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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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Abstract

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

1. Introduction

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

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

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

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

2. Material and methods

2.1. Bacterial strains

The three *Lactobacillus* strains used in this research work were previously isolated from Lebanese vaginal microbiota (Al Kassaa, Hamze, Hober, Chihib, & Drider, 2014), and recently selected for their probiotic traits (Belguesmia et al., 2016). Previously named CMUL34, CMUL67 and CMUL80, they were re-identified by 16S rDNA sequencing and registered in the Institut Charles Viollette Laboratory Collection, under *L. gasseri* ICVB392, *L. reuteri* ICVB395 and *L. gasseri* ICVB396 strains respectively. The strains were also associated in equal amount within the multi-strain cocktail designated during this study as 3RG consortium. Before each experiment, the strains were grown for 18-24 h at 37°C in de Man-Rogosa-Sharpe (MRS) medium (De Man, Rogosa, & Sharpe, 1960). Two additional 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 $x10^9$
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 $\mu g.mL^{-1}$), 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% CO_2. 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

2.3. Epithelial barrier model

The human colon epithelial cell line Caco-2 clone TC7 (Chantret et al., 1994) was used to study the impact of the lactobacilli on trans-epithelial electric resistance (TEER). The Caco-2 cells were grown at 37°C with 10% CO_2 in DMEM supplemented with 5% heat-inactivated foetal calf serum (FCS, Gibco, Life technologies, Ghent, Belgium), 1% Non-Essential Amino Acids (Gibco, Life Technologies, Ghent, Belgium), 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin (Gibco, Life Technologies, Ghent, Belgium) and 2mM L-glutamine (Gibco, Life Technologies, Ghent, Belgium).

For the permeability test, polarized Caco-2 monolayers were prepared by growing the epithelial cells on 12-wells Transwell® insert filters (polycarbonate membrane with 3 µm pore size, 12 mm diameters, Costar, Corning Life Science, Kennebunk, ME, USA) at a density of 10^5 cells per cm^2. The medium was changed every two days until 14 days when optimal trans-epithelial resistance (TEER ≥ 1800 Ω/cm^2) was reached (which was measured every 2 days 1 h after changing medium using a millicell-ERS (Electrical Resistance System; Millipore, Billerica, MA, USA). At day 14, fresh medium without FCS was added and cells were treated, in the apical compartment with bacteria (or not) at a bacteria-to-cell ratio of 10:1, 30 min before the addition of hydrogen peroxide (H_2O_2) in both basal and apical compartment (at 100µM final concentration). TEER was measured before H_2O_2 addition (T0) and every 30 min until 120 min. The results were compared to non-treated cells. Three different experiments were performed including duplicates of each condition and results were expressed in % TEER compared to T0 ± SEM.

2.4. Lipid accumulation in adipocytes

The effect of lactobacilli on lipid accumulation in adipocytes was studied using the pre-adipocyte 3T3-L1 murine cell line. The 3T3-L1 cells were grown at 37°C with 5% CO_2 in

DMEM supplemented with 10% heat-inactivated FCS, 100 U.mL^{-1} penicillin and 100 µg.mL^{-1} streptomycin and 2 mM L-glutamine. Cells were used between the 10th and the 16th passage. The 3T3-L1 cells were distributed in 12-wells plates at a concentration of 3500 cells per wells and were differentiated in adipocytes according to the protocol described by (Zebisch, Voigt, Wabitsch, & Brandsch, 2012). Briefly, cells were grown in medium supplemented with 0,5 mM 3-isobutyl-1methylxanthine, 1 µg.mL^{-1} insulin and 0.25 µM dexamethasone. After 48h, fresh medium supplemented with only insulin (1 µg.mL^{-1}) was replaced. Basal medium was changed every two days for 10 days, until cells were differentiated in mature adipocytes. Cells were then stimulated for 24h with bacteria (or not) at a bacteria-to-cell ratio of 10:1, in fresh medium in the presence of 150 µg.mL^{-1} gentamicin. Lipid accumulation was quantified by Oil- Red-O staining. Briefly, cells were stained using 1 ml ready-to-use Oil-red O solution (DiaPath, Martinengo, Italy) for 15 min., washed 3 times with PBS (Gibco, Life Technologies, Ghent, Belgium). Oil-Red-O was eluting with isopropanol for 30 min incubation. Optic density was measured at 490 nm by a spectrophotometer (EL$_x$808, Biotech instruments). The percentage of Oil-red-O stained cells relative to control cells without bacteria was calculated as (A490nm [probiotic sample]/A490nm[control])*100.

2.5. Gut hormones secretion study

The STC-1 murine cell line, derived from the intestinal tumour of double transgenic mice, gratefully received from Dr. C. Roche (INSERM U865, Lyon, France), was used for gut hormones study. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, France), supplemented with 10% foetal bovine serum, 5 mM of L-glutamine and 100 U.mL^{-1} of penicillin and streptomycin, at 37°C in 5% CO_2-95% air atmosphere. The STC-1 cells were passed twice a week, and were used between the 60th and the 65th passage for the different assays. STC-1 cells were seeded in 24 wells plate at 40,000 cells/well and

were grown in DMEM culture medium for 48-72h. Cells were washed twice with Hepes buffer without glucose (NaCl 140 mM, Hepes 20 mM, KCl 4.5 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1.2 mM, adjusted to pH 7.4 with NaOH 3M) and then co-incubated with 10^8 CFU.mL^{-1} of the selected *Lactobacillus* strains, alone or in combination, for 8 hours at 37°C in 5% CO_2-95% air atmosphere. Purified peptidoglycan from *L. acidophilus* (Macho-Fernandez et al., 2011), and purified flagellin from *Salmonella enterica* serovar Typhimurium (gratefully supplied by Dr Jean Claude Sirard from Institut Pasteur de Lille) were tested at 50 and 10 µg.mL^{-1} respectively, to evaluate their impact on gut hormones secretion. The resulting supernatants were centrifuged (8000 g for 10 min) and were kept at -20°C. GLP-1 and CCK quantifications were realized by Radio-Immuno Assay (RIA) using EMD Milipore (USA) and Cisbio International (France) kits for each hormone, respectively.

To examine the capacity of bacteria to degrade the gut hormones, pure active GLP-1 1-26 (EMD Milipore, USA) and CCK8S (Sigma Aldrich Merk, Germany) peptide hormones were used at initial concentration of 800 pM and 600 pM, respectively. Lactobacilli (at 10^7 CFU.mL^{-1}) were incubated in Hepes buffer solution containing active GLP-1 or CCK8S, in presence or absence of DPP-IV enzyme inhibitor (Ile-Pro-Ile, Sigma-Aldrich Germany) at 1 mg.mL^{-1}, during 8 h at 37°C. The supernatants were recovered by centrifugation at 8000 g, 4°C for 10 min, and quantification of remaining active GLP- 1 or CCK8S in the supernatants was performed by RIA as previously described. Negative control without bacteria was incubated in the same conditions for both hormones. The results were expressed in percentage of initial concentration of active GLP-1 or CCK8S.

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

and *4*-aminomethylbenzoic acid (AMBA-4, Sigma Aldrich, Merck Germany) were used at a final concentration of 25 µM, 50 µM and 10 mM in Hepes Buffer, respectively. The STC-1 cells were washed after reaching 80% confluence culture and were incubated for 15 minutes at 37°C in 5% CO_2-95% air atmosphere with 100 µL of described above solutions. Then the treated STC-1 cells were incubated with lactobacilli in the same conditions as described above.

2.6. RT-PCR analysis

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

2.7. Statistical analysis

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

3. Results

3.1 Immunomodulatory capacities of the lactobacilli

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

3.2 Capacity of the strains to strengthen the epithelial barrier

We evaluated the capacity of the strains to restore the gut barrier function using an *in vitro* model of H_2O_2-sensitized Caco-2 cells monolayers, as previously reported (J. Alard et al., 2018). As expected, H_2O_2 sensitization induced permeability as shown by a significant and time-dependent drop in the trans-epithelial electric resistance (TEER). All the strains were

able to restore the epithelial barrier, as shown by an increase in the TEER as compared to H_2O_2-sensitized control cells (Figure 2), which was however not significant for *L. gasseri* ICVB396 and *L. reuteri* ICVB395 which was able to maintain the TEER at the level of untreated control only until 60 min. Interestingly, the 3RG consortium and the *L. gasseri* ICVB392 strain not only restored but even reinforced the epithelial barrier, the TEER being higher than the H_2O_2 non-sensitized control monolayer.

3.3 Impact on lipid accumulation in adipocytes

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

3.4 Capacity of the lactobacilli strains to modulate gut hormones secretion

3.4.1 Effects on active-GLP-1 secretion

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

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 leaded 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 (GPRC6*A*), 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-1with 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

of the control level, respectively. Regarding the effect of the GPRC6A antagonist, we observed more or less the same effect with major decrease for the ICVB392 and ICV395 strains, and moderate for the 3RG consortium. However the decrease of GLP-1 level measured for the ICV396 pre-treated STC-1 was not significant (Figure 4A). Interestingly Pept-1 inhibitor induced a reduction of GLP-1 secretion for all strains and the 3RG consortium, nearly to the same levels obtained with GPRC6A inhibitor, except for the strain ICVB395 for which GLP-1 secretion was not impacted by AMBA-4 Pept-1 inhibitor.

The results obtained for CCK were slightly different, as we observed a statistically significant effect just for the ICVB396 strains and 3RG consortium after pre-treatment with CaSR antagonist. GPRC6A and Pept-1 antagonist and inhibitor didn't appear to affect the CCK secretion level in a significant manner in all cases studied in this work, excepted for a slight effect which was observed with Pept-1 inhibitor and GPRC6A antagonists on the effect of ICVB392 strain and the 3RG consortium respectively (Figure 4B).

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

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

In the presence of the tripeptide (Ile-Pro-Ile), a DPP-IV inhibitor (grey bars), the consumption 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 IFNγ 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 H_2O_2-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_2O_2-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

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

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

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

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

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

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

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

Interestingly, peptides and amino acids are generally sensed by EEC via the proton-coupled peptide-transporter PepT1, this nutrient-sensing being linked to hormone secretion (Zietek & Rath, 2016). We previously demonstrated that the muropeptide M-tri-Lys, derived from the peptidoglycan of a selected *Lactobacillus* strain, was able to protect mice from colitis in a NOD2-dependant manner, while the M-tri-Lys-N was not, suggesting that only the M-tri-Lys could be internalized by PepT1 or PepT2 transporters into the cell to interact with the 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 insulinotropic 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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Figures captions:

Figure. 1 Immunomodulation capacities of the selected strains

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

Figure. 2 Capacity of the selected lactobacilli to restore or strengthen epithelial barrier in polarized Caco-2 monolayers sensitized with hydrogen peroxide (H_2O_2).

Caco-2 monolayers were treated at the apical side with the bacteria (at 10:1 bacteria/cell ratio) 30 mn before the addition of H_2O_2 (100 µM). Changes in trans-epithelial electrical resistance (TEER) across Caco-2 cell monolayers were measured before the addition of H_2O_2 (T0) and every 30 mn until 120 mn. Results were expressed as % TEER compared to T0. Values represent the mean of 3 repeated experiments. Means without a common letter are different ($p<0.05$) using one way ANOVA with Tukey *post hoc* test for pairwise comparisons.

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

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

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

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

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

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

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

Figure 1.

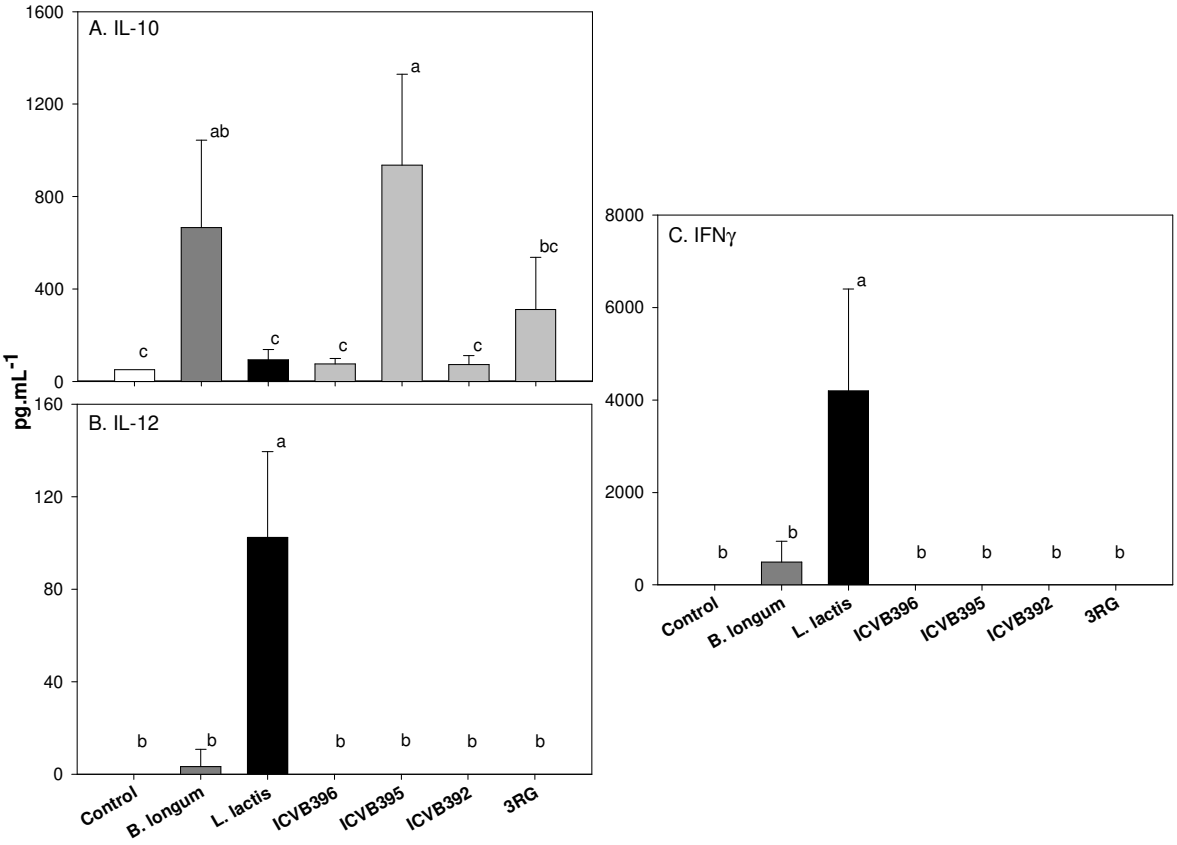

A. IL-10
B. IL-12
C. IFNγ
pg.mL-1
1600
1200
800
400
0
160
120
80
40
8000
6000
4000
2000
a
ab
bc
c
b
Control
B. longum
L. lactis
ICVB396
ICVB395
ICVB392
3RG

Figure 2.

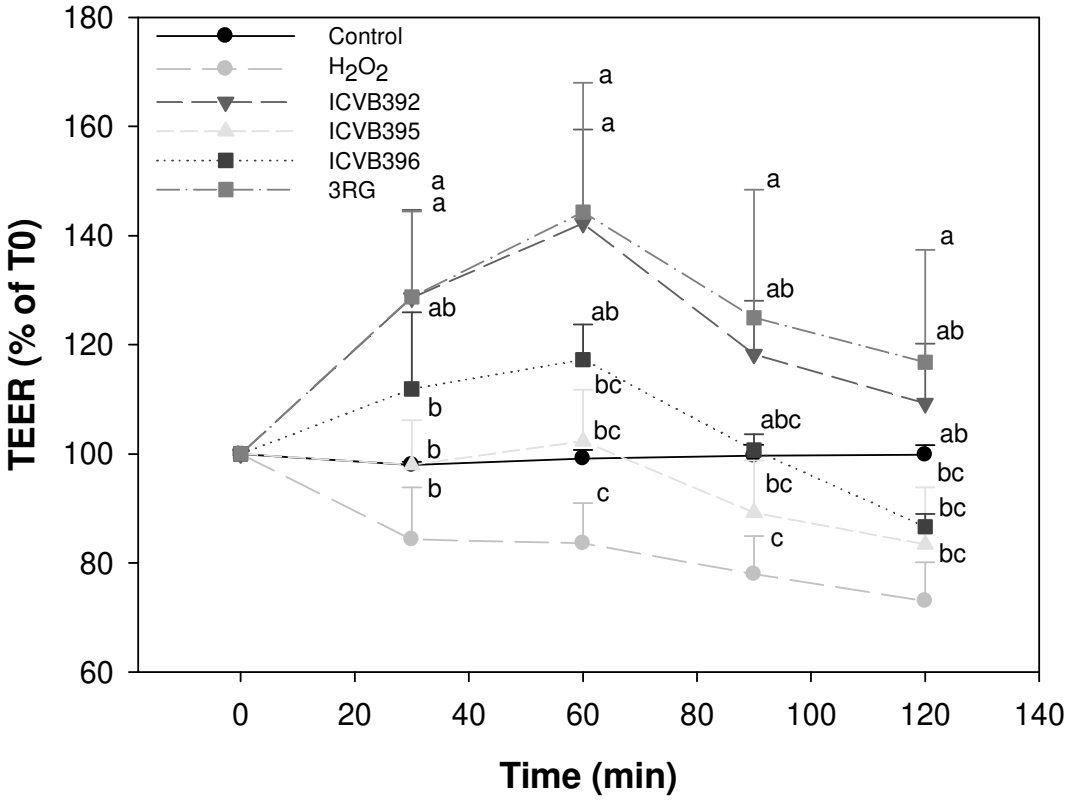

Control
H2O2
ICVB392
ICVB395
ICVB396
3RG
TEER (% of T0)
Time (min)
180
160
140
120
100
80
60
0
20
40
60
80
100
120
140
a
a
a
a
a
a
ab
ab
ab
ab
b
bc
bc
abc
ab
b
b
bc
bc
bc
c
c
bc

Figure 3.

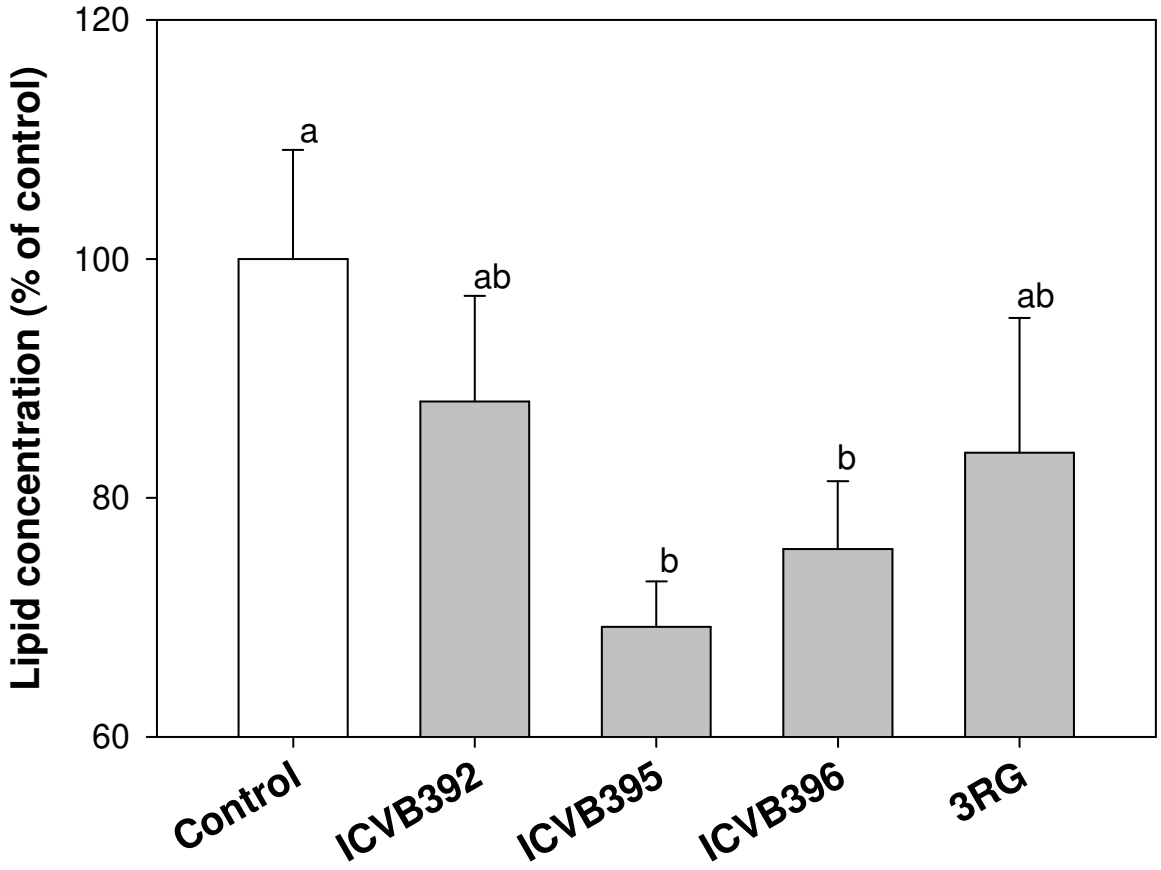

Lipid concentration (% of control)
120
100
80
60
a
ab
b
b
ab
Control
ICVB392
ICVB395
ICVB396
3RG

Figure 4.

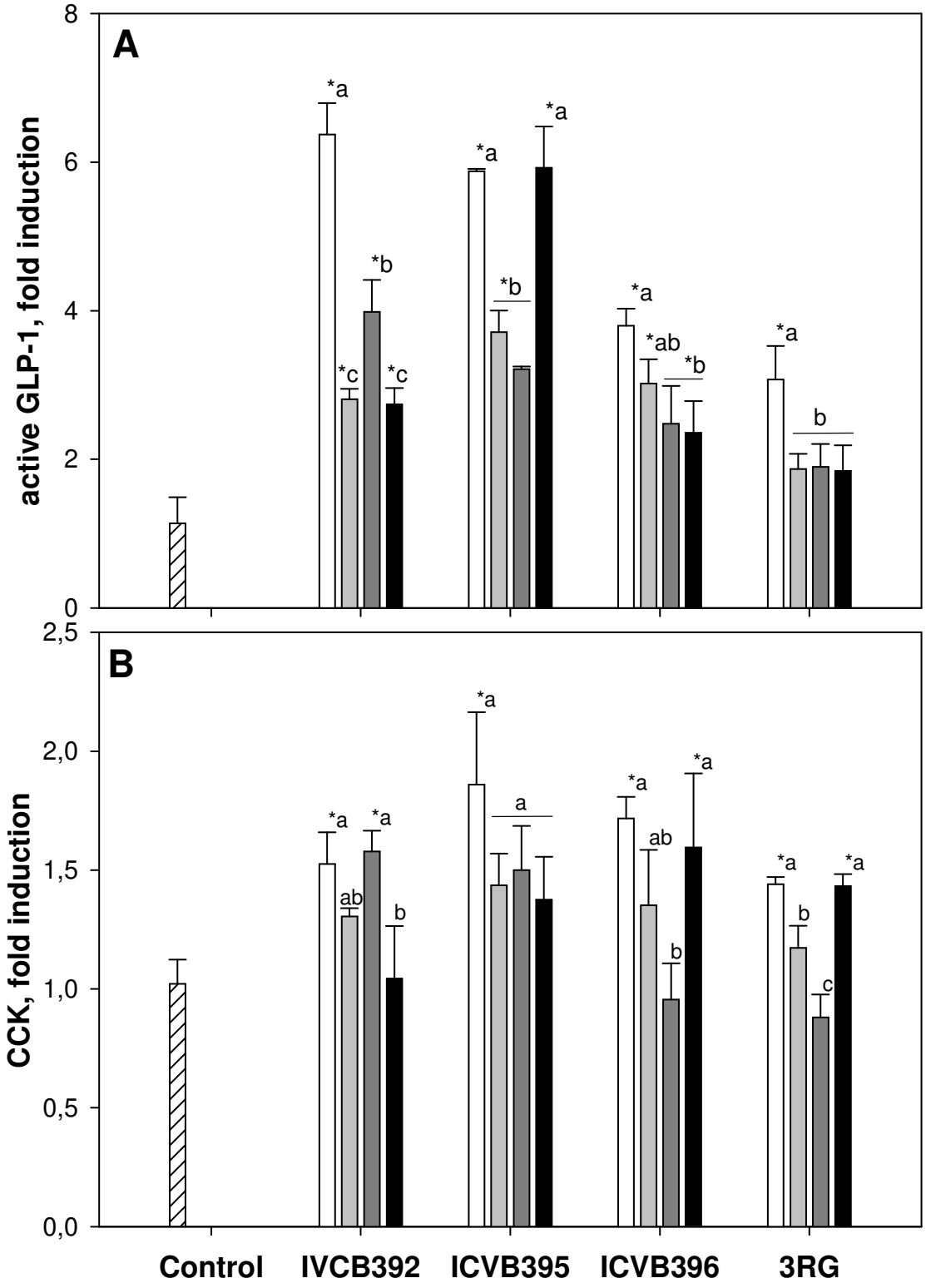
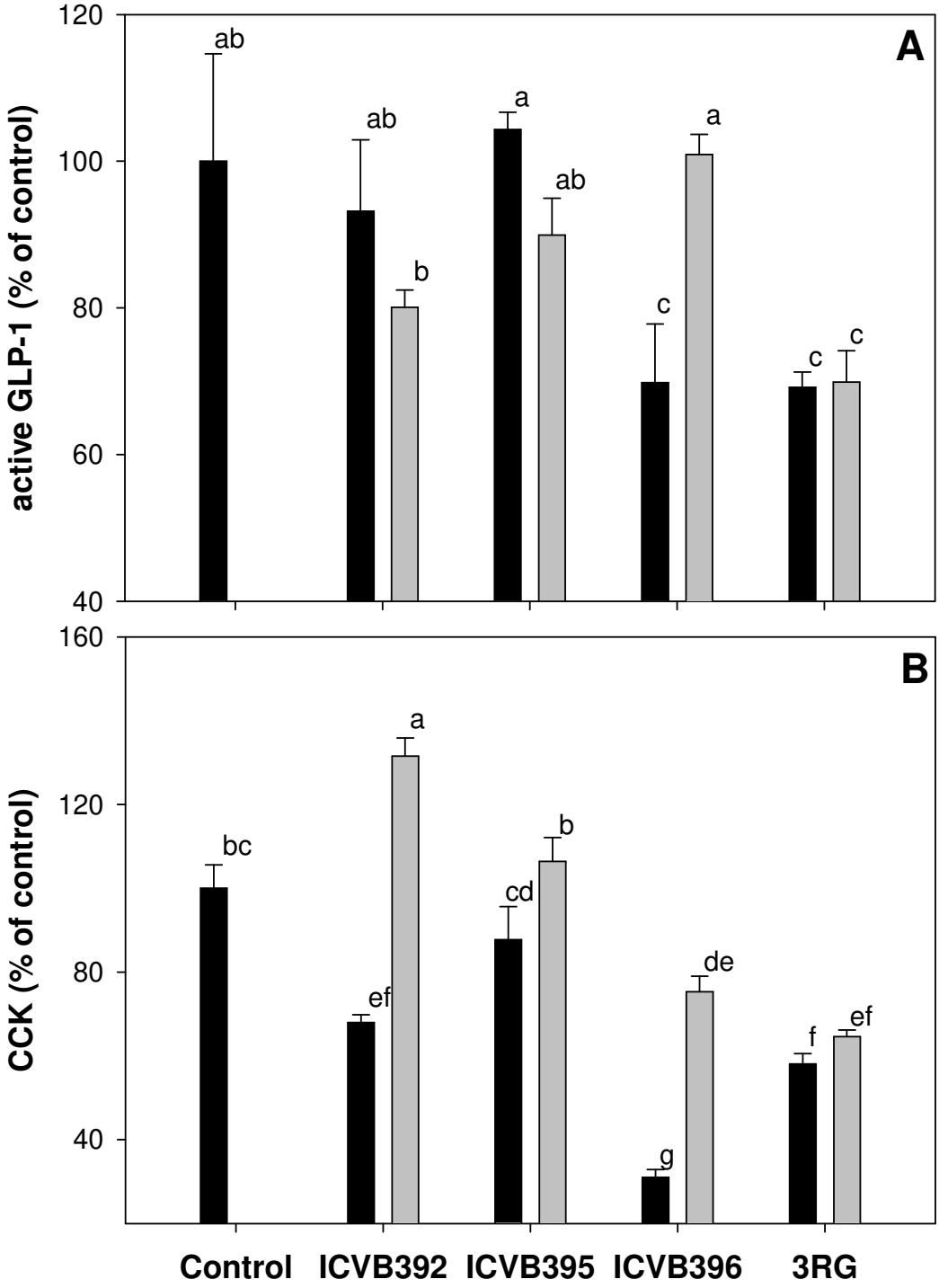

A
active GLP-1, fold induction
8
6
4
2
0
*a
*c
*b
*c
*a
*b
*a
*a
*ab
*b
*a
b
B
CCK, fold induction
2,5
2,0
1,5
1,0
0,5
0,0
*a
ab
*a
b
*a
a
*a
ab
b
*a
*a
*a
b
c
*a
Control
IVCB392
ICVB395
ICVB396
3RG

Figure 5.


A
active GLP-1 (% of control)
120
100
80
60
40
ab
ab
b
a
ab
c
a
c
c
B
CCK (% of control)
160
120
80
40
bc
ef
a
cd
b
g
de
f
ef
Control
ICVB392
ICVB395
ICVB396
3RG