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# Formation of conjugated linoleic acid by a *Lactobacillus plantarum* strain isolated from an artisanal cheese: Evaluation in miniature cheeses

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ABSTRACT

Among 129 lactic acid bacteria previously isolated from raw-milk starter-free cheeses manufactured in Galicia (NW Spain), two strains of *Lactobacillus plantarum* were definitely recognised as producers of conjugated linoleic acid (CLA). Gas chromatography analysis identified *cis*-9, *trans*-11 C18:2 as the predominant CLA isomer formed in MRS broth supplemented with linoleic acid. A centrifugation-based model for the manufacture of miniature cheeses was used to evaluate the formation of CLA by *Lb. plantarum* L200, the highest producer of CLA in MRS broth. The miniature cheeses made with the addition of the L200 strain showed significantly ($P < 0.05$) higher contents of *cis*-9, *trans*-11 CLA than those of the control cheeses (1.09 versus 0.69 percentage of total fatty acids, respectively). These results suggest that *Lb. plantarum* L200 strain could be used as an adjunct culture to slightly increase the concentrations of CLA in short-ripened cows' milk cheeses.



## 1. Introduction

Conjugated linoleic acid (CLA) isomers have attracted great interest in recent years because of their attributed functional and health promoting properties, including anticarcinogenic, antiatherogenic, antiobesity, antiinflammatory and antidiabetic effects (Hennessy, Ross, Devery, & Stanton, 2011; Yang et al., 2015). The main CLA isomers recognised with these beneficial activities are *cis*-9, *trans*-11 C18:2, *trans*-10, *cis*-12 C18:2 and *trans*-9, *trans*-11 C18:2 (Renes et al., 2017; Yang et al., 2015).

Dairy products from ruminants are the most important source of CLA in a diet, and may contribute to around 60% of the total dietary CLA intake (Chin, Liu, Storkson, Ha, & Pariza, 1992). The *cis*-9, *trans*-11 isomer, also called rumenic acid, is the principal form of dairy CLA, representing approximately 90% of the total CLA (Chin et al., 1992; Prandini, Sigolo, Tansini, Brogna, & Piva, 2007). Conjugated linoleic acid content in milk and milk products ranges between 0.1% and 2.9% of total fat, with the highest amounts found in cheeses from sheep milk (El-Salam & El-Shibiny, 2014).

A daily intake of 3 g per day for a person weighing 70 kg has been recommended to achieve the highest health benefits of CLA (Ip, Scimeca, & Thompson, 1994). Consequently, increasing the concentration of CLA in dairy products has been the focus of several studies with a view to improve their beneficial properties on health and to develop functional food products (Ozer, Kilic, & Kilic, 2016). In this sense, feeding lactating ruminants on natural pasture and oil-supplemented rations seems to be the factor that most significantly increases the CLA levels of milk and derived dairy products (El-Salam & El-Shibiny, 2014; Van Nieuwenhove, Oliszewski, & González, 2009).

Some lactic acid bacteria (LAB), especially *Lactobacillus*, and *Bifidobacterium* strains may produce CLA by isomerisation of linoleic acid (LA) using linoleate isomerase enzyme (Rodríguez-Alcalá, Braga, Malcata, Gomes, & Fontecha, 2011; Yang et al., 2017). Incorporation of such CLA-forming bacteria as starters or adjunct cultures into cultured dairy products offers a viable and natural strategy for increasing CLA content (Andrade et al., 2012). Therefore, the selection of LAB isolates able to produce CLA in milk by biological fermentation processes constitutes a meaningful purpose for the food industry in relation to cheese and fermented dairy products (Ozer et al., 2016; Rodríguez-Alcalá et al., 2011).

To assay the properties of different microbial strains in cheese making, to predict cheese yield or to evaluate variations in processing conditions, simple protocols which use small milk samples,

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fixed times between rennet addition and cutting, and centrifugation for whey separation have been developed (Bachmann, Kruijswijk, Molenaar, Kleerebezem, & van Hylckama Vlieg, 2009; Cipolat-Gotet et al., 2016). In these miniature cheese-making procedures, a small volume (1.7–10 mL) of milk contained in glass tubes or in the wells of a microplate is coagulated and centrifuged (instead of drained, moulded and pressed) at 1000–4800× *g* in one or several stages to separate the whey from the curd. These fast and inexpensive methods show reasonably acceptable performance, with manufacturing conditions being highly reproducible, and thus they can be used in the screening of microbial strains for the expression of specific enzymatic activities or flavour-forming abilities (Bachmann et al., 2009).

In this context, the aims of the present study were: (i) to screen 129 LAB isolates obtained from traditional raw-milk starter-free cow cheeses for their ability to produce CLA from free LA in synthetic culture media; and (ii) to test selected LAB strains for the formation of CLA in ripened cow milk cheeses using a miniature laboratory cheese model.

## 2. Materials and methods

### 2.1. Bacterial strains and culture media

One hundred and twenty-nine LAB isolates (55 lactococci, 42 mesophilic lactobacilli and 32 leuconostocs) previously obtained and selected among the microbiota of raw-milk starter-free cow cheeses manufactured in Galicia, NW Spain (Garabal, Rodríguez-Alonso, & Centeno, 2008), were screened for their ability to convert free LA to CLA in synthetic culture media. Commercial starter cultures had never been used in the products from which the isolates originated. In addition to the cheese isolates, two reference food-derived LAB strains (*Lactobacillus plantarum* strain CECT 749/ATCC 10241 and *Lactobacillus brevis* CECT 5172/DSMZ 6235) obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain) were used as positive controls. Stock cultures were maintained at −30 °C in 11% sterile reconstituted skim milk with 20% (v/v) glycerol added, and activated by subculturing twice at 30 °C for 24 h in MRS broth (Oxoid, Basingstoke, Hampshire, UK) for lactobacilli and leuconostocs, or in Elliker broth (BD Difco, Franklin Lakes, NJ, USA) for lactococci.

### 2.2. Screening of LAB for CLA production from free LA

The ability to convert free LA to total CLA was initially investigated in MRS broth for lactobacilli and leuconostocs and in Elliker broth for lactococci supplemented with 1% (w/v) Tween 80 (polyoxyethylene sorbitan monooleate; Scharlau, Sentmenat, Barcelona, Spain) and 0.25 mg $mL^{-1}$ free LA (99% purity; Sigma–Aldrich, St. Louis, MO, USA). The activated bacterial strains were transferred at 2% (v/v) to the culture medium (10 mL) and aerobically incubated at 30 °C for 48 h. All assessments were carried out in triplicate.

Lipid extraction from culture media was performed as described by Rodríguez-Alcalá et al. (2011). The total CLA contained in the supernatants was estimated in accordance with the rapid screening UV-spectrophotometric method proposed by Barrett, Ross, Fitzgerald, and Stanton (2007). Absorbance values at 233 nm were determined in a Lambda 650 UV/Vis spectrophotometer (PerkinElmer Ltd, Beaconsfield, UK). For each isolate, 2 mL of lipid extract in hexane were placed into quartz cuvettes and analysed. A calibration curve was built for the absorbance at 233 nm versus *cis*-9, *trans*-11 CLA isomer (96% purity; Sigma–Aldrich) concentration (0–50 μg $mL^{-1}$). The assumed isomerisation rate of LA into CLA in the culture medium was calculated by the formula: CLA concentration/initial LA concentration × 100.

### 2.3. CLA production and quantification by gas chromatography

The 15 isolates (11 *Lactococcus lactis*, 2 *Lactobacillus paracasei*, and 2 Lb. *plantarum*) showing the ability to convert free LA to total CLA with assumed isomerisation rates higher than 10% in accordance with the preliminary screening method were subsequently assayed by gas chromatography (GC). The isolates were tested in a LA emulsion in bovine serum albumin (BSA) (Lin, 2006) to avoid any potentially positive effects caused by Tween 80 on the growth and production of CLA by LAB (Corcoran, Stanton, Fitzgeral & Ross, 2007; Li et al., 2011).

The selected bacteria were activated in MRS or Elliker broth as previously indicated and then inoculated at 2% (v/v) in (100 mL) MRS broth prepared without Tween 80 and supplemented with 0.25 mg $mL^{-1}$ free LA (Sigma–Aldrich) and 0.1 mg $mL^{-1}$ BSA (≥95% pure Sigma–Aldrich), and incubated at 30 °C on a rotary shaker at 120 rpm for 48 h. The cultures were then centrifuged at 5000× *g* for 10 min at room temperature. The fat was extracted from the culture supernatant fluid and from the bacterial pellet independently, according to the method described by Yang et al. (2014).

Fatty acids from 0.5 mL hexane layers were esterified and fatty acid methyl esters were extracted as described by Ledoux et al. (2005). Separation, identification and quantification of the methyl esters of: *cis*-9, *trans*-11; *trans*-10, *cis*-12; and *trans*-9, *trans*-11 CLA isomers were performed with the aid of a Trace GC Ultra (Thermo Finnigan, Austin, TX, USA) chromatograph equipped with a flame ionisation detector (FID), under the conditions described by Méndez-Cid, Centeno, Martínez, and Carballo (2017). All samples and standards were injected in triplicate.

### 2.4. Manufacture and analysis of miniature cheese models for testing CLA production by adjunct LAB

A protocol for the manufacture of miniature laboratory cheeses that meet the requirements for gross composition and pH of both the industrial PDO Arzúa-Ulloa and Tetilla cheese varieties was designed. Both cheeses combined represent about 60% of the total annual production of unmixed cow milk PDO cheeses manufactured in Spain, and have quite similar characteristics regarding flavour and texture. Industrial Arzúa-Ulloa and Tetilla cheese making includes a curd washing step, similar to Dutch-type cheeses. Therefore, the protocol designed in this study was based on that described by Bachmann et al. (2009) for the production of miniaturised Gouda-type cheeses, even though the volumes were larger to facilitate the analytical procedures, and cheeses were ripened in an environmental atmosphere. All the information concerning the preparation and curdling of cheese milk, operations for whey drainage and cheese ripening can be found in the Supplementary material. Two cheese making trials were carried out.

#### 2.4.1. Bacterial strains for the manufacture of the miniature laboratory cheeses

The commercial starter used in the manufacture of the miniature cheeses was the freeze-dried direct-vat-set Choozit MM100 (Danisco® Food Ingredients, Sassenage, France), a mesophilic D-starter containing *L. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* strains. The starter was maintained at −25 °C until use. The strains *Lb. paracasei* L45 (non-CLA forming) and *Lb. plantarum* L200 (CLA-forming) were employed as adjunct cultures in the manufacture of the miniature cheeses. Frozen cultures were grown on MRS broth at 30 °C for 24 h, and then subcultured at 2% (v/v) in sterile (110 °C, 15 min) reconstituted skim milk (Oxoid) and incubated for 48 h at 30 °C. The absence of antimicrobial activity of the L45 and L200 strains against the commercial culture MM100 was previously

confirmed by the agar well diffusion assay, as described by Centeno, Gaya, Medina, and Nuñez (2002).

*2.4.2. Cheese sampling and physicochemical analyses*

Three groups of cheese were obtained: control cheeses, L45 cheeses (made with the non-CLA forming adjunct culture), and L200 cheeses (made with the CLA-forming adjunct culture). All miniature cheeses were sampled on day 28 of ripening. Dry extract, fat, protein and ash content were analysed only in the control cheeses. The compositional parameters and pH were determined as previously described (Centeno, Rodríguez-Alonso, Carballo, & Garabal, 2015). To perform each of the analyses, the number (between 1 and 3) of cheeses (1.10 ± 0.12 g weight) needed to yield sufficient material was pooled. All analyses were carried out in duplicate and the results averaged for each cheese making trial.

*2.4.3. Analysis of total fatty acids*

Miniature cheeses from the three different groups (control, L45 and L200) were sampled to determine total fatty acids, including: *cis*-9, *trans*-11; *trans*-10, *cis*-12; and *trans*-9, *trans*-11 CLA isomers. The fat from 5.00 ± 0.01 g samples (obtained by pooling 5 miniature cheeses from each of the groups) was extracted following the procedure described by Folch, Lees, and Sloane-Stanley (1957). Lipid methylation from 0.500 ± 0.001 g of the extracted fat was carried out according to Méndez-Cid et al. (2017). The separation of the fatty acids from the total lipids was performed by GC-FID, as previously described in Section 2.3, for the bacterial strains.

Individual fatty acid methyl esters were identified and quantified by comparison with the retention times and peak areas of the standard mixture of FAME Mix Supelco 37 Components (Supelco, Bellefonte, PA, USA) and of CLA isomers (Sigma–Aldrich). The CLA and fatty acid concentrations of miniature cheese samples were expressed in g 100 $g^{-1}$ fatty acids, calculated with peak areas corrected by factors according to the AOAC 963.22 method (AOAC, 2000). Two analytical replicates were made and the results were averaged for each of the trials.

*2.5. Statistical analysis*

Data obtained for the contents of the different fatty acids determined in the miniature cheeses analysed in the present study were examined by analysis of variance (ANOVA). When a significant effect was found in the ANOVA, the significance of the differences between cheeses were determined by the Tukey's test, assuming the hypothesis of equality of variances. Differences were considered significant at the $P < 0.05$ level. All statistical procedures were carried out with the software package SPSS Statistics version 23.0 for Windows (IBM SPSS Inc., Chicago, IL, USA).

## 3. Results and discussion

*3.1. Production of CLA by the LAB isolates*

Among the 129 LAB tested, 15 isolates generated LA isomerisation rates higher than 10% according to the spectrophotometric method. This number comprised 11 *Lc. lactis*, 2 Lb. *paracasei*, and 2 Lb. *plantarum* (Table 1). The highest assumed percentages of conversion were obtained for the two strains of *Lb. plantarum* L188 (mean value of 22.0%) and L200 (30.1%). These strains were isolated from raw-milk ripened (2.5 months) Cebreiro cheese (Garabal et al., 2008). The type strains *Lb. plantarum* CECT 749 and *Lb. brevis* CECT 5172 used as positive controls also isomerised LA to CLA with conversion values of 25.1% and 15.2%, respectively (Table 1). Strains of different species of *Lactobacillus* and one *Lc. lactis* have been described to produce CLA from LA in specific growth media (Bisig, Eberhard, Collomb, & Rehberger, 2007). Strains of *Lc. lactis* have also shown high CLA production in both skim and whole-fat milk supplemented with free LA (Kim & Liu, 2002; Rodríguez-Alcalá et al., 2011).

**Table 1**
Presumptive CLA producers, CLA concentrations and assumed isomerisation rates (AIR).[a]

| Isolate | Source | CLA (μg $mL^{-1}$) | AIR (%) |
|---|---|---|---|
| *Lc. lactis* subsp. *lactis* L42 | Arzúa-Ulloa cheese | 26.2 ± 10.2 | 12.4 |
| *Lb. paracasei* L45 | Arzúa-Ulloa cheese | 30.9 ± 20.1 | 10.5 |
| *Lc. lactis* subsp. *cremoris* L52 | Arzúa-Ulloa cheese | 27.9 ± 13.4 | 11.2 |
| *Lc. lactis* subsp. *lactis* L59 | Arzúa-Ulloa cheese | 31.3 ± 7.2 | 12.5 |
| *Lc. lactis* subsp. *cremoris* L65 | Tetilla cheese | 32.7 ± 23.2 | 13.1 |
| *Lc.lactis* subsp. *cremoris* L111 | Arzúa-Ulloa cheese | 27.6 ± 14.1 | 11.0 |
| *Lc.lactis* subsp. *cremoris* L131 | Tetilla cheese | 41.0 ± 24.6 | 16.4 |
| *Lc.lactis* subsp. *lactis* L132 | Tetilla cheese | 31.0 ± 12.1 | 12.4 |
| *Lc.lactis* subsp. *lactis* L134 | Tetilla cheese | 32.5 ± 20.2 | 13.0 |
| *Lc.lactis* subsp. *cremoris* L172 | Cebreiro cheese | 31.1 ± 16.2 | 12.5 |
| *Lc.lactis* subsp. *cremoris* L173 | Cebreiro cheese | 33.9 ± 17.3 | 13.5 |
| *Lc.lactis* subsp. *cremoris* L187 | Cebreiro cheese | 33.7 ± 12.5 | 13.5 |
| *Lb.plantarum* L188 | Cebreiro cheese | 55.0 ± 18.2 | 22.0 |
| *Lb.plantarum* L200 | Cebreiro cheese | 75.2 ± 35.4 | 30.1 |
| *Lb.paracasei* L221 | Cebreiro cheese | 30.9 ± 10.2 | 12.3 |
| *Lb.plantarum* CECT 749 | (Culture collection) | 62.8 ± 33.9 | 25.1 |
| *Lb.brevis* CECT 5172 | (Culture collection) | 48.0 ± 25.4 | 19.2 |

[a] CLA concentration (mean values ± standard deviations of triplicate determinations) were calculated spectrophotometrically at 233 nm from the linear trend of the calibration curve; assumed isomerisation rates, calculated according to the formula: CLA concentration/initial LA concentration × 100, were determined in MRS broth for lactobacilli or Elliker broth for lactococci supplemented with Tween 80 (1%, w/v) and free LA (0.25 mg $mL^{-1}$) after 48 h of incubation at 30 °C.

Unlike the results obtained by the spectrophotometric method, only the two *Lb. plantarum* L188 and L200 strains along with the type strains *Lb. plantarum* CECT 749 and *Lb. brevis* CECT 5172 were positive for the production of CLA when cultured in MRS broth without Tween 80 supplemented with free LA and BSA and then analysed by GC (Table 2). These differences could be partly explained because the screening UV-spectrophotometric method of Barrett et al. (2007) measures conjugated double bonds in all the fatty acids present in the supernatants obtained from the bacterial cultures. Both *Lb. plantarum* L188 and L200 strains formed *cis*-9, *trans*-11 and *trans*-9, *trans*-11 CLA isomers, and the strain L200 further converted LA to *trans*-10, *cis*-12 CLA. *Lb. plantarum* CECT 749 also yielded the three CLA isomers, while *Lb. brevis* did not convert free LA to *trans*-9, *trans*-11 CLA. Most of the CLA produced (mean estimated values between 82.6% and 98.7% of the total CLA formed by each of the strains, data not shown) was detected in the supernatant fraction of the cultures of the four strains. The highest percentages of LA conversion, calculated from the sum of each of the CLA isomer concentrations in the supernatant and in the pellet, were found for the *Lb. plantarum* L200 (12.9%) and CECT 749 (9.21%) strains in relation to the production of *cis*-9, *trans*-11 CLA isomer (Table 2). *Lb. plantarum* L200 also showed the highest conversion rate (0.73%) to *trans*-9, *trans*-11 CLA, and *Lb. brevis* CECT 5172 offered the highest percentage (1.01%) of LA converted to *trans*-10, *cis*-12 CLA isomer (Table 2).

CLA isomers produced by LAB are mainly found in the supernatant from the cultures compared with the cell pellets (Ribeiro, Stanton, Yang, Ross, & Silva, 2018; Yang et al., 2014). Several studies revealed a great variability in the CLA isomer profile produced by different LAB strains, although for most species *cis*-9, *trans*-11 C18:2 isomer represents more than 70% of the total CLA formed from LA (Kuhl & De Dea Lindner, 2016). *Lactobacillus* is the LAB genus that comprises most of the species able to produce CLA (Kishino, Ogawa, Omura, Matsumura, & Shimizu, 2002; Renes et al., 2017; Yang et al., 2014), and *Lb. plantarum* strains have been identified as

**Table 2**
CLA isomer concentrations in bacterial supernatants and pellets.[a]

| Strain | Culture fraction | *cis*9, *trans*11 CLA | | *trans*10, *cis*12 CLA | | *trans*9, *trans*11 CLA | |
|---|---|---|---|---|---|---|---|
| | | Concentration (μg mL$^{-1}$) | LA converted (%) | Concentration (μg mL$^{-1}$) | LA converted (%) | Concentration (μg mL$^{-1}$) | LA converted (%) |
| *Lb. plantarum* L188 | Supernatant | 9.78 ± 0.64 | 4.18 | nd | – | 0.57 ± 0.09 | 0.27 |
| | Pellet | 0.67 ± 0.14 | | nd | | nd | |
| *Lb. plantarum* L200 | Supernatant | 28.3 ± 1.10 | 12.9 | 0.53 ± 0.07 | 0.24 | 1.64 ± 0.33 | 0.73 |
| | Pellet | 3.97 ± 0.62 | | 0.08 ± 0.10 | | 0.19 ± 0.07 | |
| *Lb. plantarum* CECT 749 | Supernatant | 18.9 ± 2.76 | 9.21 | 0.62 ± 0.30 | 0.25 | 1.48 ± 0.90 | 0.71 |
| | Pellet | 4.11 ± 0.36 | | nd | | 0.30 ± 0.18 | |
| *Lb. brevis* CECT 5172 | Supernatant | 0.05 ± 0.06 | 0.02 | 2.48 ± 0.57 | 1.01 | nd | – |
| | Pellet | nd | | 0.04 ± 0.03 | | nd | |

[a] CLA isomer concentrations (mean values ± standard deviations of triplicate determinations; nd: not detected) were determined by GC-FID in bacterial supernatants and pellets from strains grown in MRS broth without Tween 80 supplemented with 0.25 mg mL$^{-1}$ of free LA and 0.1 mg mL$^{-1}$ of BSA after 48 h of incubation at 30 °C under stirring (120 rpm). LA converted (%) is the percentage of the sum of the CLA isomer concentrations determined in the supernatant and in the pellet fractions relative to the initial LA concentration.

the most efficient CLA-producers among food-derived LAB (Yang et al., 2014, 2017). Strains of *Lb. plantarum* isolated from foods have shown conversion rates of LA to total CLA over 50% (Kishino et al., 2002; Yang et al., 2014). Renes et al. (2017) and Ribeiro et al. (2018) described four and two, respectively, *Lb. plantarum* strains isolated from artisanal raw-milk cheeses forming 15–55 μg mL$^{-1}$ of total CLA in MRS broth supplemented with free LA. In both studies, the *cis*-9, *trans*-11 CLA isomer was the most abundant isomer generated, followed by the *trans*-9, *trans*-11 CLA. In addition, the isomer *trans*-10, *cis*-12 was detected as a minor compound. These results are comparable to those found in the present study.

Yadav, Jain, and Sinha (2007) suggested that strains of *Lb. acidophilus* and *Lb. casei* present in a traditional fermented milk product increase the production of free fatty acids through lipolysis of milk fat and produce CLA using the formed free LA. In this sense, the CLA-forming *Lb. plantarum* L200 strain assayed in the present study had previously been found to exhibit a weak lipolytic activity in tributyrin and Tween 80 agars (data not shown).

### 3.2. Compositional analysis and pH of the miniature cheeses

The experimental miniature control cheeses obtained in the present study fulfilled, after 28 days of ripening in the usual conditions, the compositional and pH criteria specified by both PDO Arzúa-Ulloa and Tetilla regulations (45–50% dry matter; ≥ 45% fat/dry matter; ≥ 40% protein/dry matter; 68–73% moisture in fat-free basis; and pH between 5.0 and 5.5). The results (mean ± standard deviation) obtained for dry matter, fat/dry matter, protein/dry matter, and moisture in fat-free basis (all expressed as %, w/w) were 47.4 ± 0.93, 52.7 ± 1.16, 42.7 ± 1.45, 1.12 ± 0.14, and 69.3 ± 0.67, respectively. The mean pH values were between 5.14 for L45 cheeses and 5.23 for L200 cheeses (Table 3).

### 3.3. Analysis of fatty acids in the miniature cheeses made with the different bacterial strains

The concentrations of the fatty acids identified in the miniature cheeses made with the addition of the different LAB strains are shown in Table 3. The most abundant fatty acids in the cheese groups were oleic acid (C18:1 n-9; 25.2–27.2 g 100 g$^{-1}$ of fat), palmitic acid (C16:0; 24.7–26.7 g 100 g$^{-1}$ of fatty acids), stearic acid (C18:0; 10.9–12.3 g 100 g$^{-1}$ of fatty acids) and myristic acid (C14:0; 10.4–11.9 g 100 g$^{-1}$ of fatty acids). The fatty acid profile is comparable with those described for other cow milk cheeses (Falchero et al., 2010; Van Nieuwenhove et al., 2009). The mean concentration of the *cis*-9, *trans*-11 CLA isomer in the group of control cheeses (0.69 g 100 g$^{-1}$ of fatty acids) is similar to that reported by Van Nieuwenhove et al. (2009) for 11 cow cheeses from NW Argentina (0.71 g 100 g$^{-1}$ fatty acids), however, it was lower than those found for cheeses made from milk of pasture grazed cows (1.61–1.75 g 100 g$^{-1}$ fatty acids) (Falchero et al., 2010; Povolo, Pelizzola, Lombardi, Tava, & Contarini, 2012).

The concentrations of myristic acid (C14:0) in the control miniature cheeses were significantly higher ($P < 0.05$) than in the cheeses made with the CLA-forming L200 strain, and the contents of palmitic acid (C16:0) in the cheeses made with the adjunct cultures were significantly higher ($P < 0.05$) than in the control cheeses (Table 3). These differences in the fatty acid profiles could be partly attributed to a different degree of lipolysis in the groups of cheese compared. Finally, the concentrations of *cis*-9, *trans*-11 CLA isomer were significantly higher ($P < 0.05$) in the miniature cheeses made with the CLA-forming L200 strain than in the cheeses in the two other groups (1.09 versus 0.69 and 0.61 g 100 g$^{-1}$ fatty acids) (Table 3). The increase in the total CLA content in the L200 cheeses could be estimated at 55% in relation to the control cheeses.

The calculated atherogenicity indexes (AI; value inversely proportional to the nutritional quality of lipid profile) were 1.94 in the control cheeses, 2.04 in the L45 cheeses and 1.90 in the L200 cheeses made with the CLA-forming strain, and the desirable fatty acid (DFA) values were of 49.7 in the control cheeses, 48.3 in the L45 cheeses and 49.3 in the L200 cheeses (Table 3). No significant differences were found between the groups of cheese with these parameters. The mean AI values determined in the present study are close to the value of 2 proposed as typical of dairy products by Bobe et al. (2004), and lower than the mean value obtained by Van Nieuwhenhove et al. (2009) for Argentinian cow cheeses (2.59). The DFA values are similar to those found by Taboada, Van Nieuwenhove, Alzogaray, and Medina (2015) in ripened (60-d) goat cheeses made with autochthonous strains (46–48 g 100 g$^{-1}$ of fatty acids); these values allow inferring the content of those beneficial fatty acids for health.

It has been suggested that the factors involved in the cheese making process such as the addition of starter cultures and ripening, could influence the lipolytic processes and consequently the variations of fatty acid composition but generally do not affect the concentration of CLA in cheese fat (Bisig et al., 2007; Prandini, Sigolo, & Piva, 2011). It has also been concluded that CLA-forming LAB may increase CLA content only under the condition that free LA is available in the medium (Bisig et al., 2007). Taboada et al. (2015) reported that the use of autochthonous cultures including two *Lb. plantarum* strains in artisanal goat cheese manufacture enhanced the CLA content, flavour and AI of the ripened (60-d) cheeses. The CLA level increased during ripening from 0.6 to 1.0 g 100 g$^{-1}$ of fatty acids, this final value being very similar to that determined in the cheeses made with the *Lb. plantarum* L200 strain in the present study. An increase

**Table 3**
pH and fatty acid composition of the miniature cheeses.[a]

| Parameter | Control cheeses | L45 cheeses | L200 cheeses | *P*-value |
|---|---|---|---|---|
| pH | 5.21 ± 0.15 | 5.14 ± 0.17 | 5.23 ± 0.12 | 0.825 |
| Fatty acids (g 100 $g^{-1}$ fat) | | | | |
| C4:0 | 2.57 ± 0.34 | 2.74 ± 0.11 | 2.83 ± 0.33 | 0.691 |
| C6:0 | 2.04 ± 0.02 | 2.10 ± 0.19 | 2.10 ± 0.17 | 0.890 |
| C8:0 | 0.72 ± 0.87 | 0.77 ± 0.03 | 0.71 ± 0.03 | 0.582 |
| C10:0 | 2.21 ± 0.09 | 2.18 ± 0.10 | 2.19 ± 0.11 | 0.957 |
| C12:0 | 2.80 ± 0.13 | 2.53 ± 0.15 | 2.81 ± 0.12 | 0.200 |
| C14:0 | 11.86 ± 0.28$^{A}$ | 11.25 ± 0.20$^{AB}$ | 10.38 ± 0.24$^{B}$ | 0.020 |
| C14:1 | 0.21 ± 0.002 | 0.19 ± 0.03 | 0.20 ± 0.01 | 0.649 |
| C15:0 | 0.87 ± 0.02 | 0.74 ± 0.05 | 0.85 ± 0.04 | 0.080 |
| C16:0 | 24.71 ± 0.06$^{B}$ | 26.66 ± 0.42$^{A}$ | 26.05 ± 0.22$^{A}$ | 0.013 |
| C16:1 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.02 ± 0.001 | 0.524 |
| C17:0 | 0.45 ± 0.01 | 0.40 ± 0.05 | 0.44 ± 0.01 | 0.407 |
| C17:1 | 0.21 ± 0.04 | 0.19 ± 0.04 | 0.20 ± 0.01 | 0.756 |
| C18:0 | 10.85 ± 1.34 | 11.79 ± 1.63 | 12.29 ± 1.43 | 0.655 |
| C18:1 n-9 | 26.93 ± 1.57 | 25.18 ± 1.75 | 27.20 ± 0.82 | 0.423 |
| C18:2 n-6 | 2.51 ± 0.18 | 2.40 ± 0.32 | 2.21 ± 0.09 | 0.466 |
| C18:3 n-6 | 0.11 ± 0.03 | 0.11 ± 0.02 | 0.10 ± 0.01 | 0.945 |
| C18:3 n-3 | 0.64 ± 0.02 | 0.61 ± 0.06 | 0.67 ± 0.02 | 0.580 |
| *c*9*t*11 C18:2 | 0.69 ± 0.03$^{B}$ | 0.61 ± 0.04$^{B}$ | 1.09 ± 0.03$^{A}$ | 0.001 |
| *t*10*c*12 C18:2 | 0.02 ± 0.001 | 0.01 ± 0.002 | 0.02 ± 0.001 | 0.265 |
| C20:0 | 0.11 ± 0.01 | 0.10 ± 0.02 | 0.10 ± 0.002 | 0.758 |
| C20:1 | 1.79 ± 0.66 | 1.72 ± 0.25 | 1.78 ± 0.20 | 0.984 |
| C20:3 n-6 | 0.22 ± 0.04 | 0.22 ± 0.02 | 0.19 ± 0.001 | 0.423 |
| C20:4 n-6 (ARA) | 0.10 ± 0.01 | 0.09 ± 0.01 | 0.10 ± 0.01 | 0.310 |
| C20:5 n-3 (EPA) | 0.19 ± 0.22 | 0.19 ± 0.13 | 0.24 ± 0.11 | 0.934 |
| C22:2 | 5.19 ± 0.76 | 4.94 ± 1.16 | 3.03 ± 0.70 | 0.165 |
| C24:0 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.04 ± 0.001 | 0.523 |
| Atherogenicity index | 1.94 ± 0.15 | 2.04 ± 0.17 | 1.90 ± 0.07 | 0.606 |
| Desirable fatty acid | 49.66 ± 4.92 | 48.26 ± 5.52 | 49.32 ± 3.45 | 0.954 |

[a] Results are means ± standard deviation obtained from two different cheese making trials analysed in duplicate and averaged; values within a row with different superscript letters are significantly different ($P < 0.05$; Tukey's test). L45 cheeses were made with the addition of the non-CLA forming *Lb. paracasei* L45 strain; L200 cheeses were made with the addition of the CLA-forming *Lb. plantarum* L200 strain. Atherogenicity index was according to Ulbricht and Southgate (1991): (C12:0 + 4C14:0 + C16:0)/(monounsaturated + polyunsaturated fatty acids); desirable fatty acid was according to Osmari, Cecato, Macedo, and Souza (2011): unsaturated fatty acids + C18:0.

in the levels of oleic acid and total CLA has also been reported in Italian Scamorza ewe cheese made with *Lb. acidophilus* (Albenzio et al., 2013). Therefore, it might be possible that selected lipolytic and CLA-forming lactobacilli could increase the CLA content of cheeses after releasing LA from fat glycerides.

## 4. Conclusions

Strains of *Lb. plantarum* appear to be the LAB with the highest ability to convert LA to CLA among the microbiota of raw-milk cheeses made in Galicia (NW Spain), and the *cis*-9, *trans*-11 C18:2 is the most abundant CLA isomer generated by these bacteria. Significantly higher concentrations of *cis*-9, *trans*-11 CLA were determined in miniature laboratory cheeses made with the CLA-forming *Lb. plantarum* L200 strain selected in this study compared with a *Lb. paracasei* strain. Although there is a need for further confirmation, the results of the present study suggest that *Lb. plantarum* L200 strain could be used as an adjunct culture to increase CLA content in short-ripened cows' milk cheeses.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.11.007.

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