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1 # Formation of conjugated linoleic acid by a *Lactobacillus*

2 # *plantarum* strain isolated from an artisanal cheese: evaluation in

3 # miniature cheeses

4

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21 **Abstract**

22 Among 129 lactic acid bacteria previously isolated from raw-milk starter-free cheeses
23 manufactured in Galicia (NW Spain), two strains of *Lactobacillus plantarum* were
24 definitely recognized as producers of conjugated linoleic acid (CLA). Gas
25 chromatography analysis identified *cis*-9, *trans*-11 C18:2 as the predominant CLA
26 isomer formed in MRS broth supplemented with linoleic acid. A centrifugation-based
27 model for the manufacture of miniature cheeses was used to evaluate the formation of
28 CLA by *Lb. plantarum* L200, the highest producer of CLA in MRS broth. The
29 miniature cheeses made with the addition of the L200 strain showed significantly ($P <$
30 0.05) higher contents of *cis*-9, *trans*-11 CLA than those of the control cheeses (1.09 vs.
31 0.69 percentage of total fatty acids, respectively). These results suggest that *Lb.*
32 *plantarum* L200 strain could be used as an adjunct culture to slightly increase the
33 concentrations of CLA in short-ripened cow's milk cheeses.

34

35 **1. Introduction**

36

37 Conjugated linoleic acid (CLA) isomers have attracted great interest in recent years
38 because of their attributed functional and health promoting properties, including
39 anticarcinogenic, antiatherogenic, antiobesity, antiinflammatory and antidiabetic effects
40 (Hennessy, Ross, Devery, & Stanton, 2011; Yang et al., 2015). The main CLA isomers
41 recognized with these beneficial activities are *cis*-9, *trans*-11 C18:2, *trans*-10, *cis*-12
42 C18:2 and *trans*-9, *trans*-11 C18:2 (Renes et al., 2017; Yang et al., 2015). Dairy
43 products from ruminants are the most important source of CLA in a diet, and may
44 contribute to around 60% of the total dietary CLA intake (Chin, Liu, Storkson, Ha, &
45 Pariza, 1992). The *cis*-9, *trans*-11 isomer, also called rumenic acid, is the principal form
46 of dairy CLA, representing approximately 90% of the total CLA (Chin et al., 1992;
47 Prandini, Sigolo, Tansini, Brogna, & Piva, 2007). Conjugated linoleic acid content in
48 milk and milk products ranges between 0.1% and 2.9% of total fat, with the highest
49 amounts found in cheeses from sheep milk (El-Salam & El-Shibiny, 2014).
50 A daily intake of 3 g per day for a person weighing 70 kg has been recommended to
51 achieve the highest health benefits of CLA (Ip, Scimeca, & Thompson, 1994).
52 Consequently, increasing the concentration of CLA in dairy products has been the focus
53 of several studies with a view to improve their beneficial properties on health and to
54 develop functional food products (Ozer, Kilic, & Kilic, 2016). In this sense, feeding
55 lactating ruminants on natural pasture and oil-supplemented rations seems to be the
56 factor that most significantly increases the CLA levels of milk and derived dairy
57 products (Van Nieuwenhove, Oliszewski, & González, 2009; El-Salam & El-Shibiny,
58 2014).

59 Some lactic acid bacteria (LAB), especially *Lactobacillus*, and *Bifidobacterium* strains
60 may produce CLA by isomerization of linoleic acid (LA) using linoleate isomerase
61 enzyme (Rodríguez-Alcalá, Braga, Malcata, Gomes, & Fontecha, 2011; Yang et al.,
62 2017). Incorporation of such CLA-forming bacteria as starters or adjunct cultures into
63 cultured dairy products offers a viable and natural strategy for increasing CLA content
64 (Andrade et al., 2012). Therefore, the selection of LAB isolates able to produce CLA in
65 milk by biological fermentation processes constitutes a meaningful purpose for the food
66 industry in relation to cheese and fermented dairy products (Ozer et al., 2016;
67 Rodríguez-Alcalá et al., 2011).
68 In order to assay the properties of different microbial strains in cheese making, to
69 predict cheese yield or to evaluate variations in processing conditions, simple protocols
70 which use small milk samples, fixed times between rennet addition and cutting, and
71 centrifugation for whey separation have been developed (Bachmann, Kruijswijk,
72 Molenaar, Kleerebezem, & van Hylckama Vlieg, 2009; Cipolat-Gotet et al., 2016). In
73 these miniature cheese-making procedures, a small volume (1.7-10 mL) of milk
74 contained in glass tubes or in the wells of a microplate is coagulated and centrifuged
75 (instead of drained, moulded and pressed) at 1000-4800 × *g* in one or several stages to
76 separate the whey from the curd. These fast and inexpensive methods show reasonably
77 acceptable performance, with manufacturing conditions being highly reproducible, and
78 thus they can be used in the screening of microbial strains for the expression of specific
79 enzymatic activities or flavor-forming abilities (Bachmann et al., 2009).
80 In this context, the aims of the present study were: (i) to screen 129 LAB isolates
81 obtained from traditional raw-milk starter-free cow cheeses for their ability to produce
82 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 before in the productions 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 ºC in 11% sterile reconstituted skim milk with 20% (v/v) glycerol added, and activated by subculturing twice at 30 ºC 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%

108 (w/v) Tween 80 (polyoxyethylene sorbitan monooleate; Scharlau, Sentmenat,
109 Barcelona, Spain) and 0.25 mg mL^{-1} free LA (99% purity; Sigma-Aldrich, St. Louis,
110 MO, USA). The activated bacterial strains were transferred at 2% (v:v) to the culture
111 medium (10 mL) and aerobically incubated at 30 ºC for 48 h. All samples were carried
112 out in triplicate.
113 Lipid extraction from culture media was performed as described by Rodríguez-Alcalá et
114 al. (2011). The total CLA contained in the supernatants was estimated in accordance
115 with the rapid screening UV-spectrophotometric method proposed by Barrett, Ross,
116 Fitzgerald, & Stanton (2007). Absorbance values at 233 nm were determined in a
117 Lambda 650 UV/Vis spectrophotometer (PerkinElmer Ltd, Beaconsfield, UK). For each
118 isolate, 2 mL of lipid extract in hexane were placed into quartz cuvettes and analysed. A
119 calibration curve was built for the absorbance at 233 nm versus *cis*-9, *trans*-11 CLA
120 isomer (96% purity; Sigma-Aldrich) concentration (0-50 µg mL^{-1}). The assumed
121 isomerization rate of LA into CLA in the culture medium was calculated by the
122 formula: CLA concentration/initial LA concentration × 100.
123
124 *2.3. CLA production and quantification by gas chromatography*
125
126 The 15 isolates (11 *Lactococcus lactis*, 2 *Lactobacillus paracasei*, and 2 *Lb. plantarum*)
127 showing the ability to convert free LA to total CLA with assumed isomerization rates
128 higher than 10% in accordance with the preliminary screening method were
129 subsequently assayed by gas chromatography (GC). The isolates were tested in a LA
130 emulsion in bovine serum albumin (BSA) (Lin, 2006) to avoid any potentially positive
131 effects caused by Tween 80 on the growth and production of CLA by LAB (Corcoran,
132 Stanton, Fitzgeral & Ross, 2007; Li et al., 2011). The selected bacteria were activated in

133 MRS or Elliker broth as previously indicated and then inoculated at 2% v:v in (100 mL)
134 MRS broth prepared without Tween 80 and supplemented with 0.25 mg mL^{-1} free LA
135 (Sigma-Aldrich) and 0.1 mg mL^{-1} BSA (≥ 95% pure Sigma-Aldrich), and incubated at
136 30 ºC on a rotary shaker at 120 rpm for 48 h. The cultures were then centrifuged at 5000
137 × *g* for 10 min at room temperature. The fat was extracted from the culture supernatant
138 fluid and from the bacterial pellet independently, according to the method described by
139 Yang et al. (2014).
140 Fatty acids from 0.5 mL hexane layers were esterified and fatty acid methyl esters were
141 extracted as described by Ledoux et al. (2005). Separation, identification and
142 quantification of the methyl esters of: *cis*-9, *trans*-11; *trans*-10, *cis*-12; and *trans*-9,
143 *trans*-11 CLA isomers were performed with the aid of a Trace GC Ultra (Thermo
144 Finnigan, Austin, TX, USA) chromatograph equipped with a flame ionization detector
145 (FID), under the conditions described by Méndez-Cid, Centeno, Martínez, & Carballo
146 (2017). All samples and standards were injected in triplicate.
147
148 *2.4. Manufacture and analysis of miniature cheese models for testing CLA production*
149 *by adjunct LAB*
150
151 A protocol for the manufacture of miniature laboratory cheeses that meet the
152 requirements for gross composition and pH of both the industrial PDO Arzúa-Ulloa and
153 Tetilla cheese varieties was designed. Both cheeses combined represent about 60% of
154 the total annual production of unmixed cow milk PDO cheeses manufactured in Spain,
155 and have quite similar characteristics regarding flavor and texture. Industrial Arzúa-
156 Ulloa and Tetilla cheese making includes a curd washing step, similar to Dutch-type
157 cheeses. Therefore, the protocol designed in this study was based on that described by

158 Bachmann et al. (2009) for the production of miniaturized Gouda-type cheeses, even
159 though the volumes were larger in order to facilitate the analytical procedures, and
160 cheeses were ripened in an environmental atmosphere. All the information concerning
161 the preparation and curdling of cheese milk, operations for whey drainage and cheese
162 ripening can be found in the supplementary files S1 (text) and S2 (figure). Two cheese
163 making trials were carried out.
164
165 *2.4.1. Bacterial strains for the manufacture of the miniature laboratory cheeses*
166
167 The commercial starter used in the manufacture of the miniature cheeses was the freeze-
168 dried direct-vat-set Choozit MM100 (Danisco® Food Ingredients, Sassenage, France), a
169 mesophilic D-starter containing *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp.
170 *cremoris* strains. The starter was maintained at –25 ºC until use. The strains *Lb.*
171 *paracasei* L45 (non-CLA forming) and *Lb. plantarum* L200 (CLA-forming) were
172 employed as adjunct cultures in the manufacture of the miniature cheeses. Frozen
173 cultures were grown on MRS broth at 30 ºC for 24 h, and then subcultured at 2% (v:v)
174 in sterile (110 ºC, 15 min) reconstituted skim milk (Oxoid) and incubated for 48 h at 30
175 ºC. The absence of antimicrobial activity of the L45 and L200 strains against the
176 commercial culture MM100 was previously confirmed by the agar well diffusion assay,
177 as described by Centeno, Gaya, Medina, & Nuñez (2002).
178
179 *2.4.2. Cheese sampling and physicochemical analyses*
180
181 Three groups of cheese were obtained: control cheeses, L45 cheeses (made with the
182 non-CLA forming adjunct culture), and L200 cheeses (made with the CLA-forming

183 adjunct culture). All miniature cheeses were sampled on day 28 of ripening. Dry extract,
184 fat, protein and ash content were analyzed only in the control cheeses. The
185 compositional parameters and pH were determined as previously described (Centeno,
186 Rodríguez-Alonso, Carballo, & Garabal, 2015). To perform each of the analyses, the
187 number (between 1 and 3) of cheeses (1.10±0.12 g weight) needed to yield sufficient
188 material was pooled. All analyses were carried out in duplicate and the results averaged
189 for each cheese making trial.
190
191 *2.4.6. Analysis of total fatty acids*
192
193 Miniature cheeses from the three different groups (control, L45 and L200) were
194 sampled to determine total fatty acids, including: *cis*-9, *trans*-11; *trans*-10, *cis*-12; and
195 *trans*-9, *trans*-11 CLA isomers. The fat from 5.00 ± 0.01 g samples (obtained by
196 pooling 5 miniature cheeses from each of the groups) was extracted following the
197 procedure described by Folch, Lees, & Sloane-Stanley (1957). Lipid methylation from
198 0.500±0.001 g of the extracted fat was carried out according to Méndez-Cid et al.
199 (2017). The separation of the fatty acids from the total lipids was performed by GC-
200 FID, as previously described in Section 2.3, for the bacterial strains.
201 Individual fatty acid methyl esters were identified and quantified by comparison with
202 the retention times and peak areas of the standard mixture of FAME Mix Supelco
203 37Components (Supelco, Bellefonte, PA, USA) and of CLA isomers (Sigma-Aldrich).
204 The CLA and fatty acid concentrations of miniature cheese samples were expressed as g
205 100 g^{-1} fatty acids, calculated with peak areas corrected by factors according to the
206 AOAC 963.22 method (AOAC, 2000). Two analytical replicates were made and the
207 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 isomerization 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 *Lactobacillus plantarum* CECT 749 and *Lactobacillus brevis* CECT 5172 used as positive controls also isomerized LA to CLA with conversion values of 25.1% and 15.2%, respectively (Table 1). Strains of different species of *Lactobacillus* and one *Lactococcus 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

234 production in both skim and whole-fat milk supplemented with free LA (Kim & Liu,
235 2002; Rodríguez-Alcalá et al., 2011).
236 Unlike the results obtained by the spectrophotometric method, only the two *Lb.*
237 *plantarum* L188 and L200 strains along with the type strains *Lb. plantarum* CECT 749
238 and *Lb. brevis* CECT 5172 were positive for the production of CLA when cultured in
239 MRS broth without Tween 80 supplemented with free LA and BSA and then analysed
240 by GC (Table 2). These differences could be partly explained because the screening
241 UV-spectrophotometric method of Barrett et al. (2007) measures conjugated double
242 bonds in all the fatty acids present in the supernatants obtained from the bacterial
243 cultures. Both *Lb. plantarum* L188 and L200 strains formed *cis*-9, *trans*-11 and *trans*-9,
244 *trans*-11 CLA isomers, and the strain L200 further converted LA to *trans*-10, *cis*-12
245 CLA. *Lactobacillus plantarum* CECT 749 also yielded the three CLA isomers, while
246 *Lb. brevis* did not convert free LA to *trans*-9, *trans*-11 CLA. Most of the CLA produced
247 (mean estimated values between 82.6% and 98.7% of the total CLA formed by each of
248 the strains, data not shown) was detected in the supernatant fraction of the cultures of
249 the four strains. The highest percentages of LA conversion, calculated from the sum of
250 each of the CLA isomer concentrations in the supernatant and in the pellet, were found
251 for the *Lb. plantarum* L200 (12.9%) and CECT 749 (9.21%) strains in relation to the
252 production of *cis*-9, *trans*-11 CLA isomer (Table 2). *Lactobacillus plantarum* L200 also
253 showed the highest conversion rate (0.73%) to *trans*-9, *trans*-11 CLA, and *Lb. brevis*
254 CECT 5172 offered the highest percentage (1.01%) of LA converted to *trans*-10, *cis*-12
255 CLA isomer (Table 2).
256 CLA isomers produced by LAB are mainly found in the supernatant from the cultures
257 compared to the cell pellets (Ribeiro, Stanton, Yang, Ross, & Silva, 2018; Yang et al.,
258 2014). Several studies revealed a great variability in the CLA isomer profile produced

259 by different LAB strains, although for most species *cis*-9, *trans*-11 C18:2 isomer
260 represents more than 70% of the total CLA formed from LA (Kuhl & De Dea Lindner,
261 2016). *Lactobacillus* is the LAB genus that comprises most of the species able to
262 produce CLA (Kishino, Ogawa, Omura, Matsumura, & Shimizu, 2002; Renes et al.,
263 2017; Yang et al., 2014), and *Lb. plantarum* strains have been identified as the most
264 efficient CLA-producers among food-derived LAB (Yang et al., 2014; Yang et al.,
265 2017). Strains of *Lb. plantarum* isolated from foods have shown conversion rates of LA
266 to total CLA over 50% (Kishino et al., 2002; Yang et al., 2014). Renes et al. (2017) and
267 Ribeiro et al. (2018) described four and two, respectively, *Lb. plantarum* strains isolated
268 from artisanal raw-milk cheeses forming 15-55 $\mu g\ mL^{-1}$ of total CLA in MRS broth
269 supplemented with free LA. In both studies, the *cis*-9, *trans*-11 CLA isomer was the
270 most abundant isomer generated, followed by the *trans*-9, *trans*-11 CLA. In addition,
271 the isomer *trans*-10, *cis*-12 was detected as a minor compound. These results are
272 comparable to those found in the present study.
273 Yadav et al. (2007) suggested that strains of *Lb. acidophilus* and *Lb. casei* present in a
274 traditional fermented milk product increase the production of free fatty acids through
275 lipolysis of milk fat and produce CLA using the formed free LA. In this sense, the
276 CLA-forming *Lb. plantarum* L200 strain assayed in the present study had previously
277 been found to exhibit a weak lipolytic activity in tributyrin and Tween 80 agars (data
278 not shown).
279
280 *3.2. Compositional analysis and pH of the miniature cheeses*
281
282 The experimental miniature control cheeses obtained in the present study fulfill, after 28
283 days of ripening in the usual conditions, the compositional and pH criteria specified by

284 both PDO Arzúa-Ulloa and Tetilla regulations (45-50% dry matter; ≥ 45% fat/dry

285 matter; ≥ 40% protein/dry matter; 68-73% moisture in fat-free basis; and pH between

286 5.0 and 5.5). The results (mean±standard deviation) obtained for dry matter, fat/dry

287 matter, protein/dry matter, and moisture in fat-free basis (all expressed as %, w/w) were

288 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

289 values were between 5.14 for L45 cheeses and 5.23 for L200 cheeses (Table 3).

290

291 *3.3. Analysis of fatty acids in the miniature cheeses made with the different bacterial*

292 *strains*

293

294 The concentrations of the fatty acids identified in the miniature cheeses made with the

295 addition of the different LAB strains are shown in Table 3. The most abundant fatty

296 acids in the cheese groups were oleic acid (C18:1 n-9; 25.2-27.2 g 100 g^{-1} of fat),

297 palmitic acid (C16:0; 24.7-26.7 g 100 g^{-1} of fatty acids), stearic acid (C18:0; 10.9-12.3 g

298 100 g^{-1} of fatty acids) and myristic acid (C14:0; 10.4-11.9 g 100 g^{-1} of fatty acids). The

299 fatty acid profile is comparable with those described for other cow milk cheeses

300 (Falchero et al., 2010; Van Nieuwenhove et al., 2009). The mean concentration of the

301 *cis*-9, *trans*-11 CLA isomer in the group of control cheeses (0.69 g 100 g^{-1} of fatty

302 acids) is similar to that reported by Van Nieuwenhove et al. (2009) for 11 cow cheeses

303 from NW Argentina (0.71 g 100 g^{-1} fatty acids), however, it was lower than those found

304 for cheeses made from milk of pasture grazed cows (1.61-1.75 g 100 g^{-1} fatty acids)

305 (Falchero et al., 2010; Povolo, Pelizzola, Lombardi, Tava, & Contarini, 2012).

306 The concentrations of myristic acid (C14:0) in the control miniature cheeses were

307 significantly higher ($P < 0.05$) than in the cheeses made with the CLA-forming L200

308 strain, and the contents of palmitic acid (C16:0) in the cheeses made with the adjunct
309 cultures were significantly higher ($P < 0.05$) than in the control cheeses (Table 3).
310 These differences in the fatty acid profiles could be partly attributed to a different
311 degree of lipolysis in the groups of cheese compared. Finally, the concentrations of *cis*-
312 9, *trans*-11 CLA isomer were significantly higher ($P < 0.05$) in the miniature cheeses
313 made with the CLA-forming L200 strain than in the cheeses in the two other groups
314 (1.09 vs. 0.69 and 0.61 g 100 g^{-1} fatty acids) (Table 3). The increase in the total CLA
315 content in the L200 cheeses could be estimated at 55% in relation to the control cheeses.
316 The calculated atherogenicity indexes (AI; value inversely proportional to the
317 nutritional quality of lipid profile) were of 1.94 in the control cheeses, 2.04 in the L45
318 cheeses and 1.90 in the L200 cheeses made with the CLA-forming strain, and the
319 desirable fatty acid (DFA) values were of 49.7 in the control cheeses, 48.3 in the L45
320 cheeses and 49.3 in the L200 cheeses (Table 3). No significant differences were found
321 between the groups of cheese with these parameters. The mean AI values determined in
322 the present study are close to the value of 2 proposed as typical of dairy products by
323 Bobe et al. (2004), and lower than the mean value obtained by Van Nieuwhenhove et al.
324 (2009) for Argentinian cow cheeses (2.59). The DFA values are similar to those found
325 by Taboada, Van Nieuwenhove, Alzogaray, & Medina (2015) in ripened (60-d) goat
326 cheeses made with autochthonous strains (46-48 g 100 g^{-1} of fatty acids); these values
327 allow inferring the content of those beneficial fatty acids for health.
328 It has been suggested that the factors involved in the cheese making process such as the
329 addition of starter cultures and ripening, could influence the lipolytic processes and
330 consequently the variations of fatty acid composition but generally do not affect the
331 concentration of CLA in cheese fat (Bisig et al., 2007; Prandini, Sigolo, & Piva, 2011).
332 It has also been concluded that CLA-forming LAB may increase CLA content only

333 under the condition that free LA is available in the medium (Bisig et al., 2007). Taboada
334 et al. (2015) reported that the use of autochthonous cultures including two *Lb.*
335 *plantarum* strains in artisanal goat cheese manufacture enhanced the CLA content,
336 flavor and AI of the ripened (60-d) cheeses. The CLA level increased during ripening
337 from 0.6 to 1.0 g 100 g^{-1} of fatty acids, this final value being very similar to that
338 determined in the cheeses made with the *Lb. plantarum* L200 strain in the present study.
339 An increase in the levels of oleic acid and total CLA has also been reported in Italian
340 Scamorza ewe cheese made with *Lb. acidophilus* (Albenzio et al., 2013). Therefore, it
341 might be possible that selected lipolytic and CLA-forming lactobacilli could increase
342 the CLA content of cheeses after releasing LA from fat glycerides.
343

344 **4. Conclusions**
345

346 Strains of *Lb. plantarum* appear to be the LAB with the highest ability to convert LA to
347 CLA among the microbiota of raw-milk cheeses made in Galicia (NW Spain), and the
348 *cis*-9, *trans*-11 C18:2 is the most abundant CLA isomer generated by these bacteria.
349 Significantly higher concentrations of *cis*-9, *trans*-11 CLA were determined in
350 miniature laboratory cheeses made with the CLA-forming *Lb. plantarum* L200 strain
351 selected in this study compared to a *Lb. paracasei* strain. Although there is a need for
352 further confirmation, the results of the present study suggest that *Lb. plantarum* L200
353 strain could be used as an adjunct culture to increase CLA content in short-ripened
354 cow's milk cheeses.
355

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357

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363

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492

493 **Table 1**. Presumptive CLA producers, CLA concentration[a] (mean values±standard
494 deviations of triplicate determination) and assumed isomerization rates determined in
495 MRS broth for lactobacilli or Elliker broth for lactococci supplemented with Tween 80
496 (1% w/v) and free LA (0.25 mg mL^{-1}) after 48 h of incubation at 30 ºC

497

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

498

499 [a]Calculated spectrophotometrically at 233 nm from the linear trend of the calibration curve.

500 [b]Calculated according to the formula: CLA concentration/initial LA concentration × 100.

501

502 **Table 2**. CLA isomer concentrations (μg mL^{-1}) determined by GC-FID in bacterial

503 supernatants and pellets (mean values±standard deviations of triplicate determination)

504 from strains grown in MRS broth without Tween 80 supplemented with 0.25 mg mL^{-1}

505 of free LA and 0.1 mg mL^{-1} of BSA after 48 h of incubation at 30 ºC under stirring (120

506 rpm)

507

Strain	Culture fraction	cis9, trans11 CLA	% LA converted[a]	trans10, cis12 CLA	% LA converted	trans9, trans11 CLA	% 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[b]		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	

508

509 [a]Percentage of the sum of the CLA isomer concentrations determined in the supernatant and in the pellet

510 fractions in relation to the initial LA concentration.

511 [b]nd: not detected.

512

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

514

	Control cheeses	L45 cheeses[b]	L200 cheeses[b]	*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±0.007	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
AI[d]	1.94±0.15	2.04±0.17	1.90±0.07	0.606
DFA[e]	49.66±4.92	48.26±5.52	49.32±3.45	0.954

515

516 [a]Results are mean values±standard deviation obtained from two different cheese making trials analysed in

517 duplicate and averaged.

518 [b]L45 cheeses were made with the addition of the non-CLA forming *Lb. paracasei* L45 strain; L200 cheeses

519 were made with the addition of the CLA-forming *Lb. plantarum* L200 strain.

520 [c]ND: not determined.

521 [d]AI: Atherogenicity index according to Ulbricht & Southgate (1991) = (C12:0 + 4C14:0 +

522 C16:0)/(monounsaturated + polyunsaturated fatty acids).

523 [e]DFA: Desirable fatty acid according to Osmari, Cecato, Macedo, & Souza (2011) = unsaturated fatty acids

524 + C18:0.

525 [A–C]Mean values within a row indicated by different superscripts are significantly different ($P < 0.05$;

526 Tukey's test).

527

528

SUPPLEMENTARY MATERIAL

S1. Manufacture of miniaturized model Arzúa-Ulloa/Tetilla industrial PDO cheeses

S1.1. Preparation and curdling of cheese milk

Two cheese making trials were carried out, by using 1 L of retail pasteurized (76 ºC, 20 s) non-homogenized whole (3.8 g fat 100 mL^{-1}) milk (Loureiro, Carballiño-Ourense, Spain) contained in polyethylene bags, for each trial. The milk used for each of the two trials was obtained during spring season from animals feeding on natural pastures, and had been pasteurized the day before. The whole process of milk inoculation and curdling was carried out in a laminar flow cabinet (Telstar mod. BV-10, Barcelona, Spain) using sterile tools and equipment.

Five hundred mL of milk were transferred aseptically to a 1000 mL screw-capped flask containing a stirring magnetic bar. Then, 150 µL of sterile 33% (w/v) $CaCl_2$ solution were added to the milk (rate of 0.01% w:v $CaCl_2$), and the contents were stirred for 30 s. The flask was subsequently tempered at 33 ºC in a thermostated water bath. The DVS starter had been previously rehydrated and strongly stirred in sterile reconstituted skim milk (Oxoid) at a ratio of 10 direct culture units per liter of milk, and then inoculated aseptically at 1% (v:v) in the cheese milk. The milk was again stirred for 30 s and placed in the thermostated bath for 20 min in order to acclimatize and allow the growth of the starter culture. Then, a fermentation-produced chymosin coagulant (Chy-Max® Plus, Chr. Hansen, ~200 international milk-clotting units per mL) diluted 10-fold with

sterile distilled water was aseptically added at a ratio of 250 μL per liter of cheese milk. The contents were stirred for 1 min.

Miniature cheeses were made using two sterilized rectangular (8.5 × 4.5 cm, 12 cm height) sterile polypropylene microplates (Ritter GmbH mod. Riplate® SW 10 ml, Schwabmünchen, Germany) containing 24 10-mL wells arranged in six (numbered) columns and four rows (Figure 1). Initially, 80 μL (1% v:v) of the adjunct (skim milk) culture of the L45 strain was added to the wells of column 5 of each plate, and 80 μL of the culture of the L200 strain was added to the wells of column 6. Eighty μL of non-inoculated sterile skim milk was also added to the wells of columns 1 to 4 (control cheeses), in order to compensate for any possible effect on the growth of LAB of the starter culture. Immediately after these additions were made, all of the wells were filled with 8 mL of the cheese milk (Figure 1A). Once the cheese milk was distributed into the wells (maximum time of 5 min after the rennet addition), the plates were manually shaken and covered with sterile polypropylene lids (13 × 9.5 cm). The covered plates were then placed into an oven set at 32 ºC, and maintained at this temperature for 45 min in order to complete milk curdling.

S1.2. Operations for whey drainage

The curds were cut and stirred using a custom-made device. This stirring device was made from aluminum and consisted of a rectangular (13 × 9 cm, 3 mm thick) plate supporting 24 (6 columns × 4 rows) fixed pins (4 cm long, 0.85 mm diameter) arranged in such a way that their position coincided with the center of the microplate wells (Figure 1B). A first cutting and stirring step of the curds was carried out with the plates inside the (32 ºC) curdling oven by horizontal (10), vertical (10) and circular (10 in a

clockwise direction and 10 in a counterclockwise direction) movements (total time of approximately 30 s) followed by a 5-minute pause. The plates were then placed in a tempering oven set at 35 ºC and stirred in the described manner, with 5 stirring cycles being carried out followed by 3-minute pauses.

After the stirring of the curds (Figure 1C), the microplates were centrifuged at 500 × *g* and 35 ºC for 5 min. The plates were then returned to the tempering oven set at 37 ºC. A washing operation was carried out by aseptically withdrawing 4 mL of whey from each of the wells (Figure 1D), and replacing it with 4 mL of sterile tap water warmed at 38 ºC. The addition of the washing water brought the temperature in the wells to approximately 36 ºC. A second stirring operation (5 stirring cycles followed by 3-minute pauses) was performed, and the plates were subsequently centrifuged at 2000 × *g* and 37 ºC for 5 min.

After the second centrifugation, the plates were taken to the initial (curdling) oven set at 30 ºC, then 7.5 mL of the whey-water mixture was withdrawn from each of the wells. One mL of a sterile 8% (w/v) NaCl solution was added to each well which, as calculated from the expected yield, resulted in approximately 0.5% salt in dry matter in the cheese. The salt was distributed in the curds using the stirring device by performing a stirring cycle in the previously described manner. The plates were kept at rest for 10 min, in order to allow diffusion of the salt into the curds, and then centrifuged at 2000 × *g* and 30 ºC for 3 min. The supernatant was removed by carefully tilting the plates resting on their longer sides to avoid cross-contamination between rows. A final centrifugation of the microplates at 2250 × *g* and 25 ºC for 90 min was carried out in order to simulate the pressing operation (Figure 1E). The plates were finally placed upside down on a tissue paper inside the oven set at 25 ºC for 2 h. Finally, the fresh miniature cheeses were removed from the plates by tapping the shorter sides on tissue

paper. The cheeses were placed using latex gloves on a 31 × 24 cm filter paper in the same order that they were arranged in on the plates (Figure 1F), and then kept in the oven set at 25 ºC for 6-6.5 h until a pH value of 5.5-5.7 was reached.

S1.3. Cheese ripening

Fresh miniature cheeses (1.30±0.14 g weight) were ripened inside two high density polyethylene (HDPE) containers (31,5 × 25,5 × 18,5 cm height) with jaw closures (Tatay mod. 1150107, Barcelona, Spain), prepared (one for each trial) for this purpose (Figure 2). The containers were filled with tap water to a height of 4 cm in order to maintain the relative humidity of the internal environment (Figure 2A). An HDPE mesh (5 mm edge opening) was cut out and fitted to the interior dimensions of the container. The mesh was held on 8 self-adhesive metal hangers, in an "L" shape, 9 cm high from the bottom of the container (Figure 2B). Subsequently, the mesh was covered almost entirely with a filter paper adjusted to its dimensions. The miniature cheeses obtained from the microplates were placed on the paper (Figure 2C), which was changed several times (daily the first three days; weekly from the 7th day) throughout the ripening period to avoid excessive soaking by the whey. On day 3, the cheeses were immersed instantaneously with the help of tweezers in an aqueous emulsion containing 1000 ppm of natamycin (Biomic Avant P1; Proquiga Biotech, A Coruña, Spain) to prevent the growth of moulds and yeasts on the cheese surface.
A digital thermohygrometer (Traceable®-pen, VWR 620-1586, Barcelona, Spain), with a maximum and minimum temperature register, was introduced into the container (Figure 2C) and placed in such a way that data could be visualized through the polyethylene wall (Figure 2D). The ripening containers were finally introduced into a

refrigerator cabinet set at 6 ºC. The cheeses were left to ripen (6±1 ºC; 85-90% relative humidity) for 28 days before further analysis.

SUPPLEMENTARY MATERIAL

Figure S2. Manufacture of the miniature cheeses

(A) Filling of the microplate wells with the cheese milk.

(B) Cutting and stirring of the curds with the manual device.

(C) Appearance of the cut and stirred curds before the first centrifugation.

(D) Withdrawing of the whey from the wells in the washing operation.

(E) Appearance of the curds after the last (fourth) centrifugation.

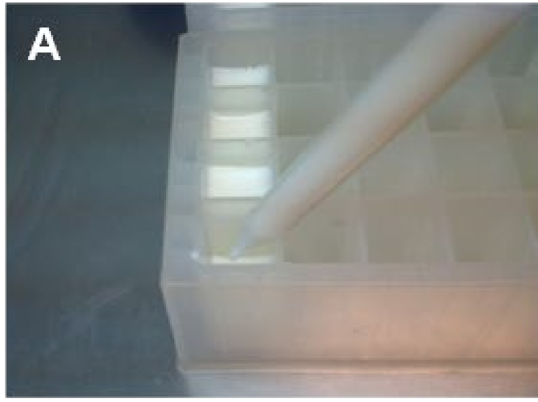
(F) Fresh miniature cheeses just after being removed from the plate wells.

(G) Ripening container filled partly with water.

(H) Placement of the HDPE mesh inside the container.

(I) Placement of the miniature cheeses and the digital thermohygrometer inside the container.

(J) Detail of the thermohygrometer screen.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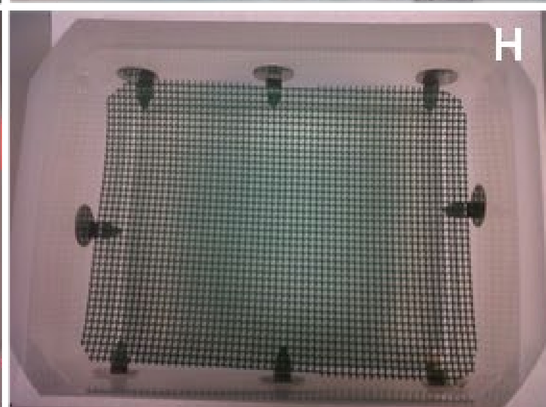
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