



MICROBIOLOGY

Antimicrobial resistance of enterococci isolated from food in South Brazil: Comparing pre- and post-RDC 20/2011

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Abstract: Antimicrobial resistance has been attributed to the overuse of antibiotics. To control the use of antibiotics, Brazil adopted the RDC 20/2011. A comparison the antibiotic-resistance profile of bacterial has provided important insights into resistance evolution. Enterococci are ubiquitous bacteria recommended to be used as a sentinel organism, in national surveillance systems, for tracking antimicrobial resistance through the food chain. The present study aimed to evaluate the diversity and antimicrobial resistance of enterococci collected from food in South Brazil in 2017 (pos-RDC 20/11) for comparison with isolated in 2007 (pre-RDC 20/11). A total of 310 enterococci were isolated from vegetables and products of animal origin, identified by PCR and MALDI-TOF, tested for antimicrobial susceptibility and screened for resistance genes. *Enterococcus casseliflavus* was dominant in vegetables and *E. faecalis* in products of animal origin. Enterococcal isolates in 2017 were mostly sensitive to ampicillin, gentamicin, chloramphenicol, and vancomycin when compared to isolated collected in 2007. While resistance levels to most compounds remained relatively stable, multidrug resistance decreased by 24% during this period. Our results suggest that RDC 20/11 had a positive outcome in controlling the spread of antimicrobial resistance. This study provides baseline data to measure future changes in the prevalence of resistant enterococci.

Key words: Enterococcal isolates, vegetable and products of animal origin, antibiotic resistance, RDC 20/2011.

INTRODUCTION

Antimicrobials are widely used to treat bacterial infections in humans and animals. Moreover, they are also used as growth promoters in animal husbandry and aquaculture. The misuse and overuse of antimicrobials are the main driving forces for the selection of resistant bacterial populations in various environments (World Health Organization 2020). The impact of the presence of antimicrobial agents or resistant bacteria in all environments is a growing global public health concern. Antibiotic-resistant bacteria can reach humans directly through

the consumption of contaminated food and/or indirectly through environmental pollution containing human or animal excrements (Kristiansson et al. 2011, Marathe et al. 2013).

In terms of transmission through food, antimicrobial resistant bacteria can contaminate animal products during slaughter and processing. Moreover, fruits and vegetables can be contaminated by direct contact with soil, water, or fertilizer containing insufficiently treated material (e.g., animal feces-based fertilizers or human sewage). Several studies have identified resistant bacteria in food products and animal samples, such as

methicillin-resistant *Staphylococcus aureus* in livestock (Price et al. 2012), extended spectrum-beta-lactamase *Escherichia coli* on vegetables, raw chicken, raw egg-surfaces, and raw meat (Rasheed et al. 2014), and antibiotic-resistant *enterococci* in various foods (McGowan et al. 2006, Gomes et al. 2008, Frazzon et al. 2009, Riboldi et al. 2009, Ben Said et al. 2015). Studies have suggested that antimicrobial-resistant bacteria and their resistance genes can be transmitted from food to humans via the food chain (Leverstein-van Hall et al. 2011, Verraes et al. 2013).

Enterococci are ubiquitous microorganisms found in the gastrointestinal tracts of humans and animals, as well as in plants, sewage, water, soil, and foods (Frazzon et al. 2009, Lebreton et al. 2014, Grassotti et al. 2018, Costa et al. 2019, Bhardwaj 2019). *Enterococcus* spp. exhibit greater environmental persistence and are regarded as robust organisms capable of tolerating a wide range of temperatures and pH levels that can grow in the presence of 6.5 % sodium chloride (NaCl) or 40% of bile salts. Additionally, they can exchange resistance determinants through gene transfer by plasmids and transposons, among themselves and with other genera (Lebreton et al. 2014). Due to their ubiquity, resilience and ability to acquire antibiotic resistance, enterococci have been recommended for integration into antimicrobial resistance-monitoring systems as sentinel bacteria (World Health Organization 2013).

Some enterococci species have been used in food and feed, such as starter-culture and probiotics, respectively (Giraffa 2002, Franz et al. 2003). However, despite their beneficial effects in foods, they can also be involved in food spoilage and their presence may also serve as an indicator of microbial contamination of fecal origin (Hanchi et al. 2018). Furthermore, enterococci are among the most important

MDR microorganisms associated with health-associated infection worldwide, causing a wide range of infections in immunocompromised and hospitalized patients (Lebreton et al. 2014, Bhardwaj 2019). Nonetheless, resistant enterococci are not restricted to clinical samples, since strains have been isolated from various environments, including food, animals, and environmental samples (Frazzon et al. 2009, Lebreton et al. 2014, Grassotti et al. 2018, Costa et al. 2019, Bhardwaj 2019).

Interventions to promote practical antibiotic use are essential to reduce the emergence and spread of antimicrobial-resistant bacteria in human healthcare and food production. Many countries have been improving the prudent use and dispensing of antimicrobials (Roca et al. 2015). Notably, the World Health Organization (WHO) has created a set of strategies to combat rising antibiotic resistance, which include, colon improving sanitation and hygiene to reduce overall infection rates, and optimizing the use (and preventing the overuse) of antibiotics in humans and animals (World Health Organization 2013). In this sense, in 2010, the Brazilian Health Surveillance Agency published the RDC nº 20/2011 (RDC 20/11) to facilitate the dispensation and control of antimicrobial use (Brasil 2011).

Studies comparing antibiotic resistance patterns in collections of clinical strains have been conducted to address the antibiotic resistance in community settings (Ventola 2015, Massot et al. 2016, Kulik et al. 2019). However, few studies have compared antibiotic-resistant strains from food collections (Alonso-Hernando et al. 2012, Tyson et al. 2017). To date, no study in Brazil has compared antibiotic-resistant enterococci isolated from food products, since the RDC 20/11 was implemented. Therefore, the present study aimed to compare the species distribution and antibiotic resistance patterns of enterococci isolated from food products in

South Brazil in 2017 (pos-RDC 20/11) with those collected and isolated in 2007 (pre-RDC 20/11).

MATERIALS AND METHODS

Enterococci from foods

Isolation: A total of 30 food samples comprising: vegetables (cassava, beetroot, potato, sweet potato, parsley, carrot and cabbage), and food of animal sources (raw chicken meat, colonial cheese type and soft cheese) were purchased from different popular markets in the Porto Alegre, South Brazil in 2017. The enterococci isolation was performed according to protocol described by Riboldi et al. (2009).

Previous collection: *Enterococcus faecalis* (n=27), *E. faecium* (n=23), *E. mundtii* (n=1) and *Enterococcus* sp. (n=5)- isolated under the same conditions in 2007 (Frazzon et al. 2009, Riboldi et al. 2009) were used to compare species distribution and antibiotic resistance patterns.

Genus and species identification

DNA extraction: Genomic DNA was extracted by boiling method, as described by Depardieu et al. (2004). Genus-specific polymerase chain reaction (PCR) assays, which targeted the *tuf* gene, performed as described by Ke et al. (1999) (Table I). The *E. faecalis* ATCC 29212 and *E. faecium* SS1274 (D'Azevedo et al. 2006) were used as positive control.

PCR assay: Isolates were screened with the species-specific PCR assay for *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. hirae* (Table I). Amplifications were carried out in a total volume of 25 μ L containing: 100 ng of template DNA, 1 X reaction buffer (Ludwig Biotechnology), 0.4 μ M of each primer (Ludwig Biotechnology), 1.5 mM MgCl₂, 200 μ M of dNTPs (Ludwig Biotechnology), 1 U *Taq* DNA polymerase (Ludwig Biotechnology), and MilliQ water. PCR amplifications were performed in the conventional thermocycler (Applied Biosystems 2720 Thermal Cycler) according to the following program: 94 °C for

Table I. Primers used in the PCR reactions carried out for identification of enterococci genus and species.

		Nucleotide sequence (5'-3')	Size ¹ (bp)	AT ² (°C)	References
Genus		TACTGACAAACCATTCATGATG	112	55	Ke et al. (1999)
		AACTTCGTCACCAACGCGAAC			
Species					
	<i>E. casseliflavus</i>	TAGGATGTTACGTCTGCGTG	139	58	Medeiros et al. (2016)
		TTGTTGGTTTGGGCTTTTCCCG			
	<i>E. faecalis</i>	CCGAGTGCTTGCACTCAATTGG	136	66	Sedgley et al. (2005)
		CTCTTATGCCATGCGGCATAAAC			
	<i>E. faecium</i>	TTGAGGCAGACCAGATTGACG	172	62	Medeiros et al. (2016)
		CGGAAGTGATGCTTCCTACTG			
	<i>E. hirae</i>	TTATGTCCCWGTWTTGAAAAATCAA	94	62	Medeiros et al. (2016)
		TATTGATAAGCTAATGCAAGCGC			

1: bp, base pairs; 2: AT, annealing temperatures.

5 min followed by 35 cycles of 94 °C for 1 min, appropriate annealing temperature for each primer for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The DNA fragments amplified were analyzed in 1.5% (w/v) agarose gels stained with SYBR® Safe DNA Gel, and visualized on a photo-documenter.

Isolates not identified by PCR were submitted to matrix-assisted laser desorption and ionization time-of-flight technique (MALDI-TOF), according to Sauget et al. (2017).

Antimicrobial susceptibility analysis

Determination of antimicrobial susceptibility was performed by disk diffusion method, according to Clinical and Laboratory Standards Institute (CLSI 2017). Eleven antibiotics commonly used in clinical and veterinary medicine were evaluated: ampicillin (AMP - 10 µg), ciprofloxacin (CIP - 5 µg), chloramphenicol (CHL - 30 µg), erythromycin (ERY - 15 µg), gentamicin (GEN - 120 µg), nitrofurantoin (NIT - 300 µg), norfloxacin (NOR - 10 µg), streptomycin (STR - 300 µg), tetracycline (TET - 30 µg), rifampicin (RIF - 5 µg) and vancomycin (VAN - 30 µg). The minimum inhibitory concentration (MIC) for streptomycin, gentamicin and vancomycin was performed using the microdilution method according to CLSI (2017). Strains resistant to three or more unrelated antibiotics were considered as multidrug resistant (MDR) (Schwarz et al. 2010).

Detection of antibiotic resistance genes by PCR

Erythromycin-, gentamicin- and tetracycline-resistant strains were tested by PCR for presence resistance genes commonly associated with clinical and environmental enterococci. The *erm(A)*, *erm(B)*, *erm(C)* genes (Sutcliffe et al. 1996), which encode resistance to erythromycin from the modification of the target preventing the binding of macrolides, and the *msrC* gene

(Werner et al. 2001), which produces low-level resistance to erythromycin through the efflux pump mechanism were tested in all erythromycin-resistant strains. To gentamicin-resistant strains, the *aac(6′)-aph(2′)* gene (Jia et al. 2014), which encode an enzymatic modification was evaluated. The *tet(M)* and *tet(S)* genes (Aarestrup et al. 2000) that encode for ribosome protection, and *tet(L)* gene (Frazzon et al. 2009) that encodes for efflux proteins were evaluated in all tetracycline-resistant strains. The primers used are listed in Table II.

Amplifications were carried out in a total volume of 25 µL containing: 100 ng of template DNA, 1 X reaction buffer (Ludwig Biotechnology), 0.4 µM of each primer (Ludwig Biotechnology), 1.5 mM MgCl₂, 200 µM of dNTPs (Ludwig Biotechnology), 1 U Taq DNA polymerase (Ludwig Biotechnology), and MilliQ water. PCR amplifications were performed in the conventional thermocycler (Applied Biosystems 2720 Thermal Cycler) according to the following program: 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, appropriate annealing temperature for each primer for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The DNA fragments amplified were analyzed in 1.5% (w/v) agarose gels stained with SYBR® Safe DNA Gel, and visualized on a photo-documenter.

Statistical analysis

The results obtained here were used to compare species distribution and antimicrobial resistant profile with our enterococcal collection isolated pre-RDC 20/11. The results obtained were submitted to statistical analysis, which was performed using Statistic Package of the Social Science (SPSS) software 18th edition, using Pearson's chi-square test (χ^2) ($p \leq 0.05$) and Fisher's exact test as evaluation parameters.

Table II. Primers used in the PCR reactions carried out for detection of resistance genes.

Resistance	gene	Nucleotide sequence (5'-3')	Size ¹ (bp)	AT ² (°C)	References
Erythromycin	<i>erm</i> (A)	TCTAAAAAGCATGTAAAAGAA	420	52	Sutcliffe et al. (1996)
		CTTCGATAGTTTATTAATATTAGT			
	<i>erm</i> (B)	GAAAAGGTACTIONCAACCAAATA	547	52	Sutcliffe et al. (1996)
		AGTAACGGTACTTAAATTGTTTAC			
	<i>erm</i> (C)	TCAAAACATAATATAGATAAA	837	52	Sutcliffe et al. (1996)
		GCTAATATTGTTAAATCGTCAAT			
<i>msrC</i>	AAGGAATCCTTCTCTCTCCG	343	52	Werner et al. (2001)	
	GTAACAAAATCGTTCCCG				
Gentamycin	<i>aac</i> (6')- <i>aph</i> (2')	CACTATCATAACCACTACCG	220	56	Jia et al. (2014)
		CCAAGAGCAATAAGGGCATA			
Tetracycline	<i>tet</i> (L)	ACTCGTAATGGTGTAGTTGC	625	58	Frazzon et al. (2009)
		TGTAACCTCCGATGTTTAACACG			
	<i>tet</i> (M)	GTAAATAGTGTCTTGGAG	657	52	Aarestrup et al. (2000)
		CTAAGATATGGCTCTAACAA			
	<i>tet</i> (S)	TGGAACGCCAGAGAGGTATT	720	58	Aarestrup et al. (2000)
		ACATAGACAAGCCGTTGACC			

1: bp, base pairs; 2: AT, annealing temperatures.

RESULTS AND DISCUSSION

Enterococcus species diversity in food

Among the 360 Gram-positive and catalase-negative cocci isolated, 310 (86.1%) were confirmed as enterococci. *E. faecalis* (57.1%) was the most abundant species detected in both groups of analyzed food, followed by *E. casseliflavus* (33.2%), *E. hirae* (5.5%), *E. faecium* (1.9%), and *E. durans* (1.0%) (Table III). Four isolates (1.3%) could not be identified at the species level and were classified as *Enterococcus* sp. Considering the ubiquitous natural distribution of enterococci, the presence of these species in food is consistent with previous studies (McGowan et al. 2006, Gomes et al. 2008, Frazzon et al. 2009, Riboldi et al. 2009, Kim et al. 2020).

In the vegetables group, *E. casseliflavus* (50.99%) and *E. faecalis* (43.56%) were the most frequent species detected in cassava, beetroot, potato, sweet potato, parsley, and cabbage. *Enterococcus hirae* (2.47%), and *Enterococcus faecium* (0.99%) were identified in low rates. In all foods of animal origin, *E. faecalis* was more frequently found (82.4%), while *E. hirae* (11.11%), *E. faecium* (3.7%) and *E. durans* (2.77%) were only isolated in soft cheeses samples, and none of the samples contained *E. casseliflavus*. Although some differences in enterococcal distribution were detected among food samples from distinct origins, not significant differences could be verified ($p > 0.05$).

The predominance of *E. casseliflavus* in vegetables is consistent with previous studies (McGowan et al. 2006, Gomes et al. 2008, Kim

Table III. Distribution of *Enterococcus* species among food samples.

Source	Food samples (n)	Number (%) of species isolated					
		<i>E. casseliflavus</i>	<i>E. durans</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. hirae</i>	<i>Enterococcus</i> sp.
Vegetables	Sweet potato (35)	21 (6.8) ^c	0 ^a	12 (3.9) ^a	1 (0.3) ^a	0 ^a	2 (0.65) ^a
	Potato (36)	19 (6.1) ^c	0 ^a	17 (5.5) ^b	0 ^a	0 ^a	0 ^a
	Beetroot (24)	24 (7.7) ^c	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	Carrot (3)	0 ^a	0 ^a	3 (1.0) ^b	0 ^a	0 ^a	0 ^a
	Cabbage (33)	5 (1.6) ^a	0 ^a	26 (8.4) ^c	1 (0.3) ^a	1 (0.3) ^b	0 ^a
	Parsley (34)	22 (7.1) ^c	0 ^a	6 (1.9) ^a	0 ^a	4 (1.3) ^b	2 (0.65) ^a
	Cassava (36)	12 (3.9) ^b	0 ^a	24 (7.7) ^b	0 ^a	0 ^a	0 ^a
	Sub-total (202)	103 (50.99)	0	88 (43.56)	2 (0.99)	5 (2.47)	4 (1.98)
Animal	Raw chicken meat (36)	0 ^a	0 ^a	36 (11.6) ^c	0 ^a	0 ^a	0 ^a
	Colonial cheese (36)	0 ^a	0 ^a	36 (11.6) ^c	0 ^a	0 ^a	0 ^a
	Soft cheese (36)	0 ^a	3 (1.0) ^a	17 (5.5) ^b	4 (1.3) ^a	12 (3.9) ^c	0 ^a
	Sub-total (108)	0	3 (2.77)	89 (82.4)	4 (3.7)	12 (11.11)	0
	Total (310)	103 (33.2)	3 (1.0)	177 (57.1)	6 (1.9)	17 (5.5)	4 (1.3)

Same downcase letters on the same column means there is no statistical difference between samples ($p > 0.05$).

et al. 2020). The presence of *E. casseliflavus*, *E. faecium*, and *E. hirae* in vegetables samples can be associated with untreated wastewater used for agriculture (mainly irrigation) and/or raw animal manure (organic fertilizers) used on vegetable crops. This finding is supported by the published study of Ben Said et al. (2015) who isolated the same species from vegetables, soil, and irrigation water samples from farms in Tunisia.

The occurrence of *E. faecalis*, *E. hirae*, *E. faecium*, and *E. durans* is consistent with the results of McGowan et al. (2006), Gomes et al. (2008), Frazzon et al. (2009), Campos et al. (2013), Camargo et al. (2014), Kim et al. (2018) and Tyson et al. (2017), who also noted these species were predominant in meats and cheese samples. The higher occurrence of *E. faecalis* in raw chicken

meat, can be justified by the manipulation and elevated susceptibility of these products to contamination with feces from slaughtered animals during the processing (Hölzel et al. 2018). The presence of *E. faecalis*, *E. faecium*, *E. durans*, and *E. hirae* in cheese samples may be related to the raw material used in cheese making process. Different enterococci species were found in artisanal cheeses made from raw or pasteurized milk from goats, sheep, buffaloes, and cows (Gelsomino et al. 2002). In cheeses, enterococci represent a major part of the microbiota and are the predominant microorganisms in the ripened cheeses (Lebreton et al. 2014).

Upon comparing the enterococci diversity observed in this study with the enterococcal collection isolated in 2007, some differences were observed. For example, in this study *E.*

casseliflavus and *E. hirae* was detected in vegetables, while Riboldi et al. (2009) and Frazzon et al. (2009) isolated only *E. faecalis* and *E. faecium* from vegetables. This difference can be related to numerous sources beyond the soil sources, fertilization and irrigation used in vegetable production (e.g., handling and storage) (Regitano & Leal 2010). On the other hand, no differences in the proportion of enterococci isolates from foods of animal origin (e.g., raw meat and colonial and soft cheeses) were observed upon isolates from 2007 and 2017. In both studies, *E. faecalis* was the most frequently isolated species.

Antimicrobial susceptibility patterns of enterococci isolated from food samples

Among the 310 strains isolated in this study, 33 (10.6%) were susceptible to all antimicrobials tested, while 277 (89.4%) showed resistance to at least one antimicrobial (Table IV). Resistance to rifampicin (54.8%) was the most commonly observed profile, followed by resistance to erythromycin (47.7%), tetracycline (31.3%), and ciprofloxacin (20%). A lower rate was found to streptomycin (14.8%), norfloxacin (12.6%), nitrofurantoin (9.3%), gentamicin (3.5%), chloramphenicol (2.3%), and ampicillin (0.6%). No resistance to vancomycin was observed. These findings are consistent with published data that also reported the presence of resistant enterococci in foods of animal and vegetable origin (McGowan et al. 2006, Ben Said et al. 2015, Ngbede et al. 2017, Kim et al. 2018).

Enterococci isolated from vegetables showed elevated rates of resistance to rifampicin (125/202, 61.88%) and erythromycin (82/202, 40.6%) were detected. Moderate rates of resistance were observed for ciprofloxacin (38/202, 18%), and tetracycline (34/202, 16.83%). Moreover, low rates of resistance were detected for norfloxacin (29/202, 14.3%), nitrofurantoin

(26/202, 12.8%), streptomycin (25/202, 12.3%), and chloramphenicol (6/202, 2.9%). Several studies conducted with vegetables have isolated antimicrobial resistant enterococci (McGowan et al. 2006, Ben Said et al. 2015, Kim et al. 2017, Hölzel et al. 2018). Ben Said et al. (2015) evaluated the antimicrobial resistance of enterococci isolated from vegetables sourced from farms and markets in Tunisia, and noted resistance for ciprofloxacin, erythromycin, tetracycline, chloramphenicol, and streptomycin. Resistant strains were also observed in vegetables from grocery stores in Athens, Georgia, USA from 2000 to 2001 (McGowan et al. 2006). Furthermore, Ngbede et al. (2017) detected ampicillin- and aminoglycosides-resistant *E. faecium* strains isolated from vegetables in Nigeria. The occurrence of erythromycin-resistant enterococci in vegetable samples observed in our study might be associated with the application of untreated irrigation water and natural fertilizers from animal manure (Regitano & Leal 2010). Erythromycin is excreted as an active metabolite via the manure or urine of livestock and remains stable in the environments such as soil and water (Gothwal & Shashidhar 2014, Dizavandi et al. 2017). Biological fluids (e.g., urine and feces) contaminated with antimicrobials or antimicrobial-resistant strains are released into the environment, especially soil, water and wastewater. Kim et al. (2017) compared in Korea fecal samples with fresh vegetable samples in Korea and observed that the vegetables can be contaminated by human and animal fecal material via environmental sources.

Regarding enterococci isolated from foods of animal origin in the present study, high rates of resistance to erythromycin (66/108, 61.11%), tetracycline (63/108, 58.3%), and rifampicin (45/108, 41.66%) were observed, while moderate rates of resistance were observed for ciprofloxacin (24/108, 22.2%), and streptomycin

Table IV. Antimicrobial resistance profiles among enterococci isolated from different type of food.

Food samples	Species (n)	Number of the resistant strains to*										
		AMP	CIP	CHL	ERY	STR	GEN	NIT	NOR	RIF	TET	MDR**
Vegetables												
Sweet potato	<i>E. casseliflavus</i> (21)	-	1 ^a	-	5 ^a	-	-	7 ^b	7 ^a	18 ^c	1 ^a	6
	<i>E. faecalis</i> (12)	-	-	-	-	-	-	3 ^b	-	12 ^c	-	-
	<i>E. faecium</i> (1)	-	-	-	-	-	-	-	-	1 ^c	-	-
	<i>Enterococcus</i> sp. (2)	-	-	-	-	-	-	1 ^b	1 ^a	1 ^c	-	1
Potato	<i>E. casseliflavus</i> (19)	-	-	-	1 ^a	-	-	-	-	7 ^b	-	-
	<i>E. faecalis</i> (17)	-	-	-	-	-	-	-	-	17 ^b	-	-
Beetroot	<i>E. casseliflavus</i> (24)	-	4 ^b	1 ^a	11 ^b	5 ^a	-	-	-	23 ^c	1 ^a	3
Carrot	<i>E. faecalis</i> (3)	-	-	-	1 ^b	-	-	2 ^b	1 ^a	3 ^b	-	1
Cabbage	<i>E. casseliflavus</i> (5)	-	3 ^c	-	4 ^c	-	-	-	-	5 ^b	-	2
	<i>E. faecalis</i> (26)	-	15 ^c	4 ^b	19 ^c	13 ^b	-	-	7 ^a	8 ^b	12 ^b	17
	<i>E. faecium</i> (1)	-	1 ^c	-	1 ^c	1 ^b	-	-	-	-	1 ^b	1
	<i>E. hirae</i> (1)	-	-	-	1 ^c	-	-	-	-	1 ^b	-	-
Parsley	<i>E. casseliflavus</i> (22)	-	2 ^b	-	6 ^a	-	-	3 ^a	4 ^a	8 ^a	11 ^b	5
	<i>E. faecalis</i> (6)	-	3 ^b	-	1 ^a	-	-	3 ^a	3 ^a	6 ^a	-	4
	<i>E. hirae</i> (4)	-	-	-	-	-	-	-	-	-	-	-
	<i>Enterococcus</i> sp. (2)	-	-	-	-	-	-	-	-	-	2 ^b	-
Cassava	<i>E. casseliflavus</i> (12)	1 ^a	4 ^b	1 ^a	12 ^c	4 ^a	1 ^a	7 ^b	1 ^a	3 ^b	3 ^a	7
	<i>E. faecalis</i> (24)	-	5 ^b	-	20 ^c	2 ^a	-	-	5 ^a	12 ^b	3 ^a	5
	Sub-total (202)	1	38	6	82	25	1	26	29	125	34	52
Animals												
Raw chicken meat	<i>E. faecalis</i> (36)	-	6 ^b	-	21 ^b	9 ^a	10 ^b	1 ^a	7 ^a	9 ^a	24 ^c	21
Colonial cheese	<i>E. faecalis</i> (36)	1 ^a	7 ^b	1 ^a	33 ^c	12 ^b	-	-	1 ^a	25 ^b	23 ^c	22
	<i>E. durans</i> (3)	-	1 ^b	-	2 ^b	-	-	2 ^a	-	-	2 ^b	2
Soft cheese	<i>E. faecalis</i> (17)	-	7 ^b	-	2 ^b	-	-	-	2 ^a	3 ^a	4 ^b	2
	<i>E. faecium</i> (4)	-	3 ^b	-	4 ^b	-	-	-	-	-	-	-
	<i>E. hirae</i> (12)	-	-	-	4 ^b	-	-	-	-	8 ^a	10 ^b	2
	Sub-total (108)	1	24	1	66	21	10	3	10	45	63	49
	Total (%)	2 (0.6)	62 (20)	7 (2.3)	148 (47.7)	46 (14.8)	11 (3.5)	29 (9.3)	39 (12.6)	170 (54.8)	97 (31.3)	101 (33.1)

*Antimicrobials: AMP: ampicillin; ERY, erythromycin; CIP, ciprofloxacin; NOR, norfloxacin; RIF, rifampicin; STR, streptomycin; GEN, gentamicin; NIT, nitrofurantoin; CHL, chloramphenicol; TET, tetracycline. ** Profile: MDR, multidrug-resistance. Intermediate and resistant strains were considered in a single category and classified as resistant strains; Same downcase letters on the same column means there is no statistical difference between samples ($p>0.05$). The statistics were divided between the different foods, and not regarding the species of each food.

(21/108, 19.4%). Moreover, low rates of resistance were detected for gentamicin (10/108, 9.2%), and norfloxacin (10/108, 9.2%). Resistant strains have also been reported in animal food sources (McGowan et al. 2006, Lebreton et al. 2014, Vaz Jr 2016). McGowan et al. (2006) isolated bacitracin, lincomycin, tetracycline and streptomycin-resistant enterococci from meat samples in Athens, Georgia in 2000 and 2001. Notably, relatively high rates of resistance for erythromycin and tetracycline were observed in our study. These profiles might be related to antibiotics that are used to treat animal infections. Although tetracycline was banned as a growth promoter in Brazil in 1998, this antibiotic class continues to be widely used to treat *Salmonella*-related infections in poultry and represents one of the main classes of antibiotics marketed and applied to the Brazilian livestock (Vaz Jr 2016). Erythromycin is also a narrow-spectrum antibiotic used by

veterinarians to treat specific types of bacterial infections in animals (Dinos 2017).

Enterococci antimicrobial profiles from the present study were compared to those of the enterococcal collection from 2007. The results showed a reduction in the prevalence of ampicillin, gentamicin, chloramphenicol, and vancomycin-resistant strains (Figure 1). Resistance to ampicillin decreased from 6.7% in 2007 to 0.6% in 2017, while gentamicin resistance decreased from 16.1% in 2007 to 3.5% in 2017, chloramphenicol resistance decreased from 23.65% in 2007 to 2.3% in 2017, and vancomycin resistance decreased from 5.4% in 2007 to 0.0% in 2017. These reductions in resistant enterococci observed in food samples over the 10-years study period might be associated with the control of the antimicrobial use under the RDC 20/11.

Resistance to erythromycin, tetracycline, ciprofloxacin, norfloxacin, and nitrofurantoin remained the same throughout the evaluated

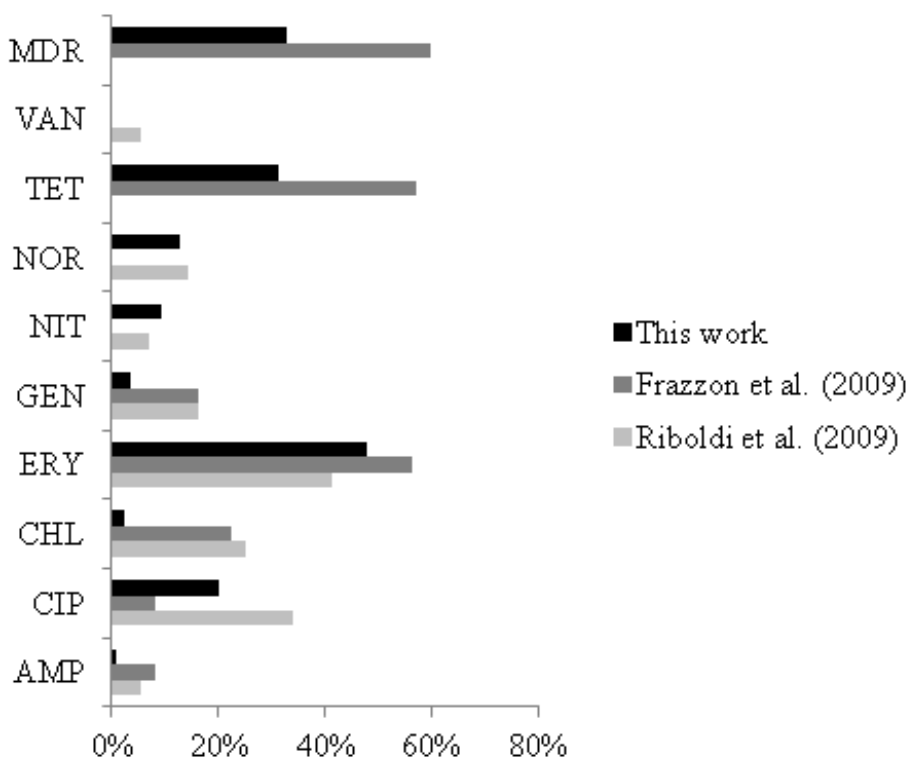


Figure 1. Distribution of resistant enterococci isolated from foods comparing pre-RDC 20/2011 (Riboldi et al. 2009, Frazzon et al. 2009) with post-RDC 20/2011 (our data). Antimicrobials: AMP: ampicillin, CIP: ciprofloxacin, CHL: chloramphenicol, ERY: erythromycin, GEN: gentamicin, NIT: nitrofurantoin, NOR: norfloxacin, TET: tetracycline, VAN: vancomycin, MDR: Multidrug resistance profile.

period. This result might be explained by the fact that these drugs being widely used in veterinary medicine. Veterinary drugs are primarily used in avian production and dairy cattle management mainly for therapy and disease prophylaxis of diseases (Novaes et al. 2017). Additionally, in Brazil there is not a controlling antimicrobial used in veterinary medicine in Brazil.

In the present study, MDR strains were identified in foods of vegetable and animal origin. A total of 101 enterococci (33.1%) strains had an MDR profile (Table II). High rates of MDR were observed in strains isolated from cabbage (20/33, 66.66%), colonial cheese (22/36, 61.11%), and chicken meat (21/36, 58.33%). Among these strains, an MDR profile was more frequently in *E. faecalis* (71.3%), followed by *E. casseliflavus* (22.8%), *E. hirae* (1.9%), *E. durans* (1.9%), *E. faecium* (0.9%), and *Enterococcus* sp. (0.9%). MDR enterococci have previously been isolated from food (McGrowan et al. 2007, Frazzon et al. 2009, Soares-Santos et al. 2015, Kim et al. 2017). Multidrug resistance decreased from 57.1% of the isolates in 2007 to 33.1% in 2017 (Figure 1). However, it is important to highlight that the emergence of MDR appears to be driven by the ubiquitous nature of *Enterococcus* spp., the plasticity of their genomes, and the widespread use of antibiotics (Prieto et al. 2016).

Frequency of antibiotic resistance genes

Of the 95 tetracycline-resistant strains, 76 (78.3%) were positive for the *tet(M)* gene and 23 (23.7%) were positive for the *tet(L)* gene. Among the 123 erythromycin-resistant strains, 63 (42.6%) were positive for the *erm(B)* gene and 7 (4.7%) were positive for the *msrC* gene. Moreover, the *aac(6')-aph(2')* gene was detected in 10 (90.9%) gentamicin-resistant strains isolated from raw chicken meat. Similar results have previously been observed by Frazzon et al. (2009) among tetracycline-resistant strains isolated from

beetroot, raw chicken meat, potato, cassava, and colonial and soft cheeses in southern Brazil in 2009. Ben Said et al. (2015) also discovered *tet(M)* and/or *tet(L)*, *erm(B)* and *aac(6')-aph(2')* genes in enterococci isolates from vegetables, soil, and irrigation water in Tunisia. According to the same authors, the potential presence of other antibiotic resistance genes cannot be ignored.

Cooking is an effective step that can be used to kill most harmful bacteria. Currently, there is a growing demand for ready-to-eat or ready-to-use products. Minimally processed fruits and vegetables are fresh fruits and vegetables processed to preserve their nutritional value and obtain their maximum benefits (Panja 2017). In the present study, we primarily focused on analyzing mainly ready-to-eat products since they are usually eaten raw, without washing or other decontamination procedures. Recently, Cruz et al. (2019) showed that minimally processed vegetables may contain pathogenic bacteria and therefore could represent a risk for consumers. Notably, the presence of antibiotic-resistant bacteria in this type of food could pose a risk to consumer. As reported by Hölzel et al. (2018), three additional situations can be connected to antimicrobial-resistant microorganisms in vegetables: (1) foodborne infectious disease due to obligate or opportunistic pathogens, (2) foodborne microbial intoxication, and (3) foodborne colonization, which could be followed by opportunistic disease after a considerable period of time. Studies have noted the transfer of antibiotic resistance genes between species, which are often passed to humans (Leverstein-van Hall et al. 2011, Nawaz et al. 2011).

CONCLUSION

In the present study, *E. casseliflavus* was predominant in vegetables, while *E. faecalis* was dominant in food sources of animal origin. Upon comparing the antimicrobial resistance profiles of enterococci recovered from food samples between 2007 and 2017, a reduction in the prevalence of resistant isolates associated with some antimicrobials used to treat human infections was observed. These results suggest that RDC 20/11 had a positive outcome in controlling the spread of antimicrobial resistance in the environment. Thus, the present study highlights that there is scope for improvement in food security through advancing food safety knowledge and practices. Therefore, it is recommended that the population should be educated on food safety and safer food practices. Ultimately, effective precautionary measures must be taken to prevent the transmission of enterococci through food. This study provides baseline data to measure future changes in the prevalence of resistant enterococci in Brazil.

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