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Vancomycin and tetracycline-resistant enterococci from from raw and processed meats: phenotypic and genotypic characteristics of isolates

Enterococos resistentes à vancomicina e tetraciclina em carnes cruas e processadas: características fenotipicas e genotipicas

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Abstract
The ubiquitous nature of enterococci and their ability to colonize different habitats account for their easy spread throughout the food chain. Here, we evaluated the distribution and antimicrobial susceptibility of *Enterococcus* isolates from meats obtained from different supermarkets. We acquired and cultured 100 products (raw chicken meat, raw pork, and boiled meats) to screen for the presence of *Enterococcus* spp. In total, 194 isolates were recovered from the samples, with contamination rates of 63.6% in the chicken samples, 31% in the raw pork meat, and 1.4% in the boiled meat samples. PCR amplification with specific primers was performed to screen the DNA of *Enterococcus* spp. (95/96), *E. faecalis* (66/96), *E. faecium* (30/96), and *E. casseliflavus/E. flavescens* (3/96). The antimicrobial susceptibility tests showed that all the isolates were resistant to at least one of the antibiotics. All *E. faecium* isolates were resistant to vancomycin, streptomycin, ciprofloxacin, norfloxacin, erythromycin, and tetracycline. The *E. casseliflavus/E. flavescens* isolates were resistant to gentamicin, streptomycin, ciprofloxacin, norfloxacin, erythromycin, and tetracycline. *E. faecalis* isolates were resistant to ciprofloxacin, tetracycline, and erythromycin (92%), norfloxacin (83%), vancomycin, and streptomycin (50%). The resistance genes tetL and vanB were detected by genotyping. The presence of these antimicrobial-resistant microorganisms in food might pose problems for public health.
Keywords: Antimicrobials, PCR, vancomycin-resistant enterococci

Resumo
A natureza ubíqua dos enterococos e sua capacidade de colonizar diferentes habitats são responsáveis pela sua fácil disseminação pela cadeia alimentar. No presente estudo, avaliamos a distribuição e a susceptibilidade antimicrobiana de isolados de *Enterococcus* provenientes de produtos cárneos. Cem produtos (carne de frango cru, carne de porco crua e carne cozida) foram adquiridos e cultivados para a presença de *Enterococcus* spp. No total, 194 amostras foram avaliadas, com taxas de contaminação de 63,6% nas amostras

de frango, 31% na carne de porco crua e 1,4% nas amostras de carne cozida. A amplificação por PCR foi realizada para confirmar a presença de *Enterococcus* spp. (95/96), *E. faecalis* (66/96), *E. faecium* (30/96) *E. casseliflavus*/*E. flavescens* (3/96). Resultados de susceptibilidade mostraram que 100% dos isolados foram resistentes a pelo menos um antibiótico, sendo 100% de *E. faecium* resistentes a vancomicina, estreptomicina, ciprofloxacina, norfloxacina, eritromicina e tetraciclina. *E. casseliflavus* / *E. flavescens* resistentes a gentamicina, estreptomicina, ciprofloxacina, norfloxacina, eritromicina e tetraciclina. *E. faecalis* foram resistentes a ciprofloxacina, tetraciclina e eritromicina (92%), norfloxacina (83%), vancomicina e estreptomicina (50%). Na genotipagem, foram detectados os genes tetL e vanB. A presença desses microrganismos resistentes aos antimicrobianos nos alimentos pode causar problemas para a saúde pública.
Palavras-chaves: Antibióticos, enterococci vancomicina-resistante, PCR.

Introduction

Enterococci are intestinal commensal bacteria of humans and animals and cause diseases in immunocompromised patients that are admitted in intensive care units for long periods or have severe underlying sicknesses[1]; they can survive in a variety of environments, such as soil, water, plants, and food[2;3]. Enterococci are frequent contaminants of poultry meat and are also well-known agents in the food chain[4]. Although the significance of antimicrobial-resistant enterococci in food has not been well elucidated, bacteria that present with resistance-related characteristics in the environment may be considered genetic reservoirs of these genes[5].

Enterococci have strikingly high levels of resistance to medically important antibiotic classes, particularly to those approved by the FDA for use in animal production, including macrolides, chloramphenicol, aminoglycosides, and vancomycin[6;7]. Vancomycin is an important agent for the treatment of serious infections caused by enterococcal species; however, the therapeutic efficacy of this antibiotic has been limited by the emergence of vancomycin-resistant enterococci (VRE)[8]. The most important vancomycin resistance gene is *van*A. Several other species of enterococci, including *E. durans, E. hirae, E. gallinarum, E. casseliflavus, E. raffinosus, E. avium*, and *E. mundtii* possess this gene[9].

Antibiotic-resistant enterococci, isolated from animals and foods, have been described in Europe and the USA[10; 11]. In Brazil, these bacteria have been reported in the southern states[12; 13]. Previously, researchers[14] have reported the presence of antimicrobial-resistant *Enterococcus* species in soft cheese from the southern part of Brazil, although the data on the occurrence of resistance and resistance genes in enterococci from the Paraná state are sparse. Nevertheless, the presence of enterococci in food is a matter of debate, as some *Enterococcus* species are involved in clinical infections, such as endocarditis, bacteremia, urinary tract infections, and neonatal sepsis[3]. Although strains of VRE isolated from animals have been studied extensively, little is known regarding the combined resistance in these microorganisms.

Thus, our present study on *Enterococcus* spp., associated with raw and boiled meats, is expected to provide insight into the biodiversity of the species and the potential resistance of these bacteria, which are of utmost importance to ensure food safety and public health.

Materials and methods

In total, 100 food samples from different supermarkets were obtained: 66 raw chicken meat samples, 14 raw pork meat samples, 10 samples of processed pork-based products (chopped pork, ham, frankfurter, baloney, and cooked and uncooked sausages), and 10 chicken meat-based products (sausage, spread, breaded chicken, and baloney). Briefly, the samples (25 g) were aseptically collected, placed in 225 ml of brain heart infusion broth (BHI), homogenized, and incubated for 24 h at 37°C. After pre-incubation, 0.1 mL of the primary enrichment was streaked on kanamycin esculin azide agar (KEA) and incubated for 24 h at 37°C. Typical colonies, suggestive of *Enterococcus*, were randomly selected from each of the primary isolation cultures on the KEA plates and were submitted to microscopic examination, Gram staining, a catalase test, and growth in BHI broth containing 6.5% NaCl at 10°C and 45°C[15].

Genomic DNA was extracted using the boiling method[14]. The identification of enterococci and the detection of resistance genes were confirmed by the polymerase chain reaction (PCR) using specific, targeted primers, which have been previously reported[16] (Table 1). All reactions in the thermocycler (Bio-Rad Thermal Cycles T100™) were carried out with a final volume of 20 µL and consisted of 2 µL of total DNA (20 ng mL^{-1}), 11.5 µL ultrapure water, 2 µL Taq buffer (10X), 1 µL $MgCl_2$ (2.5 mM), 1.4 µL dNTPs (0.17 mM), 1µL of each the forward and reverse primer (20 pmol) (Table 1), and 0.1 µL Taq DNA polymerase (1 U) (Invitrogen). The cycle program used consisted of 5 min of denaturation at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 56°C for annealing, and 30 s at 72°C, and a final extension for 5 min at 72°C, followed by a cool down to 4°C until further analysis. The amplicons were separated by electrophoresis on 1% agarose gels containing ethidium bromide, and visualization by UV light was performed by a computerized photographic system (BioDoc-it System, UVP). A 100 bp DNA marker (100 bp DNA ladder, New England Biolabs/USA) was used to estimate the size of the amplified product.

All the isolates were characterized by antimicrobial susceptibility testing with 12 antibiotics by use of the disc diffusion method, in accordance with the Clinical and Laboratory Standards Institute guidelines[17]. The 12 antibiotics tested included ampicillin (10 mg), ciprofloxacin (5 mg), chloramphenicol (30 mg), erythromycin (15 mg), streptomycin (300 µg), gentamicin (120 µg), norfloxacin (10 µg), penicillin (10 µg), tetracycline (30 mg), teicoplanin (30 mg), and vancomycin (30 mg). The antibiotic imipenem (100 mg) was also tested. The minimum inhibitory concentration (MIC) of vancomycin was determined by the broth microdilution technique, and the antibiotic breakpoints used were those specified by the CLSI guidelines. *Staphylococcus aureus* ATCC 25923 samples (American Type Culture Collection) were used for quality control

for the susceptibility tests.

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

	Gene	Gene Primers sequence (5'- 3')	Product size (bp)	reference
Enterococcus sp.	*tuf*	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	16
E. gallinarum	*vanC-1*	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	16
E. casseliflavus/ E. flavencens	*vanC-2/vanC-3*	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	439	16
E. faecalis	$ddl_{E.faecalis}$	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941	16
E. faecium	$ddl_{E.faecium}$	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	550	16
Vancomycin	*vanA*	GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA	231	
	vanB	GCCGACAATCAAATCATCCTC GCCGACAATCAAATCATCCTC	330	35
Tetracycline	*tet*	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCACCTAA	696	2

Results and discussion

This study provides the first description of antimicrobial-resistant enterococci isolated from raw and processed meats from the Paraná State. Enterococci constitute an interesting group of microorganisms because they are commensals of humans and animals that occur and grow in a variety of foods and have also been implicated in nosocomial infections. Among the 100 raw and processed meat samples collected from different supermarket sources in the Paraná state, 194 presumptive enterococcal isolates were found from 96 food samples, based on Gram staining, an absence of catalase activity, and growth in BHI broth with 6.5% NaCl at temperatures of 10°C and 45°C. Using PCR amplification with specific primers, *Enterococcus* spp. (95/96), *E. faecalis* (66/96), *E. faecium* (30/96), and *E. casseliflavus/E. flavescens* (3/96) were identified based on the obtained amplicon size[14; 18] (Figure 1).

In contrast to some reports, *E. casseliflavus/E. flavescens* were found in our food samples. Our results likely represent the presence of an enrichment step. The relative prevalence of *Enterococcus* species in food fell within the range of a previous estimate from animals and their meat, with the most frequent being *E. faecalis,* followed by *E. faecium* [4;12;19].

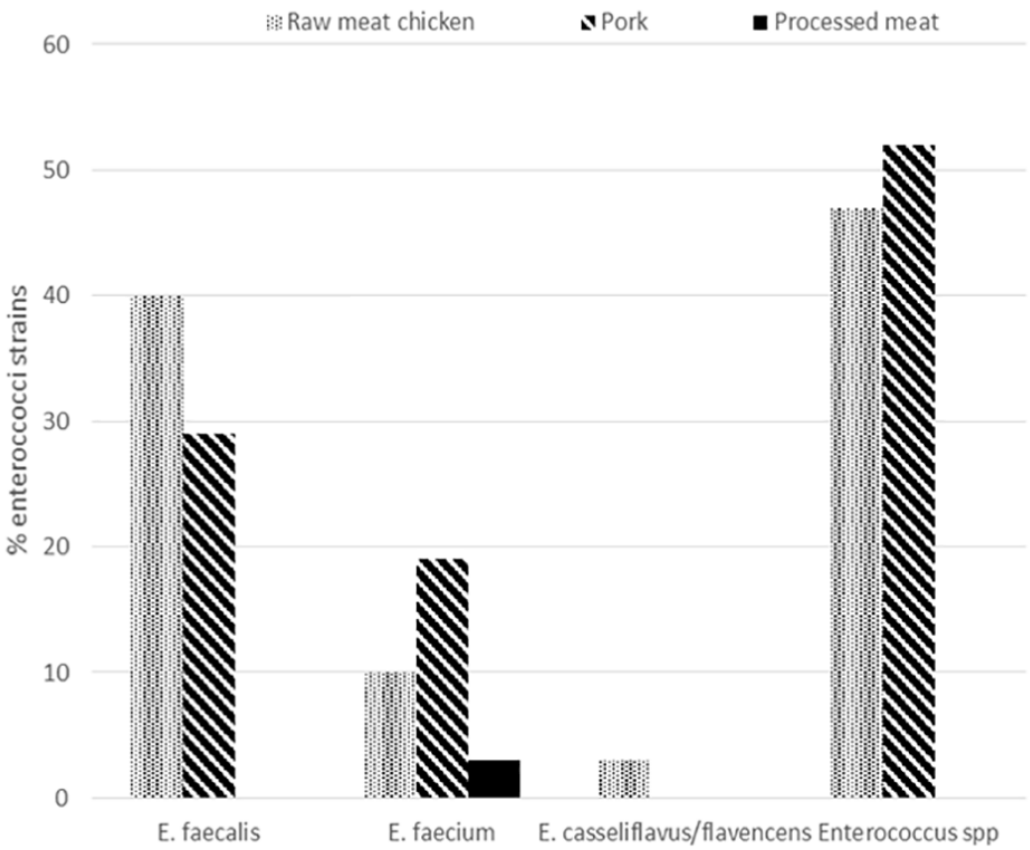

Figure 1. - Enterococci strain distribution in different meat products.

Although the species *E. casseliflavus/E. flavescens* has not been isolated as frequently; a number of isolates were found in our study; therefore, this species is important because it has intrinsic resistance to vancomycin[20]. The presence of the *E. gallinarum* species could not be confirmed among the analyzed food samples.

The data presented here reveal new avenues to study a possible role for the presence of *Enterococcus* spp. in the food chain, especially in raw and processed meat. It is, therefore, essential to investigate additional food samples to determine the potential risk to the population.

Table 2 shows the antimicrobial resistance and resistance genes present in each strain of *Enterococcus* spp. isolated from raw and processed meats. In comparison to other species, the prevalence of the resistance determinants was lower among the *E. faecium* and *E. casseliflavus/flavescens* isolates. All isolates that showed vancomycin resistance were subjected to a MIC determination. As a result, 100% of the isolates showed intermediate resistance to this antimicrobial (MIC ≥ 8 µg/ml). Regarding antimicrobial resistance, the study results showed that the isolates of *Enterococcus* spp., such as *E. faecalis* and *E. faecium,* from raw chicken meat samples, were more frequently resistant to the tested antimicrobials than the isolates from raw pork meat and processed meat

products.

Table 2. Antibiotic resistance and resistence genes of enterococci isolated from different meat products

Strain	Antimicrobial	Resistance (% of strains)	Resistance genes
E. faecium	Streptomycin, Ciprofloxacin, Norfloxacin,	100	*van*B
E. faecalis	Ciprofloxacin, Tetracycline and Erythromycin	92	*van*B, *tet*L
	Norfloxacin	83	
	Vancomycin and Streptomycin	50	
E. casseliflavus / *E. flavescens*	Gentamycin, Streptomycin, Ciprofloxacin, Norfloxacin, Erythromycin and Tetracycline	100	*tet*L
Enterococcus sp.	Ciprofloxacin	100	*van*B, *tet*L
	Norfloxacin	86	
	Erythromycin and Tetracycline	93	
	Streptomycin and Vancomycin	79	
	Imipenem	64	
	Ampicillin	50	

The multidrug resistance distribution was 46%, 40%, 10%, and 3.3% for the *Enterococcus* spp., *E. faecalis*, *E. faecium,* and *E. casseliflavus/E. flavescens* isolates, respectively. All the *Enterococcus* isolates showed different multidrug resistance profiles, of which 35.7% of the isolates showed simultaneous resistance to the 12 antimicrobials tested. Similar to our results, these authors[21] reported that *E. faecalis* strains isolated from food were more resistant to antibiotics than other species. On the other hand,[22] it was determined that *E. faecalis* and *E. faecium* strains isolated from ready-to-eat foods of animal origin showed similar antibiotic resistance patterns. Conversely, some researchers have reported that *E. faecium* strains were more resistant to antibiotics than other species[23; 24].

The highest rates of intermediate resistance to the antimicrobial chloramphenicol were observed for the *E. casseliflavus/E. flavescens* isolates (100%), followed by *E. faecium* (67%), *E. faecalis* (50%), and *Enterococcus* spp. (28%). Among all samples of *E. faecalis*, 33% showed intermediate resistance to norfloxacin, followed by 17% to ciprofloxacin and chloramphenicol. Among the *Enterococcus* spp. isolates, higher percentages of

intermediate resistance were shown for norfloxacin and tetracycline (18%), followed by erythromycin and chloramphenicol (9%). None of the *E. faecium* isolates showed intermediate resistance to the antimicrobials tested.

Among the *E. faecalis* isolates, 17% were resistant to erythromycin and tetracycline, and 25% of the *E. faecium* isolates showed resistance to erythromycin. In the other *Enterococcus* spp. isolates, a resistance profile was not detected.

In the processed meat product samples, isolates corresponding to the *E. faecium* species were identified only in the chicken meat samples; these isolates presented a sensitivity profile for all the antimicrobials tested. None of the enterococci isolates from the raw pork meat or processed meat products showed a multidrug resistance profile.

The susceptibility profile that was predominant among the isolates from raw chicken meat samples was that of resistance. Similar resistance results for isolates obtained from foods were described previously[25]. Reports of intermediate resistance should be considered as a warning because intermediate strains are likely to migrate to the group of resistant strains following the inappropriate use of antimicrobials[13]. In this study, we observed that *E. faecalis* is more intrinsically resistant to multiple antimicrobial agents, in particular, to several β-lactam antibiotics, and clinical doses of aminoglycosides. In Brazil, there is not much information regarding the antimicrobial resistance of enterococci from food [26].

The *Enterococcus* spp. strains displayed resistance to 2–8 antibiotics. The resistance of enterococci to multiple antibiotics is not surprising. Several researchers have reported that it is common for enterococci isolated from fermented meat products to exhibit resistance to multiple antibiotics[27; 28], as also confirmed in this study. [27] It was also shown that *Enterococcus* strains isolated from fermented meat products exhibited a high rate of resistance to multiple antibiotics.

Antibiotic resistance and multidrug resistance are public health problems, as they may cause the failure of treatments for enterococcal infections. Although the causes of resistance development are contradicting, evidence based on research data suggests a potential link between antimicrobial resistance and veterinary practices, such as growth promotion, prophylaxis, and treatment[10; 29;30].

The data on multidrug resistance are extremely concerning, mainly because of the risk of acquisition of these microorganisms and their mobile genetic elements, either through the food chain, from direct contact with animals, or even from environmental contamination[4;31], all of which further hinder the development of treatment alternatives for human infections. A resistant bacterium may infect a carcass during slaughter operations and resist the existing barriers present during food production in industries and/or during home preparation; then, it can reach the gastrointestinal tract of the consumers and modify the resistance profile of their intestinal microbiota[30].

In the present study, the genotypic basis for tetracycline and vancomycin resistance and/or sensitivity phenotype found in the food isolates of the *Enterococcus* strains was investigated by PCR, based on the detection of *tet* and *van* genes. The distribution of the resistance gene *tet*L, among the isolates from food, was found more frequently in the

isolates from raw chicken meat samples. The presence of the *van*B gene was evident in the isolates from raw chicken and pork meat. The *van*A gene was not detected in any of the isolates studied.

The frequency with which these genes were detected in this study coincides with that in the literature[32; 33]. The source of VRE is not known, although potential reservoirs of these organisms are hospitals and food.

Five isolates of *Enterococcus* spp. did not have the *tet*L, *van*A, or *van*B genes, and these isolates were still resistant to the tested antimicrobials, indicating that other resistance genes might be present in these isolates.

The presence of a resistance gene that did not confer resistance to these antimicrobials indicates that the gene is likely to exhibit its resistance at any time. These genes, called silent genes, can be activated by environmental factors, such as the physical conditions of the gastrointestinal tract or the synergistic effects of the bacterial microbiota[34].

Vancomycin resistance in enterococci isolated from food has a variable pattern both in Brazil and in other countries, depending on the region of their origin[26]. It should be considered that the distribution of resistance genes reported in this study is based on a relatively limited set of isolates, which highlights the need for regular monitoring of diverse strains to obtain a broader view of the prevalence of these genes in food-associated enterococci.

Conclusion

Enterococci are relatively abundant and resistant to environmental adversity; hence, they have been proposed as bacterial indicators of antimicrobial resistance, as well as of hygienic quality. However, enterococci have emerged as important opportunistic pathogens with a remarkable capacity to express resistance to several groups of antimicrobial agents, thus limiting the number of therapeutic options. We used antibiotic disc diffusion tests and PCR to screen for some resistance genes from all the isolates considered in this study.

The transmission of *Enterococcus* spp. occurs via food, and little information is available regarding the diversity and distribution of resistance in the *Enterococcus* species isolated from the area north of Paraná-Brazil. This study provides the first detailed analysis of antibiotic resistance in a variety of enterococci isolated from raw and processed meats in that state. Enterococci should not only be considered potential pathogens, but also reservoirs of genes that encode antibiotic resistance, which can be transferred to other microorganisms. Continuous monitoring of their incidence and emerging resistance is important to identify foods that potentially represent a risk to the population and to ensure effective treatment of human enterococcal infections.

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