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An integrated proteomic and physiological approach to understand the adhesion mechanism of the probiotic *Lactobacillus reuteri* Lb2 BM DSM 16143

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ABSTRACT

The adhesion ability of the probiotic *Lactobacillus reuteri* Lb2 BM DSM 16143 was tested to both enterocyte-like Caco-2 cells and to extracellular matrix proteins (laminin, fibronectin and collagen I and IV). The adhesiveness was lost after an alkaline treatment known to release moonlighting proteins from lactobacillar cell surface. To characterize the putative adhesive molecules, a 2-DE experiment in the p*I* range 4-7 was performed on the extracellular proteins. The expression of several moonlighting proteins involved in adhesion (*i.e.* GAPDH, EF-Tu, phosphoglycerate kinase) was demonstrated. Some of the identified adhesins were able to bind plasminogen (Plg), but did not convert it into plasmin (Plm), in absence of exogenous activators. This indicates that the moonlighting proteome of *L. reuteri* Lb2 BM DSM 16143 can contribute to adhesion processes.

Keywords: extracellular matrix protein; bacterial adhesion; moonlighting proteins; 2DE; plasminogen binding.

1. Introduction

Lactobacillus reuteri was demonstrated to possess several positive features supporting its employment as a probiotic. First it produces several antimicrobial compounds, useful to treat or prevent infectious diseases, such as: i) reuterin, biosynthesized during anaerobic catabolism of glycerol and active against a broad range of pathogenic microorganisms [1,2] ii) reutericin, a bacteriocin of 2,7 kDa displaying lytic activity [3] iii) reutericyclin, a highly hydrophobic tetramic acid derivative, with a molecular mass of 349 Da exhibiting a broad inhibitory spectrum [4]. Additional antimicrobial features have recently been described in this species like the ability to neutralize toxins produced by fungi [5], to modulate expression of toxins in different bacterial species [6] and to co-aggregate with toxinogenic *Staphylococcus aureus* [7]. Furthermore *L. reuteri* displays anti-inflammatory properties linked to a factor secreted by the biofilm-forming cells [8]. All these features highlight a strong attitude of this bacterial species not only to counteract infections but also to attenuate their severity.

More recently the use of a *Lactobacillus reuteri* Lb2 BM DSM 16143, the strain investigated in this study, has been suggested as nutraceutical supplement, for oxidative stress protection in the human host [9,10].

Besides the most common pre-requisites to define a strain

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as a probiotic (pH, gastric enzymes and bile salt resistance) additional important features are adhesion and metabolic/ biochemical safety (GRAS status).

Adhesion of probiotics to the mucosal surface is critical for exerting beneficial effects to the host organism [11]. It is first driven by weak forces, like Coulomb and Van der Waals attractions, and mediated by several bacterial binding-effectors such as polysaccharides [12], teichoic and lipoteichoic acids [13] followed by the specific production of proteins named adhesins [14]. Several studies have demonstrated that in some *Lactobacillus* species, glycolytic enzymes (such as GAPDH, phosphoglycerate kinase and mutase), protein folding and stress responses-involved proteins (GroEL and DnaK), as well as transcription and translation proteins (elongation factor Tu, Ts and trigger factor) can exert an adhesive function when they are secreted and surface-exposed [15,16,17]. Some of these adhesins also play a role in plasminogen (Plg) binding. In pathogenic bacteria (*Staphylococcus aureus* and *Streptococcus pneumoniae*) this constitutes a problem since they also biosynthesize enzymes (staphylokinases and streptokinases) able to convert Plg to plasmin (Plm), its proteolytic active form, that can damage tissues opening the way for blood colonization and body invasion. To date, although Plg binding capability has also been described for probiotic bacteria [18], their intrinsic potential (kinases) for Plg activation to Plm has never been demonstrated.

The adhesins cited above are defined "moonlighting proteins", *i.e.* proteins displaying different functions according to their subcellular localization [19]. They lack any anchoring motif or surface retention domain [20] and can be easily released from bacteria, either due to the normal cell-wall turnover [21] or to disturbances in cell wall permeability resulting from pH-stress [18] or exposure to host antimicrobial peptides [22]. In LAB no classical signal peptide responsible for moonlighting protein export has been identified so far. Nevertheless, it has been demonstrated in *Bacillus subtilis* that these proteins contain one additional alpha-helix responsible for their secretion [23].

A proteomic approach applied on the cell wall and extracellular proteomes can confirm the external location of such proteins. This information could not be obtained by a classical genome-transcriptome-based characterization since these adhesins, belonging to the main metabolic pathways, are constitutively expressed.

In the present study the adhesive ability of *L. reuteri* Lb2 BM DSM 16143 has been tested both on Caco-2 cells and extracellular matrix (ECM) proteins. These experiments were combined in a wide integrated approach, with classic 2DE proteomic experiments on the extracellular proteins in order to verify the presence of moonlighting proteins involved in adhesion. In parallel, other experiments were performed to evaluate if the detachment of such proteins from the cell wall was pH-dependent and if it could result in a decrease in the adhesion ability of the strain. Finally to ensure safety we investigated the intrinsic ability of the strain to activate proteolytic cascades.

2. Material and Methods

2.1 *Bacterial strain and culture conditions*

Lactobacillus reuteri Lb2 BM DSM 16143 was isolated from a human faecal sample and it belongs to the collection of BioMan life science S.r.l. It was maintained in a modified MRS (de Man, Rogosa and Sharpe) medium (10 g/L Tryptone enzymatic digest from casein, 8 g/L Peptone from soybean, 10 g/L Yeast extract, 10 g/L Sucrose, 1 ml/L Tween80, 2 g/L Potassium phosphate dibasic, 5 g/L Sodium acetate, 2 g/L Ammonium citrate tribasic anhydrous, 0.2 g/L Magnesium sulfate, 0.05 g/L Manganese sulfate) at - 24°C in 0.5 mL aliquots with 0.5 mL of 40% glycerol.

The cultures were grown in closed screw cap bottles, at 37 ° C, without shaking. The pH of the medium was adjusted at 6.4 before the inoculum. The bacterial growth was monitored by 600 nm optical density (OD_{600}) measurement.

For all the cultures three biological replicates were performed.

2.2 *Adhesion assay on Caco-2 cells*

The adhesion ability of *Lactobacillus reuteri* Lb2 BM DSM 16143 to human intestinal cells was evaluated using enterocyte-like Caco-2 cells (ATCC HTB37). The cells were grown in six-well plates in a monolayer with DMEM (Dulbecco's modified Eagle's medium, Gibco TM life technologies) and incubated for 15 days at 37°C, in a 10% CO_2 atmosphere. The culture medium was replaced every 48 hours and the monolayers of Caco-2 cells were used at post-confluence when they were fully differentiated. Intestinal cells were used between passages 25 and 35. Before starting the adhesion trial, Caco-2 cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and then a bacterial pellet, harvested during the exponential growth phase and resuspended in 1 mL of PBS was added onto Caco-2 cells. The cells were incubated for 1 h at 37°C in 10% CO_2. Then the monolayers were washed five times with sterile PBS to remove all the non-adhered bacterial cells. The washed monolayers were treated with 1 mL of 0.05% Triton X-100 PBS solution for 10 min to detach the Caco-2 cells with the adhered bacteria from the plate. The recovered solution was immediately centrifuged (4000 *x g*, 15 min, 4°C) to pellet cells and bacteria in order to avoid potential bacterial lysis due to Triton X-100 action. The number of colony forming units/mL (CFU/mL) was determined by plating serial 10-fold dilutions onto MRS agar before and after adhesion and incubated for 48 h at 37°C. The adhesion capacity was described as the percentage of bacteria adhered to Caco-2 cells relative to the total number of bacteria added. For all the cultures three technical replicates and two biological replicates were performed. The same experiment was performed on the positive control *Lactobacillus rhamnosus* GG which is known for its adhesive proper-

ties [24].

2.3 *Adhesion assay on extracellular matrix proteins*

Adhesion ability of *L. reuteri* LB2 BM DSM 16143 to extracellular matrix proteins and pH-dependent variations were tested at pH 4 and pH 8. After growth in modified MRS broth bacteria were collected (4000 *x g*, 10 min), washed twice with either 50 mM Tris-HCl buffer at pH 4 or pH 8 and finally resuspended at a concentration of 1 x 10^9 bacteria/mL in the same Tris-HCl buffer. *Lactobacillus* adherence to surface coated laminin (Sigma), fibronectin (Collaborative Biomedical Products), collagen types I and IV (Sigma) were performed as already described using 2.5 pmol surface concentration [25,26]. Bovine Serum Albumin (BSA) (Sigma), a control protein, was coated on glass surface from a solution of 25 μg/mL. The bacteria were incubated with coated target proteins on diagnostic slides for 2 h at room temperature. After incubation the slides were washed with 50 mM Tris-HCl buffer either at pH 4 or pH 8. The adherent bacteria were stained with methylene blue and slides were analyzed with light microscopy using NIH image software (Research Services Branch, National Institute of Health) [27]. The number of bacteria in 20 microscopic fields of 1.6 x 10^4 μm^2 were counted.

2.4 *SDS-PAGE of surface associated proteins*

L. reuteri Lb2 BM DSM 16143 was grown in modified MRS medium until the exponential growth phase. Bacteria were centrifuged at 4000 *x g* for 20 minutes and supernatant was removed. Pellets were resuspended in 200 mL of 50 mM Tris -HCl pH 8, and incubated one hour at 37°C in shaking mode (GallenKamp Orbital Incubator). After a centrifugation at 4000 *x g* for 20 minutes (4°C, Thermo Scientific SL 16R), supernatants were filtered with Stericup filters (Millipore) and Trichloroacetic Acid 16% w/v was added to promote proteins precipitation under shaking over night at 4°C. The obtained suspensions were then ultracentrifuged (35000 *x g*, 90 min, 4°C). Pellets were dried, pulverized and resuspended in 50 mM Tris-HCl pH 7.3. 1 mL aliquots of the obtained sample were subjected to phenol extraction as described before [28]. Briefly, 1 mL phenol was added and the mixtures were incubated for 10 min at 70°C and for 5 min at 0°C and centrifuged (7000 *x g*, 10 min, room temperature). The upper phase was discarded and 1 mL of MilliQ water was added to the lower phase, which was then incubated for 10 min at 70° C and for 5 min at 0°C and centrifuged (7000 *x g*, 10 min, room temperature) again. The upper phase was discarded and 1 mL of ice cold acetone was added to the lower phase before incubating over night at -20°C. Precipitated proteins were recovered by centrifuging (15000 *x g*, 20 min, 4°C) and washed with ice cold acetone (15000 *x g*, 20 min, 4°C). Pellets were pulverized, resuspended in Laemmli loading dye [29] and loaded on a 9.5% resolving gel and run at 120V for 1 hour.

2.5 *Protein identification of SDS-PAGE bands*

Bands were excised from the dried gels and rehydrated with MilliQ water. They were washed twice with 50% v/v ACN in a 25 mM NH_4CO_3 and once in 100% v/v ACN and vacuum-dried. The proteins were in-gel digested with sequencing-grade, modified porcine trypsin (Promega, Madison, WI, USA) and added to a MALDI target plate as described by Hewitson *et al.* [30]. Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of m/z 800–4000 and monoisotopic masses were obtained from centroid of raw, unsmoothed data. Finally, the mass spectra were internally calibrated using the tryptic autoproteolysis products at m/z 842.509 and 2211.104. CID-MS/MS was performed on the 20 strongest peaks with an S/N greater than 40. A source 1 collision energy of 1 kV was used for CID-MS/MS, with air as the collision gas. The precursor mass window was set to a relative resolution of 50, and the metastable suppressor was enabled. Default calibration was used for the MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points across a peak and a polynomial order of 4); the peak detection used a minimum S/N of 5, a local noise window of 50 m/z, and a minimum peak width of 2.9 bins. S/N 20 and 30 filters were used to generate peak lists from the MS and MS/MS spectra, respectively. The mass spectral data from the protein spots were submitted to a database search using a locally running copy of the MASCOT programme (Matrix Science, version 2.1). Batch-acquired MS/MS data were submitted to an MS/MS ion search through the Applied Biosystem GPS explorer software interface (version 3.6) with MASCOT. The search parameters allowed a maximum of one missed cleavage, the carbamidomethylation of cysteine, the possible oxidation of methionine, peptide tolerance of 100 ppm and an MS/MS tolerance of 0.1 Da. The spectra were searched against a recent version of the NCBI non-redundant protein database. The significance threshold was set at $p<0.05$, and identification required that each protein contain at least one peptide with an expect e-value <0.05.

2.6 *Preparation of extracellular protein extracts*

L. reuteri Lb2 BM DSM 16143 was grown in modified MRS medium in biological triplicate. The biomass of middle exponential phase was separated by centrifugation (4000 *x g*, 20 min, 4°C) and culture supernatants were filtered in stericup 0.22 μm filters (Millipore). The supernatant was treated as already described in the paragraph "SDS-PAGE of surface associated proteins". The obtained pellets were pulverized, resuspended in rehydration solution (6.5 M urea, 2.2 M thiourea, 4% w/v CHAPS, 5 mM Tris-HCl, pH 8.8, 0.5% IPG buffer (GE-Healthcare), 100 mM DTT) and stored at -20°C. Protein concentration was determined by Bradford assay (Bio Rad).

2.7 *2-DE*

Isoelectrofocusing (IEF) was performed as previously described [31]. Two hundred and seventy-five µg of proteins were separated in 13 cm IPG strips (GE Healthcare) with a linear gradient ranging from 4 to 7 using IPGphor (GE Healthcare) at 20°C, with 83000 Vh, after 10 h rehydration. After IEF, the strips were incubated at room temperature in 6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 8.6, enriched at first with 2% w/v DTT for 15 min and afterwards with 4.5% w/v iodoacetamide for 15 min. They were then sealed at the top of the 1.0 mm vertical second dimension gels. For each sample, SDS-PAGE was carried out on 11.5% T and 3.3% C acrylamide (Biorad Acrylamide) homogeneous gels. The running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS. The running conditions were 11°C, 600V constant voltage, 20 mA/gel, 60W for 15 min and 11° C, 600 V constant voltage, 40 mA/gel, 80W for about 2.5 h. The molecular weight markers were from the Low Mr Electrophoresis Calibration Kit (GE-Healthcare). The gels were automatically stained using Processor Plus (Amersham Biosciences) with freshly prepared Neuhoff stain (Colloidal Coomassie Blue) [32] and, after image acquisition, they were dried in a GD 2000 Vacuum Gel Drier System (GE Healthcare).

2.8 *Image analysis and statistical analysis*

2-DE gels were digitized with Personal Densitometer SI (Amersham Biosciences). Image analysis and spot detection were performed with Progenesis PG200 software (Non Linear Dynamics). Spot detection was automatically performed by using the algorithm named "2005 detection". After the establishment of some user seeds, matching was automatically performed and manually verified. Two analytical replicates of 2-DE maps of extracellular proteins obtained from each of the three biological replicates were performed. Only spots present in at least five out of six replicates were identified.

2.9 *Protein identification of 2DE gels*

Enzymatic digestion was carried out with 200 ng of trypsin in 50 µL of 10 mM NH_4CO_3 buffer, pH 7.8. Gel pieces were incubated at 37°C overnight. Peptides were then extracted by washing the gel particles with 10 mM NH_4CO_3 and 1% formic acid in 50% ACN at room temperature. The resulting peptide mixtures were filtrated using 0.22 PVDF filter from Millipore. The peptide mixtures were analysed by nanoLC-chip MS/MS**,** using a CHIP MS 6520 QTOF equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture (8 µL in 0.1% formic acid) was first concentrated and washed at 4 µL/min in 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid as eluent. The sample was then fractionated on a C18 reverse-phase capillary column (75 µm x 43 mm in the Agilent Technologies chip) at flow rate of 400 nL/min with a linear gradient of eluent B (0.1% formic acid in 95% ACN) in A (0.1% formic acid in 2% ACN) from 7 to 60 % in 50 min. Doubly and triply charged peptides were selected and analyzed using data-dependent acquisition of one MS scan (mass range from 300 to 2,000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Collision energy (CE) applied during peptide fragmentation is calculated by the sequent empirical equations: CE= 4V/100Da -2,4V. Raw data from nanoLC–MS/MS were analyzed and converted in common spectral file formats (.mgf mascot generic file), using Qualitative Analysis software (Agilent MassHunter Workstation Software, version B.02.00). MASCOT software (www.matrixscience.co) version: 2.4.0 was used for the protein identification against NCBInr database (NCBInr_20120920.fasta; 21582400 sequences; 7401135489 residues), with the taxonomy restriction to *Other Firmicutes* (2926062 sequences). The MASCOT search parameters were: "trypsin" as enzyme allowing up to 3 missed cleavages, carbamidomethyl on cysteine residues as fixed modification, oxidation of methionine and formation of pyroGlu N-term on glutamine were selected as variable modifications, 10 ppm MS/MS tolerance and 0.6 Da peptide tolerance. By data analysis, threshold provided to evaluate quality of matches for MS/MS data was found to be 41.

2.10 *Plasminogen activation assay*

tPA-specific plasminogen activation assay in the presence of *L. reuteri* Lb2 BM DSM 16143 cells or its surface associated proteins were measured as described by Lähteenmäki *et al.* [33]. Overnight grown bacteria were divided into two aliquots: bacteria of the first aliquot were collected and resuspended in PBS (pH 7.1) at a concentration of 1 x 10^{10} bacteria/mL. Bacteria of the second aliquot were incubated in 50 mM Tris-HCl pH 8 for 1 h at 37 °C to release surface associated proteins; after pelleting the bacteria, supernatant was filtrated through a 0.2 µm filter to remove the bacterial cells. Bacteria (20 µl) or surface associated proteins (50 µl) were incubated with 4 µg of human Glu-Plg (American Diagnostica), 2 ng of tPA (Biopool) and 0.45 mM chromogenic substrate of plasmin S-2251 (Kabivitrum) in a total volume of 200 µl. The plasmin formation was verified by evaluation of the increase in plasmin activity assessed by measuring the Optical Density at 405 nm every 30 minutes for a 4-hour long period. Three biological replicates were tested and for every culture two technical replicates were performed. Mean values were subtracted to the values measured at time 0.

3. Results and Discussion

The ability of a probiotic to adhere to the epithelial mucosa is crucial for intestinal colonization [34]**.** It is the first step required for competitive exclusion of entero-pathogens and for intestinal micro-ecology modification. Microbial coloni-

zation of gut epithelium requires that bacteria first bind to extracellular secreted constituents, and then adhere to cell-surface membranes or to extracellular matrix (ECM) proteins [35]. Therefore the adhesiveness of *L. reuteri* Lb2 BM DSM 16143 was tested in two biological models: the enterocyte-like Caco-2 cells and the purified proteins of the ECM.

3.1 *L. reuteri Lb2 BM DSM 16143 adhesion on enterocyte-like Caco-2 cells*

An *in vitro* binding assay was performed to evaluate the adhesion capacity of *L. reuteri* Lb2 BM DSM 16143 on enterocyte-like Caco-2 cells, a model for the intestinal epithelium that displays a good correlation to *in vivo* conditions [36]. The adhesion capacity (AC) is described as the percentage of bacteria adhered to Caco-2 cells relative to the total number of bacteria added. The obtained value for the strain in study is equal to 0,82 ± 0,17 % in comparison to the value 3,2 ± 0,41 % obtained for the positive control *Lactobacillus rhamnosus* GG. Therefore *L. reuteri* Lb2 BM proved to be a moderate adhesive strain and these results prompted us to go more in depth in the evaluation of its adhesion capability.

3.2 *L. reuteri Lb2 BM DSM 16143 adhesion assays on ECM proteins*

The first targets for bacteria attachment are the main extracellular matrix proteins: laminin, fibronectin and collagens I and IV. Therefore, the adhesiveness of *L. reuteri* Lb2 BM DSM 16143 was tested using the purified matrix proteins. The adhesive process is mainly mediated by surface-associated proteins expressed by microorganisms on their cell wall. In the literature this association has been proposed to be pH-dependent: surface proteins are positively charged at acidic pH values and therefore remain associated with the bacterial cell-wall by means of electrostatic interactions. Conversely moonlighting proteins of *L. crispatus* are released from cell surface in neutral or cationic buffers, which also reduces bacterial adhesiveness to ECM proteins [18]. For this reason the experiment was performed both at pH 4 and 8. As shown in Figure 1A, at pH 4 the strain can efficiently adhere to ECM proteins. Some differences in adhesiveness towards the four tested proteins can be observed: adhesion was stronger to collagen I, collagen IV and laminin than to fibronectin. As expected, a strong reduction of *L. reuteri* ability to adhere to ECM proteins was detected at pH 8 (Figure 1B), confirming two hypotheses: 1. the adhesion is mediated by the proteins associated to the cell wall; 2. this association is pH-dependent.

3.3 *SDS-PAGE of L. reuteri Lb2 BM DSM 16143 surface-associated proteins after detachment by alkaline treatment*

In order to further analyze the proteins released at high pH and involved in the adhesion process, bacteria were subjected to an alkaline treatment (1 hour, pH 8) to induce the release of surface-associated proteins. After SDS-PAGE and MALDI TOF-TOF analysis, fourteen different proteins were identified (Table 1). Among these, ten are reported to be surface associated in different bacterial species; furthermore ornithine carbamoyltransferase, elongation factor Tu, putative elongation factor Tu and phosphopyruvate hydratase (α-enolase) are directly involved in the adhesion process. Ornithine carbamoyltransferase is an arginine deiminase (ADI) pathway enzyme that was previously found on the surface of the opportunistic *Staphylococcus epidermidis* [37] and the pathogenic *Clostridium perfrigens* [38], where it acts as a fibronectin-binding adhesin. Elongation factor Tu (EF-Tu), a guanosine nucleotide-binding protein involved in protein synthesis, is responsible for adhesion when surface-exposed.

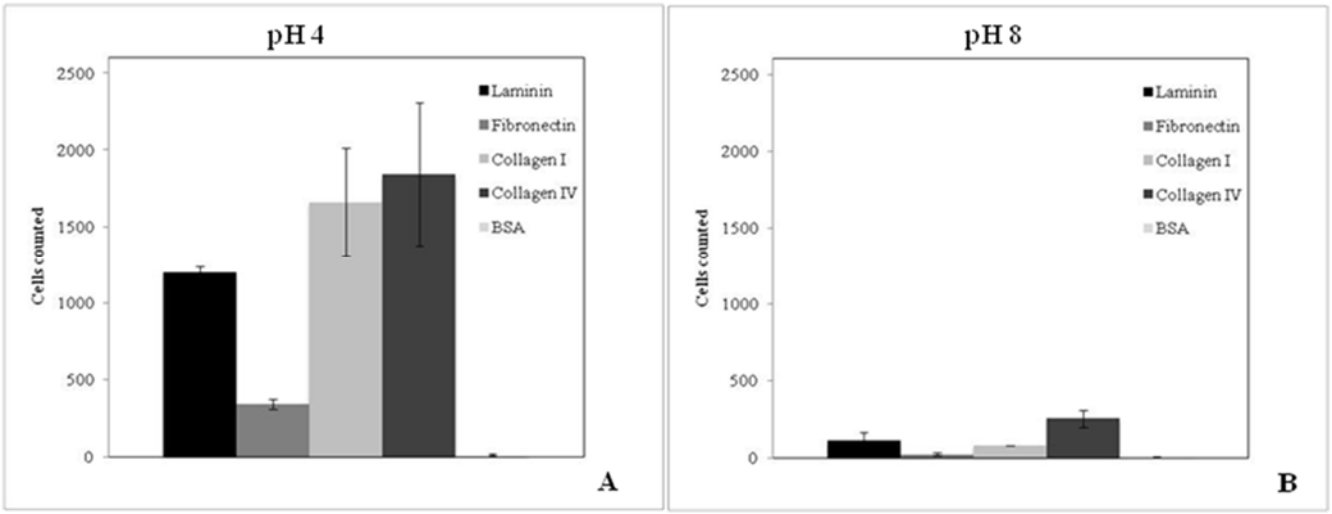

Figure 1. *Extracellular matrix adhesion assay.* Adhesion ability (reported as number of cells recovered after incubation with coated slides for 2 hours) of *L. reuteri* Lb2 BM DSM 16143 on ECM (extracellular matrix) proteins at pH 4 (A) and pH 8 (B). Values are reported as mean ± SEM.

Table 1. MALDI-TOF TOF identifications of weakly bound surface-associated proteins detached after alkaline (pH 8) treatment derived from SDS-PAGE bands. Bands 4, 6, 7 and 8 contained more than one protein, as demonstrated by the MS identification.

Bands	Score	Molecular weight	Identified protein	NCBI nr ID	Identified peptides
1	407	49920	Phosphopyruvate hydratase	gi|18415303	LGANAILAVSLAAAR GIHSFYNLSQQAR GNPTVEAEVYTEAGGVGR GIVPSGASTGEHEAVELR VDFQEFMIMPVGAPTVR
2	59	46790	NLP/P60 protein	gi|148544580	QSQWGDWYLFGNDGR
3	842	43405	Elongation factor Tu	gi|148543883	GISHDQIQR TLDLGEAGDNVGVLLR HYAHIDAPGHADYVK TKPHVNIGTIGHVDHGK GITINTAHVEYETEKR DLLSEYDFPGDDVPVVR TDLVDDDELVDLVEMEVR
4	226	37650	Ornithine carbamoyltransferase	gi|148543661	SFLTLADFNTR VLGGMFDGIEYR EMEVTDEVFESEHSVVFR
	225	36615	Alcohol dehydrogenase	gi|158544709	TVLDGIIVAGSLVGTR VGGVHAAVVTAVSASAFDQAVDSLRPDGK
	95	36284	Mannitol dehydrogenase	gi|45268465	EEIPADAYDIVVEAVGLPATQEQALAAAAR
5	70	31999	Elongation factor Ts	gi|148543917	DVAMHVAAINPEFMTR
6	148	29115	30S ribosomal protein S2	gi|14853916	FLGGIEDMPR
	216	28078	Putative elongation factor Tu	gi|22266054	TLDLGEAGDNVGVLLR
7	73	26162	Phosphoglyceromutase	gi|148543385	YGDEQVHIWR
	115	25849	Dehydratase, medium subunit	gi|148544952	IHYQAISAIMHIR
	57	24681	30S ribosomal protein S3	gi|148544690	IESYSDGTVPLHTLR
8	57	23947	Propanediol utilization protein	gi|148544946	SENFTLGIDAPIR
	156	22929	30S ribosomal protein S4	gi|148543735	QFSNLFVR EGTHGANFMALLER

Both the cell wall/extracellular localization and the involvement in the adhesion process of EF-Tu are well documented [39]: in *Mycoplasma pneumoniae* EF-Tu binds to fibronectin [40], and in *Lactobacillus johnsonii* it is able to bind mucin and thus intestinal epithelial cells, also displaying immune-modulatory properties [13]. Also α-enolase has widely been reported to be surface located in both commensal and pathogenic bacteria: in *L. plantarum* LM3 it is responsible for specifically binding human fibronectin [41], while in streptococci it mediates laminin binding [42]. Both EF-Tu and α-enolase, together with phosphoglycerate mutase, were also proved to be involved in plasminogen (Plg) binding: EF-Tu binds Plg in *Mycobacterium tuberculosis* [43] while in commensal bacteria, such as *L. crispatus* ST1

and *Bifidobacterium lactis* BI07, this function is mediated by α-enolase [18] and phosphoglycerate mutase [44] respectively.

3.4 *L. reuteri Lb2 BM DSM 16143 2DE extracellular proteome*

Adhesive proteins are mainly surface-located, but, as demonstrated in the previous paragraphs and in the literature, both pH [18] and physiological cell-wall turnover during the logarithmic growth phase [20,21], can give rise to a dynamic exchange of proteins between bacterial surface and extracellular space. The analysis of the extracellular proteome of a probiotic may shed light on its adhesion mechanisms and also confirm the safety of the strain in the tested conditions.

Figure 2 shows the extracellular proteome of *L. reuteri* Lb2 BM DSM 16143 in the 4-7 p*I* range. The 59 detected spots were all identified by nanoLC-chip MS/MS (Table 2, Table S1).

Considering p*I* isoforms, 21 proteins were identified. It is possible to hypothesize that spots 1, 2 and 15 are p*I* isoforms derived from post translational modifications, *i.e.* phosphorylations.

It is interesting to underline that seven of these proteins (phosphopyruvate hydratase, Nlp/P60 protein, elongation factor Tu, elongation factor Ts, alcohol dehydrogenase, mannitol dehydrogenase, phosphoglycerate mutase) were also identified as surface-proteins detached by the treatment at pH 8 described in the previous paragraph. This observation is a further evidence of the dynamic protein exchange between extracellular space and cell-wall.

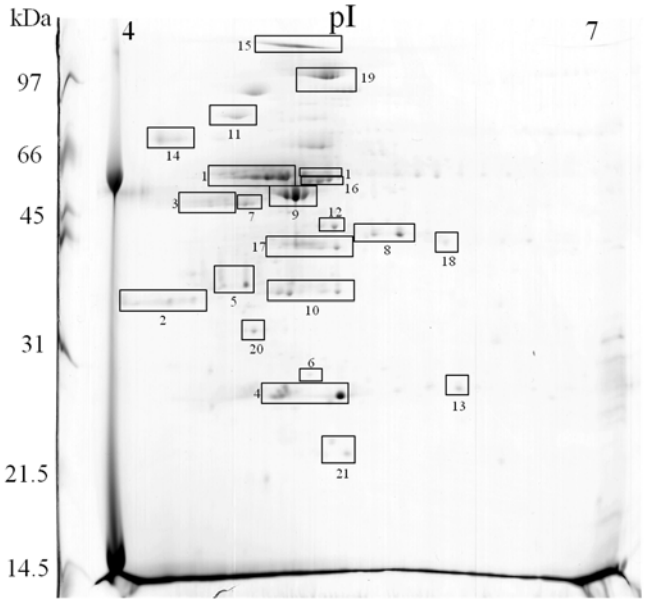

Figure 2. *2DE map of extracellular proteins*. Extracellular proteins maps in the acidic (4–7) p*I* range of *L. reuteri* Lb2 BM DSM 16143 grown in a modified MRS medium and collected at the middle exponential phase. Twenty-one proteins were identified by nanoLC-chip MS/MS from fifty-nine detected spots.

Identified proteins were divided into three main functional families and listed in Table 2: i) cell wall processing enzymes; ii) adhesion-involved proteins; iii) other proteins.

The first functional family is constituted by extracellular cell-wall processing enzymes. Bacterial cell wall is a very dynamic structure, especially during logarithmic growth and cell division [45]: all the proteins and protein domains detected are involved in the cell-wall re-arrangement and in the control of the cell shape during division. This finding is in agreement with the recovery of the bacterial cells during the middle exponential growth phase.

Mannosyl-glycoprotein endo-beta-N-acetylglucosamidase (spot 1) is a widespread enzyme in bacteria and catalyzes the hydrolysis of the glycosidic bond between *N*-acetyl-beta-D-glucosamine residues and the adjacent monosaccharides in peptidoglycan [46]. Nlp/P60 (spot 3) is an endopeptidase family with various roles in the dynamics of the bacterial cell wall such as the control of cell morphology and cell separation process [47]. Apf1-like protein (spot 6), is similar to Apf1 that was originally identified as one of the most abundant proteins in the supernatant of *L. gasseri* 4B2 [48]. It is directly related to the bacterial cell shape: an overproduction produces twisted cells, while a down-regulation causes no visible cell separation sites giving to the cells an elongated shape [49]. The peptidoglycan-binding LysM (spot 2, 4), is a repetitive domain consisting of 40 amino acids present in several peptidoglycan-binding enzymes [50]. It allows the non-covalent attachment of the majority of the extracellular proteins expressed by gram-positive bacteria to the cell-wall. Generally LysM domain is never present in Apf1 proteins. Bath and coworkers [51], nevertheless, demonstrated by bioinformatic analyses, that a LysM domain is present in *L. reuteri* ATCC 55730 Apf1-like protein. Interestingly in the same paper [51] it has been reported that the unknown extracellular protein lr1267 (spot 5), found also in this investigation, is an Apf-like protein.

It has to be taken into account that some bacterial cell-wall lysis enzymes are not only involved in autolysis and cell-wall rearrangement, but also in bacterial interspecies interactions, since they act as general cell-wall lysis factors, also degrading the cell-wall of bacterial species different from the producer one [52]. This is an appreciated feature for a probiotic strain, since it can be useful in killing competitive pathogenic bacteria, sharing the same ecological niche. Therefore their detection in the exoproteome of *L. reuteri* Lb2 BM can add consistence to the hypothesized antibacterial potential of this species [2,3,4].

The second functional group consists of adhesion-involved proteins. It is a well established matter that these proteins play important roles in both pathogenic and probiotic strains for their ability to interact with gut epithelial mucosa [15,20,21]. Most adhesins are cytosolic enzymes having well-proved moonlighting function when surface-bound or extracellularly secreted.

The evidences of the adhesive role of the surface-anchored

Table 2. The table lists the 21 identified proteins from extracellular 2DE maps of *L. reuteri* Lb2 BM 16143 divided in 3 different functional groups: cell wall processing enzymes, adhesion involved proteins and other proteins.

Functional role	Spot	Score	Molecular Weight	Identified protein	NCBI nr ID	N. of peptides	Sequence coverage (%)
Cell wall processing enzymes	1	1991	53593	mannosyl-glycoprotein endo-beta-N-	gi|148544583	41	87
		642	58147	N-acetylmuramoyl-L-alanine amidase	gi|148544581	19	47
	2	367	24886	peptidoglycan-binding LysM	gi|148544536	7	34
	3	796	46733	NLP/P60 protein	gi|148544580	24	55
	4	874	21648	peptidoglycan-binding LysM	gi|148543651	18	75
	5	435	26683	unknown extracellular protein lr1267	gi|68160846	9	34
	6	117	21600	Apf1-like protein	gi|33112857	4	16
Adhesion-involved proteins	7	1095	48010	phosphopyruvate hydratase (α-enolase)	gi|194468183	28	76
	8	1411	35971	glyceraldehyde 3-phosphate dehydrogenase	gi|184153036	38	94
	9	1518	43405	elongation factor Tu	gi|148543883	38	73
	10	1096	31999	elongation factor Ts	gi|148543917	36	60
	11	1529	67171	molecular chaperone DnaK	gi|148543938	57	77
	12	687	42934	phosphoglycerate kinase	gi|184153037	30	74
	13	234	26105	phosphoglyceromutase	gi|148543385	8	48
	14	1432	48717	trigger factor	gi|148543884	43	60
Other proteins	15	3558	167804	dextransucrase	gi|184153923	109	73
	16	523	55934	sucrose phosphorylase	gi|148544754	18	47
	17	500	35941	mannitol dehydrogenase	gi|45268465	12	49
	18	857	36102	alcohol dehydrogenase	gi|148544709	26	55
	19	1130	91346	phosphoketolase	gi|148544892	32	39
	20	233	27144	hypothetical protein Lreu_0552	gi|148543787	7	25
	21	383	20771	ribosome recycling factor	gi|148543919	17	85

elongation factor Tu has already been described in the previous paragraph. It is interesting to underline that EF-Tu (spot 9) has been detected also in the extracellular proteome of *Bifidobacterium animalis* subsp. *lactis* where it acts as moonlighting protein with adhesion roles contributing to the probiotic features of the strain [53]. EF-Ts (spot 10) has been classified as signal peptide-lacking exoprotein in both *Staphylococcus aureus* [54] and *Bacillus anthracis* [55] and as surface protein in *Lactococcus lactis* NZ9000 [56]. Also trigger factor (spot 14) was described to be exposed on the surface of *Lactobacillus plantarum* 299v [17]. It has been reported that surface adhesin P1 from *Streptococcus mutans* cannot efficiently work in the absence of both trigger factor and DnaK (spot 11) [57]. Furthermore, in *L. reuteri* NCIB 11951, a collagen I-binding protein that shares high sequence homology with *E. coli* trigger factor has been described [58].

The remaining identified proteins with adhesive function are moonlighting glycolytic enzymes. GAPDH (spot 8) is able to bind fibronectin, mucin and plasmin [59,60]; it has been found exposed on the surface of several *Lactobacillus* species (*L. gallinarum, L. gasseri, L. johnsonii, L. amylovorus, L. acidophilus* and *L. crispatus*) where it exerts the activity of fibronectin binding [61]. Phosphoglycerate kinase (PGK) (spot 12) has been referred to be extracellularly locat-

ed in *Lactobacillus rhamnosus* GG [62] and surface-associated in *Lactococcus lactis* IL1403 [63]. In eukaryotic cells it acts in the extracellular district as disulphide reductase and plasmin reductase [64]. Both activities are of interest in a probiotic strain since this enzyme (supposing the same biological function in prokaryotes) could contribute to the antioxidant effect of *L. reuteri* Lb2 BM and to the depletion from the extracellular environment of plasmin, a metabolite potentially able to activate proteolytic cascades [65].

Among the third protein group, ribosome recycling factor (spot 21) was previously described in streptococci as an extracellular located protein [66], while sucrose phosphorylase (spot 16) and dextransucrase (spot 15) are correlated to the probiotic potential of LAB. In *Lactobacillus acidophilus* NCFM sucrose phosphorylase expression is induced by sucrose. This enzyme is involved in the utilization and catabolism of human undigested sugars like fructooligosaccharides (FOS), considered prebiotic compounds, thus stimulating the growth of the producing strain and promoting competition of beneficial bacteria in the human gut [67]. Very recently [68] sucrose phosphorylase has been reported as a clue enzyme in generating fructose units, building blocks for the biosynthesis of the prebiotic functional sugars (*i.e*. FOS). Dextransucrase is an extracellular enzyme produced by several genera of lactic acid bacteria such as *Leuconostoc* and *Streptococcus* [69]. It is a glycosyltransferase catalyzing the cleavage of sucrose into glucose and fructose and the following polymerization of these glucosidic units into prebiotic molecules [70]. The finding of these proteins in the extracellular environment of *L. reuteri* Lb2 BM DSM 16143 underlines the probiotic potential of this strain for successfully colonizing the gut ecological niche.

The safety of the strain in the tested conditions is confirmed by the absence of potentially dangerous proteins such as extracellular serine protease and gelatinase recently described in the extracellular space of a strain of *Enterococcus faecalis*, a lactic acid bacterium whose use in food industry is still controversial [28].

3.5 *Potential of L. reuteri Lb2 BM DSM 16143 surface-associated proteins in plasminogen activation*

As reported in the previous paragraphs, some of the adhesive proteins identified in both the cell-wall and the extracellular space, also display the capability to bind plasminogen (Plg). Once localized to the bacterial surface, plasminogen can act as a cofactor in adhesion, or, following activation to plasmin (Plm), provide a source of potent proteolytic activity [71]. The activation to Plm may lead to damages to host tissues, opening the way for invasion by potential pathogenic bacteria, present in the same ecological niche. The conversion of Plg into Plm is a typical feature of several pathogenic bacteria, like staphylococci and streptococci, that possess staphylokinases and streptokinases which can act as specific activators [72].

The ability to convert Plg into Plm of *L. reuteri* Lb2 BM DSM 16143 was tested for both the whole bacterial cells and for surface-associated proteins recovered after cell treatment at pH 8. As shown in Figure 3 neither the whole cells nor the released proteins were able to induce Plm formation indicating the absence of the bacterial specific activators. The same assay was performed in presence of the mammalian tissue type Plg activator (tPA). As shown in Figure 3 in this condition both the whole bacterial cells and the surface-associated proteins were able, in presence of tPA, to produce Plm. This indicates that *L. reuteri* Lb2 BM DSM 16143 cells can immobilize Plg on its surface and that this property seems to depend on the surface-anchored moonlighting proteins.

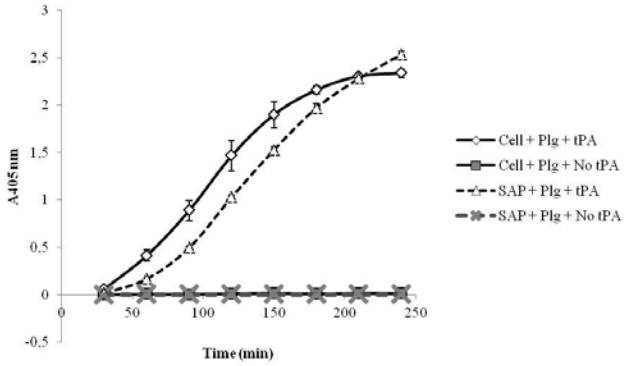

Figure 3. *Evaluation of Plg-binding ability.* Plasminogen activation assay of entire cells and surface-associated proteins (SAP) with and without mammalian tPA activator. Values were collected for 4 hours measuring absorbance at 405nm and are reported as mean ± SEM.

Even if the involvement of probiotic bacteria in the Plg/Plm system is yet to be elucidated, the proved ability of *Lactobacillus reuteri* to bind Plg on its surface could have a role in enhancing the colonization process at human epithelial surfaces, as already suggested by Candela and co-workers [44], or in localized dissolution of fibrin clots [22]. Furthermore the presence of Plg-binding proteins, either surface-bound or extracellularly released, could interfere in the interaction between Plg and gastrointestinal pathogens present in the same ecological niche, such as *Helicobacter pylori* and *Salmonella* sp., since probiotic bacteria could act as quenchers, as previously suggested by other authors [20].

4. Concluding Remarks

Integrating proteomic analyses with physiological studies proved to be a winning strategy to characterize a probiotic strain.

The ability of *L. reuteri* Lb2 BM DSM 16143 to adhere to both Caco-2 cells and extracellular matrix proteins, especially collagen and laminin, was proven by *in vitro* adhesion assays. The involvement of weakly cell-surface anchored proteins in this phenomenon was demonstrated by the drastic decrease in adhesion to ECM induced by their detachment obtained with incubation at pH 8. SDS-PAGE analysis

followed by MALDI-TOF TOF mass spectrometry revealed that the proteins involved in this process were mainly moonlighting proteins whose implication in adhesion has already been proved in both pathogenic and probiotic bacteria. The loss of adhesion ability induced by alkaline pH does not constitute a problem *in vivo* considering that the pH of colon, the main district of probiotic action, is around 6-7. However, *in vivo*, the strain adhesion potential could further be improved by the physiological activation of metabolic pathways leading to the expression of non-protein adhesive factors, such as the exopolysaccharides [73].

Extracellular proteomic profiles highlighted the presence of adhesive proteins also in this cell district. Among these are present glycolytic enzymes (GAPDH, PGM, PGK) as well as stress proteins (DnaK), trigger factor and protein synthesis enzymes (EF-Tu and Ts). The latter enzymes can also have an immune-modulating action. Since adhesion and immune system modulation are often related, the analysis of the extracellular proteomes proves to be a useful tool to obtain an overall picture of probiotic-host interaction, mainly in *L. reuteri* in which a strict link between adhesion and regulatory T-cell induction has been demonstrated [74]. Extracellular proteomics also revealed the presence of potential lytic factors, namely cell-wall hydrolases, useful in enhancing the antibacterial potential of the strain in the gut ecosystem and the total absence of virulence proteins. Furthermore proteins involved in the probiotic potential such as exopolysaccharide or fructooligosaccharide related enzyme have also been detected.

Some of the identified adhesins were reported to be able to link plasminogen; the strain was able to bind but not to activate plasminogen into plasmin without supplementation of exogenous activators like tPA. The expression of these Plg receptors is an useful feature because they can improve the adhesion ability of the tested strain and they can have a role in quenching Plg, subtracting it to pathogenic bacteria.

Although *in vivo* tests at different physiological pHs and in different individual enterotypes [75] are necessary to prove the adhesive and persistence capabilities of this strain, the present results enable us to assert that *L. reuteri* Lb2 BM can colonize the intestine, conferring to the host the benefits derived from its probiotic features.

5. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/143/0

Table S1 contains the sequences of peptides identified by nanoLC-chip MS/MS performed on the spots present in the extracellular 2DE maps.

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