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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

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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. pentosu*s 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. pentosu*s 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. pentosu*s 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. pentosu*s MP-10 to various environmental and gastrointestinal

49   conditions (e.g., acid and bile stresses).

50

1. Introduction

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

Besides the technological and health-promoting effects shown by lactobacilli with probiotic properties, such as production of antimicrobial substances and survival in gastrointestinal tracts, other requirements should be proven to justify their utility. The most important selection criteria for bacterial strains intended for use as probiotics include: 1) intrinsic resistance to antibiotics of human and veterinary importance and 2) lack of transferable resistance genes to avoid the risk of horizontal gene transfer to other 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

2. Materials and Methods

2.1. Bacterial strains and growth conditions

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

2.2. Antimicrobial agents

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

2.3. Phenotypic and genotypic antibiotic testing

2.3.1. Antibiotic susceptibility testing and MIC determination

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

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

8

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).

3. Results

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

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

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

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

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

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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

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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

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Discussion

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

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

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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.,

18

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.

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453   Jaén).

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Figure legends

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

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

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