Food Microbiology 69 (2018) 212–218

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Impact of growth temperature on exopolysaccharide production and probiotic properties of *Lactobacillus paracasei* strains isolated from kefir grains

Ana A. Bengoa [a], M. Goretti Llamas [b], Carolina Iraporda [a], M. Teresa Dueñas [b], Analía G. Abraham [a, c], Graciela L. Garrote [a, *]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, UNLP-CONICET-CIC.PBA), Calle 47 y 116, La Plata 1900, Argentina
[b] Dpto. de Química Aplicada, Facultad de Ciencias Químicas, Universidad del País, Vasco (UPV/EHU), Paseo Manuel de Lardizabal 3, 20018 San Sebastián, País Vasco, Spain
[c] Área Bioquímica y Control de Alimentos, Facultad de Ciencias Exactas, UNLP, Calle 47y 115, La Plata 1900, Argentina

ARTICLE INFO

Article history:
Received 30 March 2017
Received in revised form
15 August 2017
Accepted 18 August 2017
Available online 19 August 2017

Keywords:
Lactobacillus paracasei
Exopolysaccharide
Kefir
Probiotics
Growth temperature

ABSTRACT

EPS-producing LAB are widely used in the dairy industry since these polymers improve the viscosity and texture of the products. Besides, EPS might be responsible for several health benefits attributed to probiotic strains. However, growth conditions (culture media, temperature, pH) could modify EPS production affecting both technological and probiotic properties. In this work, the influence of growth temperature on EPS production was evaluated, as well as the consequences of these changes in the probiotic properties of the strains. All *Lactobacillus paracasei* strains used in the study showed changes in EPS production caused by growth temperature, evidenced by the appearance of a high molecular weight fraction and an increment in the total amount of produced EPS at lower temperature. Nevertheless, these changes do not affect the probiotic properties of the strains; *L. paracasei* strains grown at 20 °C, 30 °C and 37 °C were able to survive in simulated gastrointestinal conditions, to adhere to Caco-2 cells after that treatment and to modulate the epithelial innate immune response. The results suggest that selected *L. paracasei* strains are new probiotic candidates that can be used in a wide range of functional foods in which temperature could be used as a tool to improve the technological properties of the product.

1. Introduction

The consumption of some lactic acid bacteria (LAB), in particular from the genera *Lactobacillus*, has proven to be beneficial for human health and as a consequence those strains are usually considered as probiotics that are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO-WHO, 2006). Certain LAB are able to produce extracellular polysaccharides (EPS) that can be either tightly associated with the cell surface forming a capsule, or loosely attached to the outer cell structures or secreted into the environment (Ruas-Madiedo et al., 2008). The major role of EPS is to protect the cells in the environment against toxic metals, host innate immune factors, phage attack and desiccation (Ryan et al., 2015; Zannini et al., 2016). Furthermore, EPS layer is thought to be involved in the protection against adverse environmental conditions of gastrointestinal tract (GIT) including low pH, bile salts, gastric and pancreatic enzymes (Ryan et al., 2015). Additionally, it has been suggested that EPS might play a role in bacterial aggregation, biofilm formation and interaction with intestinal epithelial cells (IEC) (Dertli et al., 2015; Živkovićet al., 2016); the last being of great importance to improve the persistence of the microorganism in the human gut and their interaction with gastrointestinal immune system and gut microbiota (Pennacchia et al., 2006). Probiotics, which are usually administered orally, must be able to survive the gastrointestinal conditions in order to reach and colonize the human gut to exert their health benefits (Amund, 2016) and the presence of EPS layer around the bacteria could imply an advantage for that purpose. In addition, LAB's EPS might be responsible for several health benefits attributed to probiotic strains. A remarkable feature that certain EPS present is their capacity to modulate the host's immune response either stimulating it in order to improve

* Corresponding author.
E-mail address: ggarrote@biol.unlp.edu.ar (G.L. Garrote).

http://dx.doi.org/10.1016/j.fm.2017.08.012
0740-0020/

Abbreviations

CCL20	Chemokine-ligand-20
DMEM	Dulbecco's Modified Eagle's Minimum Essential Medium
EPS	Exopolysaccharide
FliC	Flagellin
HMW	High molecular weight
IEC	Intestinal epithelial cells
LAB	Lactic acid bacteria
Mw	Molecular weight
PBS	Phosphate buffered saline
TEM	Transmission electron microscopy

the barrier against pathogens (Balzaretti et al., 2016; Patten et al., 2014) or suppressing the response in inflammatory disorders (Bleau et al., 2010; Nikolic et al., 2012). Among the beneficial effects attributed to these biopolymers it can also be mentioned antitumor activities, cholesterol lowering capability, antihypertensive activities, epithelium protection from intestinal pathogenic microorganisms and fecal microbiota modulation (Hamet et al., 2016; Patten and Laws, 2015; Ruas-Madiedo et al., 2008).

Moreover, EPS producing-LAB are widely used in the dairy food industry since these biomolecules are able to improve the rheological properties, texture and taste (mouth feel) of fermented milks, yoghurts and cheeses (Ryan et al., 2015). Thus, these beneficial EPS are usually included in the food matrix either as additives or by *in situ* production (Caggianiello et al., 2016) for the development of functional foods with improved technological properties (Torino et al., 2015; Zannini et al., 2016).

The yield, composition and structure of the EPS that is synthesized *in situ* by the bacteria might be significantly influenced by culture conditions including temperature, medium composition and incubation time (Sanchez et al., 2006; Vera Pingitore et al., 2016; Xu et al., 2010). Although these changes in EPS production could lead to modifications in the physico-chemical properties of the fermented product, the impact that it may have on the probiotic properties of bacteria has not been studied in detail yet.

Lactobacillus paracasei CIDCA 8339, CIDCA 83123 and CIDCA 83124 were isolated from kefir grains and have been previously described as EPS-producing strains (Hamet et al., 2015) with probiotic potential (Zavala et al., 2016). Considering that growth temperature is one of the factors that could modify the production of EPS, the aim of this work was to evaluate the effect of growth temperature on the EPS produced by *L. paracasei* strains in MRS medium, and study the possible consequences of those changes on their probiotic properties.

2. Materials and methods

2.1. Bacterial strains and culture conditions

EPS-producing *Lactobacillus paracasei* strains CIDCA 8339, CIDCA 83123 and CIDCA 83124 isolated from kefir grains were stored at −80 °C in sterile skim milk and reactivated by incubation in MRS broth at 30 °C for 24 h. Then, the strains were grown in MRS broth or agar (Difco Laboratories, Detroit, MI, USA) at 20 °C (48 h), 30 °C (24 h) and 37 °C (24 h) under aerobic conditions (Hamet et al., 2013).

2.2. EPS extraction, quantification and molecular weight determination

EPS production and extraction were assessed according to Ruas-Madiedo et al. (2010). In brief, 150 μl of 24 h cultures were spread on MRS agar plates and incubated for 7 days at 20 °C, 30 °C or 37 °C. For EPS isolation, 2.5 ml of ultrapure water were added to each plate to collect cell biomass. After the addition of 1 volume of NaOH 2 M, the cell suspension was stirred overnight at 25 °C and then centrifuged (5200×*g*, 30 min, at 20 °C) to remove bacteria. The supernatant was collected and 2 vol of cold ethanol were added for EPS precipitation (16 h, at −20 °C) and then centrifuged at 5200×*g* for 30 min at 4 °C. Finally, EPS pellets were dissolved in hot distilled water and lyophilized in an Heto FD4 equipment (Lab Equipment, Denmark). The EPS concentration was determined by anthrone method involving a measurement of absorbance at 620 nm using glucose solutions as standards. The absence of other sugars was determined by thin-layer chromatography (Rimada and Abraham, 2003). The average molecular weight (Mw) of the polysaccharides was determined by high-performance size exclusion chromatography (HPLC-SEC, Agilent 1100 Series System,Hewlett-Packard, Germany) associated to a refractive index (RI) detection system (Ibarburu et al., 2015). Dextran of Mw range from 10^3 to 2×10^6 Da (Sigma-Aldrich) was used as standard.

2.3. Tolerance to simulated gastrointestinal conditions

L. paracasei strains grown at different temperatures in MRS were harvested and resuspended in a simulated gastric juice (NaCl 125 mM, KCl 7 mM, $NaHCO_3$ 45 mM, pepsin 3 g/l, pH adjusted to 2.5) at OD_{590} 0.5 (~10^8 CFU/ml). The suspensions were incubated at 37 °C for 1.5 h and washed twice with PBS buffer pH = 7. Bacteria were then resuspended in simulated intestinal fluid (NaCl 22 mM, KCl 3.2 mM, $NaHCO_3$ 7.6 mM, pancreatin 0.1% w/v, bovine bile salts 0.15% w/v, final pH adjusted to 8.0) and incubated at 37 °C for 3 h. After treatment, samples were diluted in 0.1% p/v tryptone and plated on MRS agar to determine bacterial viability (Grimoud et al., 2010). The tolerance of the strains to simulated gastrointestinal conditions was determined by comparison with number of viable cells washed with PBS and incubated in gastric and intestinal solutions for the same interval and temperature.

2.4. Evaluation of bacterial-surface EPS by transmission electronic microscopy (TEM) and alcian blue staining

The presence of surface EPS in 24 h cultures (30 °C) was evaluated before (unwashed) and after one or three successive washes with PBS. The samples were analysed by optic microscopy and TEM. Alcian blue staining of EPS was performed according to Novelli (1953). The samples were stained for 2.5 min with alcian blue 1% alcoholic solution 10 times diluted in water, rinsed with water and dried. Next, a diluted safranin solution (0.1%) was added and stained for 1 min. TEM analysis was assessed according to Lee et al. (2016). Briefly, 5 μl of the sample was added on an electron microscopy copper grid (400 mesh) for 5 min. Subsequently, a negative stain with 2% phosphotungstic acid for 20 s was carried out and then analysed under a transmission electron microscope JEM 1200 EX II (JEOL Ltd., Tokio, Japón) stabilized at 100 kV.

2.5. Bacterial adhesion to intestinal epithelial cells

Caco-2/TC-7 cells that model the mature enterocytes of the large intestine were routinely grown following the procedure described by Zavala et al. (2016). After simulated gastrointestinal treatment, bacteria were washed twice with PBS and suspended in serum-free