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

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Abstract

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

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

1. Introduction

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

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

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

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

The incorporation of probiotic microorganisms within a food matrix presents significant challenges, not only because of the interactions of the cells with a variety 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).

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

2. Materials and methods

2.1 Strain maintenance and growth

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

2.2 Cell immobilization

Wheat grains were used as immobilization support. Prior to use, they were boiled and sterilized at 130 °C for 15 min. Cell immobilization was carried out as 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 ^{o}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 ^{o}C in an
171 atmosphere of 5 % CO_2 and 95 % relative humidity. After 21 days, the six-well tissue

culture plate was ready to use as the cells were fully differentiated. One day prior to the adhesion assay, the Caco-2 cells were fed with the above culture medium, but without the presence of antibiotics. Before adhesion, the Caco-2 monolayer was washed twice with Dulbecco's phosphate buffered saline (DPBS, Lonza) in order to remove all traces of the medium. The number of the fully differentiated cells was counted using a Nikon microscope (Kingston upon Thames, UK) and was found to be around 5×10^5 cells/ml in all cases. Then, 10 ml of free or 1 g of immobilized bacterial cells (wet or freeze dried) were washed once with DPBS and then were re-suspended in DPBS to obtain an approximate concentration of around 10^8-10^9 CFU/ml and 1 ml of the bacterial suspension was added to each well. The plates were incubated at 37 ^oC in 5 % CO_2 and 95 % air. After 1 h, the supernatant was removed from the wells and the wells were washed twice with DPBS. Subsequently, 1 ml of DPBS was added to each well and the monolayer was scalped and transferred to 9 ml of ¼ Ringer's solution. The number of probiotic cells bound to the Caco-2 cells was determined after serial dilutions and plating in MRS agar plates. Adhesion of *Lactobacillus* strains was expressed as the number of viable bacteria adhering to 100 Caco-2 cells. Each experiment was conducted in triplicate.

2.5 Adhesion to *n*-hexadecane

The microbial adhesion to *n*-hexadecane (MATH assay) was employed to study the hydrophobicity of free and immobilized cells and was carried out as described previously (Deepika et al., 2009). In brief, 10 ml of free or 1 g of immobilized bacteria (wet or freeze-dried) were washed with PBS and suspended in 10 mM KH_2PO_4 to obtain an $OD_{600} \sim 0.8$. The pH was adjusted to 3.0 with 1 M HCl and then 2 ml of the bacterial suspension were mixed with equal volume of *n*-hexadecane. The mixture was vortexed for 1 min and was allowed undisturbed to rest

for 20 min to achieve a complete phase separation. The aqueous phase was removed and the OD_{600} was measured. The level of adhesion to *n*-hexadecane was expressed as the % percentage adhesion according to the following equation:

$$\%\ Adhesion\ to\ hexadecane = \left(\frac{1 - A_1}{Ao}\right) \times 100$$

where A_0: initial absorbance, A_1: absorbance after 20 min of incubation. The MATH assay was conducted in triplicate for each sample.

2.6 Trans-epithelial electrical resistance (TEER) assay

TEER was used to measure the levels of tight junction in intestinal epithelial cells according to a method described previously (Commane et al., 2005). Cell culture inserts (0.4 μm, Becton Dickinson) were coated with 0.1 % type I rat-tail collagen (Sigma-Aldrich Company Ltd., Dorset, UK) and left to dry overnight under a UV light in six-well plates (Becton Dickinson). Caco-2 cells were seeded into the inserts at 2.5 mL aliquots per well with a concentration of 5 x 10^5 cells/ml, as described above (Section 2.4). Eagle's Minimum Essential Medium (EMEM, Lonza) supplemented with 10 % Fetal Bovine Serum (FBS), 1 % mixture penicillin-streptomycin solution and 1 % non-essential amino acid solution (2 ml of culture medium) was added to the basal compartment of each well. The cells were grown at 37 ^oC in an atmosphere of 5 % CO_2 and 95 % relative humidity. The culture medium was refreshed every 2 days. After 12 days of culturing, the integrity of the monolayer was evaluated by measuring the TEER using an EVOM epithelial voltohmmeter chopstick electrode (World Precision Instruments, Stevenage, UK). Readings were performed every 24 h until the TEER values stabilized (day 16). TEER at time zero was determined before bacterial samples were added to the monolayer. Then, the culture medium from both the apical and basal compartments was removed and immediately 2 ml bacterial samples were added in the apical side (10^8-10^9 CFU/ml),

while 2 ml medium were added in the basal compartments. The cells were maintained for 1 h at 37 ^{o}C in an atmosphere of 5 % CO_2 and 95 % relative humidity. Then, the culture medium and the bacterial sample were removed and the apical side was washed with 2 ml DPBS. Thereafter, in both the apical and basal compartments, 2 ml of culture medium were applied and TEER readings were recorded after 1, 1.5 and 17 h in order to estimate the potential increase in TEER. One insert/well in every falcon was left without bacteria as a negative control. The percentage change in TEER was expressed according to the following equation:

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

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

2.7 Scanning electron microscopy (SEM)

Monolayers of Caco-2 cells were grown on glass coverslips as described previously (Chauviere, Coconnier, Kerneis, Fourniat, & Servin, 1992). Briefly, Caco-2 cells were prepared on glass coverslips which were placed in six-well tissue culture plates. Cells were grown in culture medium at 37 ^{o}C in 5 % CO_2 and 95 % air. After the bacterial adhesion assay, the cells were fixed with 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 1 h at room temperature. Then, washing with 2 ml of the phosphate buffer was performed and the cells were osmicated for 30 min with 2 % OsO_4 and washed again 3 times with the same buffer. The coverslips were gradually dehydrated with 30 % ethanol for 15 min, 50 % for 15 min, 70 % for 15 min, 80 % for 15 min, 90 % for 15 min, and 3x100 % for 15 min to achieve absolute dry samples. The cells were further dried in a critical-point dryer (Balzers CPD 030) and coated with gold. The samples were imaged using a FEI Quanta 600 FEG scanning electron microscope. Bacterial attachment was evaluated by scanning

electron microscopy using a fixed number of monolayer cells ($5x10^5$ cells/well) and bacteria (8.2 log CFU added per well).

2.8 Experimental design and statistical analysis

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

3. Results and Discussion

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

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

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

3.1 Immobilization and freeze-drying of lactobacilli

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

3.2 Adhesion to Caco-2 cells

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

this is the recommended concertation for probiotics in order to exert a beneficial effect in the gut.

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

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

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

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

3.3 Adhesion to *n*-hexadecane

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

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

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

3.4 Trans-Epithelial Electrical Resistance Assay

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

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

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

3.5 Scanning electron microscopy (SEM)

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

4. Conclusions

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

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

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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

		Free		Immobilized	
Strain	**Time (h)**	**Wet**	**Freeze-dried**	**Wet**	**Freeze-dried**
L. plantarum 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	$25.7\pm5.3^{c,e}$	18.9 ± 0.6^{f}	111.6 ± 38.8^{g}	47.3 ± 23.1^{h}
	17.0	58.4 ± 10.3^{h}	$73.6\pm29.9^{g,h,i,j,k}$	$150.8\pm54.6^{i,l,p}$	$150.7\pm33.1^{i,l,p}$
L. casei 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}	$35.2\pm9.4^{c,e,k}$
	17.0	$43.6\pm10.9^{h,k}$	$73.7\pm22.9^{g,h,i,j}$	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.

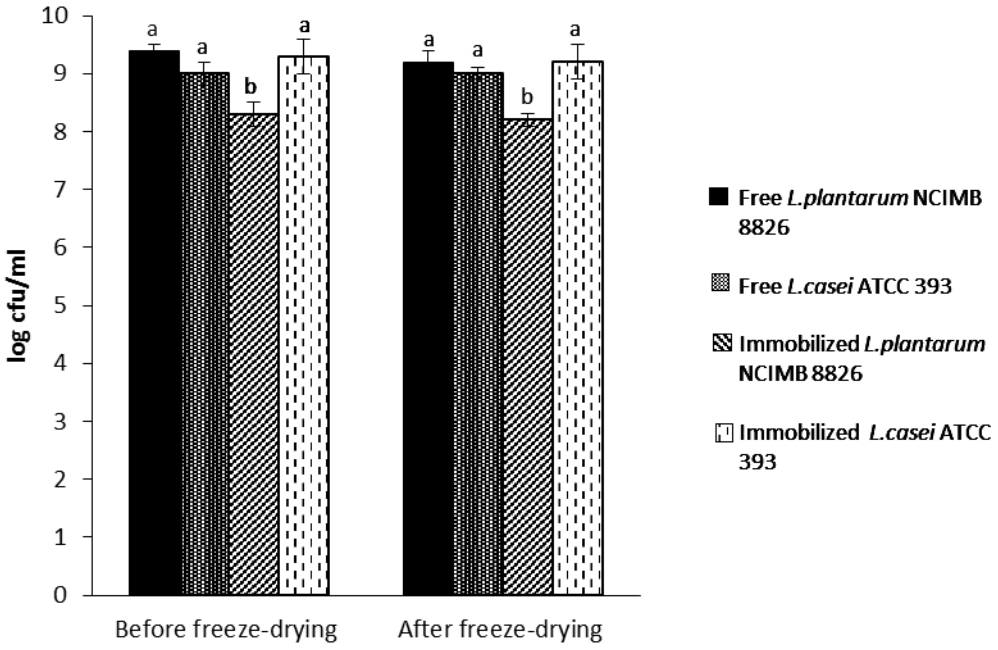
695

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.

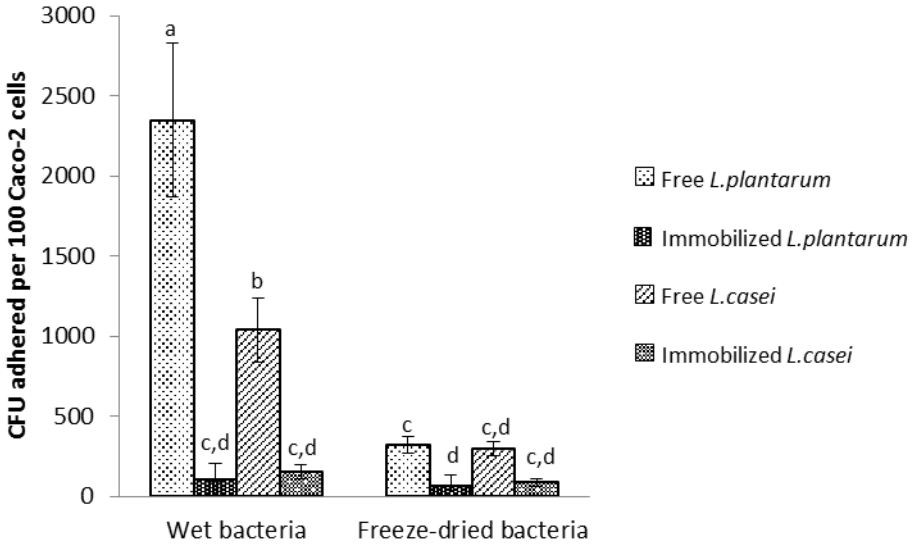
704



705

706 **Fig. 1**

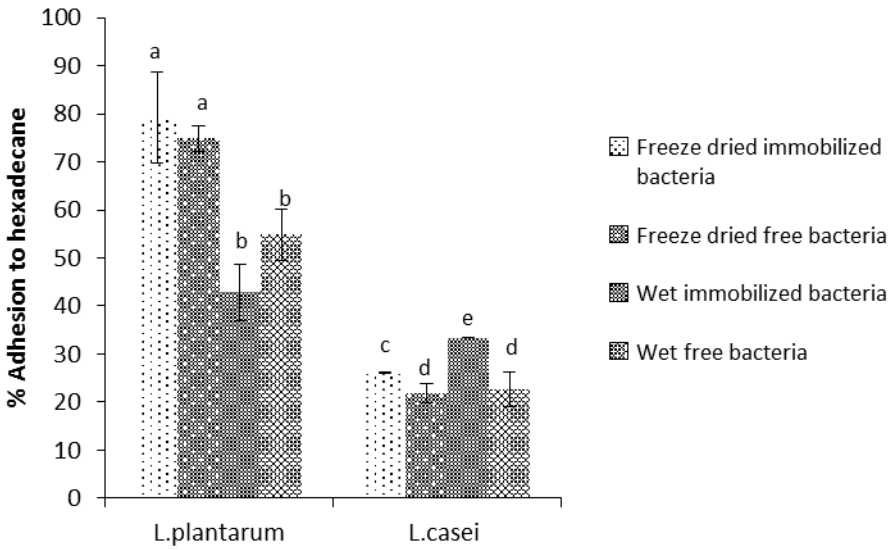
707



708

709 **Fig. 2**

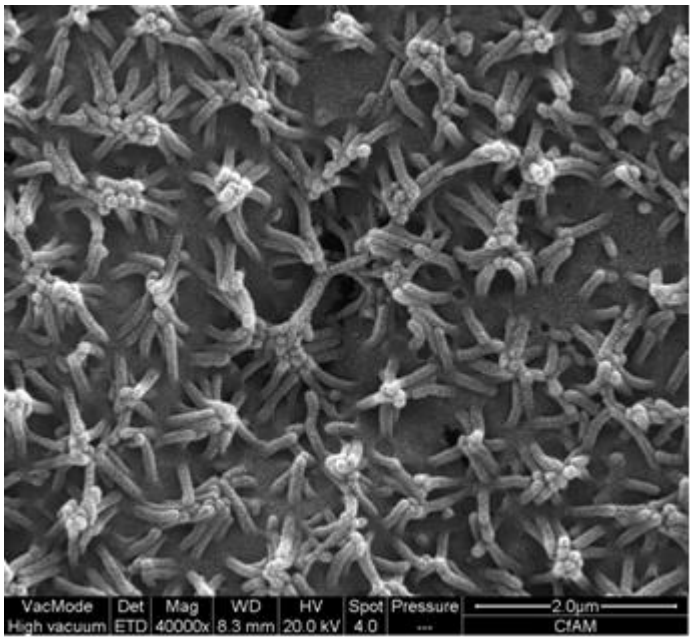
710



711

712 **Fig. 3.**

713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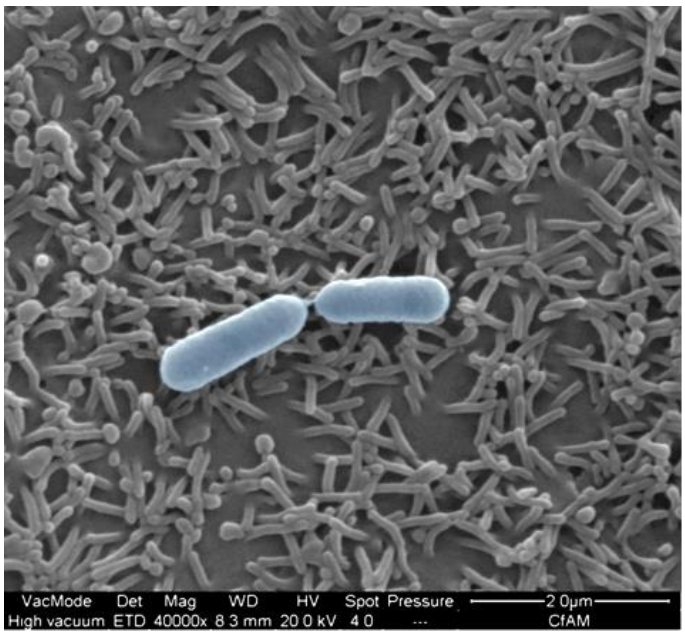
High vacuum ETD 40000x 8.3 mm 20.0 kV 4.0 --- CfAM

Fig. 4a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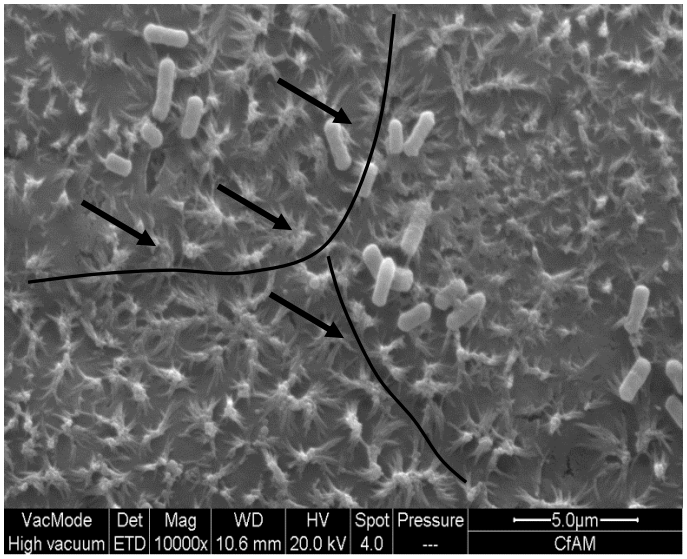
High vacuum ETD 40000x 8.3 mm 20.0 kV 4.0 --- CfAM

Fig. 4b

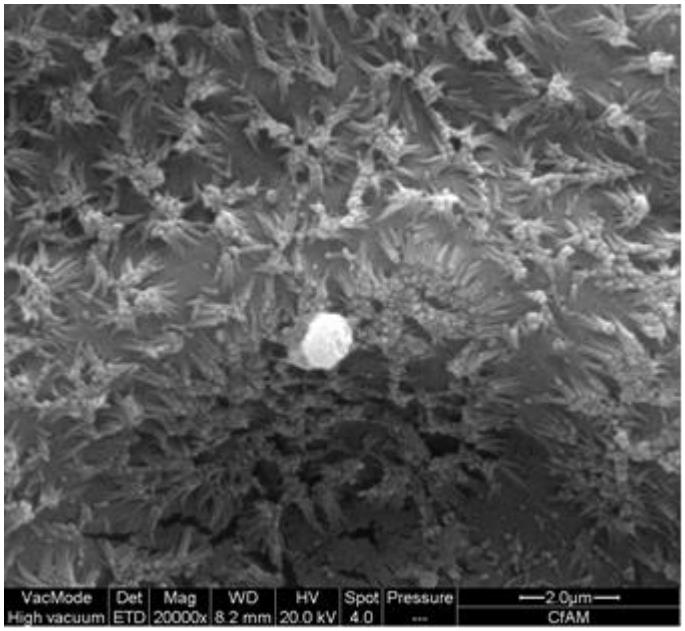


722

723 **Fig. 4c**

724

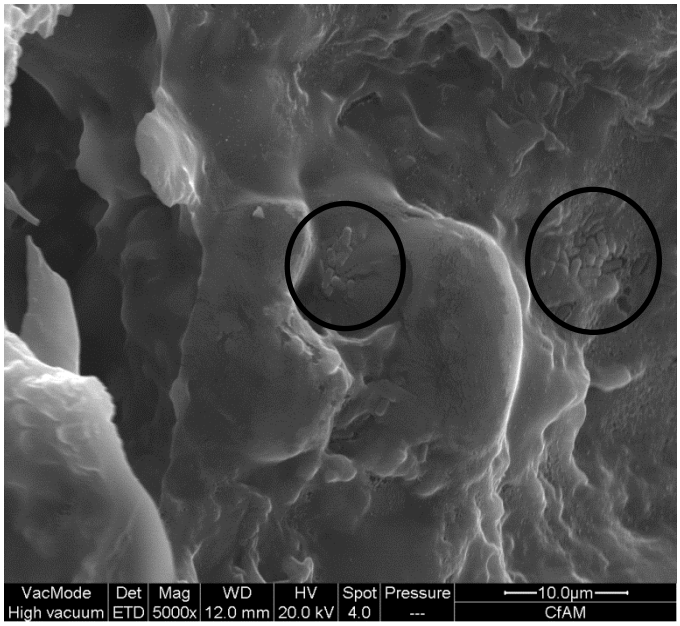
725



726

727 **Fig. 4d**

728

729 

730 **Fig. 4e**

731

732