UNIVERSIDADE FEDERAL DO TRIÂNGULO MINEIRO

Enterococcus ssp. de origem clínica e ambiental: perfil de sensibilidade aos antimicrobianos, fatores de virulência e caracterização molecular

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Uberaba – Minas Gerais
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Orientador: Prof. Dra. Adriana Gonçalves de Oliveira

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Defesa de tese e exame final de **FÁBIO EDERSON LOPES CORREA**, candidato (a) ao grau de Doutor (a) em Ciências – Medicina Translacional.

A Comissão Examinadora comunica à Coordenadoria do PGCS/UFTM, para os devidos fins, que procedeu, nesta data, ao exame da Tese de Doutorado **"Enterococcus ssp. DE ORIGEM CLÍNICA E AMBIENTAL: PERFIL DE SENSIBILIDADE AOS ANTIMICROBIANOS, FATORES DE VIRULÊNCIA E CARACTERIZAÇÃO MOLECULAR"** do Programa de Pós-Graduação em Ciências da Saúde, bem como ao exame final.

O (a) candidato (a) foi considerado (a) APROVADO

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DEDICATÓRIA

A minha mãe Nejma Lopes Ali Miyashiro, meu irmão Leandro Lopes Correa, meu padrasto Hélio Massanobu Miyashiro, minha cunhada Gislaine Santana, meu sobrinho Gael, meu pai Roni Clederson Correa (*in memorian*), minha avó Ramona do Carmo Correa (*in memorian*) e toda minha família, por serem minha maior riqueza, a base de tudo, e sempre em mim acreditarem e apoiarem.

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RESUMO

Os enterococos são encontrados na microbiota intestinal de seres humanos e de outros animais, no solo, água, plantas, vegetais e alimentos. Todavia, nas últimas décadas esses microrganismos têm se destacado como agentes de infecções hospitalares devido a sua resistência intrínseca e adquirida aos diversos antimicrobianos. O uso inadequado de antimicrobianos na agropecuária contribui para o surgimento de isolados multirresistentes no meio ambiente os quais podem ser transmitidos aos seres humanos. O objetivo do presente estudo foi investigar a presença de espécies de enterococos em diferentes amostras não-clínicas (alimentos, animais, água e esgoto) e caracterizá-las quanto ao perfil de sensibilidade aos antimicrobianos e fatores de virulência. Foi investigado também se isolados de *Enterococcus faecalis* penicilina-resistente/ampicilina-sensível (EFPRAS), que foram previamente obtidos de seres humanos, encontram-se disseminados no meio ambiente e se eles seriam geneticamente semelhantes. Amostras de diversas fontes não clínicas (alimentos, água, fezes de animais, esgoto) foram cultivadas para isolamento de enterococos. Os isolados foram identificados fenotipicamente em espécies e submetidos aos testes de sensibilidade a antimicrobianos (diluição em caldo e disco difusão) de acordo com o CLSI (*Clinical and Laboratory Standards Institute*). Foi feita a pesquisa da produção de gelatinase e hemolisinas. Isolados da espécie *E. faecalis* foram ainda comparados geneticamente pela técnica de PFGE (eletroforese em campo pulsado) com isolados clínicos obtidos previamente de pacientes do hospital de clínicas da UFTM. Foi feita a detecção do gene *aac(6')-Ie/aph(2")-Ia*, que confere alto nível de resistência à gentamicina (HLRG). As espécies mais prevalentes em amostras não clínicas de origem vegetal foram *E. casseliflavus* (28,6%) e *E. faecalis* (19,0%), mas, dentre as de origem animal foram *E. faecalis* (26,2%) e *E. faecium* (23,8%). Foi observada alta prevalência de resistência a eritromicina, tetraciclina, norfloxacina e ciprofloxacina. Dentre os isolados não clínicos de *E. faecalis*, 3 (2,9%) eram EFPRAS; todos foram obtidos do esgoto hospitalar e apresentaram HLGR. A prevalência de *E. faecalis* produtor de gelatinase de origem não clínica (50,5%) e clínica (55,8%) foi semelhante, porém, a produção de hemolisinas foi maior entre os de origem não clínica (63,5%) do que entre os de origem clínica (2,8%). Os *E. faecalis* submetidos ao PFGE (n=31) foram classificados em 19 pulsotipos (A-S). Os isolados de EFPRAS testados obtidos do esgoto (n=3) e de pacientes (n=4) foram classificados no mesmo pulsotipo. Um isolado de EFPRAS obtido do esgoto do hospital, o qual foi submetido ao MLST (tipagem por sequenciamento de multilocus), foi englobado no CC9 (complexo clonal), que é comumente encontrado em isolados hospitalares. Apesar da diversidade de espécies do gênero *Enterococcus*, as espécies mais prevalentes em amostras clínicas, ou seja, *E. faecalis e E. faecium*, também foram as espécies predominantes em

amostras não clínicas. Muitos isolados de *E. faecalis* de origem não clínica apresentaram resistência a diferentes antimicrobianos, no entanto, isolados apresentando o fenótipo incomum de resistência à penicilina parecem ser restritos ao ambiente hospitalar. Os resultados do presente estudo ressaltam a necessidade de tratamento do esgoto hospitalar a fim de evitar que microrganismos multirresistentes oriundos do ambiente hospitalar sejam disseminados para o meio ambiente.

Palavras Chave: *Enterococcus*; PFGE; Alimentos; Resistência a antimicrobianos; Fatores de Virulência; HLGR.

ABSTRACT

Enterococci are found in the intestinal microbiota of humans and other animals, in soil, water, plants, vegetables and food. However, in recent decades these microorganisms have been highlighted as agents of nosocomial infections due to their intrinsic and acquired resistance to various antimicrobials. Inadequate use of antimicrobials in agriculture contributes to the emergence of multiresistant isolates in the environment that can be transmitted to humans. The aim of the present study was to investigate the presence of enterococci species in different non-clinical samples (food, animals, water and sewage) and to characterize them for antimicrobial susceptibility profile and virulence factors. It was also investigated whether isolates of penicillin-resistant /ampicillin-sensitive *Enterococcus faecalis* (PRASEF), which were previously obtained from humans, are widespread in the environment and whether they would be genetically similar. Samples from a variety of nonclinical sources (food, water, animal feces, sewage) were grown for enterococcal isolation. Isolates were phenotypically identified in species and subjected to antimicrobial susceptibility testing (broth dilution and disc diffusion) according to the CLSI (Clinical and Laboratory Standards Institute). Research was made into the production of gelatinase and hemolysins. Isolates of the *E. faecalis* species were further compared genetically by the PFGE technique (pulsed field electrophoresis) with clinical isolates previously obtained from patients of the UFTM clinic hospital. The *aac(6')-Ie/aph(2")-Ia* gene that confers high-level resistance to gentamicin (HLRG) was detected by PCR. The most prevalent species in non-clinical samples of plant origin were *E. casseliflavus* (28.6%) and *E. faecali*s (19.0%), but among those of animal origin were *E. faecalis* (26.2%) and *E. faecium* (23.8%). It was observed high prevalence of resistance to erythromycin, tetracycline, ciprofloxacin and norfloxacin. Among the non-clinical isolates of *E. faecalis*, 3 (2.9%) were PRASEF; all of them were recovered from the hospital sewage and presented HLGR. The prevalence of non-clinical (50.5%) and clinical (55.8%) gelatinase-producing *E. faecalis* was similar, but hemolysin production was higher among non-clinical (63.5%). than among those of clinical origin (2.8%). *E. faecalis* submitted to PFGE (n = 31) were classified into 19 pulsotypes (A-S). The tested PRASEF isolates obtained from sewage (n = 3) and from patients (n = 4) were classified in the same pulsotype. One PRASEF isolate recovered from the hospital sewage was submitted to MLST (multilocus sequencing typing) and was encompassed into the CC9 (clonal complex), which is common among in isolates from hospitals. Despite the diversity of species of the genus *Enterococcus*, the most prevalent species in clinical samples, *E. faecalis* and *E. faecium*, were also the predominant species in non-clinical samples. Many *E. faecalis* isolates of nonclinical origin showed resistance to different antimicrobials; however, isolates

presenting the unusual phenotype of penicillin resistance appear to be restricted to the hospital environment. The results of the present study highlight the need for treatment of hospital sewage in order to prevent multidrug resistant microorganisms of hospital origin from spreading to the environment.

Keywords: Enterococcus; PFGE; Foods; Antimicrobial resistance; Virulence Factors; HLGR.

SUMÁRIO

I – INTRODUÇÃO

Os enterococos são cocos Gram-positivos que, embora possam ser encontrados na microbiota normal do trato gastrointestinal e geniturinário de seres humanos, são considerados agentes importantes de infecções hospitalares podendo causar endocardite, bacteremia e infecções intra-abdominais, urinárias e de feridas (Murray, 1990; Murray e Baron, 2007; Arias, Contreras e Murray, 2010). Os enterococos também podem ser encontrados em diversas fontes, tais como alimentos, animais e meio ambiente (Gomes *et al.*, 2008; Getachew *et al.*, 2013; Said, Ben *et al.*, 2016; Tamang *et al.*, 2017).

A espécie *Enterococcus faecalis* é a mais frequente em seres humanos, sendo isolada de 80% a 90% das infecções enterocócicas, e a espécie *E. faecium* é a segunda mais frequente, sendo encontrada em 5 a 10% das infecções (Murray *et al*., 2007). Outras espécies de importância clínica são *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. raffinosus*, *E. malodoratus e E. mundtii*. As demais espécies raramente são isoladas de amostras biológicas de seres humanos.

Os enterococos apresentam resistência intrínseca a vários antimicrobianos tais como cefalosporinas, aztreonam, clindamicina, trimetoprim-sulfametoxazol e penicilinas semissinteticas (oxacilina, carbenicilina, nafcilina e ticarcilina) e, por isso, essas drogas não são usadas no tratamento das infecções enterococicas (Pallares *et al.* 1993; Murray 1998; Rice 2001). Os enterococos também apresentam resistência intrínseca aos aminoglicosídeos, porem essa resistência e de baixo nível, o que permite o uso dessa droga em combinação com as penicilinas (ampicilina, penicilina G e ureidopenicilinas) ou com a vancomicina (glicopeptídeo). Essa combinação do aminoglicosídeo com um antimicrobiano que atua na parede celular gera um efeito bactericida, essencial no tratamento das infecções enterococicas mais graves (Rice, 2001).

Alem da resistência intrínseca, os enterococos também podem adquirir plasmídeos e transposons contendo genes de resistência. Essa resistência adquirida pode ocorrer também por mutações cromossômicas alterando o alvo de ação de algumas drogas. Existem vários relatos de isolamento de amostras com resistência adquirida aos aminoglicosídeos (alto nivel), cloranfenicol, eritromicina, fluoroquinolonas, tetraciclinas, penicilinas e glicopeptideos (vancomicina e teicoplanina) (Rice, 2001; Hollenbeck, Rice, 2012; Dadfarma *et al*., 2013). Os fenótipos de resistência adquirida considerados clinicamente mais significativos entre os enterococos são aqueles associados com a resistência aos aminoglicosídeos (alto nivel), aos glicopeptídeos e as penicilinas (ampicilina, penicilina G e ureidopenicilinas).

A resistência de alto nível (*high level resistance*- HLR) aos aminoglicosídeos em enterococos elimina o efeito bactericida promovido pelo sinergismo, que é essencial no tratamento de infecções mais graves, entre praticamente todos os aminoglicosídeos e os agentes ativos contra a parede celular (betalactâmicos ou glicopeptídeos). A HLR à gentamicina (CIM $\geq$ 500 μg/mL) leva a uma resistência cruzada à tobramicina, amicacina, canamicina e netilmicina; todavia, devido a diferenças entre a estrutura química da estreptomicina e dos demais aminoglicosídeos, amostras de enterococos resistentes à gentamicina podem ser sensíveis à estreptomicina (Rice, 2001).

O mecanismo de resistência aos aminoglicosídeos considerado mais relevante clinicamente se deve a ação de enzimas que modificam esses agentes, sendo denominadas de aminoglicosídeos acetiltransferases (AACs), adeniltransferases ou nucleotidiltransferases (ANTs) e fosfotransferases (APHs) (Vakulenko; Mobashery, 2003; Ramirez; Tolmasky, 2010). A resistência de alto nível à gentamicina (HLRG) em enterococos é devido, principalmente, à aquisição de um gene (*aac(6')-Ie/aph(2")-Ia*) que codifica uma enzima bifuncional (Vakulenko; Mobashery, 2003; Ramirez; Tolmasky, 2010).

Cabe ressaltar que a resistência aos aminoglicosídeos (alto nível) e à penicilina G é mais comum na espécie *E. faecium* do que em *E. faecalis* (Rice, 2001). Por isso, a emergência de isolados de *E. faecalis* penicilina-resistente/ampicilina-sensível (EFPRAS), os quais tendem a ser resistentes a outros antimicrobianos, merece uma investigação mais aprofundada por causa do risco aumentado de acúmulo de genes de resistência e seleção dessas linhagens multirresistentes.

Esses isolados de *E. faecalis* com o fenótipo incomum de resistência aos betalactâmicos (resistência à penicilina e sensibilidade à ampicilina), os quais tendem a ser resistentes também à gentamicina, ainda não foram descritas em outros seres vivos e nem no meio ambiente ou em alimentos, ou seja, a origem dessas amostras ainda é desconhecida (Conceição *et al.*, 2012; Metzidie *et al.*, 2006).

O uso inadequado de antimicrobianos na medicina veterinária e na cadeia produtiva contribui para o surgimento de isolados bacterianos resistentes na natureza (Loureiro *et al*.; 2016). A presença de microrganismos resistentes em animais de produção pode ameaçar a eficácia de antimicrobianos em seres humanos se estas bactérias ou seus genes de resistência forem incorporados à microbiota humana (Smith *et al*., 2002). Apesar do consumo de alimentos ser a principal forma de contaminação de seres humanos de bactérias resistentes, estudos reportaram presença de enterococos resistentes a antimicrobianos em água e solo (Ali, *et al*.,

2017; Conwell *et al*., 2017), mostrando que essa também é uma rota de exposição a bactérias resistentes.

Em ambientes aquáticos a exposição a antimicrobianos pode ocorrer por diversos fatores, dentre eles podemos citar a presença de resíduos de fármacos encontrados em estações de tratamentos de esgoto que podem atingir corpos d'água e contaminação por urina e fezes excretadas, tanto por animais quanto por seres humanos, tratados com antimicrobianos (Bila, Dezotti, 2003; Kummerer, 2004).

A resistência das bactérias aos antimicrobianos, particularmente dos enterococos, é um problema crescente mundialmente. Essa resistência diminui a eficácia dos medicamentos, aumenta o tempo de internação nos hospitais, eleva o custo do tratamento e repercute no uso de drogas menos eficientes, mais tóxicas e mais caras. Dentre as medidas para prevenir a resistência microbiana e fortalecer as ações de controle, o monitoramento constante das bactérias multirresistentes e o conhecimento da origem das linhagens resistentes bem como dos mecanismos que levam à resistência são fundamentais.

II - OBJETIVO

O presente projeto teve como objetivo investigar a presença de espécies de enterococos em diferentes amostras não-clínicas (alimentos, animais, água e esgoto) e caracterizá-las quanto ao perfil de sensibilidade aos antimicrobianos e fatores de virulência. Foi investigado ainda se isolados de EFPRAS, que foram previamente obtidos de seres humanos, encontram-se também disseminados no meio ambiente e se eles seriam geneticamente semelhantes.

Os objetivos específicos foram:

1- Isolar e identificar espécies do gênero *Enterococcus* obtidas de diversas amostras não clínicas (alimentos, fezes de animais, água e esgoto);

2- Detectar fenotipicamente o perfil de resistência aos antimicrobianos e fatores de virulência (hemolisinas e gelatinase) das espécies de *Enterococcus*;

3- Determinar a concentração inibitória mínima (CIM) dos antimicrobianos de maior relevância terapêutica (penicilina, ampicilina, gentamicina, estreptomicina e vancomicina);

4- Detectar a presença de EFPRAS simultaneamente resistentes à gentamicina isolados de amostras de origem não clínica;

5- Confirmar a HLGR em isolados de *E. faecalis* pela pesquisa do gene *aac(6')-Ie/aph(2")-Ia* ;

6- Avaliar e comparar a diversidade genética dos EFPRAS de origem clínica e não clínica por meio das técnicas de MLST (tipagem por sequenciamento de multilocus) e PFGE (eletroforese em campo pulsado).

ARTIGO 1

Antimicrobial susceptibility and virulence factors of non-clinical enterococci isolates from Brazil

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Abstract

Objectives: To identify the *Enterococcus* species found in non-clinical sources in southeastern Brazil. Moreover, the virulence factors and antimicrobial susceptibility profile of the isolates were investigated.

Methods and results: It was included in this study 84 enterococci isolates, 42 from vegetable samples, 20 from animal samples and 22 from swine feces. The isolates were phenotypically identified and tested for antimicrobial susceptibility and virulence factors. Among the animal-origin samples, the most prevalent isolates were of *E. faecalis* (26%) and *E. faecium* (24%) *species*. Among the vegetable-origin samples, *E. casseliflavus* (29%) was the most frequent species while *E. hirae* (26.3%) was the most found in the feces of swine. It was observed that 33.3% of the isolates were susceptible to the eleven antimicrobials tested but a variable percentage of the isolates showed resistance to erythromycin (41.6%), norfloxacin (36.9%), tetracycline (11.9%) and ciprofloxacin (4.7%). It was observed that 53.6% of the isolates produced hemolysins and 20.2% hydrolyze gelatin.

Conclusions: Despite the diversity of enterococci species found in non-clinical samples, the *E. faecalis* and *E. faecium* species were the most prevalent, particularly in food of animal origin. In addition, although most non-clinical enterococci isolates were susceptible to the tested antimicrobials, resistant isolates showing virulence factors were detected, regardless of the sample origin.

Key words: *Enterococcus faecalis*; foods; environment; antimicrobial resistance; virulence factor.

Introduction

Enterococci are common inhabitants of the gastrointestinal tract of humans, mammals, and insects but due their ability to grow and survive under severe environmental conditions, they are also ubiquitous in nature being present in soil, plants, water, and animal products (Lebreton *et al.* 2014). Moreover, enterococci are natural component of various foods, including fermented and dairy products (Murray, 1990; Giraffa, 2002).

In the last decades, enterococci emerged as important agents of multidrug resistant hospital acquired infections mainly in urinary tract, bloodstream, wounds and other sites (Arias *et a*l., 2010; Lebreton *et al.* 2014). In Brazil, species of the genus *Enterococcus* caused 6% of primary bloodstream infections associated with the use of central venous catheter in adult patients hospitalized at intensive care units, ranking seventh among the main causes of this type of infection in 2016 (Brasil, 2017). *E. faecalis* and *E. faecium* are the most frequently isolated species of *Enterococcus* from human clinical specimens (Murray, 1990; Brasil, 2017) and generally also from food products (Lebreton *et al.* 2014), although a total of 58 species and two subspecies of enterococci have been reported (LPSN, 2019).

The commensal-to-pathogen change in *Enterococcus*, although influenced by the selective advantage provided by their natural resistance to various antimicrobials and great ability to adquire new resistance genes also depends on virulence factors with components related to its pathogenicity and increased risk of acquiring infections (Gilmore, Lebreton, van Schaik, 2013; Miller *et al*., 2016).

The study of enterococci found in environment makes it possible to know their role as a reservoir of resistance coding genes and their ability to transfer these genes to human pathogens. This spread of antibiotic resistance can be accomplished in many ways, including the circulation of bacteria between humans and animals, the transfer of plasmids between

bacteria and transposon transfer, supporting the claim that bacteria found in the environment play an important role in the spread of microbial resistance *(*Bengtsson-Palme *et al.*, 2018).

Therefore, the present study aimed to identify the enterococci species found in environmental sources (foods of vegetable or animal origin and health swines feces) in southeastern Brazil. In addition, it was determined the antimicrobial susceptibility profile and detected the virulence factors of the enterococci.

Methods

Isolation and identification of enterococcal isolates

The samples of foods from vegetable origin (lettuce, potato, beet, broccoli, kale, cauliflower, corn, cabbage, arugula and tomato) and from animal origin (meatball, bacon, pork, chicken, sausage, mozzarella, cheese, sausage, sardines) evaluated in this study were obtained from the commercial area of Uberaba, MG, Brazil. Feces samples of health swines were also evaluated. A total of 25 g of vegetables, 10 g of animal foods and 1 g of feces were homogenized, respectively, with 225, 100 or 10 ml of 0.1% (wt/vol) peptone water as described elsewhere (Silva et al, 2010). The homogenates were submitted to serial 10-fold dilutions in 0.1% (wt/vol) peptone water and 100 µl of each dilution was plated on KF *Streptococcus* agar. The plates were incubated at 37^{o}C for 48 h.

The colonies suggestive of enterococci were randomly selected for phenotipic identification based on scheme proposed by Murray and American Society for Microbiology (1999). Briefly, isolates were identified at genus level by Gram staining, cellular morphology, absence of catalase production, hydrolysis of L-pyrrolidonyl-β-naphthylamide (PYR test), hydrolysis of esculin in the presence of bile salts (bile-esculin test) and growth in the presence of 6.5% NaCl. Species identification was determined based on carbohydrate fermentation tests

(mannitol, arabinose, sucrose, raffinose, melibiose, sorbitol, inulin, xylose and pyruvate), arginine hydrolysis, growth in 0.04% tellurite, yellow pigment production, and motility.

Susceptibility testing

The antimicrobial susceptibility profile was performed using disk diffusion method. The following antimicrobials agents were tested: ampicillin (10 μg), penicillin G (10 μg), streptomycin (300 μg), gentamicin (120 μg), erythromycin (15 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), chloramphenicol (30 μg), tetracycline (30 μg),vancomycin (30 μg), and teicoplanin (30 μg).

The minimal inhibitory concentration (MIC) for the most relevant therapeutic antimicrobials (ampicillin, penicillin, vancomycin, gentamicin and streptomycin) was determined using the broth dilution method. The MIC_{50} and MIC_{90} values, which killed or inhibited the growth of 50% and 90% of the isolates studied, respectively, for each of the antimicrobials was also determined.

All susceptibility tests were performed and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2018; CLSI, 2019) guidelines. Quality-control testing was performed for all susceptibility tests, using *E. faecalis* ATCC® 29212 and *E. faecalis* ATCC® 51299 (CLSI, 2018). Isolates with intermediate profile by the disk diffusion method were considered resistant in this study. Values of MIC ≥16 μg/mL, ≥32 μg/mL, ≥500 μg/mL, and ≥1,000 μg/mL indicated resistance, respectively, to ampicillin/penicillin, vancomycin, gentamicin and streptomycin.

Virulence factors

Hemolysins and gelatinase production were detected as described by Castro Porto *et al*. (2016) and Cruz and Torres (2014). For detection of hemolysins, overnight cultures were

streaked on Brain Heart Infusion (BHI) agar supplemented with 5% (vol/vol) defibrinated sheep blood. After incubation for 48 h at 36ºC, the formation of halos around the colony was verified; greenish halos indicated α-hemolysis, transparent halos indicated β-hemolysis and no halo formation indicated γ-hemolysis. For detection of gelatinase, the isolates of enterococci were incubated at 36º C for 72 h in BHI broth containing 12% (wt/vol) of gelatin; next the culture were refrigerated at 4º C for 30min. Conversion of the solid medium to liquid after the cooling period indicated gelatinase production.

Statistical analysis

Data were analyzed using the SPSS statistical software package (version 17.0; SPSS, Inc., Chicago, IL). The chi-squared test or Fisher's exact test was used to compare categorical variables. All tests were two tailed and a *p*-value ≤ 0.05 was considered statistically significant.

Results

Sixty different environmental samples of animal and vegetable origin were analyzed, but only in 49 (81.7%) samples were observed bacterial growth, being 24 from vegetable and 25 from animal (among these, 10 samples were swine feces) origin. Among the cultures with bacterial growth, 174 isolates suggestive of being enterococci were selected but only 131 isolates were confirmed as belonging to the genus *Enterococcus*. Although 3 to 5 distinct colonies were selected of each positive sample (n = 49), in 49% (n = 24) of them the enterococcal isolates belonged to the same species whereas in 32.7% (n = 16) of the samples the isolates belonged to two distinct species and only in 18.4% (n = 9) the isolates belonged to three different species of enterococci. Thus, one isolate of each species per environmental sample was subjected to the other analysis in this study, totalizing 84 isolates. Of these, 42 were recovered from vegetable foods and 42 from animal (20 from foods and 22 from swine feces).

Overall, the most frequently isolated species was *E. faecalis* (22.6%), followed by *E. faecium* (16.7%) and *E. casseliflavus* (16.7%) (Figure 1). However, considering only the enterococci isolates recovered from vegetable foods, the most frequent species were *E. casseliflavus* (28.6%), *E. faecalis* (19.0%) and *E. mundtii* (11.9%). Among those of animal origin, *E. faecalis* (26.2%), *E. faecium* (23.8%) and *E. hirae* (14.3%) were the most prevalent species (Figure 1). However, if we consider swine fecal isolates (n = 22) separately from other animal isolates, the most frequent species were *E. hirae* (26.3%; n=6), *E. faecium* (22.7%; n=5) and *E. cecorum* (18.2%; n=4) among other. The prevalence of enterococcal species isolated from foods (vegetable or animal) and from swine feces was significantly different ($p<0.01$).

Table 1 shows the MIC values according to enterococci species and origin (vegetable or animal). *E. faecalis* isolates of animal origin presented MIC_{50} of 64 µg/ml for streptomycin while those of vegetable origin presented of 16 µg/ml. *E. faecalis, E. faecium and E. casseliflavus* presented higher MIC_{50} for penicillin than the other species (Table 1). All non-clinical enterococci isolates studied were susceptible to ampicillin, penicillin, streptomycin and gentamicin by broth dilution and by disk diffusion method.

Based on the results of the disk diffusion method, it was observed that 33.3% (n = 28) of the non-clinical isolates were susceptible to all eleven antimicrobials tested. None of isolates of the *E. faecalis* and *E. gallinarum* species were susceptible to all antimicrobials tested, on the other hand, more than 80% of isolates of *E. durans* and *E. sulfureus* were susceptible to all antimicrobials tested. Overall, a variable percentage of isolates showed resistance to erythromycin (41.6%), norfloxacin (36.9%), tetracycline (11.9%) and ciprofloxacin (4.7%) (Table 2).

It was observed that 53.6% of enterococcal isolates produced hemolysins, but the β-hemolysin (40.5%) was more prevalent (Table 2). All the *E. gallinarum* (n = 4) and 92.85% (n

= 13) of the *E. casseliflavus* isolates studied had some type of hemolysis. Among the *E. faecalis* isolates, only 26.3% presented hemolysis.

Regarding the ability to hydrolyze gelatin, 20.2% (n = 17) of non-clinical enterococci isolates presented such virulence factor. The majority of the *E. faecalis* isolates of both vegetable (75.0%) and animal (54.5%) origin presented this virulence factor (Table 2). No isolate of the following species *E. hirae*, *E. durans*, *E. mundtii* or *E. gallinarum* produced gelatinase.

Among the isolates that produced α-hemolysin, none produced gelatinase and among those that did not have hemolysis, 30.8% produced gelatinase. Only two gelatinase producing isolates were susceptible to all antimicrobials tested.

Discussion

In this study, the most frequently isolated species were *E. faecalis, E. faecium, E. casseliflavus, E. hirae* and *E. durans*. Similarly, in Spain and the United Kingdom, *E. faecalis* and *E. faecium* were the most commonly isolated species in hospitals and the environment; in Denmark, however, *E. hirae* was the most commonly isolated species of slaughtered animals (Klein, 2003). In the study by Tejedor *et al.* (2001), the most isolated species in water samples in Spain was *E. faecalis*, followed by *E. faecium*, *E. hirae* and *E. durans*. Cassenego *et al.* (2011) found *E. faecalis* (33%) as the predominantly isolated species in broilers, followed by *E. faecium* (27%).

In food samples of plant origin, *E. casseliflavus* was the most frequently isolated species, similar to that reported by Klein (2003) who found predominantly *E. casseliflavus* and *E. mundtii* in this type of food. In this study, all isolates of *E. mundtii* were obtained from foods of vegetable origin. In contrast, Riboldi *et al.* (2009) identified *E. faecium* as the most abundant species in plant samples in southern Brazil.

In animal foods, *E. faecalis* and *E. faecium* were the most prevalent species, corroborating data from other studies. Riboldi *et al.* (2009) found *E. faecalis* as the most prevalent species in raw meat and colonial cheese and suggested that this finding could be explained by the fact that as these foods are handled by humans, contamination could occur during the manufacturing and handling process of these foods. Riboldi *et al.* (2009) also found *E. faecium* in processed milk, which may be explained by the ability of these microorganisms to survive during the pasteurization process. Klein (2003) also reported that *E. faecalis* and *E. faecium* are often isolated from animal-origin food samples.

E. faecalis is the most common species in humans, being isolated from 80% to 90% of enterococcal infections, and *E. faecium* is the second most common species, being found in 5 to 10% of infections (Murray, Baron, 2007). Other species of minor clinical importance are *E. avium, E. casseliflavus, E. durans, E. gallinarum, E. raffinosus, E. malodoratus* and *E. mundtii.* Other species are rarely isolated from biological samples from humans. The fact that species commonly found in clinical infections are similar to those found in animal- and vegetable-origin foods in the present study underscores the possibility of transmission of these microorganisms through the food chain, which makes it important to control exposure to antimicrobials in the environment, as whereas they can select resistant isolates making it difficult to treat infections.

Filsner *et al*. (2017) reported MIC_{50} of 2 µg/ml for penicillin in isolated animal-derived enterococci, similarly to the present study, and 4 µg/ml MIC90, lower than that observed in our study which was 8 µg/ml. For streptomycin, it was in the same study MIC50 and MIC90> 2,048 µg/ml, which are higher than the values found in our study. The low MIC of penicillin and aminoglycosides, exhibited by food-isolated enterococci in the present study, suggests that there is a low exposure to these antimicrobials in the production chain of the Uberaba-MG region.

The antimicrobials to which resistance was most erythromycin (41.6%), norfloxacin (36.9%), tetracycline (11.9%), ciprofloxacin (4.7%). In the studies by Riboldi et al. (2009) and Poeta et al. (2007) observed a large number of enterococci with resistance to erythromycin, tetracycline and ciprofloxacin. Pruksakorn *et al*. (2016), the antimicrobials for which the isolated pig enterococci presented higher resistance rate were tetracycline (86%) and erythromycin (61%).

In the present study, over 70% of the animal-origin *E. faecalis* and *E. faecium* isolates were resistant to erythromycin. Bogaard *et al*. (2000) associated the use of tylosin, an antibiotic commonly used in veterinary medicine, with increased erythromycin resistance rate. In enterococci of vegetable origin, the overall rate of resistance to erythromycin was lower than that of animal origin. However, the high rate of *E. faecalis* (87%) and *E. faecium* (50%) found in vegetables in this study suggests soil contamination, with human or animal feces, where vegetables are planted and harvested. Cooking foods of animal origin may inactivate the bacteria present, but vegetables that are eaten raw may be important vectors for the transmission of bacteria to humans. In addition, cross-contamination may occur when meat is cut without proper hygiene of the place where the vegetables were previously cut.

E. faecalis and *E. faecium* have natural resistance to various antimicrobial drugs and usually lower sensitivity to aminoglycosides and penicillin G, moderate sensitivity to ampicillin and chloramphenicol, and high sensitivity to glycopeptides. When resistant to glycopeptides, *Enterococcus* poses an epidemiological risk as treatment is difficult and genes can be transferred to other bacteria (Arthur, Courvalin, 1993).

The hemolytic activity of enterococci is generally related to the production of cytolysins, which are toxins capable of increasing the virulence of these microorganisms, especially in immunosuppressed patients. Another important virulence factor is gelatinase, which is an endopeptidase whose function is to hydrolyze gelatin, collagen, casein and other peptides,

which facilitates the dissemination of bacteria in the body and the evasion of the immune system by cleavage of complement components (Semedo *et al*., 2003)

In the present study, isolates of *E. faecalis* species had a higher prevalence of gelatinase compared to isolates of other species. Gomes *et al*. (2008) investigated gelatinase production by enterococci obtained from milk, plant and animal products, and reported that 18.2% of the isolates evaluated produced gelatinase, while Castro-Porto *et al*. (2016) detected virulence factor in a higher percentage (43%) of cheese isolates.

Among the isolates that produced α-hemolysin in the present study, none produced gelatinase and among those without hemolysis, 30.8% had such virulence factor. Only two gelatinase producing isolates were susceptible to all antimicrobials tested, as it makes relevant the actions to prevent human contamination by these isolates, since besides presenting virulence factors that aggravate the infection, they have antimicrobial resistance.

In conclusion, a variety of enterococci species may be found in environmental samples, although the species of major clinical importance in human medicine, such as *E. faecalis* and *E. faecium,* were also the most prevalent in these non-clinical sources, particularly in food of animal origin. In addition, although most non-clinical enterococci isolates were susceptible to the tested antimicrobials, resistant isolates showing virulence factors were detected, regardless of the sample origin.

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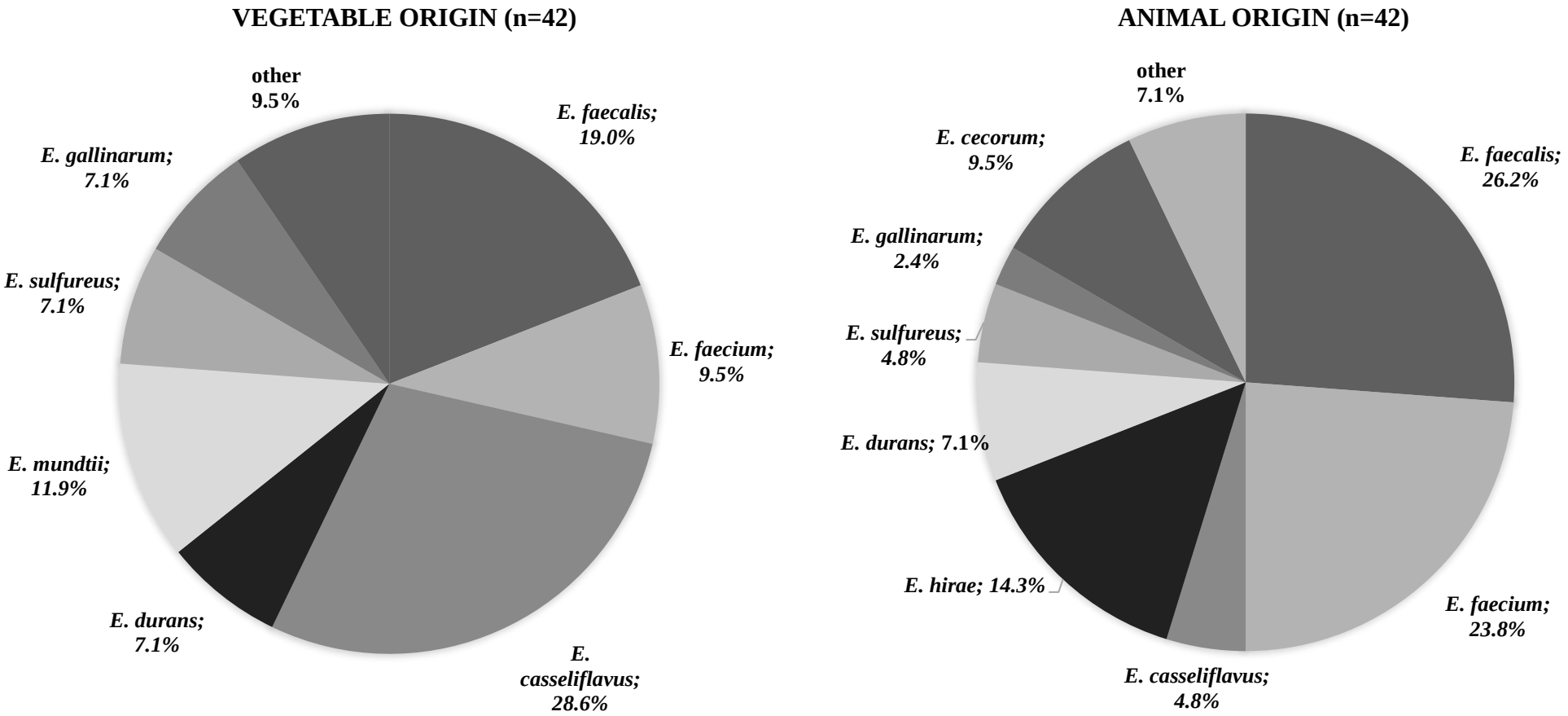

Figure 1. Species distribution of the *Enterococcus* isolates (n=84) from animal and vegetable origin in Brazil

Table 1. Values of minimal inhibitory concentration (MIC) of different antimicrobials to inhibit the growth of 50% (MIC_{50}) and 90% (MIC_{90}) of the non-clinical *Enterococcus* isolates of animal (n=42) and vegetable (n=42) origin

Origin	Species	MIC_{50} (µg/ml)					MIC_{90} (µg/ml)					MIC range (µg/ml)				
		AMP	PEN	VAN	STR	GEN	AMP	PEN	VAN	STR	GEN	AMP	PEN	VAN	STR	GEN
Animal	*E. faecalis*	0.5	4	2	64	32	0.5	8	4	128	128	0.5-1	1-8	2-4	32-256	4-128
	E. casseliflavus	0.5	4	4	32	4	0.5	4	8	64	32	0.5-0.5	4-4	4-8	32-64	4-32
	E. faecium	2	8	2	32	32	4	8	4	64	64	0.5-4	1-32	2-16	16-64	16-128
	E. hirae	0.5	1	2	16	4	2	2	2	32	16	0.5-2	1-2	2-2	8-32	4-16
	E. durans	0.5	1	2	8	4	0.5	1	2	16	8	0.5-0.5	1-1	2-2	4-16	4-8
	E. sulfureus	0.5	1	2	16	4	0.5	1	2	32	4	0.5-0.5	1-1	2-2	8-32	4-4
	E. gallinarum	0.5	1	4	16	64	0.5	1	4	16	64	0.5-0.5	1-1	4-4	16-16	64-64
	E. cecorum	0.5	1	2	16	4	0.5	1	2	32	4	0.5-0.5	1-1	2-2	8-32	4-4
	Other*	0.5	1	2	16	8	0.5	8	4	32	32	0.5-0.5	1-1	2-4	8-32	4-32
	All	0.5	2	2	32	16	2	8	4	128	64	0.5-4	1-32	2-16	4-256	4-128

Vegetable	*E. faecalis*	0.5	2	2	16	32	0,5	8	4	128	128	0.5-0.5	1-8	2-4	16-128	4-128
	E. casseliflavus	0.5	4	8	32	8	2	4	8	128	128	0.5-4	1-8	4-8	8-256	4-128
	E. faecium	0.5	8	4	16	32	0.5	8	16	128	64	0.5-0.5	4-8	2-8	16-128	16-64
	E. durans	0.5	1	2	16	4	2	1	2	32	16	0.5-2	1-1	2-2	8-32	4-16
	E. mundtii	0.5	2	4	16	32	0.5	4	8	16	64	0.5-0.5	1-4	2-8	8-128	4-64
	E. sulfureus	0.5	1	2	16	8	0.5	1	4	32	16	0.5-0.5	1-1	2-4	16-32	4-64
	E. gallinarum	0.5	1	4	64	4	0.5	8	8	64	32	0.5-0.5	1-8	4-8	8-64	4-32
	Other*	0.5	1	2	16	4	4	2	16	64	16	0.5-4	1-2	2-4	8-64	4-16
	All	0.5	2	4	32	16	0.5	8	8	128	64	0.5-4	1-8	2-8	8-256	4-128
General		0.5	2	2	32	16	2	8	8	128	64	0.5-4	1-32	2-16	4-256	4-128

**E. dispar*, *E. avium*, *E. raffinosus*, and or *E. solitarius*.

AMP, ampicillin; PEN, penicillin; VAN, vancomycin; STR, streptomycin; GEN, gentamicin.

Table 2. Antimicrobials resistance profile and virulence factors (hemolysins and gelatinase) of the non-clinical *Enterococcus* isolates of animal (n=42) and vegetable (n=42) origin

Origin	Species	Total of isolates	Number (%) of isolates						
			Producer of			Resistant to			
			α-Hemolysis	β- Hemolysis	Gelatinase	NOR	CIP	ERY	TET
Animal	*E. faecalis*	11	1 (9.1)	3 (27.3)	6 (54.5)	7 (63.6)	1 (9.1)	8 (72.7)	5 (45.45)
	E. faecium	10	2 (20.0)	4 (40.0)	1 (10.0)	2 (20.0)	1 (10.0)	7 (70.0)	1 (10.0)
	E. casseliflavus	2	1 (50.0)	1 (50.0)	0	1 (50.0)	0	1 (50.0)	0
	E. hirae	6	1 (16.6)	2 (33.3)	0	0	0	2 (33.3)	1 (16.6)
	E. durans	3	0	1 (33.3)	0	0	0	1 (33.3)	0
	E. mundtii	0	0	0	0	0	0	0	0
	E. sulfureus	2	0	1 (50.0)	0	0	0	0	0
	E. gallinarum	1	0	1 (100.0)	0	1 (100.0)	0	0	0
	E. cecorum	4	1 (25.0)	1 (25.0)	1 (25.0)	0	0	1 (25.0)	1 (25.0)
	Other*	3	0	1 (33.3)	0	0	0	1 (33.3)	0

	All	42	6 (14.3)	15 (35.7)	8 (19.0)	11 (26.2)	2 (4.7)	21 (50.0)	8 (19.0)
Vegetal	*E. faecalis*	8	0	3 (37.5)	6 (75.0)	5 (62.5)	0	7 (87.5)	1 (12.5)
	E. faecium	4	0	1 (25.0)	0	2 (50.0)	0	2 (50.0)	0
	E. casseliflavus	12	2 (16.6)	9 (75.0)	1 (8.3)	7 (58.3)	1 (8.3)	2 (16.6)	0
	E. hirae	0	0	0	0	0	0	0	0
	E. durans	3	0	0	0	0	0	0	0
	E. mundtii	5	1 (20.0)	2 (40.0)	0	3 (60.0)	0	1 (20.0)	0
	E. sulfureus	3	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	0	1 (33.3)	0
	E. gallinarum	3	1 (33.3)	2 (66.6)	0	1 (33.3)	1 (33.3)	1 (33.3)	0
	E. cecorum	0	0	0	0	0	0	0	0
	Other*	4	0	3 (75.0)	1 (25.0)	1 (25.0)	0	0	1 (25.0)
	All	42	5 (11.9)	19 (45.2)	9 (21.4)	20 (47.6)	2 (4.7)	14 (33.3)	2
Total		84	11 (13.1)	34 (40.5)	17 (20.2)	31 (36.9)	4 (4.7)	35 (41.6)	10 (11.9)

**E. dispar, E. avium, E. raffinosus,* and or *E. solitarius*.

NOR, norfloxacin; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline.

ARTIGO 2

Enterococcus faecalis of hospital and environmental origin isolated in Brazil: antimicrobial susceptibility profile, virulence factors and molecular characterization

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Abstract

In the last decades, enterococci have become one of the leading agents of hospital infections worldwide. The intrinsic resistance to different classes of antimicrobials and the ability to acquire resistance to other classes favors the survival in the hospital environment, makes treatment difficult and contributes to the increase of the number of infections by these microorganisms in humans, being the *Enterococcus faecalis* the enterococcal species most prevalent. The present study aimed to investigate whether *E. faecalis* isolates showing that unusual resistance phenotype are disseminated in the environment and to compare their molecular and phenotypic characteristics with those isolates from hospital origin. Those isolates were recovered from food (n = 18), animal feces (n = 24), water (n = 28) and sewage (n = 31). Antimicrobial susceptibility testing and virulence factors (hemolysins and gelatinase) of environmental isolates (n = 101) were performed, where the results were compared with clinical isolates (n = 145). Some environmental isolates (n = 31) were selected for molecular tests: PFGE and HLGR gene detection. No isolate recovered from food showed MDR phenotype but 52.2% (n = 12) isolates from animal feces and 18.6% (n = 11) of water and sewage were MDR. Among clinical isolates, 29.9% (n = 23) and 86.8% (n = 59) of those penicillin-resistant and penicillin-susceptible (PRASEF). Three isolates with this profile PRASEF were found in hospital sewage samples. Those isolates were classified into 19 pulsotypes (A to S) and among these only 6 pulsotypes were represented by more of an isolate. The findings of this study demonstrates that E. faecalis isolates showing resistance to diverse antimicrobial including the HLGR and virulence factors are widespread in many non-clinical sources and may represent health risks to people who have contact with these sources. However, *E. faecalis* isolates showing the unusual phenotype of penicillin G resistance but ampicillin susceptibility seems to be restricted to the hospital environment only.

Introduction

In the last decades, enterococci have become one of the leading agents of hospital infections worldwide. The intrinsic resistance to different classes of antimicrobials and the ability to acquire resistance to other classes favors the survival in the hospital environment, makes treatment difficult and contributes to the increase of the number of infections by these microorganisms in humans, being the *Enterococcus faecalis* the enterococcal species most prevalent (Comerlato et al. 2013; Upadhyaya, Umapathy, and Ravikumar 2010).

In addition to human clinical samples, enterococci have been isolated from a variety of food (animal and vegetable origin) and environmental (water, soil, sewage) sources (Gomes et al. 2008; Getachew et al. 2013; Ben Said et al. 2016; Tamang et al. 2017). Enterococci isolates showing resistance to multiple antimicrobials are not common in food in contrast to those found in hospitals (Foulquié Moreno et al. 2006; Camargo et al. 2014)

The acquired resistance phenotypes considered clinically most significant among enterococci are those associated with high-level resistance to aminoglycosides and resistance to glycopeptides and penicillins (Hollenbeck and Rice, 2012). An unusual phenotype of penicillin G resistance but ampicillin susceptibility was observed in *E. faecalis* isolates from hospitals in several countries, including Brazil (Metzidie et al. 2006; Conceição et al. 2014; Guardabassi et al. 2010), evidencing the evolution capacity of these microorganisms and the lack of knowledge regarding their mechanisms of antimicrobial resistance and genetic diversity.

To date, no study has reported penicillin-resistant, ampicillin-susceptible *E. faecalis* (PRASEF) isolates in non-clinical samples. Therefore, the present study aimed to investigate whether *E. faecalis* isolates showing that unusual resistance phenotype are disseminated in the environment and to compare their molecular and phenotypic characteristics with those isolates from hospital origin.

Materials and methods

Bacterial isolates and species identification

Environmental samples were obtained from foods of vegetable and animal origin, animal feces, water and sewage. The solid samples were ground and 10 g were added in 100 ml of 0.1% (wt/vol) peptone water. For the liquid samples, 2 ml were added in 18 ml of peptone water. The animal feces (1 g) was added in 10 ml of peptone water. Thereafter, 1 ml of the homogenates was withdrawn in up to 5 serial dilutions (10^{-1} to 10^{-5}). Thus, 1 ml of each dilution was added to plates containing KF *Streptococcus* agar, which were incubated at 37^{o}C for 48 h. The water samples were incubated at 37°C, from 8 h to 12 h, before plated on KF *Streptococcus* agar (Silva et al. 2010).

Three to five colonies with typical characteristics of enterococci of each environmental sample were transferred to a selective medium for *E. faecalis* (Brain Heart Infusion-BHI agar containing 0.04% telluride) and incubated at 37°C for 24-48 h. The black non-catalase producing colonies were identified. *Enterococcus* genus was identified based on Gram staining, cellular morphology, hydrolysis of L-pyrrolidonyl-β-naphthylamide (PYR test), bile-esculin test, and tolerance to 6.5% NaCl. Species identification was determined based on tests of carbohydrate fermentation, arginine hydrolysis, mobility, yellow pigment production and growth in 0.04% tellurite (Teixeira et al. 2011)

Clinical isolates of *E. faecalis*

Of a collection of 317 non-repetitive *E. faecalis* isolates recovered from hospitalized patients at a Brazilian tertiary hospital in the period of 2006-2015 (Conceição et al., 2014; Costa et al. 2019)., 145 isolates of *E. faecalis* were randomly selected based on their penicillin susceptibility profile. Of those isolates, 68 were PRASEF and 77 were penicillin- and ampicillin-susceptible (PSASEF). The isolates were recovered from various clinical samples

such as urine (55; 30.9%), wounds (41; 23.0%) and body fluids (blood, pleural fluid, cerebrospinal fluid, and peritoneal fluid) (36; 20.2%) among other. All the isolates were preserved at -70°C in BHI broth containing 20% glycerol.

Susceptibility testing

The susceptibility profile was determined by the disk diffusion method using the following antimicrobials: ampicillin (10 µg), penicillin (10 µg), streptomycin (300 µg), vancomycin (30 µg), gentamicin (120 µg), erythromycin (5 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg) and teicoplanin (30 µg).

The broth dilution method was used to determine the minimum inhibitory concentration (MIC) of the most important therapeutic antimicrobials belonging to the following classes: beta-lactams (ampicillin and penicillin), glycopeptides (vancomycin) and aminoglycosides (gentamicin and streptomycin). The value of CIM_{50} and CIM_{90}, which killed or inhibited the growth of 50% and 90% of the isolates studied, respectively, for each of the antimicrobials was also determined.

All the susceptibility tests were performed and interpreted according to the Clinical and Laboratory Standards Institute (Clinical & Laboratory Standards Institute 2018, 2019) guidelines. Quality-control testing was performed using *Staphylococcus aureus* ATCC (American Type Culture Collection) 25923, *S. aureus* ATCC 29213, *E. faecalis* ATCC® 29212 and *E. faecalis* ATCC® 51299. MIC values ≥16 µg/mL, ≥32 µg/mL, ≥500 µg/ mL and ≥1,000 µg/mL indicated resistance to penicillins, vancomycin, gentamicin and streptomycin, respectively. Isolates with intermediate profile were considered resistant in this study.

The isolates were defined as multi-drug resistant (MDR) if they exhibited acquired resistance to at least one representative agent in three or more different classes of antimicrobial in addition to gentamicin or streptomycin (Costa et al. 2019).

Virulence factors

The production of the enzymes gelatinase, α-hemolysin and β-hemolysin was detected phenotypically. For detection of gelatinase, the isolates of enterococci were incubated at 36ºC for 72 h in BHI broth containing 12% (wt/vol) of gelatin. After that, the culture was refrigerated at 4°C for 30 min. The persistence of the presence of liquid medium after the refrigeration period indicated the production of gelatinase (hydrolysis of gelatin) (Cruz and Torres 2012). For the detection of hemolysin production, the isolates were cultured on BHI agar supplemented with 5% (vol/vol) defibrinated sheep blood. After incubation for 48h at 36ºC, the formation of halos around the colony was verified; green halos indicated α-hemolysis, clear halos indicated β-hemolysis and no halo formation indicated γ-hemolysis (Castro-Porto *et al.*, 2016).

Confirmation of species by polymerase chain reaction (PCR)

The phenotypic species identification was confirmed by PCR using specific primers as described elsewhere (Dutka-Malen, Evers, and Courvalin 1995). Bacterial DNA from the *E. faecalis* isolates and the references strains (*E. faecalis* ATCC 29212 and ATCC 51299) was extracted using the QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A fragment of 941 bp of the *ddIE* gene were amplified using the GeneAmp PCR System 9700 (Applied Biosystems, USA) thermocycler, in steps: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 52°C for 1 min and 72°C for 1 min; and final extension at 72°C for 7 min. PCR amplification products were separated by 1.5% agarose gel electrophoresis and visualized after ethidium bromide staining under UV light.

Detection of HLGR (high-level resistance to gentamicin) by PCR

The *aac(6')-Ie-aph(2")-Ia* gene was amplified by PCR using two pairs of specific primers that generated amplicons of 369 and 348 bp as described previously (Vakulenko et al. 2003).

Pulsed field gel electrophoresis (PFGE)

Genetic characterization of the clinical and non-clinical isolates was performed by the pulsed field gel electrophoresis (PFGE) methodology. The modified protocol used for this characterization is based on Zanella et al. (1999).

After cultivation, bacteria were washed with PIV buffer {10 mM TRIS (Sigma), 1M NaCl (Sigma), pH 8.0}, where 300 µL of 1.8% Low Melting Agarose (SeaKem Gold) was added to the same volume as PIV. After solidification of the plugs, they were transferred to tubes containing 1mL EC lysis buffer (6mM Tris, 1M NaCl, 100 mM EDTA, 0.2% odium Deoxycholate, N-laurylarcosine (Sigma), pH 8}, plus RNAse to a final concentration of 50 µg/mL, Lysozyme to a final concentration of 100 µg/mL, Mutanolizine of 12 U/µl and Hyaluronidase at 96 µg/µl and incubated at 37°C for 4-6 hours. After this incubation period the EC buffer was removed and 1 mL of ES solution {EDTA, N-laurylarcosine, pH 9.0} plus Proteinase K to a final concentration of 1 mg/ml (Invitrogen Tech-Line, USA) was added and the discs were incubated at 50ºC for 18-24h. After this period, the plugs were washed three times for 30 minutes (each) with TE buffer {10 mM TRIS, pH 7.5, 1Mm EDTA, pH 8.0} and kept in TE buffer solution under refrigeration.

Approximately 2 mm of the plug was cut and equilibrated with 45 µL of 1X restriction enzyme buffer (6 mM TRIS, pH 8, 20 mM KCl, 6 mM $MgCl_2$) for at least 30 minutes at room temperature. After removal of this buffer, 45 µL of the same buffer plus SmaI restriction

enzyme (SIGMA) at the final concentration of 0.6 U/µL was added. This reaction was kept at 25ºC for at least 4h for DNA digestion.

Separation of DNA fragments was performed by 1% PFGE (Sigma) agarose gel electrophoresis in 0.5X TBE buffer (0.89 M TRIS, 0.89 M boric acid; 0.25 M EDTA}. The Lambda S340 molecular weight standard (New England, Biolabs) was used for band comparison. The electrophoretic run was performed on the CHEF-DR II system (Bio-Rad Laboratories, Richmond, CA, USA) and under the following conditions: 200 volt voltage, temperature 14ºC, start time 5s, end time 35s, run time from 24 h. After the run, the gel was stained in 1% ethidium bromide solution for 30 minutes, followed by discoloration and image capture was performed by DNR Bio-Imaging Systems (Mini BIS Pro).

Genetic profile analysis

Analysis of genetic profiles was performed using the BioNumerics program (Version 5.0; Applied Maths NV, Keistraat 120, 9830 Sint-Martens-Latem, Belgium). For this analysis, we applied the Dice coefficient and the band matching method (UPGMA) to generate the dendogram of genetic relationships between the profiles, comparing them with each other. A 1.5% tolerance and 0.5% optimization and a similarity coefficient of 80% were used for this analysis (Morrison et al. 1999). Different PFGE types were designated by capital letters and subtypes by numbers.

Multi-locus sequencing typing (MLST)

PRASEF non-clinical isolates were submitted to MLST analysis. The MLST was performed using seven conserved housekeeping genes (gdh, gyd, pstS, gki, aroE, xpt, and yqiL) (Ruiz-Garbajosa et al. 2006). Those genes were amplified and sequenced as described in the MLST Database (http://efaecalis.mlst.net/). Each gene was amplified by PCR in a GeneAmp

PCR System 9700 (Applied Biosystems, USA) thermocycler, programmed in steps: 94°C for 5 min; 30 cycles of 94°C for 30 seconds, 52°C for 1 min and 72°C for 1 min; and final extension at 72°C for 7 min. The products obtained by PCR were purified using a commercial purification kit (Wizard Genomic DNA Purification, Promega) and directly sequenced on the automated sequencer (ABI PRISM 3730, Applied Biosystems) using the ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Ready Reaction Kit. The phylogenetic relationships among closely related STs were determined using the eBURST V3 algorithm (http://eburst.mlst.net/).

Results

Overall, 280 environmental samples were collected. Enterococcal suggestive colonies were observed for 209 samples (Figure 1). Representative colonies of each sample were transferred to a selective medium for *E. faecalis*. Among those, 121 were confirmed as *E. faecalis* species but only one *E. faecalis* isolate per environmental sample were included in this study, totalizing 101 isolates. Those isolates were recovered from food (n = 18), animal feces (n = 24), water (n = 28) and sewage (n = 31).

Table 1 demonstrates the rates of resistance to the antimicrobials according to the source of the *E. faecalis* isolate and Table 2 demonstrates the MIC values for the most important therapeutic antimicrobials. Only three PRASEF isolates were recovered from environment. All of them were recovered from hospital sewage (n= 3/24; 12,5%). High resistance rates were observed to erythromycin, tetracycline, ciprofloxacin, and norfloxacin mainly for those isolates recovered from animal feces. Note that all isolates from chicken (n = 10) were resistant to tetracycline and erythromycin. Moreover, 60% (6 of 10) of the isolates from animal feces showing resistance to chloramphenicol were recovered from swines.

PRASEF isolates have been shown to be more resistant to other antimicrobial classes than clinicians sensitive to penicillin, food, animal feces, water and sewage, except for

glycopeptides and streptomycin, in contrast to 89% of PRASEF isolates, are resistant to gentamicin (figure 2).

No isolate recovered from food showed MDR phenotype but 52.2% (n = 12) isolates from animal feces and 18.6% (n = 11) of water and sewage were MDR. Among clinical isolates, 29.9% (n = 23) and 86.8% (n = 59) of those penicillin-susceptible and penicillin-resistant, respectively, were MDR (Table 1). Only 4.1% (n = 6) of clinical isolates and 21.8% (n = 22) of environmental isolates were susceptible to all tested antimicrobials.

Among the sewage isolates (n = 31), 32.3% (n = 10) were classified as MDR, of these (n = 8/24) they were isolated from the hospital sewage and (n = 2/7), sewage from the treatment station of the city of Uberaba-MG.

The MIC_{50} and MIC_{90} for vancomycin were higher for the isolates from sewage. Clinical PRASEF isolates had higher MICs for gentamicin, in contrast to the streptomycin (Table 2).

Overall, the rate of clinical (55.8%; n = 81) and environmental (50.5%; n = 51) isolates that were gelatinase producers was similar; however, among the environmental isolates, a higher rate was observed among the isolates recovered from foods (Table 1). There was no significant difference in the presence of gelatinase according to the antibiotic resistance profile.

It was observed a higher frequency of β-hemolysin than α-hemolysin among the environmental *E. faecalis* isolates (Table 1). Among clinical isolates, only 2.8% (n = 4) showed β-hemolysin. Among the environmental isolates, 75% (n = 6) of those recovered of food from vegetable origin showed absence of hemolysis.

Among the 3 PRASEF isolates recovered from sewage, 2 produced β-hemolysis, while among the 68 clinical PRASEF isolates no one present hemolysis. Considering the MDR phenotype, 16.2% (n = 17) presented β-hemolysin or α-hemolysin, whereas 37.6% (n = 53) of those that were no MDR showed this virulence factor.

Thirty-one representative isolates recovered from different sources and showing various antimicrobial susceptibility profiles were subjected to PFGE analysis. Those isolates were classified into 19 pulsotypes (A to S) and among these only 6 pulsotypes were represented by more of an isolate (Figure 3).

Pulsotype A, with 85.1% similarity, is represented by 7 (22.6%) isolates and consists of 4 subtypes (A1-4), of which A1, A3 and A4 have two identical isolates each. In this pulsotype, 4 isolates were recovered from clinical samples, two in 2006 and two in 2008. The other 3 isolates were recovered in 2017 from the hospital sewage.

The pulsotype O, with 82.6% similarity, is represented by 3 similar but not identical isolates called O1, O2 and O3. The pulsotypes denominated D, P, Q and R presented 2 subtypes with one isolate each (D1-2, P1-2, Q1-2, and R1-2).

One of the 3 PRASEF isolates recovered from sewage was selected for MLST analysis. This isolate was resolved into the ST 524, which belong to CC9.

Discussion

Enterococci survive in a variety of temperature, salinity and ecological conditions and can be found in animals, vegetables, water and soil in addition to human clinical specimens (Pissetti et al. 2018; Beshiru et al. 2017). In the present study, *E. faecalis* isolates were found in animal and plant foods, in water and sewage, and droppings of cattle, swine and poultry.

Several studies with animal samples showed a high prevalence of isolates resistant to erythromycin, tetracycline and ciprofloxacin (Tejedor et al. 2001; Poeta et al. 2007; Riboldi et al. 2009; Pruksakorn et al. 2016). High rate of resistance to erythromycin among animal isolates and animal-origin foods may be related to the use of tylosin in veterinary medicine (Bogaard 2002). In Brazil, tetracycline and ciprofloxacin are prohibited as growth promoters but a study

conducted by the Paraná State Department of Health (SESA) showed that these antimicrobials, together with penicillin and norfloxacin, were been used irregularly (SESA, 2005).

Some antimicrobials used in veterinary medicine are not completely metabolized in the animal organism, being excreted in the animals' urine or feces (Sarmah, Meyer, and Boxall 2006; Kemper 2008). These residues can accumulate in the soil and be transported to water bodies or absorbed in water plant tissues, resulting in a risk to human health in the consumption of water or vegetables used as food (Boxall et al. 2006; Regitano and Leal 2010).

Notably, despite the several environmental *E. faecalis* isolates exhibiting resistance to various antimicrobials, only 3 isolates showing resistance to penicillin were found although all of them were recovered from the hospital sewage. To date, these uncommon PRASEF isolates were found only in human clinical samples (Conceição et al. 2014; Gawryszewska, Hryniewicz, and Sadowy 2012; Guardabassi et al. 2010; Metzidie et al. 2006). The presence of these isolates in environmental sources although rare is worrying as there is the possibility of hospital sewage contaminates river water and the environs. However, the survival capacity of these isolates in the environment needs to be further investigated. The MLST analysis revealed that the hospital sewage isolate belonged to CC9 that is the same CC of the PRASEF isolates recovered from patients who were also hospitalized in the period from 2006-2010 in the hospital of the present study (Conceição et al., 2014).

Important virulence factors such as gelatinase and hemolysis were present in non-clinical isolates in this and other studies (Medeiros et al. 2014; Igbinosa and Beshiru 2019; Molechan et al. 2019). There was no significant difference for gelatinase production among *E. faecalis* isolates according to their isolation source. Gelatinase is a protease capable of cleaving hemoglobulin, insulin, casein, collagen, gelatin and fibrinogen, as well as numerous peptides and proteins, becoming an important virulence factor, where there are reports that the presence

of enterococcus with gelatinase contributes to endocarditis. in animal models (Upadhyaya, Umapathy, and Ravikumar 2010).

Hemolysin is a cytolytic protein able to lyse erythrocytes; isolates producing this virulence factor are associated with increased severity of infections (Chow et al. 1993). In the present study, the hemolysins production was higher among environmental isolates than in clinical isolates. Igbinosa and Beshiru (2019) showed hemolysis in 36.2% of the isolates recovered from shrimps and (Upadhyaya, Umapathy, and Ravikumar (2010) found this virulence factor in 16.5% of clinical isolates.

The HLGR gene (*aac(6')-Ie-aph(2'')*-Ia) were found in different environmental isolates recovered from different sources (water; bacon; chicken; sausage; potato; sewage; beef, pork and chicken feces) in this study in addition to clinical isolate. Similarly, HLGR gene was found in *E. faecalis* isolates recovered from birds in Malaysia (Chan et al. 2008), from swine and wastewater (Freitas et al. 2009), from dog and cat urine (Marques et al. 2018) showing that this gene are widespread in environmental *E. faecalis* isolates.

A great genetic diversity was observed among the *E. faecalis* isolates of environmental origin while among the clinical and hospital sewage isolates, the genetic variability was smaller according to the PFGE analysis. Among non-clinical isolates, there were 15 pulsotypes and no clones but there was similarity between isolates of bacon, cabbage and beet (O1-O3) and corn and water (P1 and P2), two of lettuce (Q1 and Q2), and swine feces and water (R1 and R2). In a recent study, PFGE of 11 *E. faecalis* isolates recovered from chickens found 3 pulsotypes (Aslantaş 2019).

In other study with 28 clinical isolates, 13 pulsotypes were found but among the PRASEF isolates (n = 17) there were only 3 pulsotypes and 1 clone represented by 11 isolates, whereas among those PSASEF isolates there were 10 pulsotypes (Conceição et al. 2014). Among the three PRASEF isolates found in the hospital sewage of the present study, there were

2 pulsotypes (A2 and A4), which were compared to isolates from the study by Conceição et al (2014), where there was 85.1% genetic similarity between them but there were no clones.

Conclusion

The findings of this study demonstrates that *E. faecalis* isolates showing resistance to diverse antimicrobial including the HLGR and virulence factors are widespread in many non-clinical sources and may represent health risks to people who have contact with these sources. However, *E. faecalis* isolates showing the unusual phenotype of penicillin G resistance but ampicillin susceptibility seems to be restricted to the hospital environment only.

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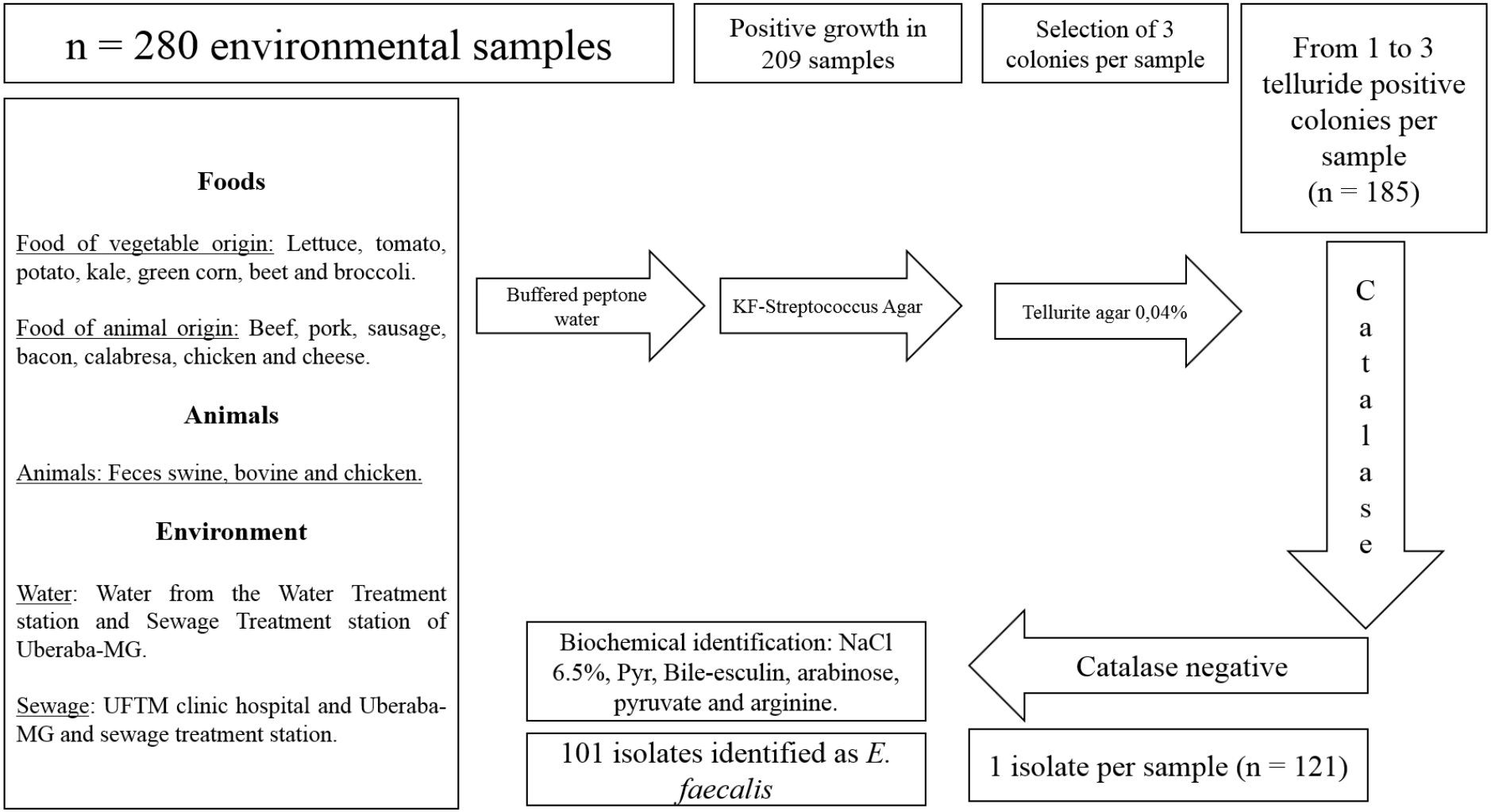


Figure 1. Flowchart for collection and identification of *Enterococcus faecalis* isolates from environmental samples

Table 1. Antimicrobial resistance profile and virulence factors of the environmental and clinical *Enterococcus faecalis* isolates

Source[a]	Isolates (n)	Percentage of isolates														
		Producers of		Resistant to[b]												
		α-hemolysin	β-hemolysin	Gelatinase	AMP	PEN	GEN	STR	VAN	TEI	NOR	CIP	CHL	ERY	TET	MDR
Food	19	5.3	26.3	63.2	0	0	0	0	0	0	68.5	5.3	0	78.9	26.3	0
Animal faeces	23	17.4	56.5	39.1	0	0	26.1	52.2	4.3	4.3	60.9	39.1	43.5	91.3	87.0	52.2
Water and Sewage	59	5.1	67.8	50.8	0	5.1	13.6	27.1	6.8	0	23.7	20.3	18.6	57.6	35.6	18.6
Clínical (S)	77	0	5.2	53.2	0	0	24.7	33.8	0	2.6	66.2	64.9	27.3	74.0	50.6	29.9
Clínical (R)	68	0	0	58.8	0	100.0	86.8	11.8	0	0	95.6	95.6	86.8	98.5	89.7	86.8

[a]Clinical (S), clinical isolates penicillin-susceptible; Clinical (R), clinical isolates penicillin-resistant.

[b]AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NOR, norfloxacin; PEN, penicillin; STR, streptomycin; TEI, teicoplanin; TET, tetracycline; Van, vancomycin.

[c]MDR, multi-drug resistance.

Table 2. Minimum inhibitory concentration (MIC) values of the most clinically important antimicrobials to inhibit the growth of 50% (MIC_{50}) and 90% (MIC_{90}) of the environmental and clinical *Enterococcus faecalis* isolates

Source	MIC_{50} (µg/ml)					MIC_{90} (µg/ml)					MIC range (µg/ml)				
	AMP	PEN	VAN	STR	GEN	AMP	PEN	VAN	STR	GEN	AMP	PEN	VAN	STR	GEN
Food (Vegetable)	0,5	1	1	32	32	8	8	8	128	128	0,5-8	1-8	1-8	16-128	8-128
Food (Animal)	4	4	1	64	32	8	8	8	128	128	0,5-8	1-8	1-8	32-250	8-128
Animal faeces	4	4	1	250	8	4	4	8	2,000	500	0,5-8	1-8	1-8	64-2,000	4-1,000
Water	0,5	1	1	128	4	4	4	8	1,000	128	0,5-4	1-4	1-8	16-1,000	4-500
Sewage	4	4	8	128	8	8	8	8	1,000	1,000	0,5-16	1-32	<-16	64-2,000	4-2,000
Clinical (S)	2	4	4	128	62.5	2	8	4	4,000	2,000	0,5-4	1-8	1-8	128-4,000	62.5-2,000
Clinical (R)	4	16	4	128	2,000	8	32	4	1,000	2,000	0,5-8	16-32	1-8	128-4,000	62.5-2,000

Clinical (S), clinical isolates penicillin-susceptible; Clinical (R), clinical isolates penicillin-resistant.

AMP, ampicillin; PEN, penicillin; Van, vancomycin; STR, streptomycin; GEN, gentamicin;

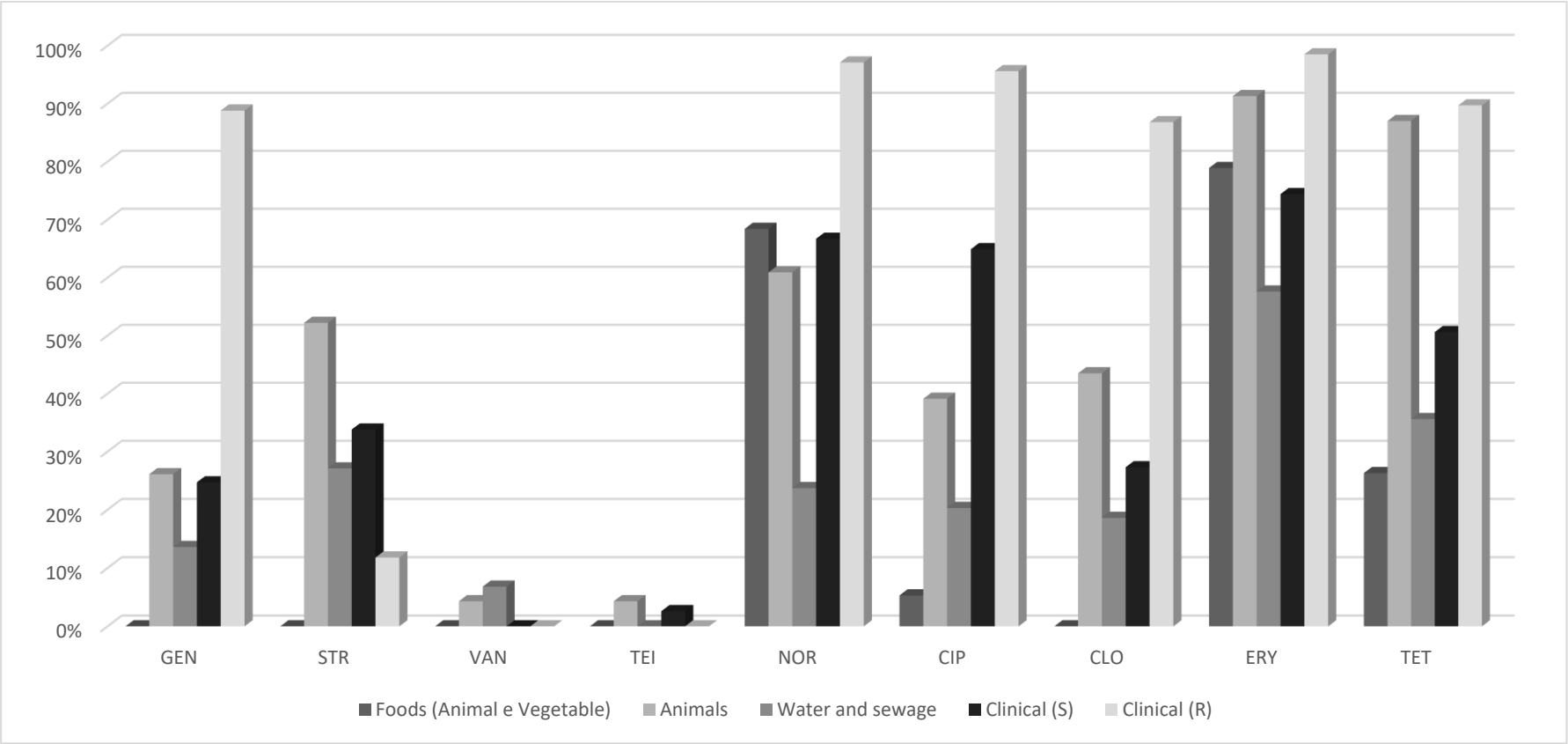
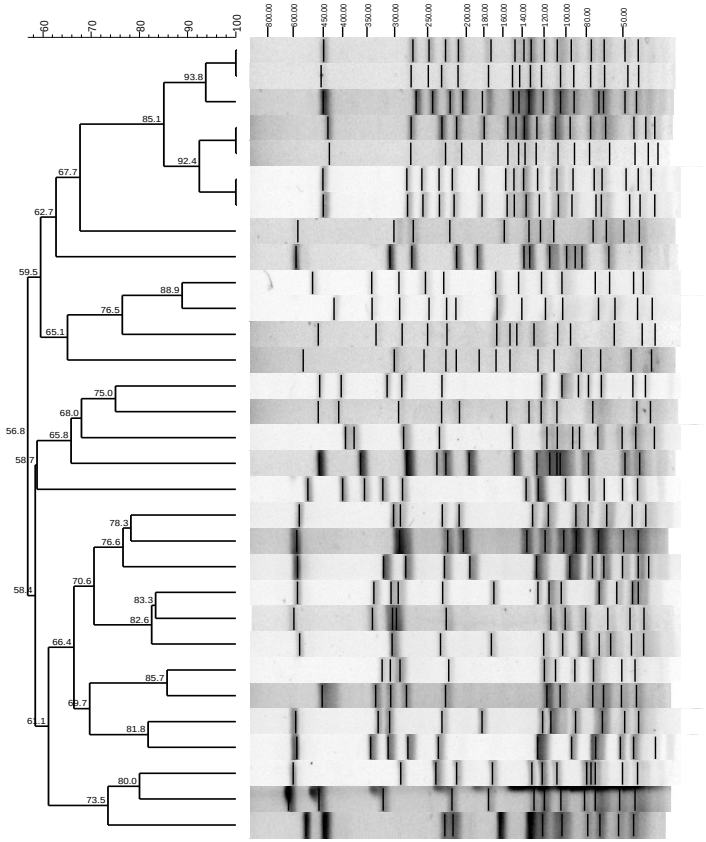


Figure 2. Comparison of the antimicrobial susceptibility profile of the *Enterococcus faecalis* isolates recovered from environmental and clinical samples. Clinical (S), clinical isolates penicillin-susceptible; Clinical (R), clinical isolates penicillin-resistant; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NOR, norfloxacin; PEN, penicillin; STR, streptomycin; TEI, teicoplanin; TET, tetracycline; Van, vancomycin.



Isolate	Source	Isolate Date	Resistance pattern	HLGR gene	Pulsotype
H20	Clinical Infection (urine)	20/03/06	Chl, Cip, Ery, Gen, Nor, Pen, Tet	+	A1
H234	Clinical Infection (wound)	03/09/08	Chl, Ery, Gen, Pen	+	A1
#68	Sewage (Hospital)	12/09/17	Chl, Cip, Gen, Nor, Pen, Tet	+	A2
H38	Clinical Infection (wound)	18/05/06	Chl, Cip, Ery, Gen, Nor, Pen, Str, Tet	+	A3
H253	Clinical Infection (blood)	15/10/08	Chl, Cip, Ery, Gen, Nor, Pen, Tet	+	A3
#34	Sewage (Hospital)	27/07/17	Chl, Cip, Ery, Gen, Nor, Pen, Tet	+	A4
#42	Sewage (Hospital)	15/08/17	Chl, Cip, Ery, Gen, Nor, Pen, Tet	+	A4
#85	Water (WTP)	26/09/17	Cip, Ery, Nor, Tet	+	B
H221	Clinical Infection (urine)	07/07/08	Cip, Ery, Gen, Nor, Str, Tet	+	C
#43	Sewage (Hospital)	18/08/17	Chl, Cip, Ery, Gen, Nor, Str	+	D1
#45	Sewage (Hospital)	23/08/17	Chl, Cip, Ery, Gen, Nor, Str, Tet	+	D2
#72	Water (WTP)	20/09/17	Chl, Cip, Ery, Gen, Nor, Tet	+	E
#94	Faeces (chicken)	14/12/17	Ery, Nor, Str, Tet	-	F
#7	Sausage	13/04/16	Nor	-	G
#77	Faeces (catlle)	18/11/17	Chl, Cip, Ery, Gen, Nor, Str, Tet	+	H
#6	Lettuce	12/04/16	Ery, Nor	-	I
#98	Faeces (chicken)	15/12/17	Ery, Nor, Tet	-	J
#13	Meat (swine)	30/04/16	Ery, Tet	-	K
#2	Potato	25/03/16	Ery, Nor	+	L
#56	Sewage (STP)	30/09/17	Ery, Str, Tet	+	M
#3	Meat (swine)	22/03/16	Nor	-	N
#9	Bacon	14/04/16	Ery, Tet	+	O1
#5	Cabbage	11/04/16	Ery, Nor	-	O2
#1	Beet	22/03/16	Ery, Nor	-	O3
#8	Corn	13/04/16	Ery	-	P1
#57	Water (STP)	17/10/17	Ery, Str, Tet	-	P2
#4	Lettuce	11/04/16	Ery, Nor	-	Q1
#12	Lettuce	15/04/16	Ery	-	Q2
#21	Faeces (swine)	15/05/17	Chl, Cip, Ery, Gen, Nor, Tet	+	R1
#57	Water (STP)	10/10/17	Ery, Str, Tet	-	R2
#48	Sewage (Hospital)	03/09/17	Chl, Cip, Ery, Gen, Nor, Tet	+	S

Figure 3. Pulsed-field gel electrophoresis (PFGE) dendrogram of SmaI-digested DNA of environmental and clinical *Enterococcus faecalis* isolates. The PFGE types are represented by uppercase letters.

IV - CONSIDERAÇÕES FINAIS

Uma diversidade de espécies do gênero *Enterococcus* foram encontradas no meio ambiente, no entanto, as espécies mais prevalentes no ambiente hospitalar, *E. faecalis e E. faecium*, também foram as mais frequentemente isoladas de fontes não clínicas.

Isolados de *E. faecalis* com resistência simultânea à penicilina e gentamicina, que são comuns no ambiente hospitalar, foram detectados raramente em fontes não clínicas. No entanto, foram encontrados vários isolados de *E. faecalis* e de outras espécies de enterococos com resistência a diversos antimicrobianos corroborando outros estudos que demonstram a presença dessas drogas no ambiente e o uso na agropecuária.

Notavelmente, os isolados da espécie *E. faecalis* obtidos no ambiente hospitalar apresentaram diversidade genética menor do que os isolados recuperados de fontes ambientais.

V – CONCLUSÃO

Uma diversidade de espécies do gênero *Enterococcus* foram isoladas de amostras não clínicas, mas, as espécies *E. faecalis e E. faecium* foram as mais prevalentes.

E. faecalis com o fenótipo penicilina-resistente/ampicilina-sensível (EFPRAS) não foi encontrado em alimentos ou animais da região de Uberaba-MG. No entanto, isolados com esse fenótipo incomum de resistência às penicilinas foram encontrados no esgoto do hospital. Os EFPRAS do esgoto são geneticamente semelhantes aos isolados de pacientes hospitalizados no mesmo hospital, mas, em período bem anterior ao do presente estudo.

E, embora não tenha ocorrido registro da presença desses isolados na Estação de Tratamento de Esgotos ou na Estação de Tratamento de Água do município, não é possível descartar o risco de disseminação desses isolados no sistema de esgoto da cidade e possibilidade de contaminação da água dos rios.

Portanto, os resultados do presente estudo ressaltam a necessidade de tratamento do esgoto hospitalar a fim de evitar que microrganismos multirresistentes oriundos do ambiente hospitalar sejam disseminados para o meio ambiente.

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