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

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Abstract

Probiotic bacterial cultures require resistance mechanisms to avoid stress-related responses under challenging environmental conditions; however, understanding these traits is required to discern their utility in fermentative food preparations, versus clinical and agricultural risk. Here, we compared the proteomic responses of *Lb. pentosus* MP-10, a potentially probiotic lactic acid bacteria isolated from brines of naturally fermented Aloreña green table olives, exposed to sub-lethal concentrations of antibiotics (amoxicillin, chloramphenicol and tetracycline) and biocides (benzalkonium chloride and triclosan). Several genes became differentially expressed depending on antimicrobial exposure, such as the up-regulation of protein synthesis, and the down-regulation of carbohydrate metabolism and energy production. The antimicrobials appeared to have altered *Lb. pentosus* MP-10 physiology to achieve a gain of cellular energy for survival. For example, biocide-adapted *Lb. pentosus* MP-10 exhibited a down-regulated phosphocarrier protein HPr and an unexpressed oxidoreductase. However, protein synthesis was over-expressed in antibiotic- and biocide-adapted cells (ribosomal proteins and glutamyl-tRNA synthetase), possibly to compensate for damaged proteins targeted by antimicrobials. Furthermore, stress proteins, such as NADH peroxidase (Npx) and a small heat shock protein, were only over-expressed in antibiotic-adapted *Lb. pentosus* MP-10. Results showed that adaptation to sub-lethal concentrations of antimicrobials could be a good way to achieve desirable robustness of the probiotic *Lb. pentosus* MP-10 to various environmental and gastrointestinal conditions (e.g., acid and bile stresses).
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
Hazards, 2010). As such, many studies have focused on genotypic methods to highlight the presence or absence of antimicrobial resistance determinants (e.g., Bautista-Gallego et al., 2013; Duran and Marshall, 2005; EFSA, 2012; Franz et al., 1999; Zhang et al., 2009). However, several aspects of bacterial fitness, which develop tolerance or resistance to different antimicrobials used in clinical setting or disinfection, remain unexplored. Bacterial adaptation to antimicrobials, which was referred by Maisonneuve and Gerdes (2014) as “bacterial persisters,” is the intermediary stage that links between sensitive and resistant phenotypes. Thus, more attention should be provided to the potential for bacterial adaptation, such as further induction of cross-resistance to other treatments and modifications in colonization or virulence (Dubois-Brissonnet, 2012). To detect the mechanisms adopted by different bacteria to resist to different drugs in various environmental niches remains important. In this respect, several studies report that physiological modifications occur during adaptation such as differential protein expression, which seems to be concomitant to increased tolerance (Dubois-Brissonnet, 2012) and cross-resistance to other environmental stressors (Karatzas et al., 2007, 2008).

In the last decade, proteomics have been used to study bacterial physiological responses to different stressors; this has progressed significantly with the availability of whole-genome sequences, progress in mass spectrometry and bioinformatics. Proteomics, as a key in post genomic era, provides useful data to identify new diagnostic markers and therapeutic targets in diseases. Recently, genomic and proteomic analyses of *Lactobacillus* genus have rapidly expanded, especially with *Lb. pentosus* having one of the largest genomes known among LAB (Abriouel et al., 2011, Maldonado-Barragán et al., 2011); however, little is known about the mechanisms adopted by *Lb. pentosus* to tolerate or resist several stressors. This information should
be of great concern since knowledge of these mechanisms could be exploited to improve the functionality of probiotic starter strains and, thus, their health promoting benefits.

The present study aimed to determine the phenotypic and genotypic antimicrobial-resistance profiles of *Lb. pentosus* MP-10 and the selected mechanisms, by which these bacteria adapt under different antimicrobial stress. We compared the proteomic profiles of this strain induced by different antimicrobials (antibiotics or biocides), each with a distinct mechanism of action. The comparative analysis provides valuable knowledge and a broad overview of the key proteins involved in antibiotic and biocide tolerance.
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 30°C 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 -80°C.

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

2.4. Tolerance induction

Tolerance to antibiotics or biocides was assessed by investigating the ability of Lb. pentosus MP-10 to grow in the presence of sub-lethal concentrations of the corresponding antimicrobials, to which the strain was originally sensitive (amoxicillin, chloramphenicol, tetracycline, benzalkonium chloride and triclosan). Tolerant phenotypes were developed by increasing the concentrations of different antimicrobials as described by Casado Muñoz et al. (unpublished data). Briefly, antimicrobial tolerance in Lb. pentosus MP-10 was induced by exposure to triclosan (1 µg/ml), benzalkonium chloride (1 µg/ml), chlamphenicol (5 µg/ml), tetracycline (10 µg/ml) or amoxicillin (0.1 µg/ml) at 30°C for 48 h; cells were then harvested by centrifugation (Casado Muñoz et al., unpublished data). All Lb. pentosus isolates were stored in 20% glycerol at -80°C until use. Isolates were streaked onto MRS-agar; a single colony was selected and subsequently used to inoculate MRS-broth for 24h at 30°C. The resulting culture was used to inoculate fresh MRS-broth at a dilution of 1:100. Cultures (both induced and non-induced controls) were harvested at mid-logarithmic growth phase (OD₆₀₀nm = 0.6).
2.5. Whole cell protein extraction

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

2.6. 2-D gel electrophoresis

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

2.7. Image analysis

Image analysis of the 2D-GE gels was performed using PD Quest 8.0.1 software (Bio-Rad). Three gels were produced from independent cultures of each condition, and only spots that were present on the three gels were selected for inter-condition
comparison. Spot intensities were normalized to the sum of intensities of all valid spots in one gel. For analysis of changes in protein expression during antimicrobial exposure, a protein was considered to be under- or over-produced when changes in normalized spot intensities were at least 1.5-fold at a significance level of $p < 0.05$ (Student's $t$ test for paired samples), as previously described (Sánchez et al., 2007). Regarding proteome comparisons between different culture conditions of *Lb. pentosus* MP-10, proteins were considered differentially produced when spot intensities passed the threshold of a twofold difference (one-way ANOVA, $p$-value $< 0.05$), as described previously (Izquierdo et al., 2009).

2.8. Protein identification

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

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

To determine the growth rate of antimicrobial-induced *Lb. pentosus* MP-10 in
comparison with control (without induction), overnight cultures were diluted 1/1000 in MRS broth and viable counts were determined by serial dilutions on MRS-agar plates after 4 and 8 hours of incubation at 30°C. Increase in growth rate was determined by the difference between \( \log_{10} \text{CFU/ml} \) at time X h (4 or 8 h) and \( \log_{10} \text{CFU/ml} \) at time 0 h.

To test if antimicrobial induction of \textit{Lb. pentosus} MP-10 improved its tolerance to acid and bile concentrations, overnight cultures were added (at 2% volume) to simulated gastric juice (pepsin and NaCl) at different conditions: pH 1.5, pH 2.5, 2% bile or 3% bile. The mixtures were incubated at 37°C for 30 min and viable counts were determined on MRS agar plates as described above. The survival rate was determined according to Bao et al. (2010) by the following equation: Survival rate (%) = (\( \log_{10} \text{CFU/ml} N_1 / \log_{10} \text{CFU/ml} N_0 \)) x 100

\( N_1 \) is the total viable count of \textit{Lb. pentosus} MP-10 after 30 min treatment (at pH 1.5, pH 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,
collected during mid-exponential growth phase, showed different proteomic profiles
depending on the antibiotic used, suggesting various antibiotic stress responses (Fig. 1).
Treatment with chloramphenicol, amoxicillin and tetracycline resulted in two, four and
six proteins (respectively) that significantly (P < 0.05) differed to the pattern from the
untreated control (Fig. 1). These proteins were individually excised from duplicate 2D-
GE gels, subjected to tryptic digestion, and identified by chip-LC-QTOF and Uniprot
database searching (summarized in Table 1). Treatment with amoxicillin or
chloramphenicol resulted in an under-expressed CTP synthase (spot 0102), an enzyme
involved in nucleotide synthesis that requires ATP for its metabolic function. On the
other hand, proteins involved in other metabolic pathways such as carbohydrate
metabolism (phosphocarrier protein HPr of the phosphotransferase system “PTS”, spot
4201), homeostasis (NADH peroxidase Npx, spot 6101) and protein synthesis (SSU
ribosomal protein S6p, spot 7202) became over-expressed in the presence of
amoxicillin. Similarly, three proteins carrying different biological functions were over-
expressed in the presence of tetracycline: 6-phosphogluconate dehydrogenase (spot
7605), involved in carbohydrate metabolism; a small heat shock protein (spot 7802)
responsible of cell protection; and LSU ribosomal protein L1p (spot 7803), implicated
in protein synthesis (Fig. 1, Table 1).

The following three proteins were only expressed in the absence of tetracycline:
pyruvate kinase (spot 3102) and NAD-dependent glyceraldehyde-3-phosphate
dehydrogenase (spot 4501), which are linked to carbohydrate metabolism, as well as
acetaldehyde dehydrogenase (spot 3104), which is involved in alcohol and fat
metabolism (Fig. 1, Table 1). In the case of chloramphenicol, the only protein not
produced, compared with the untreated control, was 6-phosphofructokinase, which is
related to carbohydrate metabolism.
3.3. Influence of biocides on protein expression levels in L. pentosus MP-10

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

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

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

Comparison of survival capacity of non-induced and antimicrobial-induced Lb. pentosus MP-10 under acid or bile (2 and 3%) stress determined that antimicrobial induction improved tolerance capacity of Lb. pentosus MP-10 at acidic conditions. The bacteria had >100% survival and they exhibited slightly greater growth than the
controls (94% and 100%, at pH 1.5 and 2.5, respectively) (Table 3). Regarding bile
tolerance, at both concentrations of 2 and 3% of bile we observed 100% survival, or
better, in benzalkonium- and triclosan-induced cells; moreover at 2% bile concentration,
chloramphenicol-induced \textit{Lb. pentosus} MP-10 also showed 100% survival (Table 3).
However, at 3% bile concentration, bacteria pre-exposed to amoxicillin,
chloramphenicol and tetracycline became less viable (Table 3).
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
cellular structure; in comparison, biocides induced fewer modifications. Adaptation to antibiotics is likely to trigger comparatively more physiological modifications than biocides; several resistance mechanisms to antibiotics have had a longer evolution process to protect bacteria, compared with the more relatively recent exposure to biocides and limited opportunity to develop resistance. Overall, several proteins involved in carbohydrate metabolism like phosphocarrier protein HPr of the PTS, as part of glycolysis-related machinery, and 6-phosphogluconate dehydrogenase of the pentose phosphate pathway were up-regulated after exposure to antibiotics (amoxicillin or tetracycline) targeting different cellular structures. Increasing the level of ATP synthesis (Wilkins et al., 2002) was either required for the increased efflux activity or compensating the low glycolytic capacity (Wouters et al., 2000), and is an important factor for survival under stress conditions. Similar results were obtained with Bifidobacterium animalis and Lactobacillus reuteri under bile stress (Lee et al., 2008; Sánchez et al., 2007). Furthermore, HPr (histidine-containing protein) protein is not only responsible for carbohydrate uptake; it also plays a regulatory role in sugar metabolism and catabolite repression, depending on protein-protein interactions with many cellular factors (Deutscher et al., 2006). Accordingly, other proteins involved in glycolysis pathways such as 6-phosphofructokinase, and pyruvate kinase and NAD-dependent glyceraldehyde-3-phosphate dehydrogenase were down-regulated in the presence of antibiotics inhibiting protein synthesis -chloramphenicol and tetracycline, respectively. Pyruvate, end product of glycolysis, is a metabolic key molecule that can be used in a number of different reactions to increase the ATP levels, thus antibiotic stress induced regulation of metabolism by down- or up-regulation of enzymes involved in energy production. These data suggest that, to ensure survival under antibiotic stress, *Lb. pentosus* MP-10 physiology may be altered to achieve a higher cellular energy gain.
via up- or down-regulation of carbohydrate metabolism (pentose and glycolysis pathways). Under antibiotic stress and subsequent limited energy conditions, PTS transport systems are used rather than ABC transporters (Taranto et al., 1999). These systems are, in fact, more energy efficient as the phosphorylated substrate can directly enter glycolysis or pentose phosphate pathways, conserving ATP. Similarly, Lin et al. (2014) reported that fluctuation of metabolic pathways may represent an antibiotic-resistance mechanism under chlortetracycline stress in *Escherichia coli*.

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

On the other hand, protein synthesis in *Lb. pentosus* MP-10, exposed to amoxicillin and tetracycline, was up-regulated. However, it has been reported that the proteins involved in cell growth, such as ribosomal proteins, were markedly under-regulated under stress conditions as an energy-saving strategy necessary for protection mechanisms in the cell (Rezzonico et al., 2007). In spite of the fact that ribosomal run-
off and transit times are slower upon stressor exposure, stress-regulatory factors are preferentially associated with ribosomes, suggesting increased translation and protein synthesis (Sherman and Qian, 2013). Enhanced protein synthesis may be required to compensate for the proteins damaged as a result of the interaction of antibiotics with the membrane or cytoplasmic proteins, regardless of their cellular target. Some may be involved in metabolism or defense (SOS response and heat shock response). Similarly, Mangalappalli-Illathu and Korber (2006) reported that higher levels of ribosomal proteins associated with increased protein synthesis were important for reduced susceptibility to quaternary ammonium compounds like benzalkonium chloride.

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

On the other hand, the adaptation of Lb. pentosus MP-10 to biocides (benzalkonium chloride or triclosan) induced physiological modifications that are, in part, similar to those caused by antibiotics such as up-regulation of protein synthesis, and down-regulation of carbohydrate metabolism and energy production (Fig. 3). In fact, cross-resistance between antibiotics and biocides was widely reported in literature (e.g.,
Fraise, 2002; Moken et al., 1997; Randall et al., 2007). Also, in a previous study, pre-
adapted *Lb. pentosus* MP-10 to low concentrations of biocides showed increased
antibiotic MICs (Casado Muñoz et al., unpublished data), suggesting that the
physiological modifications triggered by either a biocide or an antibiotic may provide
resistance to the other. Benzalkonium chloride, a disinfectant known to cause membrane
damage, specifically induced an over-expression of ribosomal subunit interface protein
related to protein synthesis. However, triclosan caused over-expression of glutamyl-
tRNA synthetase, which is considered a key enzyme required for protein biosynthesis.
Furthermore, triclosan caused down-regulation of proteins involved in carbohydrate
metabolism (phosphocarrier protein HPr) and energy production (oxidoreductase). As
stated previously with antibiotics, cells adapted to antimicrobials tended to lower
carbohydrate metabolism and energy production, while those involved in protein
synthesis were up-regulated to possibly compensate for protein damage as a result of the
interaction of biocides with the membrane. Moreover, benzalkonium chloride and
triclosan exhibited different adaptation responses, which may be attributed to different
mechanisms of action; triclosan acts by inhibiting the enoyl reductase enzyme in fatty
acid synthesis (Heath et al., 2002), while benzalkonium chloride has multiple targets in
microbial cells (Beumer et al., 2000).

In conclusion, we obtained a better understanding of the proteomic responses of a
probiotic bacterium, such as *Lb. pentosus* MP-10 to different antimicrobial stressors. In
this sense, we confirmed that antimicrobial stress could enhance bacterial resistance to
environmental and gastrointestinal stresses such as acid and bile. Thus, viable counts of
some antimicrobial-induced *Lb. pentosus* MP-10 were higher than the non-induced
strain. From this information, one could develop strategies to improve the persistence
and resistance of this bacterium under different environmental conditions. It has been
previously shown that adaptation to different stresses (salt, low pH, bile, high
temperature, etc.) could be used as a strategy to enhance the technological performance
of probiotic lactobacilli (Corcoran et al., 2006; Desmond et al., 2001; Mills et al., 2011).
In our study, pre-stressed *Lb. pentosus* MP-10 exhibited greater viability than those
without previous induction (except few cases) and had increased tolerance to acidic and
high-bile environments than the controls. Here, we describe for the first time that
antimicrobial stress adaptation could improve the resistance and robustness of potential
probiotic *Lb. pentosus* MP-10 with the aim to withstand conditions where sub-lethal
concentrations of antimicrobials and stress conditions (e.g., at low pH or high-bile
concentration) may be present, such as the food chain, the environment, or the
gastrointestinal tract. On the other hand, this fact is greatly concerning since pathogenic
bacteria, as they can develop antimicrobial resistance after exposure to antimicrobials,
could possibly develop resistance to intestinal conditions. Our results show that *Lb.
pentosus* MP-10 responds to the exposure of biocides and antibiotics by adjusting its
proteomic arsenal as a survival strategy: up-regulating protein synthesis, including
stress proteins, and down-regulating carbohydrate metabolism and energy production
(Fig. 3). Further studies are required to elucidate which proteins are involved in acid
and bile tolerance. These aspects should be further emphasized with the aim to achieve
desirable robustness of probiotic bacteria in relation to various environmental and
gastrointestinal conditions.

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Jaén).
References


EFSA Panel on Biological Hazards (BIOHAZ), 2010. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update). EFSA J. 8, 1944.


European Food Safety Authority (EFSA), 2011. Scientific Opinion on the safety and efficacy of Lactobacillus pentosus (DSM 14025) as a silage additive for all animal species. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA J. 9, 2449.


Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoude-Werner,
or the identification of key proteins in bile tolerance. BMC Microbiol. 11, 63.


Figure legends

**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.

**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.

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