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Biocide tolerance, phenotypic and molecular response of lactic acid bacteria isolated from naturally-fermented Aloreña table to different physico-chemical stresses

María del Carmen Casado Muñoz [a], Nabil Benomar [a], Leyre Lavilla Lerma [a], Charles W. Knapp [b], Antonio Gálvez [a], Hikmate Abriouel [a, *]

[a] Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071, Jaén, Spain
[b] Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, Scotland, United Kingdom

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ABSTRACT

Lactic acid bacteria (LAB) isolated throughout the fermentation process of Aloreña table olives were found to be resistant at least to three antibiotics (Casado Muñoz et al., 2014); however, most were sensitive to the biocides tested in this study (with minimum inhibitory concentrations [MIC] below the epidemiological cut-off values). 2–15% of the isolates were found to be biocide resistant: *Leuconostoc Pseudomesenteroides*, which were resistant to hexachlorophene, and *Lactobacillus pentosus* to cetrimide and hexadecylpiridinium.

We analyzed the effect of different physico-chemical stresses, including antimicrobials, on the phenotypic and genotypic responses of LAB, providing new insights on how they become resistant in a changing environment. Results indicated that similar phenotypic responses were obtained under three stress conditions: antimicrobials, chemicals and UV light. Susceptibility patterns to antibiotics changed: increasing MICs for ampicillin, chloramphenicol, ciprofloxacin, teicoplanin and tetracycline, and decreasing the MICs for clindamycin, erythromycin, streptomycin and trimethoprim in most strains. Statistically, cross resistance between different antibiotics was detected in all stress conditions. However, expression profiles of selected genes involved in stress/resistance response (*rpsL*, *recA*, *uvrB* and *srtA*) differed depending on the stress parameter, LAB species and strain, and the target gene.

We conclude that, despite the uniform phenotypic response to stresses, the repertoire of induced and repressed genes differs. So, a search for a target to improve stress tolerance of LAB, especially those of importance as starter/protective cultures or probiotics, may depend on the individual screening of each strain, even though we could predict the antibiotic phenotypic response to all stresses.

1. Introduction

Lactic acid fermented foods have been consumed globally for millions of years because of their nutritional value, extensive shelf-life and especially their cultural value. Lactic acid bacteria (LAB), a heterogeneous group of Gram-positive bacteria found widespread in many environments (Schleifer and Ludwig, 1995), are the main microorganisms carrying out the fermentation processes on the vegetables, dairy and meats. They are also used as starter or protective cultures (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004; Wood and Holzapfel, 1995) and as probiotics (Kechagia et al., 2013; Servin, 2004) due to their long history of safe use (Anadon et al., 2006; EFSA , 2007) and several strains having "QPS" (Qualified Presumption of Safety) status (EFSA , 2007). Foodborne LAB are vehicles of antibiotic resistance (AR) genes similar to those found in human pathogens (Korhonen, 2010; Mathur and Singh, 2005; Teuber et al., 1999). As such, fermented foods also represent vectors by which AR bacteria can be spread to humans (Franz et al., 2005; Klein et al., 1988; Ross et al., 2002; Reid et al., 2003; Picard et al., 2005). In fact, international organizations have launched criteria addressing the biosafety concerns of starter cultures and probiotic microorganisms; however, nothing could be done with spontaneous fermentations that rely on indigenous

* Corresponding author. Present address: Área de Microbiología, Departamento de Ciencias de la Salud. Facultad de Ciencias Experimentales, Edif. B3, Universidad de Jaén, Campus Las Lagunillas s/n, 23071, Jaén, Spain.
E-mail address: hikmate@ujaen.es (H. Abriouel).

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microbiota. LAB generally exhibit intrinsic resistance to many antibiotics (e.g., via lowered permeability, enzymatic inactivation and alteration of the target compounds) (Poole, 2002), but also acquired resistance due to chromosomal mutation and mobile genetic elements (e.g., plasmids, transposons, and integrons). The greatest concern is the horizontal gene transfer (Ammor et al., 2007, 2008) due to the higher potential for horizontal dissemination of AR genes (Khachatourians, 1998; European Commission, 2008) to other species.

The emergence of AR has mainly been due to the over-use or the misuse of antimicrobial agents for improper infection control, animal husbandry, and agriculture (Wegener, 2003; Munsch-Alatossava and Alatossava, 2007; Dixon, 2000; Feinman, 1999; SCAN , 1996), including the prophylactic use of antibiotics as animal growth promotion over the last few decades (nowadays banned in several countries), which have contributed to the generation of resistant bacteria in animals and environment. Furthermore, the increased use of biocides as disinfectants (e.g., in clinical setting, industry, and home) at concentrations below the minimum inhibitory concentration (MIC) of targeted bacteria (Holah, 2000) may generate pressures for cross-resistance with antibiotics (Russell, 2000; Meyer and Cookson, 2010; Lavilla Lerma et al., 2015). Thus, these compounds contribute to AR emergence and decreased treatment efficacy (Chapman, 2003). Sub-inhibitory concentrations of antimicrobials trigger several bacterial responses to ensure their survival: adaptation, mutation, acquisition of mobile resistance genes by horizontal gene transfer (e.g., transformation, conjugation and transduction), over-expression of resistance genes, and efflux pumps (Pearce et al., 1999; Poole, 2002, 2004, 2007; Huet et al., 2008). The genetic basis for the development of AR depends on the genetic fitness of each bacterial strain and available response strategies.

Here, we evaluated biocide tolerance of several antibiotic resistant LAB (Casado Muñoz et al., 2014) isolated from naturally-fermented Aloreña table olives during its fermentation process. Furthermore, we investigated the phenotypic and genotypic responses of LAB to different physico-chemical stressors, including antimicrobials. This is of great relevance in order to understand and explain the increased resistance of these bacteria under changing environmental conditions.

2. Material and methods

2.1. 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 30 °C either 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 −80 °C for long-term storage.

2.2. Antimicrobial agents

Antimicrobial agents (Tables 1 and 2) included various biocides used in food industry and clinically relevant antibiotics. All antimicrobials were purchased from Sigma Aldrich (Madrid, Spain), except triclosan, which was obtained from Fluka (Madrid, Spain).

2.3. Biocide susceptibility testing, MIC and ECOFF determination

The MICs of the aforementioned biocides were measured in a concentration range from 0.001 μg/ml to 10 μg/mL 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). MICs of all biocides were 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 growth of the strain. All MIC determinations of each antimicrobial against each strain were carried out in triplicate, and reliable results were taken if at least two of the three replicates were in agreement.

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

2.4. Induction of resistance by different physico-chemical stresses

For induction experiments, seven 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 we also included the potentially probiotic strain *Lb. pentosus* MP-10 isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2011). Antimicrobials (antibiotics or biocides) were selected on the basis of susceptibility results.

Overnight cultures of different cells were diluted 1:100 in fresh MRS broth and challenged against either triclosan (1 μg/mL), benzalkonium chloride (1 μg/mL), chloramphenicol (5 μg/mL), tetracycline (10 μg/mL) or amoxicillin (0.1 μg/mL). Cells were incubated at 30 °C for 48 h and then centrifuged.

Induction of SOS response, a global response to DNA damage involving DNA repair and mutagenesis, was carried out by exposing overnight culture (2 ml) to germicidal UV light (254 nm) for 1, 5 and 10 min, and incubated for 3 h at 30 °C. After incubation, induced cells were harvested 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 cryogenically stored in 20% glycerol at −80 °C until use.

2.5. Antimicrobial susceptibility of induced cells

MICs of antibiotics tested in this study were determined as reported by Casado Muñoz et al. (2014). The ECOFFs of antibiotics used in this study were reviewed by Casado Muñoz et al. (2014) as 2 μg/ml for ampicillin in *Lb. pentosus* and *Lc. pseudomesenteroides*; 8 and 4 μg/ml for chloramphenicol in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 4 and 32 μg/ml for ciprofloxacin in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 2 and 1 μg/ml for clindamycin in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 16 μg/ml for erythromycin in *Lb. pentosus* and *Lc. pseudomesenteroides*; >256 and 64 μg/ml for streptomycin in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 32 μg/ml for teicoplanin in *Lb. pentosus* and *Lc. pseudomesenteroides*; 32 and 8 μg/ml for tetracycline in *Lb. pentosus* and *Lc. pseudomesenteroides*, respectively; 8 μg/ml for trimethoprim in *Lb. pentosus* and *Lc. pseudomesenteroides*. Regarding biocides, MICs were determined as described above. There are no data are available about the ECOFF in LAB and the maximum residue level (MRL) recommended by EFSA for each biocide used as disinfectant or pesticide were of 0.01 mg/kg for cetrimide and hexachlorophene, 0.1 mg/kg for benzalkonium chloride and didecyldimethylammonium bromide, 0.2% for triclosan and 5 μg/ml for chlorhexidine (Database of MRLs in the EU; EFSA, 2014).