

Short Communication

Prevalence and phenotypic characterization of *Enterococcus* spp. isolated from food in Brazil

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Abstract

We evaluated the frequency of enterococci from food and found 95.2% of positivity, being *E. faecium* and *E. faecalis* the most frequent species. High-level streptomycin resistance was observed, as well as gelatinase and hemolysis activity, showing the potential role of environmental strains as reservoir of virulence and resistance traits.

Key words: *Enterococcus* spp., food, antimicrobial resistance.

Enterococci are lactic acid bacteria and although this genus comprises more than 25 species, *E. faecium* and *E. faecalis* are the main species isolated from food (Giraffa, 2002; Foulquié Moreno *et al.*, 2006) and clinical samples (Cetinkaya *et al.*, 2000). These bacteria can also be used as starter in the food industry due to their capacity to produce lipase, protease and volatile compounds ensuring desirable organoleptic features in some specific kinds of food. Despite their importance in food technology, certain features, such as the ability to growth over a wide range of temperature, salinity and pH make these organisms able to multiply in several foods and even spoil them (Giraffa, 2002; Foulquié Moreno *et al.*, 2006).

Enterococcus have already been isolated from several kinds of food, such as vegetables, meat, milk and dairy foods (Giraffa, 2002; Hayes *et al.*, 2003; Foulquié Moreno *et al.*, 2006; Gomes *et al.*, 2008). The frequency of isolation ranged from 52.5 to 99% (Hayes *et al.*, 2003; Johnston and Jaykus, 2004; Gomes *et al.*, 2008), depending on the kind of food, as well as the seasonal and manufacture conditions during their processing. The later aspects influence the bacterial survival, especially on cheese manufacturing and ripening (Foulquié Moreno *et al.*, 2006).

Besides the high prevalence in food, several virulence factors and antimicrobial resistance have been identified in these enterococci (Franz *et al.*, 2001; Barbosa *et al.*, 2010).

The presence of virulence and resistance factors in enterococci is quite variable (Jett *et al.*, 1994; Mundy *et al.*, 2000) and the occurrence of these bacteria in food is a matter of debate (Franz *et al.*, 2003). Differentiation between safe and non-safe strains is not easy, due to their capacity to exchange genetic elements with each other (Eaton and Gasson, 2001; Messi *et al.*, 2006). The real role of virulence and antimicrobial resistance factors of food enterococci is not well elucidate, but bacteria presenting such factors in the environment may be understood as a genetic reservoir of virulence (Hayes *et al.*, 2003). In this study we aimed to isolate enterococci species from different kinds of food evaluating some virulence factors and antimicrobial resistance.

We analyzed samples of poultry (35), pork (20), cheese (35) and vegetables (15) commercially available in eight supermarkets located in Botucatu, SP, Brazil. Samples were allocated in sterile plastic bags and transported in isothermal box under refrigeration during the way to the laboratory to be processed at the same day. Twenty-five

grams of each sample was homogenized in 225 mL of bile esculin azide broth (BBL) in Stomacher Lab Blender 400 for 30 s. Next, 0.1 and 0.01 mL of the initial dilution were spread onto the surface of bile esculin azide agar plates (BBL) and incubated at 45 °C/24 and 48 h. The initial dilutions were also incubated at 45 °C/48 h, and a loop of the broth was streaked onto the surface of bile esculin azide agar plates, followed by incubation at 45 °C/24 and 48 h (Hayes *et al.*, 2003). In order to guarantee the absence of contamination, typical enterococci colonies (black) on bile esculine azide agar (from either direct growth or after the enrichment step) were streaked onto blood agar (Oxoid) plates prepared with 5% of defibrinated sheep blood and incubated at 37 °C/24 and 48 h.

Identification of presumptive enterococci was confirmed using the tests proposed by Facklam *et al.* (2007): Gram staining, hemolysins, catalase, salt tolerance, esculin hydrolysis, pyrrolidonyl arylamidase (PYR), arginine decarboxylation, mannitol, arabinose, sorbitol and raffinose fermentation, pigment production, motility and tetrazolium reduction test. For determination of virulence traits, hemolysins were detected in blood agar base plates with 5% of defibrinated sheep blood after incubation at 37 °C/24 h and 5 °C/48 h. α -Hemolysis was defined by the presence of a viridant halo around isolate colonies, while β -hemolysis was defined by translucent halo. Gelatinase assay was carried out as described by Su *et al.* (1991). Briefly, a spot of freshly cultured enterococci was seeded onto the surface of gelatin agar and the plate was incubated at 37 °C/48 h; next the Petri dishes were kept at 4 °C/4 h, and a precipitation halo around the spot denoted a positive result. For antimicrobial susceptibility testing, vancomycin and high-level aminoglycoside (gentamicin and streptomycin) resistance was screened by disk diffusion assay (120- μ g gentamicin disk and 300- μ g streptomycin disk) assay and confirmed by minimal inhibitory concentration (MIC) determined by agar dilution (MIC above 500 and 2000 μ g/mL for gentamicin and streptomycin, respectively). Based on halos and MIC measures, the isolates were categorized as susceptible, intermediate and resistant according to the Clinical and Laboratory Standards Institute (2011). Categorical variables were compared by chi-square or Fisher's exact tests. Differences were considered statistically significant when $p \leq 0.05$.

Hundred fifty-eight strains of enterococci were isolated from 100 samples out of 105 analyzed food samples (positivity ratio = 95.2%). Enterococci was less frequently isolated from vegetable (73.3%) than cheese (100%), poultry (100%) and pork (95%) ($p = 0.001$). Samples of all the eight evaluated supermarkets were positive. *E. faecium* was the most frequent species isolated (41.8% of overall strains) followed by *E. faecalis* (20.3%). Other species were also less frequently identified (Table 1).

Antimicrobial susceptibility showed that all of strains were susceptible to vancomycin and high-level gentamicin. Only two strains presented high-level streptomycin resistant (confirmed MIC > 2000 μ g/mL), both of them were identified as *E. faecalis* and isolated from poultry and pork meats purchased at the same supermarket, but with a gap of almost three months between the evaluation of two samples. Seventy-two percent of enterococci isolated from foods showed hemolysis; this characteristic was associated to *E. faecium* species ($p = 0.016$). Gelatinase production, however, was associated with *E. faecalis* species ($p < 0.0001$). More detailed data on source, frequencies and phenotypic characterization of enterococci strains are presented at Table 1.

In this study, enterococci were isolated from 95.2% of the 105 food samples. High frequency of enterococci in foods has also been previously reported in North America (Hayes *et al.*, 2003; Johnston and Jaykus, 2004), Europe (Koluman, 2009), and Latin America (Morales *et al.*, 2004). In Brazil, there is no much available information about it: Fracalanza *et al.* (2007) analyzed 50 samples of meat and milk, and 86.6% of them were positive for enterococci. Later, Gomes *et al.* (2008) observed the presence of enterococci in 120 samples of raw and pasteurized cheese, meat, and vegetables and found out a lower frequency compared with our results (52.5% of positivity), probably due to the absence of an enrichment step.

We observed that *E. faecalis* and *E. faecium* together figured more than 60% of the overall isolated strains, but it is remarkable the diversity of species isolated from food samples (Table 1). Particularly *E. haemoperoxidus* (isolated from cheese, poultry, pork and lettuce samples) and *E. sanguinicola* (from cheese and poultry samples) species have rarely been recovered from food samples (Martín *et al.*, 2009). Although more than 25 species have been described into *Enterococcus* genus, *E. faecium* and *E. faecalis* are the most widespread species isolated from food samples (Hayes *et al.*, 2003, 2004; Abriouel *et al.*, 2008; Gomes *et al.*, 2008; Koluman, 2009). Our difficult to identify the species of a few strains was due to the similarity of phenotypic features; this limitation resulted in some isolates that have been only identified as "group" of strains according to Facklam *et al.* (2007) scheme. Hayes *et al.* (2004) have also had the same limitation to identify isolates from commercial poultry production environments.

In contrast with previous reports, including Brazilian studies (Fracalanza *et al.*, 2007; Gomes *et al.*, 2008), *E. casseliflavus* and *E. gallinarum* were not found in our food samples. These species, and *E. flavescens*, exhibit intrinsic resistance to vancomycin mediate by the chromosomal gene *vanC*. For this reason, in the absence of a confirmatory PCR test, CLSI recommends that strains with intermediate inhibition halos for vancomycin (15-16 mm) should be submitted to vancomycin MIC, motility test and pigment production. Even though none of our isolates has shown values

Table 1 - Prevalence, species frequency and distribution of virulence and resistance traits in *Enterococcus* spp. isolated from Brazilian foods.

Source (n / %+)	Species	Strains (n)	Number (%) of strains showing					
			α -Hemolysin	β -Hemolysin	Gelatinase	Resistance to		
						VAN	HLG	HLS
Cheese (35/100) ^a	<i>E. faecium</i>	29	28 (96.6)	0	1 (3.4)	0	0	0
	Group 3	20	20 (100.0)	0	0	0	0	0
	<i>E. haemoperoxidus</i>	3	3 (100.0)	0	0	0	0	0
	<i>E. faecalis</i>	2	1 (50.0)	0	1 (50.0)	0	0	0
	<i>E. sanguinicola</i>	1	1 (100.0)	0	0	0	0	0
Poultry (35/100) ^a	<i>E. faecium</i>	19	12 (63.2)	1 (5.3)	5 (26.3)	0	0	0
	<i>E. faecalis</i>	15	2 (13.3)	0	12 (80.0)	0	0	1 (6.7)
	<i>E. haemoperoxidus</i>	7	6 (85.7)	0	1 (14.3)	0	0	0
	Group 2*	6	5 (83.3)	0	1 (16.7)	0	0	0
	<i>E. sanguinicola</i>	6	4 (66.6)	0	1 (16.7)	0	0	0
	Group 3	4	4 (100.0)	0	0	0	0	0
	<i>E. avium</i>	1	1 (100.0)	0	0	0	0	0
	Group 1	1	0	1 (100.0)	0	0	0	0
Vegetable (15/73.3) ^b	Group 3	5	5 (100.0)	0	0	0	0	0
	<i>E. faecalis</i>	3	0	0	2 (66.7)	0	0	0
	<i>E. faecium</i>	3	3 (100.0)	0	0	0	0	0
	Group 5	1	1 (100.0)	0	0	0	0	0
	<i>E. haemoperoxidus</i>	1	1 (100.0)	0	0	0	0	0
	<i>E. collumbae/raffinosis</i>	1	1 (100.0)	0	0	0	0	0
Pork (20/95.0) ^a	<i>E. faecium</i>	15	10 (66.7)	0	4 (26.7)	0	0	0
	<i>E. faecalis</i>	12	1 (8.3)	0	6 (50.0)	0	0	1 (8.3)
	Group 3	2	2 (100.0)	0	0	0	0	0
	<i>E. haemoperoxidus</i>	1	1 (100.0)	0	0	0	0	0
All foods (105/95.2)	<i>E. faecium</i>	66	53 (80.3)	1 (1.5)	10 (15.2)	0	0	2 (3.0)
	<i>E. faecalis</i>	32	4 (12.5)	0	21 (65.6)	0	0	0
	Group 3	31	31 (100.0)	0	0	0	0	0
	<i>E. haemoperoxidus</i>	12	11 (91.7)	0	1 (9.1)	0	0	0
	<i>E. sanguinicola</i>	7	5 (71.4)	0	1 (14.3)	0	0	0
	Group 2*	6	5 (83.3)	0	1 (16.7)	0	0	0
	<i>E. avium</i>	1	1 (100.0)	0	0	0	0	0
	<i>E. collumbae/raffinosis</i>	1	1 (100.0)	0	0	0	0	0
	Group 1	1	0	1 (100.0)	0	0	0	0
	Group 5	1	1 (100.0)	0	0	0	0	0
All isolates		158	112 (70.9)	2 (1.3)	34 (21.5)	0	0	2 (1.3)

a, b: different letters represent significant statistical differences ($p = 0.001$).

VAN: vancomycin; HLG: High-level gentamicin [Minimal Inhibitory Concentration (MIC) > 500 $\mu\text{g}/\text{mL}$]; HLS: High-level streptomycin (MIC > 2000 $\mu\text{g}/\text{mL}$).

*: Other than *E. casseliflavus* and *E. gallinarum*, confirmed by absence of motility (hanging drop technique) (Facklam *et al.*, 2007).

of inhibition halos lower than 17 mm, the motility and pigment production tests were performed for all isolates. The strains presented neither motility nor pigment production, confirming the absence of *E. casseliflavus* and *E. gallinarum* in our samples.

Vancomycin resistance in enterococci isolated from food has a variable pattern both in Brazil and abroad (Franz

et al., 2001; Giraffa, 2002; Johnston and Jaykus, 2004; Fracalanza *et al.*, 2007; Gomes *et al.*, 2008). The source of vancomycin resistant strains appears to be different according to the geographic region where they emerge. In Europe it is attributed to the use of antimicrobial agents as growth promoter whereas in United States, it is attributed to the

wide hospital usage of vancomycin (Woodford, 1998; Wegener *et al.*, 1999).

The concern of high-level aminoglycoside resistance (HLAR) in enterococci is well documented. For instance, Donabedian *et al.* (2003) showed the transmission of HLAR strains among farm animals and humans. Although enterococci present intrinsic low level resistance to aminoglycosides, the association of high-levels of such drugs with a cell inhibitor antibiotic was showed to be efficient against enterococci *in vivo* (Murray, 1990). Resistance to high-level gentamicin (HLGR) and streptomycin (HLSR) was evaluated because HLGR predicts resistance to all aminoglycosides but streptomycin (Chow, 2000), which can be modified by other aminoglycoside acetyltransferase (Aac) family enzymes. By examining both aminoglycosides it is possible to predict high-level resistance to aminoglycosides (Chow, 2000; Clinical and Laboratory Standards Institute, 2011).

Resistance to high-level gentamicin was not observed, whereas only two (1.3%) strains isolated from poultry and pork samples showed high-level streptomycin resistance. In previous reports, HLSR was also more frequent than HLGR (Franz *et al.*, 2001; Johnston and Jaykus, 2004; Fracalanza *et al.*, 2007). In opposition, Teuber *et al.* (2009) found out 80% of enterococci isolated from cheese with high-level gentamicin resistance, and, in Brazil, Gomes *et al.* (2008) reported 22% of *E. faecalis* with high-level gentamicin resistance. The low prevalence of HLAR we have observed may be explained by the fact that in Brazil, the use of antimicrobial agents was prohibited as growth promoters in farms (Brasil, 1998) until some years ago (Brasil, 2009).

Virulence markers are also cause of concern among enterococci from food (Eaton and Gasson, 2001; Foulquié Moreno *et al.*, 2006). Several factors such as aggregation proteins to eukaryotic cells and adhesins, biofilm production, extracellular proteases (collagenases, gelatinase), cytolysins (bacteriocins and hemolysins), leukocytary evasion proteins and sex pheromones have been associated with enterococci pathogenicity (Jett *et al.*, 1994; Eaton and Gasson, 2001). Hemolysin production seems to be associated with virulence in experimental models, as well as the gelatinase (Mundy *et al.*, 2000) and the expression of these characteristics was associated with *E. faecalis* and *E. faecium*, according to previous study (Mundy *et al.*, 2000).

In this study we observed higher frequency of α -hemolysin (79.8%) than β -hemolysin (12.6%) in the food enterococci, in agreement with previous reports (Gomes *et al.*, 2008; Barbosa *et al.*, 2010). The gelatinase production was present in about 20% of the strains, most of them belonging to *E. faecalis* species. Most of these strains were isolated from chicken meat, diverging from Franz *et al.* (2001) that found a high incidence of gelatinase producing *E. faecalis* in cheese samples. It is remarkable that both foods are rich in protein contents and that gelatinase pro-

ducing strains can use these substrata as amino acid source (Franz *et al.*, 2001). In our samples, 65.6% of the *E. faecalis* strains were gelatinase producers, while 15.2% of the *E. faecium* strains showed this virulence factor. Gelatinase-producing enterococci were isolated from meat and it could cause the food degradation (Gomes *et al.*, 2008).

Several studies have investigated the occurrence of virulence factors and antimicrobial resistance in enterococci from different sources as environment, water, food and infectious diseases (Semedo *et al.*, 2003). For instance, Abriouel *et al.* (2008) have found virulence traits both in clinical and food, water and soil isolated strains. In the same way, Eaton and Gasson (2001) found a higher proportion of virulent enterococci strains in clinical isolates compared with those isolated from food or employed as starter culture in the food industry. The starter culture should present proteases and lipases in order to metabolize volatile compounds while they should be free of virulence determinants (Foulquié Moreno *et al.*, 2006). The concomitant presence of virulence factors in clinical and environmental samples, however, hinders the classification as safe or non-safe strains (Eaton and Gasson, 2001; Franz *et al.*, 2001).

In summary, we observed the presence of *Enterococcus* spp. in almost all the samples evaluated as well as hemolysin and gelatinase production in those strains. Antimicrobial resistance was very rare. The remarkable finding of enterococci in foods is their ability to exchange virulence and drug resistance with potential pathogenic strains (Eaton and Gasson, 2001; Messi *et al.*, 2006). It suggests us that environmental and food strains can represent a natural reservoir of these features, enhancing the development of more dangerous clinical strains.

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