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Role of EfrAB efflux pump in biocide tolerance and antibiotic resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolated from traditional fermented foods and the effect of EDTA as EfrAB inhibitor

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ABSTRACT

Enterococcus faecalis and *Enterococcus faecium* isolated from various traditional fermented foods of both animal and vegetable origins have shown multidrug resistance to several antibiotics and tolerance to biocides. Reduced susceptibility was intra and inter-species dependent and was due to specific and unspecific mechanisms such as efflux pumps. EfrAB, a heterodimeric ABC transporter efflux pump, was detected in 100% of multidrug resistant (MDR) *E. faecalis* strains and only in 12% of MDR *E. faecium* strains. EfrAB expression was induced by half of minimum inhibitory concentration (MIC) of gentamicin, streptomycin and chloramphenicol. However, expression of *efrA* and *efrB* genes was highly dependent on the strain tested and on the antimicrobial used. Our results indicated that 3 mM EDTA highly reduced the MICs of almost all drugs tested. Nevertheless, the higher reductions (>8 folds) were obtained with gentamicin, streptomycin, chlorhexidine and triclosan. Reductions of MICs were correlated with down-regulation of EfrAB expression (10–140 folds) in all three MDR enterococci strains. This is the first report describing the role of EfrAB in the efflux of antibiotics and biocides which reflect also the importance of EfrAB in multidrug resistance in enterococci. EDTA used at low concentration as food preservative could be one of the best choices to prevent spread of multidrug resistant enterococci throughout food chain by decreasing EfrAB expression. EfrAB could be an attractive target not only in enterococci present in food matrix but also those causing infections as well by using EDTA as therapeutic agent in combination with low doses of antibiotics.

1. Introduction

Enterococci are normal inhabitants of the gastrointestinal tract (Koch et al., 2004) of humans and many animals (about 10^7/gram of stool) and also in the genitourinary tract, being also present in a variety of foods of animal and vegetable origins, soil and water. Their ubiquitous nature due to the ability to withstand various adverse environmental conditions determines their frequent finding in foods as contaminants being their elimination a challenging and difficult task. Thus, enterococci have emerged as important nosocomial pathogens with high-level resistance to antibiotics (Murray, 2000). *Enterococcus faecalis* and *Enterococcus faecium* are the main enterococci species causing nosocomial infections of about 75 and 20% of the enterococcal-related infections (Conde-Estevez et al., 2011; Peel et al., 2011) such as urinary tract, endocarditis, bacteremia, catheter-related infections, wound infections, and intra-abdominal and pelvic infections (Koch et al., 2004; Leung et al., 2000; Poh et al., 2006; Svanborg and Godaly, 1997). While some enterococci are known for their pathogenic implications (Edmond et al., 1999; Kayser, 2003; Murray, 1997; Vergis et al., 2001), other species are used as starter cultures in food fermentations (Foulquie Moreno et al., 2006) or as probiotics (Franz et al., 2003) being only some *E. faecium* stains authorized by the European Food Safety Authority (EFSA, 2012) as feed additives.

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The high survival capacity exhibited by enterococci in a changing environment with a broad-range of bacteria including non-pathogenic species -especially in food matrix- and their capacity in biofilm formation (Creti et al., 2006; Tendolkar et al., 2006) caused a large exposition to gene exchange such as virulence factors and antibiotic resistance (Eaton and Gasson, 2001; Teuber et al., 2003; Vankerckhoven et al., 2008). Foods of animal origin have been shown to act as reservoir of multiple resistances (Lavilla Lerma et al., 2013; Toomey et al., 2010; Valenzuela et al., 2013; Witte, 2000) to antibiotics which were commonly used in animal husbandry to cure or prevent the onset of bacterial infections and also as growth promoters over decades. Furthermore, multiple resistances were also shown to biocides which were extensively used as disinfectants in food industry. The high-level resistance of enterococci to several antibiotics of clinical importance such as ampicillin, cephalosporins, aminoglycosides, and glycopeptides has been previously reported (Kacmaz and Aksoy, 2005; Murray, 1990) and could be due to intrinsic or acquired mechanisms (Hummel et al., 2007; Klare et al., 2003; Murray, 1990). Multidrug resistance of enterococci of food origin is well documented (Leclercq, 2009; Peters et al., 2003; Vignaroli et al., 2011) and is often associated with the over-expression of efflux pumps which exclude compounds that are structurally and functionally unrelated to prevent the cellular entry of drugs from the cell or the cytoplasmic membrane (Putman et al., 2000). Furthermore, other resistance mechanisms were reported in enterococci including mutational alteration of the target such as the replacement of the second D-Ala residue from peptidoglycan termini by a D-lactase or D-serine (responsible for vancomycin resistance) or the alteration of penicillin-binding proteins (PBPs), and the enzymatic inactivation of drugs (Levine, 2006; Rybkine et al., 1998). The most characterized efflux pumps in enterococci are: EmeA -a member of the major facilitator superfamily (MFS) and a homolog of NorA- (Jonas et al., 2001; Lee et al., 2003a) and EfrAB -belonging to the ATP-binding cassette (ABC) superfamily of multidrug efflux transporters- (Lee et al., 2003b). The ABC multidrug efflux pump EfrAB was detected in *E. faecalis* (Lee et al., 2003b; Valenzuela et al., 2013) but also for the first time in *E. faecium* strains as reported by Valenzuela et al. (2013).

In the present work, we explored the role of EfrAB efflux pump in tolerance/resistance to biocides and antibiotics in MDR *E. faecalis* and *E. faecium* isolated from different traditional fermented foods. Next, we examined the effect of EDTA on EfrAB expression and changes in MICs of different antimicrobials. Inhibition of efflux pump expression is a good alternative to decrease susceptibility of MDR enterococci to commonly used disinfectants and clinical antibiotics. Furthermore, knowledge of antibiotic resistance in enterococci of food origin and their resistance mechanisms is crucial to provide key informations about how to contain the antimicrobial resistance problem in food safety by using different strategies such as inhibitors of efflux pumps mediating resistance to several antimicrobials.

2. Materials and methods

2.1. Bacterial strains and media

A total of 122 enterococci strains belonging to the species of *E. faecium* and *E. faecalis* were isolated from different traditional fermented foods including fermented milk (Morocco and Spain), meat (Morocco), and vegetable products (Morocco, Spain, and Republic of Congo). Of them, 67 strains were isolated in the present study and 55 strains were isolated by Valenzuela et al. (2008, 2010, 2013) from traditional foods and water used for traditional food preparation. All strains were maintained and stored in brain-heart infusion (BHI) broth (Scharlab, Barcelona, Spain) containing 20% glycerol at −80 °C. For routine use, enterococcal strains were cultivated on BHI broth at 37 °C.

2.2. Identification of E. faecium and E. faecalis strains

2.2.1. DNA extraction

Total DNA was extracted from cultures by the method described by De los Reyes-Gavilan et al. (1992). This DNA preparation was used in further polymerase chain reactions (PCR).

2.2.2. Species-specific PCR

Presumptive *Enterococcus* sp. were identified at species level by species-specific PCR to detect the *ddlE.faecalis* and *ddlE.faecium* genes as described elsewhere (Abriouel et al., 2005).

2.3. Antibiotic and biocide phenotypic susceptibility

The antibiotic susceptibility of enterococci strains was determined by using ATB ENTEROC 5 strips (BioMérieux, Marcy-l'Etoile, France) as described elsewhere (Valenzuela et al., 2013). The tests were performed according to the manufacturer's instructions by using the following antibiotics: Ampicillin (AMP), Penicillin (PEN), Erythromycin (ERY), Tetracycline (TET), Chloramphenicol (CMP), Rifampicin (RFA), Ciprofloxacin (CIP), Levofloxacin (LVX), Vancomycin (VAN), Teicoplanin (TEC), Nitrofurantoin (FUR), Gentamycin (GEN), Streptomycin (STR) and Quinupristin/Dalfopristin (QDA). Results were recorded after 24 h of incubation at 37 °C and were evaluated according to the manufacturer's instructions.

Regarding disinfectants, the susceptibility of enterococci was assayed against the following biocides: Benzalkonium chloride (BC; Sigma Aldrich), Cetrimide (CE; Sigma Aldrich, USA), Chlorhexidine dihydrochloride (CHX; Sigma Aldrich, US), Hexadecylpyridinium chloride monohydrate (HDP, Sigma Aldrich, USA) and Triclosan (TC; Fluka, Italy). The Minimum Inhibitory Concentration (MIC) of biocides was determined in Trypticase Soya Broth (TSB, Scharlab) as described by Fernández-Fuentes et al. (2012). Results were recorded after 24–48 h of incubation by visual reading and optical density (OD 595 nm) determination in an iMark Microplate Reader (BioRad, Madrid, Spain). All susceptibility tests were performed in triplicate and the mean MIC was determined.

2.4. PCR amplification for the detection of antibiotic resistance genes

PCR amplification of well-known structural genes of antibiotic resistance: erythromycin (*erm*A,B,C; *mef*A,E; *msr*A,B; and *ere*A,B), tetracycline [*tet*(M), *tet*(O), *tet*(S), *tet*(K) and *tet*(L)] and chloramphenicol (*cat*) was performed as reported by Hummel et al. (2007). PCR of *efrA* and *efrB* genes was done according to Lee et al. (2003b).

2.5. Expression of efrA and efrB genes in MDR enterococci

2.5.1. RNA extraction

The expression of *pheS*, *efrA* and *efrB* genes by MDR enterococci was determined in TSB supplemented with or without the corresponding biocides or antibiotics. Total RNA was isolated from three biological repeats by the Direct-Zol RNA Miniprep (Zymo Research, California, USA) according to the manufacturer's instructions. 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/μl and frozen at −80 °C until required.

2.5.2. Quantitative real-time PCR of efrA and efrB genes

For quantitative real-time PCR (qRT-PCR), mRNAs were reverse transcribed into cDNA using the iScript cDNA synthesis kit (Biorad, Madrid, Spain). Phenylalanyl-tRNA synthase alpha-subunit (*pheS*) gene was used as a housekeeping gene and a no template control (NTC) was used as negative control. Primers for the *pheS* housekeeping gene were described by Naser et al. (2005) while the primers for quantitative PCR of *efrA* and *efrB* genes were described in this study: efrAqRT-fw (5′-TTGGCTTTATGACGCCAGT-3′) and efrAqRT-rev (5′-ATGCGCGTATTACCCGCAA-3′) for *efrA* gene, and efrBqRT-fw (5′-TAGTGATGATGTTCTTAATCAA-3′) and efrBqRT-rev (5′-ATTGACTTGTTTAAAGCCTTCA-3′) for *efrB* gene. Quantitative PCRs (qPCRs) were performed in triplicate on a CFX96 Touch™ Real-Time PCR Detection System from BioRad using 2 × *Power* SYBR green chemistry. PCR-grade water served as a negative control.

2.6. Effect of different antimicrobials on EfrAB efflux pump

To assess the effect of antibiotics and biocides on mRNA expression of EfrAB, *E. faecalis* (qE14 and Ac8-2) and *E. faecium* (VP501) strains carrying EfrAB efflux pump were treated with different drugs at half of MIC for 24 h (until the bacterial growth reached 0.5–0.6 OD at 595 nm). Following treatment, total mRNA isolation, subsequent cDNA synthesis and quantitative RT-PCR were done as described above. The strains without treatment with drugs served as controls.

2.7. Effect of EDTA on EfrAB efflux pump

2.7.1. Phenotypic tests

To investigate the effect of ethylenediamine tetra acetic acid (EDTA) on antibiotic and biocide MICs in MDR enterococci, 3 mM EDTA (below the inhibitory concentration of growth determined in MDR enterococci as 5 mM) was added to cultures and the MIC of both antimicrobials (biocides and antibiotics) was determined in TSB as described above.

2.7.2. Effect of EDTA on EfrAB expression

To study the effect of EDTA on mRNA of EfrAB expression, MDR enterococci positive for EfrAB efflux pump were supplemented in TSB with 3 mM EDTA for 24 h. Following treatment, total RNA extraction and quantitative real-time PCR of *efrA* and *efrB* genes were done as described in Section 2.5. Controls without EDTA were used.

2.7.3. Sequencing of efrA and efrB genes before and after EDTA treatment

With the aim to investigate the effect of EDTA on EfrAB at genetic level, MDR enterococci supplemented in TSB with or without 3 mM EDTA for 24 h were subjected to DNA extraction as described above. EfrAB encoding regions of MDR enterococci were amplified using the primers described by Lee et al. (2003b). PCR-amplified products were purified using Exo Star kit (GE-Healthcare) and then sequenced bidirectionally with their corresponding primers (Lee et al., 2003b) and also with efrA-2F (5′-ATGATGTATCA-GAAGGTG-3′) and efrB-2F (5′-ATTGTTGACCATTGCCCGA-3′). EfrA and EfrB deduced amino acid sequences of controls and EDTA-treated MDR enterococci were aligned by using DNAStar CLUSTAL W multiple alignment tool Lasergene programme, version 5.05 (DNASTAR, Inc., Madison, WI, USA).

2.7.4. Nucleotide sequence accession numbers

Nucleotide sequences of *efrA* and *efrB* genes of the selected enterococci strains determined here were deposited in GenBank with the following accession numbers HG970098 to HG970103.

2.8. Statistical analysis

Statistical analysis was performed using the Student's *t*-test and expressed as means ± standard deviation (SD).

3. Results

3.1. Biocide susceptibility of E. faecalis and E. faecium strains

The results obtained showed that all enterococci isolated from fermented foods were inhibited by lower concentrations of the biocide compounds tested such as BC, HDP and CE, with MIC's below 0.1 mg/l (Table 1). However, for the guanine CHX, 5.26% of *E. faecalis* strains (qE-14 and Mz4Sth, Table 1) and 36% of *E. faecium* strains (30 strains, Table 1) of different origins were inhibited at 0.25 mg/l, but 16% of *E. faecalis* (AC 5-2, AC 8–2, H_2O P7, MZ4sth, VP5 02 and YA8, Table 1) and 5% of *E. faecium* strains (AL6, H_2O P5 03, Tg 6 and YA5, Table 1) required still higher biocide concentration for inhibition (2.5 mg/l). Concerning Triclosan, the MICs of enterococci strains were of 0.1 mg/l for 4% of *E. faecium* strains (H_2O P3-A, H_2O P3–B and H_2O P5, Table 1), 0.2 mg/l for 10.53% and 42% of *E. faecalis* (H_2O P7, PE1-1, VP5 02 and YA8) and *E. faecium* (35 strains, Table 1) strains respectively, and 0.25 mg/l for 5.26% of *E. faecalis* strains (AC5-2 and AC8-2) and 13.1% of *E. faecium* strains (AC5-1, AC6-1, AC8-1, AC10-1, AC11-1, AC12–1, C2-3, H_2O P5 03, KAA1, KAA3 and KAA4, Table 1).

3.2. Antibiotic susceptibility and selection of MDR E. faecalis and E. faecium

On the basis of the phenotypic resistance profile of *E. faecium* and *E. faecalis* strains to chlorhexidine and triclosan, antibiotic susceptibility of resistant strains was evaluated (Fig. 1, Table 2) in this study and also by Valenzuela et al. (2010, 2013) as described previously. The incidence of phenotypic antibiotic resistance amongst biocide-tolerant enterococci was highly dependent on the species analyzed (Fig. 1), so 87.5% of *E. faecalis* strains showed phenotypic resistance to rifampicin (RFA), 50% to quinupristin/dalfopristin (QDA) and 37.5% to ciprofloxacin (CIP) and erythromycin (ERY). However, the most important antibiotics to which *E. faecium* strains showed phenotypic resistance were RFA (57.5%), ERY (55.3%), CIP (29.8%), levofloxacin (LVX) (25.5%) and nitrofurantoin (FUR) (21.2%). Concerning the food origin of isolation, biocide-tolerant enterococci exhibited phenotypic resistance to all antibiotics mentioned above being isolated from different fermented foods (vegetable, meat and fish products) and water.

Considering the phenotypic resistance of all strains, MDR enterococci were detected in 50% and 35% of *E. faecalis* and *E. faecium* strains, respectively. However the genotypic resistance detected by PCR (Table 2) indicated that such resistance determinants such as *ermB*, *tetM*, *tetL*, *cat*, *msrA/B* and *efrA/B* were only present in some strains. Nevertheless, Table 2 showed clearly that all *E. faecalis* strains with tolerance to chlorhexidine/triclosan or both exhibited the presence of *efrA* and *efrB* efflux pump (8 strains, 100%), while only 6 out of 49 (12%) *E. faecium* strains showed the presence of this efflux pump.

3.3. Effect of different drugs on expression of EfrAB efflux pump in E. faecalis and E. faecium

We determined the effect of different antimicrobials (antibiotics or biocides) on the expression of *efrA* and *efrB* genes coding for EfrAB efflux pump in MDR enterococci strains. In general, no effects were observed following treatment with most drugs in different MDR enterococci (Fig. 2). However, significant changes in mRNA

Table 1
Minimum inhibitory concentrations of biocides against enterococcal isolates from foods.

Biocide	*E. faecalis* strains with MIC's of (in mg/l)					*E. faecium* strains with MIC's of (in mg/l)				
	0.1	0.2	0.25	2.5	5	0.1	0.2	0.25	2.5	5
Benzalkonium chloride	0	0	0	0	0	0	0	0	0	0
Hexadecylpyridinium chloride	0	0	0	0	0	0	0	0	0	0
Chlorhexidine	0	0	qE14, MZ4KAA	AC5-2, AC8-2, H_2O P7, MZ4sth, VP5 02, YA8	0	0	0	AC 5-1, AC 6-1, AC10-1, AC 11-1, AC 12-1, AL 2, AL 3, AL 4, CH 1-2, H_2O P5 01, H_2O P8-A, LA 1-2, M 2-2, PE 2-1, PE 2-2, PEF 2-2, Sln 1, Sln 2, KAA 1, KAA 3, VP3 01, VP3 02, VP5 01, VP5 03, YA 2, YA 6, YA 7, YA 9-A, YA 9-B, YA 10	AL6, H_2O P5 03, Tg6, YA5	0
Cetrimide	0	0	0	0	0	0	0	0	0	0
Triclosan	0	H_2O P7, PE1-1, VP5 02, YA8	AC8-2, AC5-2	0	0	H_2O P3-A, H_2O P3, H_2O P5	AL 2, AL 3, AL 4, AL 6, CH 1-2, CHG 2-1, CHG 2-2, CHG 2-3, H_2O P501, H20 P8-A, KAA 2, KAA entC, LA 1-2, M2-1, M2-2, PE 1-2, PE 2-1, PE 2-2, PE 3-2, PEF 2-2, Sln 1, Sln 2, Sln entC, Tg6, VP3 01, VP3 02, VP5 01, VP5 03, YA 2, YA 5, YA 6, YA 7, YA 9-A, YA 9-B, YA 10	AC5-1, AC6-1, AC8-1, AC10-1, AC11-1, AC12-1, C2-3, H_2O P5 03, KAA 1, KAA 3, KAA 4	0	0

levels were detected in gentamicin and streptomycin treated *E. faecalis* Ac 8-2 (Fig. 2A), gentamicin treated *E. faecalis* qE-14 (Fig. 2B) and chloramphenicol treated *E. faecium* VP5 01 (Fig. 2C) increasing the expression levels of *efrA* and *efrB* genes by 2–3 folds compared to the corresponding controls (Fig. 2). On the other hand, triclosan decreased the expression of *efrA* and *efrB* genes by 2.3 and 1.9 folds, respectively in *E. faecalis* Ac 8-2 (Fig. 2A).

3.4. Effect of EDTA on EfrAB activity

3.4.1. Effect of EDTA on MIC

The addition of 3 mM EDTA to all three MDR enterococci resulted generally in a decrease in MIC values of different antimicrobials tested in this study (Table 3). The results indicated that MICs of antibiotics and biocides decreased by 2–3500 and 10->100 folds, respectively in the presence of EDTA. Furthermore, in some cases some antimicrobials (erythromycin and both biocides) at very low concentration in combination with 3 mM EDTA produced growth inhibition of MDR enterococci (Table 3).

3.4.2. Effect of EDTA on EfrAB expression

Our results revealed that treatment with 3 mM EDTA caused a decrease in antibiotic and biocide MICs. In an attempt to understand if EDTA had an inhibitory effect on EfrAB efflux pump in MDR enterococci or only had a synergistic effect with different antimicrobials (antibiotics or biocides), RNA expression levels of *efrA* and

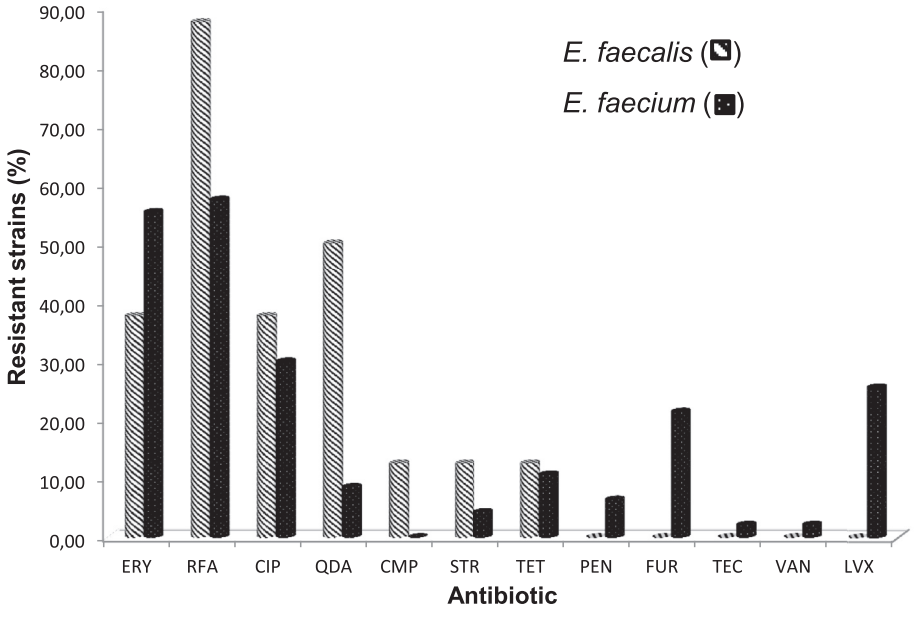

Fig. 1. Incidence of antibiotic resistance in MDR *E. faecalis* (▨) and *E. faecium* (■) isolated from traditional fermented foods.

Table 2
Incidence of antibiotic resistance in biocide-tolerant enterococcal isolates from different foods.

Isolate	Food source	Antibiotic resistance (identified resistance determinants)	Biocide tolerance
E. faecalis Ac 5-2	Olives	ERY, RFA (*ermB*, *msrA*, *efrA*, *efrB*)	CH, TC
E. faecalis Ac 8-2	Olives	CIP, QDA, RFA** (*efrA*, *efrB*)	CH, TC
E. faecalis Mz4 sth	Cow meat salami	CIP, QDA, RFA** (*efrA*, *efrB*)	CH
E. faecalis H_2O P7	Water	ERY, RFA, TET (*efrA*, *efrB*, *tetM*, *tetL*)	CH, TC
E. faecalis qE14	Cheese	ERY, CMP, QDA, RFA, STR, TET** (*ermB*, *cat*, *efrA*, *efrB*, *tetM*)	CH
E. faecalis PE1-1	Fish	QDA (*efrA*, *efrB*)	TC
E. faecalis VP5 02	Palm wine	RFA (*efrA*, *efrB*)	CH, TC
E. faecalis YA8	Fermented milk	CIP, RFA (*efrA*, *efrB*)	CH, TC
E. faecium AC5-1	Olives	ERY, PEN, RFA	CH, TC
E. faecium AC6-1	Olives	ERY, PEN, RFA	CH, TC
E. faecium AC8-1	Olives	ERY, RFA	TC
E. faecium AC10-1	Olives	ERY, RFA (*ermB*)	CH, TC
E. faecium AC11-1	Olives	ERY, PEN, RFA (*ermB*)	CH, TC
E. faecium AC12-1	Olives	ERY, RFA	CH, TC
E. faecium AL2	Fish	RFA	CH, TC
E. faecium AL3	Fish	RFA	CH, TC
E. faecium AL4	Fish	RFA	CH, TC
E. faecium AL6	Fish	RFA	CH, TC
E. faecium C2-3	Fish	RFA, FUR	TC
E. faecium CH 1-2	Fish	ERY, FUR	TC
E. faecium CHG 2-1	Fish	ND	TC
E. faecium CHG 2-2	Fish	ERY, FUR	TC
E. faecium CHG 2-3	Fish	ERY	TC
E. faecium LA 1-2	Fish	ND	CH, TC
E. faecium M2-1	Fish	ERY	TC
E. faecium M2-2	Fish	ERY, FUR	CH, TC
E. faecium PE 1-2	Fish	ND (*efrA*, *efrB*)	TC
E. faecium PE 2-1	Fish	FUR, RFA	CH, TC
E. faecium PE 2-2	Fish	CIP, FUR, LVX, RFA*	CH, TC
E. faecium PE 3-2	Fish	ERY, FUR	TC
E. faecium PEF 2-2	Fish	ERY	CH, TC
E. faecium KAA entC	Fish	ND	TC
E. faecium Sln entC	Fish	FUR, RFA	TC
E. faecium H_2O P3-A	Water	ND	TC
E. faecium H_2O P3	Water	ERY, QDA, RFA, TEC, VAN** (*msrA/B*, *efrA*, *efrB*)	TC
E. faecium H_2O P5	Water	ND	TC
E. faecium H_2O P5 01	Water	ND	CH, TC
E. faecium H_2O P5 03	Water	ERY, RFA (*efrA*, *efrB*)	CH, TC
E. faecium H_2O P8-A	Water	ND	CH, TC
E. faecium YA 2	Fermented milk	CIP, ERY, LVX, QDA, RFA** (*msr*A/B, *ermB*)	CH, TC
E. faecium YA 5	Fermented milk	QDA, RFA (*efrA*)	CH, TC
E. faecium YA 6	Fermented milk	CIP, ERY, LVX** (*msr*A/B, *ermB*)	CH, TC
E. faecium YA 7	Fermented milk	ERY, RFA	CH, TC
E. faecium YA 9-A	Fermented milk	CIP, ERY, LVX** (*msr*A/B, *ermB*)	CH, TC
E. faecium YA 9-B	Fermented milk	CIP, ERY, LVX** (*msr*A/B, *ermB*)	CH, TC
E. faecium YA 10	Fermented milk	ERY, RFA (*ermB*)	CH, TC
E. faecium KAA 1	Blood sausage	CIP, ERY, FUR, STR, TET** (*msr*A/B, *ermB*, *tetM*, *tetL*)	CH, TC
E. faecium KAA 2	Blood sausage	CIP, LVX	TC
E. faecium KAA 3	Blood sausage	CIP, FUR, LVX, RFA, STR, TET** (*tetM*, *tetL*)	CH, TC
E. faecium KAA 4	Blood sausage	CIP, ERY, LVX, RFA, TET** (*msr*A/B, *ermB*)	TC
E. faecium Sln 1	Blood sausage	CIP, LVX, RFA, TET** (*tetM*, *tetL*)	CH, TC
E. faecium Sln 2	Blood sausage	CIP, FUR, LVX, TET** (*tetL*)	CH, TC
E. faecium Tg6	Ginger beer	CIP, ERY, QDA, RFA** (*msr*A/B, *efrA*, *efrB*)	CH, TC
E. faecium VP3 01	Palm wine	CIP, ERY, LVX, RFA** (*msr*A/B)	CH, TC
E. faecium VP3 02	Palm wine	CIP, LVX, RFA**	CH, TC
E. faecium VP5 01	Palm wine	ERY, RFA (*efrA*, *efrB*)	CH, TC
E. faecium VP5 03	Palm wine	ND	CH, TC

ND: no resistance was detected.
* and **: Antibiotic resistance phenotypes and molecular determinants were determined by Valenzuela et al. (2010, 2013), respectively.
CIP, ciprofloxacin; CHX, chlorhexidine; CMP, chloramphenicol; ERY, erythromycin; FUR, nitrofurantoin; GEN, gentamycin; LVX, levofloxacin; QDA, quinupristine; RFA, rifampicin; STR, streptomycin; TC, triclosan; TEC, teicoplanin; TET, tetracycline; VAN, vancomycin.

efrB genes coding for EfrAB efflux pump were quantified in the presence and absence of EDTA. The results obtained showed that the expression of both *efrA* and *efrB* genes coding for EfrAB efflux pump was highly inhibited in the presence of 3 mM EDTA regardless the enterococci strain used (Fig. 3). In all cases, the down-regulation of the expression of EfrAB efflux pump caused by EDTA was more remarkable in *E. faecalis* qE-14 with 140 fold reductions as compared with the control without EDTA (Fig. 3).

3.4.3. Molecular effects of EDTA on efrA and efrB genes

Analysis on *efrA* and *efrB* gene sequences showed 99.4–99.6% of homology with those of *E. faecalis* deposited in database, however deduced amino acid sequences of EfrA and EfrB proteins were 100% identical (data not shown). Furthermore, analysis of the deduced amino acid sequences of EfrA and EfrB proteins before and after treatments with EDTA showed that no mutation occurred after EDTA treatment in all MDR enterococci studied.

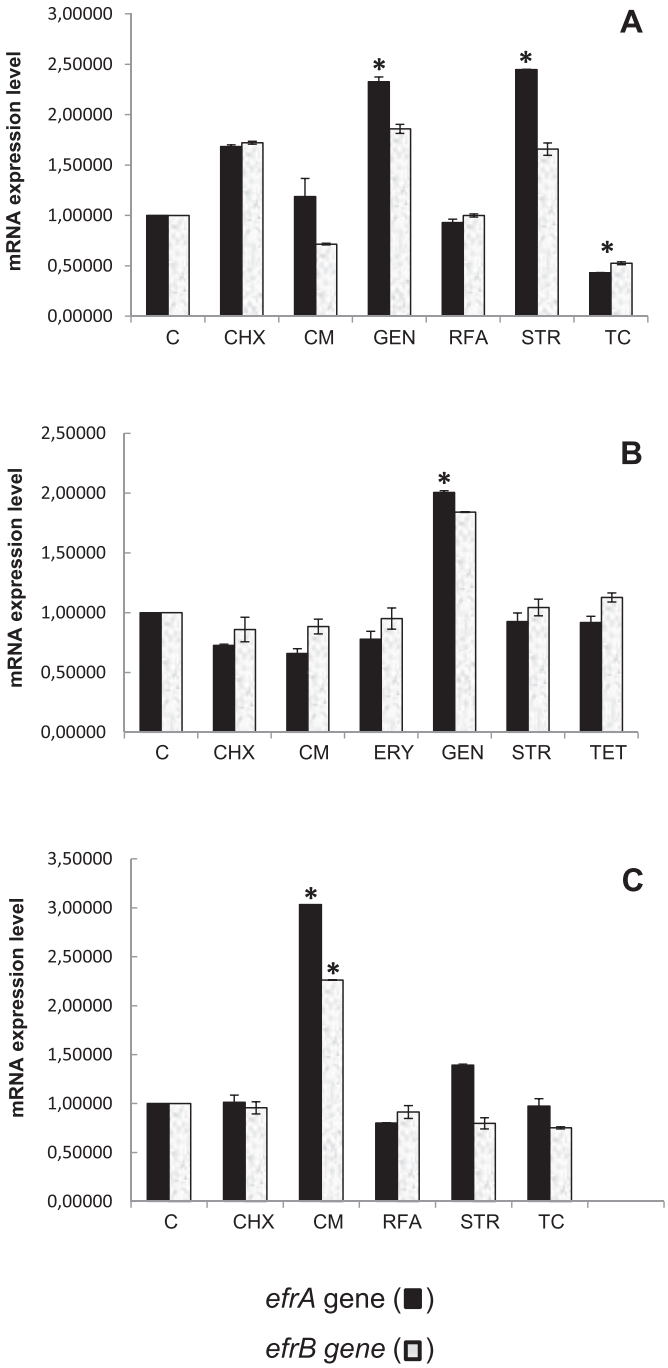

Fig. 2. Effect of half minimum inhibitory concentration (MIC) of different drugs on the expression of EfrAB efflux pump genes (*efrA* "solid bars" and *efrB* "gray bars") in *E. faecalis* Ac8-2 (A), *E. faecalis* qE-14 (B) and *E. faecium* VP5 01 (C). The following drugs were used: CHX, chlorhexidine; CM, chloramphenicol; ERY, erythromycin; GEN, gentamicin; RFA, rifampicin; STR, streptomycin; TC, triclosan and TET, tetracycline.

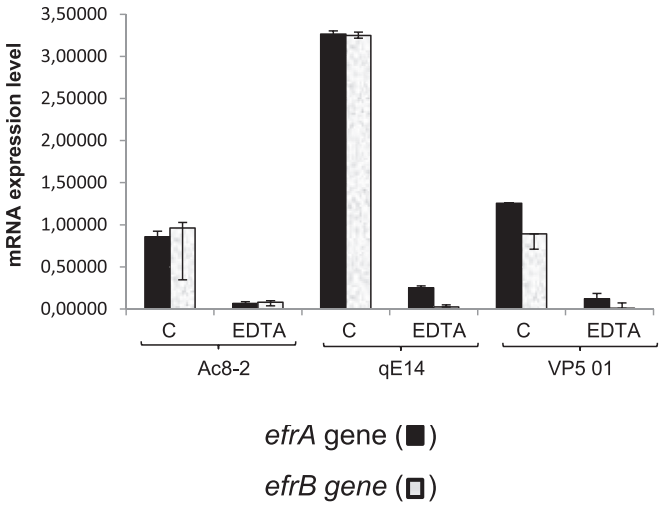

Fig. 3. Effect of EDTA on expression of *efrA* (solid bars) and *efrB* (gray bars) genes in MDR *E. faecalis* (Ac8-2 and qE-14 strains) and *E. faecium* (VP5 01 strain).

4. Discussion

Reduced susceptibility to disinfectants (such as chlorhexidine and triclosan) and antibiotics among bacteria is due to specific and unspecific mechanisms which often involves the action of active or over-expressed efflux pumps (Poole, 2005). *E. faecalis* is a highly stress resistant opportunistic pathogen able to colonize the hostile environments and to resist to several antimicrobials. The remarkable intrinsic ruggedness of *E. faecalis* is partially due to the presence of multidrug resistance efflux pumps such as EmeA which belongs to the major facilitator superfamily (MFS) and the ABC-type multidrug resistance transporters (Jonas et al., 2001; Lee et al., 2003a). Among multidrug resistance ABC transporters, EfrAB is a heterodimeric ABC transporter, being elevated drug resistance was only observed when *efrA* and *efrB* genes were coexpressed (Lubelski et al., 2007). Few studies showed that the established substrates for EfrAB efflux pump were several toxic compounds such as acriflavine, ethidium bromide, safranin O, DAPI, daunomycin, doxorubicin, novobiocin, arbekacin, doxycycline and norfloxacin (Davis et al., 2001; Lubelski et al., 2007) in *E. faecalis*. However, EfrAB was also detected in *E. faecium* isolated from fermented foods as reported for the first time by Valenzuela et al. (2013). In the present study, the incidence of antibiotic and biocide resistance/tolerance was highly dependent on the species analyzed and only some MDR enterococci exhibited resistance determinants such as *efrA*/*B* (100% and 12% of *E. faecalis* and *E. faecium* strains, respectively). With the aim to elucidate if EfrAB was implicated in antibiotic and/or biocide resistance/tolerance, we investigated the expression levels of EfrAB in the presence of different drugs. The results obtained in this study indicated that the expression of EfrAB was highly dependent on the bacterial strain and also on the drug used. In this context, some antibiotics at half of MIC increased the expression of *efrA* and *efrB* coding genes for EfrAB efflux pump such as gentamicin or streptomycin in *E. faecalis* Ac 8-2, gentamicin in *E. faecalis* qE-14 and chloramphenicol in *E. faecium* VP5 01, which suggested that efrAB expression was induced by the mentioned antibiotics. In this

Table 3
Effect of 3 mM EDTA on MIC fold reduction of different drugs in MDR enterococci.

Strains	Fold reduction in MIC							
	CMP	ERY	GEN	RFA	STR	TET	CHX	TC
E. faecalis Ac 8-2	4	S	16	1	8	>4	S	S
E. faecalis qE-14	2	1	3500	1	6	4	S	S
E. faecium VP5 01	4	S	32	4	32	2	10	100

S: sensitive to the corresponding antimicrobial at very low concentration used (>100 folds).
The antimicrobials used in the current study are: CMP, chloramphenicol; ERY, erythromycin; GEN, gentamycin; RFA, rifampicin; STR, streptomycin; TET, tetracycline; CHX, chlorhexidine; TC, triclosan.

sense, we can suggest that efrAB was involved in the efflux of chloramphenicol, gentamicin and streptomycin in the corresponding bacteria and consequently the emergence of resistance to such antibiotics could occur. However, no phenotypic resistance was reported in those strains to the corresponding antibiotics being other mechanisms involved to enhance their susceptibility to the mentioned antibiotics. On the other hand, mRNA expression of EfrAB was found to be decreased significantly with triclosan in *E. faecalis* Ac 8-2 which could be occurred for several reasons such as the disturbance of the regulatory pathway essential for expression of the efflux pump, alteration of the structure of efflux pump components, or disturbance of energy essential for pump activity as occurred in Gram-negative bacteria with other antimicrobials (Poole and Lomovskaya, 2006; Pagès and Amaral, 2009). Triclosan is a phenolic compound used in many everyday products including toothpaste, soaps, and lotions (Nester et al., 2007) to inhibit bacterial growth by the inhibition of enoyl-ACP-reductase involved in bacterial fatty acid synthesis. This interaction may cause the disruption of bacterial cell membrane integrity and thus inhibition of growth (Ellison and Champlin, 2007). Data obtained in the present study indicated that EfrAB was inhibited by triclosan which interact with enoyl-reductase associated membrane and causes the disorganization of membrane, and thus the hindrance of assembly of EfrAB efflux pump components could occur. This fact was only observed in *E. faecalis* Ac 8-2 but not in *E. faecium*. Thus, the observed decreased susceptibility to triclosan in *E. faecalis* Ac 8-2 could imply other mechanisms than EfrAB to pump out triclosan. Rational use of antiseptics such as triclosan could also have a positive effect on prevention of antimicrobial resistance/tolerance by reducing the expression of EfrAB efflux pump and consequently decreasing the antibiotic selective pressure caused by the use of systemic antibiotics. Considering the membrane organization and structure which are inherently dependent on the bacterial strain, the effect of antimicrobials targeting specific molecules depends highly on the strain tested. Those data revealed clearly that the interaction of drug with EfrAB efflux pump is strain specific, so in a complex food matrix different interactions may occur and the balance of such interactions reflect the resistance panorama observed in foodborne bacteria.

On the other hand, in the present study we explored the effect of EDTA on EfrAB efflux pump and subsequent changes in antimicrobial susceptiblility and expression. Our data revealed that the addition of a non-inhibitory concentration of EDTA (3 mM) reduced the MICs of almost all drugs used (antibiotics or biocides). Higher reductions of MICs were observed with gentamicin, streptomycin, chlorhexidine and triclosan in the presence of 3 mM EDTA. This reduction may be due to the synergistic effect of different drugs and EDTA since 3 mM EDTA alone did not cause any inhibition. EDTA is a chelating agent of divalent cations of the outer membrane of Gram-negative bacteria, and has also a little effect on Gram-positive bacteria but without increasing their susceptibility to antimicrobial agents (Russel and Furr, 1977). EDTA sequesters divalent cations, which are essential nutrients for bacterial cells and for maintenance of cell structures (like the outer cell membrane in Gram-negative bacteria). Thus, scarcity of divalent cations may affect several cellular processes, including efflux activity and others. Since the levels of expression of antibiotic resistance may depend on the physiological state of the cell, cells stressed by other factors such as decreased availability of divalent cations could possibly be impaired not only in their efflux activity (resulting in accumulation of higher antibiotic concentrations in the cytoplasm) but also in the levels of gene expression/regulation making them more sensitive to antimicrobials to which they are resistant under normal physiological conditions.

Sequestration of divalent cations by EDTA may impair EfrAB efflux pump functions. Furthermore, the binding capacity of EDTA to proteins of an ABC-type transporter was previously shown by Zhang et al. (2007). One probable reason for the reduced MICs observed in our study could be the inactivation of EfrAB efflux pump by EDTA (as efflux pump inhibitor), so the accumulation of drug may occur within the bacterial cells which in turn lead to impairment of cellular functions, including possibly the inhibition of EfrAB gene expression. To confirm this hypothesis, mRNA expression was done in the same conditions and the data obtained showed that EDTA caused significant changes in EfrAB expression in all enterococci strains tested. EDTA highly inhibited EfrAB expression in all *E. faecalis* and *E. faecium* strains tested, so EfrA and EfrB exhibited 10–140 fold down-regulation in the gene expression with 3 mM EDTA. The down-regulation of the EfrAB mRNA expression following treatment with EDTA occurred in a dose dependent manner from 1 to 3 mM (data not shown), however the best results were obtained with 3 mM EDTA. EDTA is a widely used chemical as food preservative due to its GRAS (Generally Recognized As Safe) status approved by Food and Drug Administration (FDA) since 1998 (FDA, 1998), also it is used in personal care, skin care, cosmetic preparations, and in medical, engineering, agricultural and industrial applications as well. . The down-regulation of EfrAB expression in the presence of EDTA is mainly responsible of MIC reduction of almost all antimicrobials tested in this study at neutral pH. The effect of pH on the cell envelope, its constituents, genes that regulate growth and metabolism has been largely reported in several studies, however the effect of EDTA on EfrAB efflux pump expression in milieus of acidic pHs was not studied since EDTA at acidic pH (pH 5) caused a complete growth inhibition of all enterococci strains (data not shown). Furthermore, EfrAB showed energy-dependent efflux rather than pH as reported by Lee et al. (2003b) for EfrAB in *E. faecalis* and Martins et al. (2011) for MsbA (an ABC transporter similar to EfrAB) in *Escherichia coli*. Consequently, we can confirm that EfrAB efflux pump is generally implicated in the efflux of different antibiotics and biocides (CMP, ERY, GEN, RFA, STR, TET, CHX and TC) in enterococci isolated from fermented foods, however intra- and inter-species differences were detected because of the membrane organization and composition of each strain. Furthermore, the results obtained in this study corroborate also the importance of EfrAB in multidrug-resistance in foodborne enterococci strains.

Therefore, it is reasonable to suggest that the use of EDTA at lower concentration (3 mM) should be evaluated in foods to avoid the emergence of resistance to clinically relevant antibiotics mediated by different efflux pumps as occurred in the current study with EfrAB in MDR enterococci isolated from fermented foods. The presence of EDTA enhanced susceptibility to several antibiotics and biocides to which enterococci strains were resistant such as chloramphenicol and tetracycline in *E. faecalis* qE-14, and erythromycin and rifampicin in *E. faecium* VP5 01. Furthermore, recent studies (Chaudhary and Payasi, 2012; Chaudhary et al., 2012) indicated that EDTA as efflux pump inhibitor enhanced the susceptibility of *E. coli* and *Pseudomonas aeruginosa* strains to several antibiotics by inhibiting AcrAB-TolC and MexA-MexB-OprM efflux pumps, respectively. This fact encourage the use of EDTA -at concentration regarded as safe- not only in food industry (maximum permissible levels of EDTA in foods is of 800 ppm which is almost equivalent to 3 mM tested in this study; Food and Drug Administration "http://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm091048.htm") but also in clinical infections (EDTA up to 3.33 mg/kg body weight corresponding to 12 mM is safe to human when administered intravenously; Chaudhary et al., 2012) to avoid or minimize further development of multidrug resistant strains.

5. Conclusions

The data obtained in the present study indicate that EfrAB pump expression is induced by some antibiotics in MDR enterococci isolated from fermented foods which could lead to resistance. Furthermore, EfrAB expression was highly dependent on the enterococcal strain and also on the drug used, being involved in the efflux of several antibiotics and biocides. To enhance susceptibility of MDR enterococci, EDTA was used at sub-inhibitory concentration of 3 mM as efflux pump inhibitor down-regulating EfrAB efflux pump and thus allowing higher decrease in MICs of different drugs. These results presents new findings of the correlation between the level of expression of the *efrA/B* genes and MIC reduction of different antibiotics and biocides in the presence of 3 mM EDTA. This may be a good alternative since EDTA is used as food preservative which could prevent antimicrobial resistance by reducing the expression of EfrAB efflux pump and consequently decrease the antibiotic selective pressure caused by the use of systemic antibiotics and disinfectants.

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