

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

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22 **Abstract**

23

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

42

43 **Keywords:**

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

46

## 47 1. Introduction

48

49 Strains of *Lactobacillus pentosus* are versatile bacteria, members of lactic acid  
50 bacteria (LAB) group, ubiquitously distributed in the nature (environment, mucosal  
51 membranes of animals and humans, as well as plant material) and are largely associated  
52 with fermented foods and feed products (Todorov and Dicks, 2004). Their high  
53 prevalence in naturally-fermented Aloreña (Abriouel et al., 2011, 2012) and Spanish-  
54 style green table olives (Maldonado-Barragán et al., 2011) and their prolonged history  
55 of safe use in other fermented products are at the origin of their deserved reputation as  
56 ideal starter cultures for fermentations (Anukam and Olise, N.A., 2012; Holzapfel,  
57 2002; Garrigues et al., 2013; Giraffa et al., 2010; Rodríguez-Gómez et al., 2014; Ruiz-  
58 Barba and Jiménez-Díaz, 2012). Moreover, *L. pentosus* also exhibits probiotic  
59 properties (Izumo et al., 2011; Kotani et al., 2010), and being of vegetal origin, has  
60 gained more attention in the last decade as a species capable to adapt to gastrointestinal  
61 tracts and changing environmental conditions (Abriouel et al., 2012, 2017; Granato et  
62 al., 2010; Pérez Montoro et al., 2016; Ranadheera et al., 2010), but also to accommodate  
63 dietary preferences of host organism.

64 According to guidelines by FAO/WHO working group (2006), acid resistance is one  
65 of the two *in vitro* tests commonly used for probiotic screening, along with tolerance to  
66 bile components. The aciduric or acidophilic property of lactobacilli allows them to  
67 endure acid stress in the environment, in foods and in the gastrointestinal tract, which  
68 involves a plethora of physiological activities (Broadbent et al., 2010). However, the  
69 molecular strategies that probiotic lactobacilli display to tolerate or resist gastric stresses  
70 remain poorly understood, since probiosis is inherently linked to species, or even  
71 individual strains (Hill et al., 2014). For this reason, proteomics are often utilized to  
72 elucidate the molecular mechanisms involved in different physiological processes, such  
73 as stress tolerance or antibiotic resistance (Casado Muñoz et al., 2016; Sánchez et al.,  
74 2007a, b).

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

81 pumps for intracellular pH (pHi) homeostasis, repair proteins for DNA damage, changes  
82 in cellular envelope and altered metabolism (De Angelis and Gobbetti, 2004). In the  
83 present study, to gain insight into the acid resistance of potentially probiotic *L. pentosus*  
84 strains, we used comparative proteomics via two-dimensional gel electrophoresis (2-  
85 DE) and tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS)  
86 analysis under standard and acid-stress conditions with the aim of detecting, for the first  
87 time, key proteins involved in their acid resistance. Furthermore, we investigated  
88 whether acid-stress on *L. pentosus* strains had any consequences on desired probiotic  
89 features such as auto-aggregation or co-aggregation with pathogens.  
90

## 91 2. Materials and Methods

92

### 93 2.1. Bacterial cultures and growth conditions

94

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

104

### 105 2.2. Sample preparation, protein extraction, two-dimensional gel electrophoresis and 106 image analysis

107

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

123

124

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  $\mu\text{m} \times 5 \text{ mm}$  (ID  $\times$   
147 length), 5  $\mu\text{m}$  particle size, 100  $\text{\AA}$  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  $\mu\text{m} \times 500 \text{ mm}$  (ID  $\times$   
151 length), 2 mm particle size, 100 $\text{\AA}$  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.

158 Identification of peptides and proteins was performed using PEAKS 8.0, using the  
159 reference sequence of *L. pentosus* KCA1 (<http://www.uniprot.org/uniprot/I8R8S7>) and  
160 *L. pentosus* DSM 20314 (<http://www.uniprot.org/uniprot/A0A0R1FPQ6>) downloaded  
161 in FASTA format from UniprotKB and contained 12272 protein sequences annotated  
162 with Tremb identifiers. The search parameters included parent mass error tolerance of  
163 10.0 ppm and monoisotopic parent mass, fragment mass error tolerance of 0.02 Da,  
164 trypsin was used as enzyme cleavage, the maximal number of missed cleavage was 3  
165 and only tryptic peptides were considered during the search. Carbamidomethylation for  
166 reduced and alkylated cysteine was used as fix, while methionine oxidation was used as  
167 variable modification, with 6 maximal variable post-translational modification per  
168 peptide. Results were considered with false discovery rate (FDR)  $\leq$  1% at PSM,  
169 peptide and protein levels.

170

#### 171 2.4. Effect of acid stress on the probiotic profile of *L. pentosus* strains

172

173 Acid-stressed cells of *L. pentosus* strains (AP2-15, AP2-18 and LP-1) were tested for  
174 auto-aggregation capacity as described by Vizoso Pinto et al. (2007). Overnight cultures  
175 (2 ml) of lactobacilli in MRS broth (pH 4.0 or 6.5) broth were harvested, washed and  
176 resuspended in PBS. After 2 h at room temperature, 100  $\mu$ l were taken from the top of  
177 the suspension and were transferred to a cuvette containing 900  $\mu$ l PBS. The auto-  
178 aggregation percentage is expressed as:  $\text{Agg}\% = [(1 - OD_1/OD_0)] \times 100$ , where  $OD_0$  and  
179  $OD_1$  represent the absorbance values measured at 580 nm at times (0 h) and 2 h,  
180 respectively.

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

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 37°C. 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 -80°C 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



### 218 3. Results and Discussion

219

220 Proteomic analysis can provide valuable information about stress response and  
221 physiological mechanisms in bacteria. Our main objective was to identify the key  
222 proteins involved in acid resistance in *L. pentosus* under acid stress conditions. Among  
223 31 *L. pentosus* strains, we selected three strains with different levels of acid resistance at  
224 pH 1.5: *L. pentosus* LP-1 (SP), *L. pentosus* AP2-18 (IP) and *L. pentosus* AP2-15 (RP).  
225 When exposed to pH 4.0, the three strains could recover from the acid shock and grow  
226 but at different growth rates. They were harvested at early-stationary growth phase (16-  
227 20 h) depending on the strain and also on culture conditions (absence or presence of  
228 acid stress), which coincided with an OD<sub>600nm</sub> of 0.6 (data not shown). As such, cultures  
229 under both standard and acid-stress conditions reached the early-stationary phase (same  
230 OD) in comparable physiological states, thereby reducing protein pattern differences  
231 that are due to the growth phase.

232

#### 233 3.1. Comparative proteomic analysis of *L. pentosus* strains under standard conditions

234

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

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

322 Two proteins were repressed in resistant strain after acid exposure: ATP synthase  
323 subunit beta (isoform, spot 1203) involved in ATP synthesis coupled proton transport  
324 and a 60 kDa chaperonin (spot 1303) involved in protein refolding (Figure 1A, B; Table  
325 2A). However, ATP synthase subunit beta (isoform, spot 5206) was over-produced in  
326 both resistant and intermediate phenotypes in constitutive proteomes. These variations  
327 in expression levels suggest that this protein is not relevant with regard to acid  
328 resistance (Table 1).

329 For sensitive and intermediate phenotypes (*L. pentosus* LP-1 and *L. pentosus* AP2-  
330 18, respectively), protein expression patterns were highly different when comparing  
331 standard and stressing conditions (Figure 1C-F, Tables 2B and C). For example, *L.*  
332 *pentosus* AP2-18 (IP) exhibited down-regulation of proteins involved in several  
333 metabolic pathways: translational termination (spot 9102), DNA-templated  
334 transcription, termination-nucleoside metabolic process-regulation of transcription (spot  
335 9101), phosphoenolpyruvate-dependent sugar phosphotransferase system (spot 9303),  
336 fructose 1,6-bisphosphate metabolic process and glycolytic process (spot 5205), and  
337 suppressed expression of proteins involved in DNA binding (phosphorelay signal  
338 transduction system, DNA-templated regulation of transcription, DNA-templated  
339 transcription; spot 5204), ATP binding (protein folding; spot 7206) and UTP:glucose-1-  
340 phosphate uridylyltransferase activity (biosynthetic process, UDP-glucose metabolic  
341 process; spot 9305) (Figure 1C-D, Table 2B). However, *L. pentosus* LP-1, exhibiting  
342 the sensitive phenotype for acid resistance, had greater expression of proteins involved  
343 in ATP synthesis coupled proton transport (ATP synthase subunit beta; isoform, spot  
344 1602) and a manganese-dependent inorganic pyrophosphatase (spot 1505), and lower  
345 protein expression related to DNA transcription and termination, nucleoside metabolic  
346 process, regulation of DNA transcription and translation (spots 9101 and 9102) (Figure  
347 1E-F, Table 2C). This suggests that manganese-dependent inorganic pyrophosphatase  
348 (spot 1505) over-production in both resistant and sensitive phenotypes under acid  
349 challenge is not linked to acid resistance. Furthermore, the expression of ATP synthase  
350 subunit beta (isoform, spot 1602) exhibited variations in its expression between  
351 constitutive and stress conditions, and thus it could not be considered as a biomarker  
352 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

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

394

#### 395 **4. Conclusions**

396

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

411

412

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414

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418

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420

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528

529 **Figure legends**

530

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

540

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