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In vitro effects of live and heat-inactivated *Bifidobacterium animalis* subsp. *lactis*, BB-12 and *Lactobacillus rhamnosus* GG on Caco-2 cells

Vivian M. Castro-Herrera[1*], Christine Rasmussen[2], Anja Wellejus[2] and Elizabeth A. Miles[1] and Philip C. Calder[1,3]

[1]School of Human Development and Health, Faculty of Medicine, University of Southampton, Southampton SO16 6YD, United Kingdom
[2]Human Health Innovation, Chr. Hansen A/S, 2970 Hoersholm, Denmark
[3]NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust and University of Southampton, Southampton SO16 6YD, United Kingdom
*Correspondence: vmch1m14@soton.ac.uk

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Abstract: Probiotic-host interaction can be cell-to-cell or through metabolite production. Dead (inactive) organisms could interact with the host leading to local effects and possible health benefits. This research examined the effects of live and heat-inactivated *Bifidobacterium animalis* subsp*. lactis,* BB-12 (BB-12) and *Lactobacillus rhamnosus GG* (LGG) on cultured Caco-2 cells focusing on epithelial integrity and production of inflammatory mediators. Live organisms increased transepithelial electrical resistance (TEER), a barrier-integrity marker with LGG having a greater effect than BB-12. When mildly heat-treated, both organisms had a more modest effect on TEER than when alive. When they were heat-inactivated both organisms had only a limited effect on TEER. Neither live nor heat-inactivated organisms affected production of six inflammatory mediators produced by Caco-2 cells compared to control conditions. Pre-treatment with heat-inactivated LGG or BB-12 did not alter the decline in TEER caused by exposure to an inflammatory cocktail of cytokines. However, pre-treatment of Caco-2 cells with heat-inactivated organisms alone or their combination decreased the production of interleukin (IL)-6, IL-18, and vascular endothelial growth factor. To conclude, while the live organisms improve the epithelial barrier using this model, neither live nor heat-inactivated organisms directly elicit an inflammatory response by the epithelium. Pre-treatment with heat-inactivated BB-12 or LGG can reduce some components of the response induced by an inflammatory stimulus.

Keywords: Probiotic; Gut epithelium; Inflammation; *B. animalis* subsp. *lactis;* BB-12; *L. rhamnosus GG;* heat-inactivation

1. Introduction

The World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. This definition assumes that probiotics need to be alive to interact with the host in order to exert benefits on health. Certainly, interactions of live probiotics with the host's gastrointestinal epithelium and immune system are key to many of the ascribed clinical benefits, such as remission of active ulcerative colitis [2] and control of pathogenic intestinal bacterial overgrowth [2, 3]. The probiotic-host interaction can be a direct cell-to-cell physical one (e.g. through pili interactions with host cells) [4, 5] or can be as a result of metabolites or other products released by the probiotic organisms [6, 7]. However, live organisms may also have adverse effects, as reported in case studies of microbial appearance in liver biopsies in older individuals [8] and systemic infections with probiotic organisms [9]. Dead or inactive organisms could also interact with the host and be functional, so conferring health benefits, and would not carry the risk of infection. Both live and heat-killed organisms (a mixture of probiotics

consisting of *Lactobacillus plantarum*, *L. bulgaricus*, *L. casei*, *L. acidophilus*, and *Bifidobacterium breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *Streptococcus salivarus* subsp. *thermophilus*) had significant anti-inflammatory effects through the reduction of interleukin (IL)-6 in an experimental model of colitis in rats [10]. *In vitro* studies showed that the anti-inflammatory effects of live and heat-killed *B. breve* were comparable in peripheral blood mononuclear cells isolated from patients with ulcerative colitis [11]. One reason that heat-killed or inactivated organisms may retain activity is that they keep the integrity of the cell wall components involved in interactions with the host [12]. In humans, particularly in vulnerable subgroups such as the frail elderly or critically ill, heat-inactivated probiotics may be a safer alternative to live organisms as they can elicit local benefits [13]. Theoretically, local effects of heat-inactivated probiotics can translate into systemic benefits, but this requires further exploration. Here we examine the effects of live and heat-inactivated *B. animalis* subsp. *lactis*, BB-12 and *L. rhamnosus* GG on cultured Caco-2 cells (a human colonic enterocyte cell line) with a focus on barrier integrity and production of inflammatory mediators. Both the direct effects of the organisms and the effects of pretreatment with the organisms on the subsequent response to inflammatory stimulation are examined. We chose to investigate the effects of *B. animalis* subsp. *lactis*, BB-12 and *L. rhamnosus* GG because they are widely used in the food industry and because the combination of these two organisms is currently being tested in a large clinical trial [14]. A previous study noted that the ability of probiotics to interact with Caco-2 cells is strain dependent but that lactobacilli and bifidobacteria could directly elicit a low level inflammatory response in Caco-2 cells [15] . We used Caco-2 cells because they are considered suitable to assess the interaction between microorganisms and the gut epithelium [16].

2. Materials and Methods

Two experiments were carried out to study the effect of viable and heat-inactivated *L. rhamnosus* GG and *B. animalis subsp. lactis*, BB-12 on transepithelial electrical resistance (TEER) and secretion of inflammatory mediators under control and inflammatory conditions.

2.1. Preparation of heat-inactivated and live L. rhamnosus GG and B. animalis subsp. lactis, BB-12

L. rhamnosus GG and *B. animalis* subsp. *lactis,* BB-12 (Chr. Hansen A/S, Hoersholm, Denmark) were inactivated by exposure to heat; different durations of exposure were used in order to identify the best condition to inhibit colony formation, indicative of inactivation. *L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12 were inoculated from frozen stock and cultured overnight at 37°C in De Man, Rogosa and Sharpe (MRS) broth, pH 6.5 (Difco™) with 0.05% cysteine hydrochloride monohydrate (CyHCl) under anaerobic conditions with AnaeroGen pads (Oxoid). Ten-fold dilution series were prepared from the overnight cultures and incubated overnight at 37°C under anaerobic conditions. For each strain, late exponential/early stationary phase were selected based on measures of optical density at 600 nm (OD600). Bacterial cultures were washed twice in 37°C preheated Hanks' balanced salt solution (HBSS; Gibco™) and once in antibiotic free cell culture medium (Minimum Essential Medium (MEM; Gibco™) including 20% heat-inactivated foetal bovine serum (FBS; Gibco™) and 1% MEM non-essential amino acids (Biowest)). Samples were centrifuged at 3500 x g for 5 minutes. The medium was removed and the cultures were resuspended in 5 mL antibiotic free cell culture medium. OD600 was adjusted to 3.8 and each cell suspension was divided into multiple samples of 1.5 mL in Eppendorf tubes. One vial of viable *B. animalis subsp. lactis,* BB-12 and *L. rhamnosus* GG were used directly in experiments. Heat inactivation of *B. animalis* subsp. *lactis,* BB-12 and *L. rhamnosus* GG was tested at 62.3°C for 0, 2, 4 , 6 and 8 min and also heat inactivation of *L. rhamnosus* GG was tested at 70°C for 1, 3 and 5 min by the use of a temperature-controlled waterbath. The degree of heat inactivation was assessed by subsequent growth on MRS agar (Difco™) and counting the colony forming units. In short, 1 mL of the resuspended bacterial cells was diluted in 9 mL of MRD Maximum Recovery Diluent (Dilucup®; Lab Robot), and a 10-fold dilution series was prepared using Dilucups and the Dilushaker system. For each dilution, duplicate MRS agar plates were prepared by adding 1 mL of sample from the Dilucup and deep seeding in melted MRS agar including 0.05% CyHCl. Plates were incubated anaerobically with AnaeroGen pads for 2 days at 37°C, and colonies

96 were counted. Only plates with colony counts between 20 and 300 colonies were used for calculating
97 CFU. *2.2. Experiment 1*
98
99 In this experiment *L. rhamnosus* GG and *B. animalis* subsp. *lactis,* BB-12 were tested as viable and
100 heat-inactivated cells; TEER was assessed and the concentrations of inflammatory mediators in the
101 medium from the apical and basolateral sides of Caco-2 cell cultures were measured.

102 2.2.1. Culturing of Caco-2 cells

103 The human intestinal epithelial Caco-2 cell line (DSMZ ACC 169, Leibniz-Institut DSMZ-
104 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was
105 cultured in MEM (Gibco™) supplemented with 20% heat-inactivated FBS (Gibco™), 1% MEM non-
106 essential amino acids (Biowest) and 1% Pen-Strep-Amp B (Biological Industries) at 5% CO_2 and 37°C.
107 When the cells were approximately 50% confluent the medium was removed, and the cells were
108 washed twice in Hanks' balanced salt solution (HBSS; Gibco™). The cells were trypsinized by adding
109 2 mL of TrypLE Express Enzyme (Gibco™) and left for 4 min in the CO_2 incubator at 37°C.
110 Approximately 10 mL of medium was added to the trypsinized cells; they were counted and a
111 concentration of 1×10^5 cells/mL in supplemented MEM was prepared. A volume of 500 µL of cell
112 suspension was used to seed each apical compartment of Transwell®-Clear Inserts, Polyester
113 Membranes (12 mm, 0.4 µM, Corning®), and then 1.5 mL of supplemented MEM was added to the
114 basolateral compartment. Cells (Passage 3) were cultured on the inserts for 21 days with change of
115 medium twice a week. After 21 days the transwells were moved to the CellZscope2 (NanoAnalytics,
116 Germany). The CellZscope is a computer-controlled multi-well module with dynamic measuring of
117 the TEER without removing the cells from the incubator; TEER is measured by applying weak
118 alternating current voltage, unharmful to the cells, over the Caco-2 layer. The medium was changed
119 to antibiotics (Abx) free medium adding 1.65 mL and 0.76 mL of Abx-free medium in the basolateral
120 and apical compartments, respectively. The CellZscope2 was placed overnight in a CO_2 incubator
121 (5%) at 37°C and TEER ($\Omega \times cm^2$) was measured every hour using automated data collection. This
122 overnight measurement of TEER before the experimental start allowed for determination of baseline
123 TEER in each well and served as a quality control of a stable electrical resistance.

124 2.2.2. Stimulation of Caco-2 cells with live and heat-inactivated *B. animalis* subsp. *lactis,* BB-12 and *L.*
125 *rhamnosus* GG

126 *B. animalis* subsp. *lactis,* BB-12 heat-inactivated for 4 and 6 min at 62.3°C and *L. rhamnosus* GG
127 heat-inactivated for 1, 3 and 5 min at 70°C as well as live bacteria were selected for testing in the
128 CellZscope2. In order to stimulate the Caco-2 cells with bacteria, CellZscope2 measurements were
129 paused, the CellZscope2 was removed from the CO_2 incubator and 100 µL of apical medium was
130 removed from each transwell. A 100 µL of bacteria suspension (final OD600nm of 0.5) or medium
131 control (Abx-free MEM) was added to the apical side of the relevant wells (each in triplicate). The
132 CellZscope2 were transferred back to the CO_2 incubator and the TEER measurements were resumed
133 and continued overnight. Changes in TEER during bacterial stimulation were calculated relative to
134 the latest value recorded immediately prior to the stimulation (baseline measurement, set to 100%).
135 Area under curve (AUC) was calculated for each condition. Once TEER measurements were
136 completed, after 24 hours, apical and basolateral media were collected to analyze the concentrations
137 of an inflammatory panel consisting of six inflammatory mediators: IL-6, IL-18, IL-8, interferon
138 gamma-induced protein 10 (IP-10), vascular endothelial growth factor (VEGF), and intercellular
139 adhesion molecule-1 (ICAM-1) (Magnetic multiplex immunoassay – Bio-Rad Luminex Analyzer).

140 *2.3. Experiment 2*

141 In this experiment, heat-inactivated *L. rhamnosus* GG and *B. animalis* subsp. *lactis,* BB-12 were
142 tested; TEER was assessed and the concentrations of inflammatory mediators in the medium from

143 the apical and basolateral sides of Caco-2 cell cultures were following stimulation with an
144 inflammatory cocktail. 2.3.1. Culturing of Caco-2 cells
145 Caco-2 cells were obtained from the European Collection of Authenticated Cell Cultures (a
146 Culture Collection of Public Health England, CACO-2 ECACC 86010202, Human colon
147 adenocarcinoma) and were grown in Dulbecco's modified Eagle's medium supplemented with 10%
148 foetal bovine serum, 1% nonessential amino acids, 2% L-glutamine, and 1% penicillin- streptomycin
149 (Sigma Aldrich, Gillingham, UK), at 37°C in an atmosphere of 5% CO_2 and 95% air, using polystyrene
150 cell culture flasks (Sigma-Aldrich) according to methods described elsewhere [17, 18]. Confluent cell
151 cultures (Passage 46 to 48) were used after 19 days. Cells were detached from flasks using 2.5%
152 trypsin in Hank's balanced salt solution containing 0.2 g ethylenediaminetetraacetic acid per liter (all
153 from Sigma-Aldrich). Subsequently, trypsin was neutralized with pre-warmed supplemented
154 medium and the cells were transferred to 12 insert transwell plates (12 mm^2 with 0.4 μm clear pore
155 size). Supplemented medium was added to both the apical (500 μl) and basolateral sides (1500 μl) of
156 the cultures, which were then placed at 37°C in an atmosphere of 5% CO_2 and 95% air. Medium on
157 both sides of the transwell was replaced every second day.

158 2.3.2. Effect of pre-treatment of Caco-2 cells with heat-inactivated *L. rhamnosus* GG and *B. animalis*
159 subsp. *lactis*, BB-12 on Caco-2 cell response to an inflammatory cocktail

160 Caco-2 cell monolayers (7 x 10^4 cells/well) were incubated in transwell plates with heat-
161 inactivated *L. rhamnosus* GG (heat-inactivated for 3 min at 70°C) or *B. animalis* subsp. *lactis,* BB-12
162 (heat-inactivated for 6 min at 62.3°C) or the combination of *L. rhamnosus GG* + *B. animalis* subsp. *lactis,*
163 BB-12 (same heat inactivation conditions) for 24 hr; the bacteria were added on the apical side of the
164 wells at a multiplicity of infection of 10:1. Then an inflammatory cocktail of tumour necrosis factor
165 (TNF)-α (1 ng/ml), interferon (IFN)-γ (10 ng/ml) and IL-1β (1 ng/ml) was added as a pre-warmed
166 mixture in supplemented medium on the basolateral side of the cells. The cultures were incubated
167 for 24 hr; control cultures were not pretreated with heat-inactivated probiotics. TEER was measured
168 using "chopstick" electrodes (see below) and apical and basolateral media were collected to analyse
169 the concentrations of an inflammatory panel consisting of six mediators: IL-6, IL-18, IL-8, IP-10, VEGF
170 and ICAM-1 (Magnetic multiplex immunoassay - Bio-Rad Luminex Analyzer).

171 2.3.3. TEER measurement

172 In this experiment TEER was measured using an epithelial voltohmeter; this instrument uses a
173 pair of electrodes ("chopsticks") which are placed in the transwell (Milicell ERS-2 Voltohmmeter;
174 World Precision Instruments, Hitchen, UK). One electrode is in contact with the basolateral culture
175 medium and the shorter electrode is placed on top of the actual membrane where cells are seeded.
176 Cells are never in contact with the electrodes. The calibrations of the instruments and performance of
177 the technique were carried out according to the manufacturer's instructions.

178 2.4. Measurement of inflammatory mediators

179 Cell supernatants were kept at -80°C until processing. When ready to use, supernatants were
180 defrosted, vortexed and centrifuged for 30 seconds to remove any particulate matter. Cell
181 supernatants were diluted 1:2 in buffer immediately before assay. Microparticles were resuspended
182 in buffer and read using a pre-calibrated Bio-plex Luminex Analyzer (Bio-Plex 200, Bio-Rad, Watford,
183 UK). The inflammatory panel assessed consisted of six inflammatory mediators whose sensitivity
184 values (pg/ml) were: IL-6 (1.7), IL-18 (1.93), IL-8 (1.8), IP-10 (1.18), VEGF (2.1) and ICAM-1 (87.9).
185 Measurements were carried according to the manufacturer's instructions (Magnetic multiplex
186 immunoassay; R&D Systems, Abingdon, UK).
187

188 *2.5. Statistics*

189 Data were analyzed by one-way analysis of variance by ranks (ANOVA) performed using
190 GraphPad Prism 8.0. Dunnet's test was used to make pairwise post-hoc comparisons. In all cases, a
191 value for $p < 0.05$ was considered to indicate statistical significance.

192 **3. Results**

193 *3.1. Heat-inactivation of L. rhamnosus GG and B. animalis subsp. lactis, BB-12*

194 Table 1 shows the degree of inactivation of *L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12
195 that was achieved at the selected temperatures and timepoints. Treatment at 62.3°C for 4 min knocked
196 down *B. animalis* subsp. *lactis*, BB-12 by more than 3 logs whereas 6 min exposure at this temperature
197 resulted in no live *B. animalis* subsp. *lactis*, BB-12 remaining. In the case of *L. rhamnosus* GG, 62.3°C
198 for 6 min reduced live numbers by 85%, while after 8 min live numbers were reduced by 97%. Thus,
199 *B. animalis* subsp. *lactis*, BB-12 is more heat sensitive than *L. rhamnosus* GG. Incubating *L. rhamnosus*
200 GG at 70°C for 1 min reduced live numbers by 38% while incubation for 3 min resulted in no live *L.*
201 *rhamnosus* GG remaining.

202 **Table 1.** Effect of heat-treatment for different times on numbers of *L. rhamnosus* GG and *B. animalis*
203 subsp. *lactis*, BB-12.

Strain	Heat-treatment at 62.3°C	CFU/mL
B. animalis subsp. *lactis*, BB-12	0 minutes	9.4×10^8
B. animalis subsp. *lactis*, BB-12	2 minutes	3.9×10^8
B. animalis subsp. *lactis*, BB-12	4 minutes	1.2×10^5
B. animalis subsp. *lactis*, BB-12	6 minutes	0
B. animalis subsp. *lactis*, BB-12	8 minutes	0
L. rhamnosus GG	0 minutes	7.5×10^8
L. rhamnosus GG	2 minutes	6.8×10^8
L. rhamnosus GG	4 minutes	3.9×10^8
L. rhamnosus GG	6 minutes	1.1×10^8
L. rhamnosus GG	8 minutes	2.2×10^7
Strain	**Heat-treatment at 70°C**	**CFU/mL**
L. rhamnosus GG	0 minutes	8.9×10^8
L. rhamnosus GG	1 minute	5.5×10^8
L. rhamnosus GG	3 minutes	0
L. rhamnosus GG	5 minutes	0

204 *3.2. Effect of live and heat-inactivated L. rhamnosus GG and B. animalis subsp. lactis, BB-12 on TEER in*
205 *Caco-2 cell monolayers*

206 Figure 1 shows the TEER area under the curve (AUC) from 0-22 hr. *L. rhamnosus* GG induced a
207 greater TEER effect than *B. animalis* subsp. *lactis,* BB-12. Heat inactivation of both organisms reduced
208 the ability to increase TEER compared to the live strains, but TEER was still significantly improved
209 compared to control conditions. TEER remained higher with heat-inactivated *B. animalis* subsp. *lactis,*
210 BB-12 than with heat-inactivated *L. rhamnosus* GG.

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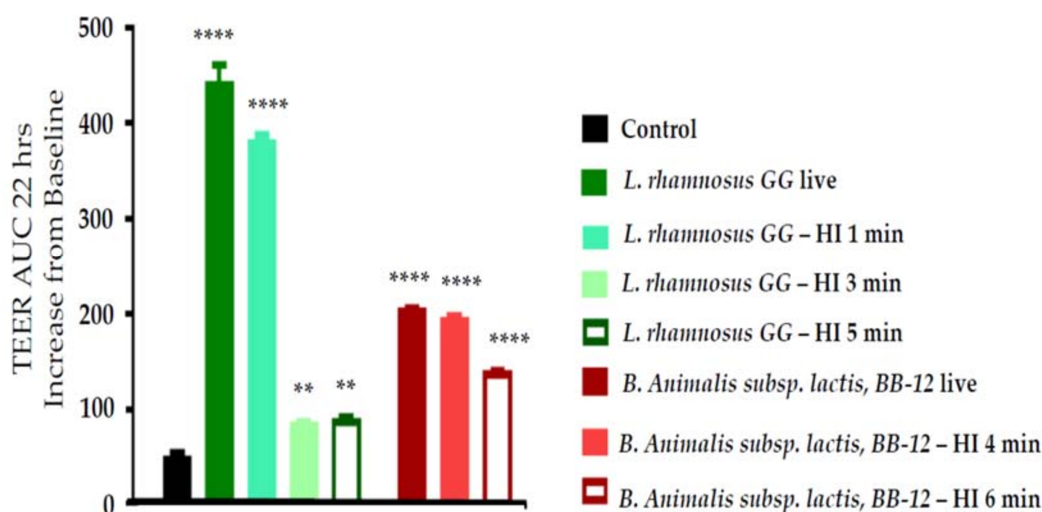

212 **Figure 1.** TEER area under curve (AUC) change after treatment of Caco-2 cells on the apical side with live or
213 heat-inactivated (HI) *L. rhamnosus* GG or *B. animalis* subsp. *lactis,* BB-12 for 22 hr. Data are mean + SD (n = 3).
214 One-way ANOVA p value < 0.0001. ** p < 0.01; **** p < 0.0001 vs control.

215 *3.3. Effect of live and heat-inactivated L. rhamnosus GG and B. animalis subsp. lactis, BB-12 on*
216 *inflammatory mediator production in Caco-2 cell monolayers*

217 Six inflammatory mediators (IL-6, IL-8, IL-18, IP-10, VEGF and sICAM-1) were measured in
218 culture medium collected from the apical and basolateral sides of Caco-2 monolayers treated with
219 live or heat-inactivated *L. rhamnosus* GG or *B. animalis* subsp. *lactis*, BB-12 for 22 hrs (Figure 2). IL-6
220 and IL-18 concentrations were similar on both apical and basolateral sides. IL-8, IP-10 and sICAM-1
221 concentrations were higher on the apical than the basolateral side. VEGF concentrations were higher
222 on the basolateral than the apical side. Neither live nor heat-inactivated *L. rhamnosus* GG or *B. animalis*
223 subsp. *lactis*, BB-12 altered the concentration of these mediators (Figure 2).
224

225 *3.4. Effect of pre-exposure to heat-inactivated L. rhamnosus GG and B. animalis subsp. lactis, BB-12 on the*
226 *Caco-2 cell TEER response to an inflammatory cocktail*

227 Incubation with the inflammatory cocktail reduced TEER by 30% at 24 hr and by 50% at 48 hr
228 (both p <0.001) (Figure 3). Pre-incubation for 24 hr with heat-inactivated *L. rhamnosus* GG or heat-
229 inactivated *B. animalis* subsp. *lactis,* BB-12 or their combination did not alter the effect of the
230 inflammatory cocktail on TEER (Figure 4).
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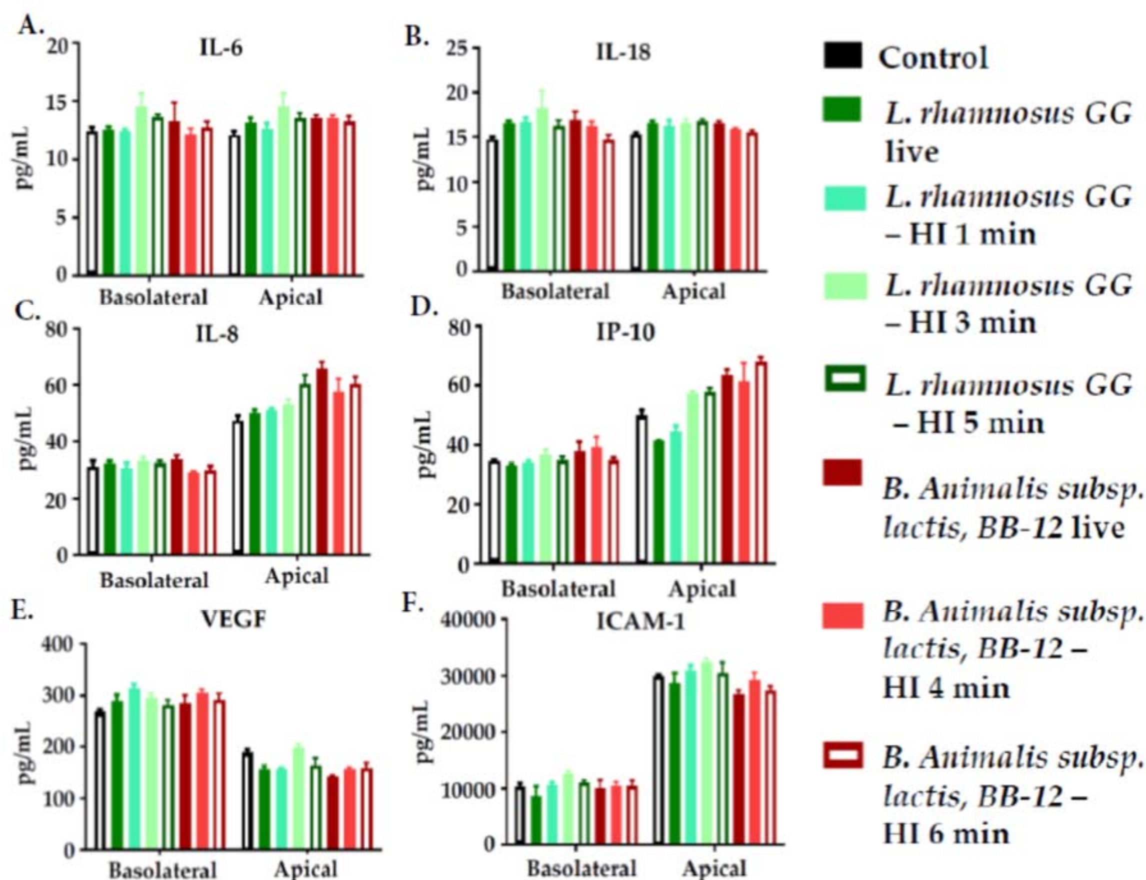

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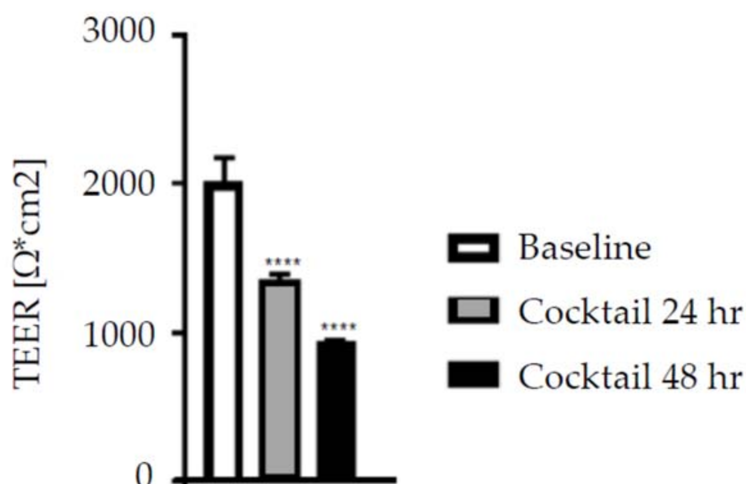
233 **Figure 2.** Concentrations (pg/mL) of IL-6 (A), IL-18 (B), IL-8 (C), IP-10 (D), VEGF (E) and sICAM-1 (F) in the
234 apical and basolateral medium of Caco-2 cells treated on the apical side with live or heat-inactivated (HI) *L.*
235 *rhamnosus* GG or *B. animalis* subsp. *lactis*, BB-12 for 22 hr. Data are mean + SEM (n=4). Data for apical and
236 basolateral sides were analysed separately by one-way ANOVA (all $p > 0.05$).



237
238 **Figure 3.** TEER measurements of Caco-2 cell cultures exposed to a cocktail of inflammatory cytokines on the
239 basolateral side for 24 or 48 hr. Data are mean + SEM (n=9). **** $p < 0.0001$ vs Baseline.

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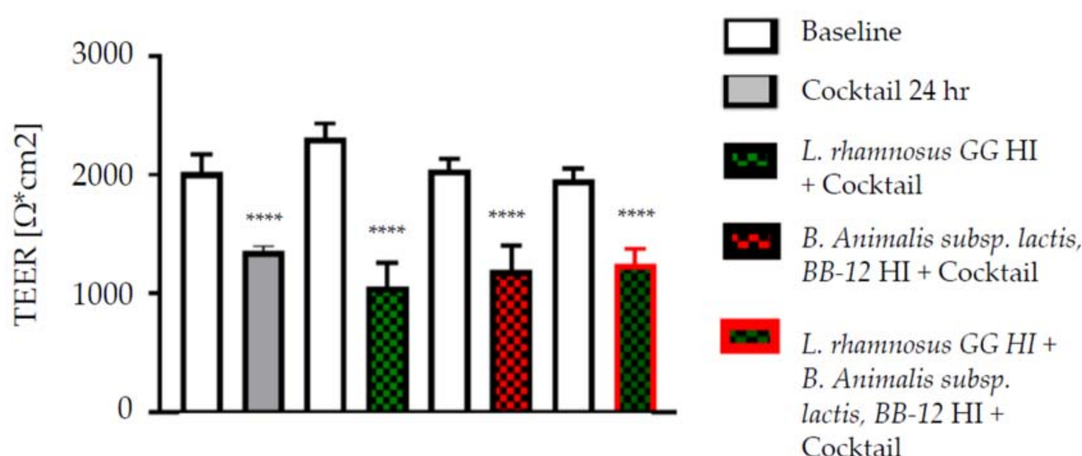

Figure 4. TEER measurements of Caco-2 cell cultures exposed to a cocktail of inflammatory cytokines following 24 hr pretreatment with heat-inactivated (HI) *L. rhamnosus* GG or *B. animalis* subsp. *lactis*, BB-12 or their combination. *L. rhamnosus* GG was heat-inactivated for 3 minutes at 70°C while or *B. animalis* subsp. *lactis*, BB-12 was heat-inactivated for 6 minutes at 62.3^{o}C. Data are mean + SEM (n=9). **** $p < 0.0001$ vs baseline.

3.5. Effect of pre-exposure to heat-inactivated L. rhamnosus GG and B. animalis subsp. lactis, BB-12 on the production of inflammatory mediators by Caco-2 cells in response to an inflammatory cocktail

The inflammatory cocktail significantly increased production of all inflammatory mediators measured (Figure 5). IL-6, IL-18 and IP-10 concentrations were similar on both apical and basolateral sides. IL-8, VEGF and sICAM-1 concentrations were higher on the apical than the basolateral side. IL-8 concentration was higher on the basolateral than the apical side.

IL-6 and IL-18 concentrations were decreased significantly in both the basolateral and apical medium by pre-treatment of Caco-2 cells with heat-inactivated *L. rhamnosus* GG, heat-inactivated *B. animalis* subsp. *lactis,* BB-12 or their combination prior to stimulation with the inflammatory cocktail (Figure 6). Heat-inactivated *L. rhamnosus* GG and heat-inactivated *B. animalis* subsp. *lactis,* BB-12 had similar effects. There were also some significant effects of heat-inactivated *L. rhamnosus* GG and the combination of heat-inactivated *L. rhamnosus* GG and heat-inactivated *B. animalis* subsp. *lactis,* BB-12 on VEGF concentration on the apical and basolateral sides, respectively. IL-8, IP-10 and sICAM-1 concentrations were not affected by heat-inactivated *L. rhamnosus* GG, heat-inactivated *B. animalis* subsp. *lactis,* BB-12 or the combination.

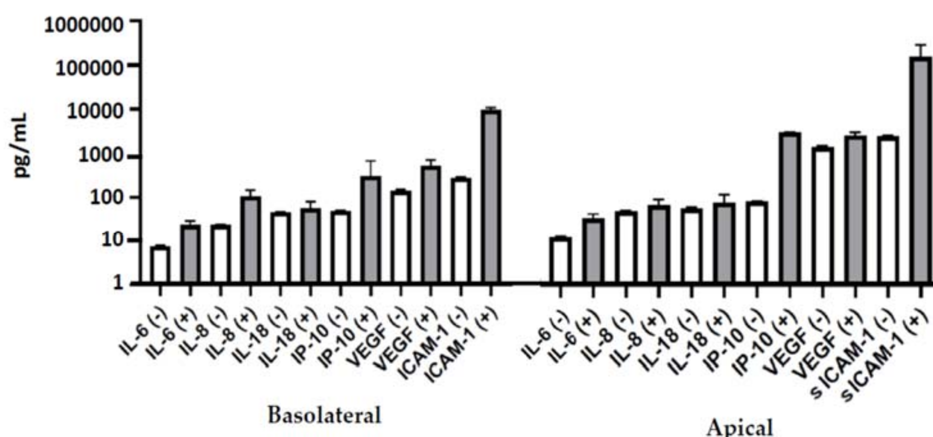

Figure 5. Cytokine concentrations (pg/mL) in the basolateral and apical medium of Caco-2 cells without (white bars "(-)") or with (grey bars "(+)") 24 hr treatment with an inflammatory cocktail on the basolateral side.

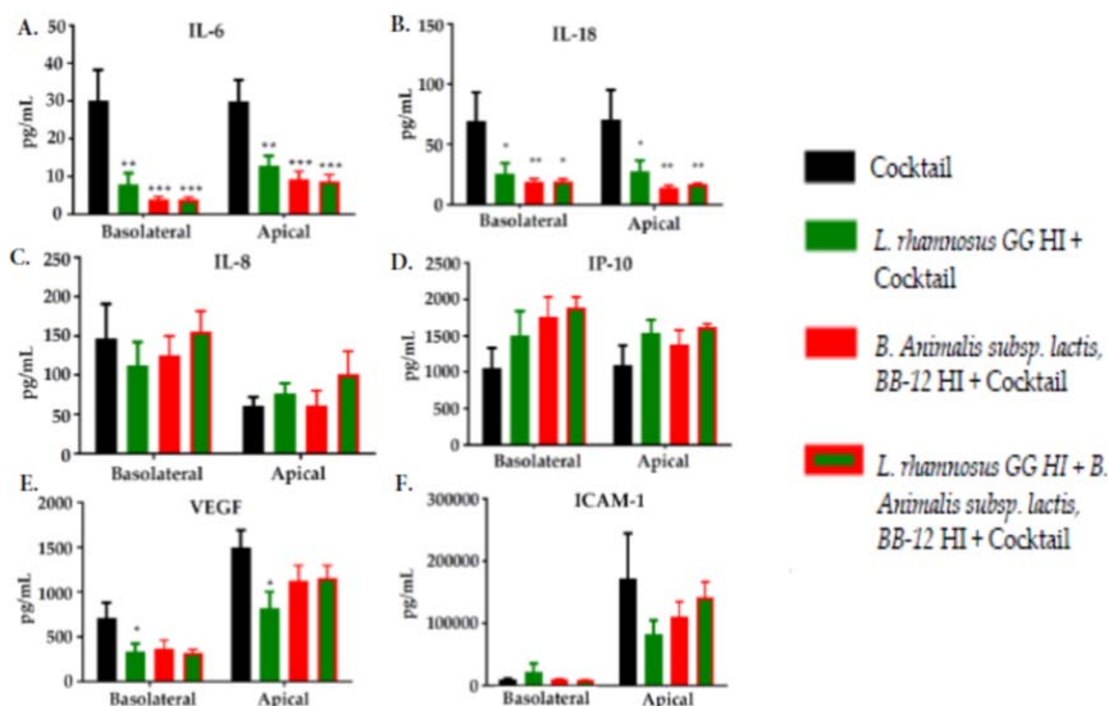

Figure 6. Cytokine concentrations (pg/mL) in the basolateral and apical medium of Caco-2 cells incubated on the apical side with heat-inactivated (HI) *L. rhamnosus GG*, *B. animalis* subsp. *lactis*, BB-12 or their combination for 24 hr prior to 24 hr treatment with an inflammatory cocktail on the basolateral side. *L. rhamnosus* GG was heat-inactivated for 3 minutes at 70°C while or *B. animalis* subsp. *lactis*, BB-12 was heat-inactivated for 6 minutes at 62.3°C. Data are mean + SEM (n=9). One-way ANOVA p value 0.0003 (IL-6 both basolateral and apical), 0.012 (IL-18 basolateral), 0.008 (IL-18 apical), 0.05 (VEGF basolateral), 0.07 (VEGF apical), > 0.15 (all others). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs cocktail alone.

4. Discussion

Both probiotics used here (*L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12) increased TEER values in Caco-2 cell monolayers after 22 hr of culture, indicating a strengthening of the epithelial monolayer when compared with the control condition. The effect was much greater for live organisms than for heat-inactivated organisms. This suggests a direct benefit from the live organisms on the epithelium, one that heat-inactivated organisms cannot fully exert, and that for an optimal beneficial effect on the epithelium live organisms are required. The difference in effect between live and heat-inactivated organisms suggests that there may be two separate mechanisms of interaction of these bacteria with the epithelium, one physical (and seen with heat-inactivated organisms) and one metabolic (and so requiring live organisms). Despite the clear effect of *L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12 on TEER, neither organism, either live of heat-inactivated, affected production of six inflammatory markers by unstimulated cultured Caco-2 cells.

Probiotics produce a number of metabolic products such as bacteriocins, acetaldehydes, and short-chain fatty acids which contribute to the maintenance of enterocyte integrity [19-21]. This allows probiotics to exert biological activity not only by inhibiting the pathogenic growth of microbes in the host (bacteriocins), but through the strengthening of tight junctions, as described by others [22, 23] and as suggested by the enhancement of TEER seen in the current research. The biological basis for any effect of heat-killed organisms has been described as an activity exerted through components in their cell walls, such as lipoteichoic acids [24] and peptidoglycan [25]. The active interaction between the bacterial strains and the host mucosal immune system and enterocytes differs and is specific according to bacterial properties. The differences observed between *L. rhamnosus* GG and *B.*

293 *animalis* subsp. *lactis*, BB-12, confirming the observations of others [26], are likely due to intrinsic
294 properties of each organism, such as the presence of the pili in *L. rhamnosus* GG [27], the composition
295 of the cell wall with components such as lipoteichoic acid [24] and the presence of proteins which
296 contribute to coping with stress conditions such as heat-inactivation [28].

297 The effect of live *L. rhamnosus* GG on TEER was stronger than that of live *B. animalis* subsp. *lactis*,
298 BB-12. One of the mechanisms by which *L. rhamnosus* GG seems to interact with epithelial cells in a
299 more effective manner than *B. animalis* subsp. *lactis*, BB-12 is through its pili structure, mainly because
300 the pili allow closer and stronger interaction with the enterocytes, while *B. animalis* subsp. *lactis*, BB-
301 12 lack this structure [5]. The findings suggest that the pili structure in the viable microorganism is
302 partially responsible for a better interaction with the enterocytes. Although it has been suggested that
303 the heat-inactivation does not destroy this structure [28], it might potentially reduce its ability to
304 function. Another mechanism of action by which *L. rhamnosus* GG is acknowledged to enhance
305 barrier function is through major secreted proteins p40 and p75 shown to protect against epithelial
306 barrier disruption *in vitro* and *ex vivo* [29, 30]. Live organisms are also known to release trophic factors
307 that interact with enterocytes (e.g. lactic acid and short chain fatty acids) which may play a significant
308 part in maintaining barrier integrity. For *L. rhamnosus* GG the results of the current study suggest that
309 those metabolic products may be more important for the interaction with epithelial cells than the
310 physical interaction with components of the bacterial cell wall. For *B. animalis* subsp. *lactis*, BB-12, on
311 the other hand, the TEER only dropped from an AUC of 207 to 142 when exposing Caco-2 cells with
312 viable compared to completely heat-inactivated bacteria. This indicates that for *B. animalis* subsp.
313 *lactis*, BB-12 the cell wall components also present in the heat-inactivated bacteria may play an
314 important role when interacting with epithelial cells.

315 Although it has been claimed that these strains in their inactivated form are safer than the
316 active form in immunocompromised individuals [31, 32], precisely due to their lack of metabolic
317 activity and lack of potential overgrowth, the findings from the TEER measurements indicate that
318 heat-inactivated microorganisms have reduced interaction with epithelial cells, at least from the
319 epithelial barrier integrity standpoint. The lack of effect of *L. rhamnosus* GG and *B. animalis* subsp.
320 *lactis*, BB-12 on inflammatory mediator production even when alive, suggests that the nature of the
321 interaction that increases TEER does not enhance or suppress the inflammatory response of gut
322 epithelial cells. *In vivo*, the gut barrier includes a significant immune cell component which might
323 respond differently from the epithelial cells [33]. Future *in vitro* experiments should explore these
324 other interactions. Furthermore, adding immune cells like dendritic cells into a co-culture system
325 with the Caco-2 cells can allow a better picture of inflammatory/immune responses to probiotic
326 bacteria as these may require such immune-epithelial cross-talk [34].

327 This research did not explore bacterial modifications following heat-inactivation or bacterial
328 components present in the co-culture media. Others have reported the effect of heat treatment of *L.*
329 *rhamnosus* on its structure and physical features[35, 36], although they used more severe conditions
330 than in the current study. Nevertheless, it has been suggested that the adhesion of inactivated *B.*
331 *animalis subsp. lactis,* BB-12 is considerable even upon inactivation at 80°C [37]. Moreover, a "probiotic
332 paradox" theory proposes that both live and dead cells are able to induce advantageous biological
333 responses [38]. Sugahara *et al*. compared immune-modulating properties of live and heat-inactivated
334 *B. breve* in a mouse model and showed that suppression in production of pro-inflammatory cytokines
335 and altered gene expression were seen for both bacterial forms [39].

336 The current study did not test mechanisms associated with direct contact of probiotic
337 organisms with the epithelial cells or metabolites produced in cultures or whether the enhancement
338 of TEER relates to an increase in junctional proteins. Despite the clear effect of *L. rhamnosus* GG and
339 *B. animalis* subsp. *lactis*, BB-12 on TEER, neither organism, either live or heat-inactivated, affected
340 production of six inflammatory mediators by unstimulated cultured Caco-2 cells. This suggests a
341 divergence in the signaling mechanisms that lead to tight junction integrity and to production of
342 inflammatory mediators, and that *L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12 can affect the
343 former but not the latter.

344 Having established effects of *L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12 on TEER
345 but not on inflammatory mediator production, the ability of the two organisms, when heat-
346 inactivated, to prevent the effects of an inflammatory stimulus (a cocktail of three cytokines) on these
347 outcomes was investigated. In preliminary experiments we tested the effect of the individual
348 components of the cytokine cocktail and of various combinations of these components using cytokine
349 production as the readout; these experiments showed that the cocktail of three cytokines used here
350 gave a greater responses than the individual components or combination of two components (data
351 not shown). The inflammatory cocktail itself decreased TEER, suggesting a breakdown in epithelial
352 integrity, and increased production of inflammatory mediators especially IL-6, IL-8, IP-10, and
353 ICAM-1. In these experiments, the Caco-2 cells were exposed to the heat-inactivated probiotics for 24
354 hr and then exposed to the inflammatory cocktail for a further 24 hr. Pre-treatment with the heat-
355 inactivated organisms did not prevent the effects of the inflammatory cocktail on TEER. However,
356 inflammatory cocktail induced production of IL-6 and IL-18, in particular, were decreased. Once
357 again, these observations suggest a divergence in the signaling mechanisms that lead to tight junction
358 integrity and to the production of inflammatory mediators. In these experiments the effects of heat-
359 inactivated *L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12 were similar. The findings suggest
360 that heat-inactivated *L. rhamnosus* GG or *B. animalis* subsp. *lactis*, BB-12, or their combination, could
361 be used to protect the intestine from an inflammatory insult. We did not test whether the organisms
362 could promote recovery from a pre-existing inflammatory state, but that would be very interesting
363 in the context of treatment of a range of gastrointestinal conditions with probiotics.

364 Other researchers have suggested that heat-inactivated *L. rhamnosus* GG enhances epithelial
365 barrier integrity through increased expression of the tight junction protein zonula occludens and
366 therefore increased junctional complexes [40]. The mentioned research was conducted in an animal
367 model of colitis, where an examination of the mucus layer was also performed. It is plausible that the
368 mechanism behind this benefit was the stimulation of mucus-producing cells by heat-inactivated *L.*
369 *rhamnosus* GG, so that the increased integrity was observed via increased mucus production. Further
370 experiments using the Caco-2 cell model could be performed by adding mucus-producing cells to
371 examine their interaction. It has been shown that a reduction in pro-inflammatory cytokine
372 production by Caco-2 cells is a contributory factor in the protection of the barrier and reduction of its
373 disruption [41]. The current study identified a reduction in cytokine production but no effect on
374 epithelial integrity as assessed by TEER and so indicates that these two responses may not always be
375 linked.

376 The results of the current study showed that the heat-inactivated organisms caused a
377 reduction in the appearance of IL-6 on both basolateral and apical sides of the Caco-2 cells when
378 added before the cytokine cocktail. TNF-α and IL-1β, components of the stimulating cytokine

cocktail, activate the transcription factors that induce IL-6 gene expression [42]. The observed reduction in IL-6 production suggests that the Caco-2 cell response to the heat-inactivated bacteria interferes with that signaling pathway. IL-6 is a pleiotropic cytokine with several effects on the immune response in the gut [43], as IL-6 receptors are expressed in intestinal cells [44]. This cytokine has been found in gut biopsies of patients with inflammatory disease and coeliac disease as well as in healthy controls. In these biopsies the protein was predominantly found in enterocytes [45]. These observations implicate IL-6 in intestinal damage and disease. However, other investigations have shown that IL-6 is also implicated in tissue repair, as some therapies blocking the effects of IL-6 cause damage to the intestine [46]. Thus, although the effects of the heat-inactivated organisms on IL-6 production may be viewed as anti-inflammatory and therefore of benefit, a clear conclusion of their health or clinical impact cannot be made.

IL-18 was also decreased by heat-inactivated *L. rhamnosus* GG and *B. animalis* subsp. *lactis*, BB-12 on both the basolateral and apical sides when the inflammatory cocktail was added. Inflammasomes are a complex of proteins that emerge during infections or tissue damage; they are induced by inflammatory cytokines [47]. Inflammasomes rapidly activate the release of IL-18 [47, 48]. The observations made here suggest that the Caco-2 cell response to the heat-inactivated bacteria interferes with the signaling pathway that leads to inflammasome activation or activity. Generally, IL-18 is relevant in the signaling within intestinal epithelial cells that activates further inflammatory responses [49].

VEGF production was decreased on the basolateral side by both heat-inactivated organisms and on the apical side by heat-inactivated *L. rhamnosus* GG. Release of VEGF by epithelial cells indicates a relationship with, and regulation of, endothelial cells [22]. VEGF has shown to be increased in the intestinal mucosa of patients with active inflammatory bowel disease, Crohn's disease and ulcerative colitis [50]. Thus, a reduction in VEGF production by enterocytes could be clinically relevant.

The current study used the Caco2 cell line, perhaps the most widely studied model of gut epithelial cells. However, it is important to recognize that these cells are epithelial colorectal adenocarcinoma cells. Nevertheless, they do possess many features of native gut epithelial cells: they spontaneously differentiate to a polarized columnar epithelium and express the functional characteristics of mature intestinal enterocytes. Polarized Caco-2 cell monolayers show TEER values that are more similar to the *in vivo* situation than seen with some other gut epithelial cell lines. Caco-2 cells also express most receptors, transporters and drug metabolizing enzymes found in normal gut epithelium. However, there are some limitations to the Caco-2 cell model. Firstly, as mentioned above, the normal epithelium contains more than one cell type (i.e. not only enterocytes), although this would be a limitation of any epithelial cell grown in isolation. Secondly, when using the Caco-2 cell model, no mucus and unstirred water layer is present.

5. Conclusions

L. rhamnosus GG and *B. animalis* subsp. *lactis,* BB-12 act on Caco-2 cells to increase TEER, an indicator of epithelial integrity, but they do not directly affect inflammatory mediator production of otherwise unstimulated cultures. Heat-inactivation of *L. rhamnosus* GG and *B. animalis* subsp. *lactis,* BB-12 markedly decreases their ability to increase TEER. Heat-inactivated *L. rhamnosus* GG and *B. animalis* subsp. *lactis,* BB-12 partially prevent the inflammatory cytokine-induced production of IL-6 and IL-18, and to a lesser extent VEGF, by Caco-2 cells, but do not prevent the inflammation-induced decline in TEER. Heat-inactivated *L. rhamnosus* GG and *B. animalis* subsp. *lactis,* BB-12 can therefore

423 have biological actions most likely due to structural features that are preserved after heat-
424 inactivation. Inflammatory signaling to interrupt epithelial integrity and to elicit inflammatory
425 cytokine production follow divergent pathways and only the latter is sensitive to heat-inactivated *L.*
426 *rhamnosus* GG and *B. animalis* subsp. *lactis,* BB-12. These findings are practically relevant because they
427 indicate that live and, to a lesser extent, heat-inactivated *L. rhamnosus* GG and *B. animalis* subsp. *lactis,*
428 BB-12 may strengthen the gut epithelial barrier and that the heat-inactivated organisms may prevent
429 adverse inflammatory responses of the gut epithelium. Thus, heat-inactivated *L. rhamnosus* GG and
430 *B. animalis* subsp. *lactis,* BB-12 may have food and nutraceutical applications.

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