Proteomic analysis of *Lactobacillus pentosus* for the identification of potential markers involved in acid resistance and their influence on other probiotic features

Beatriz Pérez Montoro[1], Nabil Benomar[1], Natacha Caballero Gómez[1], Said Ennahar[2], Peter Horvatovich[3], Charles W. Knapp[4], 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

Acidity often prevents the undesirable microbial colonization both in fermented foods and under gastric conditions. Thus, the acid resistance of *Lactobacillus pentosus* strains used as starter cultures and/or probiotics requires further understanding. This was investigated by means of comparative proteomic approach using three strains representing the phenotypes: resistant (AP2-15), intermediate (AP2-18) and sensitive (LP-1) to acidic conditions. Proteomic analysis of constitutive phenotypes revealed that the intrinsic resistance of *L. pentosus* is associated with the over-production of three principal proteins: 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 (PGAM-d), elongation factor G and 50S ribosomal protein L10, and additionally on ATP synthase subunit beta and chaperone protein DnaK; they are associated with metabolic pathways of proteins and carbohydrates, energy production and stress responses. Suggested protein biomarkers for acid resistance in *L. pentosus* include elongation factor G and PGAM-d, both being abundantly found in the constitutive proteome of the resistant phenotype under standard and acidic conditions. Furthermore, *L. pentosus* strains pre-exposed to acids displayed enhanced probiotic function such as auto-aggregation ability via surface proteins. We conclude that pre-exposure of probiotic *L. pentosus* strains to acid may strategically enhance their performance as starter cultures and probiotics.

Keywords:

Comparative proteomics; *Lactobacillus pentosus*; Probiotics; acid stress; Auto-aggregation.

1. Introduction

Strains of *Lactobacillus pentosus* are versatile bacteria, members of lactic acid bacteria (LAB) group, ubiquitously distributed in the nature (environment, mucosal membranes of animals and humans, as well as plant material) and are largely associated with fermented foods and feed products (Todorov and Dicks, 2004). Their high prevalence in naturally-fermented Aloreña (Abriouel et al., 2011, 2012) and Spanish-style green table olives (Maldonado-Barragán et al., 2011) and their prolonged history of safe use in other fermented products are at the origin of their deserved reputation as ideal starter cultures for fermentations (Anukam and Olise, N.A., 2012; Holzapfel, 2002; Garrigues et al., 2013; Giraffa et al., 2010; Rodríguez-Gómez et al., 2014; Ruiz-Barba and Jiménez-Díaz, 2012). Moreover, *L. pentosus* also exhibits probiotic properties (Izumo et al., 2011; Kotani et al., 2010), and being of vegetal origin, has gained more attention in the last decade as a species capable to adapt to gastrointestinal tracts and changing environmental conditions (Abriouel et al., 2012, 2017; Granato et al., 2010; Pérez Montoro et al., 2016; Ranadheera et al., 2010), but also to accommodate dietary preferences of host organism.

According to guidelines by FAO/WHO working group (2006), acid resistance is one of the two *in vitro* tests commonly used for probiotic screening, along with tolerance to bile components. The aciduric or acidophilic property of lactobacilli allows them to endure acid stress in the environment, in foods and in the gastrointestinal tract, which involves a plethora of physiological activities (Broadbent et al., 2010). However, the molecular strategies that probiotic lactobacilli display to tolerate or resist gastric stresses remain poorly understood, since probiosis is inherently linked to species, or even individual strains (Hill et al., 2014). For this reason, proteomics are often utilized to elucidate the molecular mechanisms involved in different physiological processes, such as stress tolerance or antibiotic resistance (Casado Muñoz et al., 2016; Sánchez et al., 2007a, b).

Gastric acidity is of utmost importance in preventing undesirable microbial colonization, since it plays a major role in reducing the survival rates of microorganisms, including pathogens, during gastrointestinal transit ($<10^4$ bacteria/gram of stomach content; O´Hara et al., 2006). Thus, probiotic lactobacilli, those highly tolerant to acid exposure, strategically utilize different cellular responses and mechanisms to withstand hostile stomach conditions, such as F1 F0-ATPase proton

pumps for intracellular pH (pHi) homeostasis, repair proteins for DNA damage, changes in cellular envelope and altered metabolism (De Angelis and Gobbetti, 2004). In the present study, to gain insight into the acid resistance of potentially probiotic *L. pentosus* strains, we used comparative proteomics via two-dimensional gel electrophoresis (2-DE) and tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) analysis under standard and acid-stress conditions with the aim of detecting, for the first time, key proteins involved in their acid resistance. Furthermore, we investigated whether acid-stress on *L. pentosus* strains had any consequences on desired probiotic features such as auto-aggregation or co-aggregation with pathogens.

2. Materials and Methods

2.1. Bacterial cultures and growth conditions

Three *L. pentosus* strains (AP2-15, AP2-18 and LP-1), originally isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2012), were selected based on their levels of acid resistance at pH 1.5 (i.e., sensitive, intermediate and resistant) as determined in a previous study (Pérez Montoro et al., 2016). *L. pentosus* LP-1 (sensitive phenotype, SP), *L. pentosus* AP2-18 (intermediate phenotype, IP) and *L. pentosus* AP2-15 (resistant phenotype, RP) were routinely cultured at 37ºC in either de Man, Rogosa and Sharpe (MRS) broth or agar (Fluka, Madrid, Spain) under aerobic (atmospheric) conditions for 24–48 h. Strains were kept in 20% glycerol at −80ºC for long-term storage.

2.2. Sample preparation, protein extraction, two-dimensional gel electrophoresis and image analysis

Sample preparation was carried out under two bacterial growth conditions: standard (MRS broth, pH 6.5) and acid-stress (MRS broth, adjusted with HCl to pH 4.0) conditions. Thus, all bacteria exhibiting different acid-resistance phenotypes were subcultured successively three times (1% v/v inoculation) in both standard and stressing conditions at 37ºC under aerobic conditions and then harvested at early stationary growth phase ($OD_{600\ nm}$ = 0.6). In this way, early-stationary phase was reached after a culture time ranging from 16 to 20 h depending on the strain and also on culture conditions (presence or absence of acid stress) as determined from their respective growth curves at 600 nm (data not shown). Harvested cells were washed three times with sterile phosphate buffered saline (PBS) before protein extraction. Whole-cell proteins were extracted for two-dimensional electrophoresis (2-DE) as previously reported (Hamon et al., 2013). For each strain and each condition (standard or stressing), three independent experiments were performed in triplicate to determine any significant differences in protein expression. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) according to the manufacturer's instructions.

2-DE was conducted using 150 µg of protein extracts which were loaded onto 17 cm long strips with a pH range of 3 to 10 (Bio-Rad), focused for 60,000 V h, and then

125 separated on a 12% SDS-polyacrylamide gel as reported previously (Izquierdo et al.,
126 2009). Three independent gels per strain and per condition were stained with Bio-Safe
127 Coomassie brilliant blue G-250 (Bio-Rad) and scanned on a GS-800 Calibrated
128 Densitometer (Bio-Rad). Image analysis was performed using PDQuest 2D analysis
129 7.4.1 software (Bio-Rad) accounting only the spots present on three gels. Normalization
130 of spot intensities and the analysis of changes in protein expression during acid
131 exposure were performed as reported by Hamon et al. (2013). Briefly, a protein was
132 considered to be either under- or over-produced when normalized spot intensities were
133 at least 1.5-fold different at a significance level (P-value < 0.05; Student's t test for
134 paired samples) (Sánchez et al., 2007b). With respect to proteome comparisons between
135 different *L. pentosus* strains, proteins were considered differentially produced when spot
136 intensities passed the threshold of a twofold difference (one-way *ANOVA*, P-value <
137 0.05), as described previously (Izquierdo et al., 2009). Thus, the spots were detected and
138 quantified with the PDQuest 2D analysis 7.4.1 software (Bio-Rad), which highlighted
139 statistically significant changes in the levels of spots.
140
141 *2.2. Mass spectrometry analysis and peptide/protein identification*
142 Spots of interest were subjected to tryptic in-gel digestion, as described by Izquierdo
143 et al. (2009). The peptide mix was injected into an Ultimate 3000 nano-LC–MS/MS
144 system (Dionex, Amsterdam, The Netherlands), in line connected to an Q Exactive Plus
145 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The sample mixture
146 was loaded on a trapping column (Acclaim PepMap, C18, 300 μm × 5 mm (ID ×
147 length), 5 μm particle size, 100 Å porosity, Thermo Scientific) and washed. After 3 min,
148 the mixture was separated using a 40 min linear gradient from 5 % of 0.1 % of formic
149 acid (FA) in water to 90 % of 0.1 % FA in acetonitrile at a flow rate of 250 nl/min on an
150 analytical nanoLC column (Acclaim PepMap RSLC, C18, 75 μm × 500 mm (ID ×
151 length), 2 mm particle size, 100Å porosity, Thermo Scientific). The mass spectrometer
152 was operated in data-dependent mode, automatically switching between MS and
153 MS/MS acquisition for the eight most abundant multiple charged ions (2, 3, and 4
154 times). Full-scan MS spectra were acquired from m/z 300 to 1650 at a target value of
155 $3 \cdot 10^6$ with a resolution of 70,000 at 200 m/z. Peptides were analyzed in the orbitrap
156 with a resolution of 35,000 at 200 m/z. The scan range for MS/MS was set to m/z 200–
157 2000.

Identification of peptides and proteins was performed using PEAKS 8.0, using the reference sequence of *L. pentosus* KCA1 (http://www.uniprot.org/uniprot/I8R8S7) and *L. pentosus* DSM 20314 (http://www.uniprot.org/uniprot/A0A0R1FPQ6) downloaded in FASTA format from UniprotKB and contained 12272 protein sequences annotated with Tremb identifiers. The search parameters included parent mass error tolerance of 10.0 ppm and 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.

2.4. Effect of acid stress on the probiotic profile of L. pentosus strains

Acid-stressed cells of *L. pentosus* strains (AP2-15, AP2-18 and LP-1) were tested for auto-aggregation capacity as described by Vizoso Pinto et al. (2007). Overnight cultures (2 ml) of lactobacilli in MRS broth (pH 4.0 or 6.5) broth were harvested, washed and resuspended in PBS. After 2 h at room temperature, 100 µl were taken from the top of the suspension and were transferred to a cuvette containing 900 µl PBS. The auto-aggregation percentage is expressed as: Agg% = $[(1 - OD_1/OD_0)] \times 100$, where OD_0 and OD_1 represent the absorbance values measured at 580 nm at times (0 h) and 2 h, respectively.

To test whether acid adaptation of *L. pentosus* strains (AP2-15, AP2-18 and LP-1) had any effect on co-aggregation capacity with pathogenic bacteria (*Listeria innocua* CECT 910, *Staphylococcus aureus* CECT 4468, *Escherichia coli* CCUG 47553, and *Salmonella* Enteritidis UJ3449), overnight cultures (10 ml) of all lactobacilli in MRS (pH 6.5 or pH 4.0) broth and pathogenic bacteria in TSB broth at 37°C were harvested, washed, resuspended in sterile PBS and their OD_{600} was adjusted to 1 according to Vlková et al. (2008). The OD_{600} of upper cell suspension mixture consisting of 3 ml of each bacteria (*L. pentosus* strain and one pathogenic strain) was measured at starting time 0 h and after 1 h incubation at room temperature. The percentage of co-aggregation was expressed as: Co-Agg% = [1 - (OD_{600} of upper suspension at time 1 h/OD_{600} of total bacterial suspension at starting time of 0 h)] × 100.

192

193 *2.5. Characterization of auto-aggregates using Scanning Electron Microscope*

194

195 The auto-aggregates formed under standard and acidic conditions were examined
196 using scanning electron microscope (SEM) according to the methods described by
197 Nyenje et al. (2012) with some modifications. For this, sterile stubs were introduced in
198 MRS broth, adjusted at either pH 4 or 6, inoculated with 4% of each *L. pentosus* strain
199 (AP2-15, AP2-18 and LP-1) in individual, sterile centrifuge tubes and then incubated
200 for 48 hours at 37ºC. Further, the stubs were removed, and the bacteria were fixed using
201 4% formaldehyde for 1 h at room temperature and then dehydrated in a series of 20, 40,
202 60, 80, and 100% ethanol solutions (15 min each). Finally, the stubs were frozen
203 at -80ºC overnight, freeze-dried for 4 hours and sputter-coated with Gold palladium
204 using Elko 1B.3 ion coater before viewing with the SEM (FESEM, MERLIN de Carl
205 Zeiss, Oxford).

206

207 *2.6. Statistical Analysis*

208

209 All analyses were performed in triplicate. Statistical analyses were conducted using
210 Excel 2007 (Microsoft Corporation, Redmond, Washington, US) program to determine
211 averages and standard deviations. Statistical evaluation of auto-aggregation data was
212 conducted by analysis of variances (*ANOVA*) using Statgraphics Centurion XVI
213 software (Statpoint Technologie, Warrenton, Virginia, US). The same software was
214 used to perform Shapiro–Wilk and the Levene tests to check data normality and to
215 perform 2-sided Tukey's multiple contrast to determine the pair-wise differences
216 between strains, where level of significance was set at *P*-value of <0.05.

217

3. Results and Discussion

Proteomic analysis can provide valuable information about stress response and physiological mechanisms in bacteria. Our main objective was to identify the key proteins involved in acid resistance in *L. pentosus* under acid stress conditions. Among 31 *L. pentosus* strains, we selected three strains with different levels of acid resistance at pH 1.5: *L. pentosus* LP-1 (SP), *L. pentosus* AP2-18 (IP) and *L. pentosus* AP2-15 (RP). When exposed to pH 4.0, the three strains could recover from the acid shock and grow but at different growth rates. They were harvested at early-stationary growth phase (16-20 h) depending on the strain and also on culture conditions (absence or presence of acid stress), which coincided with an OD_{600nm} of 0.6 (data not shown). As such, cultures under both standard and acid-stress conditions reached the early-stationary phase (same OD) in comparable physiological states, thereby reducing protein pattern differences that are due to the growth phase.

3.1. Comparative proteomic analysis of L. pentosus strains under standard conditions

Differences between constitutively expressed whole-cell proteomes of the three strains were first investigated under non-stress conditions. The aim was to establish a link between the strains´ constitutive proteome and their levels of phenotypic acid resistance, and to identify key protein markers involved in intrinsic resistance to acid. Figures 1A-C show representative 2-DE patterns for *L. pentosus* strains under standard (non-stress) conditions. All differentially expressed proteins appeared to be encoded by highly conserved genes in *L. pentosus* species (as compared with *L. pentosus* KCA1 and *L. pentosus* DSM 20314) (Table 1). The proteins differentially produced in *L. pentosus* AP2-15 (resistance phenotype) were three proteins not directly linked to acid resistance; rather, they included proteins involved in ribosome biogenesis and translation (50S ribosomal protein L10; spot 3002), gluconeogenesis and glycolytic process (PGAM-d; spot 3102), and protein biosynthesis (elongation factor G; spot 4003) (Table 1). As such, the intrinsic resistance to acids in *L. pentosus* appear to be mediated by components of the central metabolism such as glucose metabolism (biosynthesis and utilization), which influence energy production and intracellular redox potential, and protein biosynthesis. Gluconeogenesis and glycolysis equilibrate intracellular sugar, ATP and NAD^+ levels and other compounds necessary for strain survival under acidic

252 conditions. With respect to protein biosynthesis, this is a general feature that may
253 involve stress proteins implicated in the protection of cells against acids.
254 Other mechanisms that may contribute to (at least) intermediate resistance, as
255 detected in both resistant and intermediate phenotypes of *L. pentosus* strains (AP2-15
256 and AP2-18), included the over-production of proteins related to ATP synthesis coupled
257 proton transport (ATP synthase subunit beta; isoform, spot 5206) and protein folding
258 (chaperone protein DnaK; spot 7206) (Table 1). Furthermore, chaperone DnaK, which
259 plays a role in protein folding and stress resistance, was also over-produced in both *L.*
260 *pentosus* AP2-15 and AP2-18 strains (Table 1). Both proteins, however, were under-
261 produced (chaperone protein DnaK; spot 7206) or not produced (ATP synthase subunit
262 beta; isoform, spot 5206) in the sensitive phenotype (Table 1). Moreover *L. pentosus*
263 LP-1, the sensitive phenotype, under-produced proteins involved in ribosome
264 biogenesis and translation (50S ribosomal protein L10, spot 3002) and protein folding
265 (chaperone protein DnaK, spot 7206) (Table 1). However, 2,3-bisphosphoglycerate-
266 dependent phosphoglycerate mutase 2 (spot 3102), elongation factor G (spot 4003) and
267 ATP synthase subunit beta (isoform, spot 5206) were not detected in sensitive
268 *Lactobacillus* strain (Table 1), but these proteins were clearly over-produced in resistant
269 and/or intermediate phenotypes.
270 Overall, the intrinsic resistance of *L. pentosus* may rely on the over-production of
271 five principal proteins: 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2
272 (spot 3102), elongation factor G (spot 4003) and 50S ribosomal protein L10 (spot
273 3002), and additionally on ATP synthase subunit beta (isoform, spot 5206) and
274 chaperone protein DnaK (spot 7206). Intrinsic acid resistance in *L. pentosus* may differ
275 from the responses of its phylogenetically related neighbor *L. plantarum* (Hamon et al.,
276 2013), since in this case acid resistance in *L. pentosus* mainly relied on metabolic
277 pathways of proteins and carbohydrates, and energy production as physiological
278 strategies for acid resistance. Additionally, other stress-response proteins also
279 contributed in the intermediate acid resistance of *L. pentosus*. As comparison, Hamon et
280 al. (2013) on the other hand reported that the main proteins contributing to the intrinsic
281 resistance of *L. plantarum* are involved in cell protection activities and in the
282 modulation of membrane composition (molecular chaperones GrpE and ClpL, as well
283 as FabF), along with other proteins representing key components of central metabolism.
284

285 *3.2. Comparative proteomic analysis of L. pentosus strains under stress conditions*
286 *versus standard conditions*
287
288 To confirm the data obtained by the constitutive analysis of *L. pentosus* proteomes,
289 additional proteomic analysis was performed by comparing changes in protein
290 production within each *L. pentosus* strain under acid-stress versus standard conditions.
291 Figures 1 (D-F) show the 2-DE patterns for all *L. pentosus* strains following acid stress
292 and standard condition, and the difference in protein patterns were detected and
293 identified (Table 2). Some of these proteins were represented by more than one spot
294 indicating the presence of protein isoforms in *L. pentosus* strains (Tables 1 and 2). The
295 expression of protein isoforms depended on the strain and culture conditions, and
296 showed varied expression.
297 Following the acid challenge, *L. pentosus* AP2-15 (RP) over-produced proteins
298 involved in: cell division (cell division protein sufi, spot 2604), protein biosynthesis
299 (elongation factor G, spot 2606), gluconeogenesis and glycolytic process (PGAM-d,
300 spot 3109) and a manganese-dependent inorganic pyrophosphatase (spot 1505) (Figure
301 1A, B; Table 2A) indicating higher protein translation rates in *L. pentosus* following
302 acid stress. When we compared the data between the constitutive analysis and the stress
303 challenge for the each *L. pentosus* strain, we observed that elongation factor G
304 (isoforms, spot 2606 and 4003) and 2,3-bisphosphoglycerate-dependent
305 phosphoglycerate mutase 2 (isoforms, spot 3102 and 3109) – responsible for protein
306 biosynthesis and gluconeogenesis-glycolytic process, respectively – were abundant in
307 constitutive proteome of the resistant phenotype and also under acid stress, thus we
308 conclude that these proteins may represent potential biomarker candidates for acid
309 resistance in *L. pentosus*. Additionally, these proteins appeared as isoforms whose
310 production was equally distributed under both conditions (standard and acid stress), thus
311 further confirming their role in acid resistance. Therefore, these two proteins can be
312 used as biomarker indicating acid resistance independently from the culturing bacteria
313 conditions (stressed or standard condition). Furthermore, cell-division protein sufi (spot
314 2604) and manganese-dependent inorganic pyrophosphatase (spot 1505) were up-
315 regulated in response to acid challenge, which indicates that the bacteria increased their
316 replication under stress, although survival dogma suggests that they should reduce this
317 capacity to conserve energy. Similarly, Altaf Hussain and Kaur Natt (2014) described
318 the over-expression of proteins involved in cell division of *L. rhamnosus* in response to

lactose starvation. A different pattern of proteins involved in acid resistance was reported by Hamon et al. (2013) for *L. plantarum* which included the heat-shock protein GrpE, methionine synthase MetE and 30S ribosomal protein S2 RpsB.

Two proteins were repressed in resistant strain after acid exposure: ATP synthase subunit beta (isoform, spot 1203) involved in ATP synthesis coupled proton transport and a 60 kDa chaperonin (spot 1303) involved in protein refolding (Figure 1A, B; Table 2A). However, ATP synthase subunit beta (isoform, spot 5206) was over-produced in both resistant and intermediate phenotypes in constitutive proteomes. These variations in expression levels suggest that this protein is not relevant with regard to acid resistance (Table 1).

For sensitive and intermediate phenotypes (*L. pentosus* LP-1 and *L. pentosus* AP2-18, respectively), protein expression patterns were highly different when comparing standard and stressing conditions (Figure 1C-F, Tables 2B and C). For example, *L. pentosus* AP2-18 (IP) exhibited down-regulation of proteins involved in several metabolic pathways: translational termination (spot 9102), DNA-templated transcription, termination-nucleoside metabolic process-regulation of transcription (spot 9101), phosphoenolpyruvate-dependent sugar phosphotransferase system (spot 9303), fructose 1,6-bisphosphate metabolic process and glycolytic process (spot 5205), and suppressed expression of proteins involved in DNA binding (phosphorelay signal transduction system, DNA-templated regulation of transcription, DNA-templated transcription; spot 5204), ATP binding (protein folding; spot 7206) and UTP:glucose-1-phosphate uridylyltransferase activity (biosynthetic process, UDP-glucose metabolic process; spot 9305) (Figure 1C-D, Table 2B). However, *L. pentosus* LP-1, exhibiting the sensitive phenotype for acid resistance, had greater expression of proteins involved in ATP synthesis coupled proton transport (ATP synthase subunit beta; isoform, spot 1602) and a manganese-dependent inorganic pyrophosphatase (spot 1505), and lower protein expression related to DNA transcription and termination, nucleoside metabolic process, regulation of DNA transcription and translation (spots 9101 and 9102) (Figure 1E-F, Table 2C). This suggests that manganese-dependent inorganic pyrophosphatase (spot 1505) over-production in both resistant and sensitive phenotypes under acid challenge is not linked to acid resistance. Furthermore, the expression of ATP synthase subunit beta (isoform, spot 1602) exhibited variations in its expression between constitutive and stress conditions, and thus it could not be considered as a biomarker candidate for acid-resistance.

353

354 *3.3. Acid stress influence on probiotic properties of L. pentosus strains*

355

356 To elucidate whether acid-adapted *L. pentosus* strains exhibit different probiotic
357 potentials, we tested their ability to auto-aggregate and co-aggregate with pathogens
358 under standard- and after acid-challenged conditions. The results revealed that the three
359 strains differed in their ability to auto-aggregate ($p < 0.05$), especially between the
360 resistant phenotype on the one hand and the intermediate and sensitive phenotypes on
361 the other hand. In fact, the resistant phenotype physiologically had greater auto-
362 aggregation ability under both conditions, such natural auto-aggregation ability is not
363 linked to its natural acid resistance as shown by Pérez Montoro et al. (2016) for other
364 lactobacilli (Table 3). However, acid stress did increase auto-aggregation capacity of the
365 three strains (Table 3), while co-aggregation patterns with pathogens were not impacted
366 (data not shown). Similarly, Casado Muñoz et al. (2016) showed that stressed *L.*
367 *pentosus* strains using antimicrobials (antibiotic or biocide) displayed improved
368 tolerance to acids and biles.

369 The improved auto-aggregation ability of *L. pentosus* strains, once pre-exposed to
370 acid, could enhance probiotic function improving their adhesion to mucosal cells. SEM
371 images confirmed auto-aggregation by *L. pentosus* AP2-15 (resistant phenotype) under
372 acidic conditions, showing densely packed colonies at pH 4.0 (Fig. 2) compared with
373 the intermediate and sensitive phenotypes (data not shown). Conditions, as such present
374 in the stomach during the passage of probiotic *L. pentosus* strains, could improve the
375 bacteria colonization in the gut. The repertoire of proteins changed following acid
376 challenge especially among the moonlighting proteins: elongation factor G and PGAM-
377 d, which may be involved in the increase of auto-aggregation in the case of the resistant
378 phenotype, but not in co-aggregation with pathogens since their expression was
379 increased simultaneously with the auto-aggregation capacity of the strains. Waśko et al.
380 (2014) reported that moonlighting proteins such as glycolytic enzymes (enolase,
381 phosphoglycerate kinase, and phosphoglycerate mutase), proteins related to
382 translocation and transcription (elongation factor-Ts, 30S ribosomal protein S1, and
383 oligopeptide ABC transporter substrate binding protein), and stress-response and
384 protein-folding proteins (GroEL, DnaK) were involved in the adhesion process in *L.*
385 *helveticus* T159. Moreover, different proteins are involved in both aggregation
386 processes (auto- and co-aggregation) in *L. pentosus*. In the case of auto-aggregation, the

moonlighting proteins: elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 -responsible of protein biosynthesis and gluconeogenesis-glycolytic process, respectively- are produced in cytoplasm and may be found on the bacterial cell surface acting as adhesion-promoting factors for probiotics adhesive; Bergonzelli et al. (2006) and Candela et al. (2010) have reported moonlighting proteins in lactobacilli such as elongation factor Tu, heat shock protein GroEL, DnaK, and pyruvate kinase.

4. Conclusions

Comparative proteomics provided the first insights into the molecular mechanisms employed by *L. pentosus* strains to survive gastric acids under standard- and acid-stress conditions. Proteins identified as biomarker candidates for acid resistance in *L. pentosus* included elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 responsible for protein biosynthesis and energy production (gluconeogenesis-glycolytic process), respectively. They were not only constitutively abundant in the proteome of the resistant phenotype, but were also over-produced after acid exposure. Furthermore, *L. pentosus* strains pre-exposed to acid displayed better probiotic function, including increased auto-aggregation ability, by means of moonlighting proteins (such as elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 in the case of resistant phenotype) or other proteins not determined in this study. We can conclude that pre-exposure of probiotic *L. pentosus* strains to acids in the stomach or in food matrices may strategically enhance their performance as probiotic and also as starter culture.

Acknowledgments

We acknowledge research grants: AGL2013-43571-P (Ministerio de Economía y Competitividad, MINECO, FEDER), UJA2014/07/02 (Plan Propio UJA) and Research Team (EI_BIO01_2017).

References

Abriouel, H., Benomar, N., Lucas, R., and Gálvez, A., 2011. Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally fermented Aloreña green table olives. Int. J. Food Microbiol. 144, 487–496.

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M. Á., Pérez-Pulido, R., Gálvez, A., 2012. Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. Food Microbiol. 32, 308–316.

Abriouel, H., Pérez Montoro, B., Casimiro-Soriguer, C.S., Pérez Pulido, A.J., Knapp, C.W., Caballero Gómez, N., Castillo-Gutiérrez, S., Estudillo-Martínez, M.D., Gálvez, A., Benomar, N., 2017. Insight into Potential Probiotic Markers Predicted in *Lactobacillus pentosus* MP-10 Genome Sequence. Front. Microbiol. 8, 891.

Altaf Hussain, M., Kaur Natt, N., 2014. Comparison of changes in the cytosolic proteomes of lactose starved and acid stressed *Lactobacillus rhamnosus* cells. American J. Microbiol. Biotechnol. 1, 56-63.

Anukam, K.C., Olise, N.A., 2012. Development of a novel probiotic yogurt "PENTOYO" with a fully sequenced *Lactobacillus pentosus* KCA1 and its survival during storage at 4 °C. Trop. J. Pharm. Res. 11, 977–981.

Bergonzelli, G.E., Granato, D., Pridmore, R.D., Marvin-Guy, L.F., Donnicola, D., Corthésy-Theulaz, I.E., 2006. GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. Infect. Immun. 74, 425-34.

Broadbent, J.R., Larsen, R.L., Deibel, V., Steele, J.L., 2010. Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. J. Bacteriol. 192, 2445-2458.

Candela, M., Centanni, M., Fiori, J., Biagi, E., Turroni, S., Orrico, C., Bergmann, S., Hammerschmidt, S., Brigidi, P., 2010. DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts. Microbiology 156, 1609-18.

Casado Muñoz, M.C., Benomar, N., Ennahar, S., Horvatovich, P., Lavilla Lerma, L., Knapp, C.W., Gálvez, A., Abriouel, H., 2016. Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus* MP-10 for the identification of key

452 proteins involved in antibiotic resistance and biocide tolerance. Int. J. Food
453 Microbiol. 222, 8–15.
454 De Angelis, M., Gobbetti, M., 2004. Environmental stress responses in *Lactobacillus*: a
455 review. Proteomics 4, 106−122.
456 FAO/WHO., 2006. Evaluation of health and nutritional properties of probiotics in food
457 including powder milk with live lactic acid bacteria: Report of a Joint FAO/ WHO
458 Expert Consultation [Online] 2006. [25 Nov. 2008].
459 Garrigues, C., Johansen, E., Crittenden, R., 2013. Pangenomics—an avenue to
460 improved industrial starter cultures and probiotics. Curr. Opin. Biotechnol. 24,
461 187–191.
462 Giraffa, G., Chanishvili, N., Widyastuti, Y., 2010. Importance of lactobacilli in food
463 and feed biotechnology. Res. Microbiol. 161, 480–487.
464 Granato, D., Branco, G.F., Nazzaro, F., Cruz, A.G., Faria, J.A.F., 2010. Functional
465 foods and non dairy probiotic food development: trends, concepts, and products.
466 Comp. Rev. Food Sci. Food Safety 9, 292–302.
467 Hamon, E., Horvatovich, P., Marchioni, E., Aoudé-Werner, D., Ennahar S., 2013.
468 Investigation of potential markers of acid resistance in *Lactobacillus plantarum*
469 by comparative proteomics. J. Appl. Microbiol. 116, 134—144.
470 Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L.,
471 Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E., 2014. The
472 International Scientific Association for Probiotics and Prebiotics consensus
473 statement on the scope and appropriate use of the term probiotic. Nat. Rev.
474 Gastroenterol. Hepatol. 11, 506-14.
475 Holzapfel, W.H., 2002. Appropriate starter culture technologies for small-scale
476 fermentation in developing countries. Int. J. Food Microbiol. 75, 197–212.
477 Hutkins, R.W., Nannen, N.L., 1993. pH homeostasis in lactic acid bacteria. J. Dairy Sci.
478 76, 2354–2365.
479 Izquierdo, E., Horvatovich, P., Marchioni, E., Aoudé–Werner, D., Sanz, Y., Ennahar,
480 S., 2009. 2-DE and MS analysis of key proteins in the adhesion of *Lactobacillus*
481 *plantarum*, a first step toward early selection of probiotics based on bacterial
482 biomarkers. Electrophoresis 30, 949–956.
483 Izumo, T., Izumi, F., Nakagawa, I., Kitagawa, Y., Shibata, H., Kiso, Y., 2011. Influence
484 of *Lactobacillus pentosus* S-PT84 ingestion on the mucosal immunity of healthy
485 and *Salmonella* Typhimurium-infected mice. Biosci. Microflora 30, 27–35.

Kotani, Y., Shinkai, S., Okamatsu, H., Toba, M., Ogawa, K., Yoshida, H., Fukaya, T., Fujiwara, Y., Chaves, P.H.M., Kakumoto, K., Kohda, N., 2010. Oral intake of *Lactobacillus pentosus* strain b240 accelerates salivary immunoglobulin A secretion in the elderly: a randomized, placebo-controlled, double-blind trial. Immun. Ageing 7, 11.

Maldonado-Barragán, A., Caballero-Guerrero, B., Lucena-Padrós, H., Ruiz-Barba, J.L., 2011. Genome sequence of *Lactobacillus pentosus* IG1, a strain isolated from Spanish-style green olive fermentations. J. Bacteriol. 193, 5605.

Nyenje, M.E., Green, E., Ndip, R.N., 2012. Biofilm Formation and Adherence Characteristics of *Listeria ivanovii* Strains Isolated from Ready-to-Eat Foods in Alice, South Africa. Sci. World J. 2012, 873909.

O'Hara, A.M., Shanahan, F., 2006. The gut flora as a forgotten organ. EMBO Rep. 7, 688–693.

Pérez Montoro, B., Benomar, N., Lavilla Lerma, L., Castillo Gutiérrez, S., Gálvez, A., Abriouel, H., 2016. Fermented Aloreña table olives as a source of potential probiotic *Lactobacillus pentosus* strains. Front. Microbiol. 7, 1583.

Ranadheera, R.D.C.S., Baines, S.K., Adams, M.C., 2010. Importance of food in probiotic efficacy. Food Res. Int. 43, 1–7.

Rodríguez-Gómez, F., Romero-Gil, V., García-García, P., Garrido-Fernández, A., Arroyo-López, F.N., 2014. Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. Front. Microbiol. 5, 467.

Ruiz-Barba, J.L., Jiménez-Díaz, R., 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. Food Microbiol. 30, 253–259.

Sánchez, B., Champomier-Vergès, M.C., Collado, M.C., Anglade, P., Baraige, F., Sanz, Y., 2007a. los Reyes-Gavilán CG, Margolles A, Zagorec M. Low-pH adaptation and the acid tolerance response of *Bifidobacterium longum* biotype *longum*. Appl. Environ. Microbiol. 73, 6450–6459.

Sánchez, B., Champomier-Vergès, M.C., Stuer-Lauridsen, B., Ruas-Madiedo, P., Anglade, P., Baraige, F., los Reyes-Gavilán, C.G., Johansen, E., Zagorec, M., Margolles, A., 2007b. Adaptation and response of *Bifidobacterium animalis* subsp. lactis to bile: a proteomic and physiological approach. Appl. Environ. Microbiol. 73, 6757–6767.

Todorov, S.D., Dicks, L.M.T., 2004. Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer

produced by the fermentation of maize, barley and soy flour. World J. Microbiol. Biotechnol. 20, 643–650.

Vizoso Pinto, M.G., Schuster, T., Briviba, K., Watzl, B., Holzapfel, W.H., Franz, C.M., 2007. Adhesive and chemokine stimulatory properties of potentially probiotic *Lactobacillus* strains. J. Food Prot. 70, 125–134.

Waśko, A., Polak-Berecka, M., Paduch, R., Jóźwiak, K., 2014. The effect of moonlighting proteins on the adhesion and aggregation ability of *Lactobacillus helveticus*. Anaerobe 30, 161-168.

Figure legends

Figure 1. 2-DE gels of whole-cell proteomes from *Lactobacillus pentosus* AP2-15 (A, D), *L. pentosus* AP2-18 (B, E) and *L. pentosus* LP-1 (C, F) cultured in standard (A, B, C) and acidic (D, E, F) conditions. The figure shows representative 2-DE gel images (pH range: 3-10) of whole-cell protein lysates from early stationary phase of *L. pentosus* strains. Spots exhibiting constitutive differential expression between *L. pentosus* strains in standard conditions (MRS at pH 6.0) and acid exposure (MRS at pH 4.0) were identified by LC-MS/MS analysis and database search and are labelled. Labels in red correspond to spots detected in the same *L. pentosus* strain under one of the tested conditions (standard or acidic condition).

Figure 2. Scanning electron micrographs of auto-aggregated *L. pentosus* AP2-15 under standard (A and C) and acidic (B and D) conditions. Resolution of 2.5k (A and B) and 12k (C and D) were shown.