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Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process

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ABSTRACT

Antimicrobial resistance of *Lactobacillus pentosus* (n = 59) and *Leuconostoc pseudomesenteroides* (n = 13) isolated from Aloreña green table olives (which are naturally-fermented olives from Málaga, Spain) to 15 antibiotics was evaluated. Most *Lb. pentosus* (95%) and all *Lc. pseudomesenteroides* were resistant to at least three antibiotics. Principal component analysis determined that the prevalence of antibiotic resistance in LAB throughout the fermentation process was highly dependent on the fermenter where the fermentation took place. All *Lb. pentosus* and *Lc. pseudomesenteroides* strains were highly sensitive to amoxicillin and ampicillin (MIC ≤ 2 μg/ml), and also to chloramphenicol (MIC ≤ 4 μg/ml), gentamicin and erythromycin (MIC ≤ 16 μg/ml). However, they were phenotypically resistant to streptomycin (83–100%, MIC > 256 μg/ml), vancomycin and teicoplanin (70–100%, MIC > 128 μg/ml), trimethoprim (76% of *Lb. pentosus* and 15% of *Lc. pseudomesenteroides*, MIC > 128 μg/ml), trimethoprim/sulfomethoxazol (71–100%, MIC > 4–64 μg/ml) and cefuroxime (44% of *Lb. pentosus* and 85% of *Lc. pseudomesenteroides*, MIC > 32–128 μg/ml). *Lb. pentosus* was susceptible to tetracycline and clindamycin, while 46% of *Lc. pseudomesenteroides* strains were resistant to these antibiotics. Only *Lb. pentosus* strains were resistant to ciprofloxacin (70%, MIC > 4–64 μg/ml), although no mutations in the quinolone resistance determining regions of the genes encoding GyrA and ParC were found, thus indicating an intrinsic resistance. Similarly, no genes encoding possible transferable resistance determinants for the observed phenotypic resistance were detected by PCR. In some cases, a bimodal distribution of MICs was observed for some antibiotics to which both LAB species exhibited resistance. Nevertheless, such resistances resulted from an intrinsic mechanism, non-transferable or non-acquired resistance determinants which may in part be due to chromosomally encoded efflux pumps (NorA, MepA and MdeA). Results of the present study demonstrate that all *Lb. pentosus* and *Lc. pseudomesenteroides* strains lack transferable resistance-related genes (*cat*, *bla*, *blaZ*, *ermA*, *ermB*, *ermC*, *msrA/B*, *ereA*, *ereB*, *mphA*, *mefA*, *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(L), *tet*(K), *aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa*, *ant(4′)-Ia*, *dfrA*, *dfrD*, *vanA*, *vanB*, *vanC* and *vanE*) and should therefore, according to Qualified Presumption of Safety criteria, be considered safe for future application as starter cultures or as probiotics.

1. Introduction

Lactic acid bacteria (LAB) are widely consumed along with fermented foods and beverages because of their use as starter cultures in fermentation processes (Caplice and Fitzgerald, 1999; Leroy and De Vuyst, 2004; Wood and Holzapfel, 1995). They are also known for their role as protective cultures as they are involved in producing an arsenal of antimicrobial substances such as lactic acid (and other organic acids), hydrogen peroxide, diacetyl, acetoin, reuterin, reutericyclin, antifungal peptides, and bacteriocins (Holzapfel et al., 1995; Holtzel et al., 2000). In the last decades, LAB have been used as probiotics, with *Bifidobacterium* and *Lactobacillus* being the most commonly used genera (Servin, 2004). The application of LAB as probiotics has been prompted by their beneficial properties on general health of the consumers (Kechagia et al., 2013) and their "QPS" (Qualified Presumption of Safety) status based on a long history of safe use (Anadon et al., 2006; European Commission, SCAN, 2007). In this sense, international regulatory organizations recommended specific prerequisites for approval of a determined strain as feed additive. Accordingly, the European Scientific Committee on Animal Nutrition (European Commission, SCAN, 2005) and the European Food Safety Authority (EFSA, 2012) recommended that LAB strains consumed on a daily basis worldwide should lack acquired or transferable antimicrobial resistance genes prior to considering them safe for human and animal consumption and that any probiotic strain should have QPS status.

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Table 1
MIC distribution of 15 antibiotics for *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* strains isolated from Aloreña fermented olives.

Antibiotic	Species	No. of isolates with the following MIC range (μg/ml)										ECOFF (μg/ml)
		0.002–≤0.1	>0.1–≤1	>1–≤2	>2–≤4	>4–≤8	>8–≤16	>16–≤32	>32–≤64	>64–≤128	>128	
Amoxicillin	*Lb. pentosus*	30	23	6								2[a,b]
	Lc. pseudomesenteroides		10	3								16[b]
Ampicillin	*Lb. pentosus*	14	32	13								2[c]
	Lc. pseudomesenteroides	9	3	1								2[c]
Cefuroxime	*Lb. pentosus*		11			2	2	18	**21**	**5**		≥32[e]
	Lc. pseudomesenteroides		1					1	**11**			≥32[e]
Chloramphenicol	*Lb. pentosus*	38	19		2							8[c]
	Lc. pseudomesenteroides	8	5									4[c]
Ciprofloxacin	*Lb. pentosus*	7	11			**36**			**5**			>4[d]
	Lc. pseudomesenteroides		5			8						>32[a]
Clindamycin	*Lb. pentosus*	36	22	1								2[c]
	Lc. pseudomesenteroides	6	1	**6**								1[c]
Gentamicin	*Lb. pentosus*	38	20				1					16[c]
	Lc. pseudomesenteroides	6	2				5					16[c]
Erythromycin	*Lb. pentosus*	45	10			2	2					16[c]
	Lc. pseudomesenteroides	1	3	9								16[c]
Kanamycin	*Lb. pentosus*	1	18			4	12	6	18			64[c]
	Lc. pseudomesenteroides						2	**11**				16[c]
Streptomycin	*Lb. pentosus*						1		1	8	**49***	>256[a], NR[c]
	Lc. pseudomesenteroides										**13**	64[c]
Teicoplanin	*Lb. pentosus*	3	14								**42**	≥32[e]
	Lc. pseudomesenteroides										**13**	≥32[e]
Tetracycline	*Lb. pentosus*	13	32	1		6	7					32[c]
	Lc. pseudomesenteroides		6			1	**6**					8[c]
Trimethoprim	*Lb. pentosus*	1		8		5	**13**	**8**	**8**	**8**	**8**	8[c]
	Lc. pseudomesenteroides			11						**1**	**1**	8[c]
Trimethoprim/ Sulfamethoxazole[¥]	*Lb. pentosus*			17		**26**		**2**	**14**			≥4[e]
	Lc. pseudomesenteroides					**9**		**3**	**1**			≥4[e]
Vancomycin	*Lb. pentosus*		11	1							**47**	4[d], NR[c], ≥32[e]
	Lc. pseudomesenteroides									**2**	**11**	NR[c], ≥32[e]

The microbiological breakpoint values according to Danielsen and Wind (2003)[a] and Flórez et al. (2005)[b] are given. The EU Commission breakpoint values as suggested by EFSA (2012)[c], European Commission (SCAN) (2005)[d] for *Lb. pentosus* and *Lc. pseudomesenteroides* are described, and in the case of non-described antibiotics we consider the breakpoint values suggested by CLSI (2011)[e] for staphylococci. Resistant strains with a MIC value higher than the breakpoints described in the table are indicated in bold.
ND: not determined, NR: not required.
[¥]MIC refers to trimethoprim concentrations only. Trimethoprim:sulfamethoxazole was tested in the ratio 1:19.
*The concentration tested of streptomycin was >256 μg/ml.

Recently, several foods have been considered as potential vehicles of antibiotic resistance genes (Bautista-Gallego et al., 2013; Duran and Marshall, 2005; Franz et al., 1999; Zhang et al., 2009) with fermented foods being one of the most important environments where several stresses (low pH, high salt concentration and antimicrobial compounds) and the high number of living bacteria may induce the exchange of such genes. Gene exchanges may enhance survival of LAB and pathogens and thus represent an important risk within the gastrointestinal tract for spread to other bacteria (Salyers et al., 2004; van Reenen and Dicks, 2011). The indiscriminate use of antibiotics in human medicine and animal husbandry during several decades has resulted in an important public health risk. Furthermore, the increasing use of biocides as disinfectants in hospitals and food industries has led to the emergence of cross-resistance phenotypes to clinically important antimicrobial compounds (Fraise, 2002) and to new resistance mechanisms, which impose an additional health risk for consumers or the environment. The development of antimicrobial resistance among bacteria introduced in the food chain is of great concern, thus the EFSA requires that bacteria which are to be introduced into the food chain lack acquired antimicrobial resistance determinants to prevent lateral spread of these (van Reenen and Dicks, 2011). In the present study, the susceptibility patterns and possible mechanisms determining resistance to several antibiotics in *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* strains were investigated. These strains were isolated in a previous study (Abriouel et al., 2012) from Aloreña table olives (which are naturally-fermented olives manufactured by small and medium enterprises from Málaga, Spain), with the aim to select strains that lack acquired antimicrobial resistance genes for possible application as starter cultures or as probiotics. Furthermore, this study allowed us to detect the types and degrees of antibiotic resistance present among the LAB community in the natural olive fermentation environment, and also to determine the prevalence of such phenotypic resistance in LAB throughout the fermentation processes in different producing enterprises.

Table 2
PCR primers used in this study.

Target gene	Primer pair (5′→3′)	Reference
Bla	Bla-forward: CATARTTCCGATAATASMGCC Bla-reverse: CGTSTTTAACTAAGTATSGY	Hummel et al. (2007)
Cat	Catfw1: TTAGGT TATTGGGATAAGTTA Catrev: GCATGRTAACCATCACAWAC	Hummel et al. (2007)
ermA	ErmA1: TCTAAAAAGCATGTAAAAGAA ErmA2: CTTCGATAGTTTATTAATATTAGT	Sutcliffe et al. (1996)
ermB	ErmB1: GAAAAGGTACTCAACCAAATA ErmB2: AGTAACGGTACTTAAATTGTTTAC	Sutcliffe et al. (1996)
ermC	ErmC1: TCAAAACATAATATAGATAAA ErmC2: GCTAATATTGTTTAAATCGTCAAT	Sutcliffe et al. (1996)
msrA/B	MsrA/B1: GCAAATGGTGTAGGTAAGACAACT MsrA/B2: AAGTTATATCATGAATAGATTGTCCTGTTT	Sutcliffe et al. (1996)
ereA	EreA-FW: AACACCCTGAACCCAAGGGACG EreA-RV: CTTCACATCCGGATTCGCTCGA	Sutcliffe et al. (1996)
ereB	EreB-RV: AGAAATGGAGGTTCATACTTACCA EreB-FW: CATATAATCATCACCAATGGCA	Sutcliffe et al. (1996)
mphA	MphA-FW: AACTGTACGCACTTGC Mph-RV: GGTACTCTTCGTTACC	Sutcliffe et al. (1996)
mefA/mefE	MefA/E-FW: AGTATCATTAATCACTAGTGC MefA/E-RV: TTCTTCTGGTACTAAAAGTGG	Sutcliffe et al. (1996)
lsa	Abc2-FW: GGCAATCGCTTGTGTTTTAGCG Abc2-RV: GTGAATCCCATGATGTTGATACC	Singh and Murray (2005)
aac(6′)-Ie-aph(2′)-Ia	FW: CAGGAATTTATCGAAAATGGTAGAAAAG RV: CACAATCGACTAAAGAGTACCAATC	Vakulenko et al. (2003)
aac(6′)-Ie-aph(2′)-Ia	FW: CAGAGCCTTGGGAAGATGAAG RV: CCTCGTGTAATTCATGTTCTGGC	Vakulenko et al. (2003)
aph(2′′)-Ib	FW: CTTGGACGCTGAGATATATGAGCAC RV: GTTTGTAGCAATTCAGAAACACCCTT	Vakulenko et al. (2003)
aph(2″)-Ic-	FW: CCACAATGATAATGACTCAGTTCCC RV: CCACAGCTTCCGATAGCAAGAG	Vakulenko et al. (2003)
aph(2″)-Id	FW: GTGGTTTTTACAGGAATGCCATC RV: CCCTCTTCATACCAATCCATATAACC	Vakulenko et al. (2003)
aph(3′)-IIIa	FW: GGCTAAAATGAGAATATCACCGG RV: CTTTAAAAAATCATACAGCTCGCG	Vakulenko et al. (2003)
ant(4′)-Ia	FW: CAAACTGCTAAATCGGTAGAAGCC RV: GGAAAGTTGACCAGACATTACGAACT	Vakulenko et al. (2003)
tet (L)	tet(L)-2-1: CATTTGGTCTTATTGGATCG tet(L)-2-2: ATTACACTTCCGATTTCGG	Aarestrup et al. (2000)
tet (M)	tet(M)-1: GTTAAATAGTGTTCTTGGAG tet(M)-2: CTAAGATATGGCTCTAACAA	Aarestrup et al. (2000)
tet (O)	Tet(O)-1: GATGGCATACAGGCACAGAC Tet(O)-2: CAATATCACCAGAGCAGGCT	Aarestrup et al. (2000)
tet (S)	Tet(S)-1: TGGAACGCCAGAGAGGTATT Tet(S)-2: ACATAGACAAGCCGTTGACC	Aarestrup et al. (2000)
tet(W)	TetW-FW: GAGAGCCTGCTATATGCCAGC TetW-RV: GGGCGTATCCACAATGTTAAC	Aminov et al. (2001)
vanA	FW: GGGAAAACGACAATTGC RV: GTACAATGCGGCCGTTA	Dutka-Malen et al. (1995)
vanB	FW: ATGGGAAGCCGACAGTC RV: GATTTCGTTCCTCGACC	Dutka-Malen et al. (1995)
vanC1	FW: GCTGAAATATGAAGTAATGACCA RV: CGGCATGGTGTTGATTTCGTT	Miele et al. (1995)
van(E)	VanE1: TGTGGTATCGGAGCTGCAG VanE2: GTCGATTCTCGCTAATCC	Fines et al. (1999)
Bla Z	Bla Z-1: ACTTCAACACCTGCTGCTTTC BlaZ-2: TAGGTTCAGATTGGCCCTTAG	Martineau et al. (2000)
smr	Smr-FW: ATAAGTACTGAAGTTATTGGAAGT Smr-RV: TTCCGAAAATGTTTAACGAAACTA	Bjorland et al. (2001)
Gyr (A)	GyrAfw: CAMCGKCGKATTCTTTACGGAATG GyrArev: TTRTTGATATCRCGBAGCATTTC	Hummel et al. (2007)
Par (C)	ParCfw: TATTCYAAATAYATCATTCARGA ParCrev: GCYTCNGTATAACGCATMGCCG	Hummel et al. (2007)
Dfr(A)	DfrA1: CTTTTCTACGCACTAAATGTAAG DfrA2: CATTATCAATAATTGTCGCTCAC	Liu et al. (2009)
Dfr(D)	DfrD1: GGAAGGGCTTTACCTGACAGAAG DfrD2: CGACATAAGGCAAGAACATAACATA	Liu et al. (2009)

Table 2 (*continued*)

Target gene	Primer pair (5′→3′)	Reference
Aad(E)	aadEI: GCAGAACAGGATGAACGTATTCG aadEII: ATCAGTCGGAACTATGTCCC	Klare et al. (2007)
norA	FW: TTTGTTTTCAGTGTCAGAATTTATGTTTG RV: GGCTTGGTGAAATATCAGCTATTAAAC	Patel et al. (2010)
norC	FW: CAGGCAGGATACTTATCAATTAC RV: ATACCAATGACCACAATGAATG	Patel et al. (2010)
norE	norE-F: CTGGCGGCAGCGGTAA norE-R: TGCCATACAGACACCCACCATA	Swick et al. (2011)
mdeA	FW: CTTTCAGGTTACCTTGTTGAATATTTAAAC RV: ATCAATAGGTACTTTAATTGTAGTTCCAAC	Patel et al. (2010)
acrA	acrA-F: CTCTCAGGCAGCTTAGCCCTAA acrA-R: TGCAGAGGTTCAGTTTTGACTGTT	Swick et al. (2011)
acrB	acrB-F: GGTCGATTCCGTTCTCCGTTA acrB-R: CTACCTGGAAGTAAACGTCATTGGT	Swick et al. (2011)
tolC	tolC-F: AAGCCGAAAAACGCAACCT tolC-R: CAGAGTCGGTAAGTGACCATC	Swick et al. (2011)
mdfA	mdfA-F: CATTGGCAGCGATCTCCTTT mdfA-R: TTATAGTCACGACCGACTTCTTTCA	Swick et al. (2011)
mefA	FW: AGTATCATTAATCACTAGTGC RV: TTCTTCTGGTACTAAAAGTGG	Sutcliffe et al. (1996)

2. Material and methods

2.1. Bacterial strains and growth conditions

72 LAB strains (Abriouel et al., 2012) were obtained from Aloreña green table olives naturally-fermented by four small–medium enterprises (SMEs) from Málaga (Spain) and included lactobacilli (59 *Lb. pentosus* strains) and leuconostocs (13 *Lc. pseudomesenteroides* strains). These strains were routinely cultured at 30 ºC in de Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for 24–48 h. Strains were kept in 20% glycerol at −80 ºC for long term storage.

2.2. Antibiotic susceptibility testing and MIC determination

MICs of 15 antibiotics encompassing nearly all important classes were determined 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). Pharmacological classes and specific antibiotics employed in this study were: β-lactams (amoxicillin: AMO, ampicillin: AMP and cephalosporin cefuroxime: CFX), quinolone (ciprofloxacin: CIP), lincosamide (clindamycin: CLI), aminoglycosides (gentamicin: GEN, kanamycin: KAN and streptomycin: STR), macrolide (erythromycin: ERY), glycopeptides (teicoplanin: TPL and vancomycin: VAN), sulfonamides (trimethoprim: TMP and trimethoprim/sulfamethoxazole: TMP/SMZ at ratio 1.25/23.75), chloramphenicol: CMP and tetracycline: TET. All antibiotics were from Sigma, with the exception of STR, TET and CIP (Fluka), TMP and SMZ (Almirall), CFX (Norman Laboratories). The microbiological breakpoints of the antibiotics tested (defined also as ECOFF according to the European Food Safety Authority (EFSA, 2012) are shown in Table 1. For the antibiotics not included in the EFSA listing, the microbiological breakpoints were defined according to Danielsen and Wind (2003), Flórez et al. (2005) and the European Commission (European Commission, SCAN, 2005) for *Lactobacillus* and *Leuconostoc*. Furthermore, for the antibiotics for which no breakpoints were defined for *Lactobacillus* and *Leuconostoc* spp. in the literature, such as CFX, TPL and TMP/SMZ, we used the microbiological breakpoints reported by the National Committee for Clinical Laboratory Standards (CLSI, 2011) for staphylococci. We considered MICs higher than the suggested breakpoints as evidence of phenotypic resistance (EFSA, 2012). The accuracy of susceptibility testing was

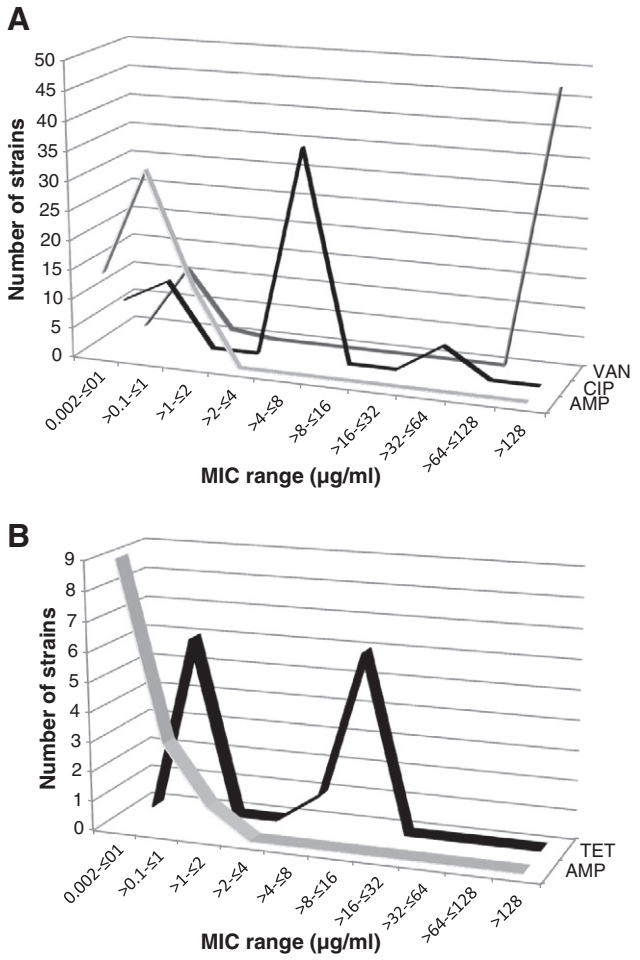

Fig. 1. Distribution of antibiotic MICs for *Lactobacillus pentosus* (A) and *Leuconostoc pseudomesenteroides* (B) strains isolated from Aloreña fermented olives.

monitored by parallel use of the quality control strain *Enterococcus faecalis* ATCC 29212.

2.3. Determination of intrinsic and acquired resistance

According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org), microorganisms without (wild type, WT) and with acquired resistance mechanisms (non-wild type, NWT) to the antibiotic in question are characterized by their MIC values as follows: WT ≤ X μg/ml and NWT > X μg/ml, X being the ECOFF (epidemiological cut-off) value of the corresponding antibiotic for the LAB species studied. The distinction between natural and acquired resistance was determined by analysis of MICs and their distributions according to Stock and Wiedemann (2001). In the case of a population with acquired resistance, plotting the MIC of a particular antibiotic for one species against the number of strains found with the respective MIC usually results in a bimodal distribution (one peak with relatively low MICs which represents the natural population, and one peak with higher MICs which represents the strains with acquired resistance).

2.4. PCR detection of antimicrobial resistance genes

PCR amplifications of well-known structural genes associated with resistance to chloramphenicol (*cat*, the chloramphenicol acetyltransferase gene), β-lactam antibiotics (*bla* and *blaZ*, the β-lactamase genes), macrolides (the *ermA*, *ermB*, *ermC*, *msrA/B*, *ereA*, *ereB*, *mphA*, *mefA*), tetracycline [*tet*(M), *tet*(O), *tet*(S) and *tet*(W), *tet*(L) and *tet*(K)], aminoglucosides [*aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa* and *ant(4′)-Ia*], sulfonamides (*dfrA* and *dfrD*) and glycopeptides (*vanA*, *vanB*, *vanC* and *vanE*) were performed using conditions described elsewhere (Aarestrup et al., 2000; Aminov et al., 2001; Dutka-Malen et al., 1995; Fines et al., 1999; Hummel et al., 2007; Klare et al., 2007; Liu et al., 2009; Martineau et al., 2000; Miele et al., 1995; Singh and Murray, 2005; Sutcliffe et al., 1996; Vakulenko et al., 2003) and primers listed in Table 2 (Supplementary Table). Table 2 also includes primers for genes mediating antibiotic resistance through other mechanisms, such as efflux pumps (mdfA, norE, acrA, acrB, tolC, mepA, norA, norC, mefA and mdeA). Template DNA for PCR reactions was prepared as in Jensen et al. (1998).

2.5. DNA sequencing of the QRDR related to ciprofloxacin resistance

To investigate whether observed phenotypic resistance to ciprofloxacin was due to mutations in the quinolone resistance determining regions (QRDR) of the *gyrA* and *parC* genes, the QRDR encoding regions were amplified (Table 2) as described by Hummel et al. (2007). PCR-amplified products purified using Exo Star kit (GE-Healthcare) were sequenced bidirectionally with their corresponding primers and the deduced amino acid sequences were aligned with those retrieved from the GenBank database by using DNAStar CLUSTAL W multiple alignment tool (Lasergene program, version 5.05 (DNASTAR, Inc., Madison, WI, USA).

2.6. Statistical analysis

Statistical analysis of data was accomplished using Excel 2007 and XLSTAT 2012 trial version (2012.4.02 Addinsoft, France) and the correlation between time of fermentation and resistance was determined by principal component analysis (PCA).

3. Results

3.1. Antimicrobial susceptibility testing and MIC distribution profiles

MIC determination of the different antibiotics was performed with 59 *Lb. pentosus* and 13 *Lc. pseudomesenteroides* from Aloreña table olives (including selected LAB with potential probiotic features according to Abriouel et al. (2012). The results obtained (Table 1) indicated that the MICs of β-lactams (amoxicillin and ampicillin), chloramphenicol,

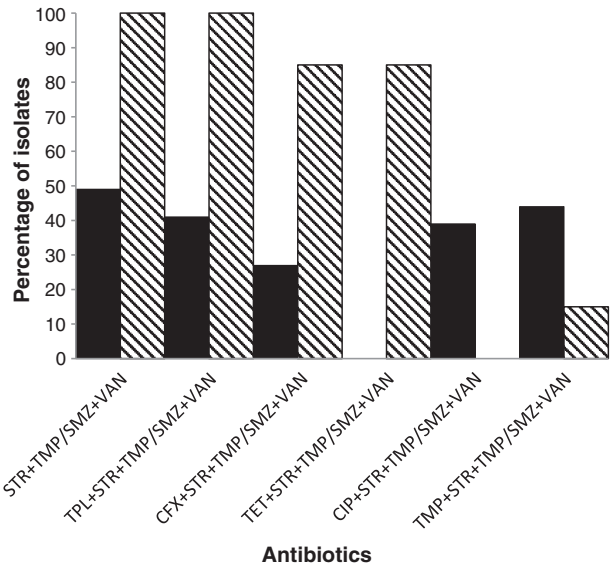

Fig. 2. Multidrug resistance observed in *Lactobacillus pentosus* (■) and *Leuconostoc pseudomesenteroides* (▨) strains isolated from Aloreña fermented olives.

Table 3
Phenotypic and genotypic antibiotic resistance of lactic acid bacteria isolated from Aloreña fermented olives.

Months of fermentation	Species	Phenotypic antibiotic resistance	Resistance gene(s) detected by PCR
Leuconostoc pseudomesenteroides			
3	CF2-26	CFX, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
6	AP2-28	KAN, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*
	CF1-31	CFX, KAN, STR, TET, TMP/SMZ, VAN	*acrA*
	CF2-31	CFX, KAN, STR, TET, TMP/SMZ, VAN	*acrA*
	CF2-33	STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-35	CFX, KAN, STR, TET, TMP/SMZ, VAN	
	CF2-38	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*mepA*
	CF2-40	CFX, KAN, STR, TET, TMP/SMZ, VAN	*mepA*
Other	SP5-11	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-12	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-17	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-18	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
	SP5-19	CFX, CLI, KAN, STR, TET, TMP/SMZ, VAN	*acrA*, *mepA*
Lactobacillus pentosus			
1	CF1-1	CFX, STR, VAN	*acrA*, *mepA*
	CF1-3	STR, TMP, TMP/SMZ, VAN	*acrA*, *mepA*
	CF1-4	CFX, CIP, STR, TMP/SMZ, VAN	*acrA*, *mepA*
	CF1-5	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-6	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-7	STR, VAN	*acrA*
	CF1-8	STR, VAN	*acrA*
	CF1-10	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF2-2	CFX, CIP, STR, TMP, TMP/SMZ	*mdeA*
	CF2-3	CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-4	CFX, TMP, TMP/SMZ	*mdeA*
	CF2-5	CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-6	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-7	STR, VAN	*mdeA*
	CF2-9	CFX, CIP, STR, TMP, TMP/SMZ	*mdeA*
	CF2-10	STR, VAN	*mdeA*
2	CF1-12	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-14	STR, VAN	*acrA*
	CF1-15	CFX, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-16	CFX, CIP, STR, TMP, VAN	
	CF1-19	CFX, CIP, STR, TMP, VAN	*acrA*
	CF1-20	STR, TMP, TMP/SMZ, VAN	*acrA*
	CF2-11	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*, *mdeA*
	CF2-12	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*, *mdeA*
	CF2-13	CFX, CIP, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF2-15P	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF2-17	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF2-18	CFX, CIP, TMP, VAN	*acrA*, *mdeA*
	CF2-19P	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*, *mdeA*
	LP-1	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	LP-5	CIP, STR, TMP, TMP/SMZ, VAN	*norA*, *mdeA*
	LP-7	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	LP-8	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
3	CF1-21	CFX, CIP, STR, TMP, VAN	*acrA*
	CF1-23	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*
	CF1-25	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-29	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-30	STR, TMP	*acrA*
	CF2-21	CIP, STR, TMP, TMP/SMZ	*acrA*, *norA*
	CF2-24	CFX, STR, TMP, TMP/SMZ, VAN	
	CF2-28	CIP, STR, TMP/SMZ, VAN	
	AP2-15	CFX, CIP, STR, TMP, VAN	
	AP2-16	CFX, CIP, STR, TMP, TMP/SMZ, VAN	
6	CF1-33	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF1-35	CIP, STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-36	CFX, STR, TMP, TMP/SMZ, VAN	*acrA*, *mdeA*
	CF1-37	STR, TMP, TMP/SMZ, VAN	*acrA*, *norA*, *mdeA*
	CF1-38	CFX, CIP, STR, TMP, TMP/SMZ, VAN	*mdeA*
	CF1-40	CIP, TMP, TMP/SMZ, VAN	
	CF1-43	CIP, TMP, TMP/SMZ, VAN	*mdeA*
	CF2-34	CIP, STR, TMP, TMP/SMZ, VAN	*norA*
	CF2-39	STR, TMP/SMZ, VAN	
Other	2C1	STR, VAN	
	2C4	CIP, STR, VAN	*acrA*
	2C5	STR	
	2C6	CIP, TMP, TMP/SMZ	
	2C7	CIP	
	5C2	STR	*acrA*
	5C3	CIP, TMP, TMP/SMZ, VAN	*acrA*

Samples CF1 and CF2 corresponded to fermenter 1 and fermenter 2 of SME1, respectively.

gentamicin (aminoglycoside) and erythromycin (macrolide) did not exceed the ECOFF suggested in most cases by the European Food Safety Authority (EFSA, 2012) for both LAB species (*Lb. pentosus* and *Lc. pseudomesenteroides*). However, in the case of amoxicillin, we used the microbiological breakpoint proposed by Flórez et al. (2005) as described in Table 1, since the official ECOFF of amoxicillin has not been designated for *Lb. pentosus* and *Lc. pseudomesenteroides* by the international organizations. Accordingly, relatively narrow unimodal MIC distributions at the low-end concentration range were shown by *Lb. pentosus* and *Lc. pseudomesenteroides* strains for amoxicillin and ampicillin (0.002–2 μg/ml). Concerning the other antibiotics to which both *Lb. pentosus* and *Lc. pseudomesenteroides* were very sensitive, they displayed either uni- or bimodal MIC distributions, depending on the antibiotic used and the LAB concerned (Table 1). Thus, chloramphenicol and erythromycin showed unimodal and bimodal MIC distributions for *Lc. pseudomesenteroides* and *Lb. pentosus*, respectively (Table 1). The bimodal MIC distributions of gentamicin observed could differentiate two sensitive subpopulations (Table 1), the major one at the low-end concentration range (MIC range 0.002–1 μg/ml) and the smallest one with intermediate MICs (>8–16 μg/ml). Regarding ciprofloxacin, only *Lc. pseudomesenteroides* strains were susceptible to this antibiotic (MIC range >0.1–8 μg/ml). However, *Lb. pentosus* showed susceptibility to clindamycin (MIC range 0.002–2 μg/ml), kanamycin (MIC range 0.002–64 μg/ml) and tetracycline (MIC range 0.002–16 μg/ml) (Table 1).

The incidence of phenotypic resistance in *Lb. pentosus* and *Lc. pseudomesenteroides* to some antibiotics varied considerably depending on the LAB genus tested and the antimicrobial used (Table 1). Generally, *Lc. pseudomesenteroides* was more resistant than *Lb. pentosus* to all antibiotics except ciprofloxacin and trimethoprim (Table 1). *Lb. pentosus* strains were phenotypically resistant to streptomycin, vancomycin, trimethoprim, trimethoprim/sulfamethoxazole, teicoplanin and ciprofloxacin in the range of 70–83% (Table 1), and cefuroxime (44%). The phenotypic antibiotic resistances observed for *Lc. pseudomesenteroides* included streptomycin, trimethoprim/sulfamethoxazole, teicoplanin and vancomycin (100%), kanamycin and cefuroxime (85%), clindamycin and tetracycline (46%), and trimethoprim (15%) (Table 1).

For cefuroxime, most strains of *Lb. pentosus* and *Lc. pseudomesenteroides* displayed broader bimodal MIC distributions, which allowed to differentiate two subpopulations: the most sensitive one with low MICs (>0.1–1 μg/ml) and the other one with intermediate-higher MIC values (>4–128 μg/ml), which included both sensitive and resistant strains (Table 1).

Concerning aminoglycosides (kanamycin and streptomycin), resistant *Lc. pseudomesenteroides* strains displayed unimodal MIC distributions (MIC range >16–32 μg/ml for kanamycin and MIC >128 μg/ml for streptomycin). However, *Lb. pentosus* strains showed bimodal MIC distribution for streptomycin, which distinguished between sensitive (MIC range 8–128 μg/ml) and resistant (MIC >256 μg/ml) subpopulations (Table 1).

For sulfonamides (trimethoprim and trimethoprim/sulfamethoxazol) and glycopeptides (teicoplanin and vancomycin), either uni- or bimodal MIC distributions were observed for both *Lb. pentosus* and *Lc. pseudomesenteroides* (Table 1), being resistant to teicoplanin, trimethoprim and vancomycin at a high MIC range (>128 μg/ml for teicoplanin and trimethoprim and >256 μg/ml for vancomycin). Bimodal distributions of teicoplanin and vancomycin for *Lb. pentosus* showed two subpopulations at the low-end (0.002–2 μg/ml) and the high-end (>64–>128 μg/ml) concentration ranges. However the bimodal distribution of trimethoprim MIC values of *Lb. pentosus* strains was broad and positioned at the intermediate-high concentration range (>4–>128 μg/ml) (Table 1).

Regarding tetracycline, only *Lc. pseudomesenteroides* was shown to be resistant to this antibiotic. The bimodal distribution obtained (Table 1, Fig. 1) indicated two subpopulations: one sensitive with low MICs (>0.1–1 μg/ml) and another one resistant with intermediate MICs (>8–16 μg/ml).

The distinction between natural and acquired resistance was determined for those strains of *Lb. pentosus* and *Lc. pseudomesenteroides* which showed phenotypic resistance to some antibiotics and displayed bi- or multimodal distribution of the MICs. Fig. 1 shows an example of the bi-/multimodal distribution of the MICs of ciprofloxacin and vancomycin for *Lb. pentosus* (Fig. 1A), and of tetracycline for *Lc. pseudomesenteroides* (Fig. 1B), in comparison with the normal unimodal MIC distribution for ampicillin to which all *Lb. pentosus* and *Lc. pseudomesenteroides* were susceptible (Fig. 1).

Multi-drug resistance (MDR, defined as resistance to 3 or more different antimicrobials) was observed in 13 *Lc. pseudomesenteroides* strains (100%) and 56 *Lb. pentosus* strains (95%) (Fig. 2, Table 3). All MDR *Lc. pseudomesenteroides* strains were resistant to at least 4 antimicrobials, and 6 were resistant to 7 of them (Fig. 2, Table 3). However, MDR *Lb. pentosus* were resistant to 3 (8 strains), 4 (9 strains), 5 (14 strains), 6 (16 strains) or 7 (9 strains) antibiotics (Fig. 2, Table 3).

3.2. Detection of resistance genes

To identify resistance determinants responsible for the resistance phenotypes observed, all strains were screened by PCR for the presence of resistance genes as described above. Analysis of tetracycline-resistant *Lc. pseudomesenteroides* showed that neither the genes encoding ribosomal protection proteins [*tet*(M), *tet*(O), *tet*(S) or *tet*(W)] nor genes encoding the tetracycline efflux pumps [*tet*(K) or *tet*(L)] were detected

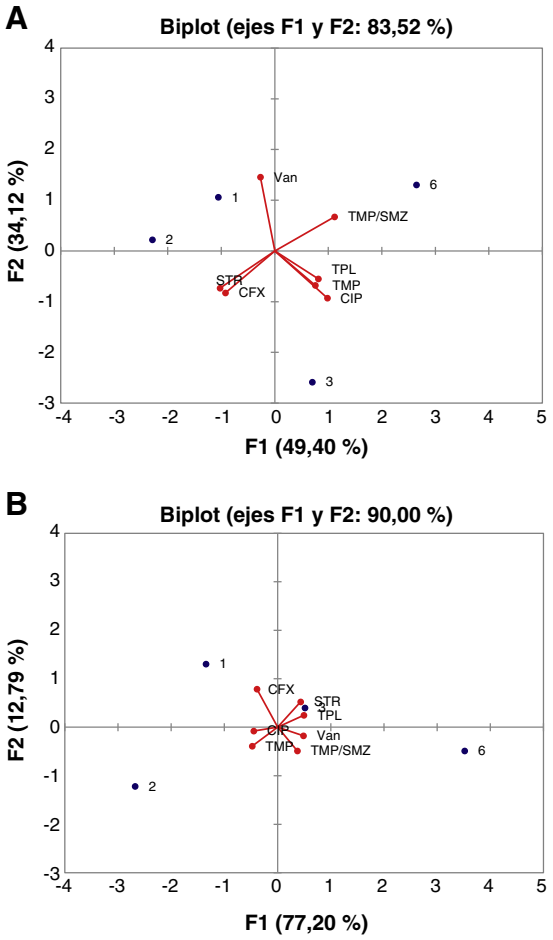

Fig. 3. Biplot of the simultaneous evaluation of the relationship of scores (antimicrobials) and sample variable (months of fermentation in fermenter 1 "A" and fermenter 2 "B" of SME1). Antimicrobials: CFX, cefuroxime; CIP, ciprofloxacin; STR, streptomycin; TPL, teicoplanin; VAN, vancomycin; TMP, trimethoprim and TMP/SMZ, trimethoprim/sulfamethoxazole.

in any strain with an MIC higher than 8 μg/ml. Similar results were obtained for the *lsa* gene responsible for resistance to lincosamides, which was not detected in clindamycin-resistant *Lc. pseudomesenteroides*. Other genes encoding for resistance to aminoglycosides [*aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa* and *ant(4′)-Ia*], sulfonamides (*dfrA* and *dfrD*), glycopeptides [*van(A)*, *van(B)*, *van(C)* and *van(E)*], and β-lactam antibiotics (*bla* and *blaZ*) were not detected in any of the LAB strains studied.

Molecular characterization of ciprofloxacin resistance was done by the comparative analysis of the deduced amino acid sequences of the highly conserved quinolone resistance-determining region (QRDR) of *Lb. pentosus* strains with either resistant or sensitive phenotypes. No substitutions could be detected within the QRDR of the GyrA subunit of DNA gyrase (Ser-83 and Asp-87) or the ParC subunit of topoisomerase IV (Ser-80 and Glu-84). Therefore, no insertion sequences, integrons or transposons flanking the genes were involved in the observed resistance to ciprofloxacin.

Concerning efflux pumps implicated in antibiotic resistance, *acrA* was the most commonly detected determinant, i.e. in 69% of *Lc. pseudomesenteroides* and 54% of *Lb. pentosus* (Table 3). Similarly, *mepA* was detected in 62% of *Lc. pseudomesenteroides*, while only 5% of *Lb. pentosus* harbored genes coding for this efflux pump. On the other hand, *mdeA* was detected in 32% of *Lb. pentosus* and only in one *Lc. pseudomesenteroides* strain. Concerning *norA*, 8% of *Lc. pseudomesenteroides* and 22% of *Lb. pentosus* gave positive results in PCR reactions of the corresponding efflux pump (Table 3). With respect to other efflux pumps (*mdfA*, *norE*, *acrB*, *tolC*, *norC* and *mefA*), none of the LAB strains showed positive results in the corresponding PCR reactions.

3.3. Principal component analysis

Principal component analysis (PCA) was used as a mathematical tool to evaluate the relationship among all antibiotic resistances tested in this study as a function of fermentation months (variable). The biplot graph shown in Fig. 3 indicates that during the first, second and six months of fermentation, vancomycin and trimethoprim/sulfamethoxazol were the most relevant antibiotic resistances in fermenter 1. Similarly, vancomycin and trimethoprim/sulfamethoxazol, and also trimethoprim and ciprofloxacin were prevalent resistances in fermenter 2 after two and six months of fermentation (Fig. 3). However, after one month fermentation in fermenter 2, cefuroxime, teicoplanin and streptomycin showed more relevant resistances, being also prevalent after 3 months of fermentation in the same fermenter (Fig. 3). On the other hand, after 3 months fermentation in fermenter 1 several antibiotic resistances were relevant such as ciprofloxacin, teicoplanin, trimethoprim, cefuroxime and streptomycin (Fig. 3).

4. Discussion

The use or rather misuse of antibiotics for decades in bacterial infection treatments, animal husbandry and agriculture (Wegener, 2003) has resulted in the increased emergence of resistant bacteria to modern antibiotics, leading to failure in therapy and causing also evolutionary and ecological problems as were reported by Gillings (2013) about the recruitment of more resistance genes into the resistome and mobilome.

Antibiotic-resistant bacteria represent a great challenge for the food industry, especially LAB which were isolated from several European fermented foods (Maietti et al. 2007; Ouoba et al. 2008; Nawaz et al. 2010; Toomey et al. 2010) such as fermented meat and dairy foods. LAB traditionally used as starter cultures and also as probiotics may act as reservoir of antibiotic-resistance genes which are similar to those found in human pathogens (Flórez et al., 2005) and potentially transferable to other pathogens either in the food matrix or in the gastrointestinal tract (Mathur and Singh, 2005). In the present study, we investigated the antibiotic susceptibility profiles of LAB isolated from a traditional and natural fermentation of Aloreña table olives. Although LAB from this food source were probably not exposed to antibiotics and were therefore not expected to harbor transferable resistances, it could not be assumed that such bacteria would be totally free of antibiotic resistances and transferable antibiotic resistance genes, and their QPS status (EFSA, 2004) would need to be confirmed for further starter culture development as demanded by EFSA (2012) for use of LAB starter strains in foods.

The antibiotic susceptibility profiles of *Lb. pentosus* and *Lc. pseudomesenteroides* strains showed that both are generally quite sensitive to clinically relevant antibiotics such as ampicillin, amoxicillin, erythromycin, chloramphenicol and gentamicin. In general, lactobacilli and leuconostocs are susceptible to antibiotics inhibiting the protein synthesis such as erythromycin, chloramphenicol, clindamycin and tetracycline (Florez et al., 2005; Ammor et al., 2007). However, only *Lb. pentosus* strains were susceptible to tetracycline and clindamycin, while 46% of *Lc. pseudomesenteroides* strains were phenotypically resistant to these antibiotics. In both cases, the bimodal distribution of MICs suggested that some *Lc. pseudomesenteroides* strains possessed acquired resistance for clindamycin and tetracycline. However, the absence of resistance determinants [*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(L) and *tet*(K)], may suggest a new mechanism of resistance which can be due either to acquired genes or to the mutation of indigenous genes (EFSA, 2012). The common resistance mechanism of erythromycin and clindamycin [the so-called macrolide–lincosamide–streptogramin (MLS) phenotype] was not responsible for the observed clindamycin resistance in resistant leuconostocs, since no *erm*, *msrA/B*, *mphA* or *mefA* genes were detected. Furthermore, *lsa* gene was not related with clindamycin resistance phenotype in *Lc. pseudomesenteroides* as this gene was absent in resistant bacteria.

The high resistance to teicoplanin and vancomycin (MICs > 128 μg/ml) exhibited by most *Lb. pentosus* strains (71–80%) and all *Lc. pseudomesenteroides* strains (100%) is in agreement with the reported intrinsic resistance of LAB to these antibiotics (Danielsen and Wind, 2003; Ammor et al., 2007; Liu et al., 2009). Glycopeptide resistance of both *Lb. pentosus* and *Lc. pseudomesenteroides* is probably due to the presence of D-Ala-D-lactate in their peptidoglycan, instead of the normal dipeptide D-Ala-D-Ala (Deghorain et al., 2007). As this is a chromosomally mediated and not transferable trait, this would explain why in the present study we did not find any vancomycin resistance gene marker such as the transferable *van* genes (*vanA*, *vanB*, *vanC* and *vanE*).

Intrinsic resistance to aminoglycosides such as streptomycin and kanamycin has been reported to be a general feature of lactobacilli (Danielsen and Wind, 2003), and is thought to result from membrane impermeability (Elkins and Mullis, 2004). The results obtained in the present study showed that *Lb. pentosus* strains (83%) were resistant only to streptomycin (MIC > 256 μg/ml). However, *Lc. pseudomesenteroides* strains (85–100%) were shown to be resistant to both aminoglycosides. The lack of aminoglycoside resistance determinants [*aad*(E), *aac(6′)-Ie-aph(2′)-Ia*, *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id*, *aph(3′)-IIIa* and *ant(4′)-Ia*] and the observed unimodal distribution of MICs at the high-end concentration range suggest an intrinsic resistance to aminoglycosides in *Lc. pseudomesenteroides* strains. Compared to kanamycin and streptomycin, gentamicin showed very effective inhibition at the low-end concentration range (0.002–1 μg/ml), possibly because this aminoglycoside is able to cross the membrane better than other aminoglycosides (Elkins and Mullis, 2004), thus resulting in lower MICs of this antibiotic for both LAB species tested.

All tested *Lc. pseudomesenteroides* and 71% of *Lb. pentosus* exhibiting bimodal or multimodal broad MIC distributions for trimethoprim/sulfamethoxazole, were shown to be resistant. However, trimethoprim displayed bimodal MIC distributions over a broad concentration range, with 76% of *Lb. pentosus* strains but only 15% of *Lc. pseudomesenteroides* strains resistant to this antibiotic. In both cases, none of the strains showed amplification with *dfrA* and *dfrD* primers related with trimethoprim resistance. The European Food Safety Authority (EFSA, 2008) considered that the resistance shown by LAB to trimethoprim, trimethoprim/

sulfamethoxazol and streptomycin is not relevant when tested on LSM medium, because of the interference of its components with the antibacterial activity of these antibiotics. Small portion of LSM broth (10% MRS) contains antagonistic components such as p-aminobenzoic acid (against sulfamethoxazole) and/or thymidine (against trimethoprim) (Klare et al., 2005; Turnidge and Bell, 2005).

The reduced susceptibility to ciprofloxacin reported here for *Lb. pentosus* (70%), exhibiting a multimodal MIC distribution with three different subpopulations, indicated an acquired resistance. However, we found no mutations in the QRDR of the genes encoding GyrA or ParC for ciprofloxacin resistance associated with insertion sequences, integrons, or transposons as reported previously (El Amin et al., 1999; Hummel et al., 2007; Petersen and Jensen, 2004), which indicated an intrinsic mechanism of resistance.

With respect to cefuroxime, LAB are usually more resistant to cephalosporins as reported by Danielsen and Wind (2003) and Coppola et al. (2005). The impermeability of the cell wall is the main mechanism of resistance to inhibitors of cell-wall synthesis, since LAB species lack cytochrome-mediated electron transport (Condon, 1983). However, the cooperation of non-specific mechanisms, such as multidrug transporters (Putman et al., 2001) and defective cell wall autolytic systems (Kim et al., 1982), may be responsible for the differences observed between the strains. In the present study, 44% of *Lb. pentosus* and 85% of *Lc. pseudomesenteroides* were resistant to this antibiotic, but none of the resistant strains harbored acquired resistance determinants (*bla* and *blaZ*).

A multi-resistance profile of LAB isolated from Aloreña table olives was shown throughout the fermentation process. Correlation of different parameters (antibiotics and months of fermentation) showed that the prevalence of antibiotic resistance in LAB was highly dependent on the fermenter where the fermentation took place, since the biotic and abiotic conditions were quite different (Abriouel et al., 2011). Green olives were placed in 6000-liter glass fiber tanks, supplemented with salt (6% wt/vol) and 0.8% (vol/vol) of acetic acid and allowed to ferment for 4 to 7 months at room temperature. Vancomycin and trimethoprim/sulfamethoxazol were the most relevant antibiotic resistances in LAB during the whole fermentation process, regardless of the fermenter sampled. On the other hand, PCA also showed that the most prevalent month was the third month of fermentation when correlated with multiple antibiotic resistance. The intrinsic resistance of LAB to several antibiotics may be partially due to genes that encode multidrug resistance efflux pumps (MDRs), which expel different types of antibiotics and also chemicals (dyes, organic solvents, detergents, biocides and metabolic products). In the present study, *norA* which confers resistance to chloramphenicol and fluoroquinolones (norfloxacin and ciprofloxacin) as reported by several authors (Neyfakh et al., 1993; Truong-Bolduc et al., 2003) was detected in LAB which were both resistant and sensitive to ciprofloxacin, suggesting that *norA* may play another role in some LAB (especially in ciprofloxacin-sensitive strains). Regarding the tripartite complex AcrAB-TolC system which is involved in the efflux of β-lactams, fluoroquinolones, chloramphenicol and tetracycline (Okusu et al., 1996), only AcrA – a fusion protein – was detected in both LAB, while AcrB – a cytoplasmic membrane transporter protein – and TolC – an outer membrane channel protein – were not detected. The absence of AcrB and TolC may hypothetically be due to a point mutation in their corresponding genes, which enabled their detection by PCR, or maybe AcrA does not play a role in antibiotic resistance in this species. NorA and MdeA, both chromosomally encoded MFS (major facilitator superfamily) pumps, and MepA, a MATE-family MDR pump that is also chromosomally encoded (Kaatz et al., 2005), were detected in both LAB species. Multidrug-resistance efflux pumps are encoded in all bacteria and can confer clinically relevant resistance to antibiotics, but it is now understood that these efflux pumps also have a physiological role (Piddock, 2006). In the present study, efflux pumps detected may confer low level resistance to several antibiotics, but they may also play a crucial role in allowing bacteria to survive in their ecological niche (high salt, low pH, antimicrobials such as phenolic compounds from olives, metabolic products).

5. Conclusions

Almost all *Lb. pentosus* (95%) and all *Lc. pseudomesenteroides* strains isolated from naturally-fermented Aloreña green table olives can be regarded as safe because of the absence of acquired resistance determinants. The intrinsic resistance to more than three antibiotics will not become a problem in a medical setting, since they were also highly sensitive to other clinically relevant antibiotics. In this study, the occurrence of intrinsic multi-resistance in both LAB species was due in part to chromosomally encoded efflux pumps such as NorA, MepA and MdeA.

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