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Antagonistic Activity, Antimicrobial Susceptibility and Potential Virulence Factors of *Enterococcus faecalis*

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Abstract: *Enterococcus faecalis* isolates (87) were phenotypically and genotypically identified and subsequently subjected to the antagonism test and antimicrobial susceptibility. The lipolitic, hemolytic and DNAse activities were identified along with the genes *gelE*, *cylL*, *cylS*, *ccf*, *cpd* and *cob* that, encode virulence determinants. Thirty seven percent of isolates inhibited *Listeria monocytogenes* (CERELA), *Listeria innocuous* (CERELA), *Staphylococcus aureus* (ATCC25932), *Lactococcus lactis* (IL1403), *Micrococcus luteus* (ATCC10240) and *Enterococcus faecalis* (ATCC29212). All strains were sensitive to the ampicillin antibiotic, but 47% were resistant to at least one antimicrobial agent and 6% of isolates presented multidrug resistance. Ninety seven percent of isolates contained the *gelE* gene, but 77% of these isolates showed gelatinase activity. Presence of *cylL* and *cylS* genes was observed in 25% of the isolates, but only 5% presented hemolytic activity. None isolates showed lipase and DNAse activities. Eight percent of isolates contained the *ccf* gene and 2% showed the presence of the *cpd* and *cob* genes. The ability to inhibit pathogenic bacteria, low resistance to antibiotics and absence of virulence factors make some of *Enterococcus faecalis* strains characterized in the present study promising for exploitation in other applications such as probiotics in aquaculture.

Key words: Bivalve mollusks, probiotics, pathogenicity, water.

1. Introduction

LABs (lactic acid bacteria) are one of the largest bacteria groups that are industrially important. *Enterococcus* spp. belongs to this group and has technological characteristics that when used in fermentation processes, contribute to maturation and flavor development in some meat and cheese products [1, 2].

The *Enterococcus* antagonistic capacity allows control of undesirable bacteria in foods [3]. The *E. faecalis* and *E. faecium* species are the most important within the *Enterococcus* genus, because they are currently the only species used to produce probiotics intended for human and animal consumption [4]. In foods, their application has been limited because they can be used as contamination indicators of fecal origin and some lines are related to nosocomial diseases, diarrhea syndromes in newborns and antimicrobial resistance [2, 5, 6].

Because they survive in environments with salt concentrations of up to 6.5%, pH between 4.0 and 9.6 and temperatures between 10 °C and 45 °C, *Enterococcus* spp. can easily be isolated from soil, water, plants, human, animal, fish and crustacean intestines [1, 7] and bivalve mollusks due to their system of feeding by filtration [8].

The inhibitory substances produced by *Enterococcus* spp. are of interest for bioconservation of fermented seafood or other foods and their use as probiotic cultures in aquaculture [9]. Probiotics are now being successfully developed isolated directly from the digestive tract of organisms and water or from environments impacted by human action because of the

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adaptation and survival capacity these organisms to environments [10]. It is also desirable that the probiotic species have specificity with host species [11].

The incidence of virulence factors as well as resistance to antimicrobials used in human therapy, have contributed for pre selection of microorganisms for use as probiotics, one of the limiting factors vancomycin resistance [12].

Knowledge of antagonistic capacity associated to the antimicrobial resistance profile and virulence mechanisms from *E. faecalis* can help to understand the complexity of these microorganisms and their potential for application in aquaculture. The authors attempted to generate information on biotechnological potential of these bacteria that are adapted to conditions of the aquatic environment and colonizing mollusk tissues, characterizing the isolates for presence of virulence markers, antimicrobial susceptibility and antagonistic activity.

2. Materials and Methods

2.1 Microorganisms, Growth Conditions and Phenotypic Identification

The *Enterococcus* cultures were previously isolated from water and bivalve mollusks (oysters and sururu) in extraction areas in the Recôncavo of Bahia, Brazil. The cultures were kept at -20 °C in medium containing glycerol 20% (v/v). The cultures were activated in brain and BHI (heart infusion broth). The indicator microorganisms *Listeria monocytogenes* (CERELA), *Listeria inocua* (CERELA), *Staphylococcus aureus* (ATCC25932), *Lactococcus lactis* (IL1403), *Micrococcus luteus* (ATCC10240) and *Enterococcus faecalis* (ATCC29212) were cultured in BHI broth.

The following biochemical tests were carried out to confirm the isolates: Gram coloring, growth at 10 °C and 45 °C, growth in the presence of 6.5% NaCl, tellurite reduction and sugar fermentation (arabinose, manitol, sorbitol and glucose with gas production) [13].

2.2 Antimicrobial Susceptibility

The antimicrobial susceptibility was performed by disk diffusion method using commercially available antibiotic-containing disks (LABORCLIN, Brazil): ampicillin (AMP, 10 μg), ciprofloxacin (CLO, 5 μg), gentamicin (GEN, 10 μg), imipenem (10 μg), nitrofurantoin (NIT, 300 μg), tetracycline (TET, 30 μg) and vancomycin (VAN, 30 μg) [14]. For control, the following reference strains were used: *Escherichia coli* ATCC 25922 e *Staphylococcus aureus* ATCC 25923. The diameters of inhibition zones were recorded to the nearest millimeter and classified as susceptible, intermediate and resistant.

MIC (minimum inhibitory concentration) was determined in strains resistant to antimicrobial test disk diffusion (Kirby-Bauer) according to the broth dilution (macrodilution) technique using Mueller-Hinton broth (Difco®) [14].

2.3 Phenotypic Virulence Tests

The following tests were made: gelatinase, lipase, hemolytic activity and DNAse [15-18]. The formation of a transparent or opalescent halo indicated test positivity.

2.4 Genotypic Identification

The isolates were cultured overnight at 37 °C in BHI broth and centrifuged at 14,000 rpm/3 min [19]. The genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit.

To amplify the ribossomal DNA 16S by PCR (polymerase chain reaction), the universal primers were used for Enterobactereaceae MD (5'CCCGGGA TCCAAGCTTAAGGAGGTGATCCA) and FD (5'CCGAATTCGTCGACAACAGAGTTTGATCCT) [19, 20]. The sequencing was carried out by the Macrogen company and the nucleotide sequences compared using sequences deposited in the GenBank.

2.5 Determining Antagonistic Activity

The screening was realized by *spot-on-the-lawn* test

[21] in BHI agar culture medium at 37 °C/24 h and an overlay was added using as culture indicators *Listeria monocytogenes* (CERELA), *Listeria inocua* (CERELA), *Staphylococcus aureus* (ATCC25932), *Lactococcus lactis* (IL1403), *Micrococcus luteus* (ATCC10240) and *Enterococcus faecalis* (ATCC29212).

Isolates that presented antagonistic activity were submitted to the *well-diffusion-agar* test. The supernatant was tested under different treatments: neutralized in pH 7.0 at 100 °C/15 min, in the presence of papain, protease and proteinase K [22].

2.6 Determining the Presence of Virulence Genes

The presence of the virulence genes was verified by PCR (polymerase chain reaction) (Table 1).

3. Results and Discussion

3.1 Identification

Of the 87 *Enterococcus* sp. strains used in the biochemical identification and analysis of rDNA 16S sequences, 13 strains were identified as *Lactococcus* sp., 3 as *E. durans* or *E. hirae*, 11 were unable to identified and 60 strains were identified as *E. faecalis,* 10 isolates from water, 14 from oysters (*Crassostrea rhizophorae*) and 36 from sururu (*Mytella guyanensis*).

Enterococcus faecalis is the dominant species in retail fish and fish-based products ready for consumption in Japan [7]. Even knowing that area suffers with runoff of domestic sewage, due to the lack of sanitation in the region, and *E. faecalis* is related to the presence of fecal contamination, the strains acquisition directly from the study environment. It has been indicated in the selection of potential probiotic bacteria application, since these have mechanisms which confer better competitive conditions [12].

3.2 Antagonistic Capacity

Microorganisms can produce several inhibitory substances, such as metabolism sub products: lactic acid, diacetyl, hydrogen peroxide, lithic agents, exotoxins and bacteriocins [23].

Twenty-two (37%) of isolates tested presented inhibitory activity. Of these, 82% (18/22) inhibited *L. lactis* (IL1403), 23% (5/22) presented anti-listerial activity, five isolates inhibited *L. inoccua* (CERELA) and one isolate inhibited *L. monocytogenes* (CERELA), 18% (4/22) presented inhibitory activity over *M. luteus* (ATCC10240), 18% (4/22) inhibited *E. faecalis* (ATCC29212) and 9% (2/22) inhibited *S. aureus* (ATCC25932) (Table 2).

The search *Enterococcus* bacteriocins producing is gaining interest they can be used to biocontrol pathogenic bacteria such as *L. monocytogenes* (*1*). Bacteriocin production in isolates from seafood was reported by Valenzuela et al. [9] and Pinto et al. [24].

The cell-free supernatant was tested in order to obtain more knowledge about the nature of the antagonistic substances produced. Inhibitory activity remained at 76% (13/22) even after passing through treatments with proteolytic enzymes and pH (7.0) neutralization. Based on the results, the nature of inhibitory compound produced cannot be stated, because the characteristics are atypical when compared with other studies [25], that is, treatment with proteolytic enzymes should promote bacteriocin

Table 1 Primers to amplify the virulence factors in *Enterococcus faecalis* isolated from water and bivalve mollusk samples.

Target gene	Sequence of primers (5'-3')	Amplicon size
gelE	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	419 pb
$cylR_{L/S}$	CAACAATTTTATGATGGAGGGTAA TCTTCCATGTAAGCACTCCTTTT	517 pb
cpb	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	782 pb
cob	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCAT	1,405 pb
ccf	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	543 pb

Table 2 Resistance and virulence among *Enterococcus faecalis* isolates that presented antagonistic potential.

Profiles					
Source	Strains	Antagonistic	Resistence	Virulence	
				Genotype	Phenotype
Water	EFA14	*LL, SA, ML*	TET	*gelE, cyl, ccf, cpd*	hem
	EFA16	*LI, EF*		*gelE, cob*	
	EFA18	*LI, EF*		*gelE, ccf*	
	EFA20	*LL, ML*	IMP*, TET	*gelE, cyl, cpd*	hem
	EFA21	*LI, LL*		*gelE, cyl, ccf, cpd*	
Oysters	EFOS26	*LL*	IMP	*gelE, cyl, cpd*	gel
	EFOS27	*LL*	VAN*	*gelE, cyl, ccf*	gel
	EFOS28	*LL*	GEN*, TET	*gelE, cyl*	gel
	EFOS29	*LL*	GEN*	*gelE, cyl*	gel
	EFOS30	*LL*	GEN*, VAN	*gelE, cyl*	gel
	EFOS31	*LL*	GEN*	*gelE, cyl*	gel
	EFOS33	*LL*		*gelE*	gel
	EFOS34	*LI*	GEN, IMP	*gelE*	gel
Mytella guyanensis	EFSU40	*LL*	CIP, GEN*	*gelE, cyl*	gel
	EFSU41	*LL*	GEN*	*gelE, cyl*	gel
	EFSU42	*ML, EF*	GEN	*gelE*	gel
	EFSU50	*LL*	CIP*, GEN	*gelE*	gel, hem
	EFSU57	*LL*	GEN, VAN*	*gelE, cyl*	gel
	EFSUP64	*LM, LI, LL, S A, ML, EF*	GEN	*gelE, cyl*	gel
	EFSUP65	*LL*	GEN*	*gelE*	gel
	EFSUP84	*LL*	GEN*	*gelE*	gel
	EFSUP87	*LL*	GEN	*gelE*	gel

LM—Listeria monocytogenes (CERELA), LI—Listeria inocua (CERELA), LL—Lactococcus lactis (IL1403), SA—Staphylococcus aureus (ATCC25932), ML—Micrococcus luteus (ATCC10240), EF—Enterococcus faecalis (ATCC29212), CIP—ciprofloxacin, GEN—gentamicin, TET—tetracycline, IMP—imipemem, VAN—vancomycin, *—intermediate resistance, gel—perfil gelatinolytic, hemolytic profile.

inactivation because they are composed of peptides [24].

All the inhibitory substances were considered heat sensitive because they lost their activity after treatment at 100 °C/15 min. *Enterococcus* bacteriocins are usually characterized as heat resistant, and activity loss in the substrate studied was attributed to the presence of a peptide compound from thermolabile fraction [26].

Bacteriacin ST15, produced by *Enterococcus mundtii*, has also been reported because it loses all its activity after treatment at 90-121 °C/10 min [27]. The bacteriocin described in the study by Pinto et al. [24] partially lost activity after treatment at different temperatures (90-120 °C).

Recently, the use of bacteria from the sea and producers of inhibitory substances against pathogenic bacteria in aqua systems [3] has become more common as an efficacious alternative to ensure the organisms health, minimizing the use of antimicrobials [12].

Table 2 shows that isolates from water and *M. guyanensis* presented similar results to the antagonistic potential, so that they were capable of inhibiting practically the same culture indicators, except for *L. monocytogenes* (CERELA) that was inhibited only by one *M. guyanensis*t isolate. It is also possible to see that the isolates that presented hemolytic activity came from these two origins. This similarity was because the aquatic environment is considered highly competitive and adverse, and the sediment where *M. guyanensis* is found buried contains a high concentration of microbial cells.

3.3 Antimicrobial Susceptibility Profile

All the isolates were sensitive only to ampicillin (100%) and nitrofurantoin (98%). Resistance to at least one antimicrobial agent was observed in 47%, although strains with high intermediate resistance were observed in 70% of the isolates (Table 3).

Enterococcus resistance to antimicrobials results from their need for survival and persistence in highly competitive environments [28]. Resistance to antimicrobial agents in aquatic microorganisms indicates ecosystem change by human action, especially when antibiotics are released into sewers through the urine, feces and eventually animal carcasses [29].

Presence of antimicrobial compounds in aquatic environments is a cause for concern, because even when they are present at low concentrations they are considered a risk to the ecosystems due to their interference in biological processes [30]. The aquatic environment is considered the most efficient niche in genetic material exchange between microorganisms due to the presence of mobile elements such as plasmids and transposons. This exchange favors the development of pathogenic and multiresistant bacteria to antimicrobials [31].

High resistance was observed to gentamicin and tetracycline. Multi-resistance was observed in 6% (3/60) of the isolates, 2% (1/60) were resistant to imipenem and tetracycline, 2% (1/60) to gentamicin and tetracycline, 2% (1/60) to gentamicin and imipenem. Incidence of multiple resistance to antimicrobials in bacteria allows a better understanding of the strains, antimicrobial consumption and their effects on cultured organisms and human diseases [32].

According to the literature, the most important resistance phenotypes are those related to the aminoglycosides, beta lactams and glycopeptides because they are used in the treatment of *Enterococcus* infections. Although the genus has been described as resistant to low levels of aminoglycosides and tetracycline in clinical isolates, the high frequency of resistance to gentamicin has been reported in several strains isolated from food and water worldwide [7, 33].

Vancomycin-resistant strains are emerging as the global menace to public health. In the present study, only one isolate presented resistance profile and 7% (4/60) were classified with intermediate resistance and are not indicated for biotechnological application (Table 3). Using antimicrobials with intermediate sensitivity selects resistant strains [34].

Intrinsic resistance of *Enterococcus* to vancomycin is due to alterations in the cell wall synthesis.

Table 3 Microbial susceptibility and MIC (minimum inhibitory concentration) of *Enterococcus faecalis* isolates in water and bivalve mollusk samples.

	% susceptibility/number of isolates			
Antimicrobials	Sensitive	Intermediate	Resistant	MIC (μg)
Aminoglycosides				
Gentamicin (10 μg)	$35_{(21/60)}$	$40_{(24/60)}$	$25_{(15/60)}$	60 μg
Betalactamics				
Ampicillin (30 μg)	$100_{(60/60)}$	0	0	0
Imipenem (10 μg)	$82_{(49/50)}$	$10_{(6/60)}$	$8_{(5/60)}$	50 μg
Glycopeptides				
Vancomycin (30 μg)	$90_{(54/60)}$	$7_{(4/60)}$	$2_{(1/60)}$	70 μg
Nitrofuran				
Nitrofurantoin (300 μg)	$98_{(59/60)}$	$2_{(1/60)}$	0	0
Quinolone				
Ciprofloxacin (5 μg)	$92_{(55/60)}$	$6_{(4/60)}$	$2_{(1/60)}$	20 μg
Tetraciclina				
Tetracycline (30 μg)	$85_{(51/60)}$	$5_{(3/60)}$	$10_{(6/50)}$	80 μg

Vancomycin resistance is a limit for their use as probiotics [1, 13].

The minimum inhibitory concentration of resistant isolates was considered low compared to the limits established by the CLSI [14], that is, the largest MIC (over five times the limit) was observed for ciprofloxacin, tetracycline, and smallest CIM (over twice the limit) was obtained for vancomycin (Table 3).

Enterococcus spp. has several intrinsic or acquired resistance mechanisms. Acquired resistance occurs by the transfer of genes present in plasmids through the conjugation of intra and interspecific bacteria [3]. The antimicrobial agents for animal and human use when discharged in the water environment alter the ecological balance [36] and increase the incidence of resistant bacteria in water environments and seafood. Although the resistance transference indices in foodstuffs and aquatic environments are lower than in clinical indices [12], microorganism accumulation in bivalve mollusks tissues may facilitate this transference.

Culture-resistant *Enterococcus* can be introduced into the food chain and the human intestine, promoting transference of resistance factors between bacteria from intestinal microflora [13]. The safe use of *E. faecalis* resistant as probiotic cultures is important to ensure that these resistant factors will not be transferred [12].

3.4 Virulence Factors

Genetic analysis showed that 97% of isolates had the structural gene *gelE* responsible for producing the gelatinase enzyme in *E. faecalis*, however gelatinolytic activity was observed in only 77%. None isolates were positive for the lipase and Dnase tests (Fig. 1).

Enterococcus spp. can produce some proteases that help them to survive in the medium where they live and are produced only when necessary [37]. Gelatinase is a metalloprotease that hydrolyses gelatin, collagen, hemoglobin and other bioactive peptides. Although this enzyme is related to the induction of inflammatory processes and biofilm production [38-40], its proteolytic action is considered advantageous for industry [9].

Birri et al. [28] who studied the gelatinolytic profile in bacteria of the *Enterococcus* genus and reported greatest prevalence in the *E. faecalis* species.

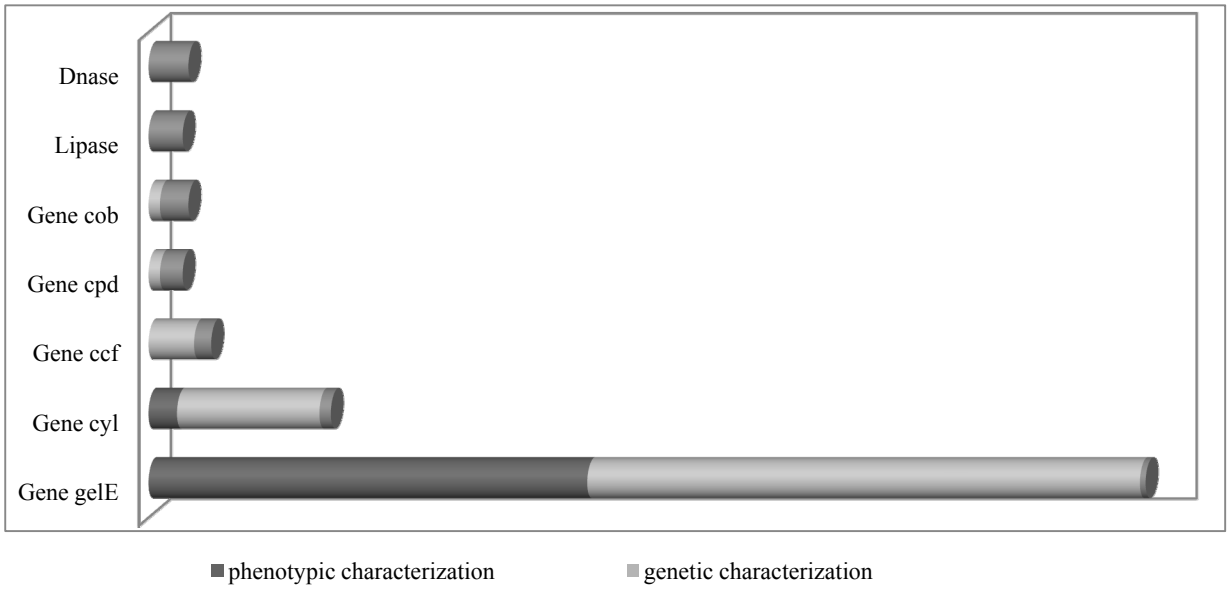

Fig. 1 Profile of the presence of pathogenicity-related genes in cultures of *Enterococcus faecalis* isolated from water and bivalve mollusk samples.

Cormelato et al. [40] compared gelatinase activity in *E. faecium* and *E. faecalis* isolates and also observed a greater presence of the *gelE* gene in *E. faecalis*. According to these authors isolates that presented the *gelE* gene were not capable of degrading gelatin that implies lack of gene expression.

Although 25% of isolates contained the $cylL_{L/S}$ gene that encodes cytolysin, only 5% presented hemolytic activity (Fig. 1). Cytolysin production is due to an operon consisting of H genes, principally the *cyl*A gene, because without it the expression of $cylL_{L/S}$ structural gene does not occur [28]. The $CylL_{L/S}$ structural gene does not appear to be an exclusive characteristic in isolates from clinical samples because it can be detected in the most diverse samples studied, including soil, water and foodstuffs [6].

Cytolysin is a peptide exclusive to the *Enterococcus* genus that has inhibitory activity against gram positive bacteria, encoded by the $CylL_{L/S}$ g structural gene usually located in the plasmodium. This gene is also responsible for causing hemolysis in humans [2]. Lines that express protein present tenfold pathogenicity, which contributes to their proliferation in the bloodstream because their activity takes place on the lysis of red blood cells, macrophages and neutrophils [38].

Hemolytic activity is not only related to the presence of *cylA* gene and other factors should be taken into consideration, such as the environment and the presence of other genes in the operon [28]. Hemolytic activity and the *cyl*A gene were not observed in a study on *E. faecium* strains isolated from seafood [9], although Valenzuela et al. [5] reported that in 17% of *E. faecalis* isolates from plants and animals that contained the *cylA* gene, there was no hemolytic activity.

Genes responsible for encoding the chemoreceptors were present in 8% (4/60), 2% (1/60) and 2% (1/60) of isolates for the genes *ccf*, *cpd* and *cob*, respectively (Fig. 1). These genes can promote the resistance acquisition to antimicrobials, virulence factors and other characteristics from *Enterococcus* spp. strains by forming cellular aggregates. Phermones mediate the conjugation facilitating the exchange of genetic information [15].

The low incidence of the *ccf*, *cpd* and *cob* genes means that they are not a concern for the safe use of *E. faecalis* strains [9].

Environmental factors can influence the genetic expression of microorganisms, so that some silent genes become active because of changes in the environment and the conditions found in the gastrointestinal tract of the host [15, 39].

The presence of a specific gene does not mean its phenotypic expression, as reported for *E. faecalis* isolates from several sources including water and foods [2, 15, 28, 35, 39]. Hemolytic activity serves as an indicator of pathogenicity potential in *Enterococcus* spp. and this is a limiting factor for its use as probiotic and/or as starter culture in foods. The probiotic potential of *E. faecalis* could be safely exploited in 30% of isolates studied, because they did not present resistance to vancomycin and hemolytic profile.

4. Conclusions

The inhibitory activity presented by the *E. faecalis* isolates is promising regarding exploitation of its biotechnological potential, especially when considering microorganisms with probiotic potential for aquaculture.

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