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

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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

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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

DMEM (GIBCO BRL Life Technologies Rockville, USA) at $OD_{590} \cong 1.0$. For adhesion assay, 500 μl of suspensions were added to Caco-2/TC-7 monolayers and incubated for 1 h at 37 °C in a 5% CO_2 − 95% air atmosphere. Then, the monolayers were washed three times with PBS and lysed by adding 500 μl of sterile distilled water. The number of bacteria adhered to Caco-2/TC-7 cell was assembled by colony counting in MRS agar (Zavala et al., 2016).

2.6. Immunomodulation assay with Caco-2-ccl20:luc reporter system

Caco-2 cells stably transfected with a luciferase reporter construction under the control of the chemokine-ligand-20 (CCL20) promoter (Caco-2 ccl20:luc) have been previously described (Nempont et al., 2008). Flagellin (FliC), obtained from *Salmonella enterica* serovar Enteritidis (Sierro et al., 2001) was used to induce the proinflammatory response.

Confluent Caco-2 ccl20:luc cells were pre-incubated 1 h with bacterial suspensions ($OD_{590} \cong 0.25$) in serum-free DMEM, stimulated with FliC 1 μg/ml and incubated for 6 h at 37 °C (5% CO_2 - 95% air). A basal condition without any treatment was included as a control lacking stimulation and a stimulated control was added to define a 100% induction of the proinflammatory response. Luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, USA) using a luciferase assay system (Promega, Madison WI, USA). Luminescence was normalized to the stimulated control cells and expressed as a percentage of the normalized average luminescence (% NAL) ± standard deviation (SD) (Iraporda et al., 2014).

2.7. Statistical analysis

One way analysis of variance (ANOVA) was performed. Differences were statistically tested using Tukey's multiple comparison test ($p < 0.05$) conducted by the GraphPad Prism® software.

3. Results and discussion

3.1. Fermentation temperature induces changes in EPS profiles

The three EPS-producing *L. paracasei* strains were plated on MRS agar and incubated at different temperatures in order to evaluate morphological changes in the colony. Fig. 1 shows *L. paracasei* CIDCA 83124 grown on MRS agar at 20 °C, 30 °C and 37 °C. Colonies with a non-ropy appearance were observed when the strain was grown at 37 °C whereas at 20 °C and 30 °C a ropy phenotype of colonies was manifested. *L. paracasei* CIDCA 83123 showed the same behavior as CIDCA 83124. On the other hand, colonies of strain *L. paracasei* CIDCA 8339 did not expressed ropy phenotype at any of tested temperatures. Morona et al. (2003) have previously evidenced that changes in the produced polysaccharide can modify the colony morphology, suggesting that qualitative and/or quantitative modifications in the EPS synthesized by *L. paracasei* CIDCA 83123 and 83124 at different temperatures could be responsible for the changes observed.

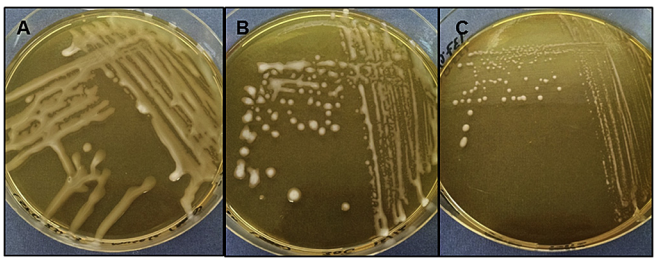

Fig. 1. *L. paracasei* CIDCA 83124 grown on MRS agar at 20 °C (**A**), 30 °C (**B**) and 37 °C (**C**).

Subsequently, EPS produced by all three strains at different temperatures on MRS agar were isolated, quantified and analyzed. The results obtained demonstrated that growth temperature affects the amount of produced EPS. In general, the three strains produce more EPS at lower temperatures. *L. paracasei* CIDCA 8339 synthesized an amount of about 1200 μg/1 × 10^8 CFU at 20 °C and 30 °C whereas it produced two times less of EPS when it was grown at 37 °C (630μg/1 × 10^8 CFU). The amount of EPS produced by *L. paracasei* CIDCA 83124 was 1170, 527 and 373 μg/1 × 10^8 CFU at 20 °C, 30 °C and 37 °C respectively. *L. paracasei* CIDCA 83123 showed a similar behavior to CIDCA 83124 but with lower EPS production being 390, 309 and 270 μg/1 × 10^8 CFU at 20, 30 and 37 °C, respectively.

The increase of EPS production at low temperature seems to be a common feature for mesophilic EPS-producing LAB (Degeest et al., 2001). When bacteria are growing under suboptimal conditions a much slower cell wall polymers' biosynthesis is exerted and consequently more isoprenoid lipid carrier precursor molecules are available for the EPS biosynthesis (Sutherland, 1972). Otherwise, the increment in polysaccharide production could also be a result of increased activities of enzymes involved in the synthesis of precursors (Kumar et al., 2007). On the contrary, Dupont et al. (2000) have reported no significant difference in the quantity of EPS produced by commercial *L. paracasei* and *L. rhamnosus* strains at 32 and 37 °C.

With regard to Mw of EPS, an influence of growth temperature was also evidenced for the three strains in a similar way (Fig. 2). At 37 °C, crude EPS was composed by only one fraction of about 0.7–2x10^4 Da, but at 20 °C, two fractions of EPS of 5–7x10^4 Da and 2.6–3x10^3 Da were synthesized. Besides, *L. paracasei* CIDCA 8339 synthesized both fractions also at 30 °C. The presence of a larger fraction at 20 °C suggests that polymerization is enhanced at this temperature. These results correlate well with the increase in the colony ropiness observed in the case of *L. paracasei* CIDCA 83123 and CIDCA 83124. On the other hand, changes in the EPS characteristics of CIDCA 8339 were not expected since no changes in the colony aspect were observed. This indicates that the differences in the EPS characteristics produced by a strain are not always reflected in the colony aspect.

In agreement with our results, several works show that low temperatures significantly increase EPS production (Sanchez et al., 2006; Vera Pingitore et al., 2016; Xu et al., 2010). Sanchez et al. (2006) proved that *L. pentosus* LPS26, a strain that produce a high

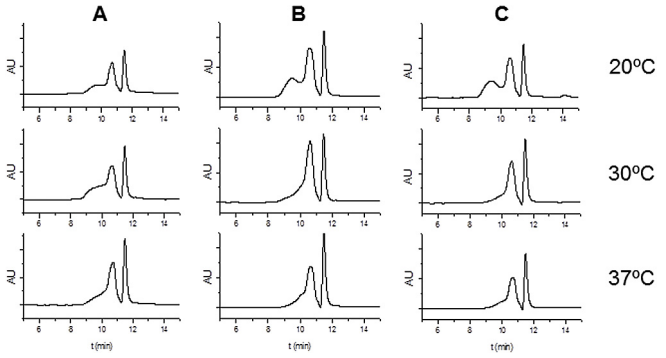

Fig. 2. High-performance size exclusion chromatograms of EPS isolated from MRS agar synthesized by *L. paracasei* CIDCA 8339 (**A**), CIDCA 83123 (**B**) and CIDCA 83124 (**C**) at 20 °C, 30 °C and 37 °C. AU: arbitrary units.

(1.9×10^6 Da) and a low Mw (3.3×10^4 Da) EPS, was influenced by growth temperature. They reported an increase in the HMW EPS at 20 °C and, as a consequence, a total polymer increment respect to 25 and 30 °C. Moreover, *L. paracasei* HTC produced the highest quantity of EPS at 27 °C whereas at 32 °C, 37 °C and 42 °C the EPS yield was significantly lower (Xu et al., 2010).

3.2. Surface associated EPS of L paracasei has a protective role against adverse gastrointestinal conditions

After ingestion, probiotics must deal with the harsh conditions of the gastrointestinal tract, including the acidic environment of the stomach as well as the presence of digestive enzymes and bile salts in the small intestine in order to reach the colon alive where health benefits are exerted (Papadimitriou et al., 2015).

As shown in Fig. 3A, *L. paracasei* strains grown at 20 °C, 30 °C or 37 °C, demonstrated good tolerance to simulated gastrointestinal conditions, since no significant reduction on the number of viable bacteria was observed after treatment. These results evidenced that strains possess ability to tolerate gastrointestinal tract conditions independently of molecular weight of EPS. When bacteria were washed with PBS immediately before the treatment they showed a higher susceptibility to the adverse conditions of acid and bile, evidenced by the 3–4 log reduction in the number of viable bacteria (Fig. 3B).

EPS is lightly attached to the cell wall so washing the strains could conduct to the partial removal of EPS layer, as it was previously described by Fernández de Palencia et al. (2009). EPS's alcian blue staining of *L paracasei* without washing or after one or three washes is shown in Fig. 4A, B and C. In this case, it can be appreciated the partial loss of the blue layer that surround the bacteria after one wash with PBS indicating that one wash is sufficient to remove a huge amount of EPS. In addition, three times washed lactobacilli cells have completely lost the EPS layer that wraps the

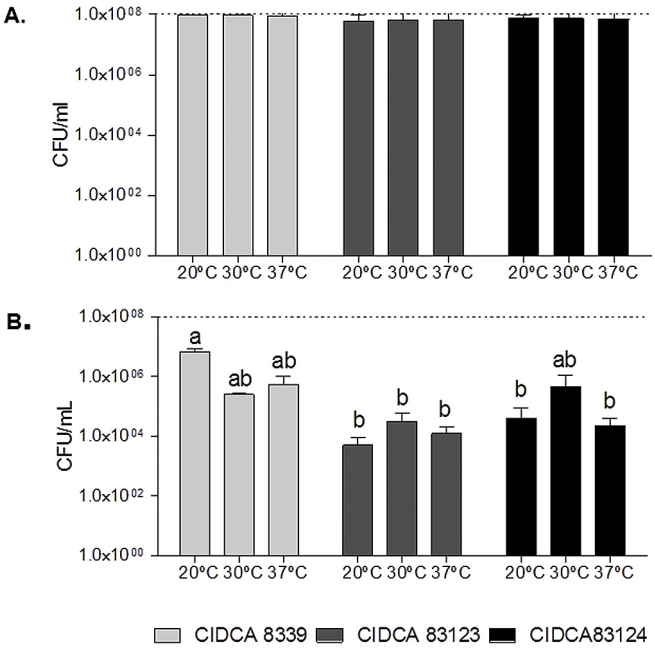

Fig. 3. Viability of *L. paracasei* strains grown at 20 °C, 30 °C and 37 °C after simulated gastrointestinal (GI) treatment. **A**: strains subjected to GI conditions without previously washing. **B**: strains washed with PBS and subsequently subjected to GI conditions. Results are expressed as mean ± SD of two independent experiments. Different letters indicate statistical differences among the data ($p < 0.05$; Tukey's multiple comparison test).

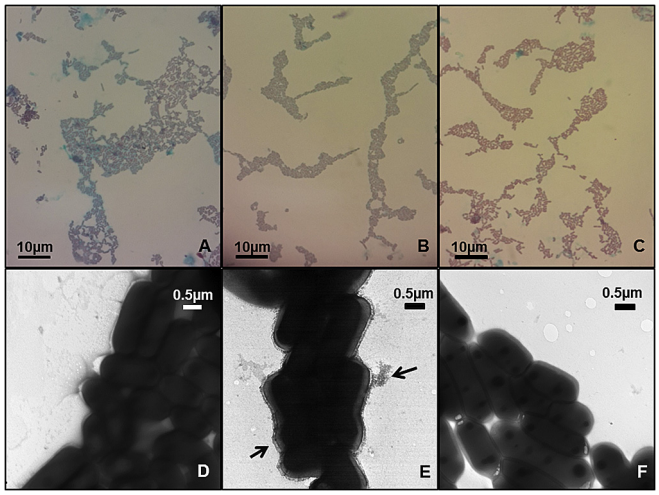

Fig. 4. Microscopic evaluation of the presence of surface exopolysaccharide of *L. paracasei* CIDCA 8339 before and after one or three successive washes by alcian blue staining (**A, B, C**) and TEM (**E, F,G**).

bacteria. Fig. 4D, E and F showed *L. paracasei* CIDCA 8339 cells analysed by TEM without washing and after one and three washes, respectively. The image evidence that three times washed lactobacilli cells possess significantly lower amount of EPS on their surface confirming alcian blue staining observations. The same behavior was obtained with *L. paracasei* CIDCA 83123 and CIDCA 83124 (data not shown). These results suggest that the loss of EPS layer could be responsible for the differences observed in gastrointestinal tolerance between washed and unwashed lactobacilli, supporting the protective role of EPS against stress conditions.

The literature indicates that LAB's tolerance to gastrointestinal conditions is strain-dependent (Papadimitriou et al., 2015). Viability of *L. paracasei* and *L. casei* strains in acidic conditions and bile has been previously studied; *L. casei* LC71 isolated from human stomach (Delgado et al., 2015) and *L. paracasei* LPC-S01 (Balzaretti et al., 2015) showed a higher tolerance to acidic conditions than *L. casei* Shirota, a strain with demonstrated tolerance to human gastrointestinal conditions (Tuohy et al., 2007). *L. paracasei* CIDCA 8339, CIDCA 83123 and CIDCA 83124 strains were able to survive treatment with a gastric juice with pepsin at pH = 2.5, followed by a 3 h exposure to an intestinal juice composed of pancreatin and bile salts that is in accordance to results obtained by Ren et al. (2014). They demonstrated that an EPS-producing *L. casei* subsp. *casei* strain isolated from yoghurt showed a good tolerance to acidic conditions with no significant changes in the number of viable bacteria at pH 2 and 3. Besides, this strain showed a high resistance to 1% bile salts. Stack et al. (2010) demonstrated that the β-glucan-producing *L. paracasei* NFBC 338 showed a significantly better tolerance than its non-EPS producing parental strain to both gastric juice and bile salts, evidencing a protective role of EPS. On the contrary, *L. plantarum* SF2A35B and Lp90 (Lee et al., 2016) and *P. parvulus* 2.6 (Fernández de Palencia et al., 2009) showed no differences in the tolerance to simulated gastrointestinal conditions when they were compared to their EPS-reduced derivatives. Nikolic et al. (2012) characterized three different non-EPS producing *L. paraplantarum* strains and observed that the percentage of survival of these strains after the gastrointestinal treatment was similar to the one obtained for EPS-producing *L. paraplantarum* strain, indicating that EPS layer did not confer any additional protection. The differences observed for EPS protective function indicate that the role of EPS layer in the protection against adverse conditions is strain-dependent and it is probably reliant on the

structure and composition of the polymer (Caggianiello et al., 2016).

Results obtained in the present work demonstrate that the partial loss of the EPS layer lead to an increase in bacterial susceptibility indicating the relevance of the EPS in the protection against acid and bile stress. This effect was similar at all temperatures assayed meaning that it is independent of the EPS amount and structure but it is important to keep the EPS loosely bound to the cells.

3.3. Fermentation temperature does not affect L. paracasei adhesion to intestinal epithelial cell nor the downregulation of epithelial innate immune response

Adherence to epithelial cells is a desirable probiotic trait, as it facilitates colonization of the human gut preventing elimination of probiotics by peristalsis and providing a competitive antagonism against pathogens (Kos et al., 2003; Papadimitriou et al., 2015).

Considering that the presence of EPS and its characteristics, such as structure and composition, could influence the ability of bacteria to adhere to the intestinal epithelium (Ruas-Madiedo et al., 2006), we proceed to evaluate the adhesion of *L. paracasei* strains grown at 20 °C, 30 °C and 37 °C to IEC after simulated gastrointestinal treatment (Fig. 5). The results indicate that the three EPS-producing *L. paracasei* strains had the ability to adhere to Caco-2/TC-7 cells. When 8 log were initially inoculated, *L. paracasei* CIDCA 8339 was able to adhere in an order of 5 log, significantly better than CIDCA 83123 and CIDCA 83124 (3–4 log). Interaction of the bacteria with epithelial cells was not affected by growth temperature despite the differences in the EPS production indicating no involvement of EPS in that interaction.

In contrast many authors have demonstrated that the presence of cell surface polysaccharide interfere with the adhesion of bacteria to intestinal epithelial cells (Mercan et al., 2015; Polak-Berecka et al., 2014; Ruas-Madiedo et al., 2006). Nikolic et al. (2012) compare the adhesion of EPS-producing strain *L. paraplantarum* BGCG11 with its non-producing derivative NB1. They observed a better binding to epithelial cells with the last one indicating that EPS layer might cover specific adhesion factors on the bacterial cell surface and/or electrostatically interfere with the binding to receptors of mucosal surface. As a consequence, the absence of exopolysaccharide allows a better exposure of cell surface proteins in bacteria improving the interaction with epithelial cells (Dertli et al., 2015). On the contrary, some studies proved that the presence of EPS improves the strain ability to adhere (Fernández de Palencia et al., 2009; Ren et al., 2014; Ruas-Madiedo et al., 2006). The differences found suggest that the effect of EPS on the ability to adhere to cell surface is variable and probably depends on differences in physicochemical and/or structural characteristics of the EPS polymers, as well as on the surface characteristics of the strains (Ruas-Madiedo et al., 2006). In this work we did not analyse the adhesive properties of EPS producing and non-producing strains but we studied the possible effects of qualitative and quantitative changes in EPS over lactobacilli adhesion capacity. Similarly, Živković et al. (2016) observed that *L. paracasei* susbp. *paracasei* BGSJ2-83, that produce an EPS composed of two fractions at 25 °C, had better capacity to adhere to Caco-2 cells than the mutant strain EPS7 that produce a polysaccharide composed of a HMW fraction only. In the present study, we observed that the differences in the structure of the EPS synthesized by the *L. paracasei* strains at different temperature do not modify the bacterial adhesion to IEC. The presence of a HMW fraction at low temperatures does not improve neither reduce their adhesion.

It has been suggested that immunomodulatory properties could be attributed to the interaction of bacterial polysaccharides with the host immune system (Caggianiello et al., 2016; Ryan et al., 2015). In order to find out the modulation of the innate immune response of the strains grown at different temperatures Caco-2-ccl20:luc cells were used. In this cells the ccl20 promoter is inducible upon pro-inflammatory stimuli such as TLR agonists, IL-1β or TNF- α, being an indicator of activation of innate response (Nempont et al., 2008), Caco-2-ccl20:luc cells were pre-incubated with *L. paracasei* strains and then stimulated with FliC. All three tested strains were able to modulate the intestinal epithelial innate response as they were able to reduce luciferase expression by 25–35% with respect to the stimulated control (Fig. 6). Besides, it was proved that growth temperature did not modify their capacity to downregulate the epithelial innate immune response. These results indicate that despite the changes in EPS described above, these strains are able to maintain their immunomodulatory properties. The finding of new probiotic strains with anti-inflammatory properties are of great interest since they can be used to prevent and/or alleviate the symptoms of inflammatory bowel diseases (Martín et al., 2013). The modulatory effect of lactic acid bacteria on intestinal epithelial innate response is strain dependent since differences among the same species have been reported (Romanin et al., 2010; Santos Rocha et al., 2012). In agreement with our results, Balzaretti et al. (2015) evaluated different *L. paracasei* strains that showed immunomodulatory properties with a significant decrease in the NF-kB-dependent production of bioluminescence. The component(s) responsible for LAB immune properties are also strain specific, being surface molecules the most frequently key

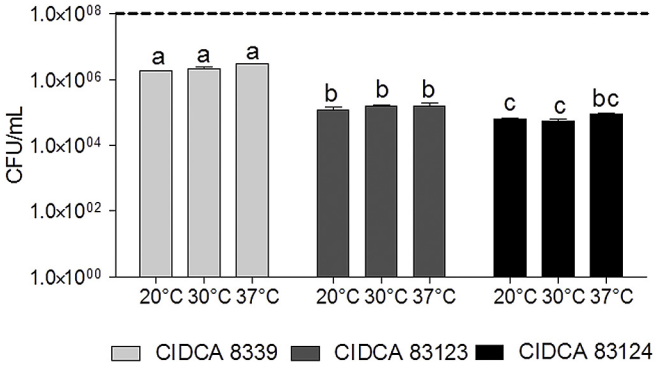

Fig. 5. Adhesion of *L. paracasei* strains grown at 20 °C, 30 °C and 37 °C to intestinal epithelial cells Caco-2/TC-7 after simulated gastrointestinal treatment. Results are expressed as mean ± SD of three independent experiments. Different letters indicate statistical differences among the data ($p < 0.05$; Tukey's multiple comparison test).

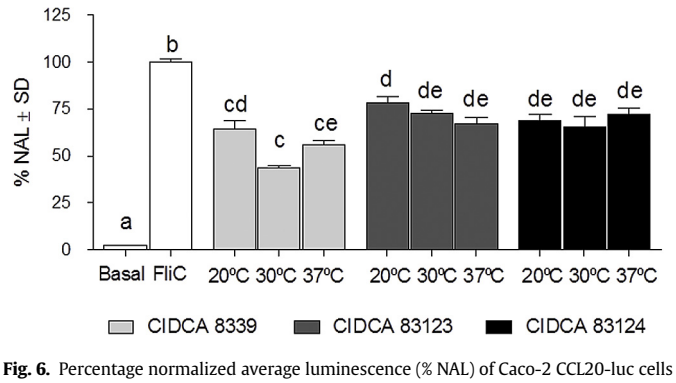

Fig. 6. Percentage normalized average luminescence (% NAL) of Caco-2 CCL20-luc cells preincubated 1 h with *L. paracasei* suspensions (OD_{590} 0.25). Bacteria were previously grown at 20 °C, 30 °C and 37 °C. Results are expressed as mean ± SD of at least three independent experiments. Different letters indicate statistical differences among the data ($p < 0.05$; Tukey's multiple comparison test).

components (Foligné et al., 2016; Taverniti and Guglielmetti, 2011). Foligné et al. (2016) demonstrated that growth medium affected the immunomodulatory properties of *Lactococcus lactis* ssp *lactis* CB460 and *Lactococcus lactis* ssp *cremoris* CB461 probably due to bacterial surface components modifications. On the contrary, we proved that modifying growth temperature, does not impact on the immunomodulatory properties of *L. paracasei* strains. It has been claimed that EPS can present immunostimulatory or immunosuppressive effects depending on their structure/molecular weight (Ryan et al., 2015). Nevertheless, we observed that the presence of a HMW fraction at low growth temperature does not modify immunomodulatory properties of the strains.

4. Conclusion

In the present study it was demonstrated that *L. paracasei* CIDCA 8339, CIDCA 83123 and CIDCA 83124 strains are able to produce EPS with different level of polymerization depending on growth temperature. These strains showed good probiotic properties such as tolerance to gastrointestinal conditions, adhesion to IEC and immunomodulatory capacity, which are not affected by growth temperature. Although the presence of the EPS loosely bound to the bacterial surface enhance its resistance to gastrointestinal stress, the differences in EPS polymerization do not affect the probiotic properties studied. The fact that growth temperature does not affect any of the probiotic properties studied represents an advantage for the food industry since it allows the employment of these EPS-producing strains for the development of a wide range of functional foods that require different manufacturing conditions enabling the use of fermentation temperature as an additional tool to improve the technological properties of the product.

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