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1 **Probiotic *Lactobacillus* strains from Mongolia improve calcium**  
2 **transport and uptake by intestinal cells in vitro**

3  
4 **Cyril Raveschot <sup>ab</sup>, François Coutte <sup>a</sup>, Marc Frémont <sup>b</sup>, Maxime Vaeremans <sup>b</sup>, Jamyan**  
5 **Dugersuren <sup>c</sup>, Shirchin Demberel <sup>c</sup>, Djamel Drider <sup>a</sup>, Pascal Dhulster <sup>a</sup>, Christophe**  
6 **Flahaut <sup>a</sup>, Benoit Cudennec <sup>a\*</sup>**

7  
8 <sup>a</sup> Univ. Lille, INRA, ISA, Univ. Artois, Univ. Littoral Côte d'Opale, EA 7394 - ICV - Institut  
9 Charles Viollette, F-59000 Lille, France

10 <sup>b</sup> VF Bioscience, Parc Eurasanté, F-59120 Loos-lez-Lille, France

11 <sup>c</sup> Probiotic Research Center, Mongolian Veterinary Institute, 17024 Ulaanbaatar, Mongolia

12  
13 **\*Corresponding author:**

14 Dr. Benoit Cudennec

15 Université de Lille, Institut Charles Viollette

16 Cité Scientifique, Bâtiment SN2

17 Avenue Mendeleiev

18 59655 Villeneuve d'Ascq, France

19 E-mail address: [benoit.cudennec@univ-lille.fr](mailto:benoit.cudennec@univ-lille.fr)

20

21 **Abbreviations**

22 ATR: Acid Tolerance Response

23 CFU: Colony Forming Unit

24 Cld-2: Claudin-2

25 Cld-4: Claudin-4

26 Cld-12: Claudin-12

27 DMEM: Dulbecco's Modified Eagle's Medium

28 LAB: Lactic Acid Bacteria

29 LDH: Lactate Dehydrogenase

30 MATS: Microbial Adhesion To Solvents

31 MRS: De Man, Rogosa, Sharpe

32 OD: Optical Density

33 PBS: Phosphate Buffered Saline

34 TEER: Trans Epithelial Electrical Resistance

35 TRPV6: Transient Receptor Potential Vanilloid 6

36 VDR: Vitamin D Receptor

37 **ABSTRACT**

38 The aim of this study was to investigate the probiotic properties of 174 *Lactobacillus* strains  
39 isolated from Mongolian dairy products, and particularly their impact on intestinal calcium  
40 uptake and absorption. All isolates underwent a first screening based on cell surface  
41 hydrophobicity, acid tolerance, tolerance to gastro-intestinal digestion, autoaggregation,  
42 adhesion and cytotoxicity against intestinal cells. Six *Lactobacillus* strains from different  
43 species (*L. casei*, *L. kefiranofaciens*, *L. plantarum*, *L. fermentum*, *L. helveticus* and *L.*  
44 *delbrueckii*) were selected, and their impact on intestinal calcium uptake and transport was  
45 investigated using Caco-2. Five strains were able to improve total calcium transport after 24h  
46 contact with a differentiated Caco-2 cell monolayer. Concomitantly the *L. plantarum* strain  
47 was able to increase cellular calcium uptake in Caco-2 cells by 10% in comparison to control  
48 conditions. To determine which pathway(s) of calcium absorption was modulated by the  
49 strains, a qPCR-based study on 4 genes related to calcium/vitamin D metabolism or tight  
50 junction integrity was conducted on mucus-secreting intestinal HT-29 MTX cells. The *L.*  
51 *plantarum* strain modulates the transcellular pathway by regulating the expression of vitamin  
52 D receptor (1.79 fold of control) and calcium transporter (4.77 fold of control) while the *L.*  
53 *delbrueckii* strain acts on the paracellular pathway by modulating claudin-2 expression (2.83  
54 fold of control). This work highlights the impact of *Lactobacillus* probiotic strains on  
55 intestinal calcium absorption and for the first time give some evidence about the cellular pathways  
56 involved

57

58 **Keywords:** Calcium intake; gut barrier; lactobacillus, probiotics, Caco-2 cells; HT29-MTX  
59 cells

60

## 61 **1. Introduction**

62 More than 99% of calcium in the human body is stored in the skeleton (Beto, 2015).

63 However, besides being the main mineral component of bones, calcium is also involved in  
64 many physiological processes such as vascular contraction, vasodilation, muscle function,  
65 neuronal transmission, intracellular signaling and hormonal secretion (Pu et al., 2016).

66 Calcium deficiency or hypocalcemia is associated with the development of bone diseases, like  
67 osteopenia and osteoporosis, but also arterial hypertension, insulin resistance, obesity and  
68 metabolic syndrome (Areco et al., 2015; Soto et al., 2014). Moreover, chronic hypocalcemia  
69 is associated with neuropsychiatric symptoms, cataract formation, and sometimes increased  
70 intracranial pressure (Hassan-Smith & Gittoes, 2017). These diseases occur predominantly in  
71 postmenopausal women, individuals with milk allergy or lactose intolerance, adolescents or  
72 elders (Beto, 2015).

73 About 90% of the overall calcium absorption occurs in the small intestine via two different  
74 pathways (Areco et al., 2015). The first one is a non-saturable process that relies on the  
75 electric gradient between lumen and intestinal mucosa, leading to the passive diffusion of  
76 calcium ions across the intestinal epithelium (Pérez et al., 2008). This transport mechanism is  
77 not fully understood but recent report showed the involvement of tight junction proteins  
78 claudin-2 and claudin-12 (Fujita et al., 2008). The second pathway is a transcellular saturable  
79 multistep process which first involves the uptake of calcium by epithelial intestinal cells via  
80 the  $\text{Ca}^{2+}$  channel protein transient receptor potential vanilloid 6 (TRPV6), followed by  
81 intracellular transport mediated by calbindin D9k proteins, and finally calcium extrusion  
82 through plasma membrane involving  $\text{Ca}^{2+}$ -ATPase PMCA and sodium-calcium exchanger  
83 NCX1 (Fleet & Schoch, 2010; Pu et al., 2016).

84 These  $\text{Ca}^{2+}$ -transport systems both depend on the steroid hormone 1,25 dihydroxyvitamin D,  
85 and its interaction with the nuclear vitamin D receptor (VDR). Vitamin D is thus crucial for

86 both calcium absorption in the intestine and metabolism in bones (Brincat et al., 2015;  
87 Bronner, 2003). Vitamin D-activated VDR acts as a transcription factor which regulates the  
88 expression of TRPV6, or TRPV5 in kidney, calbindin D9k, claudin-2 and claudin-12  
89 (Bronner, 2003; Fleet, 2017; Fujita et al., 2008; Haussler et al., 2010; Pu et al., 2016).  
90 Generally, hypocalcemia results from vitamin D or parathyroid hormone deficiency (Hassan-  
91 Smith & Gittoes, 2017). Deletion of VDR in mice causes disruption of calcium metabolism,  
92 severe hypocalcemia and elevated plasma levels of parathyroid hormone and vitamin D (Van  
93 Cromphaut et al., 2001).

94 Probiotics are defined as live microorganisms that, when administered in adequate amounts,  
95 confer a health benefit on the host. Live cultures associated with fermented foods or  
96 undefined fecal microbiota transplants are not include in this definition (Hill et al., 2014). The  
97 most common probiotics are lactic acid bacteria (LAB) such as *Lactobacillus*, *Streptococcus*,  
98 *Enterococcus*, *Bifidobacterium* (Roobab et al., 2020). The beneficial effects of probiotics are  
99 numerous and include enhancement of intestinal epithelium barrier function, production of  
100 antimicrobial substances, exclusion of pathogenic microorganisms, immunomodulation, anti-  
101 inflammatory, antidiabetic and hypolipidaemic effects (Belguesmia et al., 2019; Bermudez-  
102 Brito et al., 2012; Fernandez et al., 2011; Mousavi Khaneghah et al., 2020; Sarfraz et al.,  
103 2019; Shafi et al., 2019; Vasconcelos et al., 2019). The impact of *Lactobacillus* species on  
104 calcium metabolism was already evidenced *in vitro* (Gilman & Cashman, 2002) and *in vivo*  
105 by animal or clinical studies (Areco et al., 2015; Kruger et al., 2009; McCabe &  
106 Parameswaran, 2018; Nilsson et al., 2018; Parvaneh et al., 2014; Scholz-Ahrens et al., 2016),  
107 but the mechanisms involved are still unclear, and there has been little investigation on the  
108 impact of probiotics on calcium uptake.

109 The use of probiotics could be a safe and promising approach to treat elderly people suffering  
110 from hypocalcemia. A decrease in calcium absorption is indeed observed upon aging (Nordin

111 et al., 2004), and this is actually concomitant with a loss of microbiota diversity (Mello et al.,  
112 2016; Vaiserman et al., 2017). Calcium and vitamin D supplementations alone have failed to  
113 show any significant pharmacological effect in the treatment of osteoporosis (Brincat et al.,  
114 2015). However, probiotics may efficiently complement these supplementation strategies, by  
115 increasing calcium biodisponibility and absorption (Vaiserman et al., 2017). Consistent with  
116 this hypothesis, one publication previously reported that two strains of *L. plantarum* and *L.*  
117 *rhamnosus* could increase VDR expression in mouse and human cells (Wu et al., 2015).

118 This study aimed to evaluate the probiotic capacity of *Lactobacillus* strains isolated in three  
119 different areas of Mongolia (Raveschot et al., 2020). Production of dairies through  
120 spontaneous milk fermentation has a long history in this country. Herders use different milks  
121 (mare, cow, yak, camel, sheep, and goat) to produce a variety of fermented food such as  
122 yogurt, kefir, or airag (fermented horse milk). These products were reported to contain a wide  
123 diversity of LAB, especially *Lactobacillus* species (Watanabe et al., 2008; Yu et al., 2011). It  
124 was recently reported that calcium bioavailability in dairy products was not impacted by the  
125 food structure or components but was more dependent on the calcium source and its solubility  
126 (Lorieau et al., 2018). Nevertheless, the presence of probiotics in these products could  
127 increase the intestinal calcium absorption (Areco et al., 2015; Cavalcanti Neto et al., 2018;  
128 Chugh & Kamal-Eldin, 2020). In this study, a screening of 174 LAB isolates was carried out  
129 based on different probiotic characteristics. Criteria like cell surface hydrophobicity, acid  
130 tolerance, adhesion to intestinal cells, autoaggregation, and tolerance to gastrointestinal (GI)  
131 digestion were assessed. Then, the main objective of this study was to assess the possible  
132 impact of probiotic strains on intestinal calcium absorption. To achieve this goal, the selected  
133 strains were studied for their capacity to modulate calcium absorption using both Caco-2 and  
134 HT-29 MTX intestinal cells. This study provides evidence for the beneficial impact of  
135 Mongolian *Lactobacillus* probiotic strains on calcium metabolism and absorption.

136

## 137 **2. Material and methods**

### 138 **2.1. Mongolian *Lactobacillus* collection**

139 The screening was performed on a collection of 174 LAB strains isolated from Mongolian  
140 dairy products. Strain isolation and identification had been described previously (Raveschot et  
141 al., 2020). More than 93% of the isolates are *Lactobacillus* strains with 3 major species: *L.*  
142 *helveticus*, *L. delbrueckii* subsp. *bulgaricus* and *L. kefiranofaciens*. The other species in the  
143 collection are *L. fermentum*, *L. plantarum*, *L. kefiri*, *L. acidophilus*, *L. casei*, *Lactococcus*  
144 *lactis*, *Leuconostoc lactis* and *Streptococcus thermophilus*.

145

### 146 **2.2. Cell surface hydrophobicity determination**

147 Cell surface hydrophobicity was determined by the Microbial Adhesion To Solvents (MATS)  
148 test as described by Bellon-Fontaine et al., (1996). Briefly, strains were grown in 5 mL of  
149 MRS medium for 24h at 37°C, washed twice, by successive centrifugation at 13,000 × g for  
150 10 min and resuspension in 10 mL of phosphate buffered saline (PBS) buffer. The culture was  
151 finally resuspended in 10 mL of PBS buffer and the OD<sub>630nm</sub> of the suspension was  
152 determined. One milliliter of the suspension was mixed with 100 µL of n-hexadecane (Acros  
153 Organics, Molinons, France). The final mix was vortexed for 1 minute and let to rest for 15  
154 minutes at room temperature. One hundred microliters were then taken from the aqueous  
155 phase for OD<sub>630nm</sub> determination. Cell surface hydrophobicity was determined using the  
156 following formula:

$$157 \%H = \left( \frac{A_0 - A}{A_0} \right) \times 100$$

158 Where  $A_0$  is the initial  $OD_{630nm}$  of the bacterial suspension and  $A$  the final  $OD_{630nm}$  of the mix  
159 with n-hexadecane.

160

### 161 **2.3. Acid tolerance assay**

162 For acid tolerance assay, each strain was grown in 5 mL of MRS medium for 24h at 37°C.

163 Two hundred microliters of each culture were then diluted 1/10 in either MRS pH 6.2 (tube

164 A), or in MRS pH 2 (tube B). The tubes were incubated at 37°C for 2h and 10  $\mu$ L were

165 streaked on MRS plates followed by incubation for 48h at 37°C in anaerobic conditions. Acid

166 tolerance was evaluated by comparing the number of colonies on plates between A and B.

167 Strains were grouped in classes according to their acid tolerance: class 0, no colony on B

168 (strain not resistant), class 1, B = 0 to 30% of colonies on A (sensitive strain), class 2, B = 30-

169 80% of colonies on A (moderately sensitive strain), class 3, B > 80% of colonies on A

170 (tolerant strain).

171

### 172 **2.4. Autoaggregation assay**

173 Autoaggregation was assessed as described by Kotzamanidis et al., (2010). Briefly, strains

174 were grown in 5 mL of MRS medium for 24h at 37°C, centrifuged at  $13,000 \times g$  for 10 min

175 and resuspended in PBS buffer at  $OD_{600nm}$  of 1. Four milliliters of the suspension were then

176 vortexed for 10 seconds and let to rest at room temperature. One hundred microliters were

177 carefully removed from the upper suspension at 0, 2.5h and 5h of incubation. Samples were

178 then diluted (1/10) in PBS buffer and  $OD_{600nm}$  was measured. The autoaggregation percentage

179 was calculated according to the following formula:

180 
$$\%A = \left(1 - \frac{At}{A_0}\right) \times 100$$

181 Where  $A_t$  is the  $OD_{600nm}$  measured at  $t = 2.5$  or 5 hours and  $A_0$  the initial  $OD_{600nm}$  measured at  
182  $t = 0$  hour.

183

## 184 **2.5. Tolerance to simulated gastro-intestinal digestion**

185 The survivability of *Lactobacillus* strains was evaluated using a static *in vitro* digestion model  
186 inspired by Belguesmia et al., (2016). This model simulates mouth, stomach and small  
187 intestine steps of digestion by incubating the bacteria in fluids that mimic the physiological  
188 conditions encountered in these different compartments. Strains were grown in MRS for 24h  
189 at 37°C, washed twice and resuspended with PBS buffer at  $10^9$  CFU.mL<sup>-1</sup>. One milliliter of  
190 the bacterial suspension was used for digestion. Mouth step was simulated by adding 8 mL of  
191 PBS buffer (pH 6.8) to the bacterial suspension and incubating for 5 minutes at 37°C at  
192 constant agitation (200 rpm) in an incubation shaker (Infors HT, Basel, Switzerland). The  
193 gastric step was simulated by adding 12 mL of PBS buffer (pH 3) containing 1.56 mg.mL<sup>-1</sup> of  
194 porcine pepsin (Sigma-Aldrich, St Louis, USA). This step was conducted during 2h at 37°C  
195 and 200 rpm, the pH being maintained with HCl (1 M) or NaOH (1 M) at values ranging from  
196 3 to 3.5. Two milliliters of NaHCO<sub>3</sub> (1 M) were then added to raise the pH up to 7; finally, the  
197 small intestine step was then simulated by adding 12 mL of PBS buffer (pH 8.2) containing  
198 0.28 mg.mL<sup>-1</sup> of pancreatin from porcine pancreas (Sigma-Aldrich, St Louis, USA) and 6 mL  
199 of PBS buffer (pH 8.1) containing 60 g.L<sup>-1</sup> of bile salts (Sigma-Aldrich, St Louis, USA). This  
200 digestion step was conducted for 2h at 37°C and 200 rpm, the pH being maintained between 7  
201 and 7.5. Samples were taken after each step of the digestion process and diluted in PBS buffer  
202 (pH 7.4). One hundred microliters of adequate dilutions were streaked on MRS plates and  
203 incubated at 37°C for 48h under anaerobic conditions. The dilution factor varied from  $10^3$  to  
204  $10^8$  depending on each strain and step of the digestion process. The number of CFU was then  
205 calculated for each step of the simulated gastro-intestinal digestion.

206

## 207 **2.6. Cell lines and culture**

208 Caco-2 human colorectal carcinoma (Sigma-Aldrich, St Louis, USA) and mucus-secreting  
209 HT-29 MTX human adenocarcinoma (Sigma-Aldrich, St Louis, USA) cell lines were both  
210 used between passages 10 to 25. Both cell lines were grown in Dulbecco's Modified Eagle's  
211 Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 5 mM L-glutamine and  
212 100 U.mL<sup>-1</sup> each of penicillin and streptomycin (all from Dutscher, Brumath, France). For  
213 cytotoxicity assay and calcium uptake measurement, cells were seeded at a density of 8,000  
214 cells/well in 96-well tissue culture plate. For adhesion assay and gene expression analysis,  
215 cells were seeded at 40,000 cells/well in 24-well tissue culture plate. For calcium transport  
216 determination, cells were seeded at 50,000 cells/well on transwell permeable supports (0.4  
217 µm, polyester membrane) in 12-well tissue culture plate. Plates were incubated at 37°C under  
218 5% CO<sub>2</sub>-95% air atmosphere for 7 days for cytotoxicity and adhesion assays and for 15 days  
219 for the other experiments. Medium was replaced every 2 days after confluence (7 days).

220

## 221 **2.7. Adhesion assay**

222 *Lactobacillus* strains were grown in MRS for 24h at 37°C and washed in PBS buffer. Strain  
223 density was then adjusted to 10<sup>7</sup> CFU.mL<sup>-1</sup> in DMEM medium. After 7 days of culture, Caco-  
224 2 cells were washed twice with PBS buffer. Three hundred microliters of bacterial suspension  
225 were then added on confluent Caco-2 monolayer. After incubation for 2h at 37°C under 5%  
226 CO<sub>2</sub>, cells were washed twice with PBS buffer to remove non-adherent bacteria. Cells were  
227 then lysed by adding 100 µL of Triton X-100 in PBS 0.1% (v/v) and incubated for 15  
228 minutes. The lysate containing adherent bacteria was then diluted and streaked on MRS plates  
229 for CFU determination.

230

## 231 **2.8. Cytotoxicity assay**

232 *Lactobacillus* strain cytotoxicity was assessed on both Caco-2 and HT-29 MTX cell lines.

233 Bacterial suspensions were prepared as described in section 2.7. Cells were grown until

234 confluence (7 days) in 96-well tissue culture plate, washed twice in PBS buffer, then 150  $\mu$ L

235 of the different bacterial suspensions were added on cell monolayers. PBS buffer was used as

236 negative control. After incubation for 24h at 37°C under 5% CO<sub>2</sub>, 50  $\mu$ L of cell supernatant

237 were taken and lactate dehydrogenase (LDH) activity was determined using In Vitro

238 Toxicology Assay Kit, LDH Based (Sigma-Aldrich, St Louis, USA). Results were expressed

239 in percentage of the LDH activity of non-treated cells.

240

## 241 **2.9. Transepithelial calcium transport across Caco-2 monolayer**

242 Caco-2 cells were seeded on transwell permeable supports and grown for 15 days. Bacterial

243 suspensions were prepared as described in section 2.7. The Caco-2 cell monolayers were

244 washed twice with PBS, then 300  $\mu$ L of the bacterial suspension (or PBS buffer as control)

245 and 10  $\mu$ L of CaCl<sub>2</sub> (250 mM) were added on the cells (apical chamber) which were then

246 incubated for 24h at 37°C under 5% CO<sub>2</sub>. At 30 minutes, 7h and 24h of incubation, 100  $\mu$ L of

247 the basolateral medium were taken and kept at -20°C until calcium quantification. At the

248 same time, transepithelial electrical resistance (TEER) value of Caco-2 monolayer was

249 measured using a Voltmeter Ohmmeter MilliCell Electrical Resistance System (Merck

250 Millipore, Burlington, USA). Calcium quantification in samples taken from the basolateral

251 side of the Caco-2 cell monolayer was done using a Calcium Colorimetric Assay Kit (Sigma

252 Aldrich, St Louis, USA) following manufacturers' instructions and a microplate reader

253 spectrofluorometer (Xenius, Safas, Monaco, France). Results for both TEER and calcium  
254 quantification were expressed as fold or percentage of the initial sample (t = 30 min).

255

## 256 **2.10. Measurement of calcium uptake in Caco-2 cells**

257 Caco-2 cells were seeded in 96-well tissue culture plate and cultured for 15 days as described  
258 in section 2.6. Bacterial suspensions were prepared as described in section 2.7. Cell  
259 monolayer was washed with PBS buffer, 150  $\mu$ L of bacterial suspension were added in each  
260 well and incubated for 24h under 5% CO<sub>2</sub>. The supernatants were discarded and cells washed  
261 three times with PBS buffer. Then, calcium uptake by Caco-2 cells was determined using the  
262 FluoForte™ Calcium Assay Kit (ENZ-51017, Enzo Life Sciences, Farmingdale, USA).  
263 Briefly, the fluorogenic calcium binding dye FluoForte™ was added on Caco-2 monolayer for  
264 1 hour at room temperature until its integration in cells. Fluorescence was then monitored  
265 every second for a period of 30 seconds in a microplate reader spectrofluorometer (Xenius,  
266 Safas, Monaco, France). 25  $\mu$ L of CaCl<sub>2</sub> (250 mM) were injected in the well at 10 seconds of  
267 the kinetic. Fluorescence emission was expressed as fold of the basal fluorescence measured  
268 between 0 and 9 seconds of the kinetic.

269

## 270 **2.11. Gene expression analysis**

271 HT-29 MTX cells were grown in 24-well tissue culture plate for 15 days. Bacterial  
272 suspensions were prepared as described previously. The HT-29 MTX monolayer was washed  
273 twice with PBS buffer and 300  $\mu$ L of bacterial suspension were added in each well. PBS  
274 buffer was used as negative control (non-treated cells). After incubation for 24h at 37°C under  
275 5% CO<sub>2</sub>, cells were washed twice with PBS buffer and RNA extraction was performed using  
276 Trizol reagent (Sigma-Aldrich, St Louis, USA). Briefly, 100  $\mu$ L of Trizol reagent was added

277 in each well for cell lysis, the obtained lysates were then transferred in new sterile tubes.  
278 Twenty microliters of chloroform were added and the tubes were centrifuged 13,000 × g for  
279 15 minutes at 4°C. The RNA-containing fraction was taken and 50 µL of isopropanol were  
280 added followed by incubation at -20°C for 24h for RNA precipitation. Centrifugation for 20  
281 minutes at 13,000 × g at 4°C was then performed and the supernatants were discarded. The  
282 pellet was washed with ethanol 70% (v/v) and centrifuged for 20 minutes at 13,000 × g at  
283 4°C. RNAs were then air dried for 3h at room temperature and finally dissolved in water.  
284 Extracted total RNA were kept at -80°C. Concentration and purity of each sample was  
285 assessed on a NanoDrop Lite (ThermoScientific, Waltham, USA). cDNA was obtained by  
286 reverse transcription on a Mastercycler gradient (Eppendorf, Hambourg, Germany) using the  
287 RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoScientific, Waltham, USA).  
288 Four genes were studied: vitamin D receptor (*vdr*), claudine-2 (*cld-2*), claudine-4 (*cld-4*) and  
289 transient receptor potential selective for calcium (*trpv6*). The peptidylprolyl isomerase A  
290 (*ppia*) gene was used as reference gene (Domenger et al., 2018). For each gene, reverse  
291 transcribed cDNA was quantified by comparative Ct experiment on a CFX Connect Real  
292 Time Detection System (Bio-Rad, Hercules, USA) using the Power SYBR Green PCR Master  
293 Mix (Applied Biosystems, Life Technologies, Foster City, USA). Specific primers were used:  
294 forward 5'-GCCACCTGCTCTATGCCAAG-3' and reverse 5'-  
295 CAGGCTGTCCTAGTCAGGAGAT-3' for *vdr* gene; forward 5'-  
296 TGATGCGGCTCATCAGTGCCAGC-3' and reverse 5'-  
297 GTAGAAGTGGCCTAGCTCCTCG-3' for *trpv6* gene; forward 5'-  
298 TGGCCTCTCTTGGCCTCCAACCTTGT-3' and reverse 5'-  
299 TTGACCAGGCCTTGGAGAGCTC-3' for *cld-2* gene; forward 5'-  
300 CCACTCGGACAACCTTCCCAA-3' and reverse 5'-ACTTCCGTCCCTCCCCAATA-3' for  
301 *cld-4* gene; forward 5'-TGCTGACTGTGGACAACCTCG-3' and reverse 5'-

302 TGCAGCGAGAGCACAAAGAT-3' for *ppia* gene. Thermocycler conditions were 10  
303 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at  
304 72°C, followed by a melting curve step. mRNA fold induction was calculated according to the  
305  $2^{-\Delta\Delta C_t}$  method (Livak & Schmittgen, 2001) using *ppia* as reference gene. Results were  
306 expressed as fold of control (non-treated cells).

307

### 308 **3. Results**

309

#### 310 **3.1. Strain selection based on cell surface hydrophobicity and acid tolerance**

311 The first step of selection was conducted by assessing cell surface hydrophobicity and acid  
312 tolerance. The cell surface hydrophobicity of the Mongolian strains ranged from 0 to 74%  
313 (**Figure 1**). 50% of the studied strains showed less than 10% cell surface hydrophobicity.  
314 Acid tolerance repartition was done in classes from 0 to 3 (non resistant to highly tolerant).  
315 Among the 174 studied strains, 85 fell into class 0, 29 into class 1, 16 into class 2 and 44 into  
316 class 3 (**Figure 1**). 75% of the strains in class 0 were *L. delbrueckii*, highlighting the poor acid  
317 tolerance of this species. In contrast, all *L. fermentum* and *L. plantarum* strains fell into class  
318 3. For the 2 other species most represented in the Mongolian collection (*L. helveticus* and *L.*  
319 *kefiranofaciens*), acid tolerance appeared to be strain-dependent, as the isolates were more  
320 equally distributed between classes. **Figure 1** represents cell surface hydrophobicity versus  
321 acid tolerance for all studied strains. For the rest of this study, 8 *Lactobacillus* strains were  
322 selected i) with cell surface hydrophobicity more than 30%, ii) preferentially in class 3 for  
323 acid tolerance and iii) belonging to different *Lactobacillus* species. The 8 *Lactobacillus*  
324 strains selected were *L. casei* 9b, *L. kefiranofaciens* 15b, *L. plantarum* 46a, *L. fermentum* 48a,  
325 *L. helveticus* 49d, *L. delbrueckii* 50b, *L. delbrueckii* 56a and *L. fermentum* 62b.

326

### 327 **3.2. Strains were resistant to simulated GI digestion**

328 The 8 selected *Lactobacillus* strains were then tested for survivability upon simulated GI  
329 digestion. At the beginning of the *in vitro* digestive process the bacterial cells were added at  
330  $10^9$  CFU.mL<sup>-1</sup>. At the end of the first step (mouth conditions), the number of living bacterial  
331 cells was about  $10^9$  CFU.mL<sup>-1</sup> for all strains (**Figure 2**), which corresponds to the initial  
332 amount: there was no decrease of viability at this stage. Depending on the strain, the number  
333 of viable counts then decreased to various extents in subsequent digestion steps. The most  
334 sensitive strains were *L. plantarum* 46a and *L. delbrueckii* 56a who went down to  $10^6$   
335 CFU.mL<sup>-1</sup> at the end of the whole process. The other strains reached cell counts ranging from  
336  $10^8$  to  $10^7$  CFU.mL<sup>-1</sup>.

337 It is important to note that samples resulting from strain GI digestion were not used for the  
338 further experiments in intestinal cells because bile acids are toxic for cultured cells. Thus, in  
339 order to get closer to post-digestion conditions, the concentration of bacterial suspensions was  
340 adjusted at the mean concentration reached at the end of the digestion process ( $10^7$  CFU.mL<sup>-1</sup>).  
341

### 342 **3.3. Autoaggregation, adhesion capacity and cytotoxicity**

343 An increase of strain autoaggregation was observed for all isolates between 2.5h and 5h of  
344 incubation in PBS, except for *L. casei* 9b which had already reached its maximal value at  
345 2.5h. This strain showed the highest capacity of autoaggregation with about 95% of cells  
346 aggregated at the end of the test; for the other strains, autoaggregation scores at 5h ranged  
347 from 7.86% to 67.44% (**Table 1**).

348 *Lactobacillus* strains were tested for their adhesion capacity on Caco-2 cells monolayer.

349 Percentages of adherent bacteria varied between 0.18 and 4.63% depending on the strain

350 (**Table 1**). The 3 less adherent strains were *L. casei* 9b, *L. delbrueckii* 50b and 56a whereas  
351 the 3 most adherent strains were *L. kefiranofaciens* 15b, *L. plantarum* 46a and *L. fermentum*  
352 48a.

353 Interestingly, adhesion and autoaggregation results were negatively correlated. For instance,  
354 *L. casei* 9b strain presented a high aggregation percentage but a weak adhesion capacity  
355 whereas *L. kefiranofaciens* 15b showed a poor aggregation capacity but a high adhesion  
356 potential. This negative correlation was confirmed by the calculation of Pearson's coefficients  
357 between adhesion and autoaggregation, that were equal to -0.6 (autoaggregation values at  
358 2.5h) and -0.75 (autoaggregation values at 5h).

359 In parallel the cytotoxicity of the isolates was evaluated on both Caco-2 and HT-29 MTX  
360 cells (LDH assay). No significant cytotoxicity (one way ANOVA,  $p < 0,05$ ) was observed for  
361 all tested *Lactobacillus* strains after 24h of contact with the 2 cell lines (**Table 2**).

362 Based on these results, 6 *Lactobacillus* strains were kept for the rest of this study: *L. casei* 9b,  
363 *L. kefiranofaciens* 15b, *L. plantarum* 46a, *L. fermentum* 48a, *L. helveticus* 49d and *L.*  
364 *delbrueckii* 50b.

365

### 366 **3.4. Modification of calcium uptake and transport in Caco-2 cells**

#### 367 *3.4.1. Modification of total calcium transport across Caco-2 monolayer*

368 Modulation of total calcium transport was assessed by determination of calcium flux across  
369 fully differentiated Caco-2 monolayer upon contact with the selected *Lactobacillus* strains.

370 The TEER of Caco-2 monolayer was measured at the same time. The TEER increased  
371 significantly (two way ANOVA) during the experiment (**Figure 3A**); however there was no  
372 statistical difference between cells that were put in contact with the bacterial suspensions and

373 cells treated with PBS. This suggested that none of the tested strains had a detrimental effect  
374 on Caco-2 monolayer integrity.

375 None of the strains was found to increase total calcium transport through the Caco-2  
376 monolayer after 7h of contact (**Figure 3B**). However, after 24h of contact, 4 strains  
377 significantly increased the basal side calcium concentration compared to control condition  
378 (PBS buffer): *L. casei* 9b, *L. kefiranofaciens* 15b, *L. helveticus* 49d and *L. delbrueckii* 50b.  
379 Moreover, a tendency for calcium increase at the basal side was observed for *L. plantarum*  
380 46a strain (P value = 0.052). There was no increase of calcium transport for cells treated with  
381 *L. fermentum* 48a.

382

#### 383 3.4.2. *Lactobacillus* strains could modulate calcium uptake by Caco-2 cells

384 Calcium uptake was assessed after contact for 24h between bacterial strains and fully  
385 differentiated Caco-2 cells. After injection of CaCl<sub>2</sub>, an increase in fluorescence emission  
386 occurred showing the calcium incorporation by Caco-2 cells (**Figure 4**). In control condition  
387 (PBS), the ratio of fluorescence emission increased from 1 to 1.3-1.4 after calcium injection.  
388 The ratio increase was not significantly different upon contact with *L. casei* 9b, *L.*  
389 *kefiranofaciens* 15b, *L. helveticus* 49d and *L. delbrueckii* 50b. A significant diminution of  
390 calcium uptake was observed after contact with *L. fermentum* 48a strain compared to control  
391 condition. In contrast, calcium uptake was higher in Caco-2 cells monolayer incubated with *L.*  
392 *plantarum* 46a bacteria, than in the control monolayer: the ratio of fluorescence increased  
393 significantly from 1 to 1.4-1.5 after calcium injection. These results suggested that *L.*  
394 *fermentum* 48a and *L. plantarum* 46a modulate calcium uptake by Caco-2 cells.

395

#### 396 3.6. Change in *vdr*, *trpv6*, *cld-2* and *cld-4* RNA levels on HT-29 MTX cells

397 The effect of the 6 selected *Lactobacillus* strains on the expression of 4 genes related to  
398 calcium/vitamin D metabolism or tight junction integrity (*vdr*, *trpv6*, *cld-2* and *cld-4*) were  
399 evaluated on HT-29 MTX cells. Only 2 strains modulated the expression of *vdr* in HT-29  
400 MTX cells: *L. plantarum* 46a and *L. delbrueckii* 50b (**Figure 5A**). *vdr* mRNA level increased  
401 by 1.8 fold compared to control condition (PBS buffer). Four strains significantly increased  
402 the level of *trpv6* mRNA: *L. casei* 9b, *L. kefiranofaciens* 15b, *L. plantarum* 46a and *L.*  
403 *helveticus* 49d (**Figure 5B**). Only *L. delbrueckii* 50b lead to a significant increase of *cld-2*  
404 mRNA level (**Figure 5C**), reaching 3 fold the level seen in control condition. However, all  
405 *Lactobacillus* strains induced a significant increase of *cld-4* mRNA level (at least 2 fold of  
406 control). The highest *cld-4* mRNA level was observed by treatments with *L. casei* 9b, *L.*  
407 *kefiranofaciens* 15b and *L. helveticus* 49d strains (**Figure 5D**).

408 In summary, 4 patterns of mRNA level changes could be distinguished among the 4 tested  
409 genes. *L. fermentum* 48a modulated only *cld-4* gene. Three *Lactobacillus* strains modulated  
410 *cld-4* and *trpv6* mRNA levels: *L. casei* 9b, *L. kefiranofaciens* 15b and *L. helveticus* 49d. *L.*  
411 *delbrueckii* 50b lead to an increase of *vdr*, *cld-2* and *cld-4* gene expressions. Finally, *L.*  
412 *plantarum* 46a strain modulated expression of *vdr*, *trpv6* and *cld-4* genes.

413

414

#### 415 **4. Discussion**

416 The first objective of this study was to evaluate the probiotic characteristics of 174 LAB  
417 strains isolated from Mongolian dairy products. Before looking at biological effects *per se*,  
418 the screening focused on the analysis of parameters related to the capacity of the strains to  
419 settle and survive in the GI tract, and interact with intestinal epithelial cells without exerting  
420 any cytotoxicity.

421 Acid tolerance determination was used as a first evaluation of the bacterial cell capacity to  
422 resist GI digestion (particularly the gastric step) and reach the intestine in a viable and active  
423 state (Angmo et al., 2016; Lo Curto et al., 2011). The acid tolerance response (ATR) of  
424 *Lactobacillus* is known to be variable (Sengupta et al., 2013). The differences observed in our  
425 study were, to some extent, species-specific. All *L. fermentum* and *L. plantarum* strains tested  
426 were tolerant to acid condition. These species were already reported to be tolerant to pH 2  
427 (Ramos et al., 2013). In contrast, all *L. delbrueckii* isolates were weakly tolerant, which is also  
428 consistent with previous reports (Charteris et al., 1998; Vinderola & Reinheimer, 2003).

429 Cell surface hydrophobicity was also analyzed. It is thought to be a determinant of cell  
430 adhesion capacity (Van Loosdrecht et al., 1987). Adherence to intestinal cells is considered as  
431 a major criteria for the selection of probiotic strain (Vinderola et al., 2004), as it may in some  
432 cases mediate the biological activities observed (Di Cerbo et al., 2016; Sengupta et al., 2013).

433 A high variability was observed between the Mongolian isolates, even within the same  
434 species. Such variability was already observed; it reflects the fact that hydrophobicity depends  
435 on a complex pattern of cell wall components (peptidoglycan, teichoic acids, cell wall  
436 polysaccharides, cell surface proteins) (Angmo et al., 2016; Sengupta et al., 2013).

437 Isolates presenting the highest hydrophobicity values (29.2% for *L. fermentum* 62b, up to  
438 69.1% for *L. casei* 9b), and showing strong acid tolerance, were further considered for the  
439 screening (*L. casei* 9b, *L. kefiranofaciens* 15b, *L. plantarum* 46a, *L. fermentum* 48a, *L.*  
440 *helveticus* 49d and *L. fermentum* 62b). Two *L. delbrueckii* strains (50b, 56a) were also kept  
441 owing to their high cell surface hydrophobicity (36.3% and 48.9%, respectively).

442 Consistent with the high hydrophobicity values, most of the selected strains showed a good  
443 percentage of adhesion to Caco-2 cells, ranging from 1.48 to 4.63%. These values are in  
444 agreement with Tuomola and Salminen (1998) which obtained adhesion percentages ranging  
445 from 2.6 to 14.4%. Both *L. delbrueckii* strains (50b and 56a) make a notable exception, with

446 low adhesion percentages (0.18% and 0.22%). Adhesion to intestinal cells depends on the  
447 strain cell wall composition which varies between *Lactobacillus* strains and species (Dimitrov  
448 et al., 2014; Sengupta et al., 2013).

449 Another parameter analyzed was autoaggregation. This trait is also thought to relate to the  
450 capacity of the bacteria to interact with intestinal cells (Abushelaibi et al., 2017; Kotzamanidis  
451 et al., 2010). Autoaggregation results were highly variables between the strains. Six of the 8  
452 tested strains showed high autoaggregation percentages after 5h incubation in PBS, the values  
453 were similar in range to those obtained by Kotzamanidis et al., (2010). Surprisingly, a  
454 negative correlation was observed between autoaggregation and adhesion percentage. This  
455 observation was not in agreement with a previous report of a positive correlation  
456 (Kotzamanidis et al., 2010). However, other authors did not find any correlation between  
457 aggregation abilities and adhesion, suggesting that interaction of probiotic bacteria with  
458 intestinal cells involves more complex mechanisms (García-Cayuela et al., 2014; Ramos et  
459 al., 2013).

460 Resistance to GI digestion is an important criteria for probiotic selection (Shewale et al.,  
461 2014). In addition to the acid tolerance assay, a static *in vitro* digestion model was used  
462 (Belguesmia et al., 2016), mimicking the impact of different GI digestion effectors (digestive  
463 enzymes, bile salt and electrolytes). The tested *Lactobacillus* strains displayed good tolerance  
464 abilities, reaching a high number of viable cells after the intestinal phase (at least  $10^6$   
465 CFU.mL<sup>-1</sup>). The most deleterious step was the gastric phase with the combined effect of low  
466 pH and gastric enzymes except *L. casei* 9b, that was unaffected by all steps of GI digestion,  
467 and *L. plantarum* 46a for which a significant decrease was only observed at the end of the  
468 whole process. Protocols used to simulate GI digestion (pH, enzyme concentrations,  
469 incubation times) differ between studies, comparing our findings with published results is  
470 therefore difficult. In most studies however, a loss 1 to 3 points of log(CFU/mL) was seen

471 following simulated GI digestion (Belguesmia et al., 2016; Fernandez et al., 2003; Pitino et  
472 al., 2010), which is consistent with our results.

473 Finally, no cytotoxic effect was reported for the tested *Lactobacillus* strains on both Caco-2  
474 and HT-29 MTX intestinal cells. This is in agreement with previous reports, supporting the  
475 recognition of *Lactobacillus* strains as safe bacteria (Er et al., 2015; Messaoudi et al., 2012).

476 Based on the results obtained, 6 *Lactobacillus* strains were kept for further studies on calcium  
477 transport modulation: *L. casei* 9b, *L. kefiranofaciens* 15b, *L. plantarum* 46a, *L. fermentum*  
478 48a, *L. helveticus* 49d and *L. delbrueckii* 50b. Two isolates were eliminated, *L. delbrueckii*  
479 56a for its poor tolerance to GI digestion and *L. fermentum* 62b for its adhesion ability lower  
480 than *L. fermentum* 48a.

481 Modulation of calcium metabolism by probiotic bacteria was mostly studied *in vivo* on bones;  
482 usually probiotic treatment resulted in increased bone calcium content and reduced rate of  
483 bone loss (Areco et al., 2015; Kruger et al., 2009; McCabe & Parameswaran, 2018; Parvaneh  
484 et al., 2014; Scholz-Ahrens et al., 2016). In this study, the effect of *Lactobacillus* strains on  
485 intestinal total calcium transport was assessed *in vitro* using Caco-2 cells. The Caco-2  
486 monolayer is considered as a relevant model for calcium transport experiments (Giuliano et  
487 al., 1991); indeed, these cells possess an enterocyte like phenotype after differentiation, a  
488 functional vitamin D receptor and both saturable and non-saturable calcium transport  
489 pathways (Fleet & Wood, 1999).

490 Assessment of TEER values confirmed that contact with the bacteria for 24h did not affect the  
491 integrity of the Caco-2 membrane, nor impact the paracellular transport of nutrients. Actually,  
492 a reinforcement of membrane integrity was observed for all tested conditions. In previous  
493 studies, *Lactobacillus* strains improved or did not change TEER values on Caco-2 cells (Alard  
494 et al., 2018; Belguesmia et al., 2019; Ramos et al., 2013). In the same experiment, calcium

495 concentration in the basal side of the monolayer was assessed. Except for *L. fermentum* 48a,  
496 all strains increased calcium concentration in the basal compartment after 24h of incubation,  
497 suggesting an improvement of total calcium transport by Caco-2 cells. However, calcium  
498 uptake by Caco-2 was only improved significantly by *L. plantarum* 46a strain. Calcium  
499 uptake by enterocytes is the first step of the saturable pathway of calcium absorption (Pérez et  
500 al., 2008). It has been observed that a strain of *Lactobacillus salivarius* improved calcium  
501 uptake in Caco-2 cells (Gilman & Cashman, 2002) but to our knowledge, this is the first  
502 report showing an impact of probiotic *Lactobacillus* on intestinal calcium transport *in vitro*.

503 In order to understand the mechanism by which *Lactobacillus* strains improved calcium  
504 uptake and/or transport, the expression of four genes related to calcium/vitamin D metabolism  
505 or tight junction integrity was assessed on HT-29 MTX cells. HT-29 MTX cells, because of  
506 their growth characteristics, are not well suited for experiments requiring monolayer  
507 formation; but the presence of a mucus layer on differentiated cells, which mimics the  
508 conditions of the intestinal mucosa, makes them an excellent model to study bacteria/gut cell  
509 interactions. The four genes investigated were the genes coding for VDR and TRPV6, which  
510 are directly involved in the saturable transcellular pathway of calcium transport (Bronner,  
511 2003; Wood et al., 2001), and the genes coding for claudin-2 and claudin-4. Although the  
512 paracellular pathway is not completely elucidated, it seems that claudin-2, as a component of  
513 tight junctions, drives the selective paracellular transport of calcium through the intestinal  
514 mucosa (Amasheh, 2002; Fujita et al., 2008). Claudin-4 is involved in tight junction integrity  
515 and plays a critical role in regulating paracellular permeability (Lu et al., 2013).

516 All tested strains lead to an increase of *cld-4* mRNA level, suggesting improvement of  
517 membrane integrity (Lechuga & Ivanov, 2017), which is consistent with the TEER results on  
518 Caco-2 cells. *L. fermentum* 48a did not modulate any of the other genes, which is in  
519 accordance with its absence of effect on total calcium transport. *L. casei* 9b, *L.*

520 *kefiranofaciens* 15b and *L. helveticus* 49d, all of which improved total calcium transport, had  
521 a moderate effect on *trpv6* gene expression but did not modulate the expression of *vdr* or *cld-*  
522 2. Based on these results, it is difficult to identify the pathway(s) by which these 3 strains  
523 were able to improve intestinal calcium absorption. One possibility is the reduction of apical  
524 (luminal) pH, by production of lactic acid or short chain fatty acids (mostly acetate,  
525 propionate and butyrate), which contributes to increase calcium solubility and absorption  
526 (Pérez et al., 2008; Scholz-Ahrens et al., 2007). Experiments should be conducted to analyze  
527 the metabolites produced by these strains in intestinal condition. *L. delbrueckii* 50b improved  
528 total calcium transport and upregulated both *vdr* and *cld-2* mRNA levels. Claudin-2 serves as  
529 transport channel for ions and water (Lu et al., 2013). Together with claudin-12, it plays a role  
530 in paracellular calcium diffusion under the control of vitamin D (Fujita et al., 2008).  
531 Therefore, our results suggested that *L. delbrueckii* 50b strain improves calcium absorption *in*  
532 *vitro* by modulation of the paracellular pathway.

533 The last strain tested in this study was *L. plantarum* 46a. This strain increased both *vdr* and  
534 *trpv6* mRNA levels, and there was a tendency for improvement of calcium transport.  
535 Interestingly it was the only strain tested which significantly increased calcium uptake by  
536 Caco-2 cells. These two results are consistent, because TRPV6 is responsible for calcium  
537 uptake by the cell; the suggested mechanism is therefore a modulation of the transcellular  
538 calcium absorption pathway, involving upregulation of TRPV6 (Pérez et al., 2008). It is  
539 important to note that the involvement of a calcium transporter (TRPV6) makes the  
540 transcellular pathway a saturable pathway. This actually prevents intracellular calcium  
541 concentration to reach unacceptable levels (Bronner, 2003; Fleet & Schoch, 2010). In the  
542 meantime, this may also explain why the increase of total calcium transport by Caco-2 cells  
543 upon contact with *L. plantarum* 46a was relatively moderate.

544 In conclusion, by screening a collection of 174 Mongolian LAB isolates, we were able to  
545 identify 5 *Lactobacillus* strains displaying good probiotic characteristics, and able to modulate  
546 calcium absorption by intestinal cells. The calcium absorption pathway actually modulated  
547 appeared to depend on the tested strain. *L. casei* 9b, *L. kefiranofaciens* 15b and *L. helveticus*  
548 49d were able to increase total calcium transport by Caco-2 cells most probably through an  
549 improvement of calcium solubility. *L. delbrueckii* 50b impacted the paracellular pathway of  
550 calcium absorption by up-regulation of the *cld-2* gene. Finally, *L. plantarum* 46a seemed to  
551 improve intestinal calcium uptake and absorption through the transcellular pathway involving  
552 VDR and TRPV6. This work provides new insights regarding the effects of probiotics on  
553 calcium absorption, and their potential use in the prevention or treatment of hypocalcemia.  
554 These results will be confirmed by *in vivo* experiments.

555

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563

## 564 **Conflict of interest**

565 CR, MF and MV are affiliated with VF Bioscience SAS, France, a company commercializing  
566 lactic acid bacteria strains for use as probiotics or dairy starters.

567 **References**

- 568 Abushelaibi, A., AlMahdin, S., El-Tarabily, K., Shah, N. P., & Ayyash, M. (2017).  
569 Characterization of potential probiotic lactic acid bacteria isolated from camel milk. *LWT*  
570 *- Food Science and Technology*, 79, 316–325. <https://doi.org/10.1016/j.lwt.2017.01.041>
- 571 Alard, J., Peucelle, V., Boutillier, D., Breton, J., Kuyllé, S., Pot, B., Holowacz, S., &  
572 Grangette, C. (2018). New probiotic strains for inflammatory bowel disease management  
573 identified by combining in vitro and in vivo approaches. *Beneficial Microbes*, 9(2), 317–  
574 331. <https://doi.org/10.3920/BM2017.0097>
- 575 Amasheh, S. (2002). Claudin-2 expression induces cation-selective channels in tight junctions  
576 of epithelial cells. *Journal of Cell Science*, 115(24), 4969–4976.  
577 <https://doi.org/10.1242/jcs.00165>
- 578 Angmo, K., Kumari, A., Savitri, & Bhalla, T. C. (2016). Probiotic characterization of lactic  
579 acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT - Food*  
580 *Science and Technology*, 66, 428–435. <https://doi.org/10.1016/j.lwt.2015.10.057>
- 581 Areco, V., Rivoira, M. A., Rodriguez, V., Marchionatti, A. M., Carpentieri, A., & Tolosa de  
582 Talamoni, N. (2015). Dietary and pharmacological compounds altering intestinal  
583 calcium absorption in humans and animals. *Nutrition Research Reviews*, 28(2), 83–99.  
584 <https://doi.org/10.1017/S0954422415000050>
- 585 Belguesmia, Y., Alard, J., Mendil, R., Ravallec, R., Grangette, C., Drider, D., & Cudennec, B.  
586 (2019). In vitro probiotic properties of selected lactobacilli and multi-strain consortium  
587 on immune function, gut barrier strengthening and gut hormone secretion. *Journal of*  
588 *Functional Foods*, 57(April), 382–391. <https://doi.org/10.1016/j.jff.2019.04.028>
- 589 Belguesmia, Y., Domenger, D., Caron, J., Dhulster, P., Ravallec, R., Drider, D., & Cudennec,

590 B. (2016). Novel probiotic evidence of lactobacilli on immunomodulation and regulation  
591 of satiety hormones release in intestinal cells. *Journal of Functional Foods*, 24, 276–286.  
592 <https://doi.org/10.1016/j.jff.2016.04.014>

593 Bellon-Fontaine, M. N., Rault, J., & Van Oss, C. J. (1996). Microbial adhesion to solvents: A  
594 novel method to determine the electron-donor/electron-acceptor or Lewis acid-base  
595 properties of microbial cells. *Colloids and Surfaces B: Biointerfaces*, 7(1–2), 47–53.  
596 [https://doi.org/10.1016/0927-7765\(96\)01272-6](https://doi.org/10.1016/0927-7765(96)01272-6)

597 Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., Gomez-Llorente, C., & Gil, A.  
598 (2012). Probiotic mechanisms of action. *Annals of Nutrition and Metabolism*, 61(2),  
599 160–174. <https://doi.org/10.1159/000342079>

600 Beto, J. A. (2015). The Role of Calcium in Human Aging. *Clinical Nutrition Research*, 4(1),  
601 1. <https://doi.org/10.7762/cnr.2015.4.1.1>

602 Brincat, M., Gambin, J., Brincat, M., & Calleja-Agius, J. (2015). The role of vitamin D in  
603 osteoporosis. *Maturitas*, 80(3), 329–332. <https://doi.org/10.1016/j.maturitas.2014.12.018>

604 Bronner, F. (2003). Mechanisms of intestinal calcium absorption. *Journal of Cellular*  
605 *Biochemistry*, 88(2), 387–393. <https://doi.org/10.1002/jcb.10330>

606 Cavalcanti Neto, M. P., Aquino, J. de S., Romão da Silva, L. de F., de Oliveira Silva, R.,  
607 Guimarães, K. S. de L., de Oliveira, Y., de Souza, E. L., Magnani, M., Vidal, H., & de  
608 Brito Alves, J. L. (2018). Gut microbiota and probiotics intervention: A potential  
609 therapeutic target for management of cardiometabolic disorders and chronic kidney  
610 disease? *Pharmacological Research*, 130, 152–163.  
611 <https://doi.org/10.1016/j.phrs.2018.01.020>

612 Charteris, Kelly, Morelli, & Collins. (1998). Development and application of an in vitro

613 methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and  
614 *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied*  
615 *Microbiology*, 84(5), 759–768. <https://doi.org/10.1046/j.1365-2672.1998.00407.x>

616 Chugh, B., & Kamal-Eldin, A. (2020). Bioactive Compounds Produced by Probiotics in Food  
617 Products. *Current Opinion in Food Science*. <https://doi.org/10.1016/j.cofs.2020.02.003>

618 Di Cerbo, A., Palmieri, B., Aponte, M., Morales-Medina, J. C., & Iannitti, T. (2016).  
619 Mechanisms and therapeutic effectiveness of lactobacilli. *Journal of Clinical Pathology*,  
620 69, 187–203. <https://doi.org/10.1136/jclinpath-2015-202976>

621 Dimitrov, Z., Gotova, I., & Chorbadjiyska, E. (2014). In vitro characterization of the adhesive  
622 factors of selected probiotics to Caco-2 epithelium cell line. *Biotechnology and*  
623 *Biotechnological Equipment*, 28(6), 1079–1083.  
624 <https://doi.org/10.1080/13102818.2014.969948>

625 Domenger, D., Cudennec, B., Kouach, M., Touche, V., Landry, C., Lesage, J., Gosselet, F.,  
626 Lestavel, S., Goossens, J.-F., Dhulster, P., & Ravallec, R. (2018). Food-derived  
627 hemorphins cross intestinal and blood-brain barriers in vitro. *Frontiers in Endocrinology*,  
628 9(APR), 159. <https://doi.org/10.3389/fendo.2018.00159>

629 Er, S., Koparal, A. T., & Kivanç, M. (2015). Cytotoxic effects of various lactic acid bacteria  
630 on Caco-2 cells. *Turkish Journal of Biology*, 39(1), 23–30. [https://doi.org/10.3906/biy-](https://doi.org/10.3906/biy-1402-62)  
631 1402-62

632 Fernandez, E. M., Pot, B., & Grangette, C. (2011). Beneficial effect of probiotics in IBD: Are  
633 peptidoglycan and NOD2 the molecular key effectors? *Gut Microbes*, 2(5), 280–286.  
634 <https://doi.org/10.4161/gmic.2.5.18255>

635 Fernandez, M. F., Boris, S., & Barbés, C. (2003). Probiotic properties of human lactobacilli

636 strains to be used in the gastrointestinal tract. *Journal of Applied Microbiology*, 94(3),  
637 449–455. <https://doi.org/10.1046/j.1365-2672.2003.01850.x>

638 Fleet, J., & Schoch, R. (2010). Molecular mechanisms for regulation of intestinal calcium  
639 absorption by vitamin D and other factors. *Crit Rev Clin Lab Sci*, 47(4), 181–195.  
640 <https://doi.org/10.3109/10408363.2010.536429>.Molecular

641 Fleet, J C, & Wood, R. J. (1999). Specific 1,25(OH)2D3-mediated regulation of transcellular  
642 calcium transport in Caco-2 cells. *Am J Physiol*, 276, G958-964.

643 Fleet, James C. (2017). The role of vitamin D in the endocrinology controlling calcium  
644 homeostasis. In *Molecular and Cellular Endocrinology* (Vol. 453, pp. 36–45). Elsevier  
645 Ireland Ltd. <https://doi.org/10.1016/j.mce.2017.04.008>

646 Fujita, H., Sugimoto, K., Inatomi, S., Maeda, T., Osanai, M., Uchiyama, Y., Yamamoto, Y.,  
647 Wada, T., Kojima, T., Yokozaki, H., Yamashita, T., Kato, S., Sawada, N., & Chiba, H.  
648 (2008). Tight Junction Proteins Claudin-2 and -12 Are Critical for Vitamin D-dependent  
649 Ca<sup>2+</sup> Absorption between Enterocytes. *Molecular Biology of the Cell*, 19(5), 1912–  
650 1921. <https://doi.org/10.1091/mbc.E07-09-0973>

651 García-Cayuela, T., Korany, A. M., Bustos, I., P. Gómez de Cadiñanos, L., Requena, T.,  
652 Peláez, C., & Martínez-Cuesta, M. C. (2014). Adhesion abilities of dairy Lactobacillus  
653 plantarum strains showing an aggregation phenotype. *Food Research International*, 57,  
654 44–50. <https://doi.org/10.1016/j.foodres.2014.01.010>

655 Gilman, J., & Cashman, K. D. (2002). The effect of probiotic bacteria on transepithelial  
656 calcium transport and calcium uptake in human intestinal-like caco-2 cells. *Current*  
657 *Issues in Intestinal Microbiology*, 7(1), 1–6.  
658 [http://sfx.scholarsportal.info/mcmaster?sid=OVID:embase&id=pmid:&id=doi:&issn=14](http://sfx.scholarsportal.info/mcmaster?sid=OVID:embase&id=pmid:&id=doi:&issn=1466-531X&isbn=&volume=7&issue=1&spage=1&pages=1-)  
659 [66-531X&isbn=&volume=7&issue=1&spage=1&pages=1-](http://sfx.scholarsportal.info/mcmaster?sid=OVID:embase&id=pmid:&id=doi:&issn=1466-531X&isbn=&volume=7&issue=1&spage=1&pages=1-)

660 6&date=2006&title=Current+Issues+in+Intestinal+Microbiology&atitle=The+effect+of  
661 +probiotic+bacteria+on+transepithelial+calcium+t

662 Giuliano, A. R., Franceschi, R. T., & Wood, R. J. (1991). Characterization of the vitamin D  
663 receptor from the Caco-2 human colon carcinoma cell line: effect of cellular  
664 differentiation. *Archives of Biochemistry and Biophysics*, 285(2), 261–269.  
665 <http://www.ncbi.nlm.nih.gov/pubmed/1654769>

666 Hassan-Smith, Z., & Gittoes, N. (2017). Hypocalcaemia. *Medicine (United Kingdom)*, 45(9),  
667 555–559. <https://doi.org/10.1016/j.mpmed.2017.06.006>

668 Haussler, M. R., Haussler, C. A., Whitfield, G. K., Hsieh, J. C., Thompson, P. D., Barthel, T.  
669 K., Bartik, L., Egan, J. B., Wu, Y., Kubicek, J. L., Lowmiller, C. L., Moffet, E. W.,  
670 Forster, R. E., & Jurutka, P. W. (2010). The nuclear vitamin D receptor controls the  
671 expression of genes encoding factors which feed the “Fountain of Youth” to mediate  
672 healthful aging. *Journal of Steroid Biochemistry and Molecular Biology*, 121(1–2), 88–  
673 97. <https://doi.org/10.1016/j.jsbmb.2010.03.019>

674 Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani,  
675 R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert  
676 consensus document: The international scientific association for probiotics and prebiotics  
677 consensus statement on the scope and appropriate use of the term probiotic. *Nature*  
678 *Reviews Gastroenterology and Hepatology*, 11(8), 506–514.  
679 <https://doi.org/10.1038/nrgastro.2014.66>

680 Kotzamanidis, C., Kourelis, A., Litopoulou-Tzanetaki, E., Tzanetakis, N., & Yiangou, M.  
681 (2010). Evaluation of adhesion capacity, cell surface traits and immunomodulatory  
682 activity of presumptive probiotic *Lactobacillus* strains. *International Journal of Food*  
683 *Microbiology*, 140(2–3), 154–163. <https://doi.org/10.1016/j.ijfoodmicro.2010.04.004>

684 Kruger, M. C., Fear, A., Chua, W.-H., Plimmer, G. G., & Schollum, L. M. (2009). HN001 on  
685 mineral absorption and bone health in growing male and ovariectomised female rats.  
686 *Dairy Science and Technology*, 89, 219–231. <https://doi.org/10.1051/dst/2009012>

687 Lechuga, S., & Ivanov, A. I. (2017). Disruption of the epithelial barrier during intestinal  
688 inflammation: Quest for new molecules and mechanisms. In *Biochimica et Biophysica*  
689 *Acta - Molecular Cell Research* (Vol. 1864, Issue 7, pp. 1183–1194). Elsevier B.V.  
690 <https://doi.org/10.1016/j.bbamcr.2017.03.007>

691 Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-  
692 time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods*, 25(4), 402–408.  
693 <https://doi.org/10.1006/meth.2001.1262>

694 Lo Curto, A., Pitino, I., Mandalari, G., Dainty, J. R., Faulks, R. M., & John Wickham, M. S.  
695 (2011). Survival of probiotic lactobacilli in the upper gastrointestinal tract using an in  
696 vitro gastric model of digestion. *Food Microbiology*, 28(7), 1359–1366.  
697 <https://doi.org/10.1016/j.fm.2011.06.007>

698 Lorieau, L., Le Roux, L., Gaucheron, F., Ligneul, A., Hazart, E., Dupont, D., & Floury, J.  
699 (2018). Bioaccessibility of four calcium sources in different whey-based dairy matrices  
700 assessed by in vitro digestion. *Food Chemistry*, 245(October 2017), 454–462.  
701 <https://doi.org/10.1016/j.foodchem.2017.10.108>

702 Lu, Z., Ding, L., Lu, Q., & Chen, Y.-H. (2013). Claudins in intestines. *Tissue Barriers*, 1(3),  
703 e24978. <https://doi.org/10.4161/tisb.24978>

704 McCabe, L. R., & Parameswaran, N. (2018). Advances in Probiotic Regulation of Bone and  
705 Mineral Metabolism. *Calcified Tissue International*, 102(4), 480–488.  
706 <https://doi.org/10.1007/s00223-018-0403-7>

707 Mello, A. M., Paroni, G., Daragjati, J., & Pilotto, A. (2016). Gastrointestinal Microbiota and  
708 Their Contribution to Healthy Aging. *Digestive Diseases (Basel, Switzerland)*, *34*(3),  
709 194–201. <https://doi.org/10.1159/000443350>

710 Messaoudi, S., Madi, A., Prévost, H., Feuilleley, M., Manai, M., Dousset, X., & Connil, N.  
711 (2012). In vitro evaluation of the probiotic potential of *Lactobacillus salivarius*  
712 SMXD51. *Anaerobe*, *18*(6), 584–589. <https://doi.org/10.1016/j.anaerobe.2012.10.004>

713 Mousavi Khaneghah, A., Abhari, K., Eş, I., Soares, M. B., Oliveira, R. B. A., Hosseini, H.,  
714 Rezaei, M., Balthazar, C. F., Silva, R., Cruz, A. G., Ranadheera, C. S., & Sant’Ana, A. S.  
715 (2020). Interactions between probiotics and pathogenic microorganisms in hosts and  
716 foods: A review. *Trends in Food Science and Technology*, *95*(November 2019), 205–  
717 218. <https://doi.org/10.1016/j.tifs.2019.11.022>

718 Nilsson, A. G., Sundh, D., Bäckhed, F., & Lorentzon, M. (2018). *Lactobacillus reuteri*  
719 reduces bone loss in older women with low bone mineral density: a randomized,  
720 placebo-controlled, double-blind, clinical trial. *Journal of Internal Medicine*, 307–317.  
721 <https://doi.org/10.1111/joim.12805>

722 Nordin, B. C., Need, A. G., Morris, H. A., O’Loughlin, P. D., & Horowitz, M. (2004). Effect  
723 of age on calcium absorption in postmenopausal women. *Am J Clin Nutr*, *80*(4), 998–  
724 1002. <http://ajcn.nutrition.org.buproxy.bastyr.edu:2048/content/80/4/998.long>

725 Parvaneh, K., Jamaluddin, R., Karimi, G., & Erfani, R. (2014). Effect of probiotics  
726 supplementation on bone mineral content and bone mass density. *The Scientific World*  
727 *Journal*, 2014. <https://doi.org/10.1155/2014/595962>

728 Pérez, A. V., Picotto, G., Carpentieri, A. R., Rivoira, M. A., Peralta Lopez, M. E., & Tolosa  
729 De Talamoni, N. G. (2008). Minireview on regulation of intestinal calcium absorption:  
730 Emphasis on molecular mechanisms of transcellular pathway. *Digestion*, *77*(1), 22–34.

731 <https://doi.org/10.1159/000116623>

732 Pitino, I., Randazzo, C. L., Mandalari, G., Lo Curto, A., Faulks, R. M., Le Marc, Y.,  
733 Bisignano, C., Caggia, C., & Wickham, M. S. J. (2010). Survival of *Lactobacillus*  
734 *rhamnosus* strains in the upper gastrointestinal tract. *Food Microbiology*, *27*(8), 1121–  
735 1127. <https://doi.org/10.1016/j.fm.2010.07.019>

736 Pu, F., Chen, N., & Xue, S. (2016). Calcium intake, calcium homeostasis and health. *Food*  
737 *Science and Human Wellness*, *5*(1), 8–16. <https://doi.org/10.1016/j.fshw.2016.01.001>

738 Ramos, C. L., Thorsen, L., Schwan, R. F., & Jespersen, L. (2013). Strain-specific probiotics  
739 properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis*  
740 isolates from Brazilian food products. *Food Microbiology*, *36*(1), 22–29.  
741 <https://doi.org/10.1016/j.fm.2013.03.010>

742 Raveschot, C., Cudennec, B., Deracinois, B., Frémont, M., Vaeremans, M., Dugersuren, J.,  
743 Demberel, S., Drider, D., Dhulster, P., Coutte, F., & Flahaut, C. (2020). Proteolytic  
744 activity of *Lactobacillus* strains isolated from Mongolian traditional dairy products: A  
745 multiparametric analysis. *Food Chemistry*, *304*(August 2019), 125415.  
746 <https://doi.org/10.1016/j.foodchem.2019.125415>

747 Roobab, U., Batool, Z., Manzoor, M. F., Shabbir, M. A., Khan, M. R., & Aadil, R. M. (2020).  
748 Sources, formulations, advanced delivery and health benefits of probiotics. *Current*  
749 *Opinion in Food Science*, *32*, 17–28. <https://doi.org/10.1016/j.cofs.2020.01.003>

750 Sarfraz, F., Farooq, U., Shafi, A., Hayat, Z., Akram, K., & Rehman, H. U. (2019).  
751 Hypolipidaemic effects of synbiotic yoghurt in rabbits. *International Journal of Dairy*  
752 *Technology*, *72*(4), 545–550. <https://doi.org/10.1111/1471-0307.12618>

753 Scholz-Ahrens, K. E., Ade, P., Marten, B., Weber, P., Timm, W., Açil, Y., Glüer, C.-C., &

754 Schrezenmeir, J. (2007). Prebiotics, probiotics, and synbiotics affect mineral absorption,  
755 bone mineral content, and bone structure. *The Journal of Nutrition*, *137*(3 Suppl 2),  
756 838S-46S. <https://doi.org/137/3/838S> [pii]

757 Scholz-Ahrens, K. E., Adolphi, B., Rochat, F., Barclay, D. V., de Vrese, M., Açıllı, Y., &  
758 Schrezenmeir, J. (2016). Effects of probiotics, prebiotics, and synbiotics on mineral  
759 metabolism in ovariectomized rats — impact of bacterial mass, intestinal absorptive area  
760 and reduction of bone turn-over. *NFS Journal*, *3*, 41–50.  
761 <https://doi.org/10.1016/j.nfs.2016.03.001>

762 Sengupta, R., Altermann, E., Anderson, R. C., McNabb, W. C., Moughan, P. J., & Roy, N. C.  
763 (2013). The Role of Cell Surface Architecture of Lactobacilli in Host-Microbe  
764 Interactions in the Gastrointestinal Tract. *Mediators of Inflammation*, *2013*, 1–16.  
765 <https://doi.org/10.1155/2013/237921>

766 Shafi, A., Naeem Raja, H., Farooq, U., Akram, K., Hayat, Z., Naz, A., & Nadeem, H. R.  
767 (2019). Antimicrobial and antidiabetic potential of synbiotic fermented milk: A  
768 functional dairy product. *International Journal of Dairy Technology*, *72*(1), 15–22.  
769 <https://doi.org/10.1111/1471-0307.12555>

770 Shewale, R. N., Sawale, P. D., Khedkar, C. D., & Singh, A. (2014). Selection criteria for  
771 probiotics: A review. *International Journal of Probiotics and Prebiotics*, *9*(1–2), 17–22.

772 Soto, A. M., Morales, P., Haza, A. I., García, M. L., & Selgas, M. D. (2014). Bioavailability  
773 of calcium from enriched meat products using caco-2 cells. *Food Research International*,  
774 *55*, 263–270. <https://doi.org/10.1016/j.foodres.2013.10.038>

775 Tuomola, E. M., & Salminen, S. J. (1998). Adhesion of some probiotic and dairy  
776 Lactobacillus strains to Caco-2 cell cultures. *International Journal of Food*  
777 *Microbiology*, *41*(1), 45–51. [https://doi.org/10.1016/S0168-1605\(98\)00033-6](https://doi.org/10.1016/S0168-1605(98)00033-6)

778 Vaiserman, A. M., Koliada, A. K., & Marotta, F. (2017). Gut microbiota: A player in aging  
779 and a target for anti-aging intervention. *Ageing Research Reviews*, 35, 36–45.  
780 <https://doi.org/10.1016/j.arr.2017.01.001>

781 Van Cromphaut, S. J., Dewerchin, M., Hoenderop, J. G. J., Stockmans, I., Van Herck, E.,  
782 Kato, S., Bindels, R. J. M., Collen, D., Carmeliet, P., Bouillon, R., & Carmeliet, G.  
783 (2001). Duodenal calcium absorption in vitamin D receptor-knockout mice: Functional  
784 and molecular aspects. *Proceedings of the National Academy of Sciences*, 98(23),  
785 13324–13329. <https://doi.org/10.1073/pnas.231474698>

786 Van Loosdrecht, M. C., Lyklema, J., Norde, W., Schraa, G., & Zehnder, A. J. (1987). The role  
787 of bacterial cell wall hydrophobicity in adhesion. *Applied and Environmental*  
788 *Microbiology*, 53(8), 1893–1897. [https://doi.org/0099-2240/87/081893-05\\$02.00/0](https://doi.org/0099-2240/87/081893-05$02.00/0)

789 Vasconcelos, F. M., Silva, H. L. A., Poso, S. M. V., Barroso, M. V., Lanzetti, M., Rocha, R.  
790 S., Graça, J. S., Esmerino, E. A., Freitas, M. Q., Silva, M. C., Raices, R. S. L., Granato,  
791 D., Pimentel, T. C., Sant’Ana, A. S., Cruz, A. G., & Valença, S. S. (2019). Probiotic  
792 Prato cheese attenuates cigarette smoke-induced injuries in mice. *Food Research*  
793 *International*, 123(April), 697–703. <https://doi.org/10.1016/j.foodres.2019.06.001>

794 Vinderola, C. G., Medici, M., & Perdigón, G. (2004). Relationship between interaction sites  
795 in the gut, hydrophobicity, mucosal immunomodulating capacities and cell wall protein  
796 profiles in indigenous and exogenous bacteria. *Journal of Applied Microbiology*, 96(2),  
797 230–243. <https://doi.org/10.1046/j.1365-2672.2004.02158.x>

798 Vinderola, C. G., & Reinheimer, J. A. (2003). Lactic acid starter and probiotic bacteria: A  
799 comparative “in vitro” study of probiotic characteristics and biological barrier resistance.  
800 *Food Research International*, 36(9–10), 895–904. [https://doi.org/10.1016/S0963-](https://doi.org/10.1016/S0963-9969(03)00098-X)  
801 [9969\(03\)00098-X](https://doi.org/10.1016/S0963-9969(03)00098-X)

802 Watanabe, K., Fujimoto, J., Sasamoto, M., Dugersuren, J., Tumursuh, T., & Demberel, S.  
803 (2008). Diversity of lactic acid bacteria and yeasts in Airag and Tarag, traditional  
804 fermented milk products of Mongolia. *World Journal of Microbiology and*  
805 *Biotechnology*, 24(8), 1313–1325. <https://doi.org/10.1007/s11274-007-9604-3>

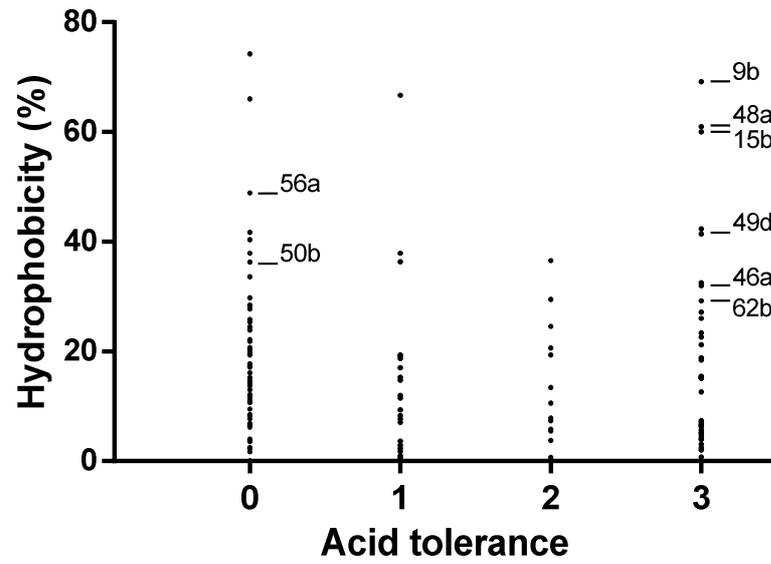
806 Wood, R. J., Tchack, L., & Taparia, S. (2001). 1,25-Dihydroxyvitamin D3 increases the  
807 expression of the CaT1 epithelial calcium channel in the Caco-2 human intestinal cell  
808 line. *BMC Physiol*, 1(1), 11. <https://doi.org/10.1186/1472-6793-1-1>

809 Wu, S., Yoon, S., Zhang, Y., Lu, R., Xia, Y., Wan, J., Petrof, E. O., Claud, E. C., Chen, D., &  
810 Sun, J. (2015). Vitamin D receptor pathway is required for probiotic protection in colitis.  
811 *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 309(5), G341–  
812 G349. <https://doi.org/10.1152/ajpgi.00105.2015>

813 Yu, J., Wang, W. H., Menghe, B. L. G., Jiri, M. T., Wang, H. M., Liu, W. J., Bao, Q. H., Lu,  
814 Q., Zhang, J. C., Wang, F., Xu, H. Y., Sun, T. S., & Zhang, H. P. (2011). Diversity of  
815 lactic acid bacteria associated with traditional fermented dairy products in Mongolia.  
816 *Journal of Dairy Science*, 94(7), 3229–3241. <https://doi.org/10.3168/jds.2010-3727>

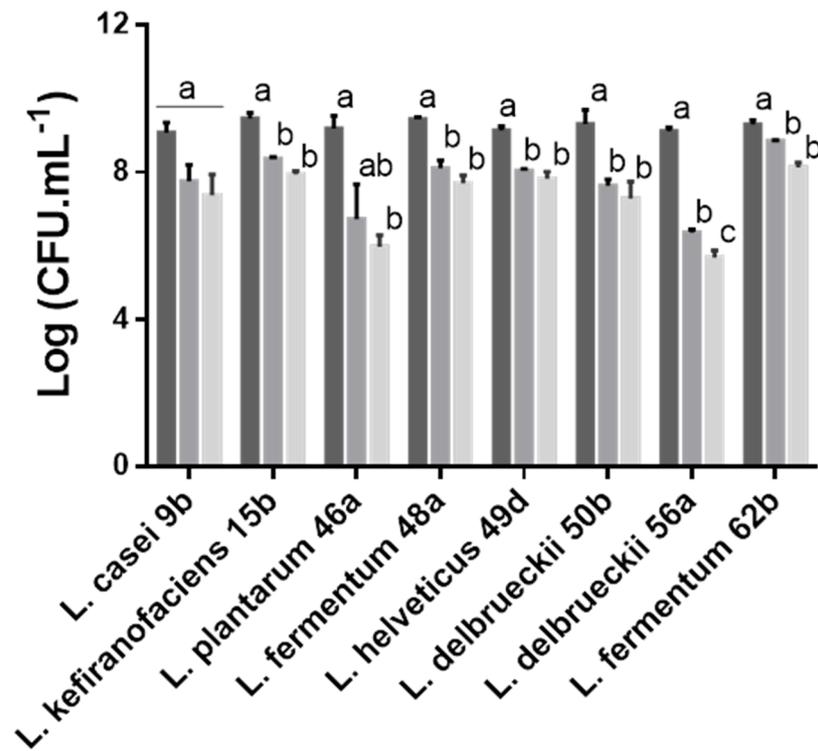
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**Figure 1:**



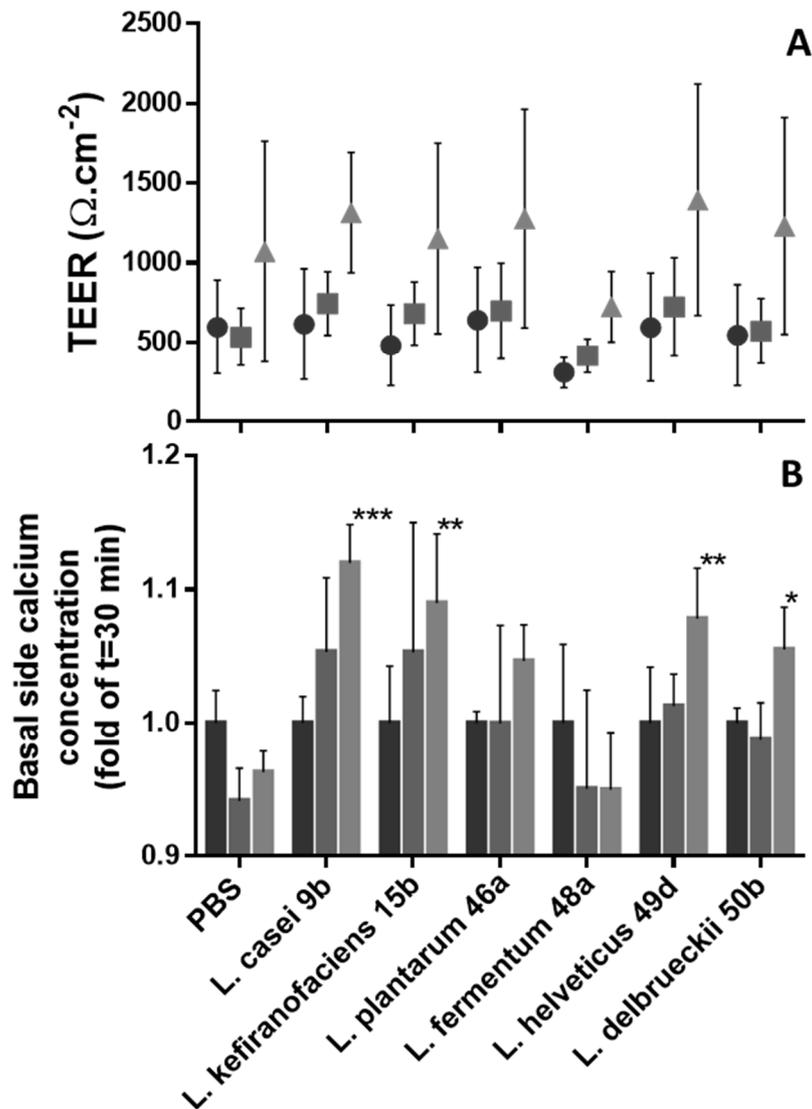
**Fig. 1.** Percentage of hydrophobicity versus acid tolerance for each Lactobacilli strain. Each strain is represented by a plot according to hydrophobicity and acid tolerance. Classes of acid tolerance: 0, strain not resistant, 1, sensitive strain, 2, moderately sensitive strain and 3, tolerant strain. The eight selected strains are reported on the graph: *L. casei* 9b, *L.kefiranofaciens* 15b, *L. plantarum* 46a, *L. fermentum* 48a, *L. helveticus* 49d, *L. delbrueckii* 50b, *L. delbrueckii* 56a and *L. fermentum* 62b. Values are means of three replicates.

Figure 2:



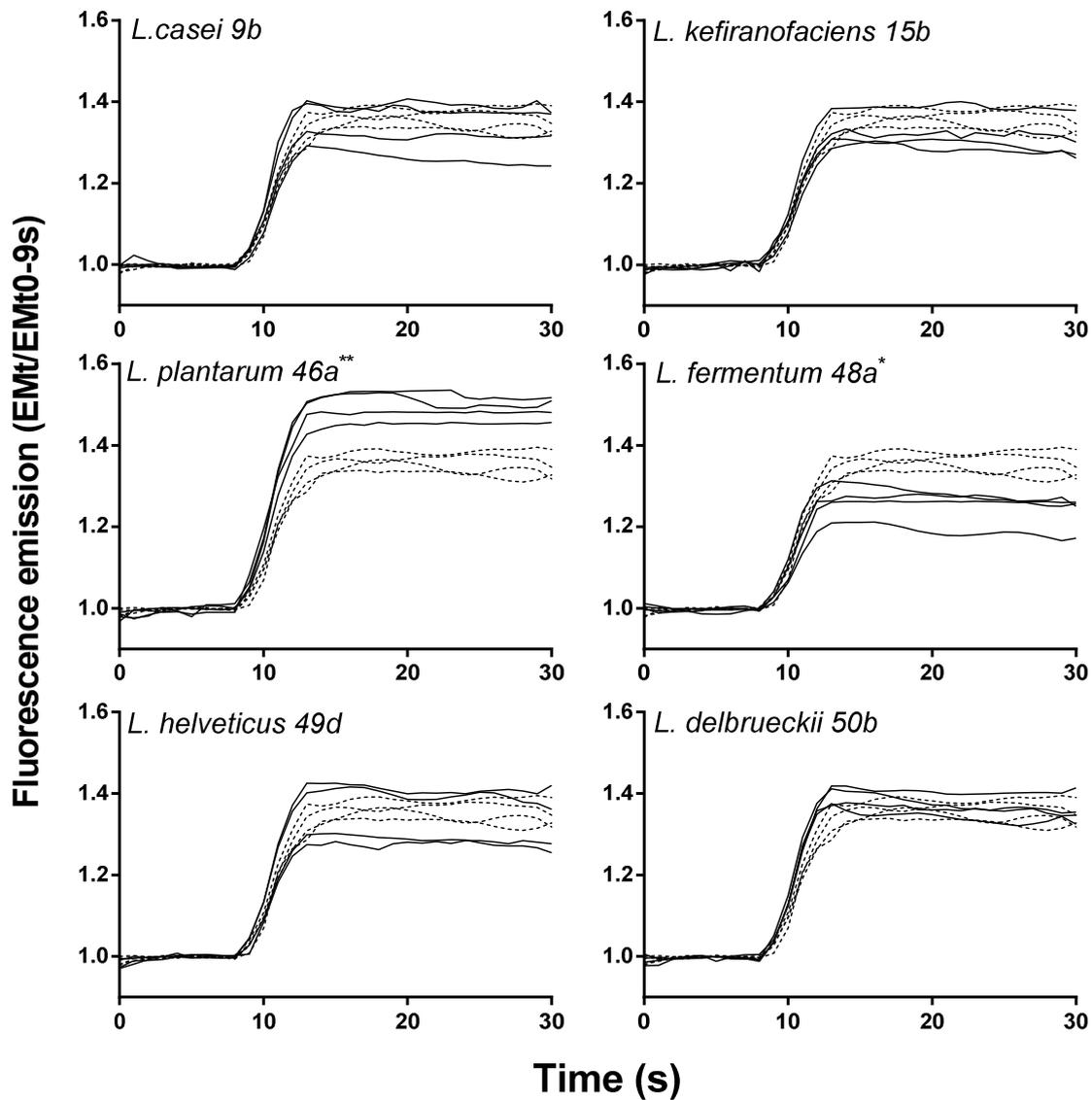
**Fig. 2.** Number of *Lactobacillus* after incubation in simulated GI tract compartments (static *in vitro* digestion model): mouth (dark grey), stomach (medium grey) and duodenum (light grey). Samples were taken after each step and the number of CFU.mL<sup>-1</sup> was evaluated by plating on MRS medium. Values are means  $\pm$  SD. \*Statistical analysis using one way ANOVA and Tukey's post-hoc test for each strain. Mean with different letter differed significantly (P value < 0.05).

**Figure 3:**



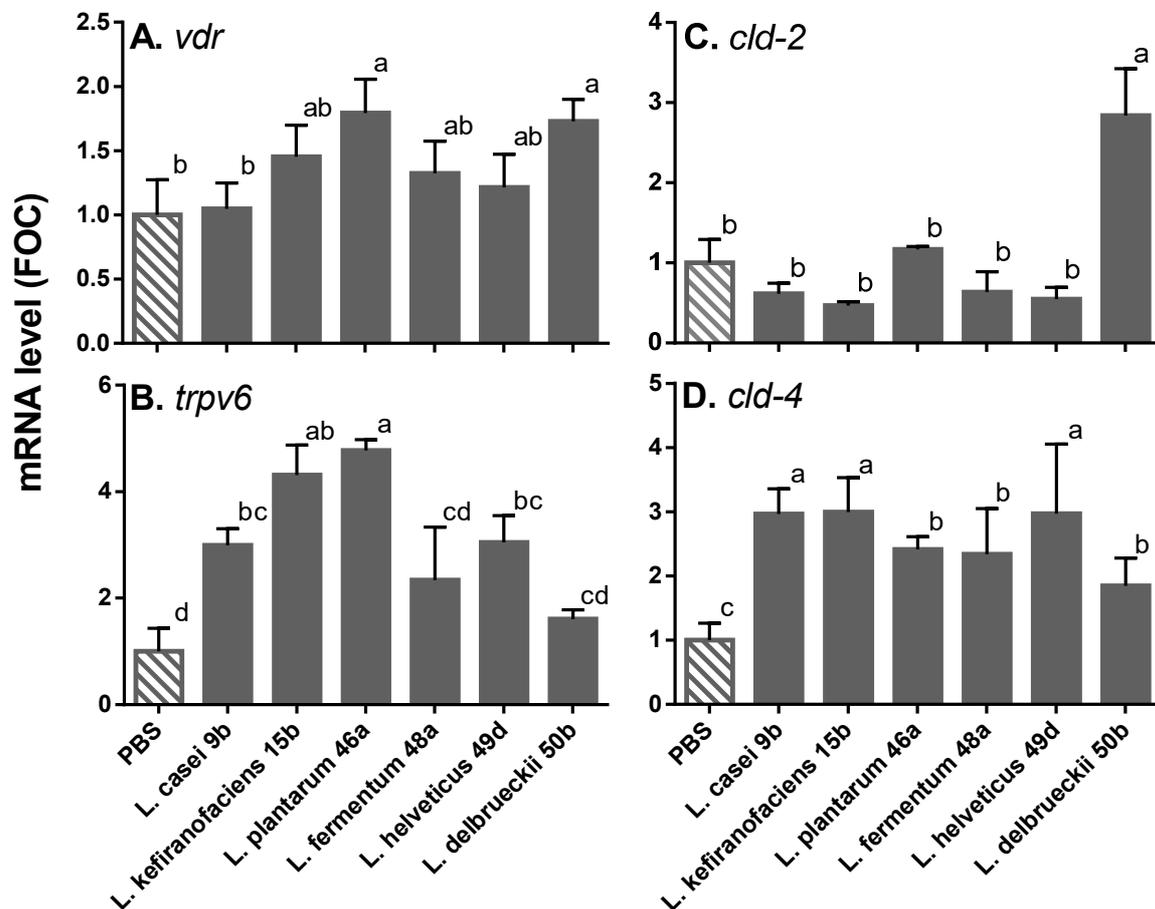
**Fig. 3.** Change in trans-epithelial electrical resistance (TEER) across Caco-2 cell monolayers, and in calcium concentration at the basal side of the monolayer, following contact with *Lactobacillus* strains for 24h. (A) Evolution of TEER in  $\Omega \cdot \text{cm}^{-2}$  at time t=30 min (●), 7h (■) and 24h (▲). (B) Change in calcium concentration at the basal side of Caco-2 membrane monolayers. Samples were taken at time t=30 min (dark grey), 7h (medium grey) and 24h (light grey) from the basal side of Caco-2 cell monolayers. Results are expressed as a fold ratio compared to t=30 min values. Values are means  $\pm$  SD of three repeated measurements. Means without a common symbol are different (\*\*\* P value < 0.001, \*\* P value < 0.01, \* P value < 0.05) using one way ANOVA with Dunnet's post-hoc test vs PBS condition as reference.

Figure 4:



**Fig. 4.** Change in the intracellular calcium concentration in Caco-2 cells after injection of 25  $\mu$ L of  $\text{CaCl}_2$  (250 mM) at 9 seconds of the kinetic (arrow). Modulation of the fluorescent  $\text{Ca}^{2+}$  sensitive molecule is assessed for 30 seconds after the  $\text{CaCl}_2$  injection. Caco-2 cells were incubated with *Lactobacillus* strains for 24h before the experiment. Each graph represent experiments for one tested strain (full line) compared to control condition (PBS) (dotted line) (N=4). Values are expressed as ratio of emission (EMIt) by emission mean from 0 to 9 seconds of kinetic (EMIt0-9s). Statistical analysis between 15 and 30 seconds of kinetic using one way ANOVA with Dunnet's post-hoc test with PBS condition as reference (\*\* P value < 0.01, \* P value < 0.05).

Figure 5:



**Fig. 5.** mRNA expression levels of four genes in HT-29 MTX cells following 24h contact with *Lactobacillus* strains. (A) Vitamin D Receptor (*vdr*), (B) transient receptor potential selective for calcium (*trpv6*), (C) claudin-2 (*cld-2*) and (D) claudin-4 (*cld-4*). Results are reported in mean fold of control (FOC, PBS buffer)  $\pm$  SD of three replicates. Within the same graph, means with a different letter are significantly different (P value < 0.05) using one way ANOVA and Tukey's post-hoc test for pairwise comparisons.

**Table 1.** Percentage of autoaggregation in PBS buffer and percentage of *Lactobacillus* cells showing adhesion to Caco-2 cell monolayer.

<i>Lactobacillus</i> strains	Autoaggregation (%)		Adherent cells (%)
	2,5 hours	5 hours	2 hours
<i>L. casei</i> 9b	95,25 ± 2,23 <sup>d</sup>	95,23 ± 0,92 <sup>d</sup>	0,95 ± 0,53 <sup>ab</sup>
<i>L. kefiranofaciens</i> 15b	3,53 ± 5,12 <sup>a</sup>	7,86 ± 3,57 <sup>a</sup>	4,11 ± 2,2 <sup>c</sup>
<i>L. plantarum</i> 46a	12,74 ± 3,15 <sup>ab</sup>	28,27 ± 9,7 <sup>ac</sup>	4,63 ± 1,22 <sup>c</sup>
<i>L. fermentum</i> 48a	1,97 ± 6,28 <sup>a</sup>	22,49 ± 3,19 <sup>ab</sup>	4,39 ± 0,33 <sup>c</sup>
<i>L. helveticus</i> 49d	5,83 ± 5,24 <sup>a</sup>	67,44 ± 6,99 <sup>cd</sup>	1,48 ± 0,41 <sup>ab</sup>
<i>L. delbrueckii</i> 50b	33,33 ± 14,8 <sup>bc</sup>	66,67 ± 17,97 <sup>cd</sup>	0,18 ± 0,13 <sup>a</sup>
<i>L. delbrueckii</i> 56a	39,29 ± 6,97 <sup>c</sup>	46,73 ± 22,13 <sup>ac</sup>	0,22 ± 0,09 <sup>a</sup>
<i>L. fermentum</i> 62b	11,72 ± 2,22 <sup>ab</sup>	18,04 ± 9,94 <sup>ab</sup>	2,76 ± 0,81 <sup>bc</sup>

For autoaggregation assay, samples were taken at 2.5h and 5h of incubation from the top of the *Lactobacillus* suspensions for OD<sub>600nm</sub> determination. For adhesion test, the percentage of *Lactobacillus* cells adhering to Caco-2 monolayer was assessed after contact with bacterial suspension for 2h. Values are expressed as percentage of OD<sub>600nm</sub> or number of CFU.mL<sup>-1</sup> at 0h and are means ± SD of three replicates. \*Statistical analysis using one way ANOVA and Tukey's post-hoc test. Mean values in the same column without a common letter differ significantly (P value < 0.05).

**Table 2.** Survival percentage of Caco-2 and HT-29 MTX cells after contact with PBS buffer or *Lactobacillus* strains for 24h.

	Caco-2	HT-29 MTX
PBS	100.00 ± 8.07	100.00 ± 4.16
<i>L. casei</i> 9b	91.99 ± 9.07	93.67 ± 7.50
<i>L. kefiranofaciens</i> 15b	95.74 ± 10.91	107.63 ± 4.34
<i>L. plantarum</i> 46a	101.34 ± 2.95	105.49 ± 8.37
<i>L. fermentum</i> 48a	98.66 ± 21.04	107,91 ± 6.57
<i>L. helveticus</i> 49d	97.58 ± 7.96	103.07 ± 1.05
<i>L. delbrueckii</i> 50b	97.16 ± 4.84	90.42 ± 4.84
<i>L. delbrueckii</i> 56a	93.84 ± 8.21	97.84 ± 2,93
<i>L. fermentum</i> 62b	85.80 ± 6.04	95.80 ± 2,68

The survival of Caco-2 and HT-29 MTX cells was assessed by determination of lactate dehydrogenase activity. Values are expressed in percentage of control (PBS) and are means ± SD of six replicates. No significant differences were observed between treatments after statistical analysis using one way ANOVA and Tukey's post-hoc test (P value < 0.05).

