Proteomic analysis of *Lactobacillus pentosus* for the identification of potential markers of adhesion and other probiotic features

Beatriz Pérez Montoro[1], Nabil Benomar[1], Natacha Caballero Gómez[1], Said Ennahar[2], Peter Horvatovich[3], Charles W. Knapp[4], Esther Alonso[1], Antonio Gálvez[1], Hikmate Abriouel[1*]

[1]Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071-Jaén, Spain.
[2]Equipe de Chimie Analytique des Molécules Bio-Actives, UMR 7178, IPHC-DSA, Université de Strasbourg, CNRS, 67400 Illkirch-Graffenstaden, France.
[3]Department of Analytical Biochemistry, Centre for Pharmacy, University of Groningen, Groningen, The Netherlands.
[4]Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, Scotland, United Kingdom.

[*]**Correspondence:** Dr. Hikmate Abriouel, Área de Microbiología. Departamento de Ciencias de la Salud. Facultad de Ciencias Experimentales. Edif. B3. Universidad de Jaén. Campus Las Lagunillas s/n. 23071-Jaén, Spain. Tel.: 34-953-212003; fax: 34-953-212943.
hikmate@ujaen.es

Abstract

We analyzed the adhesion capacity to mucus of 31 *Lactobacillus pentosus* strains isolated from naturally fermented Aloreña green table olives using an immobilized mucin model. On the basis of their adhesive capacity to mucin, three phenotypes were selected for cell-wall protein proteomic analysis to pinpoint proteins involved in the adhesion process: the highly adhesive *L. pentosus* CF1-43N (73.49% of adhesion ability), the moderately adhesive *L. pentosus* CF1-37N (49.56% of adhesion ability) and the poorly adhesive *L. pentosus* CF2-20P (32.79% of adhesion ability). The results revealed four moonlighting proteins over-produced in the highly adhesive *L. pentosus* CF1-43N, which were under/not produced in the other two *L. pentosus* strains (CF1-37N and CF2-20P). These proteins were involved in glycolytic pathway (phosphoglycerate mutase and glucosamine-6-phosphate deaminase), stress response (small heat shock protein) and transcription (transcription elongation factor GreA). Furthermore, the relative fold change in gene expression analysis showed significant up-regulation of the genes coding for these four moonlighting proteins in the highly adhesive *L. pentosus* CF1-43N versus the poorly adhesive *L. pentosus* CF2-20P and also in response to mucin for 20 h which clearly indicate the significant role of these genes in the adhesion capacity of *L. pentosus*. Thus, these proteins could be used as biomarkers for mucus adhesion in *L. pentosus*. On the other hand, mucin exposure induced other probiotic effects in *L. pentosus* strains, enhancing their co-aggregation ability with pathogens and possible inactivation.

Keywords:

Proteomics; *Lactobacillus pentosus*; Probiotics; Mucus; Adhesion; Biomarkers; qRT-PCR.

1. Introduction

The *Lactobacillus* genus belongs to the lactic acid bacteria (LAB) group and includes bacteria of great importance in food fermentation (e.g., dairy, meat and vegetables) and also potential probiotic bacteria as evidenced by their large and diverse genomes harboring genes with functional properties for such traits (Abriouel et al., 2016; Bonatsou, Tassou, Panagou &, Nychas, 2017; Maldonado-Barragán et al., 2011; Panagou et al., 2008). In particular, the probiotic potential of lactobacilli from vegetable origins have attracted considerable attention in the last decade especially those isolated from naturally fermented table olives as they are frequently consumed as part of the meditrerranean diet. As such, *L. pentosus* and *L. plantarum*, frequently isolated from fermented table olives, have been characterized with regard to their probiotic properties and their role as starter cultures in olive fermentation (Abriouel et al., 2012; Blana, Grounta, Tassou, Nychas, & Panagou, 2014; Grounta, Doulgeraki, Nychas, & Panagou, 2015; Hurtado, Reguant, Bordons, & Rozès, 2010; Rodríguez-Gómez et al., 2014).

Table olive surface is colonized during fermentation and storage by biofilm-forming lactic acid bacteria (LAB), especially *Lactobacillus* spp. (Randazzo, Rajendram, & Caggia, 2010) which are responsible for the fermentation process in cooperation with yeasts (Abriouel, Benomar, Lucas, & Gálvez, 2011; Arroyo-López, Durán-Quintana, Ruiz-Barba, Querol, & Garrido-Fernández, 2006; Nisiotou, Chorianopoulos, Nychas, & Panagou, 2010). As reported by Faten et al. (2016), *L. plantarum* cells adhere to the olive surface, forming a protective biofilm ($10^{6.1}$ - $10^{8.1}$ CFU/g) which could be considered beneficial because their presence appeared to effectively inhibit the adhesion of undesirable microorganisms during storage. Similarly, Grounta, Doulgeraki, Nychas, and Panagou (2015) showed that multifunctional starter *L. pentosus* B281 formed biofilms ($10^{5.5}$ - $10^{6.5}$ CFU/g) during controlled fermentation of *Conservolea* natural black olives for 153 days. Furthermore, olives are considered as good providers of probiotics for the host (Lavermicocca et al., 2005; Martins et al., 2013; Pérez Montoro et al., 2016); for one reason, the molecular adhesion properties of these lactobacilli to intestinal mucosa (reviewed in Bonatsou, Tassou, Panagou, & Nychas, 2017) is presumed to be similar to those for the olive surface (De Bellis, Valerio, Sisto, Lonigro, & Lavermicocca, 2010; Domínguez-Manzano et al., 2012; Faten et al., 2016). Besides acid and bile tolerance, adhesion to intestinal epithelium is among the main criteria for

the selection of probiotic strains, since adequate host-interaction is required for successful colonization of mucosal surfaces by probiotic bacteria and thus the provision of health benefits including pathogen exclusion and stimulation of the mucosal immune system (Cross, 2002; Ouwehand, Salminen, & Isolauri, 2002). Since the primary target is the mucus layer protecting gastrointestinal tract (GIT) (Fuller, 1989), the bacteria in GIT become directly exposed to mucin.

In this study, we examined the surface proteome of potentially probiotic *L. pentosus* strains isolated from naturally fermented Aloreña green table olives (Abriouel et al., 2012; Pérez Montoro et al., 2016) to determine the molecular mechanisms involved in their adhesion to mucus. For the first time, proteins are identified which may serve as adhesion biomarkers to discriminate *L. pentosus* strains with regard to their probiotic potential. Furthermore, we explored whether the interaction of *L. pentosus* strains with mucin impacted other probiotic features.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Thirty-one *Lactobacillus pentosus* strains isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2012) were used in this study. Strains were cultured in de Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) at 30°C for 24 h. Cultures were maintained in 20% glycerol at -20°C and -80°C for short and long term storage, respectively.

2.2. In vitro mucus adhesion assay

In vitro evaluation of the mucin-adhesion ability of *L. pentosus* strains was performed as previously reported by Valeriano, Parungao-Balolong, and Kang (2014) with some modifications. Porcin mucin (Sigma) solution was prepared at 1 mg/ml in sterile phosphate buffered saline (PBS) and stored at -20°C until use. 100 µl of porcin mucin solution was immobilized on a 96-well polystyrene microtiter plate for 1 h, and then incubated overnight at 4°C. Wells were washed twice with 200 µl of sterile PBS and then added with 100 µl of a bacterial suspension (10^8 CFU/ml) prepared from an overnight culture, which was washed twice and resuspended in PBS. Microtiter plates were incubated for 1 h at 37°C, after which they were washed five times with 200 µl of sterile citrate buffer to discard unbound bacteria and added with 200 µl of 0.5% Tween 80 (v/v) to collect adhered bacteria. Viable cell count was determined by plating onto MRS agar plates in triplicate. The percentage of adhesion was estimated using the formula (Collado, Meriluoto, & Salminen, 2008):

$$\%\ \text{Relative Adhesion} = \frac{\text{CFU/ml after adhesion}}{\text{CFU/ml before adhesion}} \times 100\%$$

2.3. Cell wall protein extraction

Extraction of cell-wall protein fraction was done according to Izquierdo et al. (2009) with some modifications. Cell surface exposed proteins were extracted by lysozyme cell shaving. Briefly, selected strains were inoculated (1%) into 40 ml MRS in triplicate and incubated for 18-20 h (stationary phase) at 37°C. Bacterial cultures were harvested by centrifugation (5000 rpm, 5 min, 4°C), washed three times with PBS, and the obtained pellets were resuspended in 2 ml of the extraction solution previously prepared (100 mM Tris-HCl, pH 8.0, 5 mM EDTA and 1 mg/ml lysozyme). The mixtures were

incubated for 2 h at 37°C under moderate agitation, and then centrifuged (8000 g, 15 min, 4°C). Supernatants were collected and 0.3 ml of a homogeneized solution (50:50, v/v) consisting of a strong cation exchanger phase (SCX, International Sorbent Technology, Tucson, USA) previously equilibrated with a solution 100 mM Tris-HCl, pH 8.0 and 5 mM EDTA was added. The mixtures were incubated under strong agitation for 30 min at 37°C, centrifuged (5000 rpm, 2 min, 4°C) and the resulting supernantants were collected, filtered through a 0.45 µm pore size filter (Crhomafil PET; Macherey-Nagel, Hoerdt, France) and the proteins were precipitated by adding 10 ml of ice-cold acetone. Protein pellets were collected by centrifugation (9000 rpm, 10 min, 4°C) and purified by adding 0.5 ml of Trizol (Euromedex, Souffelweyersheim, France) and 0.1 ml of chloroform, followed by vortex agitation and incubation for 5 min at room temperature. After centrifugation (12000 rpm, 15 min, 4°C), the upper aqueous phase was discarded, 0.15 ml of ethanol was added and the content was mixed by inversion. Samples were centrifugued (2000 rpm, 5 min, 4°C) and the proteins present in the supernatant were precipitated by adding 2 ml of ice-cold acetone. Protein pellets were harvested by centrifugation (12000 rpm, 15 min, 4°C), washed twice with acetone/water (80:20, v/v) at -20°C and resuspended in 0.2 ml of buffer solution (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris, pH 8.5) prior quantification of the protein concentration using Bradford protein assay (Bio-Rad).

2.4. Protein separation by 2-DE gel electrophoresis

A buffer solution IEF (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris, pH 8.5, 1 M DTT, 0.2% carrier ampholites, pH 3.0–10.0, 0.5 % bromophenol blue) was used for sample dilution (about 150 µg of protein). Diluted samples were loaded onto 17 cm, pH 3-10 IPG strips for rehydratation at room temperature for 12-15 h at 50 V, focused for 60000 V × h using a Protean® IEF Cell (Bio-Rad) and separated on a 12% SDS-polyacrylamide gel as previously reported by Izquierdo et al. (2009). The gels were stained with Coomassie brilliant blue (Fluka, Steinheim, Germany) and scanned on a GS-800 Calibrated Densitometer (Bio-Rad). With each bacterial strain, three analyses from three independent cultures were carried out. Only proteins whose presence was confirmed on the nine gels were considered as proteins of interest and selected for further analysis.

169 *2.5. Image analysis and trypsin digestion*

170 Gel imaging, spot detection, matching, and quantitation were carried out using
171 PDQuest 8.0.1 data analysis software for 2D gel electrophoresis (Bio-Rad). Spots
172 intensities were normalized to the total intensity of valid spots, and both qualitative and
173 quantitative analyses were performed. To determine the differential production of a
174 protein, a protein was considered under- or overporduced when spot intensities passed
175 the threshold of at least a twofold difference (one-way ANOVA, p-value < 0.05), as
176 previously described by Izquierdo et al. (2009). Spots of interest, corresponding to
177 statistically significant changes in the levels of protein expression, were excised and
178 subjected to tryptic digestion according to Izquierdo et al. (2009).
179
180 *2.6. Tryptic digest analysis and protein identification*

181 Tryptic digests were analyzed using an Ultimate 3000 nano-LC-MS/MS system
182 (Dionex, Amsterdam, The Netherlands), in line connected to an QExactive Plus mass
183 spectrometer (Thermo Fisher Scientific, Bremen, Germany). The simple mixture was
184 loaded on a trapping column (Aclaim PepMap, C18, 300 µm × 5 mm (ID × length),
185 5 µm particle size, 100 Å porosity, Thermo Scientific). After washing, the mixture was
186 separated using a 40 min linear gradient from 5% of 0.1% of formic acid (FA) in water
187 to 90% of 0.1% FA in acetonitrile at a flow rate of 250 nl/min on an analytical nanoLC
188 column (Acclaim PepMap RSLC, C18, 75 µm × 500 mm (ID × length), 2 mm particle
189 size, 100 Å porosity, Thermo Scientific). The mass spectrometer was operated in data-
190 dependent acquisition mode, automatically switching between MS and MS/MS
191 acquisition for the eight most abundant multiple charged ions (2, 3, and 4 times). Full-
192 scan MS spectra were acquired from m/z 300 to 1650 at a target value of 3×10^6 with a
193 resolution of 70,000 at 200 m/z. MS/MS spectra were obtained with a resolution of
194 35,000 at 200 m/z. The scan range for MS/MS was set to m/z 200–2000.
195 The identification of peptides and proteins was performed using PEAKS 8.0, using
196 the reference sequence of *Lactobacillus pentosus* KCA1
197 (http://www.uniprot.org/uniprot/I8R8S7) and *Lactobacillus pentosus* DSM 20314
198 (http://www.uniprot.org/uniprot/A0A0R1FPQ6) downloaded in FASTA format from
199 UniprotKB and contained 12272 protein sequences annotated with Tremb identifiers.
200 The search parameters included parent mass error tolerance of 10.0 ppm and
201 monoisotopic parent mass, fragment mass error tolerance of 0.02 Da, trypsin was used

as enzyme cleavage, the maximal number of missed cleavage was 3 and only tryptic peptides were considered during the search. Carbamidomethylation for reduced and alkylated cysteine was used as fix, while methionine oxidation was used as variable modification, with 6 maximal variable post-translational modification per peptide. Results were considered with false discovery rate (FDR) <= 1% at PSM, peptide and protein levels.

2.7. Characterization of adhesive L. pentosus strains using Scanning Electron Microscope

The mucin-adhesion capacity of each *L. pentosus* strain (i.e., highly, moderately and poorly adhesive phenotypes) was examined using scanning electron microscope (SEM) according to the methods described by Nyenje, Green, and Ndip (2012) with some modifications. For this, sterile stubs were introduced in sterile centrifuge tubes with 5 ml of Porcin mucin solution (1 mg/ml) in PBS, as described above, for 1 h at room temperature, and then incubated overnight at 4°C. Further, the stubs were removed, washed twice with 5 ml of sterile PBS and then added with 5 ml of a bacterial suspension (10^8 CFU/ml) prepared as described above (paragraph 2.2). The stubs were incubated for 1 h at 37°C and then they were washed five times with 5 ml of sterile citrate buffer to discard unbound bacteria. Then, the stubs were removed, and the bacteria were fixed using 4% formaldehyde for 1 h at room temperature and then dehydrated in a series of 20, 40, 60, 80, and 100% ethanol solutions (15 min each). Finally, the stubs were frozen at -80ºC overnight, freeze-dried for 4 hours and sputter-coated with Gold palladium using Elko 1B.3 ion coater before viewing with the SEM (FESEM, MERLIN de Carl Zeiss, Oxford).

2.8. Effect of mucin exposure on gene expression

To test the changes in the expression of genes -selected as biomarkers by proteomic analysis- in the presence and absence of mucin, MRS broth (5 ml) added or not with porcin mucin solution at 0.1% (w/v) was inoculated with 1% of an overnight culture of *L. pentosus* strains with high or poor adhesion capacity to mucin. After 20 h incubation at 37ºC, cells were centrifuged and subjected to RNA extraction using Direct-zol™ RNA Miniprep (Zymo Research, California, USA) according to the manufacturer´s

instructions. RNA quantification and quality assessment were carried out by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNAs were adjusted to a concentration of 500 ng/ml and frozen at -80 ºC until required for analysis.

The expression of selected genes was determined by quantitative, real-time PCR (qRT-PCR) using SensiFASTTM SYBR & Fluorescein One-Step Kit (BIOLINE). Phenylalanyl-tRNA synthase alpha-subunit (*pheS*) gene was used as a housekeeping gene (Naser et al., 2005), and a no-template control (NTC) was used as negative control. Primers and annealing temperatures used in this study are described in Table 1. Quantitative PCRs (qPCRs) were performed in triplicate on a CFX96 TouchTM Real-Time PCR Detection System from BioRad using 2 Power SYBR green chemistry. PCR-grade water served as a negative control.

2.9. Evaluation of the effect of mucin on the probiotic profile of L. pentosus strains

To test whether mucin had any effect on *L. pentosus* strains auto-aggregation or co-aggregation capacity with pathogenic bacteria: *Listeria innocua* CECT 910, *Staphylococcus aureus* CECT 4468, *Escherichia coli* CCUG 47553, and *Salmonella* Enteritidis UJ3449. All *L. pentosus* strains were cultured separately overnight at 37ºC in the presence of 0.1% mucin (prepared as described above, paragraph 2.2.). After incubation, the auto-aggregation and the co-aggregation capacities versus controls prepared in MRS broth without mucin were determined following the procedures reported by Pérez Montoro et al. (2016). The auto-aggregation percentage is expressed as:

$$\text{Agg\%} = \left(1 - \frac{OD_1}{OD_0}\right) \times 100\%,$$

where OD_0 and OD_1 represent the absorbance values measured at 580 nm at times t = 0 h and t = 2 h, respectively. The percentage of co-aggregation was expressed as:

$$\text{Co} - \text{Agg\%} = \left(1 - \frac{OD_{600} \text{ of upper suspension at time of 0h}}{OD_{600} \text{ of total bacteria suspension at starting time of 0h}}\right) \times 100\%.$$

2.10. Statistical analysis

All analyses were performed in triplicate. Statistical analyses were conducted using Excel 2007 (Microsoft Corporation, Redmond, Washington, US) program to determine averages and standard deviations. Statistical evaluation of *in vitro* adhesion, auto-

aggregation and co-aggregation assays were conducted by analysis of variance (*ANOVA*) using Statgraphics Centurion XVI software (Statpoint Technologie, Warrenton, Virginia, US). The same software was used to perform Shapiro–Wilk and the Levene tests to check data normality and to perform 2-sided Tukey's multiple contrast to determine the pair-wise differences between strains, where level of significance was set at *P*-value of <0.05.

3. Results

3.1. Adhesion capacity of L. pentosus strains

The relative adhesion capacity to mucin was investigated among 31 *L. pentosus* strains with the aim of selecting three phenotypes for further proteomic examination: highly (HA), moderately (MA) and poorly (PA) adhesive strains. Strains exhibited adhesion capacities ranging from 32.79% to 73.49% showing that adhesion is a strain specific property (Table 2). We therefore selected three strains with significant ($p < 0.05$) differences in adhesion ability: *L. pentosus* CF2-20P, with a poor adhesion (32.79%), *L. pentosus* CF1-37N, with a moderate adhesion (49.56%), and *L. pentosus* CF1-43N, with a high adhesion (73.49%) (Table 2). The three strains were further investigated by comparative analyses of their cell-wall proteomes.

3.2. Comparative cell wall proteomic analysis of L. pentosus strains

The objective of this investigation was to pinpoint proteins involved in the adhesion of *L. pentosus* strains to mucus. Figure 1 shows representative 2-D elecrophoresis patterns of cell surface proteome extracted by lysozyme cell shaving of *L. pentosus* CF2-20P (PA), CF1-37N (MA) and CF1-43N (HA) strains. Overall, cell wall proteomes of highly and poorly adhesive *L. pentosus* strains were very similar; however, the moderately adhesive *L. pentosus* strain showed several disctinctive proteins (Fig. 1). Eleven of the observed proteins displayed differential production levels; among them, nine were over-produced in *L. pentosus* CF1-43N (HA), seven in *L. pentosus* CF1-37N (MA) and one in *L. pentosus* CF2-20P (PA) (Table 3, Table S1). All proteins were identified using *L. pentosus* DSM 20314 or *L. pentosus* KCA1 proteome (Table 3). Among the differentially expressed proteins, four could be linked to the ability of *L. pentosus* strains to adhere to mucus, as they were found in higher amounts in the cell-wall proteome of the highly adhesive strain CF1-43N, as compared to strains CF2-20P and CF1-37N (Table 3, Table S1). These proteins were phosphoglycerate mutase "PGM" (spot 7203), glucosamine-6-phosphate deaminase "GNPDA" (spot 5301), transcription elongation factor GreA "GreA" (spot 2102) and the small heat-shock protein (spot 1102). These four proteins are involved in the glycolytic pathway (PGM and GNPDA), stress response (small heat shock protein) and transcription (GreA).

3.3. SEM analysis of adhesion capacity of L. pentosus strains

To elucidate variations in adhesion capacity of *L. pentosus* strains, SEM images revealed differences in adhesion to mucin especially between the highly and the poorly adhesive strains (Fig. 2). Microscopy revealed that the poorly adhesive *L. pentosus* CF2-20P used other adhesion mechanisms since mucin induced biofilm formation (Fig. 2 F-G), which was not evident in the HA strain. As such, it could be presumed that the highly adhesive strain relied on surface properties for attachment.

3.4. Expression of genes selected as "biomarkers" in response to mucin exposure

The genes corresponding to proteins differentially produced in the highly and poorly adhesive *L. pentosus* strains CF1-43N and CF2-20P, respectively were *pgm* (coding for phosphoglycerate mutase), *nagB* (coding for glucosamine-6-phosphate deaminase), *greA* (coding for transcription elongation factor GreA) and *shsp* (coding for the small heat-shock protein). The relative expression of all genes in the absence of mucin was high in the highly adhesive *L. pentosus* CF1-43N in comparison with the poorly adhesive *L. pentosus* CF2-20P except for *nagB* gene (Fig. 3). The highly adhesive *L. pentosus* CF1-43N (without mucin) was considered as control for mucin exposure and set to one. The fold changes in the expression of *pgm, nagB, greA* and *shsp* genes in response to mucin exposure in the highly and poorly adhesive strains CF1-43N and CF2-20P, respectively were shown in Figure 3. There was a significant up-regulation of *pgm, nagB* and *greA* genes in the highly adhesive *L. pentosus* CF1-43N when exposed to mucin for 20 h and the fold change was ranging from 2.6 to 5. However, only *pgm* was up-regulated in the poorly adhesive *L. pentosus* CF2-20P, while *shsp* gene was under-regulated in the presence of mucin (Fig. 3).

3.5. Mucin influence on probiotic properties of L. pentosus strains

Pre-exposure of *L. pentosus* strains to mucin decreased their auto-aggregation capacity except for *L. pentosus* CF1-37N (MA) (Table 4). However, there was an increase in the co-aggregation ability of *L. pentosus* CF1-43N (HA) with *Listeria innocua* CECT 910 and *Escherichia coli* CCUG 47553 and of *L. pentosus* CF1-37N (MA) with *Salmonella* Enteritidis UJ3449 (Table 4). *L. pentosus* CF2-20P on the other hand did not exhibit improved co-aggregation with these bacteria after mucin exposure (Table 4).

4. Discussion

Lactobacillus spp. of vegetal origin are potentially probiotic and could confer health benefits by promoting healthy digestion (simple and complex carbohydrates, and also prebiotics), inhibiting pathogens (via production of acids and plantaricins, auto-aggregation, or co-aggregation with several pathogens) and strenghtening the intestinal barrier (interaction with host cells) (Pérez Montoro et al., 2016). Thus, selection of potential probiotic strains with the capacity to colonize, even transiently, the intestinal tract, should be based on the survivability under harsh gastro-intestinal conditions (e.g., exposures to acid and biles) and also on the ability to adhere to host cells and mucosa - a presumed requisite for sufficient host-interaction and health effects. As such, the adhesion of probiotic bacteria to intestinal mucosa could very importantly help to guarantee efficient colonization and persistance in the intestinal tract. Several reports have highlighted the importance of the molecular adhesion mechanisms by *Bifidobacterium* spp. and *Lactobacillus* spp., the two most known and used probiotic groups (Gilad, Svensson, Viborg, Stuer-Lauridsen, & Jacobsen, 2011; Izquierdo et al., 2009; Sánchez, Bressollier, & Urdaci, 2008). Furthermore, Kleerebezem et al. (2010) reported that an important part of *Lactobacillus'* health-promoting interactions with the host (intestinal) system involves effector molecules existing in the bacterial cell envelope. Therefore, cell-wall proteomic approaches could bring insight into the molecular mechanisms involved in adhesion process and help identify key molecules underlying a strain's ability to colonize, persist and exert beneficial health effects.

Intriguingly, part of the cytoplasmic housekeeping proteins detected in the extracellular proteomes have been defined as moonlighting proteins. Moonlighting proteins display a dual role depending on their subcellular localization, as they perform metabolic functions inside the cell but also could be transported to the cell-wall surface to contribute to secondary biochemical functions (Huberts & van der Klei, 2010). Such proteins include glycolytic proteins (such as PGM, phosphoglycerate kinase, pyruvate kinase, GAPDH, glucose 6-phosphate isomerase, enolase), protein folding and stress responses-involved proteins (GroEL and DnaK), as well as transcription and translation proteins (elongation factor Tu, elongation factor Ts, several ribosomal proteins) (Bergonzelli et al., 2006; Castaldo et al., 2009; Granato et al., 2004; Kainulainen et al., 2012; Kinoshita et al., 2008). These protein could be found on the surface of *Lactobacillus* spp. where they act as adhesion promoting factors. Izquierdo et al. (2009) suggested that the over-production of EF-Tu, GroEL chaperonin, molecular chaperone

DnaK, GroES co-chaperonin and GAPDH may contribute to the high adhesion ability of *L. plantarum* WHE 92 to mucin. Overall, in this study the cell-wall of the highly adhesive *L. pentosus* showed different moonlighting protein patterns when compared with *L. plantarum,* although they have similar key functions (i.e., glycolytic enzymes and stress response).

PGM, a key enzyme of the central metabolism, which catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis and gluconeogenesis has been shown to contribute to *Bifidobacterium*'s ability to adhere to plasma components (Candela et al., 2007). Furthermore, *in silico* analysis of *L. pentosus* MP-10 isolated from Aloreña green table olives revealed different gene copies of PGM in its genome, which indicates that the gene products may accomplish other functions as moonlighting proteins (Abriouel et al., 2017). In this study, PGM was over-produced in the highly adhesive *L. pentosus* CF1-43N as compared to the other two strains: *L. pentosus* CF2-20P and *L. pentosus* CF1-37 each with poorly and moderately adhesive capabilities, respectively. This fact suggests that this surface-associated protein plays a key role in the adhesion of *L. pentosus* to mucus in addition to other functions such as resistance to acids. Pérez Montoro et al. (2018) showed that 2,3-bisphosphoglycerate-dependent PGM 2 (PGAM-d) was among the key markers of acid resistance in *L. pentosus*, as it was also over-produced by resistant strains in response to acids.

GNPDA, which catalyzes the reversible isomerization-deamination of glucosamine 6-phosphate (GlcN6P) to form fructose 6-phosphate (Fru6P) and ammonium ion during carbohydrate metabolic process, was also over-produced in *L. pentosus* CF1-43N (HA), but it was down-regulated in *L. pentosus* CF1-37N (MA) and not expressed in *L. pentosus* CF2-20P (PA). No previous reports could however be found about a possible role of this protein in the adhesion process, but Koskenniemi et al. (2011) showed however that GNPDA/isomerase was strongly up-regulated after addition of bile.

GreA is another moonlighting protein which was over-produced in the highly adhesive *L. pentosus* CF1-43N as compared to *L. pentosus* CF2-20P (PA), and was not found in *L. pentosus* CF1-37N (MA). This protein has multiple roles in *E. coli* enhancing the resistance of host cells to environmental perturbations and may have functional chaperone roles during resistance response to various stressors (Li et al., 2012). Similarly, GreA was up-regulated under low pH growth of *Streptococcus mutans* (Len, Harty, & Jacques, 2004) and also under cell-wall targeted antibiotic stress in *S. aureus* (Singh, Jayaswal, & Wilkinson, 2001).

405 Regarding stress proteins, small heat shock protein was present in higher amounts in
406 the highly adhesive *L. pentosus* CF1-43N than the other two strains. This protein is
407 involved in survival and stress tolerance, but it is also associated with probiotic
408 interactions with the host (Candela et al., 2009; Gilad, Svensson, Viborg, Stuer-
409 Lauridsen, & Jacobsen; Izquierdo et al. 2009; Le Maréchal et al., 2014; Sánchez et al.,
410 2005). The small heat-shock protein is involved in the irreversible protein denaturation
411 prevention in response to cellular stresses (Narberhaus, 2002); in this study, the small
412 heat shock protein of Hsp20 family was involved in the adhesion of *L. pentosus* to
413 mucin in a similar way as reported by Le Maréchal et al. (2015) for *Propionibacterium*
414 *freudenreichii*.
415 Analysis of the expression of genes coding for PGM, GNPDA, GreA and small heat-
416 shock protein in response to mucin exposure in the highly and poorly adhesive *L.*
417 *pentosus* strains CF1-43N and CF2-20P, respectively confirmed the role of these
418 proteins in mucin adhesion. Differences were detected between the highly and poorly
419 adhesive *L. pentosus* in the absence of mucin, since *L. pentosus* CF1-43N showed
420 higher fold change with 2.2-9 compared with *L. pentosus* CF2-20P. After exposure to
421 mucin, *nagB, greA* and *pgm* were upregulated in the highly adhesive *L. pentosus* CF1-
422 43N while *shsp* gene didn´t change. However, when compared to the poorly adhesive *L.*
423 *pentosus* CF2-20P, *shsp* gene was up-regulated in *L. pentosus* CF1-43N. The expression
424 change in response to mucin exposure clearly indicates the significant role of *nagB,*
425 *greA, pgm* and *shsp* genes in the adhesion capacity of *L. pentosus*.
426 SEM images confirmed that *L. pentosus* CF1-43N (HA) cells were more tightly
427 adhered to mucin than *L. pentosus* CF2-20P (PA) cells, and the mechanisms employed
428 by the highly and the poorly adhesive strains seem to be different. As such, the
429 lactobacilli used different mechanisms to interact with the host cells; here, we observed
430 that the differential surface proteins in the highly adhesive strain likely contributed to
431 more efficient mucin interaction and greater number of adhered cells. On the contrary,
432 mucin-adhesion of the poorly adhesive *L. pentosus* strain was mediated by biofilm
433 formation (exopolysaccharides and/or fimbrial interactions). Fimbriae are proteinaceous
434 extensions involved in mucus adhesion of lactobacilli as reported by Van Tassel &
435 Miller (2011). Thus, lactobacilli from Aloreña green table olives exhibited a variety of
436 molecular mechanisms mediating host-adhesion, which reflects niche specialization
437 since surface proteins are essential for host colonization as for LAB (e.g., review by
438 Nishiyama, Sugiyama, & Mukai, 2016). The interaction with mucin, whether by

fimbria, exopolysaccharide or surface proteins of probiotic *L. pentosus* strains, could improve their antimicrobial effect in the gut since their co-aggregation ability was enhanced in the presence of mucin. Conditions, as those present in the gut after interaction of probiotic *L. pentosus* strains, could improve the protection of mucosal barrier via exclusion of pathogens.

Further, the probiotic potential of *L. pentosus* strains could be enhanced by a previous stress (e.g, acids, bile salts, antimicrobials, mucin) since a plethora of genes involved in survival and interaction with host can be expressed as a consequence. A previous study by Casado Muñoz et al. (2016) showed that adaptation to sub-lethal concentrations of antimicrobials could promote the desirable increased robustness of probiotic *L. pentosus* MP-10 to many environmental and gastrointestinal conditions (e.g., acid and bile stresses). Similarly, Pérez Montoro et al. (2018) described that pre-exposure of *L. pentosus* strains to acids enhanced their probiotic function such as auto-aggregation ability via surface proteins. In the present study, PGM was over-produced in the highly adhesive *L. pentosus* strain in the absence and presence of mucin and also under acidic conditions in *L. pentosus* strains (Pérez Montoro et al., 2018).

5. Conclusions

Cell wall proteome analysis identified, for the first time, key protein biomarkers involved in mucus adhesion of *L. pentosus* strains. The results revealed the presence, in higher amounts, of four moonlighting proteins in the highly adhesive *L. pentosus* CF1-43N than in the other two *L. pentosus* strains. The genes coding for these proteins were up-regulated in response to mucin in the highly adhesive *L. pentosus* CF1-43N which clearly indicates the significant role of *nagB, greA, pgm* and *shsp* genes in the adhesion capacity of *L. pentosus*. These proteins were involved in glycolytic pathway (PGM and GNPDA), stress response (small heat shock protein) and transcription (GreA). They could be used as biomarkers for the adhesion ability of *L. pentosus* strains and probably also for other probiotic effects, such as the co-aggregation with pathogens, which was enhanced following exposure to mucin.

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Figure legends

Figure 1. 2-DE gels of cell-wall proteomes from *Lactobacillus pentosus* CF2-20P (A), *L. pentosus* CF1-37N (B) and *L. pentosus* CF1-43N (C) with poorly, moderately and highly adhesive capacity, respectively. The figure shows representative 2-DE gel images (pH range: 3-10) of cell-wall protein lysates from early stationary phase of *L. pentosus* strains. Spots exhibiting differential production between *L. pentosus* strains were identified by LC-MS/MS analysis and database search.

Figure 2. Scanning electron micrographs of mucin-adhered *L. pentosus* CF1-43N (A-C) and *L. pentosus* CF2-20P (D-G) with highly and poorly adhesive capacity, respectively. Resolution of 10k (A and D), 30k (B and E), 82.53k (G) and 200k (C and F) were shown.

Figure 3. The effect of mucin on the expression of *greA, nagB, pgm* and *shsp* genes in *L. pentosus* CF1-43N and *L. pentosus* CF2-20P. The relative expression level in control for the highly adhesive *L. pentosus* CF1-43N (without mucin exposure) was set to one for fold expression analysis in other experimental groups. Each bar represents mean value and standard deviation as error bar of three independent experiments. The samples CH and CP corresponded to controls (without mucin) of *L. pentosus* CF1-43N and *L. pentosus* CF2-20P, respectively; the samples MH and MP corresponded to mucin-exposed *L. pentosus* CF1-43N and *L. pentosus* CF2-20P, respectively. *significant differences between controls of both strains without mucin ($P < 0.05$). **significant differences between the same *L. pentosus* strain in the presence and absence of mucin ($P < 0.05$).