1 **Comparative proteomic analysis of a potentially probiotic *Lactobacillus pentosus***
2 **MP-10 for the identification of key proteins involved in antibiotic resistance and**
3 **biocide tolerance**

4

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28 **Abstract**

29 Probiotic bacterial cultures require resistance mechanisms to avoid stress-related
30 responses under challenging environmental conditions; however, understanding these
31 traits is required to discern their utility in fermentative food preparations, versus clinical
32 and agricultural risk. Here, we compared the proteomic responses of *Lb. pentosus* MP-
33 10, a potentially probiotic lactic acid bacteria isolated from brines of naturally
34 fermented Aloreña green table olives, exposed to sub-lethal concentrations of antibiotics
35 (amoxicillin, chloramphenicol and tetracycline) and biocides (benzalkonium chloride
36 and triclosan). Several genes became differentially expressed depending on
37 antimicrobial exposure, such as the up-regulation of protein synthesis, and the down-
38 regulation of carbohydrate metabolism and energy production. The antimicrobials
39 appeared to have altered *Lb. pentosus* MP-10 physiology to achieve a gain of cellular
40 energy for survival. For example, biocide-adapted *Lb. pentosus* MP-10 exhibited a
41 down-regulated phosphocarrier protein HPr and an unexpressed oxidoreductase.
42 However, protein synthesis was over-expressed in antibiotic- and biocide-adapted cells
43 (ribosomal proteins and glutamyl-tRNA synthetase), possibly to compensate for
44 damaged proteins targeted by antimicrobials. Furthermore, stress proteins, such as
45 NADH peroxidase (Npx) and a small heat shock protein, were only over-expressed in
46 antibiotic-adapted *Lb. pentosus* MP-10. Results showed that adaptation to sub-lethal
47 concentrations of antimicrobials could be a good way to achieve desirable robustness of
48 the probiotic *Lb. pentosus* MP-10 to various environmental and gastrointestinal
49 conditions (e.g., acid and bile stresses).

50

51 **1. Introduction**

52 *Lactobacillus pentosus* is the most prevalent species of lactic acid bacteria (LAB)
53 found in naturally-fermented Aloreña table olives (Abriouel et al., 2011, 2012) and
54 Spanish-style green fermented olives (Maldonado-Barragán et al., 2011). Furthermore,
55 these versatile bacteria have been detected in various environmental niches such as plant
56 materials, silage, fermented foods (dairy, vegetable and meat), as well as the oral
57 cavities, gastrointestinal tracts (GIT), and vaginas of humans and animals (Anukam et
58 al., 2013; Okada et al., 1986; Tajabadi et al., 2011; Todorov and Dicks, 2004). Due to
59 their wide distribution and beneficial effects, special and deserved attention was
60 recently given to the application of lactobacilli, especially of vegetable origin, as a
61 starter culture in different fermentations (Rodríguez-Gómez et al., 2014; Ruiz-Barba
62 and Jiménez-Díaz, 2012), as a probiotic in silage (EFSA, 2011), dairy (Anukam and
63 Olise, 2012) and fermented olives (Rodríguez-Gómez et al., 2014), as they provide bio-
64 therapeutic benefits via bacterial pathogen inhibition and improved immune system.
65 More specifically, *Lb. pentosus* MP-10 isolated from brine of naturally fermented
66 Aloreña olives (Abriouel et al., 2011, 2012) could be used as a probiotic strain due to
67 their ability to inhibit pathogenic bacteria and tolerate low pH (1.5) and bile salts (3%)
68 in the gastrointestinal environment.

69 Besides the technological and health-promoting effects shown by lactobacilli with
70 probiotic properties, such as production of antimicrobial substances and survival in
71 gastrointestinal tracts, other requirements should be proven to justify their utility. The
72 most important selection criteria for bacterial strains intended for use as probiotics
73 include: 1) intrinsic resistance to antibiotics of human and veterinary importance and 2)
74 lack of transferable resistance genes to avoid the risk of horizontal gene transfer to other
75 bacteria in the food chain and environment (EFSA, 2008, EFSA Panel on Biological

76 Hazards, 2010). As such, many studies have focused on genotypic methods to highlight
77 the presence or absence of antimicrobial resistance determinants (e.g., Bautista-Gallego
78 et al., 2013; Duran and Marshall, 2005; EFSA, 2012; Franz et al., 1999; Zhang et al.,
79 2009). However, several aspects of bacterial fitness, which develop tolerance or
80 resistance to different antimicrobials used in clinical setting or disinfection, remain
81 unexplored. Bacterial adaptation to antimicrobials, which was referred by Maisonneuve
82 and Gerdes (2014) as "bacterial persisters," is the intermediary stage that links between
83 sensitive and resistant phenotypes. Thus, more attention should be provided to the
84 potential for bacterial adaptation, such as further induction of cross-resistance to other
85 treatments and modifications in colonization or virulence (Dubois-Brissonnet, 2012). To
86 detect the mechanisms adopted by different bacteria to resist to different drugs in
87 various environmental niches remains important. In this respect, several studies report
88 that physiological modifications occur during adaptation such as differential protein
89 expression, which seems to be concomitant to increased tolerance (Dubois-Brissonnet,
90 2012) and cross-resistance to other environmental stressors (Karatzas et al., 2007,
91 2008).

92 In the last decade, proteomics have been used to study bacterial physiological
93 responses to different stressors; this has progressed significantly with the availability of
94 whole-genome sequences, progress in mass spectrometry and bioinformatics.
95 Proteomics, as a key in post genomic era, provides useful data to identify new
96 diagnostic markers and therapeutic targets in diseases. Recently, genomic and proteomic
97 analyses of *Lactobacillus* genus have rapidly expanded, especially with *Lb. pentosus*
98 having one of the largest genomes known among LAB (Abriouel et al., 2011,
99 Maldonado-Barragán et al., 2011); however, little is known about the mechanisms
100 adopted by *Lb. pentosus* to tolerate or resist several stressors. This information should

101 be of great concern since knowledge of these mechanisms could be exploited to
102 improve the functionality of probiotic starter strains and, thus, their health promoting
103 benefits.

104 The present study aimed to determine the phenotypic and genotypic antimicrobial-
105 resistance profiles of *Lb. pentosus* MP-10 and the selected mechanisms, by which these
106 bacteria adapt under different antimicrobial stress. We compared the proteomic profiles
107 of this strain induced by different antimicrobials (antibiotics or biocides), each with a
108 distinct mechanism of action. The comparative analysis provides valuable knowledge
109 and a broad overview of the key proteins involved in antibiotic and biocide tolerance.
110

111 **2. Materials and Methods**

112 *2.1. Bacterial strains and growth conditions*

113 *Lactobacillus pentosus* MP-10, isolated from naturally-fermented Aloreña green
114 table olives (Abriouel et al., 2011, 2012), was routinely cultured at 30ºC in Man Rogosa
115 and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for
116 24-48 h. The strain was stored long-term in 20% glycerol at -80ºC.

117 *2.2. Antimicrobial agents*

118 The antimicrobial agents used in this study were clinically relevant antibiotics:
119 amoxicillin "AMX", ampicillin "AMP", cefuroxime "CFX", chloramphenicol "CMP",
120 ciprofloxacin "CIP", clindamycin "CLI", erythromycin "ERY", gentamicin "GEN",
121 kanamycin "KAN", streptomycin "STR", sulfamethoxazole/trimethoprim "SMZ/TMP",
122 teicoplanin "TC", trimethoprim "TMP", tetracycline "TET" and vancomycin "Van";
123 and biocides commonly used in food industry: benzalkonium chloride "BC" and
124 triclosan "TC". All antibiotics and benzalkonium chloride were purchased from Sigma
125 Aldrich (Madrid, Spain); however, triclosan was obtained from Fluka (Madrid, Spain).

126 *2.3. Phenotypic and genotypic antibiotic testing*

127 *2.3.1. Antibiotic susceptibility testing and MIC determination*

128 The MICs of the above-mentioned antibiotics were determined for *Lb. pentosus* MP-
129 10 as described by Casado Muñoz et al. (2014) in LSM broth [a mixture of 90% IST
130 broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka,Madrid, Spain)] (Klare et al.,
131 2005) according to the ISO 10932/IDF 233 standard (International Organization for
132 Standardization, 2010).

133 *2.3.2. PCR detection of antibiotic resistance genes*

134 PCR amplifications of well-known genes determinants associated with resistance to
135 β-lactam antibiotics (*bla* and *blaZ*, the β-lactamase genes), sulfonamides (*dfrA* and
136 *dfrD*) and glycopeptides (*vanA*, *vanB*, *vanC* and *vanE*) were performed using conditions
137 described elsewhere (Dutka- Malen et al., 1995; Fines et al., 1999; Hummel et al., 2007;
138 Liu et al., 2009; Martineau et al., 2000; Miele et al., 1995). Furthermore, PCR of genes
139 mediating antibiotic resistance through other mechanisms, such as efflux pumps (*mdfA*,
140 *norE*, *acrA*, *acrB*, *tolC*, *mepA*, *norA*, *norC*, *mefA* and *mdeA*), were also performed in the
141 present study. Template DNA for PCR reactions were prepared as reported in Jensen et
142 al. (1998).

143 *2.4. Tolerance induction*

144 Tolerance to antibiotics or biocides was assessed by investigating the ability of *Lb.*
145 *pentosus* MP-10 to grow in the presence of sub-lethal concentrations of the
146 corresponding antimicrobials, to which the strain was originally sensitive (amoxicillin,
147 chloramphenicol, tetracycline, benzalkonium chloride and triclosan). Tolerant
148 phenotypes were developed by increasing the concentrations of different antimicrobials
149 as described by Casado Muñoz et al. (unpublished data). Briefly, antimicrobial tolerance
150 in *Lb. pentosus* MP-10 was induced by exposure to triclosan (1 µg/ml), benzalkonium
151 chloride (1 µg/ml), cholamphenicol (5 µg/ml), tetracycline (10 µg/ml) or amoxicillin
152 (0.1 µg/ml) at 30°C for 48 h; cells were then harvested by centrifugation (Casado
153 Muñoz et al., unpublished data). All *Lb. pentosus* isolates were stored in 20% glycerol
154 at -80ºC until use. Isolates were streaked onto MRS-agar; a single colony was selected
155 and subsequently used to inoculate MRS-broth for 24h at 30°C. The resulting culture
156 was used to inoculate fresh MRS-broth at a dilution of 1:100. Cultures (both induced
157 and non-induced controls) were harvested at mid-logarithmic growth phase ($OD_{600\ nm}$ =
158 0.6).

159 *2.5. Whole cell protein extraction*

160 The cell pellets obtained, as described above, from isogenic mutants were
161 resuspended in 2 ml of PBS and dispersed into liquid nitrogen with a 200-µl
162 micropippette to obtain cryobeads. Whole-cell protein extraction was done as described
163 by Caballero-Gómez et al. (2013). The bacterial beads were ground in liquid nitrogen
164 using a cryogenic grinder (6870 Freezer/ Mill, SpexCertiPrep, Stanmore, UK) with
165 three steps of 3 min at a rate of 24 impacts/s. The samples were centrifuged at 5000 ×g
166 for 5 min (at 4 °C), and the resultant supernatants were filtered through a 0.45-µm pore
167 size filter (Chromafil PET; Macherey-Nagel, Düren, Germany). Proteins were extracted
168 from the filtered supernatants with Trizol reagent (Euromedex, Souffelweyersheim,
169 France) as previously described (Izquierdo et al., 2009). Protein concentrations were
170 determined using Bradford protein assay (Bio-Rad) according to the manufacturer's
171 instructions.

172 *2.6. 2-D gel electrophoresis*

173 Protein extracts (150 µg) were loaded onto 17-cm strips with a pH range of 3 to 10
174 (Bio-Rad), focused for 60,000 V h, and then separated on a 12% SDS-polyacrylamide
175 gel as reported previously (Izquierdo et al., 2009). The gels were stained as described by
176 Candiano et al. (2004) using Bio-Safe Coomassie brilliant blue G-250 (Bio-Rad), which
177 has a reported detection limit of 1 ng for BSA, and scanned on a GS-800 Calibrated
178 Densitometer (Bio-Rad).

179 *2.7. Image analysis*

180 Image analysis of the 2D-GE gels was performed using PD Quest 8.0.1 software
181 (Bio-Rad). Three gels were produced from independent cultures of each condition, and
182 only spots that were present on the three gels were selected for inter-condition

183 comparison. Spot intensities were normalized to the sum of intensities of all valid spots
184 in one gel. For analysis of changes in protein expression during antimicrobial exposure,
185 a protein was considered to be under- or over-produced when changes in normalized
186 spot intensities were at least 1.5-fold at a significance level of $p < 0.05$ (Student's t test
187 for paired samples), as previously described (Sánchez et al., 2007). Regarding proteome
188 comparisons between different culture conditions of *Lb. pentosus* MP-10, proteins were
189 considered differentially produced when spot intensities passed the threshold of a
190 twofold difference (one-way ANOVA, p-value < 0.05), as described previously
191 (Izquierdo et al., 2009).

192 *2.8. Protein identification*

193 Spots of interest were subjected to tryptic in-gel digestion as described by Izquierdo
194 et al. (2009) and analyzed by chip-liquid chromatography–quadrupole time-of-flight
195 (chip-LC-QTOF) using an Agilent G6510A QTOF mass spectrometer equipped with an
196 Agilent 1200 Nano LC system and an Agilent HPLC Chip Cube, G4240A (Agilent
197 Technologies, Santa Clara, CA, USA), as described previously (Hamon et al., 2011).
198 Protein identification was performed against the genome of *Lb. pentosus* KCA1
199 available at the NCBI Website (http://www.ncbi.nlm.nih.gov; accessed 4th November
200 2014), using PEAKS DB search engine (Bioinformatics Solutions Inc., Waterloo,
201 Canada). Using PEAKS inChorus feature, Mascot and PEAKS searches were compared
202 to confirm protein identities and limit the risk of false positives. Scores represent
203 peptide probabilities as calculated using PEAKS DB’s Peptide-Spectrum Matching
204 Score (-10lgP).

205 *2.9. Growth and survival of antimicrobial-induced and non-induced Lb. pentosus MP-*
206 *10 following exposure to gastric juices*

207 To determine the growth rate of antimicrobial-induced *Lb. pentosus* MP-10 in

208 comparison with control (without induction), overnight cultures were diluted 1/1000 in
209 MRS broth and viable counts were determined by serial dilutions on MRS-agar plates
210 after 4 and 8 hours of incubation at 30ºC. Increase in growth rate was determined by the
211 difference between Log_{10} CFU/ml at time X h (4 or 8 h) and Log_{10} CFU/ml at time 0 h.
212 To test if antimicrobial induction of *Lb. pentosus* MP-10 improved its tolerance to acid
213 and bile concentrations, overnight cultures were added (at 2% volume) to simulated
214 gastric juice (pepsin and NaCl) at different conditions: pH 1.5, pH 2.5, 2% bile or 3%
215 bile. The mixtures were incubated at 37ºC for 30 min and viable counts were
216 determined on MRS agar plates as described above. The survival rate was determined
217 according to Bao et al. (2010) by the following equation: Survival rate (%) = (Log_{10}
218 CFU/ml N_1/Log_{10} CFU/ml N_0) x 100
219 N_1 is the total viable count of *Lb. pentosus* MP-10 after 30 min treatment (at pH 1.5, pH
220 2.5, 2% bile or 3% bile), and N_0 is the total viable count at time 0 (before treatment).

221 **3. Results**

222 *3.1. Antibiotic susceptibility and molecular detection of antibiotic resistance genes in*
223 *Lb. pentosus MP-10*

224 MIC determinations of the different antibiotics revealed that *Lb. pentosus* MP-10
225 were sensitive to amoxicillin (MIC = 0.2 µg/ml), ampicillin (MIC = 0.2 µg/ml),
226 chloramphenicol (MIC = 0.04 µg/ml), clindamycin (MIC = 0.2 µg/ml), erythromycin
227 (MIC = 0.1 µg/ml), gentamycin (MIC = 0.8 µg/ml), kanamycin (MIC = 16 µg/ml),
228 streptomycin (MIC = 150 µg/ml) and tetracycline (MIC = 8 µg/ml). However, *Lb.*
229 *pentosus* MP-10 showed resistance to cefuroxime (MIC = 100 µg/ml), ciprofloxacin
230 (MIC = 8 µg/ml), teicoplanin (MIC > 128 µg/ml), trimethoprim (MIC = 128 µg/ml),
231 trimethoprim/sulfamethoxazole (MIC = 950/50 µg/ml) and vancomycin (MIC > 128
232 µg/ml). In most cases, resistance or sensitivity was categorized based on the
233 microbiological breakpoints of the antibiotics tested (also defined as ECOFF by the
234 European Food Safety Authority; European Food Safety Authority, 2012), which was
235 reviewed by Casado Muñoz et al. (2014).

236 To identify possible genetic determinants responsible for the resistance phenotypes
237 observed in *Lb. pentosus* MP-10, PCR reactions were performed as described above.
238 However, results revealed an absence of specific resistance determinants, except *norA*
239 coding for a multidrug efflux pump was detected.

240 *3.2. Influence of antibiotics on protein expression levels in Lb. pentosus MP-10*

241 Based on antibiotic susceptibility results, amoxicillin, chloramphenicol and
242 tetracycline were selected to carry out tolerance studies. We compared the proteomes of
243 antibiotic-treated and untreated *Lb. pentosus* MP-10 to elucidate the physiological
244 changes resulting from the treatments. 2D-GE analysis of antibiotic-treated cells,

245 collected during mid-exponential growth phase, showed different proteomic profiles
246 depending on the antibiotic used, suggesting various antibiotic stress responses (Fig. 1).
247 Treatment with chloramphenicol, amoxicillin and tetracycline resulted in two, four and
248 six proteins (respectively) that significantly ($P < 0.05$) differed to the pattern from the
249 untreated control (Fig. 1). These proteins were individually excised from duplicate 2D-
250 GE gels, subjected to tryptic digestion, and identified by chip-LC-QTOF and Uniprot
251 database searching (summarized in Table 1). Treatment with amoxicillin or
252 chloramphenicol resulted in an under-expressed CTP synthase (spot 0102), an enzyme
253 involved in nucleotide synthesis that requires ATP for its metabolic function. On the
254 other hand, proteins involved in other metabolic pathways such as carbohydrate
255 metabolism (phosphocarrier protein HPr of the phosphotransferase system “PTS”, spot
256 4201), homeostasis (NADH peroxidase Npx, spot 6101) and protein synthesis (SSU
257 ribosomal protein S6p, spot 7202) became over-expressed in the presence of
258 amoxicillin. Similarly, three proteins carrying different biological functions were over-
259 expressed in the presence of tetracycline: 6-phosphogluconate dehydrogenase (spot
260 7605), involved in carbohydrate metabolism; a small heat shock protein (spot 7802)
261 responsible of cell protection; and LSU ribosomal protein L1p (spot 7803), implicated
262 in protein synthesis (Fig. 1, Table 1).

263 The following three proteins were only expressed in the absence of tetracycline:
264 pyruvate kinase (spot 3102) and NAD-dependent glyceraldehyde-3-phosphate
265 dehydrogenase (spot 4501), which are linked to carbohydrate metabolism, as well as
266 acetaldehyde dehydrogenase (spot 3104), which is involved in alcohol and fat
267 metabolism (Fig. 1, Table 1). In the case of chloramphenicol, the only protein not
268 produced, compared with the untreated control, was 6-phosphofructokinase, which is
269 related to carbohydrate metabolism.

270 *3.3. Influence of biocides on protein expression levels in L. pentosus MP-10*

271 According to biocide susceptibility pattern by *Lb. pentosus* MP-10 (Casado Muñoz et
272 al., unpublished data), we selected benzalkonium chloride and triclosan for further
273 tolerance studies. Following treatment with biocides (benzalkonium chloride or
274 triclosan), the proteomes of *Lb. pentosus* MP-10 were compared with untreated bacteria.
275 The benzalkonium chloride exposure resulted in only one protein significantly ($P <$
276 0.05) over-expressed in the induced cells: ribosomal subunit interface protein (spot
277 6603), which is related to protein biosynthesis (Fig. 2, Table 2). However, the proteome
278 of *Lb. pentosus* MP-10 treated with triclosan showed significant ($P < 0.05$) differential
279 expression among three proteins: an over-expressed glutamyl-tRNA synthetase (spot
280 5801), linked to amino acid starvation; an under-expressed phosphocarrier protein HPr
281 of the PTS (spot 4401), related to carbohydrate metabolism; and no detection of
282 oxidoreductase of the aldo/keto reductase family (spot 5301), involved in energy
283 production and conversion (Fig. 2, Table 2).
284

285 *3.4. Survival and tolerance responses of antimicrobial-induced Lactobacillus pentosus*
286 *MP-10.*

287 As shown in Table 3, the growth rate was increased in almost all antimicrobial-
288 induced *Lb. pentosus* MP-10 by 0.09-0.32 Log_{10} units after 4 or 8 h incubation at 30ºC
289 except in chloramphenicol-induced cells, which showed the same growth rate as non-
290 induced controls.

291 Comparison of survival capacity of non-induced and antimicrobial-induced *Lb.*
292 *pentosus* MP-10 under acid or bile (2 and 3%) stress determined that antimicrobial
293 induction improved tolerance capacity of *Lb. pentosus* MP-10 at acidic conditions. The
294 bacteria had >100% survival and they exhibited slightly greater growth than the

295 controls (94% and 100%, at pH 1.5 and 2.5, respectively) (Table 3). . Regarding bile
296 tolerance, at both concentrations of 2 and 3% of bile we observed 100% survival, or
297 better, in benzalkonium- and triclosan-induced cells; moreover at 2% bile concentration,
298 chloramphenicol-induced *Lb. pentosus* MP-10 also showed 100% survival (Table 3).
299 However, at 3% bile concentration, bacteria pre-exposed to amoxicillin,
300 chloramphenicol and tetracycline became less viable (Table 3).

301
302

303 **Discussion**

304 The importance of probiotic bacteria, which are mainly members of the genera
305 *Lactobacillus* and *Bifidobacterium,* has increasingly become recognized in human and
306 animal nutrition by their contributions to immunological, digestive, and respiratory
307 health. However, according to the Qualified Presumption of Safety (QPS) approach
308 proposed by the European Food Safety Authority (EFSA, 2008), the presence of
309 antibiotic resistance determinants is one of the most important safety selection criteria
310 for bacterial strains intended for use in the food industry, even among bacteria that are
311 generally recognized as "safe". Here, *Lb. pentosus* MP-10 isolated from brines of
312 naturally fermented Aloreña green table olives (Abriouel et al., 2011, 2012) could be
313 regarded as "safe" because of the absence of acquired resistance determinants. Their
314 intrinsic resistance to more than three antibiotics, which relies on chromosomally
315 encoded efflux pumps such as NorA, is unlikely to be an issue from a medical point of
316 view, since *Lb. pentosus* MP-10 remains highly sensitive to other clinically relevant
317 antibiotics.

318 However, the survival of probiotic bacteria and their beneficial probiotic effects
319 under different environmental conditions, including those encountered in the
320 gastrointestinal tract, may rely on the resistance traits. As such, knowing which proteins
321 are involved in tolerance is important to improve the functionality of probiotic strains
322 under different stress conditions. In the present study, we investigated the proteomic
323 response of probiotic bacteria *Lb. pentosus* MP-10 to antimicrobial stress conditions.
324 Antibiotics and biocides induced adaptations in *Lb. pentosus* MP-10 as evidenced by
325 modifications of its proteomic arsenal, with the observed changes being intimately
326 dependent on the antimicrobial used. Overall, antibiotics induced several physiological
327 modifications, possibly due to various mechanisms of action, each targeting a defined

328 cellular structure; in comparison, biocides induced fewer modifications. Adaptation to
329 antibiotics is likely to trigger comparatively more physiological modifications than
330 biocides; several resistance mechanisms to antibiotics have had a longer evolution
331 process to protect bacteria, compared with the more relatively recent exposure to
332 biocides and limited opportunity to develop resistance. Overall, several proteins
333 involved in carbohydrate metabolism like phosphocarrier protein HPr of the PTS, as
334 part of glycolysis-related machinery, and 6-phosphogluconate dehydrogenase of the
335 pentose phosphate pathway were up-regulated after exposure to antibiotics (amoxicillin
336 or tetracycline) targeting different cellular structures. Increasing the level of ATP
337 synthesis (Wilkins et al., 2002) was either required for the increased efflux activity or
338 compensating the low glycolytic capacity (Wouters et al., 2000), and is an important
339 factor for survival under stress conditions. Similar results were obtained with
340 *Bifidobacterium animalis* and *Lactobacillus reuteri* under bile stress (Lee et al., 2008;
341 Sánchez et al., 2007). Furthermore, HPr (histidine-containing protein) protein is not
342 only responsible for carbohydrate uptake; it also plays a regulatory role in sugar
343 metabolism and catabolite repression, depending on protein-protein interactions with
344 many cellular factors (Deutscher et al., 2006). Accordingly, other proteins involved in
345 glycolysis pathways such as 6-phosphofructokinase, and pyruvate kinase and NAD-
346 dependent glyceraldehyde-3-phosphate dehydrogenase were down-regulated in the
347 presence of antibiotics inhibiting protein synthesis -chloramphenicol and tetracycline,
348 respectively. Pyruvate, end product of glycolysis, is a metabolic key molecule that can
349 be used in a number of different reactions to increase the ATP levels, thus antibiotic
350 stress induced regulation of metabolism by down- or up-regulation of enzymes involved
351 in energy production. These data suggest that, to ensure survival under antibiotic stress,
352 *Lb. pentosus* MP-10 physiology may be altered to achieve a higher cellular energy gain

353 via up- or down-regulation of carbohydrate metabolism (pentose and glycolysis
354 pathways). Under antibiotic stress and subsequent limited energy conditions, PTS
355 transport systems are used rather than ABC transporters (Taranto et al., 1999). These
356 systems are, in fact, more energy efficient as the phosphorylated substrate can directly
357 enter glycolysis or pentose phosphate pathways, conserving ATP. Similarly, Lin et al.
358 (2014) reported that fluctuation of metabolic pathways may represent an antibiotic-
359 resistance mechanism under chlortetracycline stress in *Escherichia coli*.

360 The interaction of amoxicillin and tetracycline with membrane lipids and proteins
361 induced the over-expression of stress proteins, such as NADH peroxidase Npx and a
362 small heat shock protein, respectively, as a first response of the cell to maintain
363 homeostasis and viability. Furthermore, it has been reported that, besides its role in cell
364 redox homeostasis (degradation of hydrogen peroxide to water and oxygen), Npx of the
365 Peroxidase-Oxidase-Reductase (POR) subgroup of the Flavoprotein-Disulphide-
366 Reductase (FDR) family also contributes to the regeneration of oxidized pyridine
367 nucleotides for glycolysis (Ying, 2006). Small heat shock proteins as "minichaperones"
368 have been associated with enhanced bacterial survival during stress, since they are
369 necessary for normal cellular functions, including growth and stability of DNA and
370 RNA. They also prevent the formation of inclusion bodies (Jakob et al., 1993;
371 Narberhaus, 2002; Veinger et al., 1998), but are not involved in protein re-folding as
372 chaperones.

373 On the other hand, protein synthesis in *Lb. pentosus* MP-10, exposed to amoxicillin
374 and tetracycline, was up-regulated. However, it has been reported that the proteins
375 involved in cell growth, such as ribosomal proteins, were markedly under-regulated
376 under stress conditions as an energy-saving strategy necessary for protection
377 mechanisms in the cell (Rezzonico et al., 2007). In spite of the fact that ribosomal run-

378 off and transit times are slower upon stressor exposure, stress-regulatory factors are
379 preferentially associated with ribosomes, suggesting increased translation and protein
380 synthesis (Sherman and Qian, 2013). Enhanced protein synthesis may be required to
381 compensate for the proteins damaged as a result of the interaction of antibiotics with the
382 membrane or cytoplasmic proteins, regardless of their cellular target. Some may be
383 involved in metabolism or defense (SOS response and heat shock response). Similarly,
384 Mangalappalli-Illathu and Korber (2006) reported that higher levels of ribosomal
385 proteins associated with increased protein synthesis were important for reduced
386 susceptibility to quaternary ammonium compounds like benzalkonium chloride.

387 Concerning other metabolic pathways, the enzymes involved in fatty-acid (alcohol
388 dehydrogenase) and pyrimidine biosynthesis (CTP synthase) were down-regulated in
389 the presence of antibiotics. Alterations in fatty-acid biosynthesis may lead to changes in
390 the cell membrane that would favour cell survival in the presence of tetracycline;
391 Rogers et al. (2007) obtained similar results with penicillin-exposed *Streptococcus*
392 *pneumoniae*. Regarding CTP synthase, this enzyme is required for the biosynthesis of
393 ribo- and deoxiribonucleotides for RNA and DNA replication (Jørgensen et al., 2004).
394 Lowered growth rates obtained just after exposure to antibiotics may have reflected the
395 down-regulation of proteins involved in nucleotide synthesis and fatty acids. However,
396 after antimicrobial exposure, growth rates were either similar or even increased in some
397 antimicrobial-induced cells (e.g., amoxicillin- or benzalkonium-induced cells).

398 On the other hand, the adaptation of *Lb. pentosus* MP-10 to biocides (benzalkonium
399 chloride or triclosan) induced physiological modifications that are, in part, similar to
400 those caused by antibiotics such as up-regulation of protein synthesis, and down-
401 regulation of carbohydrate metabolism and energy production (Fig. 3). In fact, cross-
402 resistance between antibiotics and biocides was widely reported in literature (e.g.,

403 Fraise, 2002; Moken et al., 1997; Randall et al., 2007). Also, in a previous study, pre-
404 adapted *Lb. pentosus* MP-10 to low concentrations of biocides showed increased
405 antibiotic MICs (Casado Muñoz et al., unpublished data), suggesting that the
406 physiological modifications triggered by either a biocide or an antibiotic may provide
407 resistance to the other. Benzalkonium chloride, a disinfectant known to cause membrane
408 damage, specifically induced an over-expression of ribosomal subunit interface protein
409 related to protein synthesis. However, triclosan caused over-expression of glutamyl-
410 tRNA synthetase, which is considered a key enzyme required for protein biosynthesis.
411 Furthermore, triclosan caused down-regulation of proteins involved in carbohydrate
412 metabolism (phosphocarrier protein HPr) and energy production (oxidoreductase). As
413 stated previously with antibiotics, cells adapted to antimicrobials tended to lower
414 carbohydrate metabolism and energy production, while those involved in protein
415 synthesis were up-regulated to possibly compensate for protein damage as a result of the
416 interaction of biocides with the membrane. Moreover, benzalkonium chloride and
417 triclosan exhibited different adaptation responses, which may be attributed to different
418 mechanisms of action; triclosan acts by inhibiting the enoyl reductase enzyme in fatty
419 acid synthesis (Heath et al., 2002), while benzalkonium chloride has multiple targets in
420 microbial cells (Beumer et al., 2000).
421 In conclusion, we obtained a better understanding of the proteomic responses of a
422 probiotic bacterium, such as *Lb. pentosus* MP-10 to different antimicrobial stressors;. In
423 this sense, we confirmed that antimicrobial stress could enhance bacterial resistance to
424 environmental and gastrointestinal stresses such as acid and bile. Thus, viable counts of
425 some antimicrobial-induced *Lb. pentosus* MP-10 were higher than the non-induced
426 strain. From this information, one could develop strategies to improve the persistence
427 and resistance of this bacterium under different environmental conditions. It has been

428 previously shown that adaptation to different stresses (salt, low pH, bile, high
429 temperature, etc.) could be used as a strategy to enhance the technological performance
430 of probiotic lactobacilli (Corcoran et al., 2006; Desmond et al., 2001; Mills et al., 2011).
431 In our study, pre-stressed *Lb. pentosus* MP-10 exhibited greater viability than those
432 without previous induction (except few cases) and had increased tolerance to acidic and
433 high-bile environments than the controls. Here, we describe for the first time that
434 antimicrobial stress adaptation could improve the resistance and robustness of potential
435 probiotic *Lb. pentosus* MP-10 with the aim to withstand conditions where sub-lethal
436 concentrations of antimicrobials and stress conditions (e.g., at low pH or high-bile
437 concentration) may be present, such as the food chain, the environment, or the
438 gastrointestinal tract. On the other hand, this fact is greatly concerning since pathogenic
439 bacteria, as they can develop antimicrobial resistance after exposure to antimicrobials,
440 could possibly develop resistance to intestinal conditions. Our results show that *Lb.*
441 *pentosus* MP-10 responds to the exposure of biocides and antibiotics by adjusting its
442 proteomic arsenal as a survival strategy: up-regulating protein synthesis, including
443 stress proteins, and down-regulating carbohydrate metabolism and energy production
444 (Fig. 3). Further studies are required to elucidate which proteins are involved in acid
445 and bile tolerance. These aspects should be further emphasized with the aim to achieve
446 desirable robustness of probiotic bacteria in relation to various environmental and
447 gastrointestinal conditions.
448

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454

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649

650 **Figure legends**

651

652 **Figure 1.** 2-DE gels of whole cell proteomes from *Lactobacillus pentosus* MP-10
653 cultured in the absence (A) or presence of amoxicillin (B), chloramphenicol (C) and
654 tetracycline (D). The figure shows representative 2-DE gel pictures (pH range: 4-7) of
655 whole-cell protein lysates from early stationary phase of *Lb. pentosus* MP-10 Spots
656 exhibiting constitutive differential expression between growth of *Lb. pentosus* MP-10 in
657 standard conditions and after induction by antibiotics were identified by peptide mass
658 fingerprinting and are labeled, and the identifications of the spots affected by antibiotics
659 are listed in Table 1.

660

661 **Figure 2.** 2-DE gels of whole cell proteomes from *Lactobacillus pentosus* MP-10
662 cultured in the absence (A) or presence of benzalkonium chloride (B) and triclosan (C).
663 The figure shows representative 2-DE gel pictures (pH range: 4-7) of whole-cell protein
664 lysates from early stationary phase of *Lb. pentosus* MP-10 Spots exhibiting constitutive
665 differential expression between growth of *Lb. pentosus* MP-10 in standard conditions
666 and after induction by biocides were identified by peptide mass fingerprinting and are
667 labeled, and the identifications of the spots affected by biocides are listed in Table 2.

668

669 **Figure 3.** Schematic representation of the effect of antibiotics and biocides on protein
670 expression in *Lactobacillus pentosus* MP-10.