# Characterization of a fibronectin-binding protein from *Lactobacillus casei* BL23

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Running headline: *fbpA* from *Lactobacillus casei*

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**ABSTRACT**

**Aims:** To characterize the functionality of the *Lactobacillus casei* BL23 *fbpA* gene encoding a putative fibronectin-binding protein

**Methods and Results:** Adhesion tests showed that *L. casei* BL23 binds immobilized and soluble fibronectin in a protease sensitive manner. A mutant with inactivated *fbpA* showed a decrease in binding to immobilized fibronectin and a strong reduction in the surface hydrophobicity as reflected by microbial adhesion to solvents test. However, minor effects were seen on adhesion to the human Caco-2 or HT-29 cell lines. Purified 6X(His)FbpA bound to immobilized fibronectin in a dose-dependent manner. Western blot experiments with FbpA-specific antibodies showed that FbpA could be extracted from the cell surface by LiCl treatment and that protease digestion of the cells reduced the amount of extracted FbpA. Furthermore, surface exposition of FbpA was detected in other *L. casei* strains by LiCl extraction and whole-cell ELISA analysis.

**Conclusions:** FbpA can be found at the *L. casei* BL23 surface and participates in cell attachment to immobilized fibronectin. We showed that FbpA is an important, but not the only, factor contributing to fibronectin binding in BL23 strain.

**Significance and Impact of the Study:** This is the first report showing the involvement of FbpA in fibronectin binding in *L. casei* BL23 and represents a new contribution to the study of attachment factors in probiotic bacteria.

**KEYWORDS:** *Lactobacillus casei*, adhesion, fibronectin, extracellular matrix, probiotics

## INTRODUCTION

Lactobacilli have been used for the fermentation of food products and they have attracted much attention as probiotic bacteria for their beneficial effects on human health. Adhesion of probiotic bacteria to the host intestinal epithelium is an important criterion for strain selection and several methods (binding to cultured epithelial cells, to immobilized tissue components or to resected tissue) have been employed for characterization and screening of new strains (Ouwehand et al. 2001; Styriak et al. 2003; Tuomola and Salminen 1998; Vesterlund et al. 2006). Adhesion is believed not only to play a role in the persistence of a particular strain in the digestive tract but also to participate in pathogen exclusion by competition and blocking of their binding sites at the mucosa (Collado et al. 2007; Lee et al. 2003; Vesterlund et al. 2006). Also, it may contribute to immunomodulation (Galdeano et al. 2007). However, some authors have hypothesized that attachment factors in lactic acid bacteria are risk factors that might be an indicative of their pathogenic potential (Vesterlund et al. 2007).

Lactobacilli can bind to mucin, a component of the mucus epithelial layer and to a variety of proteins present in the extracellular matrix (ECM), such as fibronectin, collagen and laminin, which are shed into the mucus or can be exposed to the intestinal lumen in case of trauma, infection or inflammation (Lorca et al. 2002; Styriak et al. 2003). While in most cases protein factors have been identified as responsible for this attachment, with the exception of mucin-binding proteins from lactobacilli, information about specific binding proteins is still scarce for this group of microorganisms (Vélez et al. 2007).

Fibronectin is a dimeric 454-kDa glycosylated protein which is present in soluble form in plasma and in immobilized form on the host cells surfaces and in the ECM. It is an important target for bacterial attachment in many pathogens, such as *Streptococcus pneumoniae* and *Streptococcus pyogenes*, where fibronectin-binding proteins are important pathogenic factors (Holmes et al. 2001; Jedrzejas 2007; Molinari et al. 1997). There are numerous works describing the attachment of lactic acid bacteria to fibronectin but information about molecules implicated in the mechanism of binding is limited. The surface layer protein (SlpA) from *Lactobacillus brevis* ATCC8287 is involved in fibronectin binding (de Leeuw et al. 2006). Moreover, inspection of lactobacilli genome sequences reveals that they carry genes encoding proteins homologous to fibronectin binding proteins from streptococci. *Lactobacillus casei* is a species widely used in the dairy industry. It is also a normal constituent of the intestinal microbiota in humans and probiotic capacities have been reported for many strains, for which it is included as a probiotic in food products. Some *L. casei* strains survive the passage through the digestive tract in humans and persist in it for several days (Oozeer et al. 2006). In this work we sought to analyze factors involved in interaction with host cells and colonization of intestinal mucosa in *L. casei* BL23. This strain has been widely used for genetic and physiology studies (Acedo-Felix andPerez-Martinez 2003), it showed anti-inflammatory effects in animal inflammatory bowel disease models (Foligne et al. 2007) and its genome sequence is available. We describe the characterization of a gene encoding a fibronectin-binding protein that was detected during the *in silico* analysis of putative adhesion factors encoded in the *L. casei* BL23 genome.

## MATERIALS AND METHODS

### Strains and growth conditions

*Lactobacillus casei* strains are listed in Table 1 and were grown in MRS broth (BD Difco, Le Pont de Claix, France) at 37ºC under static conditions. *Escherichia coli* DH5α was used for gene cloning and *E. coli* M15[pREP4] was used for protein purification. Both strains were grown in LB medium under agitation (200 rpm) at 37ºC. Ampicillin and kanamycin were used for *E. coli* at 100 and 25 μg $ml^{-1}$, respectively. Erythromycin was used for *L. casei* at 5 μg $ml^{-1}$. Solid medium was prepared by adding 1.8% agar. Bacterial growth curves were determined in microtiter plates (200 μl MRS broth per well) at 37ºC in a Polarstar Omega plate reader (BMG Labtech, Offenburg, Germany).

### Construction of an *L. casei* BL23 *fbpA* mutant

A 600-bp internal DNA fragment from the *fbpA* gene (LCABL_16620) was amplified by PCR using oligonucleotides FBP1 (5'-CTTAAGCTTCGCAGCGTTGTTGC) and FBP2 (5'-TGAGGTACCTGGGCAACGGCATTAC), which introduced *Hin*dIII and *Kpn*I restriction sites (underlined), using *L. casei* BL23 genomic DNA and EcoTaq DNA polymerase (Ecogen, Barcelona, Spain). The fragment was digested with *Hind*III and *Kpn*I and cloned into the integrative vector pRV300 (Leloup et al. 1997) treated with the same enzymes. The resulting plasmid, pRVfbp, was transformed by electroporation into BL23 strain by using a Gene-Pulser (Biorad) as previously described (Posno et al. 1991) and transformants were selected in solid media by erythromycin resistance. Integration at the correct locus and *fbpA* disruption was checked by southern blot on *Hin*dIII-digested genomic DNA. The probe was the pRVfbp insert labelled with digoxigenin (DIG) with the

PCR DIG-labeling mix (Roche). Hybridization and detection was performed in Hybond-N membranes (GE Healthcare) by using alkaline phosphatase-conjugated anti-DIG and the CDP-star chemiluminiscent reagent as recommended by the manufacturer (Roche). The insertional mutation was shown to be stable for at least ~40 generations in the absence of antibiotic (screening of 600 colonies after two consecutive overnight cultures gave a 100% of erythromycin resistants). Therefore, to discard interferences resulting from growth with antibiotics, bacteria used for the binding experiments in Figure 2A and for growth curves were grown in the absence of erythromycin.

**Microtitre plate binding assays**

Binding of *L. casei* to immobilized human fibronectin (Sigma) was performed in 96-well Polysorp plates (Nunc) with bacterial cells grown to late exponential phase (OD550 of 3.5-4; $1.2x10^9$ to $1.4x10^9$ cfu $ml^{-1}$). Plates were covered with 50 μg $ml^{-1}$ of fibronectin in carbonate/bicarbonate buffer 50 mmol $l^{-1}$ pH 9.6 at 4ºC overnight. Wells were washed three times with PBS and blocked for 1 h with PBS plus 1% Tween 20. One hundred μl of each strain were added to each well in PBS adjusted to an OD550nm of 1 ($7x10^8$ cfu $ml^{-1}$) and plates were incubated overnight at 4ºC. After removing non-adhered cells by three washes with 200 μl of PBS plus 0.05% Tween 20 (PBST), the plates were dried and adhered cells were detected by staining with crystal violet (1mg $ml^{-1}$ for 45 min). After washing, the colorant was released with citrate buffer 50 mmol $l^{-1}$ pH 4.0 (100 μl per well) and the absorbance at 595nm was determined in a Multiskan Ascent plate reader (Thermo-Labsystems, Helsinki, Finland). The effect of protease treatment was assayed by incubating bacterial cells at an OD550nm of 1 ($7x10^8$ cfu $ml^{-1}$) in PBS with 100 μg $ml^{-1}$ of proteinase K (Roche) at 37ºC for 1

h. After incubation, the protease was inactivated by addition of 1 mmol $l^{-1}$ phenylmethylsulfonyl fluoride followed by three washes with PBS containing 1 mmol $l^{-1}$ phenylmethylsulfonyl fluoride. Bacteria were resuspended in PBS to an OD550nm of 1 ($7x10^8$ cfu $ml^{-1}$) and used for binding assays. Control bacterial cells were treated exactly as digested bacterial cells but without the addition of proteinase K. Inhibition of binding by soluble fibronectin was assessed by adding different quantities of fibronectin (1 to 10 μg per well) to the binding assay described above. Blank wells without bound fibronectin were run as controls in all experiments and their absorbance values were subtracted from the values of wells covered with fibronectin. Experiments were carried out in triplicate three times with bacteria coming from independent cultures.

For whole-cell ELISA analysis, *L. casei* bacterial cells were bound to immunoplates in PBS buffer at an OD550nm of 0.1 overnight at 4ºC. The wells were washed and blocked with 2% BSA in PBS and the content of the wells were reacted with a 1:200 dilution of anti-FbpA mouse serum or preimmune serum followed by a 1:1000 dilution of peroxidase-conjugated anti-mouse IgG. Color was developed with the 1-Step™ Ultra TMB-ELISA substrate (Pierce).

To assay binding to soluble fibronectin, *L. casei* bacterial cells at an OD550nm of 1 ($7x10^8$ cfu $ml^{-1}$) were incubated with 100 to 500 ng of fibronectin in 1 ml of PBST containing 1% BSA for 1 h at 37ºC. After three washes with PBST, bound fibronectin was released by boiling the bacteria in SDS-PAGE buffer and detected by western blotting with a rabbit anti-fibronectin serum (Sigma).

**Adhesion to solvents test**

Microbial adhesion to solvent (MATS) test has been used to asses the surface properties of lactobacilli (Vinderola et al. 2004). This test was performed with *L.*

*casei* essentially as described (Bellon-Fontaine et al. 1996). Five ml of overnight cultures of each strain were washed with PBS and resuspended in PBS to a final OD600nm of 0.4 ($A_0$). This suspension was mixed (1:3) with different solvents (chloroform, ethyl-acetate or hexadecane) and vortexed for 1 min at full speed. After phase separation, absorbance of the aqueous phase was measured at 600nm ($A_1$). The percentage of adhesion was calculated from: $\%Adhesion=100X[1-(A_1/A_0)]$. Each experiment was done in triplicate with bacteria coming from independent cultures.

**Adhesion to Caco-2 and HT-29 cell lines**

Epithelial cells were seeded at $4x10^4$ cells $cm^{-2}$ (Caco-2) or $2x10^5$ cell $cm^{-2}$ (HT-29) in 24-well plates in DMEM medium (with Glutamax, glucose 25 mM; Gibco) supplemented with 1%(v/v) non-essential amino acids solution (Gibco), 1%(v/v) sodium pyruvate solution (Gibco), 1%(v/v) sodium bicarbonate solution (Gibco, only for HT-29 cells), 1% (v/v) of antibiotics (100 U $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin, Gibco) and 10%(v/v) fetal calf serum and incubated at 37ºC in a $CO_2$ incubator. After the cells reached confluence (incubation for 6 and 3 days for Caco-2 and HT-29, respectively), plates were incubated for additional 15 (Caco-2) or 21 (HT-29) days to allow cell differentiation and the medium was changed every two days. Log-phase *L. casei* bacterial cells were added to each well in 0.5 ml of culture medium adjusted to an OD550nm of 0.2 ($10^8$ cfu $ml^{-1}$) and the plates were incubated for 1 h at 37ºC with mild agitation. Non-adhered bacteria were removed by washing 3 times with 1 ml of PBS and the bacteria were detached by covering the monolayer with 200 μl of a 15% (v/v) solution of trypsin-EDTA (Gibco) in PBS. After addition of 300 μl of culture medium, serial dilutions were plated on MRS agar plates and the bacterial colonies were

counted after 48 h of incubation. The experiments were made in triplicate three times with bacteria coming from independent cultures. Adhesion was expressed as percentage of adhered bacteria respect to input bacteria.

**Purification of 6X(His)FbpA and binding assays**

The *L. casei* BL23 *fbpA* gene was amplified with oligonucleotides FBP3 (5'-CGGGGATCCATGTCATTTGACGGAATC) and FBP4 (5'-ACGAAGCTTTTACTTGGTAGGCGGGTTGC) which included restriction sites (underlined) and *Pfx* DNA polymerase (Invitrogene). The amplified fragment was digested with *Bam*HI and *Hin*dIII and cloned into the expression vector pQE30 (Qiagen) digested with the same enzymes. The plasmid construct was transferred to *E. coli* M15[pREP4] and cells of the transformed strain were grown in 500 ml of LB medium at 37ºC until OD550nm reached 0.6. Then, IPTG was added to 1 mmol $l^{-1}$ and induction was carried out for 3 h at 37ºC. Cells were collected by centrifugation, washed and resuspended in 10 ml of Tris-HCl 100 mmol $l^{-1}$ pH7.4, lysozyme 1 mg $ml^{-1}$, phenylmethylsulfonyl fluoride 0.5 mmol $l^{-1}$, dithiothreitol 0.5 mmol $l^{-1}$ and disrupted by sonication. The cellular debris were eliminated by centrifugation at 6000xg for 30 min at 4ºC, the supernatant containing recombinant 6X(His)FbpA was applied to a Ni-NTA column (Qiagen, 1 ml bed volume) and the recombinant protein purified according to the instructions of the manufacturer. Fractions containing 6X(His)FbpA were analyzed by SDS-PAGE and dialyzed overnight at 4ºC in Tris-HCl 50 mmol $l^{-1}$ pH8, EDTA 1 mmol $l^{-1}$, NaCl 500 mmol $l^{-1}$, glycerol 15% and stored at -80ºC until use. Protein concentrations were determined with the BioRad dye-binding assay. To test *in vitro* fibronectin binding of FbpA, different protein amounts of 6X(His)FbpA were added in 100 μl of PBS buffer plus 0.1% BSA to microwell

plates covered with fibronectin or BSA (50 μg $ml^{-1}$ in carbonate/bicarbonate buffer 50 mmol $l^{-1}$ pH9.6, overnight at 4ºC). After 1 h of incubation at 37ºC, unbound protein was removed by washing 3 times with PBST and His-tagged FbpA was detected with the HisProbe™-HRP reagent (Pierce) and 1-Step™ Ultra TMB-ELISA (Pierce) as recommended by the manufacturer.

**Preparation of antiserum to 6X(His)FbpA**

Fifteen micrograms of purified 6X(His)FbpA were intraperitonially administered to 8-weeks old female Balb/c mice (kept at the animal facilities of the University of Valencia) in 50 μl of PBS containing adjuvant. Three doses were applied at two weeks intervals. Ten days after the last administration, mice were bled and the presence of anti-FbpA antibodies in sera was tested by western-blot.

**Isolation of cellular fractions and western blot**

*L. casei* bacterial cells were grown in 50 ml of MRS to late exponential phase (OD550nm of 3.5-4; $1.2x10^9$ to $1.4x10^9$ cfu $ml^{-1}$) and washed two times with PBS. The pellet was resuspended in Tris-HCl 10 mmol $l^{-1}$ pH8, LiCl 1.5 mol $l^{-1}$ and incubated at 4ºC for 1 h. Bacteria were pelleted by centrifugation at 6000xg for 10 min and proteins in the supernatant were precipitated by adding trichloroacetic acid to 10% and incubation at 4ºC for 1 h, followed by centrifugation at 10.000xg 20 min, washing with cold 96% ethanol and resuspension of the pellet in urea 7 mol $l^{-1}$. The cell pellet was disrupted with glass beads (0.1 mm) in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK, USA ) with four cycles of 30 s at maximal speed and unbroken cells were discarded by centrifuging the supernatant three times at 6000xg for 5 min. The supernatant was then centrifuged at 22.000xg, 20 min at 4ºC. The soluble fraction was retained as the cytoplasm fraction, whereas the pellet was washed

three times at 22.000xg for 15 min with Tris-HCl 50 mmol $l^{-1}$ pH8 plus NaCl 0.5 mol $l^{-1}$ and retained as the cell-envelope fraction (cell-wall/membrane fragments). To asses the effect of protease digestion on LiCl extraction of FbpA, before extraction the bacteria were treated with protease as described above. Samples of the different fractions were separated by 10% SDS-PAGE and the gels were electro-transferred to Hybond-ECL membranes (GE Healthcare). FbpA was detected with a mouse anti-FbpA serum (1:5000) and the ECL-advance western blotting detection kit (GE Healthcare).

**Statistical analysis.**

Results are indicated as means ± standard deviation. The significance of the difference of the means in experiments carried out with wild-type *L. casei* and the *fbpA* mutant was calculated by the Student´s t-test with the PRISM 4.0 software (Graph Pad Software, San Diego, CA, USA).

## RESULTS

### Characterization of an *L. casei* BL23 strain mutated in *fbpA*

Inspection of the *L. casei* BL23 genomic sequence (Genbank FM177140) revealed the presence of a gene (LCABL_16620, designated *fbpA* from now), which encoded a protein showing homology to FBP54 (46% identity) or PavA (43% identity) from *Streptococcus pyogenes* and *S. pneumoniae*, respectively, two proteins which have been reported to mediate fibronectin binding (Courtney et al. 1994; Holmes et al. 2001). The product of *fbpA* was a 64-kDa protein which contained the typical pfam05833 and pfam05670 domains (Pfam database) present in a variety of bacterial fibronectin-binding proteins. In order to construct a mutant affected in *fbpA*, an internal fragment of the gene was cloned into the non-replicative plasmid pRV300. Electroporation of this construct (pRVfbp) in BL23 yielded erythromycin-resistant clones in which the plasmid was integrated at the *fbpA* locus leading to a disruption of the gene (Figure 1). One of such integrants was chosen and designated BL308 (*fbpA*::pRV300). Compared to the wild type, the *fbpA* mutant showed a reduced specific growth rate (0.320±0.005 and 0.290±0.01 $h^{-1}$ for BL23 and BL308 strains, respectively; P=0.0217). Both strains were able to bind fibronectin immobilized on immunoplates in a protease-sensitive manner, as treatment of the cells with proteinase K drastically reduced the binding (Figure 2A). Interestingly, the presence of the *fbpA* mutation produced a 50% reduction in binding (P=0.004) to immobilized fibronectin compared to the wild type (Figure 2A). Adding soluble fibronectin to the binding assays resulted in a decrease of bacterial binding in both the wild type and the *fbpA* mutant, suggesting that *L. casei* BL23 was interacting with immobilized as well as soluble fibronectin

(Figure 2B). This latter idea was confirmed by incubating *L. casei* cells with fibronectin. After several washings, fibronectin attached to the cell surface could be released and detected by immunoblotting (Figure 2C). In these assays, no differences between the wild-type and the *fbpA* mutant were observed and protease treatment reduced the binding in both strains (Figure 2C). In conclusion, the attachment ability to immobilized as well as soluble fibronectin probably involved surface proteinaceous substances and FbpA played a role in attachment to the immobilized form.

Two other tests were performed in order to detect changes in cell surface characteristics induced by the *fbpA* mutation. First, we measured adhesion of the strains to cultured intestinal epithelial cells lines. A small but significant increase in adhesion to the HT-29 cell line was observed in the *fbpA* mutant strain with respect to the wild type (% adhesion of 3.03±0.7 and 4.11±1.8 for the wild type and the *fbpA* mutant, respectively; P=0.02), whereas no significant changes were detected in the binding ability to Caco-2 (% adhesion of 0.96±0.6 and 1.3±0.34 for the wild type and the *fbpA* mutant, respectively; P=0.167). As a second approach we used the microbial adhesion to solvent test (MATS) with three different solvents: chloroform (acidic solvent and electron acceptor); ethyl-acetate (basic solvent and electron donor) and hexadecane (hydrophobic solvent). The results showed that the *fbpA* mutation did not induce changes in the acid-base characteristics of the cell surface; however, *fbpA*-disrupted cells showed a clear diminishing in their hydrophobicity, as reflected by a 70% decrease in the affinity for hexadecane (Figure 3).

### ***L. casei*** **FbpA binds to fibronectin**

The *fbpA* gene was cloned in *E. coli* and FbpA was purified after expression as a His-tagged protein. When the purified protein was tested for binding to fibronectin immobilized on immunoplates, it was shown that 6X(His)FbpA bound to fibronectin in a dose-dependent and saturable manner. A low binding was observed when the immunoplates were covered with the control protein BSA (Figure 4A). In inhibition experiments where soluble fibronectin was added to the binding reaction, a low inhibition (around 20%) in FbpA binding was only found at the highest fibronectin concentration (Figure 4B). These results were in agreement with the previous characterization of the *L. casei fbpA* mutant and those reported for the *S. pneumoniae* protein (Holmes et al. 2001), which showed that FbpA preferentially binds to immobilized fibronectin.

**Cellular location of FbpA**

To address the question whether FbpA was present at the *L. casei* cell surface several *L. casei* fractions were tested by western blot against an anti-FbpA serum. Results showed that a 64-kDa band, the molecular weight of FbpA, was detected in all cellular fractions (surface proteins extracted with LiCl, cell-envelope proteins and cytoplasmic proteins, Figure 5A). Additional unspecific bands were also shown to react with the antiserum. These bands were not detected in the LiCl fraction, indicating the absence of cross-contamination. The 64-kDa band disappeared in the *fbpA*-disrupted mutant (Figure 5B), thus confirming its identity as FbpA. Similar amounts of extracted proteins were loaded onto each lane, which led us to the conclusion that most of FbpA was present intracellularly. Treatment of the cells with protease reduced the amount of FbpA extracted by LiCl treatment, reinforcing the idea that part of FbpA is

surface-exposed and accessible to hydrolytic enzymes (Figure 5C). However, similar to the rest of homologue proteins, no signal peptide responsible for protein secretion was identifiable in the FbpA sequence.

**FbpA in other *L. casei* strains**

We screened a collection of *L. casei* strains from different origins (food and human isolates, including probiotic strains, Table 1) for the presence of FbpA. As expected from the presence of a gene homologous to *fbpA* in its genome (LSEI_1439), the BL90 (ATCC334) strain showed a reacting protein band similar to BL23 (Figure 5). FbpA homologue proteins were also extracted at different levels by LiCl treatment in the rest of *L. casei* strains and were also present in the corresponding surface fractions (Figure 6). The cross-reacting bands varied in size, indicating that FbpA from different *L. casei* strains are not totally identical. Furthermore, southern blot hybridization with an *fbpA* probe showed that a single copy of *fbpA* was present in all strains (data not shown). Whole-cell ELISA analysis, in which the bacterial cells were bound to microtitre plates and probed with the anti-FbpA serum, led also to the detection of FbpA (Figure 7). These results concluded that the presence of FbpA on the cell surface is a common feature in *L. casei*.

## DISCUSSION

In this work we tried to get some insight into the mechanisms which mediate interactions of lactobacilli to host cells. To this end, we have characterized FbpA from *L. casei*, a protein homologous to fibronectin-binding proteins described in other bacteria. Genome search at the NCBI database revealed that all sequenced lactobacilli genomes encode FbpA homologues with amino acid identities ranging from 41 to 60% compared to *L. casei* FbpA. In the search for host adhesion factors in *L. acidophilus* NCFB, Buck et al. (Buck et al. 2005) constructed a mutant in *fbpA* which displayed a strong reduction in Caco-2 cells attachment. These experiments established that *L. acidophilus* FbpA participates in adhesion to epithelial cells. Nevertheless, no assays on fibronectin binding were carried out in this study. Contrarily to what was expected, our results showed that, compared to the wild type, *L. casei fbpA* strain adhered slightly better to HT-29 cells, whereas no significant changes were detected on Caco-2 adherence. This striking result suggests that changes in the bacterial surface resulting from an *fbpA* mutation (as evidenced by decreased surface hydrophobicity) lead to a slightly improved capacity to attach to HT-29 cell surfaces. However, the reason for this observation is not known. *L. casei* probably utilizes other FbpA-independent mechanisms for attachment to the HT-29 and Caco-2 cell lines or the contribution of FbpA to binding in these models is low. It has been reported that FBP54, an FbpA homologue from *S. pyogenes*, had minor effects on binding to some types of epithelial cells, whereas binding to others was strongly influenced by this adhesin (Courtney et al. 1996).

Similar to *fbpA*-mutated streptococci, where reduction of bacterial fibronectin binding ranged from 50 to 25% (Christie et al. 2002; Miller-Torbert et al. 2008), disruption of *L. casei fbpA* led to only a 50% reduction in binding. This strengthens the idea that adhesion is a multifactor process and suggests the presence of additional not yet identified fibronectin-binding molecules, presumably of proteinaceous nature. In any case, inspection of the BL23 genome does not reveal the presence of genes encoding other types of fibronectin-binding proteins.

Previous studies showed that some lactobacilli were able to bind to the immobilized but not to the soluble form of fibronectin (Lorca et al. 2002). We showed that *L. casei* BL23 can bind both forms of fibronectin but FbpA is binding more efficiently to only the immobilized form. The FbpA proteins belong to an atypical group of fibronectin-binding proteins which lack the repetitive, secretion and cell-wall anchoring (LPXTG motif) sequences present in other characterized fibronectin-binding proteins (Jedrzejas 2007). Lack of conventional signal for secretion and anchoring is a common feature of numerous proteins which decorate the bacterial surface. In lactobacilli, many of the characterized attachment factors are surface “moonlighting” proteins that are implicated in other processes. These include the elongation factor Tu (EF-Tu) (Granato et al. 2004), the heat shock protein GroEL (Bergonzelli et al. 2006) and glycolytic enzymes (Hurmalainen et al. 2007; Kinoshita et al. 2008; Ramiah et al. 2008). How these proteins are transported and localized at the cell surface is still unknown.

Although *L. casei* FbpA can be found at the cell surface, the vast majority of the protein was of intracellular location. Similar results were found for the

homologue protein Fbp68 from *Clostridium difficile* (Hennequin et al. 2003). Despite of the fact that all FbpA-homologues characterized to date have a surface location, posses *in vitro* binding ability to fibronectin and are important virulence factors in pathogens (Dramsi et al. 2004; Holmes et al. 2001), some controversy exists about the exact role of FbpA. In *S. gordonii*, *fbpA* is clustered with a gene (*cshA*) encoding a distinct fibronectin-binding protein whose expression is down-regulated upon *fbpA* mutation, for which it was postulated that FbpA might play a role in the transcriptional regulation of adhesion factors (Christie et al. 2002). Likewise, a mutation in *fbpA* of *Listeria monocytogenes* reduces the amount of two virulence factors (listeriolysin O and InlB) acting at the post-transcriptional level and FbpA co-precipitates with them, therefore it was postulated that it might function as a chaperone or an escort protein for these factors (Dramsi et al. 2004). The genome context of *fbpA* in *L. casei* BL23 does not allow prediction of putative functions for FbpA. The *fbpA* gene is monocistronic and no adhesion-related genes can be found adjacent to it.

The pleiotropic effects of *fbpA* mutations largely differ between species. *S. pneumoniae pavA* (*fbpA*) mutants bound less to fibronectin and were attenuated in virulence, however, they showed no changes in cell surface physicochemical properties or in the expression of virulence factors (Holmes et al. 2001). A different situation was found in *S. gordonii* (Christie et al. 2002) and in *L. casei* BL23 *fbpA* mutants, where a clear decrease in the cell surface hydrophobicity was observed. In *S. gordonii* this decrease was related to the lower expression of CshA (Christie et al. 2002). Further research is needed to disclose the changes produced by an *fbpA* mutation on the cell surface of *L. casei* BL23. Whether FbpA directly interacts with fibronectin *in vivo* or it modulates the

expression and functionality of other interacting proteins, or both, is still unknown.

It has been reported that the adhesive capacity of lactobacilli to ECM proteins is not exclusively found in probiotics or human isolates (Vesterlund et al. 2007). Therefore, no link between attachment and probiotic character appears to exist. FbpA orthologues are encoded in the genomes of all sequenced lactobacilli and the protein can be extracted from the cell surface of all *L. casei* strains tested in this work, from both human and food origin. While the exact role of FbpA in either pathogen, commensal or probiotic bacteria is not yet understood, the study of this protein in lactobacilli may lead to a better understanding of the relations established between these bacteria and the intestinal epithelium.

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**Table 1. *L. casei* strains used in this study**

| strain | origin/reference | selected characteristics |
|---|---|---|
| BL23 | CECT* 5275 | laboratory strain; sequenced genome (Acedo-Félix and Pérez-Martínez 2003) |
| BL32 | CECT4040 | cheese isolate |
| BL82 | ATCC† 25598 | sour milk isolate |
| BL83 | CECT4043 | cheese isolate |
| BL87 | ATCC11578 | oral cavity isolate |
| BL90 | ATCC334 | cheese isolate; sequenced genome |
| BL91 | ATCC4545 | dental caries isolate |
| BL101 | laboratory stock | isolated from commercial probiotic drink |
| BL106 | laboratory stock | isolated from commercial probiotic drink |
| BL193 | laboratory stock | isolated from commercial probiotic drink |
| BL199 | CRL‡ 87 | exopolysaccharide producer (Mozzi et al. 1996) |
| BL208 | laboratory stock | human intestinal isolate |
| BL212 | CRL686 | dry cured sausage isolate (Fadda et al. 1998) |
| BL227 | laboratory stock | commercial probiotic |
| BL229 | laboratory stock | commercial probiotic |
| BL308 | BL23 *fbpA*::pRV300, $ery^r$ | this work |

*Colección Española de Cultivos Tipo; †American Type Culture Collection; ‡Centro de Referencia para Lactobacilos; $ery^r$, erythromycin resistant.

## FIGURE LEGENDS

### Figure 1

Construction of an *L. casei* BL23 mutant in *fbpA*. (A) Wild-type strain was transformed with the non-replicative pRVfbp plasmid and erythromycin-resistant colonies were selected. (B) Integration at the correct locus was verified by southern blot on *Hin*dIII-digested genomic DNA from wild-type strain (BL23) and the disrupted mutant (BL308).

### Figure 2

Binding of *L. casei* to fibronectin. (A) Effect of protease treatment and *fbpA* disruption on *L. casei* binding to immobilized fibronectin; (B) Inhibition of *L. casei* binding to immobilized fibronectin by soluble added fibronectin, (C) Binding of *L. casei* to soluble fibronectin. Cells were incubated with fibronectin, washed and bound fibronectin was detected by western-blot. In parallel experiments, cells were treated with protease prior to fibronectin binding assays. BL23 is wild-type *L. casei*; BL308 is the *fbpA* mutant strain. The bars represent standard deviations.

### Figure 3

Cell-surface characteristics of *L. casei* wild type (BL23) and the *fbpA* mutant (BL308) measured by the MATS test. The bars represent standard deviations.

### Figure 4

Binding of purified FbpA. (A) Binding of 6X(His)FbpA to fibronectin immobilized on immunoplates. After washing, the bound protein was detected with a His-tag detection reagent; (B) Inhibitory effect of soluble fibronectin on 6X(His)FbpA binding. The bars represent standard deviations.

### Figure 5

Detection of FbpA at the cell surface. (A) Western blot detection of FbpA in different cellular fractions of *L. casei* wild type (BL23). One μg of extracted proteins was loaded onto each lane. LiCl, surface proteins extracted with LiCl; E, surface proteins from the cell-envelope (cell-wall/membrane fraction); C, cytoplasmic proteins; (B) Detection of FbpA in cell extracts (1 μg total protein per lane) of *L. casei* BL23 and the *fbpA* mutant (BL308); (C) The effect of protease digestion on the LiCl extraction of FbpA from *L. casei* BL23.

**Figure 6**

Detection of FbpA in different *L. casei* strains. Equal amount of LiCl-extracted proteins (equivalent to the protein extracted from 10 absorbance units (600nm) of cells) (A) and one μg of cell-envelope proteins (B) were resolved by 10% SDS-PAGE and tested by western-blot with an anti-FbpA serum.

**Figure 7**

Whole-cell ELISA analysis to detect the presence of FbpA on the surface of *L. casei* strains. Bacterial cells were bound to the surface of immunoplates and an ELISA test was carried out with an anti-FbpA serum. Data presented are subtracted from the absorbance values of control wells incubated with a pre-immune serum. The bars represent standard deviations

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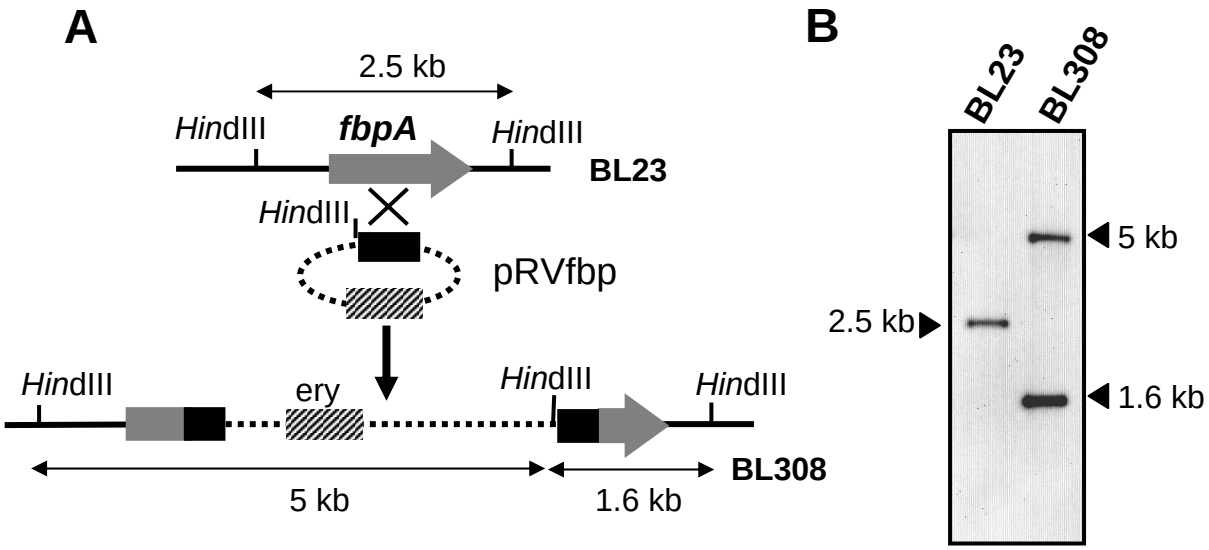
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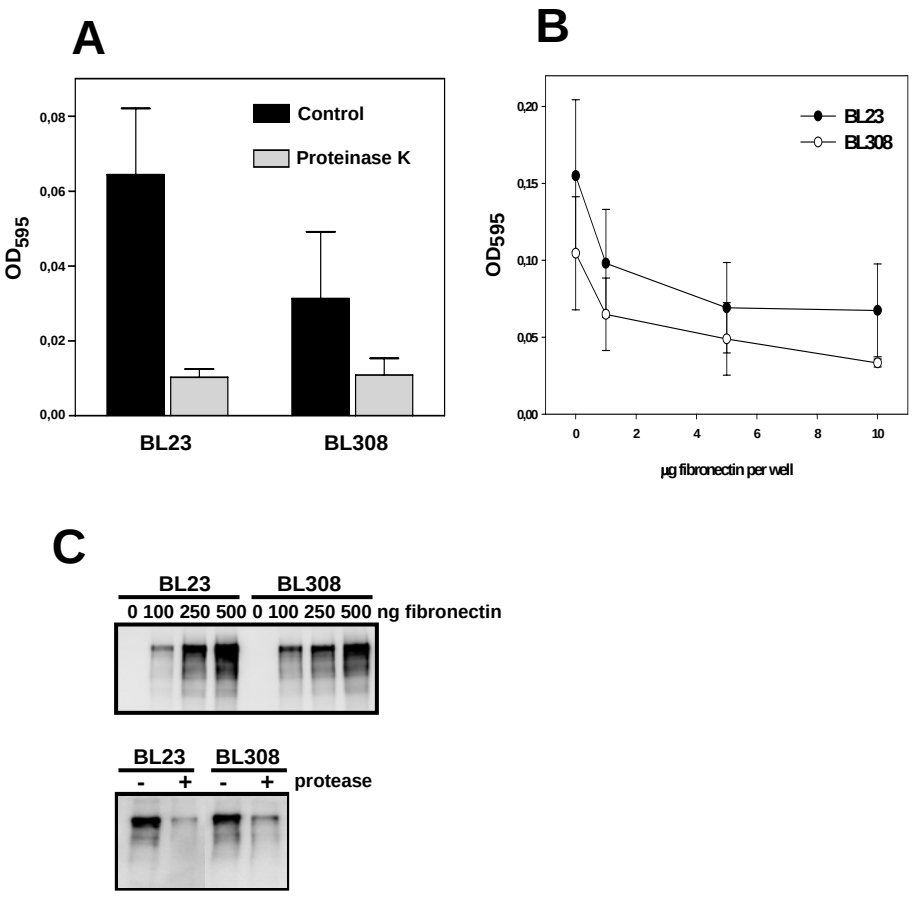
Figure 1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0,10
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2
4
6
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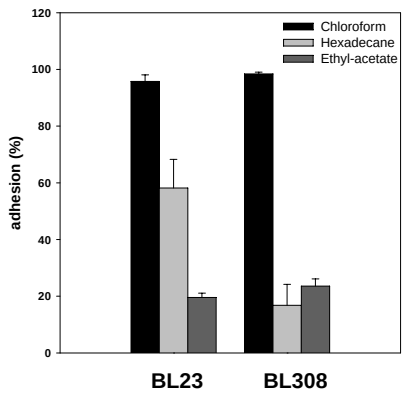
Figure 2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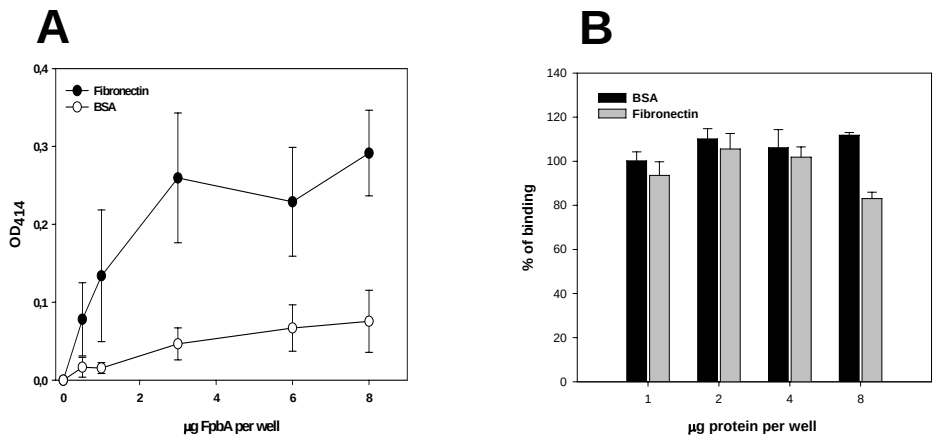
Ethyl-acetate
BL23
BL308

Figure 3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µg FpbA per well
% of binding
µg protein per well

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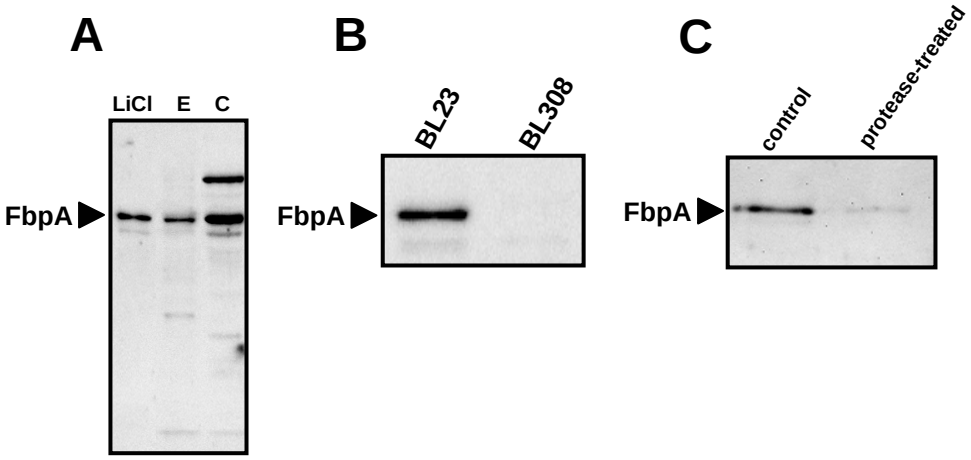
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Figure 5

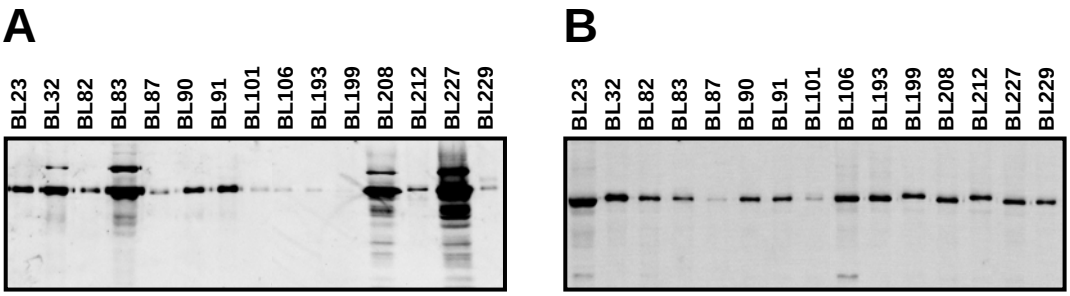
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Figure 6

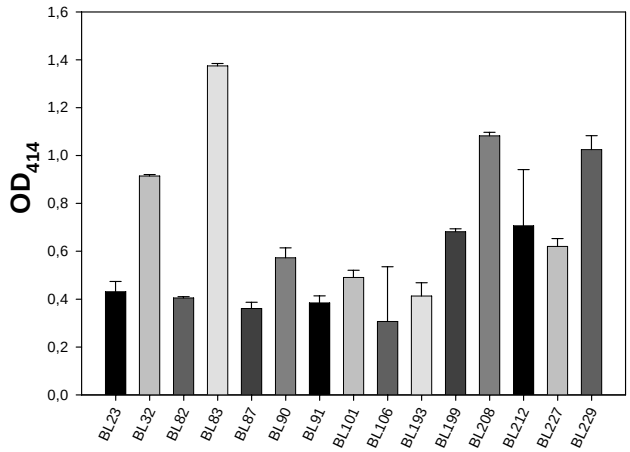
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Figure 7