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In vitro probiotic properties of selected lactobacilli and multi-strain consortium on immune function, gut barrier strengthening and gut hormone secretion

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26 **Abstract**

27

28 *Lactobacillus reuteri* ICVB395, *L. gasseri* ICVB392 and *L. gasseri* ICVB396 strains, isolated
29 from vaginal microbiota, were investigated for their probiotic traits. *L. reuteri* ICVB395 strain
30 and the 3RG consortium, associating these three *Lactobacillus* strains, showed the best anti-
31 inflammatory profile on peripheral blood mononuclear cell (PBMC) while *L. gasseri*
32 ICVB392 was the most potent together with the 3RG consortium to strengthen a Caco-2-
33 derived epithelial barrier. The three studied strains induced various secretion levels of
34 glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK) by STC-1 enteroendocrine cells,
35 whereas the 3RG consortium was globally less performing. Specific antagonists of protein G
36 receptors, CaSR (Calcium-Sensing Receptor) and GPRC6A (G protein-coupled receptor
37 family C group 6 member A), and inhibitor of the peptide transporter Pept-1 provoked
38 differential modulation of the GLP-1 and CCK secretion by STC-1 cells, indicating that
39 different mechanisms are involved in the capacity of *lactobacilli* and the 3RG to modulate gut
40 hormones secretion.

41

42 **1. Introduction**

43 The gastro-intestinal tract houses billions of microorganisms, namely the gut microbiota,
44 dominated by bacteria, and characterized by their extreme diversity and their key role in the
45 gut homeostasis (Arulampalam, Greicius, & Pettersson, 2006; Sanders, 2016; Stubbendieck,
46 Vargas-Bautista, & Straight, 2016). Nowadays this rich and opulent gut microbiota is
47 generally considered as a full microbial “endocrine organ” (Clarke et al., 2014; Jayasinghe,
48 Chiavaroli, Holland, Cutfield, & O’Sullivan, 2016). Amongst multitude roles of this
49 microbiota we can quote the protection of the host against the invasion of unwanted
50 microorganisms, the contribution to the proper functioning of the immune system, its
51 influence on glucose and lipid homeostasis, and its role on the degradation of insoluble
52 dietary fibres in short chain fatty acids known to exhibit a beneficial impact on the anti-
53 inflammatory and metabolic responses (Delzenne & Cani, 2011; Drissi, Raoult, & Merhej,
54 2017; Sánchez et al., 2017). Nonetheless the gut microbiota has also a function of “dialogue”
55 with the digestive tract, interacting with the intestinal cells (Cani & Knauf, 2016). The
56 diversity of the gut microbiota can be overthrown by drastic changes related to clinical
57 practice and external pressure, notably the mode of delivery and new-borns feeding, medical
58 treatments such as antibiotic, nutrition and health care behaviours and environmental
59 exposure. This can disrupt temporarily the balance of this complex ecosystem leading to
60 digestive discomfort, and the development of chronic diseases (Beaugerie & Petit, 2004;
61 Quigley, 2013). These perturbations are associated with a dramatic increase in incidence of
62 immune-mediated diseases including allergic and inflammatory bowel diseases but also
63 metabolic diseases including obesity and diabetes and most likely neurodegenerative and
64 psychiatric diseases (Cani & Knauf, 2016; Doré, Multon, Béhier, & participants of Giens
65 XXXII, Round Table No.2, 2017; Fernandez, Lasa, & Man, 2014). Therefore, the
66 development of approaches targeting the key features of this altered host-microbes

67 interactions are highly relevant. Probiotics precisely aimed at preserving and/or restoring the
68 balance of this delicate ecosystem and therefore respond to the precise definition emitted in
69 2002 by the Food and Agriculture Organisation (FAO) and World Health Organization
70 (WHO), establishing that probiotics are "live microorganisms which, when ingested in
71 adequate amounts, exert positive effects on health, beyond traditional nutritional effects"
72 (FAO/WHO, 2002). Probiotic bacteria, mainly belonging to the *Lactobacillus* or
73 *Bifidobacterium* genera, are natural inhabitants of the gastrointestinal (GI) tract (Vaughan &
74 Mollet, 1999). Probiotics have to survive to the passage through the stomach and the upper
75 part of the small intestine before reaching their site of action to provide their benefits (Fooks
76 & Gibson, 2002). To overcome this challenge, the selection of potential probiotics bacteria
77 mainly focus on their ability to survive to the harsh conditions of the GI tract (Morelli, 2000).
78 Probiotics could be provided as a unique individual strain or as combination of multiple
79 strains. Multi-strain cocktails could present many advantages providing more benefits
80 compared to those of microorganisms taken alone (Timmerman, Koning, Mulder, Rombouts,
81 & Beynen, 2004). Indeed, combining multiple strains belonging to different species able to
82 colonize different parts of the digestive tract could be more effective to restore gut
83 homeostasis and to express their positive effects, as these strains can work simultaneously on
84 the different potential causes of the observed disorders (Collado, Meriluoto, & Salminen,
85 2007; Timmerman et al., 2004). Use of consortium strains could be justified by the individual
86 specific response of the host, as natural probiotic predominant species can vary from one
87 individual to another. Each strain also helps to generate specific enzyme activities and can
88 stimulate the immune system by different pathways (Sánchez et al., 2017). Bacteria belonging
89 to the two main families of known probiotics are found in various locations in the intestine.
90 *Lactobacilli* are natural residents of the small intestine while the bifidobacteria are dominant
91 in the colon, their natural habitat (Collado et al., 2007; Gionchetti, Lammers, Rizzello, &

92 Campieri, 2005; Perdigon, Galdeano, Valdez, & Medici, 2003; Timmerman et al., 2004).
93 Most **lactobacilli** and **bifidobacteria species are considered as GRAS** “Generally Recognized
94 As Safe” (Hugas & Monfort, 1997). The benefits of these bacteria are well known for decades
95 and many traditional and commercial **probiotic preparations contain them** (Di Cerbo, Palmieri,
96 Aponte, Morales-Medina, & Iannitti, 2015; Drissi et al., 2017). Probiotic strains express their
97 **benefits by strengthening of the intestinal barrier, regulating sugar and lipid metabolism,**
98 **reducing inflammation or preventing pathogens invasion** (Botta, Langerholc, Cencič, &
99 Cocolin, 2014; Di Cerbo et al., 2015; Park, Oh, & Cha, 2014; Takemura, Okubo, &
100 Sonoyama, 2010). **Moreover probiotics, especially lactobacilli, synthesize a wide variety of**
101 **proteases, implied in the food maturation process such as in fermented cheeses** (Liu, Bayjanov,
102 Renckens, Nauta, & Siezen, 2010). **Amongst which, serine proteases are produced by many**
103 ***lactobacilli* species, notably by *L. gasseri*, *L. plantarum* and some *L. acidophilus* strains** (Law
104 & Haandrikman, 1997; Margono, Sumaryono, Malik, & Sadikin, 2014). Moreover they
105 produce, lactic acid promoting the balance of intestinal pH, and sometimes antimicrobial
106 substances that inhibit the growth of pathogens (Drissi et al., 2017).
107 Furthermore, recent works established that probiotic **lactobacilli** are able to interact with
108 intestinal cells and induce modulation of gut hormones; as glucagon-like peptide-1 (GLP-1);
109 cholecystokinin (CCK) and PYY peptide (Panwar et al., 2016; Yadav, Lee, Lloyd, Walter, &
110 Rane, 2013). These hormones, because of their influence in food intake regulation and
111 glucose homeostasis, represent promising lever to manage and reduce chronic metabolic
112 diseases like obesity and associated type 2-diabetes (Caron, Domenger, Dhulster, Ravallec, &
113 Cudennec, 2017).
114 During a previous work, some ***Lactobacillus*** strains, isolated from the vaginal microbiota,
115 exhibited many traits of probiotics microorganisms, including good **survivability in the harsh**
116 **conditions** of the GI tract and adhesion to epithelial cells without toxicity (Belguesmia et al.,

117 2016). During these researches some strains, including *L. gasseri* ICVB392, *L. reuteri*
118 ICVB395 and *L. gasseri* ICVB396, initially designated CMUL34, CMUL67 and CMUL80
119 respectively, and renamed after re-identification by 16S rDNA sequencing, appeared good
120 potential probiotics and were able, *in vitro*, to modulate gut hormone expression and secretion
121 in murine enteroendocrine STC-1 cells.

122 The aim of this study is to investigate further probiotic traits of these strains, alone and
123 associated in a consortium. We first evaluated their immunomodulation abilities and their
124 capacity to strengthen the intestinal barrier. Using the enteroendocrine STC-1 cell line, we
125 also studied deeper the impact of the consortium on the secretion of gut hormones, and we
126 unravelled how the bacteria interacted with the cells. Finally, we studied their capacity to
127 limit lipid accumulation in adipocytes.

128

129 **2. Material and methods**

130

131 **2.1. Bacterial strains**

132 The three *Lactobacillus* strains used in this research work were previously isolated from
133 Lebanese vaginal microbiota (Al Kassaa, Hamze, Hober, Chihib, & Drider, 2014), and
134 recently selected for their probiotic traits (Belguesmia et al., 2016). Previously named
135 CMUL34, CMUL67 and CMUL80, they were re-identified by 16S rDNA sequencing and
136 registered in the Institut Charles Viollette Laboratory Collection, under *L. gasseri* ICVB392,
137 *L. reuteri* ICVB395 and *L. gasseri* ICVB396 strains respectively. The strains were also
138 associated in equal amount within the multi-strain cocktail designated during this study as
139 3RG consortium. Before each experiment, the strains were grown for 18-24 h at 37°C in de
140 Man-Rogosa-Sharpe (MRS) medium (De Man, Rogosa, & Sharpe, 1960). Two additional
141 strains were used as control strains for immune cells stimulation: *Bifidobacterium longum* IPL

142 A7.5 was grown at 37°C in anaerobic condition (GENbag anaer, Biomérieux, France) in MRS
143 (Difco, Detroit, USA) supplemented with 0.1% (w/v) L-cysteine hydrochloride (Sigma) and
144 *Lactococcus lactis* MG1363 was cultured at 30°C, in M17 Broth supplemented with 0.5%
145 glucose (Difco, Detroit, USA). For *in vitro* studies (stimulation of PBMCs, Caco-2 epithelial
146 barrier and adipocytes), bacteria were grown overnight, washed twice in sterile phosphate
147 buffered saline (PBS) buffer pH 7.2 and resuspended at a final concentration of 2×10^9
148 cfu.mL⁻¹ in PBS.

149

150 **2.2. *In vitro* immunomodulation assays**

151 **Blood samples from five different healthy adult donors were obtained at the Etablissement**
152 **Français du Sang (French National Blood Service), in accordance with our institution**
153 **committees (INSERM, CNRS and Institut Pasteur de Lille, agreement N° DC 2013-2022).**

154 Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood as already
155 described (Foligne et al., 2007). Briefly, after Ficoll gradient centrifugation (GE Healthcare
156 Bio-Sciences, Uppsala, Sweden), mononuclear cells were collected, washed in RPMI-1640
157 medium (Gibco, Life Technologies, Ghent, Belgium), and adjusted to 2×10^6 cells per mL in
158 RPMI supplemented with gentamicin (150 µg.mL⁻¹), L-glutamine (2 mM), and 10% heat-
159 inactivated FCS (Gibco, Life Technologies, Ghent, Belgium). PBMCs were stimulated with
160 phosphate-buffered saline (PBS, Gibco, Life Technologies, Ghent, Belgium) or bacteria at a
161 bacteria-to-cell ratio of 10:1 for 24 h at 37 °C with 5% CO₂. The supernatants were collected
162 and stored at -20°C until cytokines (IL-10 IL-12 and IFN-γ) measurements performed using
163 R&D Duoset ELISA kits (R&D, Minneapolis, MN, USA). *B. longum* and *Lactococcus lactis*
164 were used as positive control for the induction of anti-inflammatory (IL-10) and Th-1/pro-
165 inflammatory (IL-12 and IFN-γ) cytokine secretion by PBMCs, respectively.

166

167 **2.3. Epithelial barrier model**

168 The human colon epithelial cell line Caco-2 clone TC7 (Chantret et al., 1994) was used to
169 study the impact of the **lactobacilli** on trans-epithelial electric resistance (TEER). The Caco-2
170 cells were grown at 37°C with 10% CO₂ in DMEM supplemented with 5% heat-inactivated
171 foetal calf serum (FCS, Gibco, Life technologies, Ghent, Belgium), 1% Non-Essential Amino
172 Acids (Gibco, Life Technologies, Ghent, Belgium), 100 U.mL⁻¹ penicillin and 100 µg.mL⁻¹
173 streptomycin (Gibco, Life Technologies, Ghent, Belgium) and 2mM L-glutamine (Gibco, Life
174 Technologies, Ghent, Belgium).

175 For the permeability test, polarized Caco-2 monolayers were prepared by growing the
176 epithelial cells on 12-wells Transwell® insert filters (polycarbonate membrane with 3 µm
177 pore size, 12 mm diameters, Costar, Corning Life Science, Kennebunk, ME, USA) at a
178 density of 10⁵ cells per cm². The medium was changed every two days until 14 days when
179 optimal trans-epithelial resistance (TEER ≥ 1800 Ω/cm²) was reached (which was measured
180 every 2 days 1 h after changing medium using a millicell-ERS (Electrical Resistance System;
181 Millipore, Billerica, MA, USA). At day 14, fresh medium without FCS was added and cells
182 were treated, in the apical compartment with bacteria (or not) at a bacteria-to-cell ratio of 10:1,
183 30 min before the addition of hydrogen peroxide (H₂O₂) in both basal and apical compartment
184 (at 100µM final concentration). TEER was measured before H₂O₂ addition (T0) and every 30
185 min until 120 min. The results were compared to non-treated cells. Three different
186 experiments were performed including duplicates of each condition and results were
187 expressed in % TEER compared to T0 ± SEM.

188

189 **2.4. Lipid accumulation in adipocytes**

190 The effect of **lactobacilli** on lipid accumulation in adipocytes was studied using the pre-
191 adipocyte 3T3-L1 murine cell line. The 3T3-L1 cells were grown at 37°C with 5% CO₂ in

192 DMEM supplemented with 10% heat-inactivated FCS, 100 U.mL⁻¹ penicillin and 100 µg.mL⁻¹
193 streptomycin and 2 mM L-glutamine. Cells were used between the 10th and the 16th passage.
194 The 3T3-L1 cells were distributed in 12-wells plates at a concentration of 3500 cells per wells
195 and were differentiated in adipocytes according to the protocol described by (Zebisch, Voigt,
196 Wabitsch, & Brandsch, 2012). Briefly, cells were grown in medium supplemented with 0,5
197 mM 3-isobutyl-1-methylxanthine, 1 µg.mL⁻¹ insulin and 0.25 µM dexamethasone. After 48h,
198 fresh medium supplemented with only insulin (1 µg.mL⁻¹) was replaced. Basal medium was
199 changed every two days for 10 days, until cells were differentiated in mature adipocytes. Cells
200 were then stimulated for 24h with bacteria (or not) at a bacteria-to-cell ratio of 10:1, in fresh
201 medium in the presence of 150 µg.mL⁻¹ gentamicin. Lipid accumulation was quantified by
202 Oil- Red-O staining. Briefly, cells were stained using 1 ml ready-to-use Oil-red O solution
203 (DiaPath, Martinengo, Italy) for 15 min., washed 3 times with PBS (Gibco, Life Technologies,
204 Ghent, Belgium). Oil-Red-O was eluting with isopropanol for 30 min incubation. Optic
205 density was measured at 490 nm by a spectrophotometer (ELx808, Biotech instruments). The
206 percentage of Oil-red-O stained cells relative to control cells without bacteria was calculated
207 as (A490nm [probiotic sample]/A490nm[control])*100.

208

209 ***2.5. Gut hormones secretion study***

210 The STC-1 murine cell line, derived from the intestinal tumour of double transgenic mice,
211 gratefully received from Dr. C. Roche (INSERM U865, Lyon, France), was used for gut
212 hormones study. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM,
213 Invitrogen, France), supplemented with 10% foetal bovine serum, 5 mM of L-glutamine and
214 100 U.mL⁻¹ of penicillin and streptomycin, at 37°C in 5% CO₂-95% air atmosphere. The
215 STC-1 cells were passed twice a week, and were used between the 60th and the 65th passage
216 for the different assays. STC-1 cells were seeded in 24 wells plate at 40,000 cells/well and

217 were grown in DMEM culture medium for 48-72h. Cells were washed twice with HEPES
218 buffer without glucose (NaCl 140 mM, HEPES 20 mM, KCl 4.5 mM, CaCl₂ 1.2 mM, MgCl₂
219 1.2 mM, adjusted to pH 7.4 with NaOH 3M) and then co-incubated with 10⁸ CFU.mL⁻¹ of the
220 selected *Lactobacillus* strains, alone or in combination, for 8 hours at 37°C in 5% CO₂-95%
221 air atmosphere. Purified peptidoglycan from *L. acidophilus* (Macho-Fernandez et al., 2011),
222 and purified flagellin from *Salmonella enterica* serovar Typhimurium (gratefully supplied by
223 Dr Jean Claude Sirard from Institut Pasteur de Lille) were tested at 50 and 10 µg.mL⁻¹
224 respectively, to evaluate their impact on gut hormones secretion. The resulting supernatants
225 were centrifuged (8000 g for 10 min) and were kept at -20°C. GLP-1 and CCK quantifications
226 were realized by Radio-Immuno Assay (RIA) using EMD Milipore (USA) and Cisbio
227 International (France) kits for each hormone, respectively.

228 To examine the capacity of bacteria to degrade the gut hormones, pure active GLP-1 1-26
229 (EMD Milipore, USA) and CCK8S (Sigma Aldrich Merck, Germany) peptide hormones were
230 used at initial concentration of 800 pM and 600 pM, respectively. *Lactobacilli* (at 10⁷
231 CFU.mL⁻¹) were incubated in HEPES buffer solution containing active GLP-1 or CCK8S, in
232 presence or absence of DPP-IV enzyme inhibitor (Ile-Pro-Ile, Sigma-Aldrich Germany) at 1
233 mg.mL⁻¹, during 8 h at 37°C. The supernatants were recovered by centrifugation at 8000 g,
234 4°C for 10 min, and quantification of remaining active GLP- 1 or CCK8S in the supernatants
235 was performed by RIA as previously described. Negative control without bacteria was
236 incubated in the same conditions for both hormones. The results were expressed in percentage
237 of initial concentration of active GLP-1 or CCK8S.

238 The effects of CaSR and GPRC6A antagonists and Pept-1 inhibitor on the secretion of GLP-1
239 and CCK hormones by STC-1 after contact with selected *Lactobacillus* strains were studied.
240 The two antagonists and the inhibitor were prepared according to the suppliers
241 recommendations. NPS 2143 (Sigma Aldrich, Merck Germany), CpD (Enamine, Ukraine),

242 and 4-aminomethylbenzoic acid (AMBA-4, Sigma Aldrich, Merck Germany) were used at a
243 final concentration of 25 μ M, 50 μ M and 10 mM in Hepes Buffer, respectively. The STC-1
244 cells were washed after reaching 80% confluence culture and were incubated for 15 minutes
245 at 37°C in 5% CO₂-95% air atmosphere with 100 μ L of described above solutions. Then the
246 treated STC-1 cells were incubated with *lactobacilli* in the same conditions as described above.
247

248 ***2.6. RT-PCR analysis***

249 The relative transcript levels of PepT1 (Peptide transporter) and β -actin were analysed by
250 quantitative real-time PCR. Briefly, total RNA was extracted from cultured cells using the
251 NucleoSpin® RNA XS (Macherey-Nagel, Germany) according to the manufacturer's
252 instructions. Concentration and purity of each sample were evaluated on a NanoDrop Lite
253 (Thermo Scientific, USA). cDNA was obtained by reverse transcription on a Mastercycler
254 gradient (Eppendorf, Germany) using the RevertAid H Minus First Strand cDNA Synthesis
255 Kit (Thermo Scientific, USA). Finally, reverse transcribed cDNAs were quantified by
256 comparative Ct experiment on a StepOne™ Plus system (Applied BioSystems, Life
257 Technologies, USA) using the Power SYBR Green PCR Master Mix (Applied BioSystems,
258 Life Technologies, USA) and specific oligonucleotides: forward (F) 5'-
259 ACACCCTTAACGAGATGGTCAC-3' and reverse (R) 5'-CCGCCGTGGTGTTTATTGTG-
260 3' for PepT1 and (F) 5'-TGCCCTGAGGCTCTTTTCCA-3' and (R) 5'-
261 GGCATAGAGGTCTTTACGGATGTC-3' for β -actin, all purchased from Eurogentec
262 (France). The cycling conditions were 10 min at 95 °C, 40 cycles of 15 sec at 95 °C, 30 sec at
263 60 or 61 °C and 30 sec at 72 °C, followed by a melting curve step.

264

265 ***2.7. Statistical analysis***

266 Data were expressed as mean \pm standard deviation (SD) calculated over three independent
267 experiments performed in triplicate. SigmaPlot 11.0 software (Germany) was used to carry
268 out statistical analysis. One-Way ANOVA followed by a pairwise comparison with Tukey's
269 test was used for comparison of data with normal distribution. p values < 0.05 were regarded
270 as significant.

271

272 **3. Results**

273 ***3.1 Immunomodulatory capacities of the lactobacilli***

274 The three *Lactobacillus* strains, incubated alone and in 3RG multi-strain consortium with the
275 peripheral blood mononuclear cells induced different cytokine secretion profiles. The two
276 strains of *L. gasseri* ICV396 and ICV392 were not able to induce significant release of IL-10
277 conversely to *L. reuteri* ICVB395 strain which induced significant levels in comparison to
278 untreated cells, reaching similar level (900 pg.mL⁻¹) observed with the control anti-
279 inflammatory strain *B. longum*. The consortium 3RG was also able to induce IL-10 secretion
280 after PBMC stimulation, but at a lower level, reaching approximately 400 pg.mL⁻¹ despite no
281 significant difference when compared to the control (Figure 1A). The three individual
282 *Lactobacillus* strains and the 3RG consortium were not able to induce detectable IL-12 and
283 IFN γ secretion by PBMC (Figure 1B, 1C), while the control *L. lactis* strain induced
284 significant amount of these two Th1/pro-inflammatory cytokines.

285

286 ***3.2 Capacity of the strains to strengthen the epithelial barrier***

287 We evaluated the capacity of the strains to restore the gut barrier function using an *in vitro*
288 model of H₂O₂-sensitized Caco-2 cells monolayers, as previously reported (J. Alard et al.,
289 2018). As expected, H₂O₂ sensitization induced permeability as shown by a significant and
290 time-dependent drop in the trans-epithelial electric resistance (TEER). All the strains were

291 able to restore the epithelial barrier, as shown by an increase in the TEER as compared to
292 H₂O₂-sensitized control cells (Figure 2), which was however not significant for *L. gasseri*
293 ICVB396 and *L. reuteri* ICVB395 which was able to maintain the TEER at the level of
294 untreated control only until 60 min. Interestingly, the 3RG consortium and the *L. gasseri*
295 ICVB392 strain not only restored but even reinforced the epithelial barrier, the TEER being
296 higher than the H₂O₂ non-sensitized control monolayer.

297

298 **3.3 Impact on lipid accumulation in adipocytes**

299 Lipid accumulation observed in 3T3-L1 derived mature adipocytes treated with the
300 **lactobacilli** were generally lower than the level found in non-treated adipocytes. The most
301 important reduction was observed with the *L. reuteri* ICVB395 and *L. gasseri* ICVB396
302 strains, which induced significant drop of lipid level comparatively to the control, reaching
303 respectively 30% and 20% decrease in comparison to the control level (Figure 3). The third
304 strain, *L. gasseri* ICVB392, as well as the 3RG consortium showed also a similar tendency
305 which was however not significant (Figure 3).

306

307 **3.4 Capacity of the *lactobacilli* strains to modulate gut hormones secretion**

308 **3.4.1 Effects on active-GLP-1 secretion**

309 We evaluated the capacity of the strains to induce the release of GLP-1 using the STC-1
310 enteroendocrine cell line. After 8 hours bacterial stimulation of STC-1 cells, the highest GLP-
311 1 secretion was obtained with *L. gasseri* ICVB392 and *L. reuteri* ICVB395, which induced
312 hormone secretion estimated to 6 fold higher than the control level. *L. gasseri* ICVB396 and
313 the 3RG consortium induced also significant GLP-1 release, which was, however lower,
314 reaching 4 fold and 3 fold of the control GLP-1 secretion level, respectively (Figure 4A).

315

316 **3.5.2 Effects on CCK secretion**

317 The *Lactobacillus* strains tested in this study exhibited moderate impact on the CCK secretion
318 by stimulated STC-1 cells (Figure 4B). *L. reuteri* ICVB395 and *L. gasseri* ICVB396 showed
319 the most important effect inducing significant CCK secretion, reaching level more than one
320 fold and half of the control level. The association of these strain with *L. gasseri* ICVB392, in
321 the 3RG consortium led to a decrease of the quantified CCK level compared to those
322 obtained with ICVB395 and ICVB396 tested alone (Figure 4B). Purified peptidoglycan from
323 *L. acidophilus* and flagellin from *S. enteritidis* serovar Thyphimirium didn't stimulate the
324 secretion of both CCK and GLP-1 by STC-1 cells (data not shown).

325

326 **3.5.3 Investigation of the different pathways involved on gut hormone secretion modulation**

327 Since GLP-1 and CCK secretion are under the influence of three major signalling pathways
328 implying protein G receptors, notably the calcium-sensing receptor (CaSR) and the GPR
329 family C group 6 member A (GPRC6A), and the di/tripeptide transporter 1 (Pept1), we
330 evaluated their respective role using CaSR and GPRC6A antagonists and Pept-1 inhibitor.

331 We first studied *Pept-1* gene expression in the enteroendocrine STC-1 cells. RT-PCR analysis
332 unveiled a significant gene expression of Pept-1 with CT value obtained of 26.47 ± 0.44 while
333 reference β -actin gene expression showed CT of 14.02 ± 0.22 (data not shown)

334 The addition of CaSR and GPRC6A antagonists induced a significant decrease of GLP-1
335 secretion induced by the stimulation of the STC-1 cells with the *lactobacilli* alone or with the
336 3RG consortium, except for GPRC6A inhibitor with ICVB396 strain (Figure 4A). Analysis of
337 effect of CaSR antagonist indicated that the most important decrease is observed for the
338 ICVB392 and ICVB395 strains for which the GLP-1 level decreased from 6 to around 4 fold
339 the control level. The GLP-1 secretion level reduction observed for the ICVB396 strain and
340 the 3RG consortium was moderate decreasing from 4 to 2.5 fold and from 3 to less than 2 fold

341 of the control level, respectively. Regarding the effect of the GPRC6A antagonist, we
342 observed more or less the same effect with major decrease for the ICVB392 and ICV395
343 strains, and moderate for the 3RG consortium. However the decrease of GLP-1 level
344 measured for the ICV396 pre-treated STC-1 was not significant (Figure 4A). Interestingly
345 Pept-1 inhibitor induced a reduction of GLP-1 secretion for all strains and the 3RG
346 consortium, nearly to the same levels obtained with GPRC6A inhibitor, except for the strain
347 ICVB395 for which GLP-1 secretion was not impacted by AMBA-4 Pept-1 inhibitor.
348 The results obtained for CCK were slightly different, as we observed a statistically significant
349 effect just for the ICVB396 strains and 3RG consortium after pre-treatment with CaSR
350 antagonist. GPRC6A and Pept-1 antagonist and inhibitor didn't appear to affect the CCK
351 secretion level in a significant manner in all cases studied in this work, excepted for a slight
352 effect which was observed with Pept-1 inhibitor and GPRC6A antagonists on the effect of
353 ICVB392 strain and the 3RG consortium respectively (Figure 4B).

354

355 **3.5.4 Capacity of selected *lactobacilli* strains to degrade GLP-1 1-26 and CCK8S**

356 When the GLP-1 1-26 (800 pM) and CCK8S (600 pM) peptide solutions (Figure 5, black
357 bars) were incubated with the selected strains and the consortium for 8h, significant decreases
358 of peptides were observed in the presence of *L. gasseri* ICVB396 and the 3RG multi-strains
359 cocktail. Resulting concentrations of around 550 pM of active GLP-1 1-26, and of around 180
360 and 340 pM of CCK8S, were measured after incubation with *L. gasseri* ICVB396 strain and
361 3RG consortium, respectively. A significant impact was also observed for *L. gasseri*
362 ICVB392 on the measured amount of CCK with a decrease from 600 to 400 pM after
363 incubation.

364 In the presence of the tripeptide (Ile-Pro-Ile), a DPP-IV inhibitor (grey bars), the consumption
365 of GLP-1 1-26 and CCK8S by *L. gasseri* ICVB392 and ICVB396 was reduced to nearly the

366 control level. However, the DPP-IV inhibitor did not significantly affect the consumption of
367 GLP-1 and CCK by the 3RG consortium (Figure 5 A, B).

368

369 **4. Discussion**

370 In the present study, we investigated additional probiotic traits of *Lactobacillus* strains
371 selected during previous works (Al Kassaa et al., 2014; Belguesmia et al., 2016). We
372 previously established, using Caco-2 cells stimulation, that the three strains, selected in the
373 present study, exhibited promising anti-inflammatory abilities, limiting the level of IL1- β -
374 induced IL-8 release and inducing the secretion of IL-10 (Belguesmia et al., 2016). It has been
375 pointed out that in vitro immunomodulation assays have to be considered carefully regarding
376 the type and the physiological state of the eukaryotic cell models. Indeed probiotic strains
377 could display different immunomodulatory profiles using epithelial cell model (i.e. HT-29 cell
378 line) and PBMC stimulation used (Kechaou et al., 2013). Taking in account this postulate and
379 in order to consolidate the results obtained on epithelial intestinal cells, we evaluated the
380 immunomodulation capacities of the selected *Lactobacillus* strains, considered alone or in
381 combination in the 3RG consortium, using in vitro PBMCs stimulation. We notably unravel
382 their ability to induce the secretion of the anti-inflammatory IL-10 versus the Th1/pro-
383 inflammatory IL-12 and IFN γ cytokines, We previously observed that in vitro
384 immunomodulation abilities of lactobacilli are strain-specific and linked to their in vivo
385 protective effects in murine models of colitis (Foligne et al., 2007).

386 We revealed during PBMC study that the *L. reuteri* ICVB395 strain was the most potent
387 strain to induce the secretion of the anti-inflammatory IL-10 cytokine, while the two other *L.*
388 *gasseri* strains, were not able to induce significant IL-10 response comparatively to the
389 negative control. The 3RG consortium was able to induce moderate but significant secretion
390 of this interleukin.

391 Nonetheless in the present study, none of the strains alone or in the 3RG consortium was able
392 to induce IL-12 or interferon γ (IFN- γ) which overall remained undetectable (Figure 1C). IL-
393 12 cytokine plays an important role in activating the Th1 immune response by promoting the
394 differentiation of naïve T cell to Th1 cells to produce interferon γ (IFN- γ) (Meijerink et al.,
395 2012; Watson, Sargianou, & Panos, 2012). In contrast, IL-10 is known to inhibit natural killer
396 (NK) and Th1 cells, by down-regulating the IL-12 production and facilitating Th2 immune
397 response (Tripp, Wolf, & Unanue, 1993; Uyemura et al., 1996). The balance between these
398 two interleukins defines the immune response and plays a major role in the anti-
399 inflammatory/pro-inflammatory state which is dysregulated during colitis, allergy, irritable
400 bowel syndrome and other inflammatory diseases. Indeed, a high IL-10/IL-12 ratio could
401 predict favourable anti-inflammatory abilities of probiotic strains, especially for **lactobacilli**
402 (Foligne et al., 2007; Meijerink et al., 2012; Watson et al., 2012). Hence the bacterial strains
403 tested showed differential abilities to influence the inflammatory state.

404 Chronic inflammatory diseases are often associated with an increased intestinal permeability
405 known as leaky gut which facilitates the translocation of commensal bacteria, thus
406 contributing to the development of a chronic inflammatory state. We then evaluated the
407 capacity of the strains to strengthen the epithelial barrier using an in vitro model of epithelial
408 barrier. All the strains were able to attenuate the H₂O₂-induced permeability. However, the
409 best ability to strengthen the epithelial barrier was observed with *L. gasseri* ICVB392 and the
410 3RG consortium which were not only able to restore the H₂O₂-sensitized monolayer but were
411 also able to reinforce the trans-epithelial resistance. Among studied probiotic properties of
412 microorganisms, TEER assay appeared as a reliable method to anticipate and establish effect
413 of potential probiotics strains on epithelium (J. Alard et al., 2018; Klingberg, Pedersen,
414 Cencic, & Budde, 2005; Messaoudi et al., 2012). Most studies showed protective effect of
415 probiotic strains, improving permeability of sensitized epithelial monolayer, with some strains

416 able also to reinforce the epithelium barrier with increased TEER values over the basal level
417 observed for untreated control epithelium (Anderson et al., 2013; Botta et al., 2014; Ramos,
418 Thorsen, Schwan, & Jespersen, 2013). Mechanisms implied in this phenomenon are still not
419 completely elucidated and seems to be strain-specific (Anderson et al., 2013; Ramos et al.,
420 2013). Some strains seem to display a negative effect (i.e. *L. fermentum* RGR1487) whereas
421 other (i.e. *L. fermentum* RGR1485) have neutral/positive effect on the TEER of Caco-2 cells
422 epithelium (Anderson et al., 2013). Ramos et al. (2013) isolated a number of probiotic
423 **lactobacilli**, belonging to *L. plantarum*, *L. brevis* and *L. fermentum* species, from different
424 Brazilian food products. Most of these strains showed enhanced TEER, but not at the same
425 level and independently from species consideration. In a previous study, we were also able to
426 select strains (*L. acidophilus* PI11, *L. helveticus* PI5 and *L. gasseri* LA806) able to restore and
427 reinforce the epithelial barrier using the same in vitro model (J. Alard et al., 2018). Recently,
428 Kawano et al., stated that the probiotic *L. gasseri* SBT2055 (LG2055) exhibits anti-obesity
429 effects by improving the intestinal integrity and thus reducing the entry of inflammatory
430 substances like endotoxin from the gut lumen, which may improve the inflammation state
431 within metabolic organs (Kawano, Miyoshi, Ogawa, Sakai, & Kadooka, 2016).

432 Another aspect of probiotic property investigated during our study is the ability of the strains
433 to influence the accumulation of lipid in adipocytes. We showed that two of the tested strains
434 (*L. reuteri* ICV39 and *L. gasseri* ICV396) induced significant reduction of lipid accumulation
435 in adipocytes. The third strain, *L. gasseri* ICVB392, doesn't reach such reduction but achieve
436 a lowering tendency. Similar behaviour was also observed for the 3RG consortium containing
437 the three strains with an intermediate effect but a lower efficiency than the *L. reuteri*
438 ICVB395 and *L. gasseri* ICVB396 strains considered alone. In previous work, Park et al.,
439 showed that a probiotic strain of *L. brevis*, designated KLEB, inhibited lipid accumulation in
440 the differentiated 3T3-L1 adipocytes by downregulating the expression of adipogenic

441 transcription factors and other specific genes involved in lipid metabolism, leading to the
442 inhibition of adipocyte differentiation, intracellular triglyceride accumulation and a decrease
443 of glycerol-3-phosphate dehydrogenase (GPDH) activity (Park et al., 2014). In an *in vivo*
444 study using high fat diet-fed C57BL/6 mice, a strain of *L. bulgaricus* N°14 was clearly able to
445 reduce adipocytes size, the weight of white adipose tissue and the serum leptin and cholesterol
446 levels (Takemura et al., 2010).

447 In a recent review, Drissi et al. analysed the results of experimental and clinical studies which
448 evaluated the impact of **lactobacilli** on animal and human body weight and reported a strain
449 specific effect. The administration of *L. reuteri*, *L. sakei*, *L. acidophilus* and *L. casei* was
450 associated with weight gain in human, while the consumption of specific strains of *L. gasseri*,
451 *L. amylovorus*, *L. plantarum* and some *L. acidophilus* strains was associated with weight loss
452 in obese humans and body fat loss in overweight healthy individuals (Drissi et al., 2017).

453 Stenman et al. also identified promising probiotics for preclinical studies including several
454 **Lactobacillus** strains, notably *L. acidophilus* NCFM, *L. gasseri* 2055, *L. reuteri* GMNL-263
455 and the multistrain consortium LGG/Bb12 and VSL#3, which showed proven benefits on
456 insulin insensitivity, fat accumulation and weight loss during *in vivo* assays in human and
457 animal trials (Stenman, Burcelin, & Lahtinen, 2016).

458 Gut hormones, notably PYY and GLP-1, released from enteroendocrine cells within the
459 gastrointestinal tract are known to play crucial role not only in the control of satiety and
460 energy balance, but also, notably for GLP-1, numerous effects as incretin hormone on glucose
461 homeostasis (Holst, 2007). GLP-1 was also recently shown to exhibit anti-inflammatory
462 effects and to promote gut barrier integrity (Lebrun et al., 2017). In the present study, using
463 the STC-1 cell line, we confirmed that the selected strains, *L. gasseri* ICVB392, *L. reuteri*
464 ICVB395 and *L. gasseri* ICVB396, were able to modulate the secretion of active GLP-1 and
465 CCK gut hormones. GLP-1 and CCK were demonstrated to be potential targets of probiotic

466 preparations to control food intake and regulate the appetite on mouse model (Yadav et al.,
467 2013). Interestingly, the two strains, *L. gasseri* ICVB392 and *L. reuteri* ICVB395, induced
468 strong GLP-1 secretion, reaching around 6 fold the control level, while the *L. gasseri*
469 ICVB396 induced lower amount however reaching 4 fold the basal level. Surprisingly,
470 mixing the 3 strains in the 3RG consortium seemed to provoke a reduction of the secreted
471 GLP-1, as compared to the levels obtained with individual strains, notably with the two
472 highest ones. Interestingly, we demonstrated that the *L. gasseri* ICVB396 was able, in contrast
473 to the two other strains, to degrade in vitro the active GLP-1 and this was also observed with
474 the mixture. We can thus hypothesize that the GLP-1 released upon STC-1 stimulation with
475 the *L. gasseri* strain, or by the 3RG consortium could be under-evaluated following its
476 degradation by proteases. This result was not surprising regarding the diversity of the
477 proteases present in **lactobacilli** (Law & Haandrikman, 1997; Liu et al., 2010). Dipeptidyl
478 peptidase IV (DPP-IV) is a prolyl oligopeptidase, member of the serine proteases, able to
479 cleave the incretin hormone GLP-1, playing thus a determinant role on its metabolic and
480 immune functions. Two forms of DPP-IV have been described, a soluble circulating form and
481 a transmembrane serine exopeptidase (Aso et al., 2012). DPP-IV hydrolyses the GLP-1(7–36)
482 amide to generate GLP-1(9–36) amide and the N-terminal histidine–alanine dipeptide
483 (Nadkarni, Chepurny, & Holz, 2014). We therefore use a competitive inhibitor of the DPP-IV,
484 the Ile-Pro-Ile tripeptide called diprotin A, to ensure that observed consumption, or
485 degradation, of GLP-1 in the STC-1 culture supernatants was not linked to the presence of
486 serine protease DPP-IV-like enzymes produced by the *Lactobacillus* strains. We showed that
487 the GLP-1 degradation was inhibited by the addition of the competitive inhibitor, while the
488 effect of the 3RG consortium on GLP-1 release seemed to be insensitive to the treatment with
489 the Ile-Pro-Ile tripeptide, maintaining the same decreased level of GLP-1, in the presence or
490 absence of the inhibitor. The peptidic nature of the DPP-IV inhibitor made it susceptible to be

491 degraded by enzymes secreted by the other *lactobacilli*, explaining the absence of inhibitory
492 effects on serine proteases implied in the degradation of GLP-1 1-26 and CCK-8S hormones
493 used in this study. However it is difficult to elucidate the potential effect of enzymes
494 interaction when the three strains consortium partners are mixed in the 3RG multi-strains
495 cocktail. Indeed this interesting observation pointed out the limits of the in vitro models used.
496 However this observation need to be relativized in in vivo conditions, in which GLP-1 is
497 secreted at the basal side of the intestinal epithelium, making the degradation of this hormone
498 by the bacteria not conceivable, since the microorganisms interact with these cells on their
499 apical side (Bohórquez & Liddle, 2011).

500 Similarly to the results observed on GLP-1, CCK secretion level varied significantly when the
501 three strains were mixed in the 3RG consortium. The three *Lactobacillus* strains appeared to
502 be able to increase the level of the CCK secreted by the STC-1 cells. Although the CCK level
503 obtained after incubation with the 3RG multi-strains cocktail was lower (by 20%) than the
504 level obtained with *L. reuteri* ICVB395 or *L. gasseri* ICVB396 tested alone. As for GLP-1
505 experiment, we estimate the ability of the *selected Lactobacilli* strains selected for this study
506 to consume or degrade the CCK8S hormone. We also observed a strong reduction of the
507 CCK8S level when co-incubated with the ICVB392 and ICVB396 strains, or the 3RG
508 consortium. Moreover as for the GLP-1 1-26, the CCK consumption by the two strains was
509 dramatically reduced by the addition of diprotin A. Again, the impact of the 3RG consortium
510 seemed to be insensitive to the addition of the serine protease inhibitor, as the level of the
511 CCK remained the same observed with and without the addition of the diprotin A (Figure 5).
512 This could be explained also in this case by the complex interactions occurring in a multi-
513 strains mixture between strains and STC-1 cells. Rose et al. established that CCK-8 can be
514 cleaved by serine peptidases, an isoform of tripeptidyl peptidase II notably present in the rat
515 brain, and producing CCK-5 and GWM as major fragments. Interestingly CCK-8 degradation

516 was limited by serine-alkylating reagents, permitting recovery of complete initial amount of
517 CCK-8 (C. Rose, Camus, & Schwartz, 1988; Christiane Rose et al., 1996). Interaction of
518 probiotic preparations with intestinal cells, in the control of gut hormone secretion, is the
519 subject of many researches. Most of them, notably using experimental murine models of
520 obesity, pointed the importance of short chain fatty acids (SCFA), which are bacterial
521 fermentation products of soluble fibers by commensal bacteria, on the food intake and weight
522 regulation (Jeanne Alard et al., 2016; Wang et al., 2015; Yadav et al., 2013). However none of
523 the selected strains used in this study are able to produce the main SCFA involved in energy
524 balance, namely butyrate, propionate or acetate, since no detectable level were measured
525 using gas chromatography analysis in the supernatant of the STC-1 cells culture after 8h of
526 incubation with the chosen *Lactobacillus* strains (data not shown). We then investigated the
527 mechanism involved in the interaction between the *lactobacilli* strains and the STC-1 cells.
528 Enteroendocrine mammalian cells (EECs) can interact with bacteria, or derived components,
529 through different receptors. The apical portion of enteroendocrine cells, in contact the gut
530 lumen, may sense bacterial inputs through pattern recognition receptors (PRR) such as toll-
531 like receptors (e.g., TLRs 4, 5, and 9), which are expressed in EECs. Stimulation with
532 bacterial ligands (e.g., LPS or flagellin) following direct contact, have been shown to promote
533 the secretion of gut hormones (Bohórquez & Liddle, 2011). However many other receptors
534 could be involved in interaction with bacteria, we therefore evaluated other possible pathways
535 which could affect the gut hormones secretion.
536 GLP-1 and CCK secretion are under the influence of three major signalling pathways
537 implying protein G receptors, notably the calcium-sensing receptor (CaSR) and the GPR
538 family C group 6 member A (GPRC6A), and the di/tripeptide transporter 1 (Pept1), involving
539 ERK 1/2 phosphorylation or proton uptake coupled to peptide transport (Caron et al., 2017).
540 These receptors were demonstrated to be sensitive to the action of peptide fragments and

541 amino-acids, and their activation led to the release of GLP-1 and CCK gut hormones in the
542 circulatory system (Caron et al., 2017).

543 Previous research established the expression of the gene encoding Pept-1 in the STC-1 cell
544 line (Liou et al., 2011), while other published work indicated low expression levels
545 (Diakogiannaki et al., 2013). We were able, using quantitative PCR, to highlight *pept1* gene
546 expression in the STC-1 cells we used. We therefore evaluate the possible implication of this
547 transporter in the interaction between the *Lactobacillus* strains and the STC-1 cells, as well as
548 the two GRPs.

549 We demonstrated that the inhibitory action of specific antagonists and inhibitors of those
550 receptors and transporter led to different effects on the impact of the tested strains ICVB392,
551 ICVB395 and ICVB396, and the consortium 3RG. The response to the two strains of *L.*
552 *gasseri* ICVB392 and the ICVB396 was modified by the antagonists of CaSR and GPRC6A,
553 and the inhibitor of the Pept1 transporter, leading globally to the decrease GLP-1 secretion in
554 the medium. Although the effect obtained with the *L. reuteri* ICVB395 strain appeared to be
555 insensitive to the action of the Pept1 inhibitor AMBA-4, interestingly the effects of the
556 inhibitor and antagonists were significant for the 3RG consortium. These observations
557 indicated differential interaction of the *Lactobacillus* strains with the STC-1 cells involved in
558 GLP-1 secretion. Conversely the effect of Pept1 inhibitors and antagonists of CasR and
559 GPRC6A on CCK secretion were more limited. However the CaSR antagonist and the Pept1
560 inhibitor affected significantly the interaction of STC-1 with *L. gasseri* ICVB392 and
561 ICVB396, involved in CCK secretion. However the ability of these strains to degrade the
562 GLP-1 and the CCK could minimise the observed induction effects on hormone secretion
563 measured in the supernatant of STC-1 cells culture. The calcium-sensing receptor (CaSR) is a
564 Class C G-protein coupled receptor which senses extracellular levels of calcium ion (Vezzoli,
565 Soldati, & Gambaro, 2009). Various metabolites could activate CaSR response leading to

566 GLP-1 and CCK secretion, like Ca⁺⁺ ions and basic L-amino acids like L-phenylalanine
567 (Caron et al., 2017). Previous studies on *L. gasseri* strains, isolated from vaginal environment,
568 established that those lactic acid bacteria, with good aggregation abilities, possess specific
569 surface protein designated aggregation promoting factor (*afp*) (Boris, Suarez, & Barbes, 1997).
570 This protein appeared to contain basic amino acids and phenylalanine residues which can
571 interact with the surface of host cells (Boris et al., 1997; Jankovic et al., 2003).

572 The GPRC6A receptor is a newly orphanized class C GPCR, for which the first allosteric
573 antagonist, based on the 2-arylindole privileged structure scaffold, was recently reported
574 (Johansson et al., 2015). GPRC6A activation led to initiating signalling cascade,
575 intermediated by Gαq subunit, which induce intracellular Ca⁺⁺ increase and GLP-1 hormone
576 secretion (Caron et al., 2017). Wellendorph et al. established that the GPRC6A was activated
577 by basic α-amino acids, L-Argenin, L-Lysine and L-Ornithine being the most active agonists
578 for this receptor. The role of these amino acids in activation of GPRC6A receptor was
579 confirmed by Oya et al. using GLUTag and STC-1 enteroendocrine cell lines. Furthermore
580 GPRC6A receptor antagonists, a phospholipase C inhibitor or an IP3 receptor antagonist,
581 suppress significantly the L-Ornithine-induced intracellular Ca⁺⁺ concentration and GLP-1
582 secretion, identifying the involvement of this pathway in the hormone secretion (Oya et al.,
583 2013; Wellendorph et al., 2005).

584 Interestingly, peptides and amino acids are generally sensed by EEC via the proton-coupled
585 peptide-transporter PepT1, this nutrient-sensing being linked to hormone secretion (Zietek &
586 Rath, 2016). We previously demonstrated that the muropeptide M-tri-Lys, derived from the
587 peptidoglycan of a selected *Lactobacillus* strain, was able to protect mice from colitis in a
588 NOD2-dependant manner, while the M-tri-Lys-N was not, suggesting that only the M-tri-Lys
589 could be internalized by PepT1 or PepT2 transporters into the cell to interact with the
590 cytosolic receptor NOD2 (Macho-Fernandez et al., 2011). The importance of the main TLR

591 adaptator MyD88 was also linked to the increase of GLP-1 and glucose-dependent
592 insulintropic polypeptide (GIP) secretion when STC-1 cells were co-cultured with
593 *Lactobacilli* strains (Panwar et al., 2016). Moreover it was established that the modulation of
594 the microbiota/SCFA/bile-acid-signalling, impacted the metabolism and the inflammatory
595 processes via GLP-1 and other markers, representing an interesting target for the treatment of
596 chronic diseases (Zietek & Rath, 2016).

597 In vitro tests realized during this study showed that the 3RG consortium exhibited different
598 response profiles on GLP-1 and CCK hormone secretion, in comparison to the strains
599 considered alone. However the association of these strains could retain our interest as it could
600 combine the potential of each strain maximizing their combined effect on the metabolism,
601 even if this was not observed in our experimental in vitro conditions. It remains thus
602 important to evaluate such potential in in vivo animal models to definitively establish the
603 beneficial impact of the proposed 3RG consortium.

604 In conclusion our study established that the selected *Lactobacillus* strains we evaluated in this
605 work, presented interesting probiotic potential regarding in vitro experiment results. Positive
606 impacts on anti-inflammatory cytokine secretion, decrease in lipid accumulation in adipocytes
607 and restoration of the epithelial barrier, were highlighted for ICVB395 strain and in a more
608 limited tendency for 3RG consortium. ICV 392 was the best potent strain to strengthen the
609 epithelial barrier together with the 3RG consortium. The impact of the *lactobacilli* on gut
610 hormone GLP-1 and CCK secretion appeared more difficult to analyse due to the limit of the
611 in vitro enteroendocrine STC-1 cell model used. We indeed established that some of the
612 *Lactobacillus* strains have the ability to degrade the GLP-1 1-26 and CCK-8S, probably,
613 through the action of serine peptidases. To resolve this question a relevant alternative would
614 be to use a polarized EEC in vitro model using insert (transwell) system, in which gut
615 hormones would be secreted at the basolateral compartment while bacteria would be added at

616 the apical surface. A recent study published by Zhang et al., described such approach using
617 secreting GLP-1 Caco-2 cells grown in vitro on inserts (Zhang, Liu, Chen, & Luo, 2018).
618 However, since all these in vitro models present certain limits, we plan to unravel the
619 probiotic potential of these strains in appropriate in vivo models to confirm their beneficial
620 capacities in the context of chronic diseases such as IBD and obesity.

621

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629

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857
858

859 **Figures captions:**

860

861 **Figure. 1 Immunomodulation capacities of the selected strains**

862 Peripheral blood mononuclear cells (PBMCs) derived from human blood were stimulated *in*
863 *vitro* with freshly cultured bacteria at a ratio of 10:1 (bacteria/cells) for 24h. *B. longum* strain
864 was added as anti-inflammatory control and *L. lactis* MG1363 strain as pro-inflammatory
865 control. Control PBMCs were only treated with PBS buffer. Levels of IL-10 (A), IL-12 (B)
866 and IFN γ (C) were measured by ELISA in the supernatant after 24 h stimulation with the
867 selected bacteria. GraphPad Prism was employed for graph preparation and statistical
868 evaluation. Differences between groups were assessed using ANOVA, followed by
869 nonparametric Mann-Whitney test. Data with p value ≤ 0.05 were considered to be significant.
870

871 **Figure. 2 Capacity of the selected *lactobacilli* to restore or strengthen epithelial barrier**
872 **in polarized Caco-2 monolayers sensitized with hydrogen peroxide (H₂O₂).**

873 Caco-2 monolayers were treated at the apical side with the bacteria (at 10:1 bacteria/cell ratio)
874 30 mn before the addition of H₂O₂ (100 μ M). Changes in trans-epithelial electrical resistance
875 (TEER) across Caco-2 cell monolayers were measured before the addition of H₂O₂ (T0) and
876 every 30 mn until 120 mn. Results were expressed as % TEER compared to T0. Values
877 represent the mean of 3 repeated experiments. Means without a common letter are different
878 (p<0.05) using one way ANOVA with Tukey *post hoc* test for pairwise comparisons.
879

880 **Figure. 3 Capacity of the strains to limit lipid accumulation.**

881 Mature 3T3-L1 derived adipocytes were treated for 24h with the bacteria (10:1 bacteria/ cell
882 ratio). **Lipid content was compared** using red-oil staining **and reported** in percentage of level
883 obtained with *lactobacilli*-treated cells versus control untreated cells **(normalized at 100%)**.
884 Values are the mean of four repeated experiments. Means without a common letter are
885 different (p<0.05) using one way ANOVA with Tukey *post hoc* test for pairwise comparisons.
886

887 **Figure. 4 Capacity of the strains to induce the release of gut peptides and impact of**
888 **specific antagonists of CasR and GPRC6A and Pept-1 inhibitor.**

889 CasR antagonist NPS 2143 (25 μ M, dark grey bars), GPRC6A antagonist CpD (50 μ M, grey
890 bars) and Pept-1 inhibitor AMBA-4 (10 mM, black bars) were added (or not, white bars) 15
891 mn prior the addition of the bacteria and active Glucagon-Like Peptide 1 “GLP-1” (A) and

892 cholecystokinin “CCK” (B), secreted by STC-1 cells were measured by RIA. Values are
893 expressed in fold of untreated control cells without inhibitors treatment and are means \pm SD
894 of three repeated experiments. Means without a common letter are different ($p < 0.05$) using
895 one way ANOVA with Tukey *post hoc* test for pairwise comparisons in each inhibitor
896 condition. * $p < 0.05$ vs. control.

897

898 **Figure. 5 Capacity of selected strains to degrade GLP-1 (A) and CCK (B).**

899 (A) GLP-1 1-26 (800 pM) and (B) CCK8S (600 pM) were treated with bacteria (at 10^7
900 CFU.mL⁻¹) in the presence (grey bars) or absence (black bars) of DDP-IV inhibitor for 8h.
901 Remaining quantities of each hormone were measured by RIA and expressed in percentage of
902 control without treatment. Values are expressed in percentage of control and are means \pm SD
903 of three repeated measurements. Means without a common letter are different ($p < 0.05$) using
904 one way ANOVA with Tukey *post hoc* test for pairwise comparisons.

905

Figure 1.

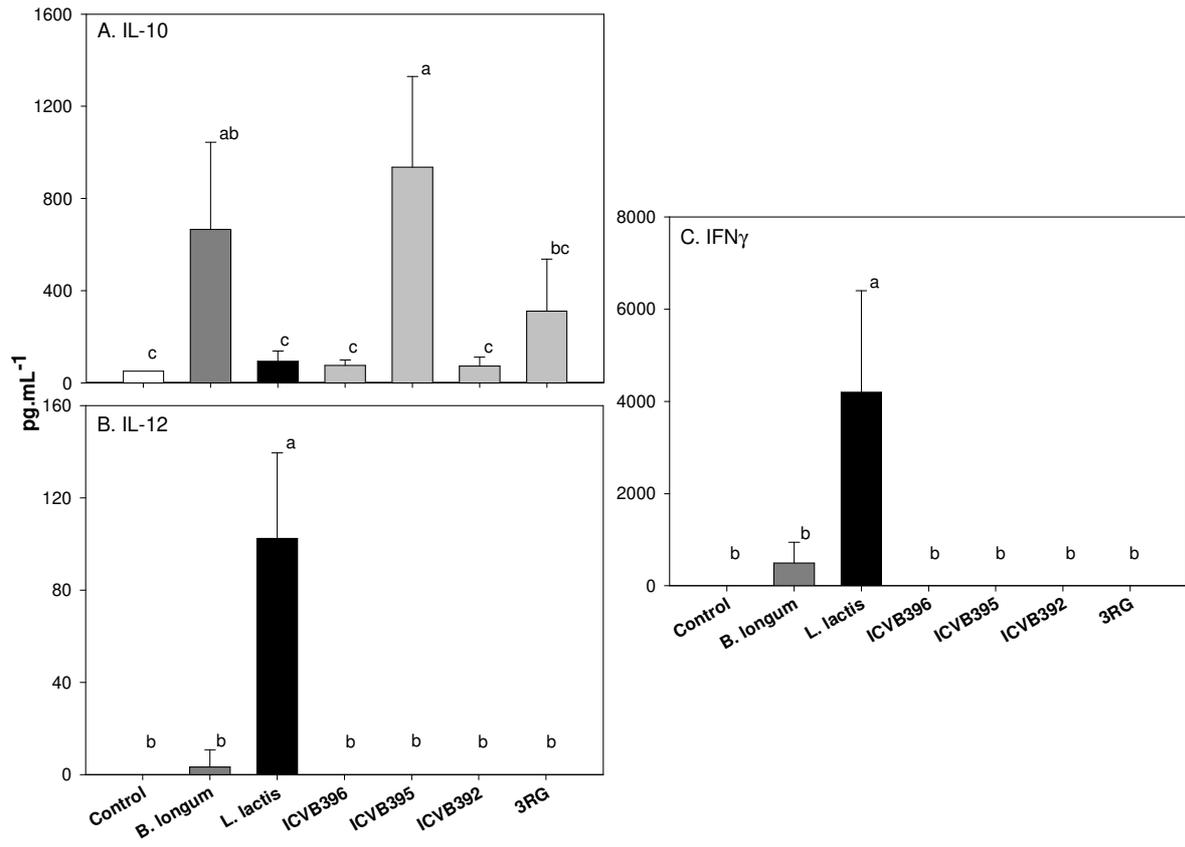


Figure 2.

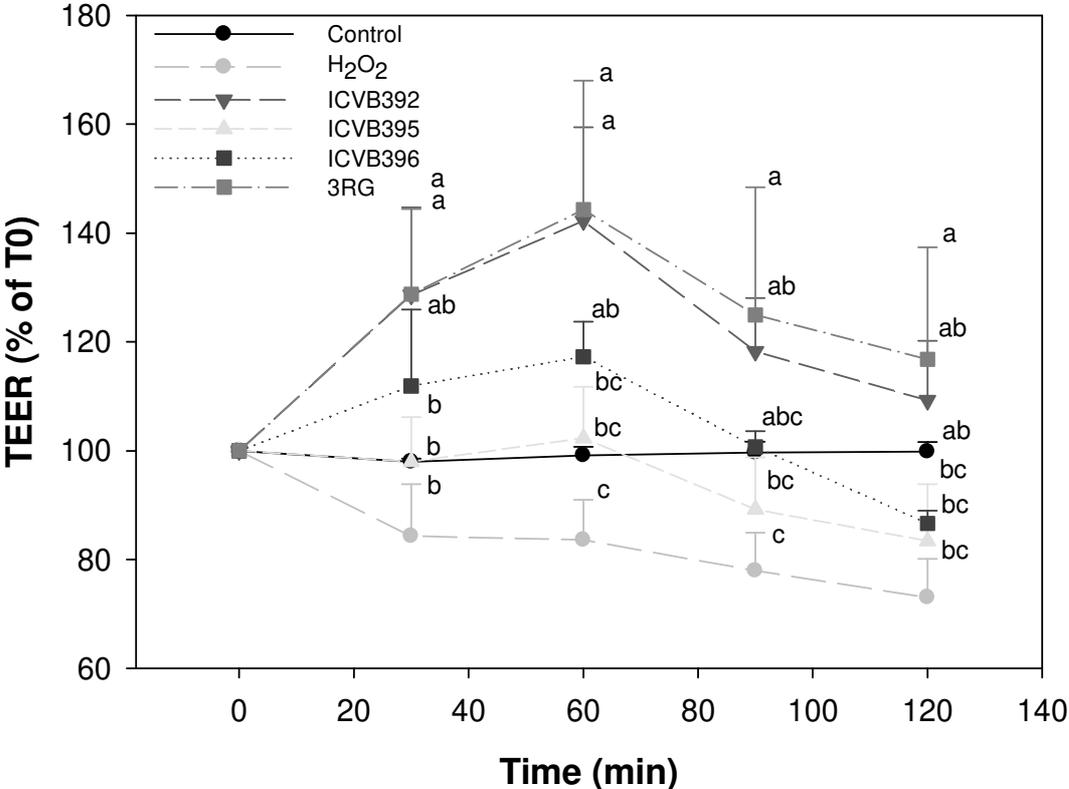


Figure 3.

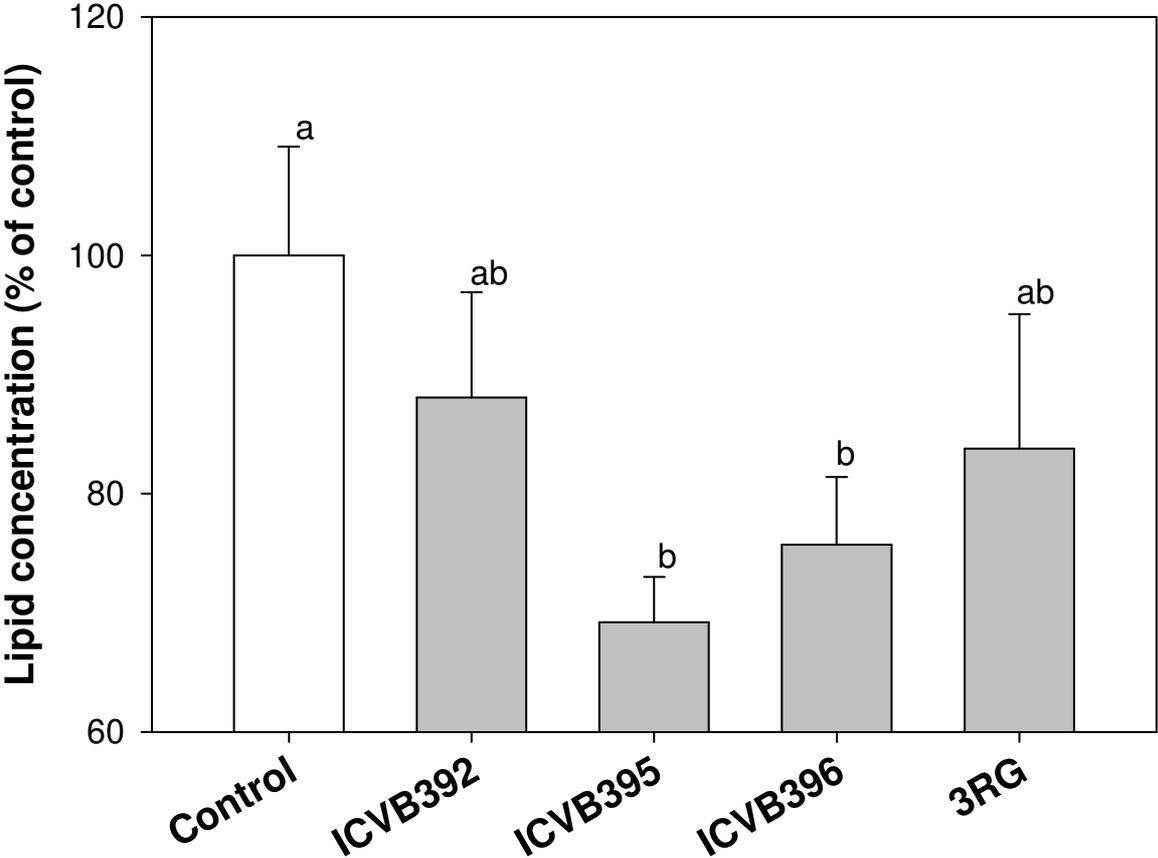


Figure 4.

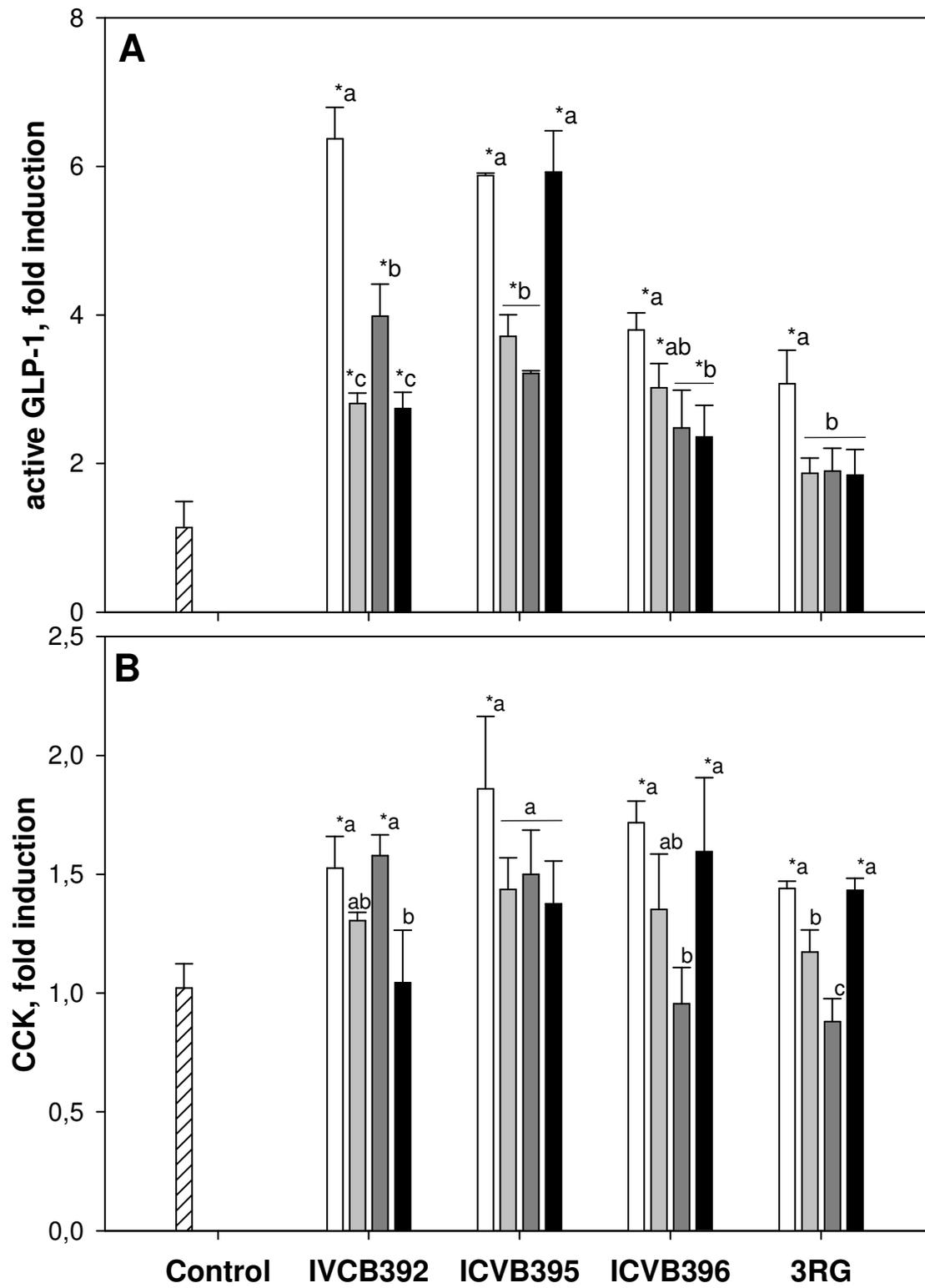


Figure 5.

