Characterization of *Enterococcus* spp. isolated from a fish farming environment in southern Brazil

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Abstract

The aim of present study is to characterize the resistance and virulence profile of enterococci isolated from aquaculture excavated ponds and masonry tanks (6 samples) in southern Brazil. Samples were cultured in selective medium, 10 colonies were randomly selected from each sample, which were identified by MALDI-TOF and tested against 13 antimicrobials. The presence of resistance (*tet*L, *tet*M, *tetS, erm*B and *msr*C) and virulence (*ace, esp, agg, cyl*A and *gel*E) genes were determined by PCR. A total of 79 enterococci were identified, and *Entecococcus faecalis* (44.3%) and *E. casseliflavus* (36.7%) were the most prevalent species isolated. Sixty-five strains (82.3%) were resistant to at least one of the antimicrobials tested, whereas 27 (34.2%) strains were multiresistant. The overall percentages of antimicrobial resistant isolates were: 58.2% to rifampicin, 40.5% to fluoroquinolones, 36.7% to erythromycin and 30.4% to tetracycline. The *tet*L and *tet*M genes were found in 57.7% of the tetracycline-resistant strains; and *msr*C in 31.01% of erythromycin-resistant strains. The most frequently detected virulence factors were *ace* and *gel*E genes. Although limited to a single farm, these data suggest that aquaculture may be a reservoir of resistant and virulent enterococci. This study is the first step towards enhancing our understandingof distribution, resistance and virulence profile in enterococci isolated from fish farming environments in the south Brazil.

Keywords: *Enterococcus* sp., continental aquaculture, microbial ecology, antimicrobial resistance, virulence genes.

Caracterização de Enterococcus spp. isolado de um ambiente de piscicultura no sul do Brasil

Resumo

O objetivo do estudo apresentado é caracterizar o perfil de resistência e virulência de enterococos isolados de viveiros escavados e tanques de alvenaria (6 amostras) de uma piscicultura no Sul do Brasil. As amostras foram cultivadas em meio seletivo, 10 colônias foram selecionadas aleatoriamente de cada amostra, que foram identificadas por MALDI-TOF e testadas contra 13 antimicrobianos. A presença de genes de resistência (*tet*L, *tet*M, *tetS, erm*B e *msr*C) e virulência (*ace*, *esp, agg, cyl*A e *gel*E) foi determinada por PCR. Foram identificados 79 enterococos, sendo *Entecococcus faecalis* (44,3%) e *E. casseliflavus* (36,7%) as espécies mais frequentes isoladas. Sessenta e cinco cepas (82,3%) eram resistentes a pelo menos um dos antimicrobianos testados, enquanto 27 (34,2%) eram multirresistentes. As porcentagens gerais de isolados resistentes a antimicrobianos foram: 58,2% para rifampicina, 40,5% para fluoroquinolonas, 36,7% para eritromicina e 30,4% para tetraciclina. Os genes *tet*L e *tet*M foram encontrados em 57,7% das cepas resistentes à tetraciclina; e *msrC* em 31,01% das cepas resistentes à eritromicina. Os fatores de virulência mais comumente detectados foram *ace* e *gel*E. Embora limitados a uma única fazenda, esses dados indicam que a aquicultura pode ser uma fonte de enterococos resistentes e virulentos. Este estudo é o primeiro passo para melhorar nosso entendimento da distribuição, resistência e perfil de virulência em enterococos isolados de ambientes de piscicultura no sul do Brasil.

Palavras-chave: *Enterococcus* sp., aquicultura continental, ecologia microbiana, resistência antimicrobiana, genes de virulência.

1. Introduction

Aquaculture is the fastest growing sector of world food-production, with a growth rate of over 8.0%. In 2017, world fish production reached 170.9 million tons, roughly 46.8% of all this production derives from aquaculture (FAO,

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2018). According to PeixeBR data, in 2018 Brazilian fish farm production equaled 722,560 tons of fish, and become the world's fourth biggest producer of Nile tilapia (*Oreochromus niloticus*). A significant portion of the fish production in Brazil is located in the Rio Grande do Sul, with a total production of 23,000 tons/year, with a high proportion of exotic fishes (74.8%), such as carp (*Cyprinus carpio*), trout (*Oncorhynchus* sp.) and panga (*Pterogymnus laniarius*), followed by Nile tilapia (*O. niloticus*) (17.8%) and native species (7.4%) (PeixeBR, 2019).

Fish farming is one of the most valuable solutions to the overexploitation of natural resource (FAO, 2018). However, due to the expansion of fish production worldwide, fish waste can impact the local environment by polluting the water and soil, considered one of the major worries of this practice. Therefore, the developments of efficient water reuse systems that reduce the environmental impacts and enable sustainable growth are considered necessary for this activity (Rocha et al., 2016).

Aquaculture requires large quantities of antimicrobial agents, which are administered as growth promoters (Novais et al., 2018). Effluents from aquaculture contaminated with antibiotic residues and other chemical agents allow selection for antibiotic-resistant organisms (Rocha et al., 2016). These effluents, when discharged into the water bodies, without a proper water treatment system, favor the process of natural selection of resistant bacteria, serving as a vehicle for transmission of antimicrobial-resistant bacteria in the environment (Di Cesare et al., 2013; Rocha et al., 2016). The presence of bacterial species carrying antimicrobial resistance profile has already been detected in fish farming systems, as well as in water effluents (Novais et al., 2018). Among microorganisms resistant isolated from different types of water, highlights the enterococci (Nachtigall et al., 2013; Lebreton et al., 2014).

Enterococcus spp. are considered commensal microbiota of the oral cavity, genitourinary and gastrointestinal tract of humans and animals, and are widely distributed in the environment, occurring in plants, soil and water. Enterococcal species have great genomic plasticity, high versatility to occupy broad ecological roles, able to grow in temperatures ranging from 10 and 45 °C, with optimum growth at 35 °C and salt tolerance (6.5% NaCl) (Lebreton et al., 2014). Currently, the genus is composed of more than 50 species, with *Enterococcus faecalis* predominant in the gastrointestinal tract of humans and other mammals, followed by *E. faecium*, *E. hirae*, *E. durans*, *E. casseliflavus*, *E. gallinarum* and *E. mundtii* (Cassenego et al., 2011; Novais et al. 2018).

Enterococci are considered opportunistic pathogens, which represent the second most common cause of hospital-acquired infections, particularly affecting the urinary tract, wounds, and soft tissues. The emergence of antibiotic resistant enterococci is a major medical and public health concern. Resistant strains are not exclusively restricted to clinically relevant species, since resistant strains have been investigated and monitored in different environments (Frazzon et al., 2010; Cassenego et al., 2011; Grassotti et al.,

2018; Novais et al., 2018; Huff et al., 2020). The presence of resistant strains in all environmental, could provide important information of ecological disturbance

Resistance to different classes of antimicrobials is a hallmark of *Enterococcus* spp., they are intrinsically resistant to sulfonamides, ertapenem, perfloxacin, penicillin and ampicillin and, high-level resistance to most cephalosporins. Enterococcus species are also resistant to low levels of aminoglycosides, due to the decreased uptake of this antibiotic class. Furthermore, they can transfer and acquire antibiotic resistance genes (through the transfer of plasmids and transposons, chromosomal exchange, or mutation) (Lebreton et al., 2014). Indeed, this ability to transfer/acquire resistance and/or virulence genes, offer a selective advantage to *Enterococcus* spp. survival and dispersal in the environment (Savaşan et al., 2016).

Due to their presence in human and animal faeces, the ability to adapt to different environmental conditions (freshwater and marine environments) and the correlation with human health, allows *Enterococcus* sp. to be used as sentinels for water quality changes (Di Cesare et al., 2013, Lebreton et al., 2014). However, specific aquaculture legislation determines standard wastewater-treatment procedure, such as CONAMA Resolutions No. 357/2005 and No. 430/2011 (Brasil, 2005; Brasil, 2011), this requirement does not include enterococci. Based on this, the present study aims to determine the occurrence, the antimicrobial susceptibility profile and the presence of virulence genes in enterococci isolated from a fish farming environment in southern Brazil.

2. Material and Methods

2.1. Sample collection

Sediment samples were collected from 6 sites in a fish farm located in the city of São Leopoldo - Rio Grande do Sul, Brazil (-29.736318, - 51.086023). Sediments were collected in 2018, in six different points named: P1, P2, P3 (excavated nurseries covered with masonry and plastic canvas), P4, P5 (excavated uncovered ponds, where water remains constantly contact with the bottom sediment) and P6 (a runoff point of the effluent produced by the farm) (Figure 1). Four replicate sediment samples were taken from each of point in different periods of the year (summer, fall, winter, and spring), totalizing 24 samples. The samples were placed in sterile containers and refrigerated to be transported to the laboratory, where the microbiology analyzes was performed.

Fish farm was located on the bank of the River dos Sinos, considered one of more polluted river in Brazil. The farm is involved in fingerling production and culture of exotic fishes species as carp (*C. carpio*) and tilapia (*O. niloticus*), besides native and ornamental fishes. The fishes are fed with the feed. The fish ponds are lined with concrete, earth or plastic, with not interconnection between them. The pond systems are designed to retain or confine water and/or waste within a pond. The fish farm has a water reuse system, where the wastewater is
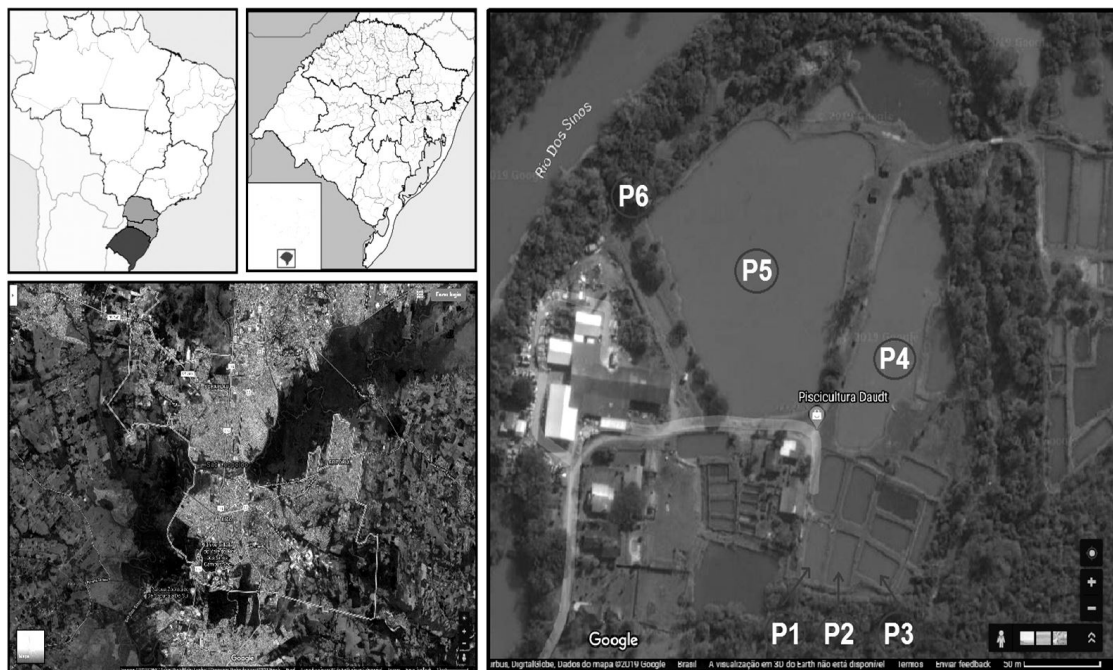
Figure 1. Sediment sampling sites along the fish farm, São Leopoldo, Rio Grande do Sul, Brazil. On the left Brazil and Rio Rio Grande do Sul (top) and Fish farm (bottom) maps. On the right-detailed map of Fish farm showing locations of collected samples site. P1, P2 and P3 (excavated nurseries covered with masonry and plastic canvas, average static volume capacity per pond is 80, 90 and 80 m³, respectively); P4, and P5 (excavated uncovered ponds, where water remains constantly contact, average static volume capacity per pond 981 e 1500 m³) and P6 (a runoff point of the effluent produced by the farm, average static volume capacity per pond 256 m³).

reused to another pond and water purification by water hyacinth (*Pontederia crassipes*). In the farm area, there is residential property, rearing of livestock (chickens, cattle and horses), and logging.

2.2. Isolation of enterococci

Isolation of enterococci was performed as previously described by Cassenego et al. (2011), with adaptations. One milliliter of each sample was added in 9 mL Buffered Peptone Water (Himedia, Mumbai, India) and incubated at 35 ± 1 °C for 24 h. After incubation, 1.0 mL was inoculated in 9.0 mL of Azida Dextrose Broth (Himedia, Mumbai, India) and incubated again at 35 ± 1 °C for 24 h. From that, 0.1 mL were inoculated in triplicate in Brain Heart Infusion Agar (Acumedia, Lansing, Michigan) supplemented with 6.5% NaCl and incubated at 35 ± 1 °C for 48 h. Five to ten colony-forming units were randomly selected from each sample. Phenotypic criteria, such as size/volume, shape, color, Gram staining, catalase production, capacity to growth at 45 °C and bile aesculine reaction, were used to separate the enterococci group and the non-enterococcal strains (Teixeira et al., 2011). Selected pure colonies were stored at -20 °C in a 10% (w/v) solution of skim milk (Difco, Sparks, MD, USA) and 10% (v/v) glycerol (Neon Comercial Ltda).

2.3. Species identification using the matrix assisted laser ionization and desorption technique (MALDI-TOF)

Collected bacteria were identified by MALDI-TOF technique applied to Enterococcus, according to Sauget et al. (2017). MALDI-TOF analysis was performed using a LT Bruker microflex mass spectrometer (Bruker Daltonik GmbH) and spectra were automatically identified using BrukerBioTyper ™ 1.1 software.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility of all strains was determined by Kirby-Bauer disk diffusion method, recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018). Thirteen antimicrobial agents were tested: Ampicillin 10 μg (AMP), Ciprofloxacin 5 μg (CIP), Chloramphenicol 30 μg (CHL), Erythromycin 15 μg (ERY), Streptomycin 300 μg (STR), Gentamicin 120 μg (GEN), Linezolid 30 μg (LNZ), Nitrofurantoin 300 μg (NIT), Norfloxacin 10 μg (NOR), Oxitetracycline 30 μg (OTC), Rifampicin 5 μg (RIF), Tetracycline 30 μg (TET) and Vancomycin 30 μg (VAN). Reference strains E. faecalis ATCC 29212 was used as control.

Intermediate and resistant-strains were included in a single category as resistant-strains. Strains were classified has single (SR), double (DR) or multidrug-resistant (MDR)

when showned resistance for one, two, and three or more antibiotics, respectively (EFSA and ECDC, 2013).

2.5. Detection of resistance and virulence related genes.

Genomic DNA was extracted by physicochemical method as previously described by Depardieu et al., (2004). The detection of resistance-related genes commonly observed in clinical and environmental enterococci: *erm*B, which encodes a ribosomal methylase that mediates macrolides, lincosamides and type B streptogramins resistance; *msrC*, which encodes for a macrolide and streptogramin B efflux pump; *tet*M and *tet*S, which encodes for tetracycline resistance via a ribosomal protection protein mechanism, and *tet*L which encodes for tetracycline resistance via efflux pumps proteins was performed using the Polymerase Chain Reaction (PCR) (Sutcliffe et al., 1996; Aarestrup et al., 2000; Werner et al., 2001; Frazzon et al., 2010, Rathnayake et al., 2011). In addition, PCR reactions were performed to detected virulence encoding genes: *ace* (collagen adhesin), *agg* (aggregating substance), *cyl*A (cytolysin), *esp* (enterococcal surface protein) and *gel*E (gelatinase) (Mannu et al., 2003; Eaton and Gasson 2001; Shankar et al., 1999).

3. Results and Discussion

3.1. Enterococcus spp. distribution in sediments of fish farm

The number of colony-forming units of *Enterococcus* spp. in sediment samples ranged from 1.9×10^3 to 2.0×10^7 CFU.g^{-1}. The current agriculture, livestock and food supply legislation in Brazil does not determine the enterococci limits in fish or aquaculture environments, but ensures values of this bacterial in recreational water use for primary contact activities (Brasil, 2005). Nevertheless, fish farming is not part of the primary contact activity; however, practices developed in fish farming can expose workers to the risk of direct contact with water for a considerable period.

The distribution of *Enterococcus* species recovered from sediment samples is shown in Table 1. The most common species found in our samples were *E. faecalis* (n = 35; 44.3%) and *E. casseliflavus* (n = 29; 36.7%), followed by *E. faecium* (n = 11; 13.9%), *E. gallinarum* (n = 2; 2.5%) and *E. hirae* (n = 2; 2.5%). These results corroborate with Di Cesare et al. (2013), Pereira et al. (2017) and Novais et al. (2018), which found these species in water and sediment samples from freshwater

and marine aquatic environments. Besides, these studies reported *E. faecalis* as the abundance specie in these environments (Di Cesare et al., 2013; Pereira et al., 2017; Novais et al., 2018).

Analyzing the distribution of species by collection point, it was observed that in P4 site there was a predominance of *E. faecium* (13/35; 37.1%) and *E. faecalis* (7/11; 63.3%) (Table 1). The P4 site is one of the largest nursery ponds for farming of carp (*C. carpio*) and tilapia (*O. niloticus*), with a high density of animals. *E. faecalis* and *E. faecium* are commonly described in human fecal samples and considered as fecal contaminants in water samples. Similar results were reported for rainbow trout (*Oncorhyncus mykiss*) in Portugal, pirarucu (*Arapaima gigas*) in Brazil and sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) in Italy, where these two species also prevailed over the isolates, further revalidating their occurrences in these environments, regardless of the fish species cultivated (Di Cesare et al., 2013; Pereira et al., 2017; Novais et al., 2018). It is important to highlights that these species are the main agents of hospital-acquired infections (Lebreton et al., 2014).

The presence of *E. casseliflavus*, *E. gallinarum* and *E. hirae* in our samples might be justified by the occurrence of the vegetation, birds and bats near to the ponds (Lebreton et al., 2014). However, Novais et al. (2018), showed results contrary to that for *E. casseliflavus* in water samples collected from trout sample that reported the lower presence of this species. The high frequency of this species in the study may be associated by the fact *E. casseliflavus* is usually isolated from larval feces of insect species. In fish farming environments there are dragonflies eggs, these animals depend on water in the larval and adult phase, also serving as food for fishes.

Our results show a low incidence of *E. gallinarum* and *E. hirae* in sediment samples from the fish farm. These results corroborate those observed by Novais et al. (2018) in sediment samples of cultivation of trout (*O. mykiss*) in southern Europe. Although not common, both species have already been reported to be responsible for human infections (Lebreton et al., 2014).

3.2. Antimicrobial susceptibility profile and resistance gene frequencies

Of the 79 strains of enterococci tested, 65 (82.3%) were resistant to at least one antimicrobial agent tested

Table 1. Distribution of enterococcal species recovery from different points at the fish farm.

Points	Species (n)	Total (%)
P1	*E. casseliflavus* (4)	4 (5)
P2	*E. casseliflavus* (5); *E. faecalis* (7)	12 (15.2)
P3	NI	0
P4	*E. faecalis* (13); *E. faecium* (7); *E. gallinarum* (1)	21 (26.6)
P5	*E. casseliflavus* (9); *E. faecium* (4); *E. gallinarum* (1)	14 (17.7)
P6	*E. faecalis* (15); *E. casseliflavus* (11); *E. hirae* (2)	28 (35.4)
		79 (100)

NI: not isolated

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(Table 2). Isolates were susceptible to STR, GEN, LNZ and VAN. In the samples collected from fish ponds, it was possible to recover *Enterococcus* spp. with resistance profile of different antimicrobial agents, such as beta-lactams, phenolics, macrolides, fluoroquinolones, nitrofurans, rifampicin and tetracyclines, considered important by the World Health Organization. Previous studies on different fish farms systems have found antimicrobial resistance strains (Di Cesare et al., 2013; Novais et al., 2018). These data can be considered worrying since these environmental bacterial populations may evolve throughout secondary genetic events to resistance levels with human clinical impact (Andersson and Hughes, 2014).

Resistance to RIF was mainly found among *E. casseliflavus* (100%) and *E. hirae* (100%), while resistance to OTC or/and TET was observed in *E. faecium* (100%), *E. hirae* (100%), *E. faecalis* (44%) and *E. casseliflavus* (24.13%). More than forty percent of the *E. casseliflavus* and *E. faecalis* analyzed are resistant to ERY and CIP, respectively. Similar results have been reported by Novais et al. (2018) who found high rates of resistance to CIP, ERY and TET in enterococci isolated from water and sediment samples from trout farming in Southern Europe, while Di Cesare et al. (2013) found

Table 2. Antimicrobial profile of *Enterococcus* species isolated from fish farm in South Brazil.

Antimicrobial	Profile*	Number of strains (%)
Ampicillin (AMP)	R	1 (1.3)
	S	78 (98.7)
Ciprofloxacin (CIP)	R	28 (35.4)
	S	51 (64.6)
Chloramphenicol (CHL)	R	2 (2.6)
	S	77 (97.4)
Erythromycin (ERY)	R	29 (36.7)
	S	50 (63.3)
Streptomycin (STR)	R	-
	S	79 (100)
Gentamycin (GEN)	R	-
	S	79 (100)
Linezolid (LNZ)	R	-
	S	79 (100)
Nitrofurantoin (NIT)	R	8 (10.1)
	S	71 (89.9)
Norfloxacin (NOR)	R	9 (11.4)
	S	70 (88.6)
Oxitetracycline (OTC)	R	20 (25.3)
	S	59 (74.7)
Rifampicin (RIF)	R	46 (58.2)
	S	33 (41.8)
Tetracycline (TET)	R	17 (21.5)
	S	62 (78.5)
Vancomycin (VAN)	R	-
	S	79 (100)

* R: resistant; S: susceptible

TET resistant enterococci in sediments collected at a fish farm in Varano lagoon. Although the farm owner denied all antibiotic use in the pond, either for therapeutic or for growth promotion purposes, the use of OTC in previous years, could partially explain the tetracyclines resistance observed in our study. Tetracycline and erythromycin are prescribed in human and veterinary medicine, and are excreted as active metabolites and remain stable in the environment (Rahardjo et al., 2011; Schafhauser et al., 2018; Rudra et al., 2018). Consequently, this class of antibiotic is considered modern pollutants in the soil and aquatic environment (Gothwal and Shashidhar, 2014; Dizavandi et al., 2016).

In this study, 34.2% (n=27) of strains were multi-resistant (Table 3). The detection of multidrug-resistant enterococci isolated from the sediments of fish ponds might represent potential environmental contamination since this fish farm is near to River of Sinos, considered one of the more polluted rivers in Brazil. Human negligence with waste, sewage, wastewater, and also industrial waste and agricultural irrigation is the main environmental impacts within the River of Sinos (Figueiredo et al., 2010). Also, Di Cesare et al. (2013) suggested that feed supplied to captive fish contributed to the presence of multidrug-resistant strains.

Among the antimicrobial resistance genes found in this study, the *tet*L and *tet*M genes were observed in 57.7% of the tetracycline-resistant strains; and the *msr*C gene (encoding) was positive in 31.01% of the erythromycin-resistant strains. None of the strains tested were positive to *tet*S and *erm*B genes. Similar results were found by Di Cesare et al. (2013) and Novais et al. (2018) in studies conducted in southern European. Authors pointed out that the *tet*M gene is of great recurrence in enterococci isolated from environmental samples, including marine aquaculture, coinciding with those found in the present study. In relation to species, the *msrC* gene was carried by all erythromycin-resistant *E. faecium* and two *E. casseliflavus;* and the *tet*L and *tet*M genes in six tetracycline-resistant *E. faecium,* five *E. faecalis,* two *E. casseliflavus* and two *E. hirae*. In spite of the fact that *msrC* gene is considered intrinsic to *E. faecium,* Werner et al. (2001), demonstrated that this gene is not an intrinsic property of all *E. faecium* isolates, and other studies have been showed the presence of this gene in other enterococci species, such as *E. hirae*, and *E. faecalis* (Grassotti et al., 2018).

Aquaculture is believed to contribute to the spread and persistence of antimicrobial resistance in the environment, and resistant bacteria have been recovered from aquaculture production areas, as previously mentioned by Di Cesare et al. (2013), Pereira et al. (2017) and, Novais et al. (2018). Not surprisingly, research reports not only resistant strains, but also resistance genes and remnants of the drug itself, reiterating that the discharge of antibiotics through effluents into natural water bodies, can lead to selective pressure and consequent increased antimicrobial resistance (Rocha et al., 2016).

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Table 3. Antimicrobial resistance phenotypic profile of *Enterococcus* spp. isolated from sediments isolated in the fish farm.

Phenotype*	Antimicrobials**	Species of enterococci resistents (n)	Total
SR	CIP	*E gallinarum* (1)	1
	ERY	*E. casseliflavus* (1)	1
	OTC	*E. faecalis* (1); *E. faecium* (1); *E. gallinarum* (1)	3
	RIF	*E. casseliflavus* (11); *E. faecalis* (5)	16
DR	CIP/NIT	*E. faecalis* (2)	2
	CIP/TET	*E. faecalis* (1)	1
	ERY /NIT	*E. casseliflavus* (1)	1
	ERY/RIF	*E. casseliflavus* (6); *E. faecalis* (1)	7
	NIT/NOR	*E. faecalis* (1)	1
	CHL/RIF	*E. casseliflavus* (1)	1
MR	CIP/NOR/ERY	*E. faecalis* (1)	1
	CIP/NOR/RIF	*E. faecalis* (2)	2
	CIP/OTC/TET	*E. faecalis* (1)	1
	AMP/CIP/NIT	*E. faecalis* (1)	1
	CIP/ERY/OTC	*E. faecium* (1)	1
	CIP/ERY/RIF	*E. faecalis* (2); *E. casseliflavus* (3)	5
	CIP/ERY/TET	*E. faecium* (1)	1
	ERY/OTC/RIF	*E. casseliflavus* (1)	1
	ERY/RIF/TET	*E. faecalis* (1)	1
	NOR/OTC/RIF	*E. hirae* (1)	1
	CIP/ERY/OTC/RIF	*E. casseliflavus* (1)	1
	CIP/ERY/OTC/TET	*E. faecium* (4)	4
	CIP/ERY/OTC/NOR	*E. casseliflavus* (1)	1
	CIP/OTC/TET/RIF	*E. faecalis* (3)	3
	CHL/ERY/NIT/RIF	*E. casseliflavus* (1)	1
	ERY/OTC/TET/RIF	*E. casseliflavus* (1)	1
	NIT/NOR/RIF/TET	*E. faecalis* (1)	1
	NOR/OTC/TET/RIF	*E. hirae* (1)	1
	CIP/NOR/OTC/TET/RIF	*E. faecalis* (1)	1
	CIP/ERY/NIT/OTC/TET	*E. faecalis* (1)	1
	CIP/ERY/OTC/TET/RIF	*E. casseliflavus* (1)	1
			65

* SR: single resistance; DR: double resistance; MR: multiresistance. ** AMP: ampicillin; CIP: ciprofloxacin; CHL: chloramphenicol; ERY: erythromycin; STR: streptomycin; NIT: nitrofurantoin; NOR: norfloxacin; OTC: oxitetracycline; RIF: Rifampicin; TET: Tetracycline.

3.3. Virulence profile of enterococcal strains

The *gel*E was the most frequently detected gene (64.6%) in enterococci strains isolated from fish farm, followed by *ace* (51.9%), *agg* (13.9%) and *cyl*A (3.8%) genes (Table 4). The *esp* gene was not detected. Strains carrying virulence genes in water could be a risk to the health of humans and other animals that inhabit aquatic environments. Ahmad et al. (2014) compared the presence of these virulence genes between enterococci isolated from seawater and river on a Malaysian beach. The authors reported that in river water samples, the *gel*E, *asa*, *esp* and *cyl*A genes were detected in 100%, 63.41%, 21.95% and 7.32% of the strains, respectively; and in beach water samples a lower frequency of these genes was observed, being present in 67.27%, 41.82%, 20% and 0% of the strains.

The *gel*E gene was observed at high frequency among strains. This result agrees with those obtained by

Table 4. Virulence genes observed in strains of *Enterococcus* spp.

Specie (n)	Number of strains positive for virulence genes				
	ace	*agg*	*cyl*A	*esp*	*gel*E
E. casseliflavus (29)	6	0	0	0	7
E. faecalis (35)	31	8	3	0	34
E. faecium (11)	2	2	0	0	8
E. gallinarum (2)	1	0	0	0	1
E. hirae (2)	1	1	0	0	1
Total (79)	41	11	3	0	51

Novais et al. (2018) who also detected high percentages of this gene in *E. faecalis* isolated from water and sediment samples from a trout fish farm in Europe. On the contrary, Savaşan et al. (2016) found low frequencies of *gel*E gene in *Enterococcus spp*. isolated from fish samples in Turkey. The *ace* gene was one of the more prevalent in our samples.

The *ace* gene contributes to the microorganisms attach to surfaces and develop biofilms (Lebreton et al., 2014). Aquaculture environments present a large flow of water movement, necessary for the renovation or filling of the ponds. Thus, it is possible to assume that the occurrence of this gene is useful to bacterial maintenance in this environment. The *agg* gene, responsible for *Enterococcus* aggregation on host surfaces, occurred in 13.9% of the isolates. Similar results were observed by Savaşan et al. (2016) with samples from farmed fish. Novais et al. (2018) did not observe the presence of the *agg* gene in fish samples in Portugal, but detected the presence of another aggregation gene in 50% of the isolates they studied. The presence of virulence factor in clinical strains of *Enterococcus* spp. is associated with the occurrence of infections; however, other authors associate the occurrence of these genes in non-clinical strains as a common characteristic, which probably contributes to enterococci persistence in the environment (Lebreton et al., 2014).

In conclusion, this study showed the occurrence of resistant enterococci as well as multiresistant strains in a fish pond that do not use antibiotics, either for therapeutic or for growth promotion purposes. Suggesting that the resistance found in these strains may be associated with environmental pollution. This study is the first step towards enhancing our understanding of distribution, resistance and virulence profile in enterococci isolated from fish farming environments in the south Brazil. The presence of resistant enterococci in waters and sediment is not only indicative of faecal contamination but may also a public health problem whether these microorganisms come into contact with humans and other animals. Our results are promising and should be validated by a large sample size.

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