days of ripening. The reported LA variations over ripening period could be related to lipolysis performed by each probiotic strain especially with *L. casei*-01; the increase of total free fatty acids was statistical significant in comparison to the corresponding control curdled milk (Rodrigues et al., 2011) being probably responsible for the increase of free fatty acids such as LA.

Studies on alternative substrates for the CLA production besides LA, have shown that these can be obtained from oleic acid (C18:1) with strains of *L. delbrueckii* (Lin, 2006) reporting production of trans-8:trans-10, trans-10:cis-12 and cis9:trans-11 C18:2 isomers. This phenomenon could explain the significant decrease of oleic acid ($p < 0.05$) in probiotic curdled milk inoculated with *L. casei*-01. Slighter decreases were observed in those samples inoculated with *B. lactis* B94 and *L. acidophilus* La-5, respectively.

4. Conclusions

The production of CLA in new potential probiotic foods could indeed be considered as an added-value in functional foods since dietary CLA has been reported as important food additive with biological properties. Therefore, a single and reliable methodology based on optical fibre analysis could fasten the screening process for determining the potential of CLA production by probiotic bacteria in milk matrices. The optical fibre-based methodology showed performance and detection limits comparable to the GC-MS while avoiding the use of more expensive and sophisticated equipment.

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