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Virulence factors, antimicrobial resistance and biofilm formation in *Enterococcus* spp. isolated from retail shrimps

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ABSTRACT

A total of 60 samples from retail raw, cooked and ready-to-eat shrimps were collected and the prevalence of *Enterococcus* spp, their virulence factors, antibiotic resistance and biofilm-forming ability was determined. Most of the strains were isolated from ready-to-eat shrimps (16/20; 80%) followed by raw shrimps (11/20; 55%) and cooked shrimps (8/20; 40%). Among the 35 isolates, *Enterococcus faecalis* (62.9%) and *Enterococcus faecium* (28.6%) were the dominant species. More than half of all investigated isolates (65.7%) were resistant to at least one class of antibiotic, and 16 (45.7%) of the strains were classified as multidrug resistant. A high percentage of isolates were resistant to tetracycline (48.6%), followed by tigecycline and fosfomycin (45.7%). All of the enterococci isolates, carried between 5 and 9 tested virulence genes. The presence of *esp* (100%), *gel*E (88.6%), *efa*A (77.1%) genes and sex pheromones *cpd* (100%) *cob* (94.3%), *ccf* (94.3%) were found most frequently in all of the tested enterococci species. Shrimp strains showed a moderate or weak ability to produce biofilm. Our data indicate that shrimps can be considered a reservoir of antibiotic resistant, virulence strains from the genus *Enterococcus*.

1. Introduction

Enterococcus spp. are one of the most common groups of bacteria present in foods. These bacteria are also dominant in shrimps and seawater (Dalgaard et al., 2003; Di Cesare et al., 2014). The ubiquity of enterococci in food is mainly a result of their resistance to adverse environmental conditions during production technology, as well as food storage conditions and their high adaptability. Because of their relative abundance and their resistance to environmental factors, enterococci have been proposed as indicator bacteria for antimicrobial resistance, as well as hygiene quality indicators for food and water (Boehm & Sassoubre, 2014).

Although they were considered to be safe microorganisms for many years, a rapid increase in the incidence of hospital infections has made them an urgent subject of interest. During the past few decades, enterococci have emerged as important healthcare-associated pathogens (Arias & Murray, 2012; Benenson et al., 2009; Gilmore, Clewell, Ike, & Shankar, 2014; Khan, Ahma, & Mehboob, 2015). The high prevalence of resistance and virulence factors makes a safety assessment of enterococcal strains a difficult task (EFSA, 2008). Enterococci are intrinsically resistant/tolerant to many antibiotics and are able to acquire drug resistance either by chromosome, transfer of plasmid or transposon acquisition containing genetic sequences that confer resistance in other bacteria (Ben Belgacem et al., 2010; Hammerum, Lester, & Heuer, 2010).

As well as transmissible antibiotic resistance plasmids, virulence factors are known to be transmissible by highly efficient gene transfer mechanisms (Eaton & Gasson, 2001). In the last decade, several virulence factors have been described in enterococci including cytolysins, gelatinase, serine protease, hyaluronidase, aggregation substance, extracellular surface protein, cell wall adhesins and biofilm formation (Barbosa, Gibbs, & Teixeira, 2010). Although the detection of virulence factors may indicate a virulence potential in food isolates, foodborne enterococcal infections have never been reported. However, the consumption of food carrying antibiotic-resistant bacterial populations is a possible transfer route.

It has been suggested that biofilms are an important factor in the pathogenesis of enterococcal infections (Creti et al., 2004; Mohamed, Huang, Nallapareddy, Teng, & Murray, 2004). Around 80% of persistent bacterial infections in the United States were found to be associated with biofilms (Janssens et al., 2008). Since they can render their inhabitants more resistant to disinfectant (Sidhu, Langsrud, & Holck, 2001), biofilms have become

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problematic in a wide range of food industries, including seafood processing. Seafood factories are vulnerable to biofilm formation on surfaces and within water distribution pipes. Biofilms result from bacterial attachment and growth in aqueous environments that produce bacteria resistant to sanitizing agents (Shikongo-Nambabi, 2011).

The presence of commensal microbiota in environmental ecosystems (Salyers & Shoemaker, 2006), human ecosystems and in food (Gazzola, Fontana, Bassi, & Cocconcelli, 2012; Wang, 2012) suggests that microorganisms can play a much more important role in transferring resistance genes to antibiotics than was initially thought and the food chain may play a key role in the transmission of resistance between the environment and humans (Marshall, Dorothy, & Levy, 2009). In recent years, a growing interest in seafoods, which are considered balanced, healthy food has been observed. Therefore, the objectives of this study were to estimate the resistance frequency in *Enterococcus* spp. isolated from retail shrimps, to analyze virulence factors and biofilm formation as well as to describe the associations between strains.

2. Materials and methods

2.1. Sampling collection

Sixty shrimp samples included 20 raw shrimps from 3 distributors (originated from Vietnam, Thailand or Bangladesh), 20 cooked shrimps (which were pre-cooked before being sold) from 2 manufacturers and 20 ready-to-eat (RTE) shrimps (such as shrimps in sauce, shrimps in oil, shrimps in marinade, etc. from 4 manufacturers) were collected in Olsztyn (in north–eastern Poland). Immediately after purchase, the samples were transported to a laboratory and analyzed.

2.2. Isolation and presumptive identification of enterococcal strains

Food samples (10 g) were homogenized in 90 ml of buffered peptone water (Merck, Germany), incubated overnight at 37 °C and streaked on selective plates containing Slanetz–Bartley agar (Merck). One or two colonies per sample, were selected with typical enterococcal morphology. Presumptive identification of isolates was carried out with the following tests: Gram staining, catalase and oxidase production, growth at 10 °C and 45 °C, growth in the presence of 6.5% NaCl, growth at pH 9.6 and growth and esculin hydrolysis on bile-esculin agar (Merck). The enterococcal isolates were deposited in the laboratory culture collection in the Department of Industrial and Food Microbiology. Prior to analysis, isolates were stored in a Microbank at −80 °C (Biocorp, Poland).

2.3. PCR identification of Enterococcus spp. strains

Identification using a Simple and Multiplex PCR was performed with primers and conditions described previously (Deasy, Rea, Fitzgerald, Cogan, & Beresford, 2000; Dutka-Malen, Evers, & Courvalin, 1995; Kariyama, Mitsuhata, Chow, Clewell, & Kumon, 2000; Knijff, Dellaglio, Lombardi, Andrighetto, & Torriani, 2001), specific for the *Enterococcus* spp., *Enterococcus faecium*, *Enterococcus faecalis*, *E. casseliflavus*, *E. gallinarum*, *Enterococcus durans* and *Enterococcus hirae* (Table 1). For DNA extraction, bacterial strains were streaked onto brain heart infusion agar (Merck) and incubated overnight at 37 °C. The colonies were suspended in a Tris–EDTA (Sigma–Aldrich, Poland) buffer and were lysed by lysozyme enzyme (0.6 mg/ml) (A&A Biotechnology, Poland). The total genomic DNA of isolated and reference strains was extracted using the Genomic Mini DNA purification kit (A&A Biotechnology) according to the manufacturer's instructions. All PCR reactions using a PCR reaction mixture were analyzed by electrophoresis through a 1.5% high resolution agarose gel (Promega, Poland) in 1 × TBE buffer pH 8.3. The sizes of the amplification products were estimated by comparison with a 100-bp molecular size ladder (Thermo Scientific, Fermentas, Poland). Gels were stained with ethidium bromide (Sigma–Aldrich) and viewed under UV light using a transilluminator (Gel-doc; Bio-Rad Hercules, USA). Each profile was visually compared with those obtained from the enterococcal reference strains: *E. faecalis* ATCC 29212; *E. faecium* ATCC 19434; *E. casseliflavus* ATCC 49605; *E. gallinarum* ATCC 700425 and *E. durans* ATCC 49479.

2.4. Antibiotic susceptibility testing

Antimicrobial susceptibility was determined using the disc diffusion method. Eighteen antibiotics commonly used in the treatment of clinical infection or in agricultural procedures were tested. Their names and respective disc contents were as follows: ampicillin 10 μg (AMP), penicillin G 10 IU (P), gentamicin 120 μg (CN), streptomycin 300 μg (S), vancomycin 30 μg (VA), teicoplanin 30 μg (TEC), norfloxacin 10 μg (NOR), levofloxacin 5 μg (LEV), ciprofloxacin 5 μg (CIP), tetracycline 30 μg (TE), tigecycline 15 μg (TGC), rifampicin 5 μg (RD), erythromycin 15 μg (E), nitrofurantoin 300 μg (F), linezolid 30 μg (LZD), fosfomycin 200 μg (FOT), chloramphenicol 30 μg (CL) and quinupristin/dalfopristin (for *E. faecium*) 15 μg (QD). Cartridges with commercially-prepared paper discs containing the appropriate antibiotic dosage were purchased from Oxoid (United Kingdom). Disk diffusion assays were performed on Mueller-Hinton Agar (Merck). An overnight culture of enterococcal isolates was spotted on the surface of the Mueller-Hinton agar. Antibiotic discs were then placed on the plates and incubated at 37 °C. Zone diameters were recorded after a 24 h incubation period. Strains were classified as resistant and susceptible according to the criteria from CLSI (2012). *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control organisms.

2.5. Screening for virulence genes

The following virulence genes: gelatinase (*gel*E), enterococcal surface protein (*esp*), enterococcal surface adhesion (*ace*), aggregation substance (*agg*), cytolysin operon (cylA), cell wall adhesins (*efa* A) and sex pheromones (*cpd*, *cob*, *ccf*) were amplified by PCR using published specific primers and conditions (Eaton & Gasson, 2001; Mannu et al., 2003; Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999; Vankerckhoven et al., 2004) (Table 1). The amplicons were evaluated by 1.5% agarose gel electrophoresis followed by staining in ethidium bromide (0.5 mg/ml) (Sigma–Aldrich) and were visualized on a UV transilluminator.

2.6. Production of gelatinase and hemolysin

For the detection of gelatinase activity, enterococci were inoculated on BHI agar plates (Merck) containing 3% gelatin (BTL, Poland), which were then incubated at 25 °C for 48 h. The presence of a turbid zone around the colonies indicated gelatinase activity.

Hemolysin activity was determined on Columbia agar (Merck, Germany) containing 5% defibrinized sheep blood. After 48 h at 37 °C incubation, the presence or absence of clearing zones around the colonies was interpreted as β-hemolysis (positive hemolytic activity) or γ-hemolysis (negative hemolytic activity), respectively. When observed, greenish zones around the colonies were interpreted as α-hemolysis and regarded as negative for the assessment of hemolytic activity (Barbosa et al. 2010).

Table 1
Oligonucleotides used in PCR reactions.

Species/gene	Primer sequence (5′→ 3′)	Amplicon size (bp)	Source
Enterococcus spp.	TCAACCGGGGAGGGT ATTACTAGCGATTCCGG	733	Deasy et al. 2000
E. faecalis	TCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	941	Dutka-Malen et al., 1995
E. faecium	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	658	Dutka-Malen et al., 1995
E. casseliflavus	CGGGGAAGATGGCAGTAT CGCAGGGACGGTGATTTT	488	Kariyama et al., 2000
E. gallinarum	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	Kariyama et al., 2000
E. durans	TTATGTCCCWGTWTTGAAAAATCAA TGAATCATATTGGTATGCAGTCCG	186	Knijff et al., 2001
E. hirae	TTTTGTTAGACCTCTTCCGGA	377	Knijff et al., 2001
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	Vankerckhoven et al., 2004
ace	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	320	Mannu et al., 2003
efaA	CGTGAGAAAGAAATGGAGGA CTACTAACACGTCCACGAATG	499	Mannu et al., 2003
gelE	AGTTCATGTCTATTTTCAC CTTCATTATTTACACGTTTG	402	Mannu et al., 2003
agg	CCAGTAATCAGTCCAGAAACAAACC TAGCTTTTTTCATTCTTGTGTTTGTT	406	Mannu et al., 2003
esp	TTACCAAGATGGTTCTGTAGGCAC CCAAGTATACTTAGCATCTTTTGG	913	Shankar et al., 1999
cpd	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	782	Eaton & Gasson, 2001
ccf	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	543	Eaton & Gasson, 2001
cob	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCAT	1405	Eaton & Gasson, 2001

2.7. Detection of biofilm formation by the microplate (MP) method

The method for assessing biofilm formation by the MP method was based on the techniques previously proposed by Stepanović, Vuković, Dakić, Savić, and Svabić-Vlahović (2000) and Stepanović et al. (2007) with some modifications. Wells of sterile, 96-well flat–bottomed polystyrene microtiter Nunc plates (Thermo Scientific, Fermentas) were filled with 200 μL of fresh sterile BHI broth (Merck, Germany). Twenty microliter of overnight cultures of each strain with a cell density of 1×10^9 cells/ml were added in triplicate, onto a 96-well. Negative control wells contained broth only. The plates were covered and incubated aerobically at 36 °C for 24 h. The bacterial suspension was aspirated and each well was washed three times with 250 μL of PBS buffer (Sigma). Following this the biofilm was fixed with 200 μL of ethanol (99%) for 15 min, and was later removed. The plates were dried at room temperature, stained with a 200 μL of crystal violet solution used for Gram staining (Merck) for 5 min, washed in running water until the unbound crystal violet was removed and dried at room temperature. The dye bound to the adherent cells was re-solubilized with 160 μL of 33% (v/v) glacial acetic acid (Sigma) per well. Absorbance was read using an Infinite M1000 PRO plate reader (Tecan) at 570 nm. The optical density (ODs) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the cut-off OD (OD_c) which was defined as three standard deviations above the mean OD of the negative control. The following classification was used for the determination of biofilm formation: no biofilm production (OD ≤ ODc), weak biofilm production (ODc < OD ≤ 2xODc), moderate biofilm production (2xODc < OD ≤ 4xODc) and strong biofilm production (4xODc < OD) (Stepanović et al. 2000).

3. Results

3.1. Isolation and identification of enterococci from shrimps

From 60 samples of shrimps, 35 enterococci strains were isolated (there were 28 positive samples). According to PCR amplification, four different species were identified. The majority of these were *E. faecalis* (62.9%, 22/35), followed by *E. faecium* (28.6%, 10/35), *E. casseliflavus* (5.7%, 2/35) and *E. gallinarum* (2.9%, 1/35). Most of the strains (n = 16) isolated from ready-to-eat shrimps included 10 *E. faecalis*, 4 *E. faecium*, 1 *E. casseliflavus* and 1 *E. gallinarum*. From 20 samples of cooked shrimps, 8 enterococcal strains, including 6 *E. faecalis* and 2 *E. faecium* were isolated. Eleven strains were isolated from raw shrimps, of which 6 were identified as *E. faecalis* and 5 as *E. faecium* (Table 2).

3.2. Antibiotic resistance

More than half of all investigated isolates (n = 23; 65.7%) were resistant to at least one class of antibiotic, and 16 (45.7%) of the strains were classified as multi-drug resistant (MDR; resistant to three or more classes of antibiotics). Overall, MDR enterococci were common among isolates from RTE shrimps and were not common among isolates obtained from raw or cooked shrimps. A high percentage of isolates were resistant to TE (n = 17; 48,6%), followed by TGC and FOT (n = 16; 45.7%), TEC (n = 13; 37.1%), E (n = 10; 28.6%), NOR (n = 6; 17.1%). Resistance to these antibiotics was higher in *E. faecalis* than in other species. Taking into account the source of isolation, strains isolated from RTE shrimps showed resistance to antibiotics more often than strains isolated from other sources. Resistance to LZD, and VAN antimicrobials, very important in human medicine, was not found in any enterococci. Resistance to CIP, also a clinically important drug, was found in 14.3% (n = 5) of strains, including three *E. faecalis* and one *E. faecium* isolated from RTE shrimps and one *E. faecalis* isolated from raw shrimps. Resistance to other antibiotics tested ranged from 8.6% to 2.9%. Since *E. faecalis* is intrinsically resistant to QD, resistance data for this antimicrobial was not reported. Resistance to QD was found only in one *E. casseliflavus* strains. Twelve enterococci strains (34.3%) were susceptible to all tested antimicrobials (Table 2).

3.3. Virulence factors

All of the enterococci isolates carried between 5 and 9 tested virulence genes. Three *E. faecalis* were positive for all tested virulence genes. *E. faecalis* strains harbor more virulence determinants than other species. All of the tested sex pheromone determinants (*cpd*, *cob*, *ccf*) were present in 31 out of 35 isolates. The gene *esp*, coding for enterococcal surface protein, was present in all isolates. The gene *gel*E, coding for extracellular metalloendopeptidase, was present in 30 out of 35 isolates. The gene *efa*A was detected only in *E. faecalis* (n = 20) and *E. faecium* (n = 7) strains. The gene *cylA* was detected only in three *E. faecalis* strains isolated from RTE shrimps and one *E. faecalis* strains isolated from raw shrimps. None of the *E. casseliflavus* and *E. gallinarum* strains harbored the surface adhesin gene (*efa*A), the aggregation protein gene (*agg*) or the cytolysin gene (*cylA*).

In comparing the results of phenotypic and genotypic analysis, it was observed that 30 isolates carried the *gel*E gene (85.7%) and 15 (42.9%) were gelatinase producers, but it is important to highlight

Table 2
The presence of virulence genes and antibiotic resistance phenotypes among enterococci isolated from retail shrimps.

Isolates	Species	Virulence genes	Gelatinase/Hemolysin activity	Biofilm formation	Phenotypic resistance	Shrimps
ERT-1	*E. faecalis*	*cpd, cop, ccf, efaA, gelE*, esp, ace*	–	negative	TE, E, TGC, TEC	ready-to-eat
ERT-4	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE, esp, ace, cylA**	gel, β-hem	moderate	TE, TGC, FOT, TEC, NOR, CIP	ready-to-eat
ERT-5	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE*, esp, ace*	–	negative	TE, E	ready-to-eat
ERT-7	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE*, esp*	–	Weak	TE, E, TGC, TEC	ready-to-eat
ERT-12	*E. faecalis*	*cpd, cop, ccf, efaA, gelE*, esp, ace*	–	negative	TE, E	ready-to-eat
ERT-13	*E. faecalis*	*cpd, cop, ccf, efaA, gelE, esp, ace*	gel	Weak	TE, TGC, FOT, C, NOR, CIP	ready-to-eat
ERT-15	*E. faecalis*	*cpd, cop, ccf, efaA, gelE, esp*	gel	Weak	TE, E, TGC, TEC	ready-to-eat
ERT-17	*E. faecalis*	*cpd, cop, ccf, efaA, gelE*, esp, ace*	–	negative	–	ready-to-eat
ERT-19	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE, esp, ace, cylA*	gel, β-hem	moderate	TE, TGC, FOT, TEC, CIP	ready-to-eat
ERT-20	*E. faecalis*	*agg, cpd, cop, ccf, gelE, esp, ace, cylA**	gel	negative	–	ready-to-eat
EC-1	*E. faecalis*	*agg, cpd, cop, ccf, efaA, esp, ace*	gel	Weak	TE, E, TGC, FOT, TEC, NOR	cooked
EC-5	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE*, esp*	–	negative	–	cooked
EC-8	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE, esp, ace*	gel	Weak	TE, TGC, FOT	cooked
EC-10	*E. faecalis*	*cpd, cop, ccf, gelE*, esp, ace*	–	negative	TE, TEC	cooked
EC-15	*E. faecalis*	*cpd, ccf, efaA, gelE,* esp, ace*	–	negative	–	cooked
EC-18	*E. faecalis*	*cpd, cop, ccf, efaA, esp, ace*	–	negative	E, FOT, TEC	cooked
ER-2	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE, esp, ace*	gel	Weak	TE, TGC, FOT, C, NOR, CIP	raw
ER-3	*E. faecalis*	*cpd, cop, ccf, efaA, gelE*, esp*	–	negative	–	raw
ER-8	*E. faecalis*	*cpd, ccf, efaA, gelE*, esp*	–	negative	–	raw
ER-11	*E. faecalis*	*cpd, cop, ccf, efaA, esp, ace*	–	Weak	RD, NOR	raw
ER-17	*E. faecalis*	*cpd, cop, ccf, efaA, gelE, esp, ace*	gel	negative	TE, TGC, FOT, TEC	raw
ER-18	*E. faecalis*	*agg, cpd, cop, ccf, efaA, gelE*, esp, ace, cylA**	–	Weak	–	raw
ERT-5	*E. faecium*	*agg, cpd, cop, ccf, efaA, gelE, esp, ace*	gel	Weak	TE, TGC, E, FOT, TEC, C	ready-to-eat
ERT-7	*E. faecium*	*agg, cpd, cop, ccf, efaA, gelE*, esp, ace*	–	Weak	E, FOT, TEC	ready-to-eat
ERT-11	*E. faecium*	*agg, cpd, cop, gelE*, esp*	–	negative	–	ready-to-eat
ERT-18	*E. faecium*	*cpd, cop, ccf, efaA, gelE, esp, ace*	gel	Weak	TE, TGC, FOT, NOR, CIP	ready-to-eat
EC-1	*E. faecium*	*cpd, cop, ccf, efaA, gelE*, esp, ace*	–	Weak	TGC, FOT, TEC	cooked
EC-10	*E. faecium*	*cpd, cop, ccf, efaA, gelE*, esp*	–	negative	–	cooked
ER-16	*E. faecium*	*agg, cpd, cop, ccf, efaA, gelE, esp, ace*	gel	Weak	TE, TGC, E, FOT	raw
ER-17	*E. faecium*	*cpd, cop, ccf, efaA, esp*	–	negative	–	raw
ER-18	*E. faecium*	*cpd, cop, ccf, gelE, esp, ace*	gel	negative	TGC, FOT	raw
ER-19	*E. faecium*	*cpd, cop, gelE*, esp, ace*	–	negative	–	raw
ER-20	*E. casseliflavus*	*cpd, cop, ccf, gelE, esp*	gel	negative	–	raw
ERT-2	*E. casseliflavus*	*cpd, cop, ccf, gelE, esp, ace*	gel	negative	TE, TGC, FOT, TEC, QD	ready-to-eat
ERT-6	*E. gallinarum*	*cpd, cop, ccf, esp, ace*	–	negative	E, RD	ready-to-eat

Abbrevations: *gel*E – gelatinase, *esp* – enterococcal surface protein, *ace* – enterococcal surface adhesion, *agg* – aggregation substance, *cylA* – the cytolysin operon, *efa*A – cell wall adhesins, *cpd*, *cob*, *ccf* – sex pheromones, TE – tetracycline, E – erythromycin, TGC – tigecycline, TEC – teicoplaine, NOR – norfloxacin, CIP – ciprofloxacin, C – chloramphenicol, RD – rifampicin, LZD – linezolid, QDA – quinupristin/dalfopristin, * – silents genes, "-" – trait has not been present.

that only 40% of them were positive for both genotypic and phenotypic characters. This confirms that some strains had "silent" genes when the presence of the determinant using PCR was observed but this was not phenotypically expressed. Moreover, one of the isolates was a gelatinase producer, but did not show the *gel*E gene.

Comparison of β-hemolysis and detection of the *cyl* gene revealed that cytolysin determinants behave as "silent" genes in two non-hemolytic isolates.

3.4. Biofilm formation

The data indicated that none of the shrimp isolates presented a strong ability to form biofilm on abiotic surfaces, but two *E. faecalis* strains isolated from RTE shrimps showed a moderate ability to produce biofilm. A weak ability to produce biofilm was shown by 13 isolates, on which 6 strains were isolated from RTE shrimps (three *E. faecalis* and three *E. faecium*), four from raw shrimps (three *E. faecalis* and one *E. faecium*) and three from cooked shrimps (two *E. faecalis* and one *E. faecium*).

The experiments also indicated that there was no association between the presence of *esp* and *gel*E and the ability to form biofilm. It was noted that although all isolates were *esp*-positive, only 15 were biofilm producers while from 31 *gel*E-positive strains, 14 were biofilm-positive. One *gel*E-negative strain had the ability to produce biofilm.

4. Discussion

Enterococci are one of the most common groups of bacteria present in foods, mainly due to their resistance to adverse environmental conditions during production technology, as well as food storage conditions and their high adaptability. The literature, has few reports on the incidence of enterococci in seafood products. Several researchers have suggested that seafood contamination occurs naturally from the environment where fish are harvested. Cross-contamination may occur during food processing or preparation where bacteria are transferred from raw fish, contaminated surfaces or from utensils to hygienically-safe seafood (Shikongo-Nambabi, 2011). Harvested shrimps are generally processed by washing the body and removing the head. They are then distributed and exported as frozen products. While processing and freezing partially reduces the bacterial levels, resistant survivors may contaminate the product sold at market and be passed onto consumers (Yano, Hamano, Satomi, Tsutsui, & Aueumneoy, 2011). 58.3% of the shrimp samples investigated in our study were positive for enterococci. Most of the strains were isolated from RTE shrimps (70% positive samples), followed by raw shrimps (45% positive samples) and cooked shrimps (30% positive samples). In RTE shrimp samples, the incidence of *E. faecalis* was higher than the incidence of *E. faecium*. This is in accordance with Hammad, Shimamoto, and Shimamoto (2014) who isolated enterococci from sashimi samples. On the other hand, 40% of *E. faecium* and only 27.2% of *E. faecalis* were isolated from raw shrimps, which is in accordance with previous reports published by Al Bulushi, Poole,

Barlow, Deeth, and Dykes (2010) and Valenzuela, Benomar, Abriouel, Canamero, and Galvez (2010), in which *E. faecium* was the most common species isolated from fresh fish. This may suggest that the RTE shrimp samples analyzed in our study were contaminated with enterococci originating from other sources than raw shrimps.

A specific cause for concern and a factor contributing to pathogenesis of enterococci is their resistance to a variety of antibiotics which can be acquired or found intrinsically. Most of the studies on antibiotic resistance and virulence of enterococci have concentrated on strains isolated from clinical samples. Recently, some researchers have suggested that environment and food could play a significant role in the transmission of resistance to humans (Barbosa et al., 2010; Chajęcka-Wierzchowska, Zadernowska, Nalepa, Sierpińska, & Łaniewska-Trokenheim, 2014; Chajęcka-Wierzchowska, Zadernowska, Nalepa & Łaniewska-Trokenheim, 2015; Gomes et al., 2008; Koluman, 2009). In the tests performed, resistance to at least one antibiotic was observed in 65.7% of the investigated strains. Multiple resistance to more than four to eight antibiotics was also reported in isolates from other aquaculture sources (Akinbowale, Peng, & Barton, 2006). The recovery of high percentages of MDR enterococci from shrimps, especially RTE shrimps, suggests that they may be an important reservoir of antimicrobial resistance phenotypes. Regardless of sample type and enterococcal species, isolates appeared to be highly resistant to the same antimicrobials. The highest resistance among all isolates in this study was to TE, TGC and FOT. Tetracyclines are broad-spectrum antibiotics frequently used in the treatment of human and animal infections (Chopra & Roberts, 2001). The high prevalence of resistance to tetracycline has been previously reported among enterococcal isolates from different sources (Chajęcka-Wierzchowska, Zadernowska, Nalepa, & Łaniewska-Trokenheim, 2012; Templer & Baumgartner, 2007). In our study of tetracycline resistant enterococci, 40% also showed resistance to tigecycline and 31% were also resistant to fosfomycin. No resistance to LZD or VA was encountered, which is in accordance with other studies of aquaculture products (Hammad et al., 2014).

Recent studies have associated enterococcal virulence with different factors, such as gelatinase (*gel*E), enterococcal surface protein (*esp*), enterococcal surface adhesion (*ace*), aggregation substance (*agg*), cytolysin operon (*cylA*), cell wall adhesins (*efa* A), sex pheromones (*cpd*, *cob*, *ccf*) and biofilm formation (Chuang, Schlievert, Wells, Manias, & Tripp, 2009). Molecular screening of the genes encoding virulence factor revealed distinct trends in the occurrence of virulence between species. *E. faecium* and *E. faecalis* strains carried more virulence-associated genes than other species, which is in accordance with reports by other authors (Han et al., 2011; Martin, Garriga, Hugas, & Aymerich, 2005).

In our study, the occurrence of the *gel*E gene was the most predominant virulence factor. The gene *gel*E is responsible for gelatinase production (Tsikrikonis et al. 2012). Gelatinase is a metalloproteinase containing zinc, which can hydrolyze casein, hemoglobulin, insulin, fibrinogen, collagen, gelatin, as well as various proteins/peptides (Giridhara Upadhyaya et al. 2009). Our results indicated that some *Enterococcus* isolates positive for *gel*E did not produce the enzyme. Indeed, according to Marra et al. (2007), the presence of *gel*E is not necessarily correlated with gelatinase activity. Some studies suggest that other genes may be associated with *gel*E expression control (Lindenstrau et al. 2011). A similar situation occurred in the case of hemolysis. Although β-hemolytic activity was not present in two of the tested isolates, they carried hemolysin-related gene *cylA*. The same situation was previously reported (Eaton & Gasson, 2001; Toğay, Keskin, Açik, & Temiz, 2010).

Most of the isolated strains harbored *efa*A and *esp* gene. Both of them seem to contribute to the colonization and persistence of enterococcal strains in ascending infections of the urinary tract. The genes *cpd*, *ccf* and *cob* were also identified in a large number of the strains. Eaton and Gasson (2001) reported that sex pheromone determinants were detected only in *E. faecalis* strains, while all of the *E. faecium* strains were clear of the sex pheromone determinants. This was in disagreement with our result, sex pheromones were identified in all species tested. Eaton and Gasson (2001) also stated that pheromone determinants sometimes occurred with, and sometimes without, the *agg* virulence gene. The current study produced similar results.

Another important factor in the pathogenesis of enterococcal infections is biofilm formation (Creti et al., 2004; Mohamed et al., 2004). Some authors have reported that an ability to form biofilm is more common among *E. faecalis* isolates than among other species, independent of their source (Baldassarri et al., 2001; Creti et al., 2004). We observed greater biofilm production among strains isolated from ready-to-eat shrimps than that isolated from raw or cooked shrimps. Some authors have indicated that mechanisms for biofilm formation are independent from *esp* (Hufnagel, Koch, Creti, Baldassarri, & Huebner, 2004; Kristich, Li, Cvitkovitch, & Dunny, 2004; Mohamed et al., 2004) while others have indicated that there is an association between the ability to form biofilm and the presence of *esp* (Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010; Tendolkar, Baghdayan, Gilmore, & Shankar, 2004). Gelatinase has also been suspected of being involved in the process of biofilm formation, by mediating signals arriving through the quorum-sensing *fsr* system (Hancock & Perego, 2004). However, Mohamed and Murray (2005) found a lack of a correlation between gelatinase and biofilm formation in a large collection of *E. faecalis* isolates. The results obtained by Mohamed et al. (2004) indicated that a more important factor than gelatinase in biofilm production may be serine protease. The results of our experiments indicated that there was no association between the presence of *esp* and *gel* and the ability to form biofilm.

5. Conclusions

Our data indicate that enterococci are widely present in retail shrimps. Many isolated strains are antibiotic resistant and carry transferable virulence factors which represent a potential source of resistance transmission to bacteria in humans.

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