



In vitro study on the cell adhesion ability of immobilized lactobacilli on natural supports

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1 ***In vitro* study on the cell adhesion ability of immobilized lactobacilli**
2 **on natural supports**

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25 **Abstract**

26 The aim of the present study was to investigate the effect of probiotic immobilization
27 onto wheat grains, both wet and freeze dried, on the adhesion properties of the
28 probiotic cells and make comparisons with wet and freeze dried free cells.
29 *Lactobacillus casei* ATCC 393 and *Lactobacillus plantarum* NCIMB 8826 were used
30 as model probiotic strains. The results showed satisfactory adhesion ability of free
31 cells to a monolayer of Caco-2 cells (>1000 CFU/100 Caco-2 cells for wet cells). Cell
32 immobilization resulted in a significant decrease in adhesion, for both wet and freeze
33 dried formulations, most likely because immobilized cells did not have direct access
34 to the Caco-2 cells, but it still remained in adequate levels (>100 CFU/100 Caco-2
35 cells for wet cells). No clear correlation could be observed between cell adhesion and
36 the hydrophobicity of the bacterial cells, measured by the hexadecane adhesion assay.
37 Most notably, immobilization enhanced the monolayer integrity of Caco-2 cells,
38 demonstrated by a more than 2-fold increase in transepithelial electrical resistance
39 (TEER) compared to free cells. SEM micrographs ascertained the adhesion of both
40 immobilized and free cells to the brush border microvilli. Finally, the impact of the
41 food matrix on the adhesion properties of probiotic bacteria and on the design of
42 novel functional products is discussed.

43

44

45 **Keywords:** Probiotics, Lactic acid bacteria, Adhesion, Caco-2 cells

46

47 **1. Introduction**

48 Nowadays, there is a growing interest in developing foods containing
49 probiotic microorganisms, such as bifidobacteria and lactic acid bacteria (LAB). Such
50 functional cultures may offer organoleptic, technological and nutritional advantages,
51 but more importantly confer a health benefit to the host. Indeed, administration of
52 probiotics has been linked to the prevention, and in some cases reduction or treatment,
53 of various diseases, including viral or bacterial diarrhea, gastroenteritis, irritable
54 bowel syndrome, inflammatory bowel disease (Crohn's disease and ulcerative colitis),
55 depressed immune function, lactose intolerance, infant allergies, *Helicobacter pylori*
56 infections, antibiotic-associated diarrhea in children and others (Deshpandea, Rao, &
57 Patolea, 2011; Hempel et al., 2012; Ritchie, & Romanuk, 2012).

58 In order to deliver the health benefits, probiotic products need to contain an
59 adequate amount of live cells (at least 10^6 - 10^7 CFU/g of product) (Boylston,
60 Vinderola, Ghoddusi, & Reinheimer, 2004), the cells should be able to survive the
61 acidic conditions of the upper gastrointestinal (GI) tract, adhere to mucosal-epithelial
62 surfaces and colonize the colon, exhibit antimicrobial activity against pathogenic
63 bacteria and bile salt hydrolase activity (Boylston et al., 2004; Kechagia et al., 2013;
64 Nagpal et al, 2012). Adhesion of probiotics to the intestinal mucosa is an important
65 prerequisite for transient colonization within the GI, and it is an area that attracts
66 considerable amount of research. To evaluate the adhesion abilities, *in vitro* methods
67 have been applied using mainly the Caco-2 cell line (Tuomola & Salminen, 1998;
68 Hilgendorf et al., 2000). *Lactobacillus casei*, *L. plantarum* and *L. rhamnosus* are
69 commonly used probiotic bacteria and have been shown to exert medium to strong
70 binding towards Caco-2 cells (Segers & Lebeer, 2014; Douillard et al., 2013; Elo,
71 Saxelin, & Salminen, 1991; Duary, Rajput, Batish, & Grover, 2011).

72 A variety of methods have been applied to determine the adhesive properties
73 of bacterial cells. The microbial adhesion to hydrocarbons (MATH) method measures
74 various forces contributing to the adhesion of bacterial cells to hydrophobic solvents,
75 including van der Waals, electrostatic and short-range interactions, as adhesion to
76 hydrocarbons reflects a number of physico-chemical interactions involved in adhesion
77 and not exclusively hydrophobicity (van der Mei, van der Belt-Gritter, Pouwels,
78 Martinez, & Busscher, 2003; Oliveira, Azeredo, Teixeira, & Fonseca, 2001). It must
79 be noted that there is very little information on how the formulation, for example the
80 food matrix, affects the physicochemical properties of the cells, and hence their
81 hydrophobicity and adhesion ability.

82 The transepithelial electrical resistance (TEER) assay determines the
83 attainment of permeability *in vitro* and also includes the measurement of electrical
84 physical resistance (Klingberg, Pedersen, Cencic, & Budde, 2005). Using the TEER
85 assay as a measure of the integrity of the tight junctions between intestinal epithelial
86 cells, studies have shown that some bacteria can enhance the intestinal barrier
87 function (Anderson, Cookson, McNabb, Kelly, & Roy, 2010b). Probiotic bacteria,
88 such as *L. plantarum* WCFS1 or *L. plantarum* MB452, *Lactobacillus rhamnosus* GG,
89 *Bifidobacterium infantis*, *Lactobacillus casei* subsp. *rhamnosus* Lcr35, have been
90 used to enhance the intestinal barrier, which is compromised in a number of intestinal
91 disorders, such as inflammatory bowel diseases, irritable bowel syndrome and some
92 types of food-borne infections (Karczewski et al., 2010; Anderson et al., 2010a; Fang
93 et al., 2010).

94 The incorporation of probiotic microorganisms within a food matrix presents
95 significant challenges, not only because of the interactions of the cells with a variety
96 of chemical components, but also because of the unfavourable conditions often

97 employed during food processing and storage which might lead to high losses in
98 viability. This is in particular evident when probiotics are incorporated into complex
99 food matrices, such as cheese and meat products (Sidira, Karapetsas, Galanis,
100 Kanellaki, & Kourkoutas, 2014a). To overcome these adversities, immobilization of
101 probiotic cells onto a solid support, prior to their inclusion into the targeted food
102 matrix, can be used to protect the cells and maintain their viability, activity and
103 functionality during processing and storage. To this end, several studies have
104 demonstrated the successful immobilization of probiotic bacteria onto various natural
105 food-grade material supports, such as starch (Mattila-Sandholm et al., 2002), fruit
106 pieces (Kourkoutas et al., 2006; Kourkoutas, Xolias, Kallis, Bezirtzoglou, &
107 Kanellaki, 2005), casein (Dimitrellou, Kourkoutas, Koutinas, & Kanellaki, 2009) and
108 wheat grains (Bosnea et al., 2009), and their application into food products, such as
109 sausages (Sidira et al., 2014a) and cheese (Kourkoutas et al., 2006). However, there is
110 limited knowledge, on how immobilization affects the functional properties of the
111 probiotic cells, and in particular their ability to adhere to epithelial cells and their
112 action as an intestinal barrier.

113 The probiotic properties of free and immobilized *L. casei* ATCC 393 were
114 previously assessed by documenting the maintenance of cell viability after transit
115 through the GI tract, adhesion at the large intestine and regulation of the intestinal
116 microbiota in rats (Saxami et al., 2012, Sidira et al., 2010). Although, it was suggested
117 that adhesion to the GI tract was transient, indicating the need for daily consumption
118 of probiotic products containing the specific strain, no information about the effect of
119 cell immobilization on adhesion properties were available. Similarly, *in vivo*
120 experiments showed that *L. plantarum* enhanced the intestinal barrier and induced
121 changes in the epithelial tight junctions (Karczewski et al., 2010).

122 Thus, the objective of the present study was to investigate the effect of
123 probiotic immobilization onto wheat grains (wet and freeze dried) on the adhesion
124 properties of lactobacilli using the model probiotic strains *L. casei* ATCC 393 and *L.*
125 *plantarum* NCIMB 8826 in comparison to free wet and freeze dried cells. *L.*
126 *plantarum* NCIMB 8826 was selected due to the strong binding characteristics
127 (Izquierdo et al., 2009; Kinoshita et al., 2008), resistance to acidic conditions
128 (Charalampopoulos, Pandiella, & Webb, 2002), survival during refrigerated storage
129 (Charalampopoulos & Pandiella, 2010) and in fruit juices (Nualkaekul &
130 Charalampopoulos, 2011), while *L. casei* ATCC 393 due to its good survival in the
131 GI tract (Saxami et al., 2012; Sidira et al., 2010) and excellent technological
132 properties (Bosnea et al., 2009; Kourkoutas et al., 2006, 2005; Sidira et al., 2014a).

133

134 **2. Materials and methods**

135 **2.1 Strain maintenance and growth**

136 *L. casei* ATCC 393 (DSMZ, Germany) and *L. plantarum* NCIMB 8826
137 (National Collection of Industrial and Marine Bacteria, UK) were stored at -20 °C in 2
138 ml cryovials containing 20 % (v/v) glycerol. The cells were initially propagated at 37
139 °C in 250 ml flasks containing 100 ml of MRS Broth (Fluka, Buchs, Switzerland) and
140 then cultivated at 37 °C in 500 ml flasks for 72 h containing 200 ml of the same
141 culture medium. The free cells were collected by centrifugation at 3200 g and 4 °C for
142 15 min.

143 **2.2 Cell immobilization**

144 Wheat grains were used as immobilization support. Prior to use, they were
145 boiled and sterilized at 130 °C for 15 min. Cell immobilization was carried out as
146 described previously (Bosnea et al., 2009). In brief, 50 g of immobilization support

147 and ~1 g (wet weight) of *L. casei* ATCC 393 or *L. plantarum* NCIMB 8826 cells were
148 added into 1 L flasks containing 500 ml of MRS broth. The culture was incubated at
149 37 °C without agitation for 48 h. The immobilized cells were collected by washing
150 twice with sterilized ¼ Ringer's solution (Sigma-Aldrich, UK) for removal of non-
151 immobilized free cells. The immobilized cells were either used directly or subjected to
152 freeze-drying.

153 **2.3 Freeze-drying of free and immobilized cells**

154 Free and immobilized cells were freeze-dried as described previously
155 (Siaterlis, Deepika, & Charalampopoulos, 2009). Briefly, the immobilized cells were
156 washed three times with ¼ Ringer's solution and transferred into 250 ml sterilin
157 polystyrene container containing 25 ml of 10 % sucrose in PBS solution used as
158 cryoprotectant. Similarly, the free cells were re-suspended in 10 ml of 10 % sucrose in
159 PBS solution. Both immobilized and free cell suspensions in sucrose solutions were
160 incubated at room temperature for 1 h and then frozen at -80 °C for 24 h. The frozen
161 cultures were then freeze-dried in a IEC Lyoprep 3000 freeze-dryer (Lyoprep,
162 Dunstable, UK) for approximately 2-4 days. The viable cell concentration were
163 measured both pre- and post-freeze-drying. The water activity of freeze-dried
164 powders in all cases was ~ 0.07. Each experiment was conducted in triplicate.

165 **2.4 Adhesion of *Lactobacillus* cells to Caco-2 cells**

166 Adhesion of the *Lactobacillus* cells to Caco-2 cells was studied as described
167 previously (Deepika, Green, Frazier, & Charalampopoulos, 2009). The Caco-2 cell
168 line was cultured in Eagle's Minimum Essential Medium (EMEM, Lonza, UK)
169 supplemented with 10 % Fetal Bovine Serum (FBS), 1 % mixture penicillin-
170 streptomycin solution and 1 % non-essential amino acid solution at 37 °C in an
171 atmosphere of 5 % CO₂ and 95 % relative humidity. After 21 days, the six-well tissue

172 culture plate was ready to use as the cells were fully differentiated. One day prior to
173 the adhesion assay, the Caco-2 cells were fed with the above culture medium, but
174 without the presence of antibiotics. Before adhesion, the Caco-2 monolayer was
175 washed twice with Dulbecco's phosphate buffered saline (DPBS, Lonza) in order to
176 remove all traces of the medium. The number of the fully differentiated cells was
177 counted using a Nikon microscope (Kingston upon Thames, UK) and was found to be
178 around 5×10^5 cells/ml in all cases. Then, 10 ml of free or 1 g of immobilized
179 bacterial cells (wet or freeze dried) were washed once with DPBS and then were re-
180 suspended in DPBS to obtain an approximate concentration of around 10^8 - 10^9
181 CFU/ml and 1 ml of the bacterial suspension was added to each well. The plates were
182 incubated at 37 °C in 5 % CO₂ and 95 % air. After 1 h, the supernatant was removed
183 from the wells and the wells were washed twice with DPBS. Subsequently, 1 ml of
184 DPBS was added to each well and the monolayer was scalped and transferred to 9 ml
185 of ¼ Ringer's solution. The number of probiotic cells bound to the Caco-2 cells was
186 determined after serial dilutions and plating in MRS agar plates. Adhesion of
187 *Lactobacillus* strains was expressed as the number of viable bacteria adhering to 100
188 Caco-2 cells. Each experiment was conducted in triplicate.

189 **2.5 Adhesion to *n*-hexadecane**

190 The microbial adhesion to *n*-hexadecane (MATH assay) was employed to
191 study the hydrophobicity of free and immobilized cells and was carried out as
192 described previously (Deepika et al., 2009). In brief, 10 ml of free or 1 g of
193 immobilized bacteria (wet or freeze-dried) were washed with PBS and suspended in
194 10 mM KH₂PO₄ to obtain an OD₆₀₀ ~ 0.8. The pH was adjusted to 3.0 with 1 M HCl
195 and then 2 ml of the bacterial suspension were mixed with equal volume of *n*-
196 hexadecane. The mixture was vortexed for 1 min and was allowed undisturbed to rest

197 for 20 min to achieve a complete phase separation. The aqueous phase was removed
198 and the OD₆₀₀ was measured. The level of adhesion to *n*-hexadecane was expressed as
199 the % percentage adhesion according to the following equation:

$$200 \quad \% \textit{ Adhesion to hexadecane} = \left(\frac{1 - A_1}{A_0} \right) \times 100$$

201 where A₀: initial absorbance, A₁: absorbance after 20 min of incubation. The MATH
202 assay was conducted in triplicate for each sample.

203 **2.6 Trans-epithelial electrical resistance (TEER) assay**

204 TEER was used to measure the levels of tight junction in intestinal epithelial
205 cells according to a method described previously (Commane et al., 2005). Cell culture
206 inserts (0.4 μm, Becton Dickinson) were coated with 0.1 % type I rat-tail collagen
207 (Sigma-Aldrich Company Ltd., Dorset, UK) and left to dry overnight under a UV
208 light in six-well plates (Becton Dickinson). Caco-2 cells were seeded into the inserts
209 at 2.5 mL aliquots per well with a concentration of 5 x 10⁵ cells/ml, as described
210 above (Section 2.4). Eagle's Minimum Essential Medium (EMEM, Lonza)
211 supplemented with 10 % Fetal Bovine Serum (FBS), 1 % mixture penicillin-
212 streptomycin solution and 1 % non-essential amino acid solution (2 ml of culture
213 medium) was added to the basal compartment of each well. The cells were grown at
214 37 °C in an atmosphere of 5 % CO₂ and 95 % relative humidity. The culture medium
215 was refreshed every 2 days. After 12 days of culturing, the integrity of the monolayer
216 was evaluated by measuring the TEER using an EVOM epithelial voltohmmeter
217 chopstick electrode (World Precision Instruments, Stevenage, UK). Readings were
218 performed every 24 h until the TEER values stabilized (day 16). TEER at time zero
219 was determined before bacterial samples were added to the monolayer. Then, the
220 culture medium from both the apical and basal compartments was removed and
221 immediately 2 ml bacterial samples were added in the apical side (10⁸-10⁹ CFU/ml),

222 while 2 ml medium were added in the basal compartments. The cells were maintained
223 for 1 h at 37 °C in an atmosphere of 5 % CO₂ and 95 % relative humidity. Then, the
224 culture medium and the bacterial sample were removed and the apical side was
225 washed with 2 ml DPBS. Thereafter, in both the apical and basal compartments, 2 ml
226 of culture medium were applied and TEER readings were recorded after 1, 1.5 and 17
227 h in order to estimate the potential increase in TEER. One insert/well in every falcon
228 was left without bacteria as a negative control. The percentage change in TEER was
229 expressed according to the following equation:

$$\% \text{ change in TEER} = \left[\frac{(T1 - T0)}{T0} \right] \times 100$$

230

231 where T0: TEER at time zero, T1: TEER after 1 h, 1.5 h and 17 h. Each experiment
232 was conducted in triplicate.

233 **2.7 Scanning electron microscopy (SEM)**

234 Monolayers of Caco-2 cells were grown on glass coverslips as described
235 previously (Chauviere, Coconnier, Kerneis, Fourniat, & Servin, 1992). Briefly, Caco-
236 2 cells were prepared on glass coverslips which were placed in six-well tissue culture
237 plates. Cells were grown in culture medium at 37 °C in 5 % CO₂ and 95 % air. After
238 the bacterial adhesion assay, the cells were fixed with 2.5 % glutaraldehyde in 0.1 M
239 sodium phosphate buffer (pH 7.4) for 1 h at room temperature. Then, washing with 2
240 ml of the phosphate buffer was performed and the cells were osmicated for 30 min
241 with 2 % OsO₄ and washed again 3 times with the same buffer. The coverslips were
242 gradually dehydrated with 30 % ethanol for 15 min, 50 % for 15 min, 70 % for 15
243 min, 80 % for 15 min, 90 % for 15 min, and 3x100 % for 15 min to achieve absolute
244 dry samples. The cells were further dried in a critical-point dryer (Balzers CPD 030)
245 and coated with gold. The samples were imaged using a FEI Quanta 600 FEG
246 scanning electron microscope. Bacterial attachment was evaluated by scanning

247 electron microscopy using a fixed number of monolayer cells (5×10^5 cells/well) and
248 bacteria (8.2 log CFU added per well).

249 **2.8 Experimental design and statistical analysis**

250 All treatments were carried out in triplicate. The experiments were designed
251 and analyzed statistically by ANOVA. Duncan's multiple range test was used to
252 determine significant differences among results (coefficients, ANOVA tables and
253 significance ($P < 0.05$) were computed using Statistica v.5.0).

254

255 **3. Results and Discussion**

256 Cell immobilization techniques are usually applied in order to maintain cell
257 viability, activity and functionality during food production, processing and storage
258 (Deepika, Rastall, & Charalampopoulos, 2011; Lopez-Rubio, Gavara, & Lagaron,
259 2006), and during passage through the GI tract (Saxami et al., 2012; Sidira et al.,
260 2010). In order to confer a health effect, high adhesion ability of the probiotic cells is
261 required (Ouweland & Salminen, 2003). Despite the considerable amount of research
262 aiming at evaluating the adhesion properties of free cells and the potential interaction
263 between probiotics and the host (Ouweland & Salminen, 2003; Tuomola & Salminen,
264 1998), there is limited knowledge on how the food matrix influences the adhesion
265 ability of probiotics (Burgain et al., 2014; Endo et al., 2014; Bove et al. 2013). This is
266 important for the design of novel foods that are able to maintain high cell viability and
267 functionality.

268 To the best of our knowledge, this is the first report concerning the
269 investigation of the effect of the immobilization support on the adhesion properties of
270 probiotic bacteria. The strategy adopted was to use the model probiotic strains *L.*
271 *casei* ATCC 393 and *L. plantarum* NCIMB 8826, which were immobilized on wheat

272 grains. Wheat grains were previously used as immobilization supports of *L. casei*
273 ATCC 393 (Bosnea et al., 2009) and the immobilized cells were incorporated in
274 traditional foods to confer probiotic properties (Sidira et al., 2014a; Sidira, Galanis,
275 Nikolaou, Kanellaki, & Kourkoutas, 2014b). In addition, the effect of freeze-drying
276 was also evaluated because wet cells are incompatible with commercial and industrial
277 needs, which require robust cultures that can maintain their availability during
278 storage.

279

280 **3.1 Immobilization and freeze-drying of lactobacilli**

281 The results concerning the effect of freeze-drying on the viability of free and
282 immobilised lactobacilli onto wheat grains are presented in Figure 1. It can be
283 observed that freeze-drying had no significant ($P>0.05$) effect on cell survival for
284 both free and immobilized cells and that high cell concentrations were obtained post
285 freeze-drying in all cases.

286 **3.2 Adhesion to Caco-2 cells**

287 The results concerning the adhesion abilities of both *L. casei* ATCC 393 and *L.*
288 *plantarum* NCIMB 8826 to Caco-2 cells are presented in Figure 2, and indicate that
289 both strains showed considerable adhesion ability to Caco-2 cells (>1000 CFU/100
290 Caco-2 cells for free wet cells). These values were higher than those reported in
291 previous studies for other lactobacilli, which ranged between 0.9 and 900 CFU/100
292 Caco-2 cells (Ren et al., 2012; Bogovic-Matijasic, Narat, & Zori, 2003; Chauviere et
293 al., 1992; Bernet, Brassart, Neeser, & Servin., 1994; Sarem, Sarem-Damerdjji, &
294 Nicolas, 1996; Coconnier, Klaenhammer, Kerneis, Bernet, & Servin, 1992). A cell
295 concentration of 8-9 log CFU/ml of *Lactobacillus* was added to the Caco-2 culture, as

296 this is the recommended concentration for probiotics in order to exert a beneficial
297 effect in the gut.

298 Although cell immobilization resulted in a significant reduction of adhesion
299 ability (>100 CFU/100 Caco-2 cells for immobilized wet cells), adhesion still
300 remained in satisfactory levels compared to values reported in literature concerning
301 free cells (0.9-900 CFU/100 Caco-2 cells) (Bogovic-Matijasic et al., 2003; Chauviere
302 et al., 1992; Bernet et al., 1994; Ren et al., 2012; Sarem et al., 1996; Coconnier et al.,
303 1992).

304 Freeze-drying had a negative effect on the adhesion properties of free cells,
305 but not of immobilized cells. The adhesion to Caco-2 cells decreased significantly for
306 free cells (86 % and 71 % reduction for *L. plantarum* and *L. casei*, respectively).
307 According to Henriksson, Szewzyk, & Conway, (1991) the adhesion of lactobacilli is
308 most probably mediated by a proteinaceous component on the bacterial surface.
309 Bacterial surface layers (S-layers) are composed of protein monomers arranged in
310 crystalline arrays (Callegari et al., 1998) and it appears that freeze-drying can induce
311 the detachment of S-layers monomers from the cell wall of lactobacilli (Ray &
312 Johnson, 1986). This cell surface damage that occurs during freeze-drying may
313 provide an explanation of the results above. Nevertheless, the adhesion values still
314 were comparable to those recorded in the literature for free dried cells, ranging from
315 3-325 CFU/100 Caco-2 cells (Bogovic-Matijasic et al., 2003; Chauviere et al., 1992;
316 Bernet et al., 1994).

317 The adoption of functional criteria (adherence to human cell lines, resistance
318 to gastric acidity and bile acids) for the *in vitro* selection of probiotic bacteria can
319 result in the isolation of strains capable of performing effectively in the GI tract and
320 that may reflect certain *in vivo* effects on the host (Dunne et al., 2001). A previous

321 study has shown how *in vitro* methods can be used for prediction of the survival
322 potential of lactobacilli in the human GI tract (Jacobsen et al., 1999), although Lebeer
323 at al., (2010a) has shown that *in vitro* studies are not related with survival *in vivo*. Our
324 results validated the suggestions of Busscher & Weerkamp (1987), according to
325 which the role of hydrophobic cells, supposed to be associated with bacterial surface
326 appendages, is suggested to be its dehydrating capacity, allowing the removal of the
327 water film and yielding a small area of direct contact between the protuberant parts of
328 the cell surface and the substrate. Noticeably, the ability of probiotics to remove
329 vicinal water depends greatly on the strain used.

330 **3.3 Adhesion to *n*-hexadecane**

331 Figure 3 shows the results from the MATH assay for wet and freeze-dried
332 cells. The results indicated that the *L. casei* strain was relatively hydrophilic compared
333 to the *L. plantarum* strain (54.8 % and 22.7 % for free cells of *L. plantarum* and *L.*
334 *casei*, respectively). Immobilization affected ($P<0.05$) positively the adhesion only
335 for *L. casei*. On the other hand, although freeze-drying resulted in increased ($P<0.05$)
336 adhesion ability for *L. plantarum* (both free and immobilized) and for immobilized *L.*
337 *casei*, it had no effect ($P>0.05$) on free *L. casei* cells.

338 Cell surface hydrophobicity has been associated with bacterial adhesion to a
339 variety of surfaces (Marin et al., 1997). The consensus is that high hydrophobicity of
340 bacteria results in greater attractive forces and higher levels of adhesion to Caco-2
341 cells, whereas smaller results in lower levels of adhesion (Rijnaarts, Norde, Bouwer,
342 Lyklema, & Zehnder, 1993). According to Reid et al. (1992), the surface
343 hydrophobicity of *Lactobacillus* strains varies greatly. In the present study, the results
344 indicated that *L. casei* ATCC 393 strain was relatively hydrophilic (33.3% adhesion to
345 *n*-hexadecane), whereas *L. plantarum* NCIMB 8826 is characterized as hydrophobic

346 (42.86% adhesion to *n*-hexadecane). Similar results were obtained by Pelletier et al.,
347 (1997) and Harty, Patrikakis, and Knox (1993) concerning the *L. casei* cells and by
348 Zavisic et al., (2011), Jamaly, Benjouad, and Bouksaim (2011) and Abdulla, Abed,
349 and Saeed (2014) concerning the *L. plantarum* cells.

350 However, no correlation between adhesion ability to the Caco-2 monolayer
351 and hydrophobicity was observed for immobilized *L. casei* ATCC 393. Although the
352 strain was characterized as relatively hydrophilic, the number of adhered immobilized
353 *L. casei* was higher than immobilized *L. plantarum* (154 to 104 CFU adhered per 100
354 Caco-2 cells, respectively). The opposite results were observed in free cells, as
355 hydrophobicity correlated well with adhesion ability (1038 and 2349 CFU adhered
356 per 100 Caco-2 cells for *L. plantarum* and *L. casei*, respectively). A possible
357 explanation for these observations might be that the immobilized cells do not have
358 direct access to the Caco-2 cells (Figure 4). The inability to correlate hydrophobicity
359 with adhesion to the Caco-2 monolayer is most likely due to many factors that are
360 involved in the adhesion of lactobacilli (Azuma & Sato, 2001). Cell surface
361 hydrophobicity is one of the physico-chemical properties that facilitate the first
362 contact between the microorganism and the host cells. This non-specific initial
363 interaction is weak and reversible and precedes the subsequent adhesion process
364 mediated by more specific mechanisms involving cell-surface proteins and
365 lipoteichoic acids (Schillinger, Guigas, & Holzapfel, 2005). Bacterial cell surface
366 macromolecules may interact with host pattern recognition receptors (PRRs) of the GI
367 mucosa and elicit specific responses in the intestinal system (Bron, Baarlen, &
368 Kleerebezem, 2012; Lebeer, Vanderleyden, & Keersmaecker, 2010b). As stated
369 above, structural damage (S-layer) of bacteria during freeze-drying can affect the
370 adhesion ability (Callegari et al., 1998) and reduce its cell surface hydrophobicity

371 significantly (Bruinsma, van der Mei, & Busscher, 2001). It therefore appears that the
372 adhesion was not caused by hydrophobic interaction. According to Schillinger et al.,
373 (2005), hydrophobicity is not a prerequisite for a strong adherence capacity.

374 **3.4 Trans-Epithelial Electrical Resistance Assay**

375 The most sensitive measure of mucosal barrier function is transepithelial
376 electrical resistance (TEER), as it reflects the degree to which ions traverse tissue
377 (Blikslager, Moeser, Gookin, Jones, & Odle, 2007). When TEER is increased, the
378 tight junction between the cells becomes stronger and the permeability of the
379 monolayer is decreased (Mattar, Drongowski, Coran, & Harmon, 2001). The TEER
380 ratio was measured before adding the bacterial inoculum (time zero), and after
381 addition and incubation of the probiotic strains in Caco-2 monolayer. The results from
382 the TEER assay are presented in Table 1. For all samples, the TEER values increased
383 with incubation time, reaching the maximum after 17 h of incubation.

384 The results indicated that both wet and freeze-dried free cells led to an
385 increase in TEER values (58.4 and 43.6 % for wet *L. plantarum* and *L. casei*,
386 respectively, and 73.6 and 73.7 % for freeze-dried *L. plantarum* and *L. casei*,
387 respectively). Similar results were also published previously reporting increases in
388 TEER by 20-158 % for *L. plantarum* (Anderson et al., 2010a, 2010b) and 6-87 % for
389 *L. casei* cells (Fang et al., 2010). However, significantly ($P<0.05$) higher increase was
390 recorded for immobilized cells (151 and 170 % for wet *L. plantarum* and *L. casei*,
391 respectively, and 151 and 121 % for freeze-dried *L. plantarum* and *L. casei*,
392 respectively). Notably, immobilized cells resulted in TEER values at least 2-fold
393 higher than free cells, highlighting the positive effects of cell immobilization in
394 enhancing the persistence of probiotic cells in the Caco-2 monolayer. It is thus
395 suggested that immobilized cells act as a protective shield against intestinal

396 permeability, which might be due to increased physical interactions between the
397 immobilised bacterial cells and the Caco-2 cells, or possibly due to increased
398 synthesis of short chain fatty acids, which are known to be important nutrients for
399 intestinal epithelial cells (Mangell et al., 2002). Improved epithelial barrier function
400 has been previously associated with synbiotic food products containing immobilized
401 probiotic microorganisms on prebiotic fibres (Commane et al., 2005). The findings of
402 this study are expected to have a significant impact in the development of novel
403 probiotic products targeting a range of GI disorders, as they indicated that cell
404 immobilization, which has been suggested for the production of probiotic products
405 (Kourkoutas et al., 2006; Bosnea et al., 2009; Sidira et al., 2014a, 2014b), can
406 potentially offer the additional benefit of increased probiotic barrier function.

407 **3.5 Scanning electron microscopy (SEM)**

408 Figure 4 shows images from electron microscopy demonstrating key features
409 of the Caco-2 cells, as well as the adhesion of the bacterial cells. It was noted that
410 both immobilized and free cells adhered to the brush border microvilli and that the
411 starch granules of wheat grains interacted with the Caco-2 monolayer, confirming the
412 results of adhesion and TEER assays. Importantly, the morphology of the monolayer
413 with the expression of microvilli, the tight-junctions of the starch granules of wheat,
414 and the immobilized cells onto wheat grains are also obvious. Similar photographs
415 were obtained for *L. plantarum* NCIMB 8826 strain and for freeze-dried cells (data
416 not shown).

417 **4. Conclusions**

418 In conclusion, the present study showed that immobilization onto wheat grains
419 decreased significantly the adhesion ability of both *L. casei* ATCC 393 and *L.*
420 *plantarum* NCIMB 8826 strains to Caco-2 monolayers most likely because

421 immobilized cells did not have direct access to the Caco-2 cells. However,
422 immobilization led to a significant enhancement of the monolayer integrity, which is
423 compromised in a number of intestinal disorders, and is an important functional
424 attribute of probiotic strains. Overall, more *in vitro* and *in vivo* research is needed in
425 order to understand the effect of immobilization, which can also occur naturally
426 within a food matrix, on the functional properties of probiotics.

427

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436

437

438 **References**

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681

682 **Table 1.** Changes in Trans-Epithelial Electrical Resistance (TEER) values of Caco-2
683 cells in the presence of *L. plantarum* NCIMB 8826 and *L. casei* ATCC 393 cells.
684 Changes are expressed as percentage change in TEER values. Significant differences
685 ($P<0.05$) are indicated by different letters in superscript.
686

Strain	Time (h)	Free		Immobilized	
		Wet	Freeze-dried	Wet	Freeze-dried
<i>L. plantarum</i> NCIMB 8826	1.0	-1.8±0.1 ^a	6.2±0.3 ^b	32.6±1.6 ^c	8.45±0.4 ^d
	1.5	25.7±5.3 ^{c,e}	18.9±0.6 ^f	111.6±38.8 ^g	47.3±23.1 ^h
	17.0	58.4±10.3 ^h	73.6±29.9 ^{g,h,i,j,k}	150.8±54.6 ^{i,l,p}	150.7±33.1 ^{i,l,p}
<i>L. casei</i> ATCC 393	1.0	13.9±0.7 ^m	1.3±0.1 ⁿ	25.9±1.3 ^e	2.6±0.1 ^o
	1.5	10.7±2.0 ^d	41.2±8.2 ^h	86.3±3.7 ^j	35.2±9.4 ^{c,e,k}
	17.0	43.6±10.9 ^{h,k}	73.7±22.9 ^{g,h,i,j}	169.8±9.9 ^l	121.1±2.2 ^p

687
688

689 **Legends to Figures**

690 **Figure 1.** Effect of freeze-drying on viability of lactobacilli. Significant differences
691 ($P<0.05$) are indicated by different letters.

692

693 **Figure 2.** Adhesion of lactobacilli to Caco-2 cells (CFU adhered per 100 Caco-2
694 cells). Significant differences ($P<0.05$) are indicated by different letters.

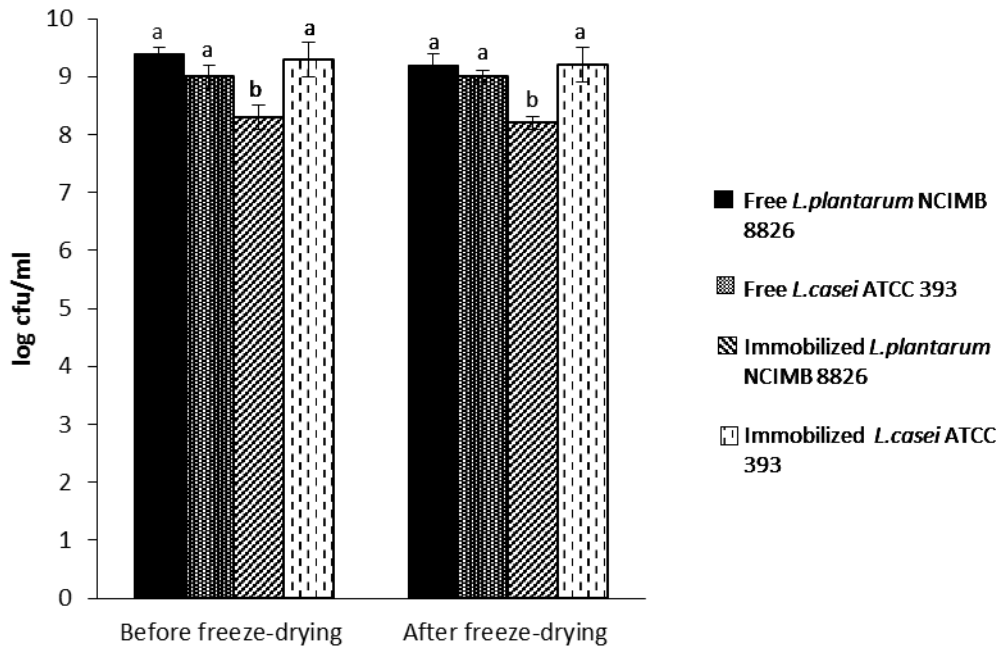
695

696 **Figure 3.** Adhesion of *L. plantarum* NCIMB 8826 and *L. casei* ATCC 393 to *n*-
697 hexadecane. Significant differences ($P<0.05$) are indicated by different letters.

698

699 **Figure 4.** SEM micrographs showing: a) the morphology of complete Caco-2
700 monolayer, b) adhesion of free *L. casei* ATCC 393 bacteria to Caco-2 cells, c)
701 adhesion of *L. plantarum* NCIMP 8826 bacteria to Caco-2 cells and the level of
702 intercellular junctions (shown by arrows), d) starch granules of wheat interacting with
703 the Caco-2 monolayer and e) immobilized *L. casei* ATCC 393 cells on wheat grains.

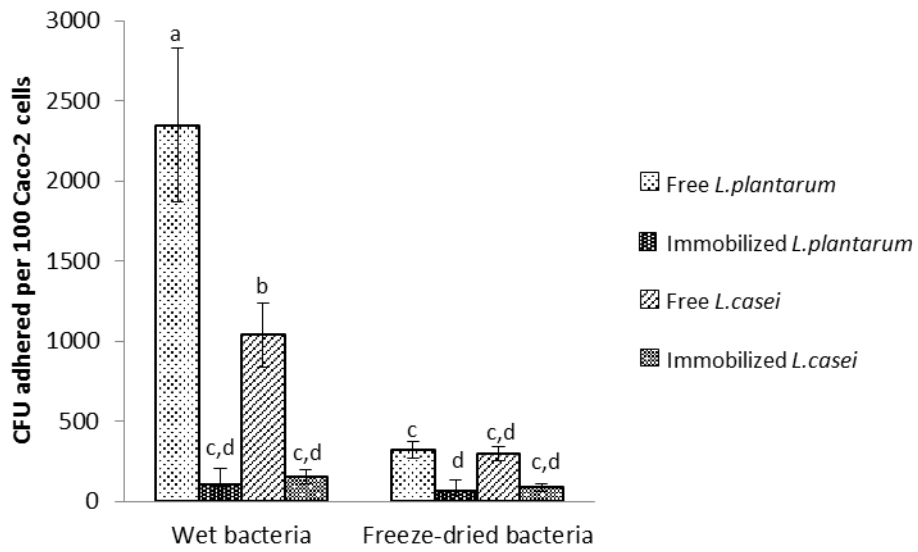
704



705

706 **Fig. 1**

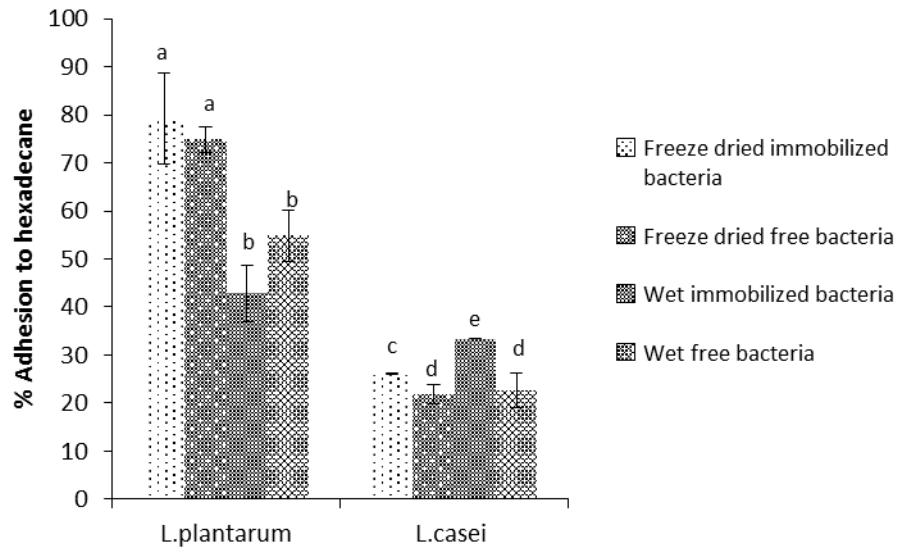
707



708

709 **Fig. 2**

710

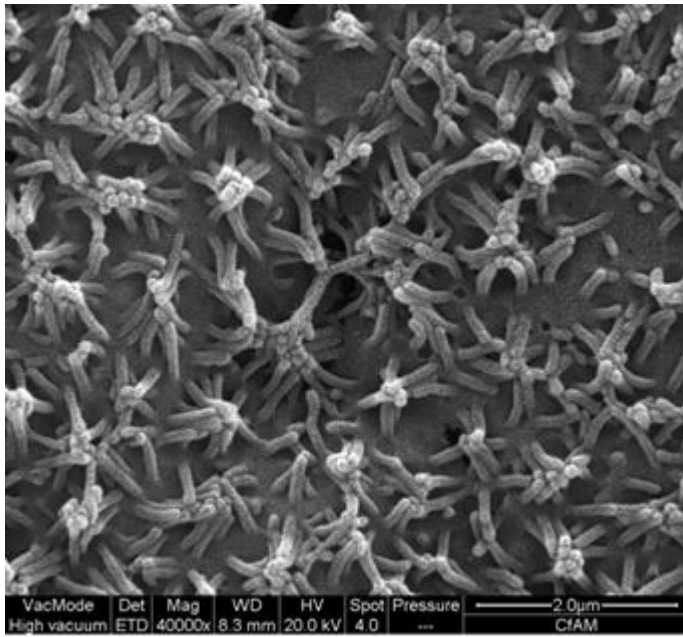


711

712 **Fig. 3.**

713

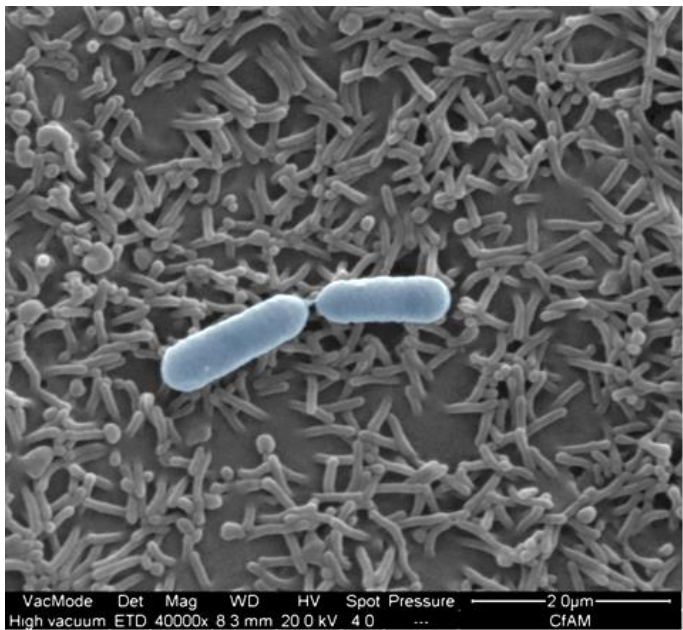
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715

716 **Fig. 4a**

717

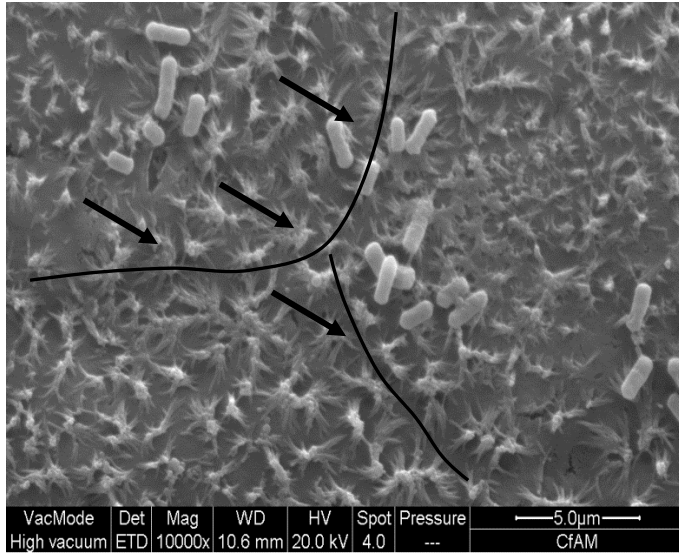


718

719 **Fig. 4b**

720

721

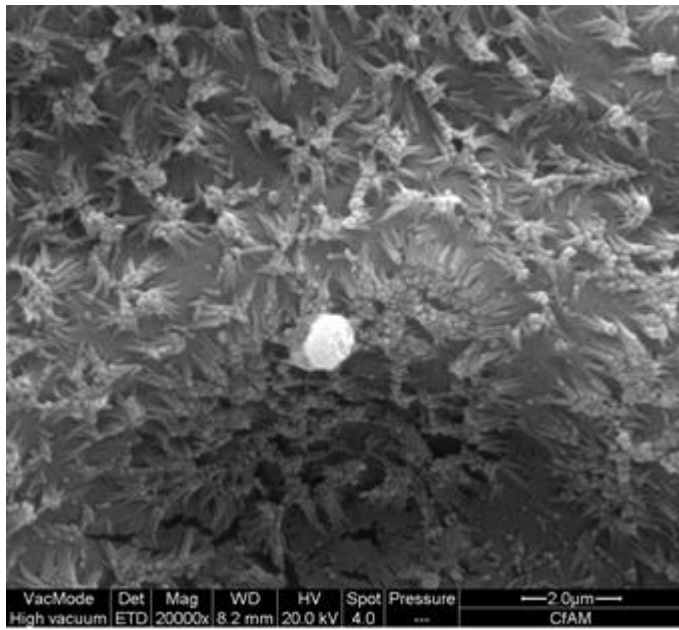


722

723 **Fig. 4c**

724

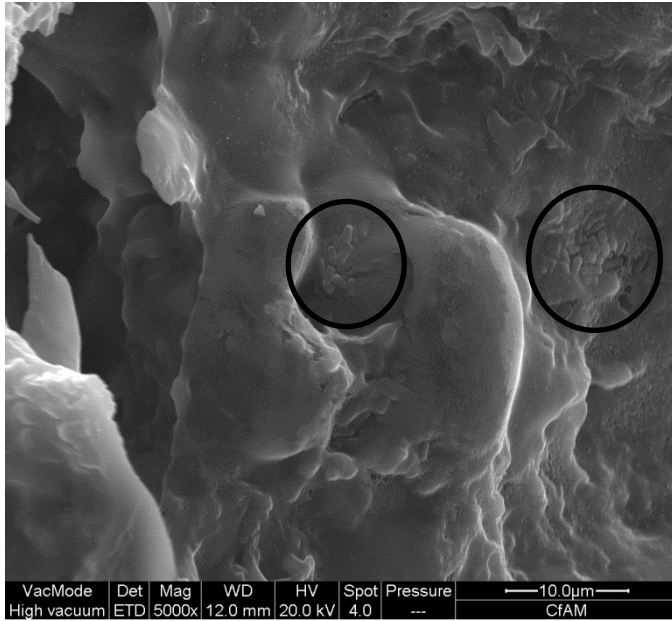
725



726

727 **Fig. 4d**

728



729

730 **Fig. 4e**

731

732