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Conjugated linoleic acid production and probiotic assessment of *Lactobacillus plantarum* isolated from Pico cheese

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Abstract

Lactic acid bacteria isolated from a traditional Azorean cheese were screened for their ability to convert free linoleic acid to conjugated linoleic acid (CLA). Two strains of *Lactobacillus plantarum* were recognized as potential CLA producers. GC analysis identified *cis*-9, *trans*-11 C18:2 as the predominant isomer (10-14 μg/mL), followed by *trans*-9, *trans*-11 C18:2 (4-6 μg/mL). The CLA producing strains demonstrated strong biofilm capacity, high cell surface hydrophobicity and good auto-aggregation ability. These strains were capable of surviving in the presence of bile salts (0.3%) and pancreatin (0.1%), but only the highest CLA producer (L3C1E8) was able to resist low pH (2.5). Moreover, the CLA-producers showed good adhesion capacity to intestinal human cells (Caco-2 and HT-29) and were able to prevent colonization of *Escherichia coli*. Of the two strains, *Lactobacillus plantarum* L3C1E8 revealed superior probiotic properties and great potential for producing food products enriched in the two CLA isomers, *cis*-9, *trans*-11 C18:2 (60%) and *trans*-9, *trans*-11 C18:2 (25%).

Keywords: Functional food, conjugated linoleic acid, CLA, lactic acid bacteria, *Lactobacillus plantarum*, probiotics.

1. Introduction

The 'functional foods' concept originated in Japan, but owing to the positive health benefits of such foods, consumer demand has spread globally. These foods are fortified with biologically active compounds that may impart beneficial effects on the body, as well as decrease the risk of certain diseases (Annunziata & Vecchio, 2011; Bigliardi & Galati, 2013). Conjugated linoleic acid (CLA) refers to a heterogeneous group of positional and geometric (*cis* or *trans*) isomers of linoleic acid (LA) with conjugated double bonds at multiple carbon positions (Pandit, Anand, Kalscheur, & Hassan, 2012). CLA isomers are considered to be beneficial functional lipids due to their biological activities and health promoting properties, such as anti-cancer, anti-atherogenic, anti-obesity and anti-inflammatory (Chinnadurai, Kanwal, Tyagi, Stanton, & Ross, 2013; Coakley et al., 2006; Hennessy, Ross, Devery, & Stanton, 2011; Kobaa & Yanagita, 2014; Shen et al., 2013; Sluijs, Plantinga, de Roos, Mennen, & Bots, 2010). Dietary CLA can be found primarily in the meat and milk of ruminants as a result of bacterial biohydrogenation of lipids in the rumen (Lin & Lee, 1997; Fuke & Nornberg, 2017), with the *cis*-9, *trans*-11 C18:2 isomer being the most prevalent (Chin, Liu, Storkson, Ha, & Pariza, 1992). Other isomers present in smaller quantities include *trans*-7, *cis*-9 C18:2, *cis*-11, *trans*-13 C18:2, *cis*-8, *trans*-10 C18:2, and *trans*-10, *cis*-12 C18:2. Nonetheless, the low concentrations of CLA found in these food products (meat, milk, and dairy products) are lower than the level required to obtain health benefits (Gaullier et al., 2007). Consequently, increasing the concentration of CLA in food products has been the target of several studies in recent years with a view to developing functional food products (Fuke & Nornberg, 2017; Ozer, Kilic & Kilic, 2016; Shingfield, Bonnet, & Scollan, 2013).

Lactic acid bacteria (LAB), especially *Lactobacillus*, may produce CLA by isomerization of linoleic acid (LA) (Alonso, Cuesta, & Gilliland, 2003; Chung et al., 2008; Coakley et al., 2003; Jiang, Björck, & Fondén, 1998; Kishino et al., 2003; Ogawa et al., 2005; Zeng, Lin, & Gong, 2009). In this regard, the production of these bioactive fatty acid metabolitesmay be considered a probiotic trait. Incorporation of such bacteria into foods offers a viable solution for increasing CLA content. Therefore, the identification of LAB cultures capable of producing CLA from a LA source is a worthwhile pursuit for the food industry, particularely in relation to fermented dairy products. (Ozer, Kilic & Kilic, 2016; Vieira et al., 2017).). In addition, CLA production in humans can be performed by the gut microbiota (Raimondi et al., 2016), as CLA production by a probiotic has been observed in the murine gut where it was linked to suppression of colitis (Bassaganya-Riera et al., 2012). Thus, the ability of CLA-producing strains to exhibit probiotic characteristics such as survival in the gastrointestinal (GI) tract is also of significance given that they may impart beneficial effects in the gut.

In order to be considered a probiotic, a bacterial strain must be able to survive in the extreme conditions of the GI tract (low pH in stomach, bile salts), adhere to the intestinal mucosa and impart beneficial effects on the host such as antimicrobial and immunomodulatory properties, amongst others (Del Piano et al., 2006; Verna & Lucak, 2010). In addition, biofilm production is considered an important characteristic leading to successful colonization (Salas-Jara, Ilabaca, Vega, & García, 2016).

Pico cheese is a traditional cheese with Protected Designation of Origin (PDO) status; it is produced from raw cow's milk from Pico Island in the Azores, without the addition of starter-cultures. In small artisanal dairy units the raw milk is coagulated with animal rennet, the curds are manually cut, placed into molds and left to ripen for approx. 20

days. Consequently, the microbial fermentation is carried out by the indigenous microbiota derived exclusively from the raw milk of grazing cows and the production environment. Therefore, Pico cheese is a fertile ground for the identification and isolation of novel LAB strains. The present study was aimed to screen LAB previously isolated from Pico cheese (Domingos-Lopes et al., 2017), for their ability to produce CLA. The highest CLA-producing strains were further evaluated for their probiotic potential, which included ability to survive to the extreme conditions of the GI tract, adhesion to intestinal cells and anti-adhesion assays against the pathogenic bacterium *Escherichia coli*.

2. Materials and methods

2.1 Microorganisms

The LAB strains under investigation in this study were previously isolated from a traditional Azorean cheese (Pico cheese) and had been phenotypically and genetically identified (Domingos-Lopes et al., 2017). One hundred and twelve LAB strains belonging to the genus *Lactococcus* (3), *Lactobacillus* (21), *Leuconostoc* (4) and *Enterococcus* (84) were selected from the bacterial culture collection isolated from this cheese. LAB cultures were activated by successive subculturing in MRS broth (Difco Laboratories, Detroit, MI) and grown at 30 °C. The strain *Escherichia coli* ATCC 25922 was used in the assays of bacterial adhesion to intestinal cells and was grown at 37 °C in Nutrient Broth under aerobic conditions (Fluka, Gillingham, England).

2.2 Screening of LAB for CLA production

LAB strains were screened for CLA production using a spectrophotometric detection method according to Barrett et al. (2007). Briefly, LAB strains were incubated in MRS

broth containing free linoleic acid (0.5 mg/mL; Sigma-Aldrich, St Louis, MO, USA) and 2% (w/v) Tween 80, at 30°C for 48 h. After incubation, 1 mL of culture was centrifuged at 20,800 × *g* for 1 min, the pellet was discarded, and the supernatant was mixed with 2 mL of isopropanol by vortexing and allowed to stand for 3 min. The fatty acids were extracted by vortexing the solution and allowing to stand for a further 3 min, following the addition of 1.5 mL of hexane. The presence of CLA in the culture supernatant was assayed by dispensing 230 µl of the fat-soluble hexane layer into a UV-transparent 96-well plate (Costar, Corning, NY) and determining the absorbance at 233 nm using a 96-well plate spectrophotometer (GENios Plus; Tecan, Medford, MA). Measurements were obtained in duplicate.

A standard curve was constructed for the absorbance at 233 nm *versus* the CLA concentration (mg/mL), using pure *cis*-9, *trans*-11 CLA isomer (Nu-Check Prep., Elysian, MN, USA), This method was used for screening LAB for CLA production. Positive results were further confirmed by gas chromatography.

2.3 CLA quantification by gas chromatography (GC)

2.3.1 Lipid extraction from bacterial supernatant fluids and pellets

CLA production by *Lactobacillus plantarum* L2C21E8 and *Lb. plantarum* L3C1E8, identified as potential CLA-producing strains from the screening in section 2.2, was quantified by gas chromatography, according to the method described by Yang et al. (2014) with some modifications. Prior to examination of the strains, each culture was subcultured twice in MRS broth. The strains were then cultured (1%) in broth containing 0.5 mg/mL free linoleic acid (Sigma-Aldrich). The stock solution consisting of linoleic acid (30 mg/mL) and 2% (v/v) Tween 80, was previously filter sterilized

149 through a 0.45 μm filter (Minisart, Sigma-Aldrich) and stored in the dark at -20°C. The
150 strains were incubated aerobically at 30°C.
151 After 48 h incubation, the LAB cultures were centrifuged at 5000 × *g* for 10 min at
152 room temperature. The fat was extracted from the culture supernatant fluid as follows:
153 An internal standard, C17:0 heptadecanoic acid (99% pure; Sigma-Aldrich), was added
154 to 5 mL of the supernatant fluid to give a final concentration of 0.75 g internal standard
155 per sample. Then, 5 mL of isopropanol was added to the supernatant fluid, and the
156 samples were vortexed for 30 s. Five milliliter of *n*-hexane was added to this mixture,
157 vortexed and centrifuged at 3260 × *g* for 5 min. The resultant hexane layer (containing
158 lipids) was dried under a stream of nitrogen. For bacterial pellet extraction, the pellet
159 from 10 mL of bacterial culture was washed in 2 mL saline solution (0.137 mol/L NaCl,
160 7.0 mmol/L K_2HPO_4 and 2.5 mmol/L KH_2PO_4). The cells were vortexed and
161 centrifuged at 3260 × *g* for 10 min, and the washing step repeated twice. The cells were
162 suspended in 1 mL saline solution and then the samples were extracted completely as
163 described above for the bacterial supernatant fluid. Fat was extracted from supernatant
164 and pellet, independently. The lipids were stored at -20° C prior to preparation of fatty
165 acid methyl esters for GC analysis. Samples were analyzed in triplicate.
166
167 **2.3.2 Preparation of fatty acid methyl esters**
168 The extracted lipids were analyzed by gas chromatography following methylation with
169 NaOH-BF_3 in methanol as described by Yang et al. (2014). *Tert*-butyl methyl ether
170 (MTBE, Sigma-Aldrich) (0.5 mL) was added to samples prepared above, together with
171 10 mL of NaOH (0.5 M) in methanol, and the mixture was vortexed for approx. 30 s
172 and incubated for 12 min at 90 °C. Then, 10 mL of BF_3 in methanol (Sigma-Aldrich)
173 was added and incubated for 12 min at 90 °C. Upon incubation, 2 mL of water saturated

with hexane (1 mL of hexane in 100 mL of water) and 4 mL of hexane were added to the mixture and vigorously vortexed for 30 s. The upper (organic) phase was collected, and again, 2 mL of water saturated hexane was added. After standing for sufficient time, the top layer was collected to a clean methylation tube containing 0.5 g of anhydrous sodium sulphate and left in the dark for 1h. Aliquots of the samples containing fatty acid methyl esters (FAME) were stored in a vial at -20 °C for further quantification of CLA content by GC.

2.3.3 Gas chromatography analysis

A gas chromatograph (3500, Varian, Harbor City, CA, USA) fitted with a flame ionization detector was used. Helium served as the carrier gas. The GC conditions for separation of CLA isomers were as described by Coakley et al. (2003). The CLA isomers were identified by comparison with the retention time of the reference CLA standard mix (Sigma-Aldrich).

2.4 Evaluation of biofilm formation

Biofilm formation by *Lb. plantarum* L2C21E8 and L3C1E8 strains was evaluated in 96-well microtiter plates following the method described by Pérez et al. (2014). Briefly, overnight LAB cultures from MRS broth were used as inoculums and incubated in a 96-well microtiter plate without shaking at 30 °C for 24, 48 and 72 h. Then, wells were washed with phosphate-buffered saline (PBS) and biofilms stained for 30 min with 200 µL 0.1% (w/v) crystal violet. The dye in the cells was then remobilized with 200 µL of 30% (v/v) glacial acetic acid, and the absorbance of the solution (A_{570}) was determined by spectrophotometer (Fluostar Omega, BMG Labtech). Based on the absorbance, the strains were classified into the following categories: no biofilm producer, weak,

moderate or strong biofilm producers. Two independent experiments were performed, each with four replicates.

2.5 Probiotic potential of CLA producers

2.5.1 Bacterial cell surface hydrophobic/hydrophilic characteristics

Cell surface characteristics of *Lb. plantarum* L2C21E8 and L3C1E8 strains were measured according to the method of Bellon-Fontaine et al. (1996). LAB strains were grown in MRS broth and harvested by centrifugation (4500 × *g*, 10 min), washed twice with 0.85% NaCl and resuspended in the same solution. Suspensions were mixed with three different solvents: chloroform, ethyl acetate and n-hexadecane (Sigma-Aldrich), and the two phase systems were mixed by vortexing for 1 min. After the complete separation of two phases, the absorbance was measured (A_1) at 600nm. The percentage of bacterial adhesion to solvents was calculated as follow: % Adhesion = $(1-A_1/A_0)$ x 100, where A_0 and A_1 were the absorbance values before and after extraction with the organic solvent. The experiment was performed in triplicate.

2.5.2 Auto-aggregation

Auto-aggregation determination was performed according to the protocol described by Todorov & Dicks (2008). *Lb. plantarum* L2C21E8 and L3C1E8 strains were grown for 24h in MRS broth, centrifuged, washed and resuspended in 0.85% sterile saline solution. After 60 min of incubation at room temperature, the cultures were centrifuged at 300 × *g* for 2 min. Auto-aggregation was calculated by the following equation: % Auto-aggregation= $[(A_0-A_1)/A_0]$ x100, whereas A_0 represents absorbance at time 0, and A_1 absorbance after 60 min. Experiments were conducted in triplicate.

2.5.3 Resistance to low pH, bile salts and pancreatin

Lb. plantarum L2C21E8 and L3C1E8 strains were tested for resistance to low pH, bile salts and pancreatin according to Argyri et al. (2013). Overnight cultures of LAB were harvested by centrifugation (10,000 × *g* for 5 min at 4 ° C) and the pellets washed with sterile phosphate-buffer saline (pH 7.3). To determine acid tolerance, the cell pellet was resuspended in PBS adjusted to pH 2.5. For bile salts and pancreatin resistance, the cell pellets were resuspended in PBS solution (pH 7.3), containing 0.3% (w/v) of bile salts (Fluka, Buchs, Switzerland) and 0.1% (w/v) of pancreatin (Sigma-Aldrich). LAB were then incubated at 37 ° C for 0, 0.5, 1, 2 and 3 h. Enumeration of viable cells were performed on MRS agar (Biokar, Beauvais, France). Assays were carried out as four independent experiments.

2.5.4 Adhesion assays

2.5.4.1 LAB adhesion capacity to intestinal human cells

The method described by Argyri et al. (2013) was followed to study adhesion of *Lb. plantarum* L2C21E8 and L3C1E8 strains to HT-29 and Caco-2 cells, with some minor modifications. HT-29 cells were grown and maintained in McCoy´s 5A medium (Sigma-Aldrich) containing 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich) and 1% (v/v) gentamicin (Sigma-Aldrich) at 37 º C in an atmosphere of 5% CO_2. Caco-2 cells were routinely cultured in Dulbecco´s modified Eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich), 1% (v/v) non-essential amino acids (Sigma-Aldrich), and 1% (v/v) gentamicin (Sigma-Aldrich). For the experiments, cells were seeded in 24-well tissue plates at 10^4 cells density and cultured until differentiation and a confluent layer was attained. The culture

medium was changed every 2-3 days. Medium without antibiotic was used 24 h before the experiments. The ratio of cells to bacteria was ≥ 1:100. Overnight cultures of LAB strains were centrifuged, washed twice with PBS and diluted in cell culture medium without antibiotic to a concentration of approximately 10^8-10^{10} CFU. After co-incubation for 2 h at 37 °C, wells were washed three times with PBS solution to remove any non-adherent bacteria. Then, 0.1 mL of trypsin-EDTA solution (Sigma-Aldrich) was added to detach cells and adhered bacteria. The cells were lysed with Triton X-100 (0.25%, Merck) and bacterial counts were carried out in MRS agar (Biokar). The adhesion ability was expressed as the number of adhered bacteria (CFU/mL). Assays were performed in triplicate and three independent experiments were carried out.

2.5.4.2 Effects of LAB on adhesion of *E. coli* to HT-29 cells

The ability of *Lb. plantarum* L2C21E8 and L3C1E8 strains to inhibit the adhesion of *E. coli* ATCC 25922 to HT-29 cells was investigated according to the method described by García-Ruiz et al. (2014). Three different assays were conducted: competition, inhibition and displacement. For the competition assay, LAB strains and *E. coli* were simultaneously added to HT-29 cells (1:1) and incubated for 60 min. In the inhibition assay, LAB strains were previously incubated for 60 min with cells before adding *E. coli*, and incubated for another 60 min with *E. coli*. For the displacement assay, *E. coli* was added firstly to cells for 60 min before addition of LAB strains for further 60 min. *E. coli* counts were performed in TBX Agar (Oxoid, Basingstoke, England). Results were expressed as the percentage of inhibition of *E. coli* adhesion to cells by each LAB strain. Three independent experiments were carried out in triplicate.

2.6. Statistical analyses

Analyses of CLA screening were performed in duplicate and results were expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was used to compare data obtained for the different strains. Two-way factorial ANOVA was used to determine the effect of strain on CLA isomers production in supernatant and pellet. Data on biofilm formation was also processed by ANOVA. Probiotic characteristics of CLA producing strains were analyzed by factorial ANOVA, the factors were time or cells (Caco-2 and HT-29) and *Lb. plantarum* strains. When a significant F was observed ($P<0.05$), differences between means were evaluated by Bonferroni multiple comparison test. Percentages of microbial adhesion to solvents, auto-aggregation, competition, inhibition and displacement of *E. coli* in the presence of *Lb. plantarum* strains were analyzed by the Kruskal-Wallis test. The significant level was set at 0.05. All statistical tests were performed using IBM SPSS Statistics, version 22 (IBM Corporation, New York, USA).

3. Results

3.1 Screening of LAB for CLA production

A standard curve was constructed for the absorbance at 233 nm *versus* CLA concentration, using pure C18:2 *cis*-9, *trans*-11. Results demonstrated that a direct relationship could be established between absorbance and CLA concentration (R^2=0.9959, data not shown), up to an absorbance of 2.8. Therefore, the CLA concentrations in culture supernatants with an absorbance at 233nm less than or equal to 2.8 could be calculated from the linear trend line of the standard curve using the equation y= 0.0575x-0.1187. The results of LAB screening for CLA production are presented in Table 1. Two strains L2C21E8 and L3C1E8, identified as *Lb. plantarum*, presented significantly ($P<0.05$) higher CLA concentrations (or the highest percentage

299 of conversion), at 17.94 and 15.36 μg/mL of CLA, respectively, compared to the other
300 CLA-producing strains. However, it is important to emphasize that this method does not
301 distinguish between isomers of CLA, since it is based on measurement of the
302 conjugated double bond in the fatty acid. Therefore, a more accurate analysis of the
303 CLA-isomers produced by these two strains was performed by gas chromatography.
304

305 **3.2 Gas chromatography analysis**

306 The chromatogram profiles obtained by GC for the cell supernatants of *Lb. plantarum*
307 strains L2C21E8 and L3C1E8 are presented in Fig.1. The CLA isomers detected by GC
308 were *cis*-9, *trans*-11 C18:2; *trans*-10, *cis*-12 C18:2; *cis*-9, *cis*-11 C18:2; and *trans*-9,
309 *trans*-11 C18:2. As expected, the *cis*-9, *trans*-11 CLA isomer (rumenic acid) was the
310 most abundant isomer generated and was mainly found in the cell supernatant (Fig.2).
311 The level of *cis*-9, *trans*-11 CLA isomer was significantly ($P<0.05$) higher than *cis*-9,
312 *cis*-11 and *trans*-10, *cis*-12 isomers. C18:2 *trans*-9, *trans*-11 was the second most
313 abundant CLA isomer detected in the cell supernatant, for both strains. In contrast, *cis*-
314 9, *cis*-11 C18:2 was found at equivalent concentrations in the pellet and the supernatant.
315 Other CLA isomers, such as *trans*-10, *cis*-12, were also generated, but as minor
316 compounds. Similar profiles were obtained for the two selected strains and no
317 significant ($P>0.05$) differences were found between them.
318

319 **3.3 Biofilm formation**

320 *Lb. plantarum* L2C21E8 and L3C1E8 strains were evaluated for their capacity to form
321 biofilms during 24, 48 and 72 h of incubation. The two *Lactobacillus* strains were able
322 to form biofilm structures on polystyrene plates after 24 h of growth. However, the
323 strains differed significantly ($P<0.05$) since *Lb. plantarum* L3C1E8 exhibited the

highest biofilm-forming ability in this regard, from the beginning of incubation (Fig.3). However, both strains can be considered as strong biofilm producers (increase of OD by four times) after 48 and 72h of incubation.

3.4 Probiotic properties of CLA producer strains

3.4.1 Bacterial cell surface characteristics and auto-aggregation ability

The hydrophobic/hydrophilic cell surface properties of *Lb. plantarum* L2C21E8 and L3C1E8 were studied using three solvents: n-hexadecane, a non-polar solvent; chloroform, an acidic solvent; and ethyl acetate, a basic solvent. The results, expressed as percentage of microbial adhesion (Fig.4), indicated that both strains presented a more hydrophobic cell surface. Strain L3C1E8 demonstrated stronger ($P<0.05$) affinity for chloroform and n-hexadecane compared to strain L2C21E8. Both strains also showed more affinity ($P<0.05$) for chloroform and n-hexadecane than ethyl acetate, a basic solvent and electron donor. The ability of strains to aggregate was studied and results are also presented in Fig.4. Both strains showed high percentages of auto-aggregation (>70%).

3.4.2 Resistance to low pH, bile salt and pancreatin

The survival of the two strains of *Lb. plantarum* under low pH, bile salt and pancreatin was evaluated. As shown in Table 2, both strains were tolerant to bile salts (0.3%, w/v) and pancreatin (0.1%, w/v), each exhibiting no change ($P>0.05$) in viability after 3 h of incubation. However, strain L3C1E8 showed significantly higher resistance ($P<0.05$) to bile salts and pancreatin than L2C21E8. Moreover, strain *Lb. plantarum* L3C1E8 was able to survive at pH 2.5 during 2 h of incubation, although a reduction of viable cell counts was observed after 2 h (from 8.84±0.23 to 2.25±0.14 log CFU/mL). On the

contrary, strain *Lb. plantarum* L2C21E8 was susceptible to acidic conditions as no viable cells were found after 0.5 h of incubation.

3.4.3 LAB adhesion capacity to intestinal human cells

The *Lb. plantarum* L2C21E8 and L3C1E8 strains were further examined for their ability to adhere to Caco-2 and HT-29 cells. Efficiency of each strain's ability to adhere to the different cell lines are presented in Table 2. In general, both strains displayed high adhesion capacity to both cell lines and no differences ($P>0.05$) were found between strains. Higher adherence ($P<0.05$) was obtained for Caco-2 cell lines, with LAB counts higher than 7 log CFU/mL. Adherence to HT-29 cells was of approx. 6 log CFU/mL for both strains.

3.4.4 Effects on adhesion of *E. coli* to HT-29 cells

Results of the anti-adhesion assays (competition, inhibition and displacement) of *E. coli* ATCC 25922 in the presence of *Lb. plantarum* L2C21E8 and L3C1E8 are shown in Fig.5. For the competition assay, when LAB strains and *E. coli* were added simultaneously, both strains were able to reduce adherence of the pathogen in comparison to the untreated control (> 50%). In the inhibition assays, when LAB strains were added before the pathogen, strain *Lb. plantarum* L3C1E8 was the most effective ($P<0.05$), presenting a high degree of inhibition (>90%). Additionally, both strains presented high inhibition percentages (>75%) in the displacement assay when *E. coli* was added to the cells before the LAB strains.

4. Discussion

Production of CLA by some LAB and bifidobacteria has been reported in recent years, raising the question of whether CLA production may be regarded as one of the mechanisms by which these bacteria exert some of their health promoting effects (Gorissen et al., 2010; Hennessy et al., 2011; Andrade et al., 2012; O'Shea, Cotter, Stanton, Ross, & Hill, 2012). In this work, LAB isolated from artisanal cheese were screened for their ability to produce CLA. The screening procedure lead to the selection of two CLA producers belonging to *Lb. plantarum* species (strains L2C21E8 and L3C1E8). Similarly, in the screening of LAB isolated from naturally fermented foods, other authors also identified *Lb. plantarum* strains as the highest CLA-producers (Liu et al., 2011; Yang et al., 2014). CLA values observed in the present assay were lower compared to values reported by other authors, but several factors could contribute to this difference, such as temperature, fermentation time, linoleic acid (LA) concentration and other media components (Kuhl & De Dea Lindner, 2016; Ye et al., 2013). Some authors revealed a positive correlation between CLA formation and the ability to tolerate free LA, which suggests that LAB convert LA to CLA as a detoxification mechanism (Adamczak, Bornscheuer, & Bednarski, 2008; Wang, Lv, Chu, Cui, & Ren, 2007). In addition, several studies indicated that bacteria usually produced more CLA in whole milk than in MRS medium (Andrade et al., 2012). The CLA produced by the *Lb. plantarum* strains under study was mainly found in the supernatant compared with the pellets (cells). This result is in agreement with other studies showing that CLA production is primarily found in the extracellular phase (Rainio, Vahvaselkä, Suomalainen, & Laakso, 2002), though it can also be found in less amounts in the cellular membrane as a structural lipid (Oh et al., 2003).

When LA is used as substrate, LAB can convert this fatty acid into 10-hydroxy-12-*trans*-octadecadienoic acid and 10-hydroxy-12-*cis*-octadecadienoic acid, ending mainly

398 with C18:2 *cis*-9, *trans*-11, although other isomers, such as *trans*-10, *cis*-12 C18:2 and
399 *trans*-9, *trans*-11 C18:2, may also be produced in lesser amounts (Kishino et al., 2003;
400 Kuhl & De Dea Lindner, 2016; Ogawa et al., 2005). The high variation of *cis*-9, *trans*-
401 11 C18:2 production in each replication was reflected on the large standard error of the
402 mean (SEM) obtained (Fig. 2). Several studies also revealed a great variability in the
403 CLA isomer profile produced by different LAB strains, although, for most
404 species/strains, *cis*-9, *trans*-11 C18:2 isomer represented more than 70% of the total
405 CLA formed from LA (Kuhl & De Dea Lindner, 2016). In our study, the selected
406 strains of *Lb. plantarum* produced mainly *cis*-9, *trans*-11 C18:2 (approx. 60% of CLA
407 isomers), followed by *trans*-9, *trans*-11 C18:2 (approx. 25% of CLA isomers). In
408 addition, small amounts of other CLA isomers, such as *trans*-10, *cis*-12 (9-11%) and
409 *cis*-9, *cis*-11 (3-5%), were also detected in the supernatant. In the pellet, these ratios
410 were maintained for the predominant isomers (*cis*-9, *trans*-11 and *trans*-9, *trans*-11),
411 but the proportion of *cis*-9, *cis*-11 increased to 9-14% of total CLA, while *trans*-10, *cis*-
412 12 reduced to 5-6%. Most of the studies of CLA isomers produced by *Bifidobacterium*
413 and *Lactobacillus* strains indicated that LA was mainly converted to the *cis*-9, *trans*-11
414 CLA, followed by *trans*-10, *cis*-12 CLA and small amounts of *trans*-9, *trans*-11 CLA
415 isomers (Hennessy et al., 2012; Gorissen et al., 2010; Gorissen, Leroy, De Vuyst, De
416 Smet & Raes, 2015; Rodríguez-Alcalá, Braga, Malcata, Gomes, & Fontecha, 2011).
417 Nevertheless, some *Lb. plantarum* strains were found to produce high proportions of
418 *trans*-9, *trans*-11 C18:2 (Ogawa et al., 2005). In addition, culture conditions, such as
419 low pH and restriction of oxygen, were shown to change the proportion of individual
420 isomers and favor the formation of *trans*, *trans* isomers (Macouzet, Lee & Robert,
421 2008; Panghyová, Kačenová, Matulová & Kiss, 2009).

Several studies have revealed that CLAs exert various health benefits, and there is increasing evidence that these effects are isomer specific (O'Shea et al., 2012). Those studies demonstrated that *trans*-9, *trans*-11 C18:2 has a much higher inhibitory and anti-proliferative effect on the growth of the human colon and breast cancer cells, than *cis*-9, *trans*-11 CLA isomer (Coakley et al., 2006; El Roz, Bard, Huvelin, & Nazih, 2013). Other studies showed that *cis*-9, *trans*-11 CLA has extra beneficial effects, such as anti-inflammatory and anti-atherogenic effects (Ecker, Liebisch, Patsch, & Schmitz, 2009; Loscher et al., 2005). Furthermore, it has been demonstrated that the mixture of the two CLA isomers (*cis*-9, *trans*-11 and *trans*-9, *trans*-11 CLA) had a synergistic anti-proliferation effect on a human colorectal carcinoma cell line (Zhong, Luo, Huang, Deng, & Lei, 2012). Interestingly, the strains of *Lb. plantarum* tested in the present work, presented the highest production of both *cis*-9, *trans*-11 and *trans*-9, *trans*-11 CLA isomers, exhibiting a great potential for application in health promoting food products.

In the present study, the capacity of the two CLA-producers to form biofilms was also examined. Based on the results obtained, both *Lb. plantarum* strains were able to form a well-structured biofilm. Aoudia et al. (2016) demonstrated that *Lactobacillus* strains are able to form biofilms in a microtiter plate biofilm assay, even under growth conditions mimicking the gastrointestinal environment. Biofilm capacity of these strains can prevent colonization of undesirable microorganisms by covering the epithelial receptors (Martin et al., 2008; Moroni, Kheadr, Boutin, Lacroix, & Fliss, 2006).

The two CLA producers were further evaluated for characteristics concerning other relevant probiotic features. These characteristics include auto-aggregation capacity, hydrophilic/hydrophobic surface properties, survival at low pH, resistance to bile salts and pancreatin, as well as, adhesion to different human cell lines. Both strains of *Lb.*

447 *plantarum* studied possess desirable probiotic characteristics, as demonstrated by the in
448 vitro studies. Firstly, we examined the hydrophobicity and auto-aggregation ability of
449 strains. Some cell wall-associated characteristics of probiotics, such as hydrophobicity
450 and auto-aggregation, can contribute to adhesion properties of bacteria to host tissues
451 (Kos et al., 2003; Naidu, Bidlack, & Clemens, 1999; Vinderola & Reinheimer, 2003).
452 Attachment of bacteria to epithelial cells depends on several factors, such as van der
453 Waals attraction, gravitational forces and surface electrostatic charges (Van Loosdrecht,
454 Norde, & Zehnder, 1990). The bacterial surface can be qualitatively assessed as either
455 polar or non-polar, by using solvents with different polarity (Ocana & Nader-Macias,
456 2002; Rosenberg, Gutnick, & Rosenberg, 1980). In our study, the *Lb. plantarum* strains
457 displayed high hydrophobicity values. These results are in agreement with the finding
458 that cell surfaces of lactobacilli are commonly of hydrophobic nature (García-Cayuela
459 et al., 2014). Bacterial aggregation is a desirable property for probiotics and plays an
460 important role in the formation of biofilms (Maria Carmen Collado, Meriluoto, &
461 Salminen, 2008). The two *Lb. plantarum* strains under study demonstrated high
462 capacities to auto-aggregate, conferring them with a survival and proliferation
463 advantage over bacteria lacking this ability (Rickard, Gilbert, High, Kolenbrander, &
464 Handley, 2003). Various lactobacilli have been described in the literature with the
465 ability to form auto-aggregates (García-Ruiz et al., 2014; Hevia et al., 2013; Lozo et al.,
466 2007).
467 Two important characteristics of potentially probiotic strains are resistance to low pH of
468 the stomach and to bile salts and pancreatin secreted into the intestine (Hyronimus, Le
469 Marrec, Hadj Sassi, & Deschamps, 2000; Vinderola & Reinheimer, 2003). In this work,
470 only the strain *Lb. plantarum* L3C1E8 exhibited ability to survive in simulated
471 gastrointestinal conditions (pH 2.5 and 0.3% w/v bile salts, 0.1% w/v pancreatin).

Nevertheless, strain *Lb. plantarum* L2C21E8 was also able to survive in the presence of bile salts and pancreatin. With respect to acid resistance, it is reported that bacteria sensitive to gastric juice may have high rates of isolation from feces (Del Piano et al., 2006). Food intake in vivo has been show to protect bacteria during gastric passage (Saito et al., 2014; Silva et al., 2015). As previously reported, LAB strains were highly resistant to low pH (2.5) after 3 h (survival rate around 100%), when incorporated into fresh cheese (Silva et al., 2015). Therefore, acid sensitive strains can resist gastrointestinal digestion with the use of a food matrix, which can offer protection.

In vitro studies on the adhesion capabilities of *Lb. plantarum* strains to human epithelial cells (Caco-2 and HT-29 lines) were also conducted. This ability is the most commonly encountered criteria for the selection of probiotic bacteria (Collado, Isolauri, Salminen, & Sanz, 2009; Lebeer, Vanderleyden, & De Keersmaecker, 2008). Implantation in the intestinal mucosa has been considered as the critical feature a strain must possess, in order to influence the intestinal environment (Del Piano et al., 2006). Colonization of the gut mucosa by probiotic strains can provide some beneficial health effects, such as the competitive exclusion of pathogens, modulation of immunity and resistance of probiotic to elimination by peristalsis (Pennacchia, Vaughan, & Villani, 2006; Rinkinen, Westermarck, Salminen, & Ouwehand, 2003). Our results showed that the two *Lb. plantarum* strains studied were able to adhere to Caco-2 and HT-29 cells. Previous studies also reported the capability of adhesion of *Lb. plantarum* strains to these two cell lines (Oguntoyinbo & Narbad, 2015; Saxami et al., 2016). The adhesion capacity of probiotic strains has been identified and characterized, and can be explained by the binding properties of bacterial cell-surface associated proteins with mucus and intestinal cells (Sánchez, Bressollier, & Urdaci, 2008; Vélez, De Keersmaecker, & Vanderleyden, 2007). Another beneficial trait is the interference of probiotic strains

with pathogenic adhesion. In this study, both *Lb. plantarum* strains were effective in inhibiting adhesion of the pathogenic bacterium *E. coli*. The high values observed could indicate competition of these strains for the same binding sites on epithelial cells and the overlying mucus layer, in a strain–specific manner (Lee & Puong, 2002; Morrow, Gogineni, & Malesker, 2012). In addition, displacement ability of pre-adhered *E. coli* presented by the *Lb. plantarum* strains studied, could also be due to the production of anti-adhesion factors (Abedi, Feizizadeh, Akbari, & Jafarian-Dehkordi, 2013).

5. Conclusion

In conclusion, two *Lb. plantarum* strains presented the ability to produce CLA isomers from free LA, mainly *cis*-9, *trans*-11 CLA and *trans*-9, *trans*-11 CLA, known for having important biological properties. Application of these strains in fermented food products can increase the CLA intake and be beneficial for human health. In addition, these strains presented high adhesion ability to colonic cells and were able to inhibit *E. coli* adhesion. These results support the probiotic character of the two strains and their potential to be used in the production of novel functional foods. However, further *in vivo* investigations are necessary in order to confirm the role of these potential probiotic strains for promoting human health.

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Figure captions

Fig. 1. GC chromatograms of fatty acids of cell supernatant fluid of *Lb. plantarum* L2C21E8 (A) and *Lb. plantarum* L3C1E8 (B) grown in MRS plus linoleic acid, after 48h.

Fig. 2. Isomers profiles (μg/mL) of supernatant and pellet of *Lb. plantarum* L2C21E8 (A) and *Lb. plantarum* L3C1E8 (B) in MRS broth supplemented with 0.5 mg/mL of linoleic acid after 48 h of incubation. Error bars indicate standard error of the mean (SEM) from four different experiments. CLA isomers marked with different letters differ significantly ($P<0.05$).

Fig. 3. Biofilm formation of *Lactobacillus plantarum* L2C21E8 and L3C1E8 after incubation at 30 °C for 24, 48 and 72h. Data are expressed as mean ± SEM. The cut-off (ODC) was defined as the mean OD value of the negative control. Based on the OD, strains were classified as non-biofilm producers ($OD \leq ODC$), weak ($ODC < OD \leq 2 \times ODC$), moderate ($2 \times ODC <$ OD $4 \times ODC$) or strong biofilm producers ($4 \times ODC <$ OD). Different letters within each incubation time represent significant differences ($P<0.05$) between strains.

Fig. 4. Percentages of microbial adhesion to solvents and auto-aggregation of strains *Lb. plantarum* L2C21E8 and L3C1E8. Results are shown as the average (± SEM) of three independent experiments. Different letters within each solvent represent significant differences ($P<0.05$) between strains.

Fig. 5. Competition, inhibition and displacement of *E. coli* ATCC 25922 in the presence of *Lb. plantarum* L2C21E8 and *Lb. plantarum* L3C1E8. The data is shown as the average (± SEM) of three independent experiments and inhibition of *E. coli* adhesion is expressed as percentage ratios. Different letters represent significant differences ($P<0.05$) between strains.

Table 1- LAB screened for potential CLA production in MRS broth after 48 h. Values of *cis*-9, *trans*-11 CLA concentration (μg/mL) and percentage of conversion are indicated. Values of CLA concentration are means ± SEM of two replicates.

Strains	Accession No.	cis9. trans11 CLA (μg/mL)	% Conversion
Lactobacillus paracasei			
L2A21R9	KM096813	3.66±0.09[a,b]	0.73
L2A1K8	KM096814	2.15±0.05[a]	0.43
L2B21R1a	KM096816	2.56±0.17[a]	0.51
L2B21R3	KM096817	2.21±0.01[a]	0.44
L2B1K8	KM096818	3.65±0.12[a,b]	0.73
L3A21R8	KM096819	2.73±0.41[a]	0.55
L3B1M2	KM096820	2.17±0.05[a]	0.43
L3B21R1	KM096821	3.00±0.29[a]	0.60
L3B21R2	KM096822	9.16±1.72[b]	1.83
L3B21R7	KM096823	3.28±0.19[a]	0.66
L3B1K1	KM096824	3.43±0.75[a,b]	0.69
L3B21K4	KM096825	9.96±1.30[b]	1.99
L3C21M6	KM096826	7.12±0.76[b]	1.42
L3C1K8	KM096827	3.10±0.55[a]	0.62
Lactobacillus otakiensis			
L3C1R1	KM096828	4.31±0.78[a,b]	0.86
Lactobacillus plantarum			
L2B21R1b	KM103932	3.32±0.20[a]	0.66
L2C21E8	KM103933	17.94±0.13[c]	3.59
L2A21R1	KM103931	7.00±2.10[b]	1.40
L3A21R6	KM103934	2.24±0.09[a]	0.45
L3C1E8	KM079361	15.36±0.15[c]	3.07
Lactobacillus paraplantarum			
L2B21R5	KM079360	4.29±0.24[a,b]	0.86
Lactococcus lactis			
L3B1M7	KM079358	2.77±0.08[a]	0.55
L3A21M1	KF193424	5.24±0.75[a,b]	1.05
Lactococcus garvieae			
L3B1M8	KM079359	2.78±0.30[a]	0.56
Leuconostoc mesenteroides			
L2A21E7	KM079353	2.06±0.00[a]	0.00
L2B21E3	KM079354	2.52±0.07[a]	0.50
L3A21M4	KM079355	4.21±0.20[a,b]	0.84
Leuconostoc citreum			
L3C1E7	KM079357	2.97±0.07[a]	0.59
Enterococcus faecalis			
L2B21K3	KF193420	2.42±0.22[a]	0.48
L3A1M6	KF193421	2.16±0.07[a]	0.43
L3A21M3	KF193425	2.44±0.27[a]	0.49
L3A21M8	KF193426	2.20±0.07[a]	0.44
L3A21K6	KF193422	4.66±0.43[a,b]	0.93
L3A21K7	KF193423	3.38±0.52[a,b]	0.68
L3B1K3	KF193427	3.37±0.32[a,b]	0.67

* Values of CLA labelled with different letters are significantly different ($P < 0.05$).

Table 2- Probiotic characteristics of CLA producing strains. Resistance to low pH (2.5), bile salts (0.3%, w/v) and pancreatin (0.1%, w/v) and adhesion to Caco-2 and HT-29 cells. Results are presented as the average values ±SEM from four independent experiments for resistance to low pH, bile salts and pancreatin assays, and three independent experiments for adhesion assays.

Probiotic characteristics	Time (h)	Bacterial counts (log CFU/mL)*	
		Lb. plantarum L2C21E8	*Lb. plantarum* L3C1E8
Resistance to pH 2.5	0	$6.74\pm0.24^{a\,A}$	$8.84\pm0.23^{a\,B}$
	0.5	$ND^{b\,A}$	$7.95\pm0.72^{a,b\,B}$
	1	$ND^{b\,A}$	$5.74\pm0.0^{b\,B}$
	2	$ND^{b\,A}$	$2.25\pm0.14^{c\,B}$
	3	ND^{b}	ND^{c}
		Lb. plantarum L2C21E8	*Lb. plantarum* L3C1E8
Resistance to bile salts (0.3%. w/v) and pancreatin (0.1%.w/v)	0	6.04 ± 0.31^{A}	7.39 ± 0.26^{B}
	0.5	5.86 ± 0.01^{A}	6.89 ± 0.25^{B}
	1	5.98 ± 0.03^{A}	7.38 ± 0.19^{B}
	2	6.25 ± 0.29^{A}	7.42 ± 0.23^{B}
	3	5.88 ± 0.07^{A}	7.35 ± 0.22^{B}
		Lb. plantarum L2C21E8	*Lb. plantarum* L3C1E8
Adhesion to cells	Caco-2	7.36 ± 0.07^{a}	7.66 ± 0.21^{a}
	HT-29	5.77 ± 0.58^{b}	5.73 ± 0.12^{b}

* ND: Counts of bacteria below detection limits.

Different lowercase letters indicate significant differences ($P < 0.05$) within columns (among time or cells, Caco-2 and HT-29), according to Bonferroni post hoc means comparison test.

Different uppercase letters indicate significant differences ($P < 0.05$) within rows (among strains), according to Bonferroni post hoc means comparison test.

Fig.2

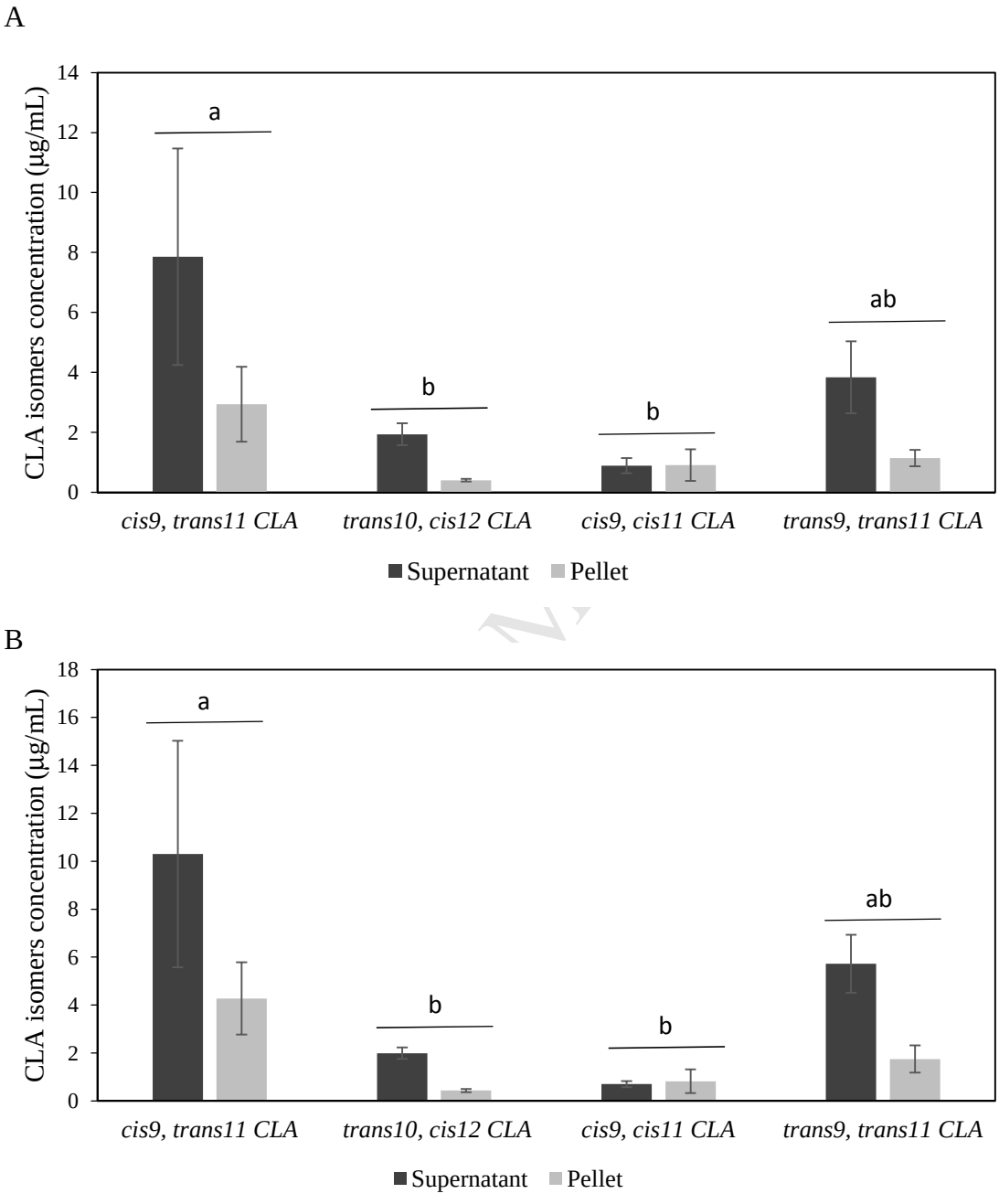

A
CLA isomers concentration (µg/mL)
14
12
10
8
6
4
2
0
a
b
b
ab
cis9, trans11 CLA
trans10, cis12 CLA
cis9, cis11 CLA
trans9, trans11 CLA
Supernatant
Pellet
B
CLA isomers concentration (µg/mL)
18
16
14
12
10
8
6
4
2
0
a
b
b
ab
cis9, trans11 CLA
trans10, cis12 CLA
cis9, cis11 CLA
trans9, trans11 CLA
Supernatant
Pellet

Fig.1

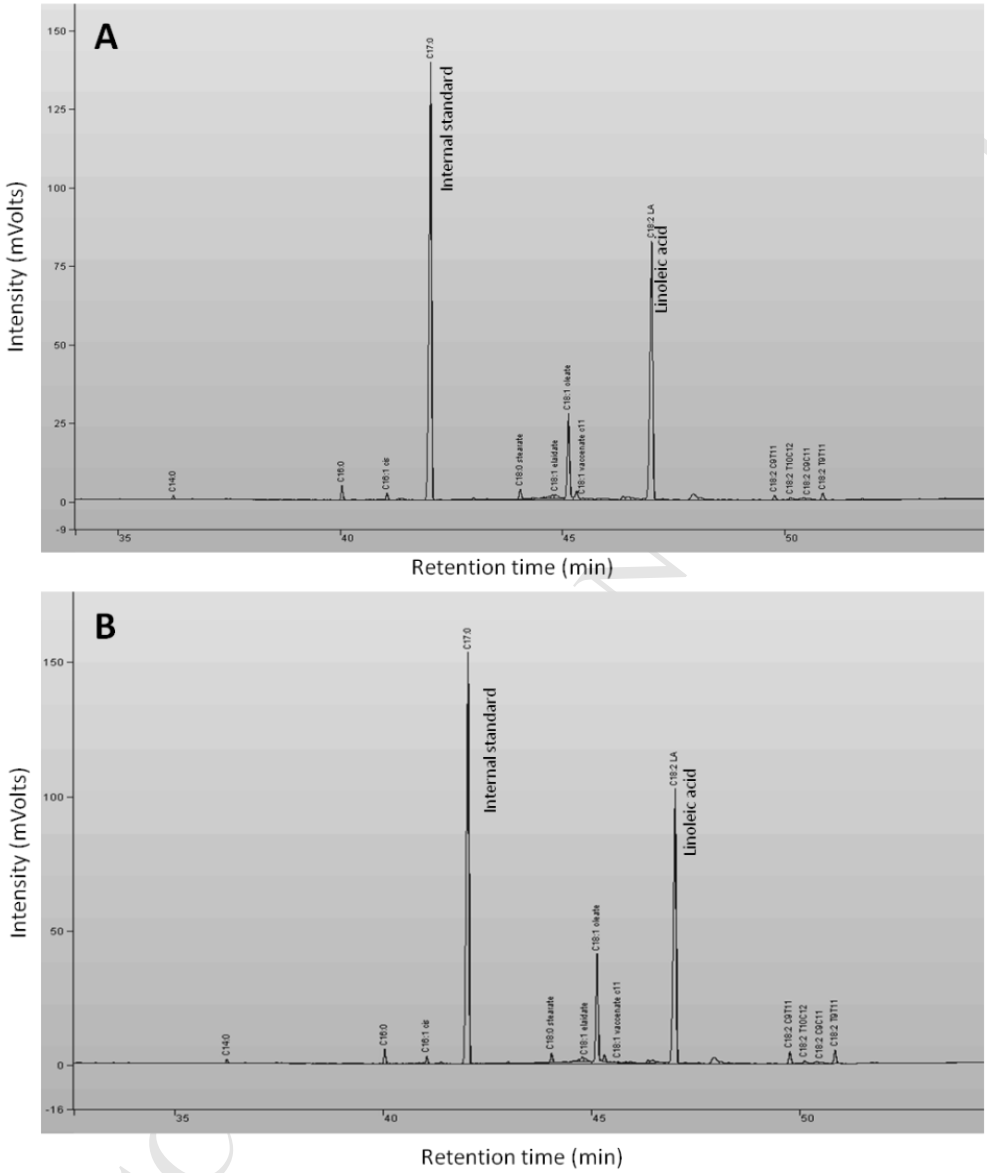

A
Internal standard
Linoleic acid
Intensity (mVolts)
Retention time (min)
B
Internal standard
Linoleic acid
Intensity (mVolts)
Retention time (min)

Fig.3

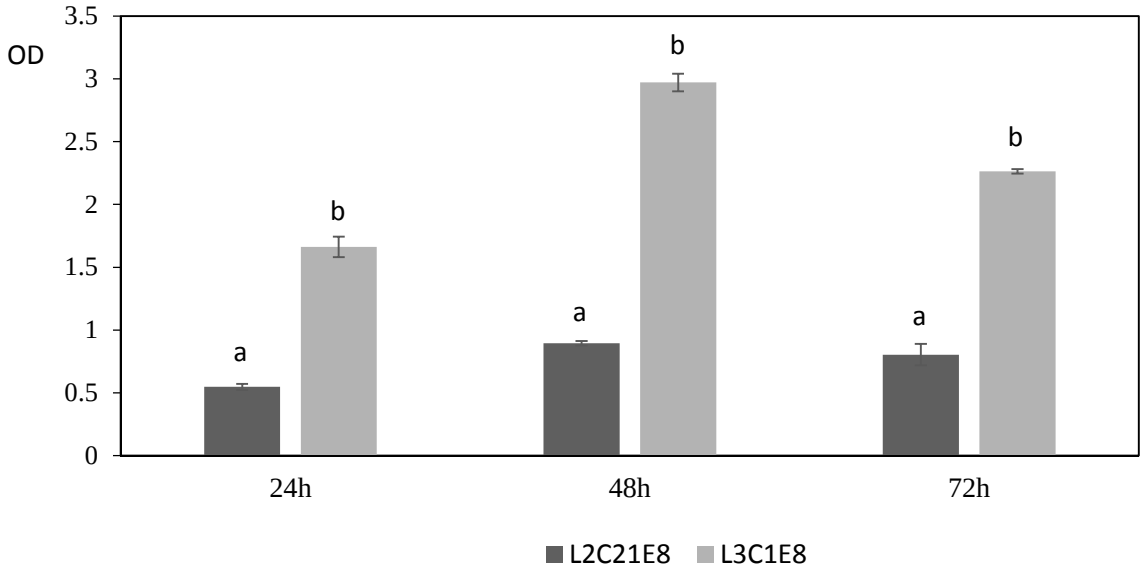


Fig.4

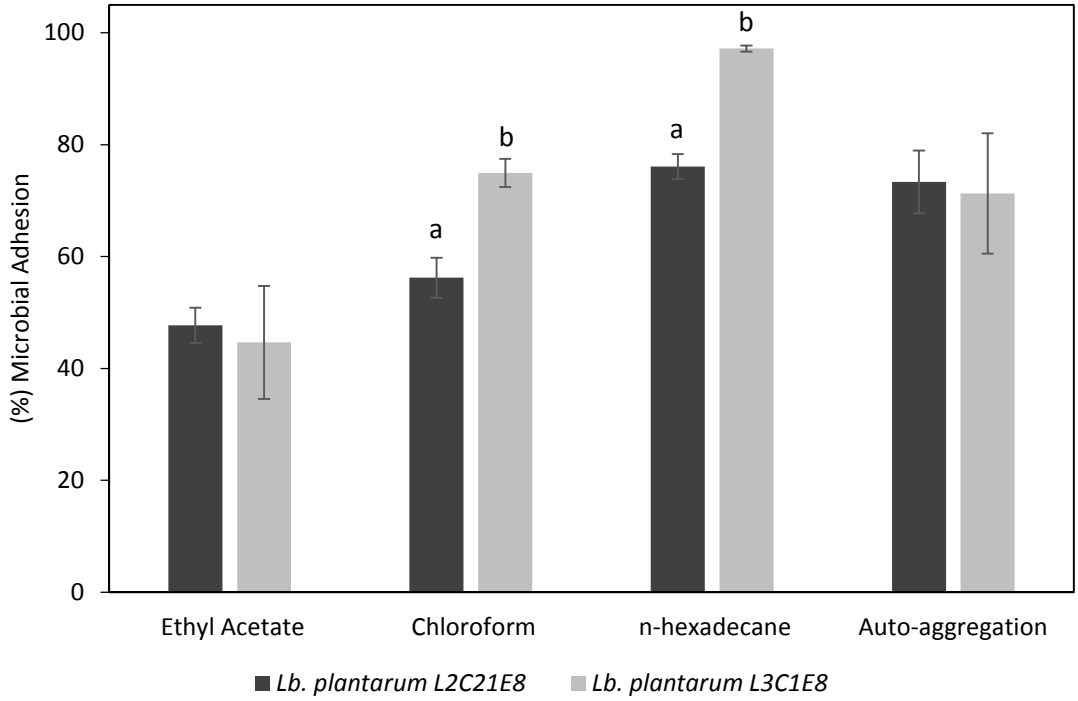
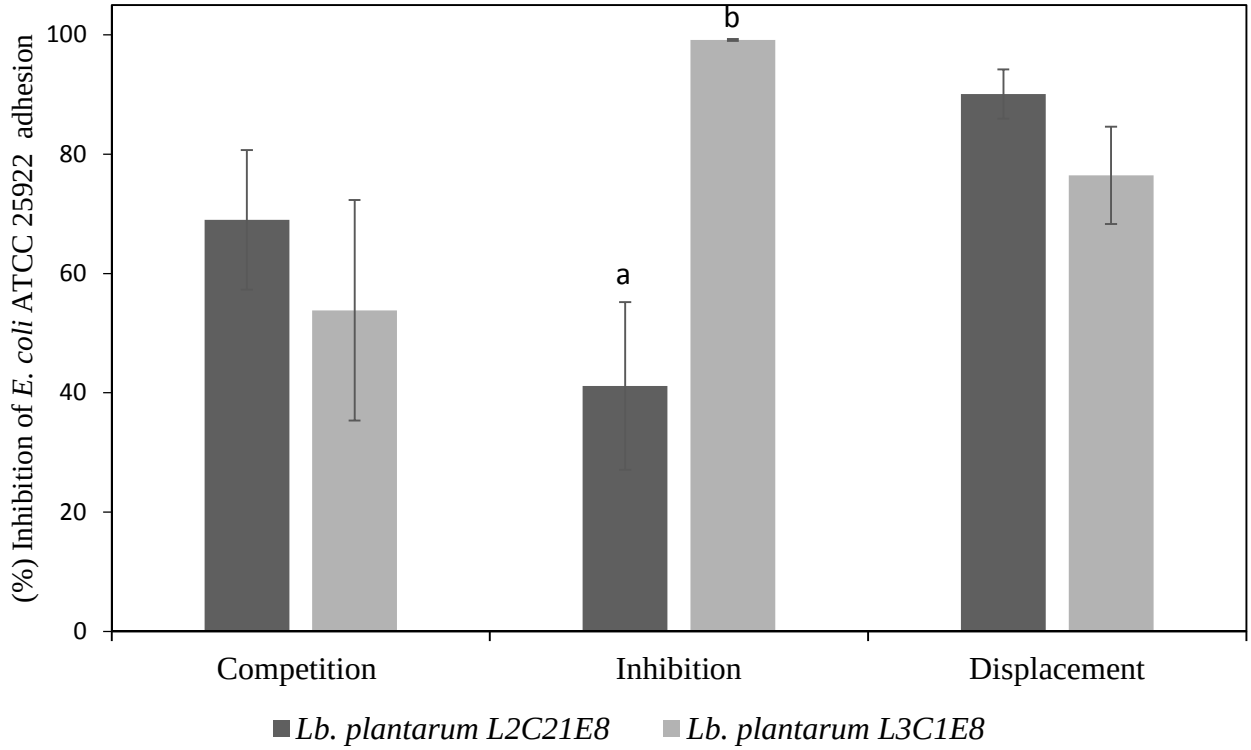


Fig.5



Highlights

- LAB strains isolated from cheese were screened for *in vitro* CLA production.
- Two *Lactobacillus plantarum* strains were selected as high CLA producers.
- Strains produced a high proportion (25%) of the uncommon *trans*-9, *trans*-11 CLA.
- CLA producer strains were evaluated for probiotic characteristics.
- CLA producers also inhibited of *E. coli* adhesion to human cells.