Biocide tolerance, phenotypic and molecular response of Lactic Acid Bacteria isolated from naturally-fermented Aloreña table olives throughout fermentation to induction by different physico-chemical stresses.

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Abstract

Lactic acid bacteria isolated from Aloreña table olives throughout fermentation process were resistant at least to three antibiotics (Casado Muñoz et al., 2014), however they were very sensitive to all biocides tested in this study (MIC below the epidemiological cut-off values "ECOFF" determined in the present study) except 2-15% of *Lc. pseudomesenteroides* which were resistant to hexachlorophene and *Lb. pentosus* to cetrimide and hexadecylpiridinium. To give new insights of how LAB become resistant in a changing environment, the effect of different physico-chemical stresses -including antimicrobials- on phenotypic and genotypic responses of LAB was analyzed in the present study. The results obtained indicated that a similar phenotypic response was obtained under all stress conditions tested (antimicrobials, chemicals and UV light) producing changes in susceptibility patterns of antibiotics (increased MICs for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, while decreased MICs were shown for clindamycin, erythromycin, streptomycin and trimethoprim in the majority of strains). By means of statistical analysis, cross resistance between different antibiotics was detected in all stress conditions. However, expression profiles of selected genes involved in stress/resistance (*rpsL*, *recA*, *uvrB* and *srtA* genes) were different depending on the stress parameter, LAB species and strain, and also the target gene. We can conclude that, in spite of the uniform phenotypic responses to several stresses, the repertoire of induced and repressed genes were different upon the stress parameter and the LAB strain. So, a search for a target to improve stress tolerance of LAB especially those of importance as starter/protective cultures or as probiotics may depend on the individual screening of each strain although, we could predict the antibiotic phenotypic response to all stresses.

Keywords:

Lactobacillus pentosus, *Leuconostoc pseudomesenteroides*, antibiotics, biocides, stress, real-time PCR, gene expression

I. INTRODUCTION

Lactic acid fermented foods are highly consumed all over the world since millions of years because of their nutritional values, their large shelf-life and especially because they are part of different cultures. Lactic acid bacteria (LAB), a heterogeneous group of Gram-positive bacteria widespread in many environments (Schleifer and Ludwig, 1995), are the main microorganisms carrying out the fermentation processes (vegetables, dairy and meat). They are used as starter or protective cultures (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004; Wood and Holzapfel, 1995) and also as probiotics (Kechagia et al., 2013; Servin, 2004) due to their long history of safe use (Anadon et al., 2006; EFSA, 2007) and that several strains can be considered to have "QPS" (Qualified Presumption of Safety) status (EFSA, 2007). In this sense, fermented foods as source of live LAB could act as important reservoirs of antibiotic resistant bacteria being spread to humans (Franz et al., 2005; Klein et al., 1988; Ross et al., 2002; Reid et al., 2003; Picard et al., 2005). Thus, foodborne LAB could be a vehicle of antibiotic resistance (AR) genes similar to those found in human pathogens (Korhonen et al., 2010; Mathur and Singh, 2005; Teuber et al., 1999). In fact, international organizations have launched several criteria addressing the biosafety concerns of starter cultures and probiotic microorganisms, however nothing could be done with spontaneous fermentation relying on indigenous microbiota. LAB generally exhibited intrinsic resistance (low permeability, impermeability, enzymatic inactivation and alteration of the target) (Poole, 2002) to many antibiotics but also acquired resistance due to chromosomal mutation and also to mobile genetic elements (plasmids, transposons, integrons) by horizontal gene transfer (Ammor et al., 2007, 2008) being those of great concern due to their higher potential for horizontal dissemination of AR genes (Khachatourians, 1998; European Commission, 2008).

The emergence of AR is mainly due to the over-use or the misuse of those antimicrobial agents such as bacterial infection treatment, animal husbandry, and agriculture (Wegener, 2003; Munsch-Alatossava and Alatossava, 2007; Dixon, 2000; Feinman, 1999; SCAN, 1996), without under-estimating the use of antibiotics as animal growth promoting over the last decades (nowadays is banned in several countries) which could be responsible of generating a pool of resistant bacteria in animals and also environment. Furthermore, the increased use of biocides as disinfectants in different areas (clinical setting, industry, home), sometimes at concentrations below the minimal

inhibitory concentration (MIC) of the target bacteria (Holah, 2000) may generate selective pressure causing crossed resistance with antibiotics (Russell, 2000; Meyer and Cookson, 2010; Lavilla Lerma et al., 2015). Thus, this fact could induce AR emergence and the treatment failure in hospitals with the corresponding agents (Chapman, 2003). Under sub-inhibitory concentrations of antimicrobials, bacteria switch on several responses via adaptation, mutation, acquisition of mobile resistance genes by horizontal gene transfer (transformation, conjugation and transduction), over-expression of resistance genes and efflux pumps with the aim to ensure survival (Pearce et al., 1999; Poole, 2002, 2004, 2007; Huet et al., 2008). The genetic basis of the development of AR may depend on the genetic fitness of each bacterial strain which could use different strategies.

In the present study, we evaluate biocide tolerance of antibiotic resistant LAB (Casado Muñoz et al., 2014) isolated from naturally-fermented Aloreña table olives throughout fermentation process. Furthermore, we investigated the effect of different physico-chemical stresses -including antimicrobials- on the phenotypic and genotypic responses of LAB. This fact is of great relevance to explain the increased resistance of those bacteria in specific environmental conditions.

II. MATERIAL AND METHODS

II.1. Bacterial strains and growth conditions. 73 LAB strains including 13 *Leuconostoc pseudomesenteroides* and 60 *Lactobacillus pentosus* strains isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2012) were used in this study. These strains were routinely cultured at 30ºC in Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or on agar under aerobic conditions for 24-48 h. Strains were kept in 20% glycerol at -80ºC for long term storage.

II.2. Antimicrobial agents. The antimicrobial agents listed in Tables 1 and 2 were used in this study and included various biocides used in food industry and clinically relevant antibiotics. Among biocides: benzalkonium chloride (BC), cetrimide (CE), chlorhexidine dihydrochloride (CH), hexachlorophene [2,20-methylenebis(3,4,6-trichlorophenol)] (CF), didecyldimethylammonium bromide (AB), hexadecylpyridinium chloride (HDP) and triclosan (TC) were used in this study. All biocides were purchased from Sigma Aldrich (Madrid, Spain) except triclosan which was obtained from Fluka (Madrid, Spain). Regarding antibiotics, a representative of each class was used such as ampicillin (AMP, beta-lactam), chloramphenicol (CHL, chloranphenicols), erythromycin (ERY, macrolides), trimethoptrim (TMP, sulphonamides), clindamycin (CLI, lincosamides), tetracycline (TET, tetracyclines), teicoplanin (TEC, glycopeptides), streptomycin (STR, aminoglycosides) and ciprofloxacin (CIP, quinolones).

II.3. Biocide susceptibility testing, MIC and ECOFF determination. The Minimum Inhibitory Concentration (MIC) of the above mentioned biocides was measured in a concentration range from 0.001 mg/L to 10 mg/L in LSM broth [90% of IST broth (Oxoid, Madrid, Spain) and 10% MRS broth (Fluka, Madrid, Spain)] (Klare et al., 2005) according to the ISO 10932/IDF 233 standard (ISO, 2010). MIC of all biocides was determined using the NCCLS broth microdilution method (NCCLS, 2000). After incubation, the MIC was read as the lowest concentration of each antimicrobial agent that inhibited the visible growth of the strain. All the MIC determinations of each antimicrobial for each strain were carried out in triplicate, and the reliable results were taken if at least two out of three replicates were in agreement.

ECOFF is defined, in a given bacterial species showing unimodal MIC distribution for a given antimicrobial, as concentration representing ≥ 95% (MIC_{95}) of the bacterial population (Pfaller et al., 2010). These values were determined as reported by Lavilla Lerma et al. (2015).

II.4. Induction of resistance by different physico-chemical stresses. To carry out the induction experiments, 7 strains of LAB [*Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* strains (CF1-16, CF1-25, CF1-35, CF2-11, CF2-15P and CF2-19P)] were selected on the basis of their phenotypic and genotypic resistance profile (Casado Muñoz et al., 2014), and also we included the potential probiotic strain *Lb. pentosus* MP-10 isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2011).

For induction with antimicrobials (antibiotics or biocides) selected on the basis of susceptibility results, overnight cultures of different cells were diluted 1:100 in fresh MRS broth and challenged with either triclosan (1 µg/mL), benzalkonium chloride (1 µg/mL), cholamphenicol (5 µg/mL), tetracycline (10 µg/mL) or amoxicillin (0.1 µg/mL). Subsequently, they were incubated at 30°C for 48 h and then centrifuged.

Induction of SOS response was carried out by overnight culture (2 ml) exposure to germicidal UV light (254 nm) in the dark during different time periods (1, 5 and 10 min) and were subsequently incubated for 3h at 30°C. After incubation, resistant cells were removed by centrifugation.

To induce the expression of genes coding for multidrug efflux proteins such as NorA and AcrA/B, 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Yu et al., 2002) or ethanol (4%) and/or sodium chloride (0.5 M) (Ma et al., 1995) were added, respectively.

In all cases, induced cells were stored in 20% glycerol at -80ºC until use.

II.5. Antimicrobial susceptibility of induced cells.

MICs of antibiotics tested in this study were determined as reported by Casado Muñoz et al. (2014). Regarding biocides, MICs were determined as described above.

II.6. Quantitative real-time PCR of stress/resistance genes after induction under different physico-chemical conditions.

II.6.1. RNA extraction. The expression of stress/resistance genes (Table 3) designed in this study (*rpsL*, *recA*, *srtA* and *uvrB*) on the basis of the annotated genome sequence of *Lb. pentosus* MP-10 (Abriouel et al., 2011) was determined after induction of cells under different conditions. Total RNA was isolated from three biological repeats by the illustra RNAspin Mini RNA Isolation Kit (GE-Healthecare, Madrid, Spain) according to the manufacturer's instructions. Controls without induction were used. RNA quantification and quality assessment were carried out by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNAs were adjusted to a concentration of 500 ng/ml and frozen at -80ºC until required.

II.6.2. RNA Quantitative real-time PCR of stress/resistance genes. For quantitative real-time PCR (qRT-PCR), mRNAs were subjected to reverse transcription into cDNA and subsequent real-time PCR in a single reaction using the SensiFast SYBR & Fluorescein One-Step kit (Bioline, Barcelona, Spain). 16S rRNA gene was used as a housekeeping gene and a no template control (NTC) was used as negative control. Primers for the 16S rRNA housekeeping gene and resistance genes were reported in Table 3. Quantitative PCRs (qPCRs) were performed in triplicate on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Madrid, Spain). PCR-grade water served as a negative control.

II.7. Statistical analysis. To investigate the degree of relationship between different physico-chemical stresses (antimicrobials, salt and ethanol), increase in MIC data of antibiotics were used. Pearson correlation coefficients (r) were calculated for the correlation between MICs of two antimicrobials by means of a computed statistical algorithm using SPSS. Pearson correlation was determined as reported by Lavilla Lerma et al. (2015). Categorization of correlations between different antimicrobials was done as mentioned by Dancey and Reidy (2004), being the strength of correlation strong when the correlation coefficient is in the range 0.7-0.9, moderate 0.4-0.6 and weak 0.1-0.3. In all analyses, a P value of <0.05 was considered significant.

III. RESULTS.

III.1. Biocide susceptibility and ECOFFs determination. LAB isolated from Aloreña green table olives were generally very sensitive to different biocides (75-100% with MIC ≤ 1 μg/ml), however up to 25% of LAB showed moderate sensitivity to different biocides (1< MIC ≤ 5 μg/ml) especially *Lb. pentosus* strains to triclosan, didecyldimethylammonium bromide and benzalkonium chloride (Table 1, Fig. 1).

ECOFF determination was in most cases similar in both LAB species (*Lb. pentosus* and *Lc. pseudomesenteroides*) except in the case of hexachlorophene. Regarding resistance, LAB analyzed in this study were in most cases sensitive and only 2-15% of resistant bacteria were detected for hexachlorophene in *Lc. pseudomesenteroides* and for cetrimide and hexadecylpiridinium in *Lb. pentosus* (Table 1, Fig. 1).

III.2. LAB phenotypic response to physico-chemical stresses. Phenotypic bacterial responses to the induction by different antimicrobials (antibiotics and biocides) were generally similar in all strains analyzed (Table 2). The results obtained showed an increase in MICs of ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline by up to 340-1000 folds especially for chloramphenicol, teicoplanin and tetracycline (Table 2). Similarly, after induction of SOS response by UV light exposure during different time periods (1, 5 and 10 min), the MICs of the mentioned antibiotics were increased by up to 340-750 folds (especially for chloramphenicol and teicoplanin) regardless the exposure time in the majority of cases (Table 4). Cells induced by IPTG or NaCl+ethanol showed similar results (Table 5), however when cells were induced by ethanol or NaCl, MIC fold increase was observed besides to the mentioned antibiotics also for clindamycin (Table 5).

On the other hand, we generally observed a decrease in MICs of some antibiotics after induction under different stresses (antimicrobials, UV and chemicals) such as clindamycin, erythromycin, streptomycin and trimethoprim (Tables 2, 4 and 5). Furthermore, ampicillin, teicoplanin and ciprofloxacin showed occasionally in some induced strains decreased MICs being highly sensitive to the corresponding antibiotics (Tables 2, 4 and 5).

III.3. Correlation between MIC of antibiotics and different stresses in LAB. Table 6 showed the strength of any linear relationship between the MIC increase of two antibiotics in LAB induced by several stresses. Pearson's correlation coefficient analysis

between all antibiotics showed a moderate-strong correlation between some antibiotics in LAB induced by several stresses especially antimicrobials (antibiotics or biocides) (Table 6A). In this sense, almost all antibiotics were significantly correlated with one or two other antibiotics except for ampicillin with three antibiotics (Table 6A). Moderate positive correlations were observed between ampicillin and ciprofloxacin, clindamycin or teicoplanin (r= 0.372-0.498), and also between clindamycin and tetracycline (r= 0.687), teicoplanin and trimethoprim (r= 0.533), and ciprofloxacin and clindamycin (r= 0.361). However, moderate negative correlations were obtained between chloramphenicol and ciprofloxacin (r= -0.409) and also between streptomycin and teicoplanin (r= -0.335) (Table 6A).

Concerning LAB induced by UV exposure, the MIC increase of some antibiotics was correlated positively (Table 6B), in this way moderate correlations were observed between chloramphenicol and teicoplanin (r= 0.682), clindamycin and trimethoprim (r= 0.649), and ampicillin and tetracycline (r= 0.528). However, moderate negative correlation was only obtained between teicoplanin and tetracycline (r= -0.511).

Induction with chemicals such as IPTG or ethanol and/or sodium chloride produced strong positive correlation (Table 6C) between chloramphenicol and teicoplanin (r= 0.739), and moderate positive correlation between ampicillin and ciprofloxacin (r= 0.535). However, a moderate negative correlation was only observed between teicoplanin and tetracycline (r= -0.483).

III.4. Genotypic response of LAB to physico-chemical stresses. We selected two strains: *Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28 to study the effect of stressors on the expression of the following genes (*rpsL*, *recA*, *uvrB* and *srtA*) involved in tolerance/resistance. The results obtained showed that responses of both bacteria were different depending on the inducer and the gene analyzed (Fig. 2). With respect to *rpsL* gene, up-regulation was observed in *Lc. pseudomesenteroides* AP2-28 after induction with different antibiotics (chloramphenicol and tetracycline), ethanol, NaCl or UV light exposure (5 and 10 min) by 4-5 folds (Fig. 2A). Similarly, up-regulation of *rpsL* gene was observed after induction of *Lb. pentosus* MP-10 by chloramphenicol, however *Lb. pentosus* MP-10 showed down-regulation of *rpsL* gene after induction by different antimicrobials (amoxicillin, benzalkonium and triclosan) (Fig. 2A).

Analysis of *recA* expression showed an opposite behavior comparing with *rpsL* gene (Fig. 2B), in this sense down-regulation of *recA* gene occurred after induction of *Lc.*

pseudomesenteroides AP2-28 with antimicrobials (amoxicillin and benzalkonium) and chemicals (IPTG, ethanol and NaCl+ethanol) and also for *Lb. pentosus* MP-10 induced by NaCl (Fig. 2B). However, up-regulation of *recA* gene was shown for *Lb. pentosus* MP-10 induced by tetracycline and UV exposure (5 and 10 min).

Concerning *srtA* gene, down-regulation was observed for both *Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* MP-10 induced by antimicrobials (chloramphenicol, tetracycline and benzalkonium), chemicals (IPTG) and UV exposure (5 and 10 min) (Fig. 2C). Furthermore, amoxicillin, ethanol and NaCl+ethanol induced down-regulation of *srtA* gene in *Lc. pseudomesenteroides* AP2-28, while NaCl did in *Lb. pentosus* MP-10 (Fig. 2C).

Down-regulation of *uvrB* gene expression was shown after induction by several stressors such as antimicrobials (amoxicillin, benzalkonium and triclosan) and chemicals (IPTG, ethanol and NaCl+ethanol) for *Lc. pseudomesenteroides* AP2-28 (Fig. 2D). However, *uvrB* gene expression was down regulated in *Lb. pentosus* MP-10 after induction by benzalkonium, IPTG and NaCl (Fig. 2D). On the other hand, we also observed up-regulation of *uvrB* gene after induction of *Lb. pentosus* MP-10 with chloramphenicol and UV exposure during 5 min (Fig. 2D).

III.5. Correlation between resistance genes in LAB induced by several physico-chemical stresses. Table 7 showed the correlations between the expression of different resistance genes (*rpsL*, *recA*, *uvrB* and *srtA*) in *Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28 induced with several physico-chemical stresses. A unique strong positive correlation was detected between *recA* and *uvrB* genes in both *Lc. pseudomesenteroides* AP2-28 (r= 0.930) and *Lb. pentosus* MP-10 (r= 0.770) regardless the stress condition. Concerning the rest of the genes, no correlations were obtained (Table 7).

Discussion

LAB can face an array of stresses (environmental challenges and chemicals) in their natural habitats, also during food processing and storage, and even during passage through gastrointestinal tract. The adaptive responses involves changes in physiology, behavior and genetic by means of several mechanisms (Chung et al., 2006; Foster, 2007; Ryall et al., 2012). Phenotypic switching is one of the main strategies adopted by bacteria to ensure their survival and persistence in various environmental conditions (Sousa et al., 2011). In this sense, LAB developed several adaptation strategies to survive to changing conditions (pH, salt concentration, temperature, nutrient concentration…etc) that involved among others alteration of cellular physiology via control of stress-response gene expression. The results obtained in the present study indicated that induction by antimicrobials (biocides or antibiotics), chemicals or UV light changed antibiotic susceptibility pattern of LAB isolated from naturally fermented Aloreña table olives. On the other hand, at genotypic level several stress genes were differentially expressed depending on the stress parameter, LAB species and strain, and also the target gene. Correlation between phenotypic and genotypic responses will give us new insights of how bacteria become resistant in a changing environment.

Development of antimicrobial resistance may frequently result from the indiscriminate use of antimicrobials as therapeutic agents and as disinfectants (Davin-Regli and Pagès, 2012; Dixon, 2000; Hawkey, 2008; McBain et al., 2002). As reported by Casado Muñoz et al. (2014) in a previous study, most *Lb. pentosus* (95%) and all *Lc. pseudomesenteroides* analyzed in the present study were resistant to at least three antibiotics (multidrug resistant). However, here we showed that they are generally sensitive to biocides (MIC below ECOFF determined in the present study) and only 2-15% of resistant bacteria were detected for hexachlorophene in *Lc. pseudomesenteroides* and for cetrimide and hexadecylpiridinium in *Lb. pentosus*. Induction of LAB with sub-lethal concentrations of antibiotics or biocides (sub-MICs) generally resulted in a completely changed susceptibility pattern with increased MICs for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, while decreased MICs were shown for clindamycin, erythromycin, streptomycin and trimethoprim in the majority of strains (being in most cases very sensitive to the corresponding antibiotics). Furthermore, Casado Muñoz et al. (2014) reported that all LAB analyzed in this study were sensitive to chloramphenicol and resistant to

streptomycin and trimethoprim, however in the present study such susceptibility pattern was inverted after induction with sub-lethal concentrations of antimicrobials being highly sensitive to streptomycin and trimethoprim and resistant to chloramphenicol (up to 1000 fold MIC). The emergence of new resistance patterns in LAB induced by sub-lethal concentrations of antimicrobials is not only important at ecological point of view but has also evolutionary consequences. Thus, sub-lethal concentrations of antibiotics could have a strong effect on mutation rates, horizontal gene transfer and biofilm formation in the natural habitat of LAB and thus may contribute to the emergence and spread of AR in the food chain and also in the environment (Laureti et al., 2013). Moreover, other stresses such as heat, salt, acidic and alkaline pH can alter significantly phenotypic antimicrobial resistance in food-related pathogens as reported by several authors (Al-Nabulsi et al., 2011; Ganjian et al., 2012; McMahon et al., 2007a, b). In this way, induction with chemicals or UV light (5 or 10 min) produced the same effects as antimicrobials concerning the increased and decreased MICs of the mentioned antibiotics. These data suggested that changes in susceptibility patterns of induced LAB may be due to alteration of cell membrane permeability to the corresponding antibiotics or expression profiles of resistance/stress genes. In general, positive correlations between antibiotics belonging to different classes and targeting different cellular structures suggested a co-resistance (resistance mechanisms carried by the same mobile genetic element such as plasmid, transposon or integron) or cross-resistance. In this study, by means of statistical analysis the positive correlations shown especially under antimicrobial stresses suggested a cross-resistance which relies on unspecific resistance mechanisms with a wide range of activity against antibiotics such as efflux pumps. Generally, similar correlations were shared by different physico-chemical stresses (antimicrobials, UV exposure and chemicals) suggesting that a repertoire of similar phenotypic and genotypic mechanisms were induced under different stress conditions. In this respect, we analyzed the expression of four genes responsible of tolerance/resistance under different stress conditions. The results of expression analysis of selected genes involved in tolerance/resistance mechanisms were shown to be different upon stressor used in challenge tests and also on LAB strain. However, phenotypic response to several stressors was generally similar under all stress conditions being involved a different repertoire of mechanisms in each case. To determine the mechanisms adopted by LAB to withstand hostile conditions, knowledge about inducible genes under each stress condition is of crucial importance since a

balance of different responses is involved in tolerance/resistance. Regarding *rpsL* gene which encodes the ribosomal protein S12 was over-expressed after induction with all stressors (antibiotics, NaCl, ethanol and UV light) except amoxicillin and biocides. This protein with polyspecific effects may act as RNA chaperone as reported by Coetzee et al. (1994) with the aim to protect the ribosome structure and then its function under stress. Increasing *rpsL* gene expression was generally responsible of the increased MICs of some antibiotics such as ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline except few cases. However, *recA* gene was only over-expressed after induction with tetracycline and UV light (5 and 10 min), while an under-expression of this gene was observed in LAB-induced by biocides, amoxicillin, IPTG, NaCl, ethanol and NaCl+ethanol. The gene *recA* encodes the RecA protein which has a role in SOS regulation system and in many DNA repair pathways such as repairing daughter strand gaps, double strand breaks and SOS mutagenesis (Cox, 2007). When the SOS response is induced, several genes involved in repair, replication, recombination and cell division are expressed such as *recA* gene, and also genes of the nucleotide excision repair (NER) system (*uvrA, uvrB, uvrC* and *uvrD* genes). In the present study, *uvrB* gene was down regulated under all stress conditions except after induction with chloramphenicol and UV light during 5 min. It is noteworthy that *recA* and *uvrB* genes were paired in their expression being involved in the same repair mechanisms that were activated only in the case of antibiotics (chloramphenicol or tetracycline) and UV light exposure. This fact was confirmed by statistical analysis in the present study. Thus, the SOS response as inducible pathway (Van der Veen and Abee, 2011) may be involved in part in the increased MICs of antibiotics after induction with chloramphenicol, tetracycline or UV light. In this way, strong positive correlations were obtained with both genes. On the other hand, *srtA* virulence gene which encodes an enzyme that anchors surface proteins to the cell wall (Mazmanian et al., 199) was under-regulated under all stress conditions. The genes *srtA* and *srtB* are responsible for cross-linking LPXTG motif-containing surface proteins to peptidoglycan, thus down-regulation of *srtA* gene under stress condition responsible of permeability alteration of the cellular membrane may be related with the decreased MICs of clindamycin, erythromycin, streptomycin and trimethoprim in the majority of cases. Similar results were obtained by Hesketh et al. (2011) regarding repression of the gene encoding sortase under drug stress.

In general, to ensure survival bacteria maintain all levels of the SOS response under tight control and select rigorously the adequate genes to be transcribed in each situation.

So, analysis of the repertoire of mechanisms adopted by two selected LAB (*Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* MP-10) may explain how they can survive in each stress condition and why they change their susceptibility pattern. Induction assays by antibiotics inhibiting protein synthesis (chloramphenicol or tetracycline) caused over-expression of *rpsL* (*Lc. pseudomesenteroides* AP2-28 and *Lb. pentosus* MP-10), *recA* and *uvrB* (*Lb. pentosus* MP-10) genes, however down-regulation of *srtA* gene was observed (*Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28). Thus, as mentioned above to survive under antibiotic stress LAB use repair and protection mechanisms switching on the involved genes such as *rpsL*, *recA* and *uvrB* depending on the antibiotic used and the LAB strain, and thus MICs of some antibiotics were increased. In parallel, down-regulation of *srtB* gene responsible of the alteration of cell surface maybe responsible of the high sensitivity of LAB to other antibiotics (clindamycin, erythromycin, streptomycin and trimethoprim). However, induction assays by biocides (benzalkonium or triclosan) caused down-regulation of *rpsL* (*Lb. pentosus* MP-10), *recA* (*Lc. pseudomesenteroides* AP2-28), *srtA* and *uvrB* (*Lb. pentosus* MP-10 and *Lc. pseudomesenteroides* AP2-28) genes. Thus, induction by antibiotics or biocides did not involve the same repertoire of genes (except for the down-regulated *srtA* gene) or mechanisms since both antimicrobials have different cellular targets, furthermore SOS response was not activated by biocide stress. The similar phenotypic response to antibiotic/biocide stress may suggest that other specific genes are involved in this similar phenotypic response or maybe unspecific mechanisms are involved such as efflux pumps (Buffet-Bataillon et al., 2012).

Regarding chemicals, induction with NaCl or ethanol or both resulted in the same expression profile over-expressing *rpsL* gene (*Lc. pseudomesenteroides* AP2-28) and down-regulating *recA, uvrB* and *srtA* genes (*Lb. pentosus* MP-10 induced by NaCl and *Lc. pseudomesenteroides* AP2-28 induced by ethanol or NaCl+ethanol). Over-expression of *rpsL* gene was attributed to its role in ribosome protection as detailed above, however down-regulation of the genes involved in SOS response may suggest that salt and ethanol stresses did not activate this system in LAB to ensure their survival. As reported for antimicrobial stress, *srtA* gene down-regulation was related with membrane permeability alteration. On the other hand, UV light exposure implied over-expression of *rpsL*, *recA* and *uvrB* genes suggesting that both repair and protection mechanisms were induced by UV exposure, while *srtA* gene was repressed as in other cases.

Conclusions

We can conclude that, in spite of the uniform phenotypic responses to several stresses, the repertoire of induced and repressed genes were different upon the stress parameter and the LAB strain, although a strong positive correlations were obtained with *recA* and *uvrB* genes under several stresses. So, a search for a target to improve stress tolerance of LAB especially those of importance as starter/protective cultures or as probiotics may depend on the individual screening of each strain, although by these data we can predict the antibiotic phenotypic response to all stresses. However, in general we can speculate that *rpsL* gene could be suggested as biomarker of robustness/resistance in LAB although further studies must be required to elucidate the correlation of induced *rpsL* gene with robustness responses.

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