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

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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

ABSTRACT

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

This study aimed to evaluate the probiotic capacity of *Lactobacillus* strains isolated in three different areas of Mongolia (Raveschot et al., 2020). Production of dairies through spontaneous milk fermentation has a long history in this country. Herders use different milks (mare, cow, yak, camel, sheep, and goat) to produce a variety of fermented food such as yogurt, kefir, or airag (fermented horse milk). These products were reported to contain a wide diversity of LAB, especially *Lactobacillus* species (Watanabe et al., 2008; Yu et al., 2011). It was recently reported that calcium bioavailability in dairy products was not impacted by the food structure or components but was more dependent on the calcium source and its solubility (Lorieau et al., 2018). Nevertheless, the presence of probiotics in these products could increase the intestinal calcium absorption (Areco et al., 2015; Cavalcanti Neto et al., 2018; Chugh & Kamal-Eldin, 2020). In this study, a screening of 174 LAB isolates was carried out based on different probiotic characteristics. Criteria like cell surface hydrophobicity, acid tolerance, adhesion to intestinal cells, autoaggregation, and tolerance to gastrointestinal (GI) digestion were assessed. Then, the main objective of this study was to assess the possible impact of probiotic strains on intestinal calcium absorption. To achieve this goal, the selected strains were studied for their capacity to modulate calcium absorption using both Caco-2 and HT-29 MTX intestinal cells. This study provides evidence for the beneficial impact of 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_{630nm} 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_{630nm} determination. Cell surface hydrophobicity was determined using the
156 following formula:

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

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

2.3. Acid tolerance assay

For acid tolerance assay, each strain was grown in 5 mL of MRS medium for 24h at 37°C. Two hundred microliters of each culture were then diluted 1/10 in either MRS pH 6.2 (tube A), or in MRS pH 2 (tube B). The tubes were incubated at 37°C for 2h and 10 µL were streaked on MRS plates followed by incubation for 48h at 37°C in anaerobic conditions. Acid tolerance was evaluated by comparing the number of colonies on plates between A and B. Strains were grouped in classes according to their acid tolerance: class 0, no colony on B (strain not resistant), class 1, B = 0 to 30% of colonies on A (sensitive strain), class 2, B = 30-80% of colonies on A (moderately sensitive strain), class 3, B > 80% of colonies on A (tolerant strain).

2.4. Autoaggregation assay

Autoaggregation was assessed as described by Kotzamanidis et al., (2010). Briefly, strains were grown in 5 mL of MRS medium for 24h at 37°C, centrifuged at 13,000 × g for 10 min and resuspended in PBS buffer at OD_{600nm} of 1. Four milliliters of the suspension were then vortexed for 10 seconds and let to rest at room temperature. One hundred microliters were carefully removed from the upper suspension at 0, 2.5h and 5h of incubation. Samples were then diluted (1/10) in PBS buffer and OD_{600nm} was measured. The autoaggregation percentage was calculated according to the following formula:

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

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

2.5. Tolerance to simulated gastro-intestinal digestion

The survivability of *Lactobacillus* strains was evaluated using a static *in vitro* digestion model inspired by Belguesmia et al., (2016). This model simulates mouth, stomach and small intestine steps of digestion by incubating the bacteria in fluids that mimic the physiological conditions encountered in these different compartments. Strains were grown in MRS for 24h at 37°C, washed twice and resuspended with PBS buffer at 10⁹ CFU.mL⁻¹. One milliliter of the bacterial suspension was used for digestion. Mouth step was simulated by adding 8 mL of PBS buffer (pH 6.8) to the bacterial suspension and incubating for 5 minutes at 37°C at constant agitation (200 rpm) in an incubation shaker (Infors HT, Basel, Switzerland). The gastric step was simulated by adding 12 mL of PBS buffer (pH 3) containing 1.56 mg.mL⁻¹ of porcine pepsin (Sigma-Aldrich, St Louis, USA). This step was conducted during 2h at 37°C and 200 rpm, the pH being maintained with HCl (1 M) or NaOH (1 M) at values ranging from 3 to 3.5. Two milliliters of NaHCO₃ (1 M) were then added to raise the pH up to 7; finally, the small intestine step was then simulated by adding 12 mL of PBS buffer (pH 8.2) containing 0.28 mg.mL⁻¹ of pancreatin from porcine pancreas (Sigma-Aldrich, St Louis, USA) and 6 mL of PBS buffer (pH 8.1) containing 60 g.L⁻¹ of bile salts (Sigma-Aldrich, St Louis, USA). This digestion step was conducted for 2h at 37°C and 200 rpm, the pH being maintained between 7 and 7.5. Samples were taken after each step of the digestion process and diluted in PBS buffer (pH 7.4). One hundred microliters of adequate dilutions were streaked on MRS plates and incubated at 37°C for 48h under anaerobic conditions. The dilution factor varied from 10³ to 10⁸ depending on each strain and step of the digestion process. The number of CFU was then 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⁻¹ 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% CO₂-95% 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 10⁷ CFU.mL⁻¹ 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 CO₂, 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 µL

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% CO₂, 50 µL 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 µL of the bacterial suspension (or PBS buffer as control)

245 and 10 µL of CaCl₂ (250 mM) were added on the cells (apical chamber) which were then

246 incubated for 24h at 37°C under 5% CO₂. At 30 minutes, 7h and 24h of incubation, 100 µL 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

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

2.10. Measurement of calcium uptake in Caco-2 cells

Caco-2 cells were seeded in 96-well tissue culture plate and cultured for 15 days as described in section 2.6. Bacterial suspensions were prepared as described in section 2.7. Cell monolayer was washed with PBS buffer, 150 µL of bacterial suspension were added in each well and incubated for 24h under 5% CO₂. The supernatants were discarded and cells washed three times with PBS buffer. Then, calcium uptake by Caco-2 cells was determined using the FluoForte™ Calcium Assay Kit (ENZ-51017, Enzo Life Sciences, Farmingdale, USA). Briefly, the fluorogenic calcium binding dye FluoForte™ was added on Caco-2 monolayer for 1 hour at room temperature until its integration in cells. Fluorescence was then monitored every second for a period of 30 seconds in a microplate reader spectrofluorometer (Xenius, Safas, Monaco, France). 25 µL of CaCl₂ (250 mM) were injected in the well at 10 seconds of the kinetic. Fluorescence emission was expressed as fold of the basal fluorescence measured between 0 and 9 seconds of the kinetic.

2.11. Gene expression analysis

HT-29 MTX cells were grown in 24-well tissue culture plate for 15 days. Bacterial suspensions were prepared as described previously. The HT-29 MTX monolayer was washed twice with PBS buffer and 300 µL of bacterial suspension were added in each well. PBS buffer was used as negative control (non-treated cells). After incubation for 24h at 37°C under 5% CO₂, cells were washed twice with PBS buffer and RNA extraction was performed using Trizol reagent (Sigma-Aldrich, St Louis, USA). Briefly, 100 µL of Trizol reagent was added

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

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

3. Results

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

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

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⁻¹. At the end of the first step (mouth conditions), the number of living bacterial
331 cells was about 10^9 CFU.mL⁻¹ 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⁻¹ at the end of the whole process. The other strains reached cell counts ranging from
336 10^8 to 10^7 CFU.mL⁻¹.

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⁻¹).
341 ¹).

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

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

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

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

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

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

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

Modulation of total calcium transport was assessed by determination of calcium flux across fully differentiated Caco-2 monolayer upon contact with the selected *Lactobacillus* strains. The TEER of Caco-2 monolayer was measured at the same time. The TEER increased significantly (two way ANOVA) during the experiment (**Figure 3A**); however there was no statistical difference between cells that were put in contact with the bacterial suspensions and

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

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

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

Calcium uptake was assessed after contact for 24h between bacterial strains and fully differentiated Caco-2 cells. After injection of CaCl₂, an increase in fluorescence emission occurred showing the calcium incorporation by Caco-2 cells (**Figure 4**). In control condition (PBS), the ratio of fluorescence emission increased from 1 to 1.3-1.4 after calcium injection. The ratio increase was not significantly different upon contact with *L. casei* 9b, *L. kefiranofaciens* 15b, *L. helveticus* 49d and *L. delbrueckii* 50b. A significant diminution of calcium uptake was observed after contact with *L. fermentum* 48a strain compared to control condition. In contrast, calcium uptake was higher in Caco-2 cells monolayer incubated with *L. plantarum* 46a bacteria, than in the control monolayer: the ratio of fluorescence increased significantly from 1 to 1.4-1.5 after calcium injection. These results suggested that *L. fermentum* 48a and *L. plantarum* 46a modulate calcium uptake by Caco-2 cells.

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

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

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

4. Discussion

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

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

Cell surface hydrophobicity was also analyzed. It is thought to be a determinant of cell adhesion capacity (Van Loosdrecht et al., 1987). Adherence to intestinal cells is considered as a major criteria for the selection of probiotic strain (Vinderola et al., 2004), as it may in some cases mediate the biological activities observed (Di Cerbo et al., 2016; Sengupta et al., 2013). A high variability was observed between the Mongolian isolates, even within the same species. Such variability was already observed; it reflects the fact that hydrophobicity depends on a complex pattern of cell wall components (peptidoglycan, teichoic acids, cell wall polysaccharides, cell surface proteins) (Angmo et al., 2016; Sengupta et al., 2013).

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

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

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

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

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

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

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

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

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

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

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

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Conflict of interest

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

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Figure 1:

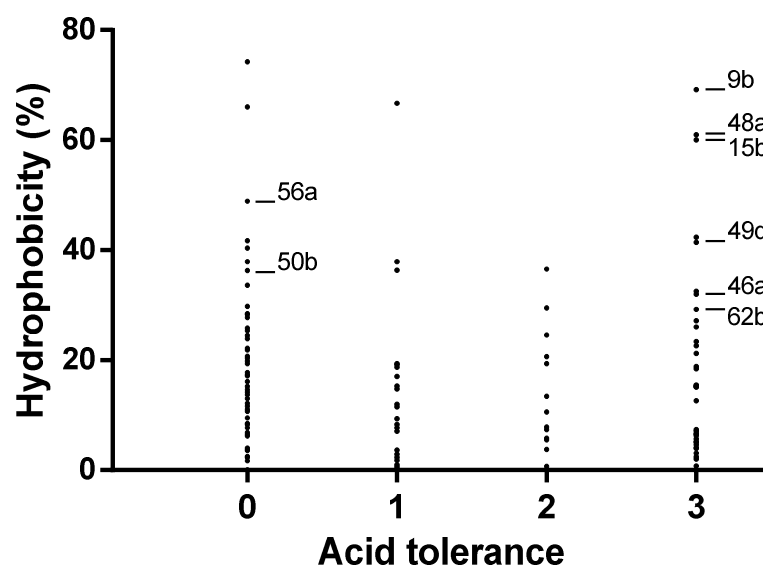


Fig. 1. Percentage of hydrophobicity versus acid tolerance for each Lactobacilli strain. Each strain is represented by a plot according to hydrophobicity and acid tolerance. Classes of acid tolerance: 0, strain not resistant, 1, sensitive strain, 2, moderately sensitive strain and 3, tolerant strain. The eight selected strains are reported on the graph: *L. casei* 9b, *L.kefiranoferiens* 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:

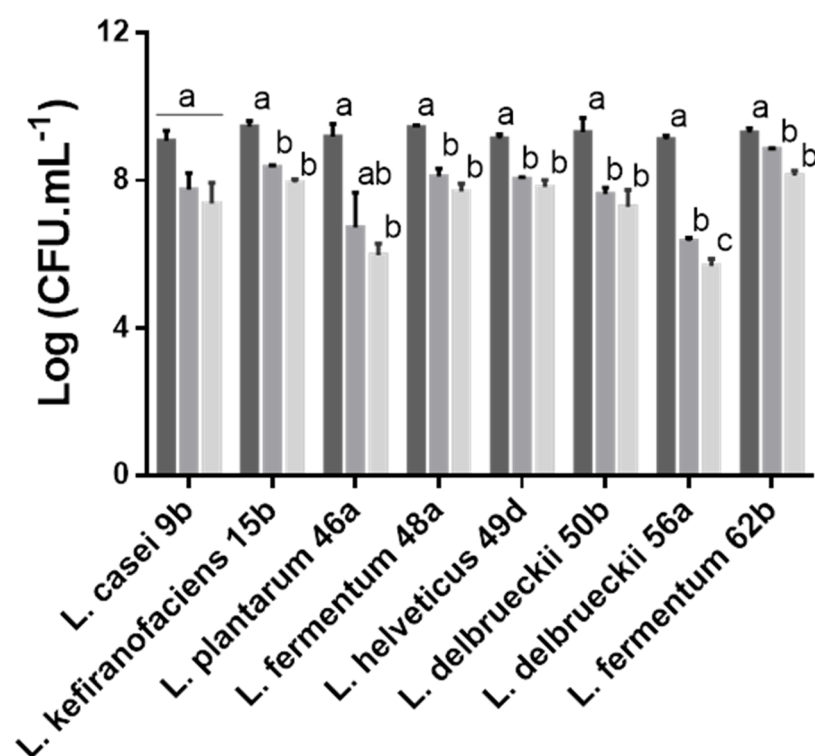


Fig. 2. Number of *Lactobacillus* after incubation in simulated GI tract compartments (static *in vitro* digestion model): mouth (dark grey), stomach (medium grey) and duodenum (light grey). Samples were taken after each step and the number of CFU.mL⁻¹ was evaluated by plating on MRS medium. Values are 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:

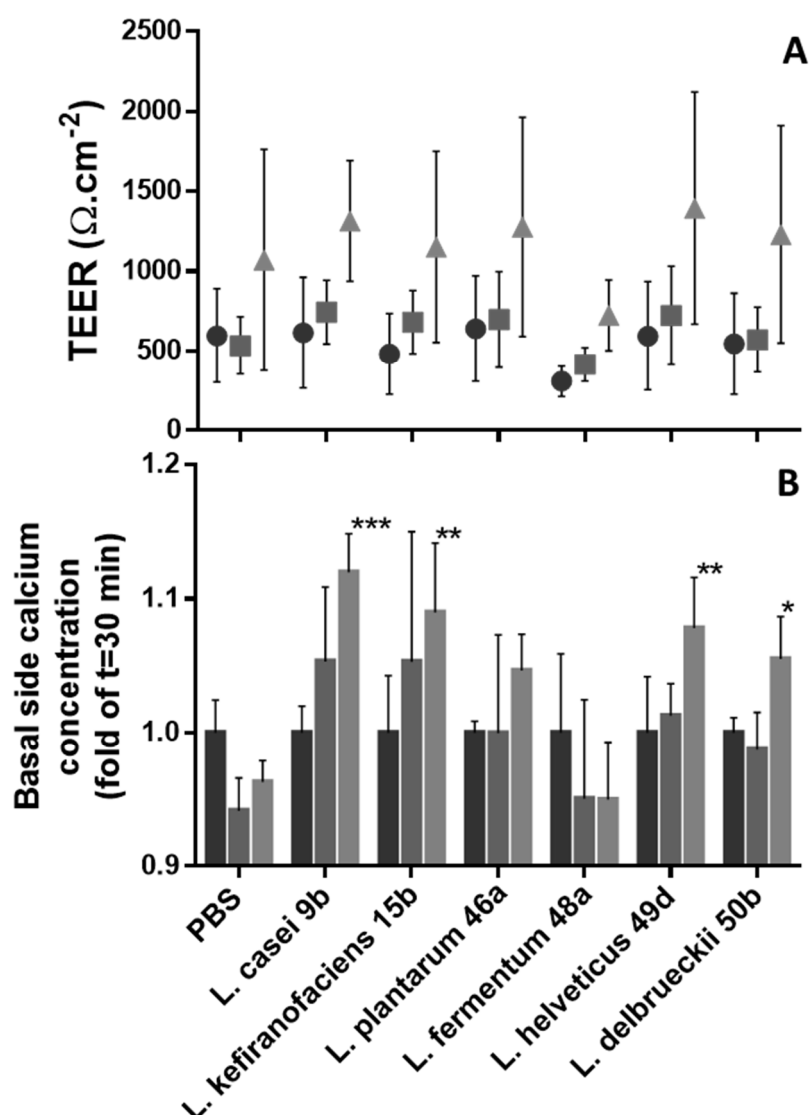


Fig. 3. Change in trans-epithelial electrical resistance (TEER) across Caco-2 cell monolayers, and in calcium concentration at the basal side of the monolayer, following contact with *Lactobacillus* strains for 24h. (A) Evolution of TEER in $\Omega \cdot \text{cm}^{-2}$ at time t=30 min (●), 7h (■) and 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:

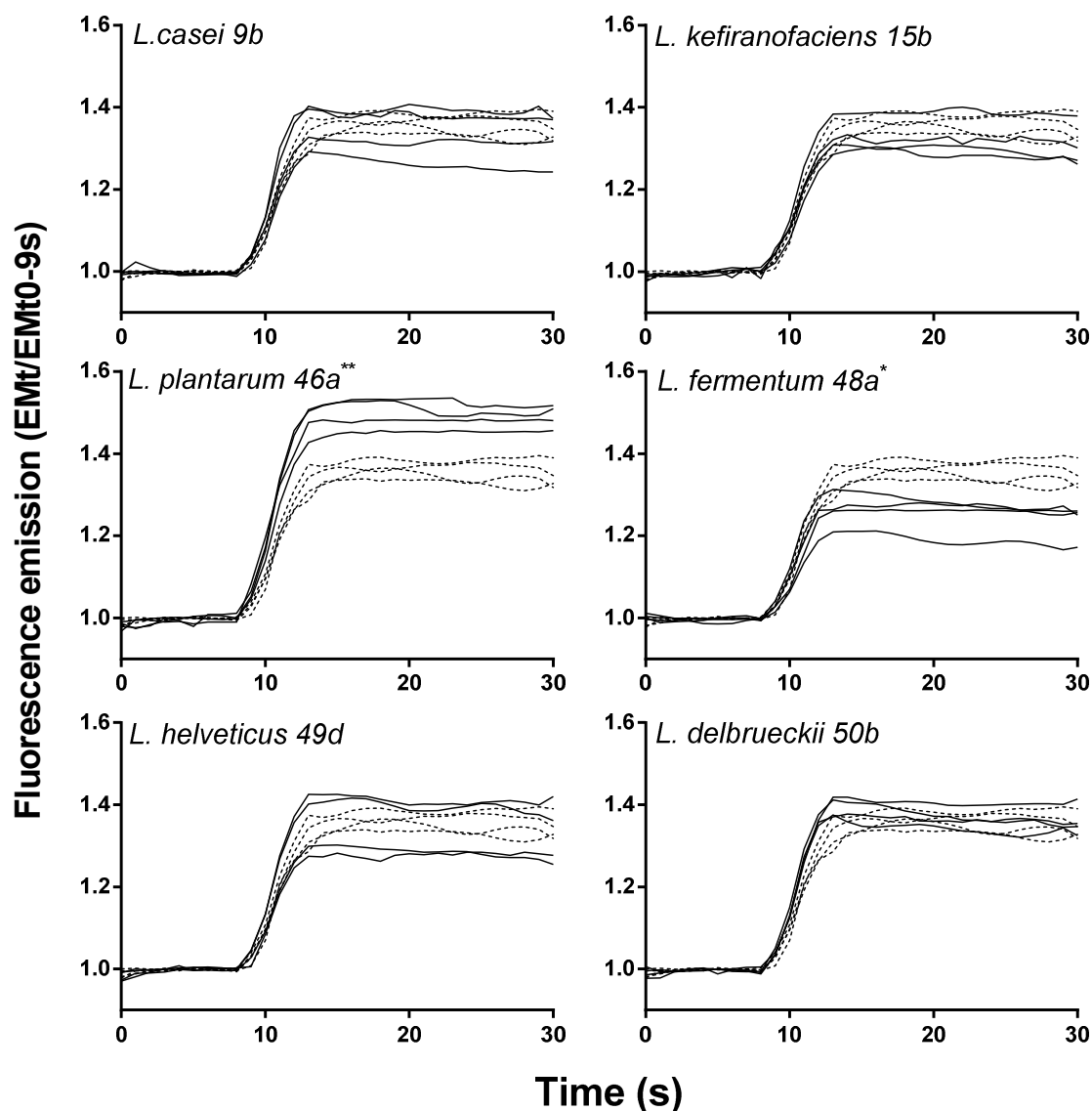


Fig. 4. Change in the intracellular calcium concentration in Caco-2 cells after injection of 25 μ L of CaCl_2 (250 mM) at 9 seconds of the kinetic (arrow). Modulation of the fluorescent Ca^{2+} sensitive molecule is assessed for 30 seconds after the CaCl_2 injection. Caco-2 cells were incubated with *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:

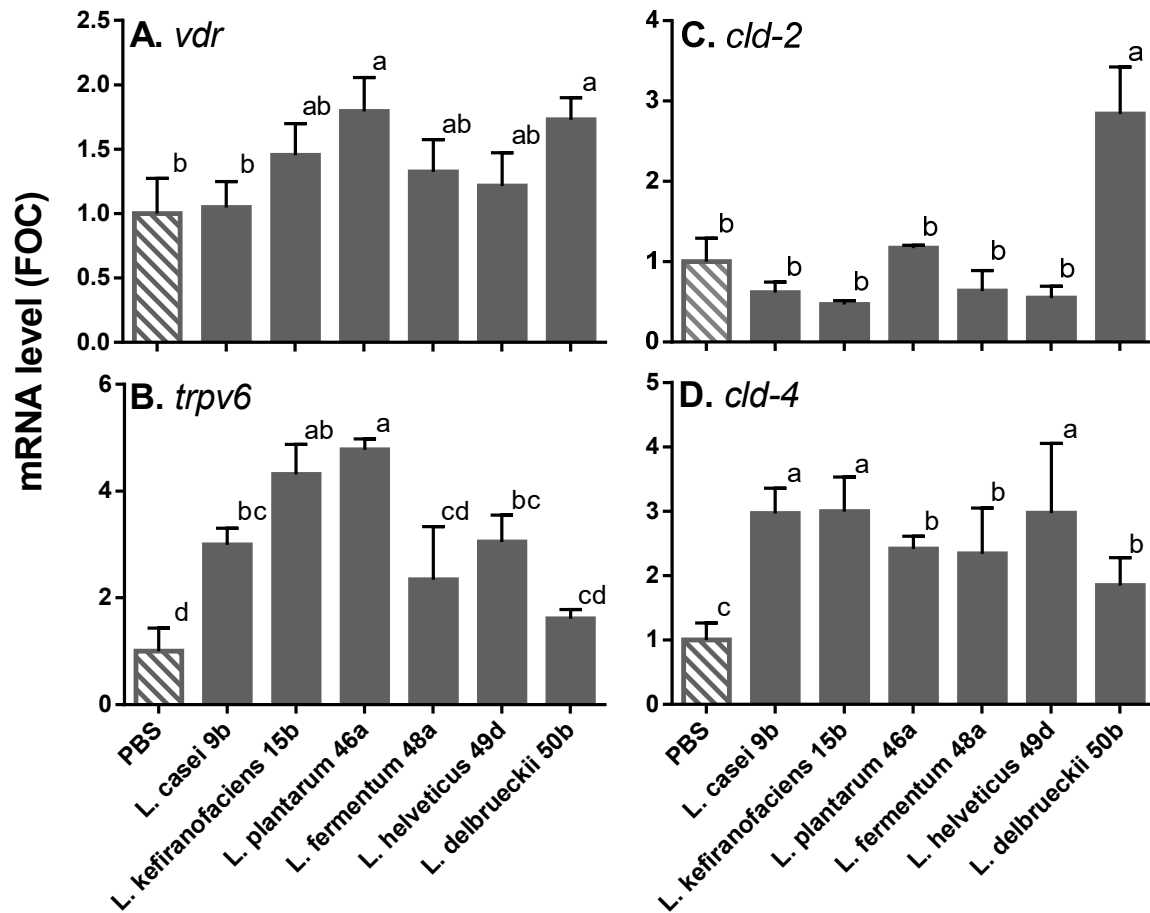


Fig. 5. mRNA expression levels of four genes in HT-29 MTX cells following 24h contact with *Lactobacillus* strains. (A) Vitamin D Receptor (*vdr*), (B) transient receptor potential selective for calcium (*trpv6*), (C) claudin-2 (*cld-2*) and (D) claudin-4 (*cld-4*). Results are reported in mean fold 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 ^d	95,23 ± 0,92 ^d	0,95 ± 0,53 ^{ab}
<i>L. kefiranofaciens</i> 15b	3,53 ± 5,12 ^a	7,86 ± 3,57 ^a	4,11 ± 2,2 ^c
<i>L. plantarum</i> 46a	12,74 ± 3,15 ^{ab}	28,27 ± 9,7 ^{ac}	4,63 ± 1,22 ^c
<i>L. fermentum</i> 48a	1,97 ± 6,28 ^a	22,49 ± 3,19 ^{ab}	4,39 ± 0,33 ^c
<i>L. helveticus</i> 49d	5,83 ± 5,24 ^a	67,44 ± 6,99 ^{cd}	1,48 ± 0,41 ^{ab}
<i>L. delbrueckii</i> 50b	33,33 ± 14,8 ^{bc}	66,67 ± 17,97 ^{cd}	0,18 ± 0,13 ^a
<i>L. delbrueckii</i> 56a	39,29 ± 6,97 ^c	46,73 ± 22,13 ^{ac}	0,22 ± 0,09 ^a
<i>L. fermentum</i> 62b	11,72 ± 2,22 ^{ab}	18,04 ± 9,94 ^{ab}	2,76 ± 0,81 ^{bc}

For autoaggregation assay, samples were taken at 2.5h and 5h of incubation from the top of the *Lactobacillus* suspensions for OD_{600nm} 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_{600nm} or number of CFU.mL⁻¹ 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).

