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

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24        barrier

25

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  $2 \times 10^9$   
148 cfu.mL<sup>-1</sup> 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  $2 \times 10^6$  cells per mL in  
158 RPMI supplemented with gentamicin (150 µg.mL<sup>-1</sup>), 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% CO<sub>2</sub>. 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% CO<sub>2</sub> 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<sup>-1</sup> penicillin and 100 µg.mL<sup>-1</sup>  
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 10<sup>5</sup> cells per cm<sup>2</sup>. The medium was changed every two days until 14 days when  
179 optimal trans-epithelial resistance (TEER ≥ 1800 Ω/cm<sup>2</sup>) 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 (H<sub>2</sub>O<sub>2</sub>) in both basal and apical compartment  
184 (at 100µM final concentration). TEER was measured before H<sub>2</sub>O<sub>2</sub> 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% CO<sub>2</sub> in

192 DMEM supplemented with 10% heat-inactivated FCS, 100 U.mL<sup>-1</sup> penicillin and 100 µg.mL<sup>-1</sup>  
193 streptomycin and 2 mM L-glutamine. Cells were used between the 10<sup>th</sup> and the 16<sup>th</sup> 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<sup>-1</sup> insulin and 0.25 µM dexamethasone. After 48h,  
198 fresh medium supplemented with only insulin (1 µg.mL<sup>-1</sup>) 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<sup>-1</sup> 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<sup>-1</sup> of penicillin and streptomycin, at 37°C in 5% CO<sub>2</sub>-95% air atmosphere. The  
215 STC-1 cells were passed twice a week, and were used between the 60<sup>th</sup> and the 65<sup>th</sup> 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, CaCl<sub>2</sub> 1.2 mM, MgCl<sub>2</sub>  
219 1.2 mM, adjusted to pH 7.4 with NaOH 3M) and then co-incubated with 10<sup>8</sup> CFU.mL<sup>-1</sup> of the  
220 selected *Lactobacillus* strains, alone or in combination, for 8 hours at 37°C in 5% CO<sub>2</sub>-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<sup>-1</sup>  
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 Merk, Germany) peptide hormones were  
230 used at initial concentration of 800 pM and 600 pM, respectively. *Lactobacilli* (at 10<sup>7</sup>  
231 CFU.mL<sup>-1</sup>) 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<sup>-1</sup>, 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  $\mu$ M, 50  $\mu$ 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% CO<sub>2</sub>-95% air atmosphere with 100  $\mu$ 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  $\beta$ -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'-CCGCCGTGGTGTATTATTGTG-  
260 3' for PepT1 and (F) 5'-TGCCCTGAGGCTCTTTTCCA-3' and (R) 5'-  
261 GGCATAGAGGTCTTTACGGATGTC-3' for  $\beta$ -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<sup>-1</sup>) 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<sup>-1</sup> 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 $\gamma$  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 H<sub>2</sub>O<sub>2</sub>-sensitized Caco-2 cells monolayers, as previously reported (J. Alard et al.,  
289 2018). As expected, H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>-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 H<sub>2</sub>O<sub>2</sub> 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  $26.47 \pm 0.44$  while  
333 reference  $\beta$ -actin gene expression showed CT of  $14.02 \pm 0.22$  (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- $\beta$ -  
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 $\gamma$  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  $\gamma$  (IFN- $\gamma$ ) 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  $\gamma$  (IFN- $\gamma$ ) (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 H<sub>2</sub>O<sub>2</sub>-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 H<sub>2</sub>O<sub>2</sub>-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 (GPC6A), 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  $\text{Ca}^{++}$  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  $\text{G}\alpha\text{q}$  subunit, which induce intracellular  $\text{Ca}^{++}$  increase and GLP-1 hormone  
576 secretion (Caron et al., 2017). Wellendorph et al. established that the GPRC6A was activated  
577 by basic  $\alpha$ -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  $\text{Ca}^{++}$  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 $\gamma$  (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  $\leq 0.05$  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 (H<sub>2</sub>O<sub>2</sub>).**

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 H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M). Changes in trans-epithelial electrical resistance  
875 (TEER) across Caco-2 cell monolayers were measured before the addition of H<sub>2</sub>O<sub>2</sub> (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 $\mu$ M, dark grey bars), GPRC6A antagonist CpD (50 $\mu$ 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<sup>-1</sup>) 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.**

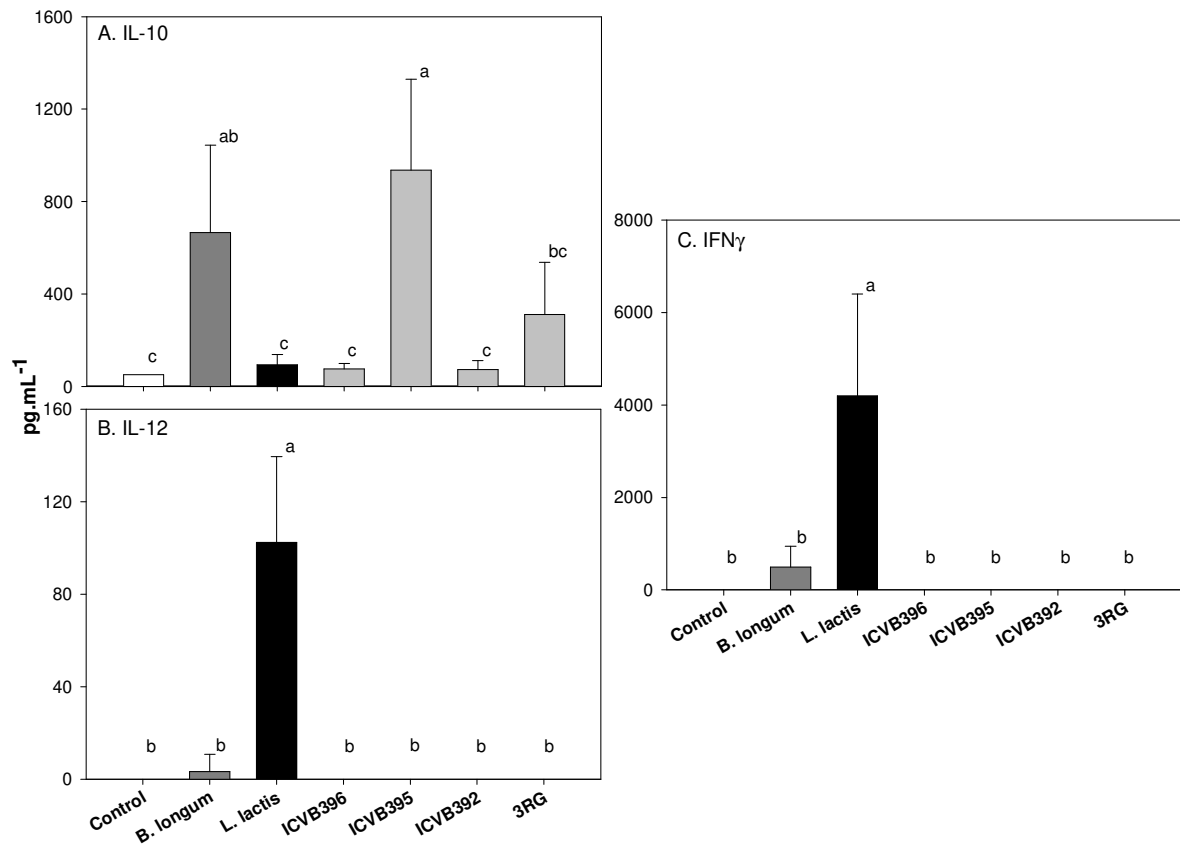


Figure 2.

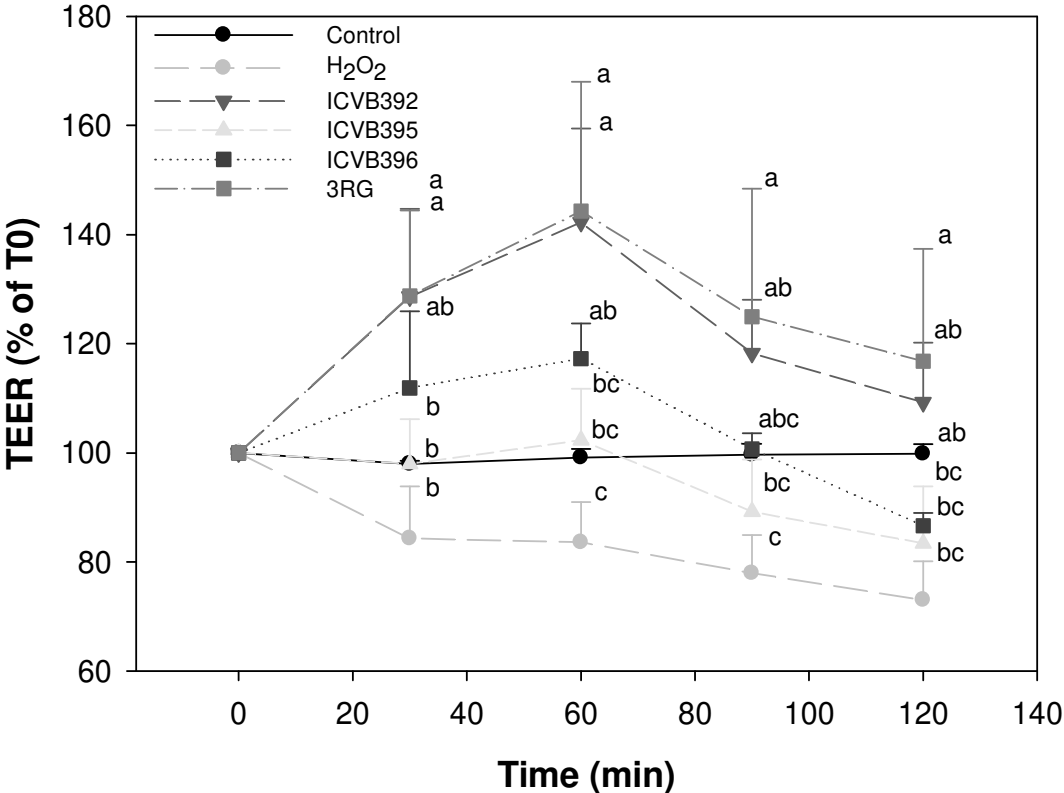




Figure 3.

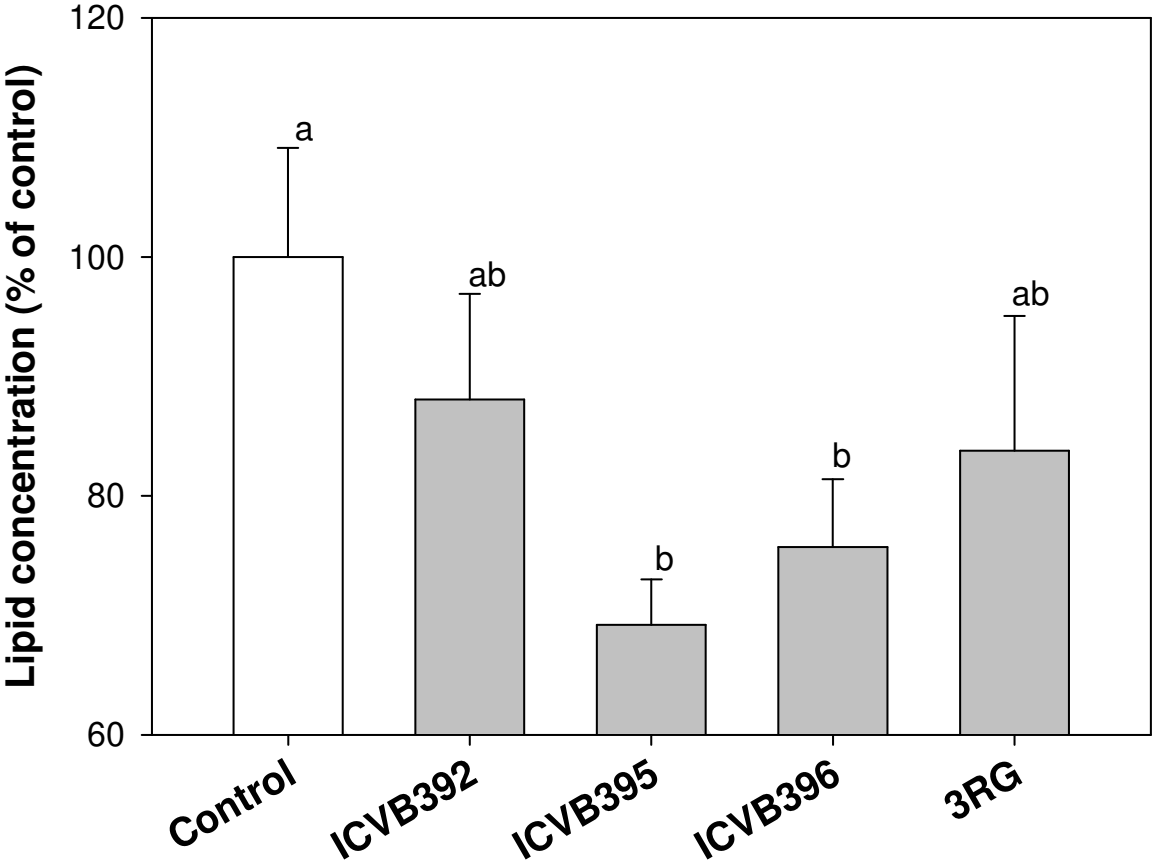


Figure 4.

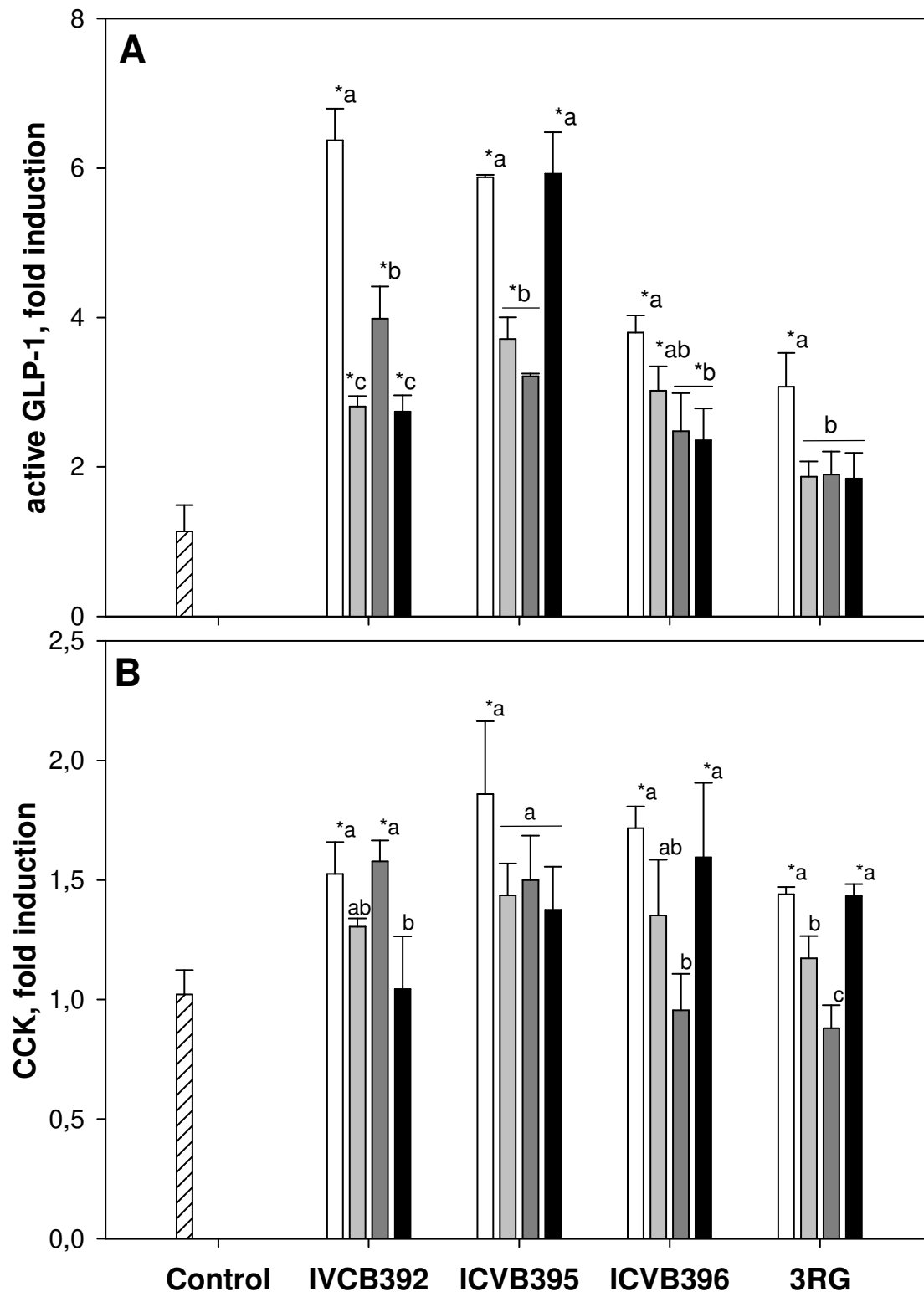


Figure 5.

