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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%).

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 (Fuke & Nornberg, 2017; Lin & Lee, 1997), 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 et al., 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

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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, Stanton, Ross, Dapkevicius, & Silva, 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, Ross, Fitzgerald, and Stanton (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 through a 0.45 μm filter (Minisart, Sigma-Aldrich) and stored in the dark at −20 °C. The strains were incubated aerobically at 30 °C.

After 48 h incubation, the LAB cultures were centrifuged at 5000 × *g* for 10 min at room temperature. The fat was extracted from the culture supernatant fluid as follows: An internal standard, C17:0 heptadecanoic acid (99% pure; Sigma-Aldrich), was added to 5 mL of the supernatant fluid to give a final concentration of 0.75 g internal standard per sample. Then, 5 mL of isopropanol was added to the supernatant fluid, and the samples were vortexed for 30 s. Five milliliter of *n*-hexane was added to this mixture, vortexed and centrifuged at 3260 × *g* for 5 min. The resultant hexane layer (containing lipids) was dried under a stream of nitrogen. For bacterial pellet extraction, the pellet from 10 mL of bacterial culture was washed in 2 mL saline solution (0.137 mol/L NaCl, 7.0 mmol/L K_2HPO_4 and 2.5 mmol/L KH_2PO_4). The cells were vortexed and centrifuged at 3260 × *g* for 10 min, and the washing step repeated twice. The cells were suspended in 1 mL saline solution and then the samples were extracted completely as described above for the bacterial supernatant fluid. Fat was extracted from supernatant and pellet, independently. The lipids were stored at −20 °C prior to preparation of fatty acid methyl esters for GC analysis. Samples were analyzed in triplicate.

2.3.2. Preparation of fatty acid methyl esters

The extracted lipids were analyzed by gas chromatography following methylation with NaOH-BF_3 in methanol as described by Yang et al. (2014). *Tert*-butyl methyl ether (MTBE, Sigma-Aldrich) (0.5 mL) was added to samples prepared above, together with 10 mL of NaOH (0.5 M) in methanol, and the mixture was vortexed for approx. 30 s and incubated for 12 min at 90 °C. Then, 10 mL of BF_3 in methanol (Sigma-Aldrich) 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 1 h. 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 Ibarreche, Castellano amd Vignolo (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,