

Antimicrobial Resistance in *Enterococcus* sp Isolated from Soft Cheese in Southern Brazil

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Received October 22, 2013; revised November 22, 2013; accepted November 29, 2013

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ABSTRACT

The aim of this research was to study the incidence of antibiotic resistance in 56 *Enterococcus* strains isolated from dairy products. The identification of enterococci was detected by polymerase chain reaction (PCR) using specific primers to *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. casseliflavus*, and antibiotic resistance was tested by the disk diffusion method. The most prevalent species was *E. faecium* with a rate of 58.33%, followed by 27.77% *E. faecalis*, 11.11% *E. casseliflavus* and 2.7% *E. gallinarum*. Distribution of resistance was found in different species. All isolates were susceptible to chloramphenicol, ampicillin, imipenem and amoxicillin/clavulanic acid. In addition, isolates resistant to tetracyclin, nalidixic acid, amikacin, erythromycin, vancomycin and cephalothin were detected. A total of 66.6% of *E. faecium* and 58.3% of *E. faecalis* strain were resistant to multiple drugs. The *van(A)* gene was detected in 100% of vancomycin resistant enterococci. Considering the results of our study, dairy enterococci can be considered a potential source for dissemination of antibiotic resistances.

KEYWORDS

Enterococci; Dairy Products; Foods; Antimicrobial Resistance

1. Introduction

Enterococci are ubiquitous bacteria that have a predominant habitat in the digestive tract of humans and animals, but may be present in soil, water's surface and in plants and vegetables. They can also be found in foods, especially in cheese [1], but their presence is unwanted in certain kinds of cheese and can cause them to be spoiled [2-4].

The reasons for the prevalence of enterococci in dairy products has long been considered a result of unhygienic conditions during milk collection and processing, together with their resistance to pasteurization temperatures and their adaptability to different substrates and growth conditions [5]. However, the presence of enterococci in cheese and other fermented foods is a matter of debate, since some enterococcal species are involved in clinical

infections such as endocarditis, bacteraemia, urinary tract infections and neonatal sepsis [6]. One of the reasons for the rise of nosocomial infections related to enterococci might be their ability to develop resistance against a wide variety of antibiotics [7,8].

Their resistance to a wide variety of antibiotics can be acquired or found intrinsically. Intrinsic antibiotic resistance of enterococci to cephalosporins, beta-lactams, sulphonamids, low levels of clindamycin and aminoglycosides may have selectively allowed them to acquire further genes of resistance against many commonly used antimicrobial agents such as aminoglycosides, penicillins, tetracycline, chloramphenicol, and vancomycin [8,9]. *Enterococcus* sp have evolved an adaptive response to the antibiotic stress and have developed drug resistance [10].

In addition, these bacteria are able to acquire resistance determinants through gene transference by plasmids and transposons [11,12], and to acquire resistance to

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many antimicrobial agents, including aminoglycosides, chloramphenicol, B-lactams, macrolides, quinolones, tetracycline, vancomycin and teicoplanin [13,14].

The occurrence of antimicrobial resistance among enterococci is not only restricted to the nosocomial setting, and therefore resistant strains carried on sources such as food may also act as potential reservoirs of antimicrobial resistance genes. The food chain can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human population [15]. More specifically, fermented dairy products that are not heat-treated before consumption provide a vehicle for antibiotic resistant bacteria with a direct link between the animal indigenous microflora and the human gastrointestinal tract.

In the present study, we investigated the incidence of antibiotic resistances among enterococci derived from soft cheese produced in the South of Brazil.

2. Materials and Methods

2.1. Strains and Phenotype Characterization

A total of 56 enterococci were isolated from samples of soft cheeses types, commercialized at several local street markets of Londrina city-Paraná State, and were collected over a period of one year from 2011 to 2012. Phenotype tests were used to separate the enterococci group and the non-enterococcal strains. Colonies with typical enterococci morphology were identified to genus level through following methods: Gramstaining, catalase production and esculin hydrolysis tests in combination with resistance to bile salts and growth at 10°C and 45°C, according to Teixeira and Facklam [16] and molecular approach.

2.2. Isolation of Enterococcal DNA and Identification by PCR

Enterococcus spp. genomic DNA was extracted through the boiling method as described by Marques and Suzart [17]. The identification of enterococci was detected by polymerase chain reaction (PCR) using specific primers targeted [18-20] (Table 1).

2.3. Antimicrobial Susceptibility Testing and Detection of Resistance Genes by PCR

The antibiotic discs (Laborclin®) were used to determine the susceptibility of the strains to ampicillin (AMP, 10 µg), nalidixic acid (NAL 30 µg), vancomycin (VAN, 30 µg), erythromycin (ERY, 15 µg), chloramphenicol (CLO, 30 µg), norfloxacin (NOR, 10 µg), tetracycline (TET, 30 µg), imipenem (IPM 10 µg), amikacin (AK 30 µg); cephalothin (CF 30 µg); ciprofloxacin (CIP 5 µg); amoxicillin/clavulanic acid (AMC 30 µg). The discs were

placed onto Mueller-Hintonagar plates overlaid with the enterococcal culture with a cell concentration corresponding to 0.5 McFarland turbidity standard. After incubation at 37°C for 18 - 24 h the diameter of inhibition haloes around the colonies was measured. Susceptibility or resistance was determined according to the recommendation of National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2002) [21]. Minimal inhibitory concentrations (MICs) of vancomycin (VAN) were also determined by the broth microdilution according to methods described by (Clinical and Laboratory Standards Institute, 2009) [22]. *E. faecalis* ATCC 29212 and *S. aureus* ATCC 25923 were used as control strains.

The detection of resistance genes was conducted by PCR in all isolates of enterococci. The presence of gene *vanA*, *aac(6')-Ie-aph(2'')*-Ia, *erm(B)* and *tet(L)* was found for vancomycin, gentamicin, eritromycin and tetracycline, respectively (Table 2).

All PCR amplifications were performed in a final volume of 20 µl containing 1 pmol of each primer (Forward and Reverse), 0.17 mM dNTPs, 2.5 mM MgCl₂, 1 U of Taq DNA polymerase (Invitrogen®), buffer of Taq, and 10 µl template DNA. An initial cycle of denaturation (94°C for 2 min) was followed by 30 cycles of denaturation (94°C for 1 min), annealing at an appropriate temperature for 1 min and elongation (72°C for 10 min). A Thermal Cycler (Techne-Tc3000) was used to carry out the PCR reactions. PCR products were analysed by gel electrophoresis in 1.2% agarose stained with ethidium bromide (0.5 g·ml⁻¹), and observed under UV transillumination and photographed by L-PIX ST (LOCCUS®).

3. Results

A total of 56 soft cheese samples that were collected from local street market were investigated for the presence of enterococci, and a conventional cultivation showed that 100% of them were harbouring *Enterococcus* spp. Genus-specific PCR with detection of *tuf* gene was in accordance with these preliminary results. According to the presumptive phenotypic characterization followed by PCR of species-specific primers for *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*, revealed that the most prevalent species was *E. faecium* with a rate of 58.33%, followed by 27.77% *E. faecalis*, 11.11% *E. casseliflavus* and 2.7% *E. gallinarum* (Figure 1).

A summary of the resistance among the *Enterococcus* strains is reported in Table 3.

A total of 56 *Enterococcus* strains displayed resistance to at least one antibiotic tested and were resistant to antibiotic agents, some of which were resistant to multiple drugs.

Table 1. List of primers and amplification conditions used in the present study.

Gene	Gene Primers sequence	Ta	Product size (bp)
<i>Tuf</i>	TACTGACAAACCATTTCATGATG AACTTCGTCACCAACGCGAAC	56	112
<i>vanC-1</i>	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	56	822
<i>vanC-2, vanC-3</i>	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	56	439
<i>ddl_{E.faecalis}</i>	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	56	941
<i>ddl_{E.faecium}</i>	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	56	550

Ta (°C) = temperature of annealing; gene *tuf*, *Enterococcus*; *vanC-1*, *E. gallinarum*; *vanC-2, vanC-3*, *E. casseliflavus*, *E. flavescens*.

Table 2. Primers used in this study for detection of resistance genes by PCR-based method.

Gene	Nucleotidesequence (5'-3') ^a	Ta (°C)	Amplicon (bp)	Reference
<i>vanA</i>	GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA	56	231	[43]
<i>aac(6')-Ieaph(2'')-Ia</i>	CAGAGCCTTGGGAAGATGAAG CCTCGTGTAATTCATGTTCTGGC	56	348	[44]
<i>erm(B)</i>	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	56	405	[45]
<i>tet(L)</i>	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCACCTAA	56	696	[46]

Ta (°C) = temperature of annealing; ^aM = A or C; R = A or G; W = A or T; *tet(L)*, tetracycline; *erm(B)*, erythromycin; *aac(6')-Ieaph(2'')-Ia*, gentamicin and *vanA*, vancomycin.

Table 3. Antibiotic resistant phenotypes of enterococcus species isolated from soft cheese.

Strain	% Resistant							
	NAL	VAN	ERI	TET	AK	NOR	CFL	CIP
<i>E. faecium</i>	61.9	14.3	14.3	9.5	9.5	9.5	9.5	4.8
<i>E. faecalis</i>	60.0	20.0	20.0	70.0	30.0	10.0	20.0	0.0
<i>E. casseliflavus</i>	0.0	25.0	25.0	25.0	0.0	0.0	25.0	0.0
<i>E. gallinarum</i>	0.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0
Total	52.8	21.9	19.4	30.6	13.9	8.3	13.9	2.8

NAL, nalidixic acid; VAN, vancomycin; ERI, erythromycin; TET, tetracycline; AK, amikacin; NOR, norfloxacin; CFL, cephalothin; CIP, ciprofloxacin.

The *E. faecalis* strains were mostly resistant to tetracycline (70%), followed by nalidixic acid (60%), amikacin (30%) and erythromycin, vancomycin and cephalothin (20%). *E. faecium* strains were mostly resistant to nalidixic acid (62%), vancomycin and erythromycin (14.3%), tetracycline, amikacin, norfloxacin and cephalothin (9.5%) and ciprofloxacin (4.8%). On the other hand, *E. casseliflavus* strains were resistant to vancomycin, erythromycin, tetracycline and cephalothin to 25% each one, and *E. gallinarum* strain were 100% resistant to vancomycin, erythromycin and tetracycline. None of the *Enterococcus sp* strains was resistant to chloramphenicol, ampicillin, imipenem and amoxicillin/clavulanic acid. A total of 66.6% of *E. faecium* and 58.3% of *E. faecalis* strain were resistant to multiple drugs. All

isolates were drug resistant for vancomycin with MICs of >32 to 64 µg·ml⁻¹.

In our study, the presence of resistance genes *erm(B)*, *tet(L)*, *vanA* and *aac(6')-Ieaph(2'')* were 86.7%, 23.3%, 80.0% and 66.7%, respectively. Several isolates harboured resistance genes to more than one antibiotic. The significant ones were *tet(L)⁺/erm(B)⁺* to *E. faecalis* (42.8%) and *erm(B)⁺/aac(6')-Ieaph(2'')*-*Ia⁺/vanA⁺* to *E. faecium* (69.6%).

The presence of the *vanA* gene was detected in twenty three isolates of *E. faecalis* and fifty-four of *E. faecium*, corresponding to 42.8% and 96.6% of the isolates, respectively. The *van(A)* gene was detected in 100% of vancomycin resistant enterococci, however, five isolates harboured the *van(A)* gene and presented vancomycin

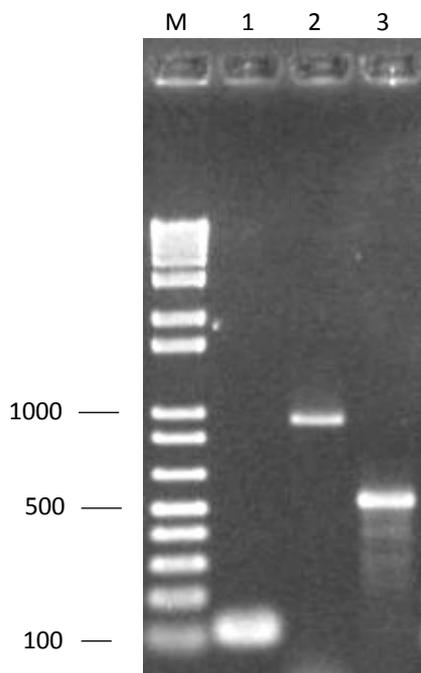


Figure 1. Amplification gel pictures characteristic of polymerase chain reaction (PCR) amplification of *Enterococcus* sp gene. Lanes: 1 *Enterococcus* spp. (112 pb); 2 *E. faecalis* (941 pb); 3 *E. faecium* (550 pb); M Ladder 1 kb plus (Invitrogen).

susceptibility phenotype.

4. Discussion

In this study, enterococci were isolated from soft cheese, the most prevalent species being *E. faecium*, followed by *E. faecalis*, *E. casseliflavus* and *E. gallinarum*. A higher prevalence of enterococci in processed foods may be attributed to their resistance to heat, extreme salinity and harsh conditions of foods [1-3,23].

Enterococci are considered as opportunistic pathogens which can cause a variety of infections in patients that have severe underlying diseases or that are immunocompromised. The major responsible for human enterococcal infections is *E. faecalis* followed by *E. faecium* and with a much lower incidence by other enterococcal species [2,6,8]. Furthermore, Enterococci are found in a variety of artisanal cheese made from raw or pasteurized milk from goats, sheep, water buffaloes, and cows [24].

A perusal of the literature revealed that *E. faecalis* and *E. faecium* were the most frequently isolated species from enterococci isolated from an artisanal cheese [25-29]. This explanation could be by the fact that these foods are manipulated by hands suggesting a contamination during the manufacturing process.

Antimicrobial susceptibility tests showed resistance phenotypes to a range of antibiotics widely administered in humans. A major concern is the presence of food

strains harbouring multiple antibiotic resistance (from 3 to 8 out of the 12 antibiotics tested). Indeed, 100% of the vancomycin resistant enterococci showed resistance also to other clinically relevant antibiotics such as erythromycin (100%), tetracycline (100%), amikacin (50%), norfloxacin (50%), cephalothin (75%) and nalidixic acid (50%) thus leaving few therapeutic options. Results showed low level and high level resistant to vancomycin antibiotic at concentration of 64 to 128 $\mu\text{g}\cdot\text{ml}^{-1}$. *Enterococcus* sp strains isolated from soft cheese did not show chloramphenicol, ampicillin and amoxicillin-resistance, a clinical relevant antibiotic since ampicillin remains the drug of choice for the treatment of enterococcal infections.

Cariolato *et al.* [30] demonstrated only one isolate of *Enterococcus* from cheese was resistant to only two antibiotics (nalidixic acid and streptomycin). All the other ones were sensitive. Mannu *et al.* [31] showed lower occurrence of dairy strains resistant to clinically relevant antibiotics among food enterococci, suggesting that enterococci from milk and cheese cannot be considered the main potential sources for dissemination of antibiotic resistances. Martín-Platero *et al.* [32] showed that no more than 13% of the isolates of *Enterococcus* sp from cheese demonstrated any antimicrobial activity.

On the other hand, Flórez *et al.* [33], Cariolato *et al.* [30], Riboldi *et al.* [28] and Frazzon *et al.* [34], showed that in colonial cheese type was detected the presence of *Enterococcus* resistant to one of ampicillin gentamicin, streptomycin, ampicillin, vancomycin and tetracycline. High incidence of enterococci resistance to tetracycline chloramphenicol and erythromycin was found among isolated raw milk cheese.

Data on the incidence of vancomycin resistance within dairy enterococci remain controversial, though several papers indicate very low or no presence of *vanA* and *vanB* resistance genes in enterococci isolated from cheese [20,23,27,35].

However, since in our study we did not apply any enrichment procedure, our data is not comparable with some results obtained in other foods, such as meat products, in which higher numbers of vancomycin resistant enterococci were isolated after a step of selective enrichment in the presence of vancomycin [36,37].

The association of resistance gene with conjugative elements is an important factor for spreading resistance in enterococci, which corroborate with the outlook that *Enterococcus* species are important sources of antibiotic resistance genes by horizontal gene transfer [38]. The *vanA* gene cluster, which confers resistance to vancomycin, is located on transposon Tn1546 [39].

Riboldi *et al.* [28] suggested that the emergence of *Enterococcus* spancomycin-resistant (VRE) strain in food samples would be explained by the massive use of

antibiotic in agriculture (e.g., avoparcin as animal growth promoters). Vancomycin-resistant indicates that *Enterococcus* is a controversial species that should not be used for probiotic applications [40].

Emergence of enterococci antimicrobial resistance and its spreading in food suggest a situation of risk for the community, and also a possible correlation between strains present in hospitals with those isolated from food must be considered [41-46].

Some studies have shown that the same resistance gene was found in bacteria isolated from both food samples and patients [37,41]. The food chain can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human population. More specifically, dairy products that are not heat-treated before consumption provide a vehicle for antibiotic resistant bacteria with a direct link between the animal indigenous microflora and the human gastrointestinal tract [15].

5. Conclusions

We have described here the first report of antibiotic resistant enterococci isolated from foods in the North of Paraná, in Southern Brazil. However, further studies are necessary in order to understand the possible role played by potential virulence factors and antibiotic resistances of dairy isolates in relation to their ecological habitat as well as the transferability of this virulence and resistance traits among strains in the cheese environment. Our results suggest that dairy enterococci can be considered as a potential source for dissemination of antibiotic resistances.

Finally, one reason that could explain the emergence of antibiotic resistant enterococci in food samples would be the use of antibiotic in agriculture, and some studies have shown that the same resistance gene was found in bacteria isolated from both food samples and patients.

Further studies are necessary in order to understand the possible role played by antibiotic resistances of dairy isolates in relation to their ecological habitat as well as the transferability of these resistance traits among strains in the cheese environment. However, this finding is the most relevant in this study and serves as a warning to authorities of public health since vancomycin is the last antibiotic alternative used for treatment of enterococcal nosocomial infections.

Acknowledgments

L.F. Maia thanks the National Council for Scientific and Technological Development (CNPq) and K.R. Rocha thanks the Brazilian Graduate Educational Federal Agency (CAPES) for the fellowship grants. Authors thank CNPq (process n. 502230/2009-6) and The State of

Paraná Research Foundation (Fundação Araucária) (process n. 185/2012) for financial support.

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