Title: Capability of exopolysaccharide-producing *Lactobacillus paraplantarum* BGCG11 and its non-producing isogenic strain NB1, to counteract the effect of enteropathogens upon the epithelial cell line HT29-MTX

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Abstract

The putative protective role of the exopolysaccharide (EPS)-producing *Lactobacillus paraplantarum* BGCG11, and its non-EPS-producing isogenic strain NB1, was tested upon HT29-MTX monolayers challenged with seven opportunistic pathogens. The probiotic strain *Lactobacillus rhamnosus* LMG18243 (GG) was used as a reference bacterium. Tested lactobacilli were able to efficiently reduce the attachment to HT29-MTX of most pathogens. *Lb. paraplantarum* NB1 and *Lb. rhamnosus* GG were more efficient reducing the adhesion of *Clostridium difficile* or *Yersinia enterocolitica* than *Lb. paraplantarum* BGCG11, while strain BGCG11 reduced, to a greater extent, the adhesion of *Escherichia coli* and *Listeria monocytogenes*. The detachment and cell lysis of HT29-MTX monolayers in the presence of pathogens alone and co-incubated with lactobacilli or purified EPS was followed. *L. monocytogenes* induced the strongest cell detachment among the seven tested pathogens and this effect was prevented by addition of purified EPS-CG11. The results suggest that this EPS could be an effective macromolecule in protection of HT29-MTX cells from the pathogen-induced lysis. Regarding innate intestinal barrier, the presence of *C. difficile* induced the highest IL-8 production in HT29-MTX cells and this capability was reinforced by the co-incubation with *Lb. paraplantarum* NB1 and *Lb. rhamnosus* GG. However, the increase in IL-8 production was not noticed when *C. difficile* was co-incubated with EPS-producing *Lb. paraplantarum* BGCG11 strain or its purified EPS-CG11 polymer, thus indicating that the polymer could hinder the contact of bacteria with the intestinal epithelium. The measurement of mucus secreted by HT29-MTX and the expression of *muc1*, *muc2*, *muc3B* and *muc5AC* genes in the presence of pathogens and lactobacilli suggested that all lactobacilli strains are weak “co-adjuvants” helping some pathogens to slightly increase the secretion of mucus by HT29-MTX, while

purified EPS-CG11 did not induce mucus secretion. Taking altogether, *Lb. paraplantarum* BGCG11 could act towards the reinforcement of the innate mucosal barrier through the synthesis of a physical-protective EPS layer which could difficult the contact of the pathogens with the epithelial cells.

Keywords: exopolysaccharide, *Lactobacillus*, pathogens, HT29-MTX, mucin, IL-8

1. Introduction

Lactic acid bacteria (LAB) have been traditionally used in foods to preserve raw materials and to control fermentative processes for obtaining safe products with reproducible organoleptic characteristics. Selection of the appropriate starter or adjunct cultures to keep the "brand identity" of any fermented product is still of pivotal relevance. However consumers demand healthier products beyond their nutritional properties; thus, nowadays microbial cultures are also selected based on their functional traits (Leroy, & De Vuyst. 2004). In this context the development of functional foods containing beneficial microbes, known as probiotics, is a trending topic in the industrial food sector, as well as in the food science and technology research areas (Vasiljevic, & Shah, 2008). Probiotics are often defined as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (WHO/FAO, 2006; Hill et al., 2014). Probiotic bacteria most commonly used for human consumption belong to genera *Lactobacillus* and *Bifidobacterium* and milk products are the most common vehicles used for their delivery (Prasanna, Grandison, & Charalampopoulos, 2014). It is worth noting that a rational selection of the best probiotic candidate(s) for a target human population(s) is highly recommended in order to obtain functional foods supporting health claims (Arboleya et al. 2012). Then these probiotics must be considered at strain level and only certain ones showing beneficial attributes should be consider for further applications. In this regard, strains having particular surface molecules that allow their interaction with the host are good candidates to be explored for probiotic formulations.

Exopolysaccharides (EPS) are carbohydrate polymers forming the external envelope of many bacteria which, apart from the ecological role for the producing bacteria, have technological, medical and industrial applications (Rehm, 2010). EPS-

77 producing LAB are currently used in the manufacture of dairy fermentations due to their
78 capability to improve the viscosity and texture of these products. Recently EPS from
79 probiotics are receiving renewed interest due to their functional properties, being related
80 in some cases with the health promoting activities of the producing bacteria. Several
81 studies *in vitro* demonstrate the biological activities of EPS such as, among others: i) the
82 capability to modulate the host immune response and the dynamic of the intestinal
83 microbiota, ii) to antagonize against pathogens and iii) to act as physical barrier against
84 toxic compounds, (Hidalgo-Cantabrana et al., 2014b). In addition, there are *in vivo*
85 evidences using different animal models proving some of these beneficial properties. As
86 an example in the context of this article, a single oral dose (9 Log CFU) of the EPS-
87 producing strain *Lactobacillus johnsonii* FI9785 administered to pathogen-free chicks
88 was enough to eliminate *Clostridium difficile* infection (Dertli, et al. 2013; La Ragione,
89 Narbad, Gasson, & Woodward, 2004).

90 In a previous study, we have showed that the EPS-producing strain
91 *Lactobacillus paraplantarum* BGCG11 isolated from a Serbian soft, white, home-made
92 cheese, and three non-EPS-producing derivative strains, had probiotic traits. They
93 survived (in enough number) to the adverse conditions of the gastrointestinal tract, they
94 adhered at different degree to three intestinal epithelial cell (IEC) lines, and they were
95 able to elicit different *in vitro* immune response upon peripheral blood mononuclear
96 cells (Nikolic et al., 2012). Besides, the parental BGCG11strain was able to synthesise a
97 ropy EPS-CG11 (Kojic, et al., 1992; Zivkovic et al. 2015), and given that derivative
98 strains lost this character, the differences in probiotic attributes were partially ascribed
99 to the presence / absence of the ropy polymer. The aim of the current work was to
100 further characterise the EPS-producing *Lb. paraplantarum* BGCG11 strain and its non-
101 EPS-CG11 producing derivative NB1, in order to assess the putative protective role that

the polymer could play upon HT29-MTX monolayers challenged with some opportunistic pathogens that inhabit, or reach the gut through the diet. Several mechanisms of probiotic action against pathogen´s activity upon IEC were explored.

2. Material and Methods

2.1. Bacterial strains and EPS purification

The bacteria and culture conditions used in this study are listed in Table 1. As standard procedure, strain stocks stored at -80ºC were spread in agar-MRS (Biokar Diagnostics, Beauvais, France) or agar-BHI (Oxoid Limited, Hampshire, UK) and incubated for 48 h under optimal conditions for each strain. A single colony was picked up to inoculate 10 ml of the corresponding broth and incubated for 24 h. The cultures were used to inoculate (2%) fresh broth media which were incubated for 18 h to harvest cells for preparation of the bacterial suspensions needed in each experiment.

The EPS produced by *Lb. paraplantarum* BGCG11 (named EPS-CG11) was purified accordingly to the procedure previously reported by Nikolic et al. (2012), consisting in an initial extraction with ethanol precipitation, dialysis and freeze-dry, followed by a second purification with sequential DNAse type-I and Pronase E treatments, protein precipitation with TCA and ending with intensive dialysis and freeze-drying.

2.2. HT29-MTX cell line culture conditions

The human colonocyte-like cellular line HT29-MTX (Lesuffleur, Barbat, Dussaulx, & Zweibaum, 1990) was used to test the capability of the two *Lb. paraplantarum* strains to counteract the effect of pathogens upon the intestinal epithelium. For this purpose, $1x10^5$ HT29-MTX cells were seed in 48-wells microplates (BD Falcon, BD Biosciences, NJ, USA) using complete-DMEM, i.e., DMEM medium supplemented with 10% foetal bovine serum and with a mixture of antibiotics (50 μg/ml

penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B). All reagents were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Microplates were incubated at 37ºC, 5% CO_2 in the CO2-Series Shel-Lab incubator (Sheldon Manufacturing Inc. OR, USA) until reach the differentiated and confluent (monolayer) state (12± 1 days post-seeding, about $1x10^7$ cells/ml). For co-cultivation with bacterial strains, HT29-MTX monolayers were incubated under same conditions in the HERAcell® 240 incubator (Thermo Electron LED GmbH, Langenselbold, Germany).

2.3. Inhibition of pathogens adhesion to HT29-MTX by lactobacilli

The capability of the seven pathogens under study, together with the three lactobacilli used as reference, to adhere to the intestinal epithelium was tested. For that, bacterial cultures were washed twice with PBS and resuspended in DMEM without antibiotics at concentration ~$1x10^8$ cfu/ml; this number was corroborated by plate counting in the agar-medium specific for each bacterium. The bacterial suspensions were independently added to the HT29-MTX monolayers at ratio (10: 1, bacteria: eukaryotic cell) and incubated at 37ºC, 5% CO_2 for 1 h. Afterwards, monolayers were gently washed twice with Dulbecco´s PBS, to remove the non-attached bacteria, and the eukaryotic cells were released using 0.25% Trypsin-EDTA solution (Sigma). The samples were diluted in Ringer solution and plated to enumerate the bacteria adhered. The percentage of adhesion was calculated as follows: 100 x cfu bacteria adhered / cfu bacteria added. Each strain was tested in duplicated wells in two replicated HT29-MTX microplates (four data per strain).

To test the capability of the two *Lb. paraplantarum* to inhibit the adhesion of pathogens to HT29-MTX monolayer, the seven pathogens were dyed with the SYTO®9 green-fluorescent nucleic acid stain (Molecular Probes, Life Technologies S.A., Madrid,

153 Spain). A working solution of 15 nM SYTO®9 was prepared in complete-DMEM and
154 added (volume / volume) to each bacterial suspension (final concentration 7.5 nM)
155 prepared as previously described. Samples were incubated at room temperature, in
156 darkness, for 2 h. To know the number of pathogens to be added to HT29-MTX, the
157 fluorescence emitted at 512 nm (after excitation at 470 nm) of these pathogen samples
158 was recorded in the Cary Eclipse fluorescence spectrophotometer (Varian Ibérica, S.A.
159 Madrid, Spain). Afterwards, the fluorescence emitted was correlated with counts (log
160 cfu) by means of linear regression equations calculated for each pathogen. The log cfu
161 were obtained after plating in the corresponding agar-media (Table 1) serial dilutions in
162 Ringer of an initial bacterial suspension of $1x10^8$ cfu/ml. The correlation coefficients
163 (R^2) of the equations obtained for the seven pathogens were 0.990±0.005. To know the
164 inhibition capability of *Lactobacillus* strains, dyed pathogens were mixed with the
165 lactobacilli suspensions (ratio 1:1). The dyed-pathogens alone were added to HT29-
166 MTX monolayers at ratio 10:1 (Fig. 1) as well as the combinations pathogen –
167 lactobacilli, thus each bacterial type was added at ratio 5:1. Microplates were then
168 incubated for 1 h at 37ºC, 5% CO_2; afterwards, wells were gently washed twice with
169 Dulbecco´s PBS and treated with 0.25% trypsine-EDTA solution. The fluorescence
170 emitted by the pathogens adhered were measured in the fluorescence spectrophotometer
171 and the corresponding log cfu were calculated using the linear regression equations.
172 Finally, the percentage of adhesion was calculated as indicated above; within each
173 pathogen, data were referred to that obtained with the pathogen alone (i.e. 100%
174 adhesion) and finally, the pathogen adhesion reduction was calculated subtracting each
175 referred value from 100. Each pair combination pathogen-lactobacilli, and the pathogen
176 alone as reference, were tested in three replicated HT29-MTX microplates.

177 *2.4. Capability of lactobacilli to counteract the toxicity of pathogens upon HT29-MTX*

178 Combinations of (non-dyed) pathogen–lactobacilli were prepared as indicated
179 above (bacterial suspensions ~1x10^8 cfu/ml in complete-DMEM, and ratio pathogen-
180 lactobacilii 1:1). In addition, each pathogen was resuspended in complete-DMEM
181 supplemented with 1 mg/ml of EPS-CG11. The HT29-MTX monolayers were co-
182 cultivated with these bacterial combinations, as well as with the seven pathogens and
183 the four probiotic factors added alone (Fig. 1), for 3 h at 37ºC, 5% CO_2. The cell line
184 was also incubated in the presence of complete-DMEM, which was used as reference
185 control. Experiments were carried out in three replicated HT29-MTX microplates. At
186 the end of the incubation, supernatants were collected and centrifugated (10,000*xg*, 4ºC,
187 10 min) to remove the detached cells, before stored at -20ºC until use.

188 *2.4.1. Detachment of HT29-MTX monolayers*

189 After supernatants collection, HT29-MTX cells were softly washed once with
190 Dulbecco´s PBS, fixed with 2% formaldehyde (in PBS) for 1 min and washed again.
191 Cells were stained with 0.13% crystal violet (in 5% ethanol and 2% formaldehyde-PBS)
192 for 20 min at room temperature. Afterwards, wells were intensively washed with PBS
193 solution, until obtain clear supernatants, and then the microplates were scanned (Ruas-
194 Madiedo et al. 2010).

195 *2.4.2. Lactate dehydrogenase activity*

196 The levels of intracellular lactate dehydrogenase (LDH) released to the
197 supernatants were quantified by means of the colorimetric Cytotoxicity Detection
198 Kit^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer
199 instructions. After enzymatic reaction, the absorbance of samples was measured at 450
200 nm in the Modulus microplate photometer (Turner Biosystems, Sunnyvale, CA, USA).
201 Data were referred to the reference control, i.e., to the values obtained in the HT29-
202 MTX wells incubated with complete-DMEM

203 *2.5. Ability of lactobacilli to modify the innate intestinal barrier*

204 *2.5.1. Production of IL-8*

205 The supernatants were also used to quantify the production of the chemokine
206 interleukin-8 (IL-8) by means of an ELISA test (eBioscience, Bender MedSystems
207 GmbH, Vienna, Austria) following the manufacturer instructions. The colorimetric
208 enzymatic reaction was measured at 450 nm in the Modulus microplate photometer and
209 data were referred to the reference control.

210 *2.5.2. Production of mucin*

211 Mucin production by Goblet cells present in HT29-MTX monolayers was
212 quantified by using the fluorescence conjugated lectin Wheat Germ Agglutinin (WGA)-
213 Alexa Fluor 488® (Molecular Probes) as follows. HT29-MTX cells were grown in 96-
214 wells fluorometry validated microplates (Optilux™ Black/Clear Bottom, BD Falcon)
215 under conditions described above until reach the monolayer state. Then bacterial
216 suspensions were added following the experimental design described in Fig. 1; each
217 condition was tested in duplicated wells in two replicated HT29-MTX microplates.
218 Incubations were also carried out for 3 h at 37ºC, 5% CO_2 and, finally, microplates were
219 washed twice with Dulbecco´s PBS.

220 For mucin detection, WGA-Alexa Fluor 488 was dissolved at 1 mg/ml in
221 ultrapure water and a working solution of 5 μg/ml was made in complete-DMEM. Then
222 100 μl of this solution was added to the washed HT29-MTX monolayer of each well
223 and incubated at 37ºC, in darkness, for 1 h. After incubation, the non-attached WGA
224 was eliminated washing twice with PBS, and finally 100 μl of PBS was added. The
225 fluorescence emitted at 518 nm by the monolayer, after sample excitation at 480 nm,
226 was recorded in the Cary Eclipse fluorescence spectrophotometer and data were referred
227 to the reference control.

228 *2.5.3. Expression of MUC genes*

229 Based on the results of mucin production, the pathogens *C. difficile* LMG21717
230 and *Escherichia coli* LMG2092 were chosen to check the expression of some genes
231 involved in the synthesis of mucin. For that purpose, HT29-MTX cells were seed in 12-
232 wells microplates (BD Falcon) and incubated until reach the monolayer state. Following
233 the experimental design described in preceding sections (Fig. 1), the five combinations
234 (pathogen-lactobacilli, pathogen-EPS or pathogen alone) were tested for each pathogen
235 in three replicated microplates. HT29-MTX monolayers grown in complete-DMEM
236 were also used as reference control. After a co-incubation for 3 h at 37ºC, 5% CO_2,
237 supernatants were removed and 0.5 ml of RNAlater solution (Ambion®, Life
238 Technologies S.A.) was added to each well to protect the RNA of the HT29-MTX cells.
239 Then released cell samples were stored at -80ºC until use.

240 Following the manufacturer´s instructions, the RNAaqueous®-PCR kit
241 (Ambion) was used for isolation of DNA-free RNA. The quantification of RNA was
242 carried out in the Epoch apparatus (BioTek Instruments, Inc., Winoskii, VT, USA). For
243 reverse-transcriptase PCR analyses 1 µg of RNA was reverse-transcribed to cDNA by
244 using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life
245 Technologies S.A.). The generated cDNA was then stored at –80ºC until further
246 analysis.

247 Real-Time PCR was performed in an ABI Prism 7500 Fast Real-Time PCR
248 System (Applied Biosystems). The TaqMan® gene Master Mix and the TaqMan® gene
249 Expression Assays (Applied Biosystems) were used for MUC1 (Hs00159357_m1),
250 MUC2 (Hs00159374_m1), MUC3B (Hs03649367_mH) and MUC5AC
251 (Hs00873638_m1) analysis; the GAPD (glyceraldehyde-3-phosphate dehydrogenase)
252 and ACTB (beta actin) were used for normalization of data. Expression levels were

253 determined by relative quantification using the ΔΔCt method (Livak & Schmittgen,
254 2001) in which the expression level in the reference control (HT29-MTX cells grown
255 alone) is arbitrarily set to 1 and the expression levels in the samples are calculated
256 relative to that of the reference control.

257 *2.6. Statistical analysis*

258 Data were statistically analysed by using the SPSS/PC 19.0 software package
259 (SPSS Inc., Chicago, IL, USA). After checking normal distribution of values,
260 independent one-way ANOVA tests and, when needed, mean comparison LSD (least
261 significant difference) tests, were used for analyses. The legend of figures or tables
262 shows the statistical comparison performed for each type of parameter.

263

264 **3. Results**

265 *3.1. Bacterial antagonism for adhesion to HT29-MTX*

266 The capability of the enteropathogens and lactobacilli strains used in this study
267 to adhere to the epithelial cell line HT29-MTX was highly variable (Fig. 2A). Most
268 pathogens showed low adhesion percentage (< 5%) and all of them, except *C. difficile*
269 and *E. coli*, adhered significantly less than the strain *Lb. rhamnosus* LMG18243 (= GG)
270 used as reference. The two *Lb. paraplantarum* strains manifested a considerable
271 difference in their adhesiveness to the colonocytes as we have previously reported
272 (Nikolic et al., 2012). The strain BGCG11, producing the ropy EPS-CG11, showed an
273 adhesion percentage similar to that of GG; the derivative NB1 strain, which has lost the
274 30 kb plasmid carrying the genes (operon) for the synthesis of the ropy EPS, almost
275 doubled its adhesion ability comparing to the parental strain.

276 The antagonism of the three lactobacilli against pathogen´s adhesion to HT29-
277 MTX monolayers varied according to the pathogenic strain (Fig. 2B). The highest

reduction of adhesion (around 45%) was detected when *Shigella sonnei* was co-incubated in the presence of the lactobacilli, although no significant differences were detected among the three strains. On the contrary, the lowest reduction (< 8%) was showed when *C. difficile* competed with the lactobacilli for adhesion to HT29-MTX. However, in this case, significant differences among lactobacilli strain used were denoted. Indeed, probiotics were able to efficiently reduce the attachment of most pathogens and the performance of the three strains was, in general, similar although their efficacy was dependent on the pathogen considered. For example, *Lb. paraplantarum* NB1 and *Lb. rhamnosus* GG were more efficient reducing the adhesion of *C. difficile* or *Yersinia enterocolitica* than *Lb. paraplantarum* BGCG11. However, the latter strain reduced in higher extent the adhesion of *E. coli* and *Listeria monocytogenes*.

3.2. Response of the cell line HT29-MTX

To test the protection that the lactobacilli or the EPS-CG11 could confer to HT29-MTX cells challenged with the seven pathogens, the monolayers were stained with crystal violet after co-cultivation of bacterial factors with eukaryotic cells (Fig. 3A). *L. monocytogenes* LMG13305 presented the stronger cell detachment of the seven pathogens added alone since only few cells remained adhered to the bottom of the microplate; other pathogens showed variable degrees of detachment. Remarkably, the purified EPS-CG11, added to the wells at the same time than the pathogens, was the most effective treatment to keep adhered the HT29-MTX cells, even better than the EPS-producing BGCG11 strain or the other two lactobacilli. Aiming to check whether the monolayer disaggregation was due to cellular lysis, the levels of the cytoplasmic LDH were determined in the supernatants of these co-cultures (Fig. 3B). Again, *L. monocytogenes* was the pathogen causing the highest LDH release and, although the

differences were not statistically significant, it seems that the presence of the purified EPS-CG11 protected HT29-MTX for the listeria-induced lysis. Similar tendency was detected with the other pathogens as well. However, it is worth noting that lactobacilli, either intact bacteria or purified EPS, were only able to significantly reduce the cellular damage induced by *C. dificille*.

When intestinal epithelium encounters pathogens, the enterocytes, acting at first barrier of the innate immune response, are able to release signalling molecules such as interleukin (IL)-8. Levels of IL-8 released by HT29-MTX, relative to the basal values (control sample), varied depending on the pathogen tested (Fig. 4). *C. difficile* alone induced the highest IL-8 production and this capability was reinforced by the presence of *Lb. paraplantarum* NB1 and *Lb. rhamnosus* GG, but no by the EPS-producing *Lb. paraplantarum* BGCG11 strain or its purified polymer. Indeed, the three probiotic lactobacilli themselves were able to induce IL-8 secretion in higher levels than some pathogens (relative IL-8 production: 3.89±0.92, 6.28±2.4 and 3.74±1.54 for BGCG11, N1, and GG strains, respectively), but the purified EPS-CG11 (0.5±0.22) was not. As stated in previous sections, it is not possible to stand out one of the three lactobacilli given that the strain BGCG11 seemed to be as good, or even better, than the other two at inducing the release of IL-8 induced by other pathogens (e.g. *L. monocytogenes*, *E. coli*, *Sh. sonnei* and *Y. enterocolitica*).

Another mechanism to keep the innate barrier at intestinal epithelium level is the secretion of glycoproteins, known as mucins, which form a protective layer (mucus) in the luminal side of the gut. The fluorescent lectin WGA was used to quantify the production of mucins by HT29-MTX in the experimental conditions of this study (Fig. 5). In general, the relative production of mucins, with respect to the basal level, was not high. The production was increased in the presence of *C. difficile* and, specially, when it

was simultaneously co-incubated with *Lb. rhamnosus* GG ($p<0.05$). This pathogen, as well as *E. coli* selected as no mucin-inducing control, was used to achieve the expression analysis of some *muc* genes (Table 2). These results confirmed that the relative expression level of *muc1*, *muc2*, *muc3B* and *muc5AC* was not high, which correlate with the few level of mucin amount detected. As expected *E. coli* did not induced the expression of any *muc* gen, whereas *C. difficile,* in combination with *Lb. rhamnosus* GG and *Lb. paraplantarum* BGCG11, promoted higher expression of *muc3B* by HT29-MTX than when the pathogen was added alone.

4. Discussion

Among the health promoting properties of probiotic strains, the capability to counteract the negative effects of pathogens is one of the desired traits (WHO/FAO, 2006). Indeed, it has been probed that probiotic formulations, containing mainly lactobacilli, are effective to treat or prevent infectious diarrhoea caused by the use of antibiotics or by recurrent *C. difficile* infections in humans (Sanders et al., 2014; Tojo et al., 2014). The probiotic antagonism against pathogens is a strain-dependent characteristic and several mechanisms of action have been proposed; probiotics might i) provide a physical barrier blocking the pathogen entry (colonization competition), ii) induce the mucus production, iii) reinforce the selective permeability of the epithelium by increasing tight-junctions, iv) produce antimicrobial factors, and/or v) stimulate the innate immune response, among others (Gareau, Sherman, & Walker, 2010; Liévin-Le Moal, & Servin, 2014). Some of these mechanisms involve the direct interaction of probiotics with the host cells which, in turns, modifies their response against the pathogens.. In this study we have explored potential mechanisms of action that could

352 explain the antagonism among two *Lb. paraplantarum* strains, differing in their
353 capability to produce a ropy EPS, and several intestinal pathogens.

354 *4.1. Lactobacilli strains reduced pathogen adhesion to HT29-MTX*

355 Regarding competition for the colonization of intestinal niche, the three
356 lactobacilli reduced in different degree the adhesion of the seven pathogens to HT29-
357 MTX monolayers having similar performance, i.e. none of the strains showed greater
358 inhibition. There are several reports in literature showing the capability of different
359 lactobacilli species to reduce the adhesion of diverse enteropathogens to intestinal cells,
360 either cellular lines (Caco2, HT29 or HT29-MTX) or IEC isolated from animals
361 (García-Cayuela et al., 2014). This capability has been attributed to the production of
362 antibacterial factors, such as bacteriocins or organic acids (Kaewnopparat et al., 2013;
363 Satish-Kumar et al., 2011), as well as to the presence of specific structural components
364 in the surface of the lactobacilli. It has been indicated that these components are cell
365 surface associated proteins (Varma, Dinesh, Menon, & Biswas, 2010) or S-layer
366 macromolecules built from proteins (Zhang et al., 2010). The putative mechanisms
367 behind the reduction of pathogen attachment to IEC are a steric hindrance of lactobacilli
368 avoiding the interaction of the pathogen (Satish-Kumar et al. 2011) and also co-
369 aggregation phenomena (Kaewnopparat et al., 2013). However, co-aggregation could
370 also increase the number of pathogens "apparently" adhered to the IEC if the
371 lactobacilli co-aggregated with the pathogen shows good adhesion properties (Ayeni et
372 al., 2011; Gueimonde, Jalonen, He, Hiramatsu, & Salminen, 2006). This is not
373 necessarily a negative trait since the formation of co-aggregates will facilitate the closer
374 antibacterial activity of lactobacilli and, if the pathogen is linked to the bacteria surface,
375 it will not be able to adhere to the IEC. This statement has been previously underlined in
376 studies carried out with EPS-producing lactobacilli and bifidobacteria interfering with

the adhesion of pathogens to human intestinal mucus (Ruas-Madiedo, Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2006a). Indeed, it was demonstrated the role that EPS plays in this interference working with the polymer purified from the Scandinavian fermented milk "viili", which is synthesized by *Lactococcus lactis* subsp. *cremoris* (Ruas-Madiedo, Gueimonde, de los Reyes-Gavilán, & Salminen, 2006b). In the current study we cannot discard that the ropy EPS-CG11 plays a role in the pathogen adhesion inhibition of some of the pathogens analysed; but its absence in the derivative strain NB1 was not an impediment to favor equal or even higher inhibition in some pathogenic strains.

4.2. Lactobacilli strains reduced HT29-MTX damage induced by some pathogens

Lactobacillus species and probiotics in general, could protect host against pathogens invasion improving the barrier of the intestinal mucosa by reinforcing the integrity of the intestinal epithelium (Nissen, Chingwaru, Sgorbati, Biavati, & Cencic, 2009). Some surface components (S-layer) from lactobacilli are able to maintain the intestinal epithelium integrity avoiding the cellular damage produced by some pathogen´s toxins (Carasi, Trejo, Pérez, De Antoni, & Serradell, 2012). In the case of the *Lb. paraplantarum* BGCG11 under study, it seems that its purified EPS could be an effective macromolecule protecting HT29-MTX cells from the lysis induced by some pathogens. The EPS could form a layer, a kind of "protective biofilm", covering the intestinal cells thus avoiding either the interaction of the toxins with their eukaryotic receptors or acting as toxin-scavenger agents (Ruas-Madiedo et al., 2010). Indeed, it was demonstrated that the EPS kefiran, purified from the fermented milk kefir, is able to abrogate the cytotoxic effect of extracellular factors released by *Bacillus cereus* upon Caco-2 cells, the effect being EPS-dose dependent (Medrano, Pérez, & Abraham, 2008).

401 Along the gastrointestinal tract, the intestinal mucosa is covered by a highly
402 viscous, complex and thick layer of mucus composed of mucin glycoproteins and other
403 molecules related with host defense against pathogens (McGuckin, Lindén, Sutton, &
404 Florin, 2011). It has been demonstrated in animal model that some lactobacilli, such as
405 *Lactobacillus fermentum*, are able to *in vivo* increase the number of Goblet mucin-
406 secretory cells as well as the expression of *muc* genes (Cao, Yang, Sun, Chanjuan, &
407 Yao, 2012). Nevertheless, most studies with probiotics explore their capability to *in*
408 *vitro* adhere to this mucus layer and, in a few of them, it was reported that the ability of
409 probiotics to inhibit pathogens adhesion is due to the up-regulation of mucus secretions
410 (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999). In some cases is enough the
411 presence of bacterial-cell extracts to stimulate the mucin production; those obtained
412 from *Lb. acidophilus* induce *muc2* expression, contributing to the inhibition of *E. coli*
413 O157:H7 attachment to HT29 intestinal epithelial cells (Kim, Kim, Whang, Kim, & Oh,
414 2008). Results obtained in our study suggest that the actobacilli, could act as weak "co-
415 adjuvants" helping some pathogens, such as *C. difficile*, to slightly increasing the
416 secretion of mucus by HT29-MTX. The poor differences detected in the expression of
417 *muc3B* gene between the two *Lb. paraplantarum* strains does not allow obtaining any
418 conclusion about the contribution of the ropy EPS-CG11 in mucus secretion. In any
419 case, the purified polymer itself was not able to improve the expression of *muc* genes,
420 nor the secretion of mucus. Thus, it seems that the EPS from *Lb. paraplantarum* is not
421 an extracellular factor inducing mucus secretion.
422 A recent review indicates that several probiotic lactobacilli diminish the
423 production of pathogen-induced IL-8 by intestinal epithelial cells (Liévin-Le Moal, &
424 Servin, 2014). However, the results obtained with both *Lb. paraplantarum* strains, as
425 well as with *Lb. rhamnosus* GG, pointed to the opposite direction; the production of IL-

8 promoted by enteropathogens was stimulated in the presence of the three lactobacilli, whereas the purified ropy EPS-CG11 had not effect itself, probably, because would difficult the contact between the bacteria and the intestinal epithelium. In this regard, we can found in literature data supporting our results, that is a lactobacilli-mediated induction of IL-8 secretion (Kim et al., 2008; Seifert et al., 2010; Vizoso-Pinto et al., 2007); but also the contrary, a down regulation of IL-8 production promoted by lactobacilli (Candela et al., 2008; Dhanani, & Bagchi, 2013; Nandakumar, Pugazhendhi, Madhu-Mohan, Jayakanthan, & Ramakrishna, 2009; O'Hara et al., 2006). These apparently contradictory results indicates that the capability to modulate the levels of this chemokine is highly dependent on the *Lactobacillus* strain considered and, probably, dependent as well on the IL-8 inductor pathogen. Besides, the influence of the intestinal epithelial cellular model used cannot be obviated (Hidalgo-Cantabrana et al., 2014a). The ability of *Lactobacillus* species to moderately increase the production of IL-8 in the presence of pathogens could be a beneficial trait since this chemokine will contribute to the recruitment of neutrophils to combat infection; therefore, this will be another probiotic mechanism to reinforce the intestinal innate defense.

5. Conclusion

The strains *Lb. paraplantarum* BGCG11 and NB1, as well as *Lb. rhamnosus* GG, seemed to induce the synthesis of cellular factors (IL-8 and mucins) by HT29-MTX monolayers challenged with pathogens which, in a physiological situation, could help to improve the intestinal barrier. The performance of the three lactobacilli was similar since we could not found any strain that behave better than the others. In addition, the ropy EPS-CG11 synthesised by the strain *Lb. paraplantarum* BGCG11 was able to partially counteract the cellular damage (release of LDH) caused by some

pathogens. Therefore, both *Lb. paraplantarum* strains are promising probiotic candidates effective against intestinal pathogens, especially *C. difficile*.

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622

623 **Table 1** Bacterial strains and culture conditions used in this study
624

Strain	Origin	Culture conditions [3]
Lactobacillus paraplantarum BGCG11	Artisanal cheese (natural isolate) [1]	MRS, 30ºC, aerobic
Lactobacillus paraplantarum NB1	Novobiocin-cured from BGCG11-derivative (lacking the 30 kb plasmid carrying the EPS operon) [1]	MRS, 30ºC, aerobic
Lactobacillus rhamnosus LMG18243 (GG)	BCCM [2]	MRS, 37ºC, anaerobic
Clostridium difficile LMG21717	BCCM	BHI, 37ºC, anaerobic
Salmonella enterica serotype Typhimurium LMG15860	BCCM	BHI, 37ºC, anaerobic
Listeria monocytogenes LMG13305	BCCM	MRS, 37ºC, aerobic
Cronobacter sakazakii LMG5740	BCCM	BHI, 37ºC, anaerobic
Escherichia coli LMG2092	BCCM	BHI, 37ºC aeration
Shigella sonnei LMG10473	BCCM	BHI, 37ºC, anaerobic
Yersinia enterocolitica LMG7899	BCCM	BHI, 37ºC, anaerobic

625 [1] Described in Kojic *et al*., 1992; Nikolic *et al*., 2012.

626 [2] Belgian Coordinated Collections of Microorganisms.

627 [3] The anaerobic conditions were achieved in the chamber MG500 (Don Whitley Scientific, Yorkshire, UK) under 80% N_2, 10% H_2, 10% CO_2 atmosphere. The
628 aeration was achieved in the Excella E24 Incubator Shaker (New Brunswick Scientific, Enfield, CT, USA).

629

630

631 **Table 2** Expression of MUC genes in HT29-MTX in the presence of pair combinations

632 pathogen and lactobacilli or EPS-CG11..

		Relative expression (mean ± SD) [2]	
Gene[1]	Probiotic factor	*C. difficile* LMG21717	*E. coli* LMG2092
muc1	Pathogen (alone)	1.34±0.11	1.17±0.29
	+ *L. paraplantarum* BCG11	1.37±0.07	1.28±0.11
	+ *L. paraplantarum* NB1	0.98±0.35	1.12±0.04
	+ *L. rhamnosus* LMG18243	1.24±0.20	1.09±0.30
	+ EPS-CG11	1.24±0.06	1.08±0.22
muc2	Pathogen (alone)	0.92±0.09	0.91±0.69
	+ *L. paraplantarum* BCG11	1.10±0.02	0.94±0.28
	+ *L. paraplantarum* NB1	1.17±0.48	0.85±0.29
	+ *L. rhamnosus* LMG18243	1.34±0.79	0.85±0.58
	+ EPS-CG11	0.57±0.03	0.62±0.03
muc3B	Pathogen (alone)	1.11±0.46 **ab**	1.47±0.37
	+ *L. paraplantarum* BCG11	1.78±0.29 **c**	1.36±0.58
	+ *L. paraplantarum* NB1	0.80±0.46 **a**	1.49±0.71
	+ *L. rhamnosus* LMG18243	1.47±0.15 **bc**	1.13±0.38
	+ EPS-CG11	0.95±0.12 **ab** *****	2.07±1.22
muc5AC	Pathogen (alone)	1.35±0.12	0.79±0.46
	+ *L. paraplantarum* BCG11	1.38±0.28	0.87±0.18
	+ *L. paraplantarum* NB1	1.11±0.75	0.76±0.14
	+ *L. rhamnosus* LMG18243	1.29±0.51	0.77±0.38
	+ EPS-CG11	1.44±0.50	1.18±0.31

633 [1] The expression of MUC genes was normalize by the housekeeping *gapdh*
634 (glyceraldehyde-3-phosphate dehydrogenase) and *actb* (actin, beta) genes and
635 calculated relative to that of the reference control (cell line grown in DMEM alone).
636 [2] Within each pathogen, data were analysed by means of one-way ANOVA test and the
637 statistical differences were annotated with asterisks (*$p<0.05$). The mean comparison
638 LSD was used to asses differences among probiotic factors; in this case, means that do
639 not share a common superscript letter are significantly different ($p<0.05$).
640

641 **Figure legends**

642 **Fig. 1** Experimental design used to test the capability of two *Lb. paraplantarum* and the
643 reference *Lb. rhamnosus* LMG18243 (GG) strains, as well as that of the EPS-CG11, to
644 counteract the effect of seven pathogens upon the intestinal cell line HT29-MTX.
645 Combinations of pathogen and lactobacilli were mixed at ratio 1:1 (final bacteria $1x10^8$
646 cfu/ml) and added to HT29-MTX confluent monolayers at ratio 10:1 (bacteria :
647 eukaryotic cell). Pathogens were resuspended in DMEM supplemented at 1 mg/ml with
648 EPS-CG11. Probiotic factors and pathogens were also tested alone and several wells
649 were kept as reference control (cultivated in DMEM without any factor added).

650

651 **Fig. 2** (A) Percentage of adhesion of the nine strains used in this study. Each strain was
652 compared with the reference *Lb. rhamnosus* LMG18243 (GG) strain by means of an
653 one way ANOVA test and asterisks denoted significant differences (* $p<0.05$, **
654 $p<0.01$). (B) Percentages of pathogen-adhesion reduction were calculated in the
655 presence of the three lactobacilli. Within each pathogen, those bars that do not share a
656 common letter are significantly ($p<0.05$) different accordingly to the mean comparison
657 LSD test.

658

659 **Fig. 3** (A) Crystal violet staining of HT29-MTX monolayers after co-cultivation with
660 different bacterial factors. (B) Relative (to the control culture) LDH (lactate
661 dehydrogenase) released to the supernatants of HT29-MTX monolayers co-cultured
662 with different bacterial factors. Within each pathogen, those bars that do not share a
663 common letter are significantly ($p<0.05$) different accordingly to the mean comparison
664 LSD test.

665

Fig. 4 Relative (to the control culture) IL-8 (interleukin) production by HT29-MTX monolayers co-cultivated with different bacterial factors. Within each pathogen, those bars that do not share a common letter are significantly ($p<0.05$) different accordingly to the mean comparison LSD test.

Fig. 5 Relative (to the control culture) mucin production by HT29-MTX monolayers co-cultivated with different bacterial factors. Within each pathogen, those bars that do not share a common letter are significantly ($p<0.05$) different accordingly to the mean comparison LSD test.

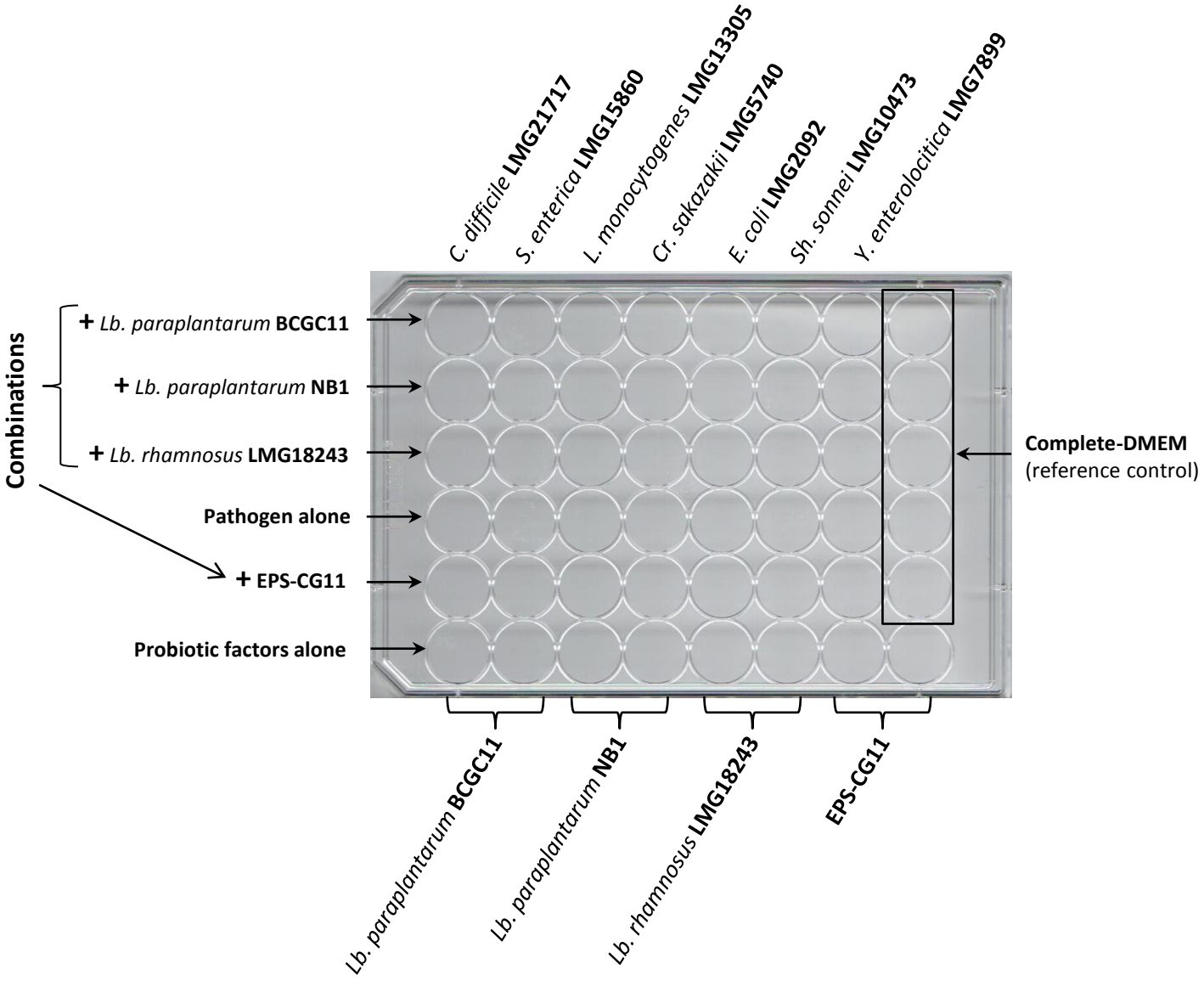
C. difficile LMG21717
S. enterica LMG15860
L. monocytogenes LMG13305
Cr. sakazakii LMG5740
E. coli LMG2092
Sh. sonnei LMG10473
Y. enterolocitica LMG7899
Combinations
+ Lb. paraplantarum BCGC11
+ Lb. paraplantarum NB1
+ Lb. rhamnosus LMG18243
Pathogen alone
+ EPS-CG11
Probiotic factors alone
Complete-DMEM
(reference control)
Lb. paraplantarum BCGC11
Lb. paraplantarum NB1
Lb. rhamnosus LMG18243
EPS-CG11

Figure 1

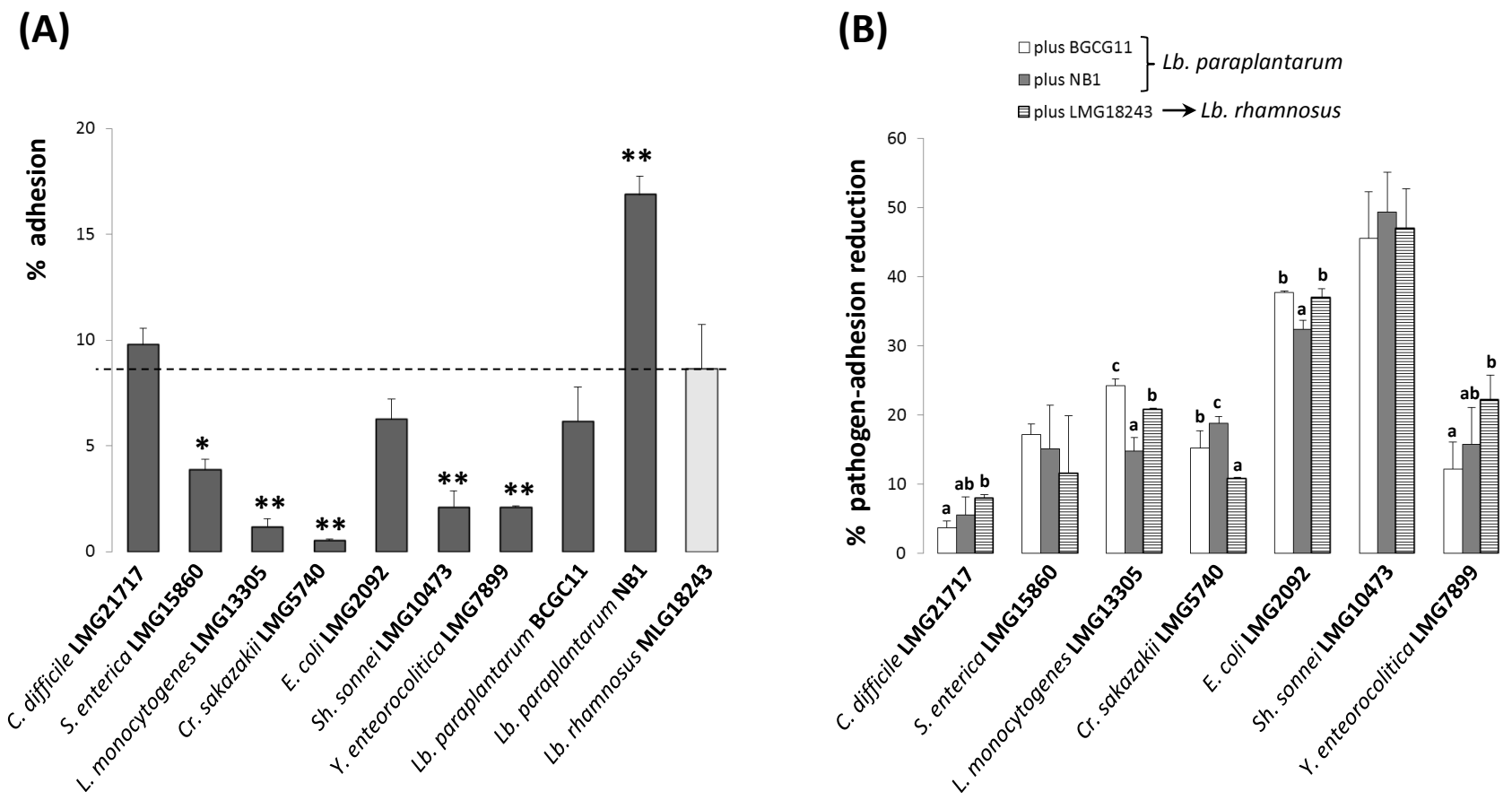
(A)
% adhesion
20
15
10
5
0
*
**
**
**
**
**
**
C. difficile LMG21717
S. enterica LMG15860
L. monocytogenes LMG13305
Cr. sakazakii LMG5740
E. coli LMG2092
Sh. sonnei LMG10473
Y. enteorocolitica LMG7899
Lb. paraplantarum BCGC11
Lb. paraplantarum NB1
Lb. rhamnosus MLG18243
(B)
plus BGCG11
plus NB1
Lb. paraplantarum
plus LMG18243
Lb. rhamnosus
% pathogen-adhesion reduction
60
50
40
30
20
10
0
a
ab
b
a
b
c
a
b
b
c
a
b
b
b
a
a
ab
b
C. difficile LMG21717
S. enterica LMG15860
L. monocytogenes LMG13305
Cr. sakazakii LMG5740
E. coli LMG2092
Sh. sonnei LMG10473
Y. enteorocolitica LMG7899

Figure 2

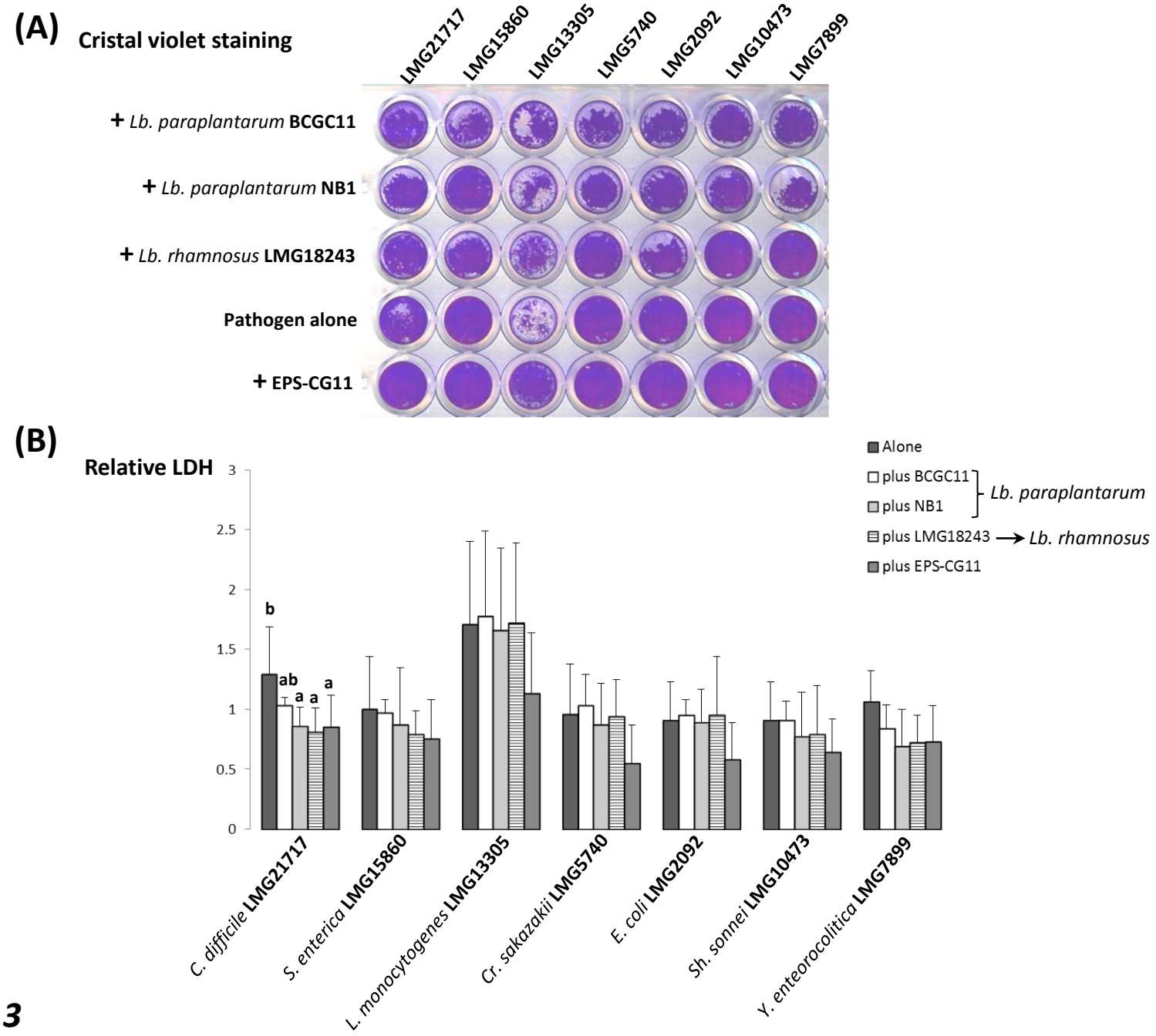
(A)
Cristal violet staining
LMG21717
LMG15860
LMG13305
LMG5740
LMG2092
LMG10473
LMG7899
+ Lb. paraplantarum BCGC11
+ Lb. paraplantarum NB1
+ Lb. rhamnosus LMG18243
Pathogen alone
+ EPS-CG11
(B)
Relative LDH
Alone
plus BCGC11
plus NB1
Lb. paraplantarum
plus LMG18243
Lb. rhamnosus
plus EPS-CG11
3
2.5
2
1.5
1
0.5
0
b
ab
a
a
a
C. difficile LMG21717
S. enterica LMG15860
L. monocytogenes LMG13305
Cr. sakazakii LMG5740
E. coli LMG2092
Sh. sonnei LMG10473
Y. enteorocolitica LMG7899

Figure 3

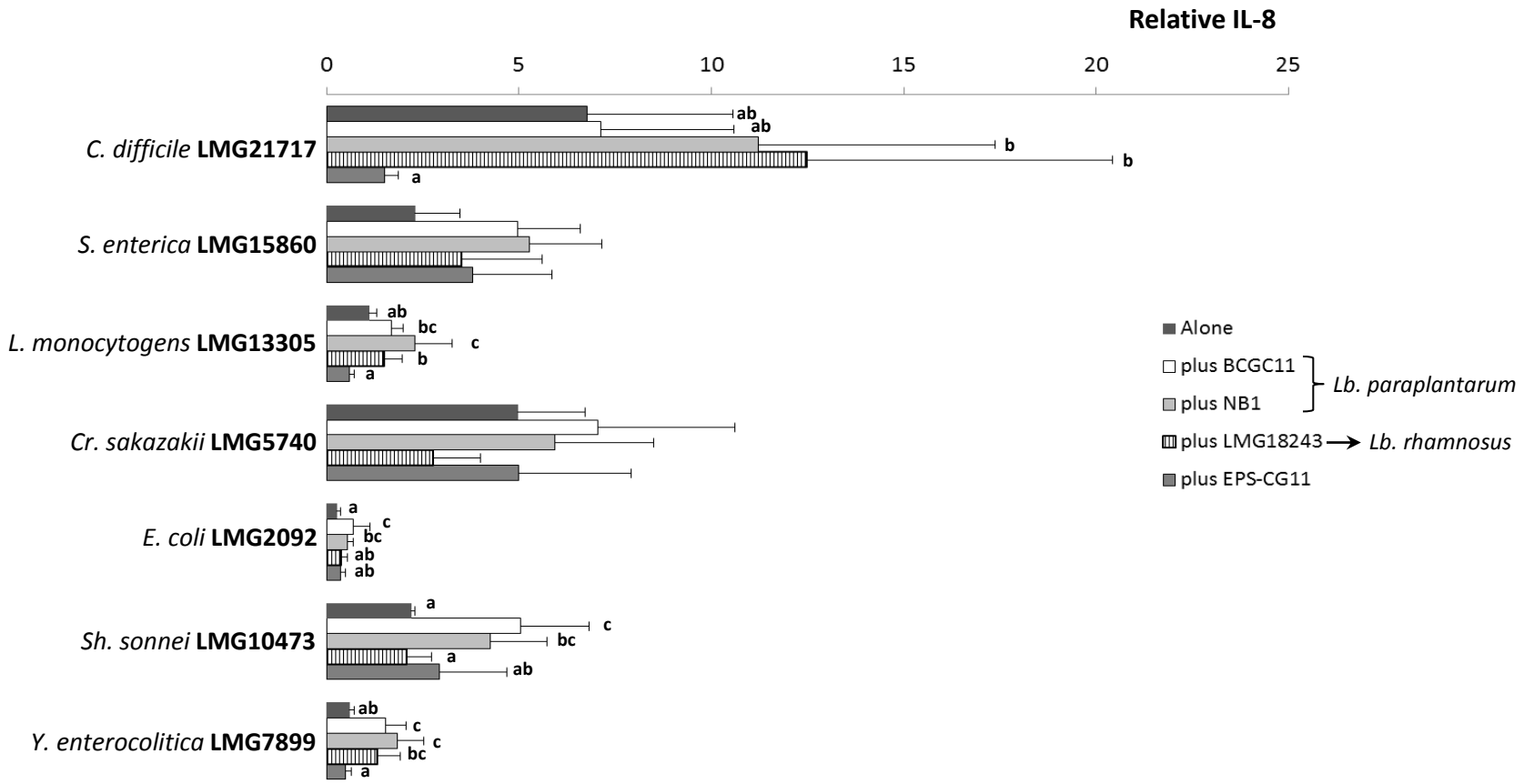
Relative IL-8
0
5
10
15
20
25
C. difficile LMG21717
ab
ab
b
b
a
S. enterica LMG15860
L. monocytogens LMG13305
ab
bc
c
b
a
Cr. sakazakii LMG5740
E. coli LMG2092
a
c
bc
ab
ab
Sh. sonnei LMG10473
a
c
bc
a
ab
Y. enterocolitica LMG7899
ab
c
c
bc
a
Alone
plus BCGC11
plus NB1
Lb. paraplantarum
plus LMG18243
Lb. rhamnosus
plus EPS-CG11

Figure 4

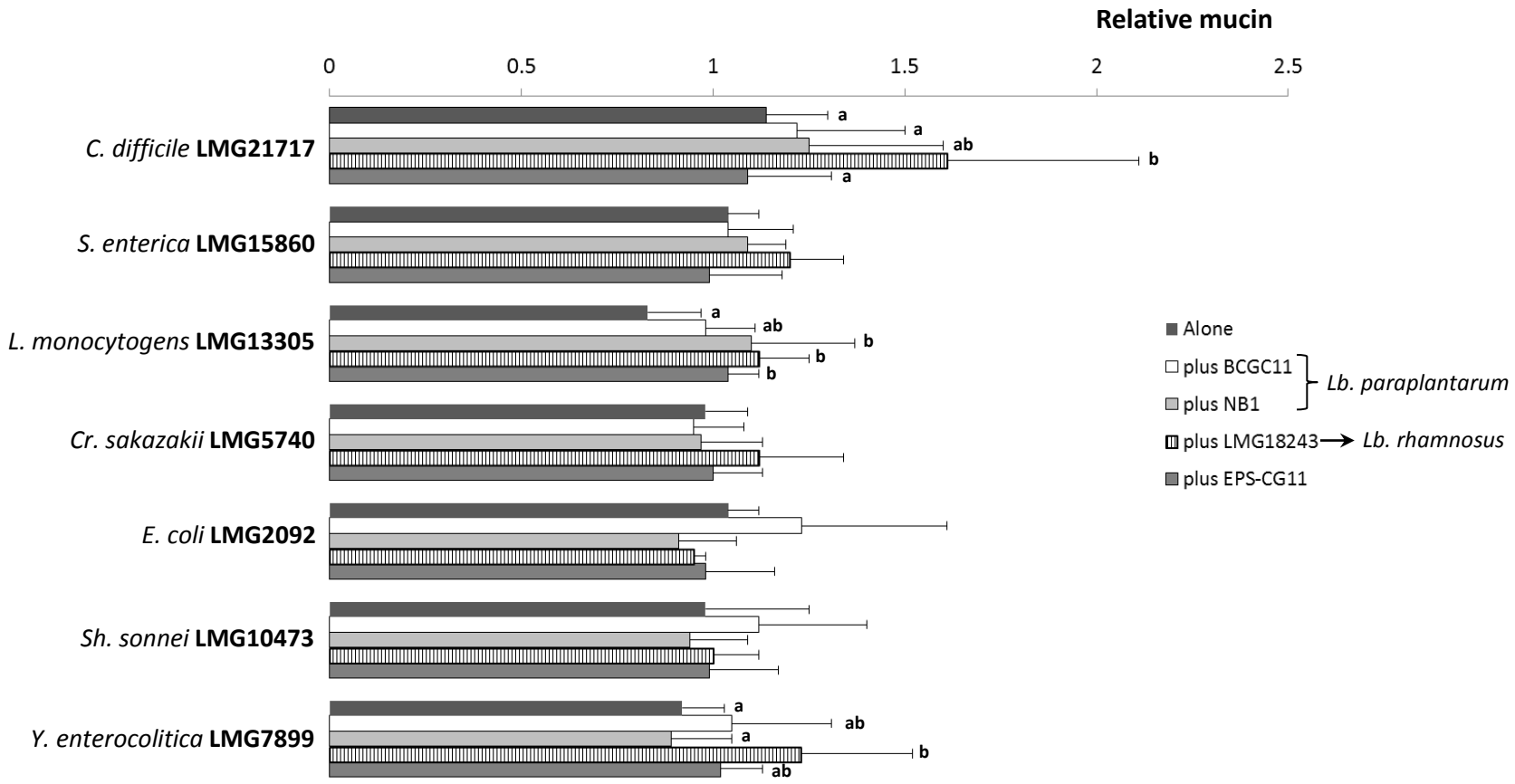
Relative mucin
0
0.5
1
1.5
2
2.5
C. difficile LMG21717
a
a
ab
b
a
S. enterica LMG15860
L. monocytogens LMG13305
a
ab
b
b
b
Cr. sakazakii LMG5740
E. coli LMG2092
Sh. sonnei LMG10473
Y. enterocolitica LMG7899
a
ab
a
b
ab
Alone
plus BCGC11
plus NB1
Lb. paraplantarum
plus LMG18243 → Lb. rhamnosus
plus EPS-CG11

Figure 5