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Antimicrobial resistance and virulence traits in *Enterococcus* strains isolated from dogs and cats

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SUMMARY

We investigated presence and prevalence of antibiotic-resistances and other biological characters in enterococci isolated from faeces of healthy dogs and cats because these microorganisms represent important human and veterinary pathogens/opportunists, and a significant burden for healthcare systems. In all samples (n=115) we detected enterococci, with a predominance of *Enterococcus faecium* (42; 36.5%) and *Enterococcus faecalis* (36; 31.3%) species, endowed with virulence traits and multidrug-resistance. The two predominant resistance patterns (erythromycin, tetracycline) were examined by polymerase chain reaction for *tet* and *erm* genes. Only *tet*M for tetracycline, and *erm*A and *erm*B for erythromycin were detected. PCR for gelatinase gene (*gelE*) was positive in 62.6% of isolates, but only 26.1% produce gelatinase suggesting the existence of silent genes. *efaAfs* and *efaAfm* genes were found in *E. faecalis* and *E. faecium* respectively. 89.6% of isolates produced bacteriocin-like substances with a prevailing action against *Listeria* genus and, among these, 33.9% were positive for the bacteriocin structural genes *entA*, *entL50* or *entP*. According to our study, pet animals can be considered a reservoir of potentially pathogenic enterococci and we cannot exclude that those microorganisms may be responsible for opportunistic infections in high-risk pet owners.

KEY WORDS: Antibiotic-resistances, Bacteriocin-like substances, Enterococci, Pets, Virulence factors.

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INTRODUCTION

Enterococcus spp. represent important human and veterinary pathogens/opportunists and a significant burden for healthcare systems worldwide. Although enterococci are commensal organisms in the gut of most animals and common in environments contaminated by human and animal faeces, they have emerged as nosocomial and community-acquired pathogens for their ability to develop high-level resistance to antimicrobials (Werner *et al.,* 2013). It is well known that the administration of antimicrobials in humans and animal for the treatment and control of infections, and in animals also as growth promoters can select resistant strains (Phillips *et al.,* 2004). One example is the possible connection in Europe between the use of avoparcin in poultry and swine industries as feed additive, and the occurrence of vancomycin-resistant enterococci in the intestine of animals and human consumers (Sundsjord *et al.,* 2001). The antibiotic-resistant enterococci that enter the intestinal tract might colonize the host or just transfer their resistance genes by conjugation to resident bacteria (Leclercq *et al.,* 1989; Schwarz *et al.,* 2001), including pathogens, during their passage through the gut (Descheemaeker *et al.,* 1999). For example, enterococci seem to be an important source of resistance genes (i.e. *vanA*) for *Listeria* spp. (Leclercq *et al.,* 1989; de Niederhausern *et al.,* 2004) and *Staphylococcus aureus* (Noble *et al.,* 1992; de Niederhausern *et al.,* 2011). In addition to antibiotic resistance, enterococci may exhibit

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biological characteristics like gelatinase and bacteriocin/cytolysin that are considered components of virulence (Eaton and Gasson, 2001; Jahan *et al.,* 2013). Furthermore, the strains that produce bacteriocins or bacteriocin-like substances have advantages in the competition for ecological niche colonization as antibacterial activity is assumed to be important in the regulation of population dynamics in bacterial ecosystems (Kalmokoff *et al.,* 1999). As many biological characteristics contributing to virulence in enterococci are frequently associated with pheromone-responsive conjugative plasmids (Laverde Gomez *et al.,* 2011), their spread can become extremely fast, in particular where the density of bacteria and the variety of species are high as in the gut of mammalians.
Many epidemiological studies on antimicrobial resistance have been conducted to better define the links between farm animals and humans. Previously most attention focused on farm animals, but more recently research has begun to investigate whether companion animals play a role as a reservoir of resistant organisms given the close physical contact between household pets and their owners (Guardabassi *et al.,* 2004; Gulhan *et al.,* 2006). Since the expression of all the above biological traits could justify an increased pathogenicity in enterococci and considering that companion animals are a potentially important reservoir of these microorganisms, the aim of the present investigation was to study the antimicrobial resistances, production of bacteriocin-like substances and virulence factors in enterococci isolated from faeces of healthy dogs and cats.

MATERIALS AND METHODS

Isolation and identification of enterococci

Faeces were collected from 79 dogs and 36 cats, all healthy, during routine visits to local veterinarians. All pets were fed with commercial food, periodically vaccinated and treated for parasites. The animals varied in sex, race, diet, and age and were not exposed to antimicrobials (including all oral, injectable and topical antibiotics) for at least one year prior to sample collection. This experiment was exempt from the institutional animal care and use committee because it did not involve direct contact with animals. Enterococci were isolated by streaking with a 10 µL loop serially diluted faeces samples on Kennel Fecal (KF)-Streptococcus agar (Difco Laboratories, Detroit, MI, USA). Plates were aerobically incubated for 48 h at 37°C. For each sample, a red colony with the typical enterococcal morphology was randomly selected and presumptively identified to the genus level by Gram staining, catalase test and bile-esculin hydrolysis. All presumptive enterococci were identified by biochemical characteristics using Api 20 Strep system (bioMérieux, Marcy l'Etoile, France). Species identification was confirmed by PCR (Dutka-Malen *et al.,* 1995; Knijff *et al.,* 2001).

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of enterococci were determined by agar dilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2011) guidelines using the EUCAST clinical breakpoints, when available, and in the other cases the breakpoints derived from CLSI (2011). The following antibacterial agents were tested: penicillin, ampicillin, amoxicillin, piperacillin, streptomycin, gentamicin, chloramphenicol, imipenem, tetracycline, erythromycin, lincomycin, rifampicin, nitrofurantoin, ciprofloxacin, teicoplanin, vancomycin, tigecycline. For each antimicrobial a twofold dilution was performed to obtain final concentrations from 0.5 to 256 µg mL^{-1}; only streptomycin concentrations were 8 to 2048 µg mL^{-1}. All compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA). *E. faecalis* ATCC 29212 was used as quality control reference strain. The isolates resistant to two or more classes of antimicrobials were considered multiresistant.

Hemolysis, gelatinase and casein hydrolysis assays

Enterococci were cultured in Tryptic Soy broth (TSB, Oxoid, Italy) at 37°C for 24 h and spotted onto Blood Agar Base (Oxoid) containing 5% of defibrinated horse blood (Oxoid). After overnight incubation at 37°C, the hemolytic activity was determined by the appearance of a clear zone of hemolysis (β-hemolysis), a partial and

greening hemolysis zone (α-hemolysis), or no activity (γ-hemolysis) around the spots.
Gelatinase production was evaluated using Todd-Hewitt agar plates containing gelatin (3% w/v) by spotting the plates with 10 µL of each suspension. After overnight incubation at 37°C, all the spots surrounded by an opaque halo were considered positive.
Casein hydrolysis was tested on Tryptic Soy agar (TSA, Oxoid) containing 3% (w/v) skimmed milk, by spotting the plates with 10 µL of each suspension followed by incubation at 37°C for 24 h. The presence of a transparent zone around the spots indicated caseinase activity.

Bacteriocin-like substance production assay
All isolates were screened for bacteriocin-like substance production by the deferred antagonism method (Kekessy and Piguet, 1970). The following strains were used as indicators: *Listeria monocytogenes* (NCTC 05105, NCTC 10888, NCTC 10890), *Listeria innocua* (ATCC BAA-680, ATCC 33090, NCTC 11288), *Enterococcus faecalis* ATCC 29212, *Lactobacillus acidophilus* ATCC 13651, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922. To eliminate inhibition due to hydrogen peroxide production, a first incubation was performed anaerobically. Antimicrobial activity was quantified by a clear zone of inhibition in the indicator lawn around the spot of the producer.

PCR detection of bacteriocin, virulence and antibiotic resistance determinants
Total DNA of strains, isolated by the rapid alkaline lysis method (Dutka-Malen *et al.,* 1990),

TABLE 1 - *Polymerase chain reaction primers and their products.*

Gene and primer	*Nucleotide sequence (5' to 3')*	*Product size (bp)*	*Ta (°C)*	*References*
gelE	TE9: ACCCCGTATCATTGGTTT TE10: ACGCATTGCTTTTCCATC	419	47	Eaton and Gasson (2001)
efaA$_{fs}$	TE5: GACAGACCCTCACGAATA TE6: AGTTCATCATGCTGTAGTA	705	47	Eaton and Gasson (2001)
efaA$_{fm}$	TE37: AACAGATCCGCATGAATA TE38: CATTTCATCATCTGATAGTA	735	46	Eaton and Gasson (2001)
agg	TE3: AAGAAAAAGAAGTAGACCAAC TE4: AAACGGCAAGACAAGTAAATA	1553	40	Eaton and Gasson (2001)
cylB	TE15: ATTCCTACCTATGTTCTGTTA TE16: AATAAACTCTTCTTTTCCAAC	843	40	Eaton and Gasson (2001)
cylM	TE13: CTGATGGAAAGAAGATAGTAT TE14: TGAGTTGGTCTGATTACATTT	742	40	Eaton and Gasson (2001)
cylA	F: TAGCGAGTTATATCGTTCACTGTA R: CTCACCTCTTTGTATTTAAGCATG	1282	55	Semedo *et al*. (2003)
entA	F: GGTACCACTCATAGTGGAAA R: CCCTGGAATTGCTCCACCTAA	138	58	Aymeric *et al*. (1996)
ent L50	F: ATGGGAGCAATCGCAAAATTA R: TAGCCATTTTTCAATTTGATC	274	56	Cintas *et al*. (1998)
ent P	F: GCTACGCGTTCATATGGTAAT R: TCCTGCAATATTCTCTTTAGC	87	56	Cintas *et al*. (1997)
erm A	5'-AAG CGG TAA ACC CCT CTG A-3' 5'-TTC GCA AAT CCC TTC TCA AC-3'	190	54	Strommenger *et al*. (2003)
erm C	5'-AAT CGT CAA TTC CTG CAT GT-3' 5'- TAA TCG TGG AAT ACG GGT TTG-3	299	54	Strommenger *et al*. (2003)
erm B	5'-CTATCTGATTGTTGAAGAAGGATT-3' 5'-GTTTACTCTTGGTTTAGGATGAAA-3'	142	54	Strommenger *et al*. (2003)
tet K	5'-GTA GCG ACA ATA GGT AAT AGT-3' 5'-GTA GTG ACA ATA AAC CTC CTA-3'	360	54	Strommenger *et al*. (2003)
tet M	5'-AGT GGA GCG ATT ACA GAA-3' 5'-CAT ATG TCC TGG CGT GTC TA-3'	158	54	Strommenger *et al*. (2003)

was subjected to PCR amplification to detect the presence of genes involved in the expression of cytolysin *(cylA, cylB, cylM*), aggregation substance (*agg*), gelatinase (*gelE*), enterococcal surface protein (*esp*), cell wall adhesins (*efaAfs* and *efaAfm*), using the primers described in Table 1. PCR amplification was also performed to detect structural genes of enterocin A (Aymerich *et al.,* 1996), enterocin P and enterocin L50 (Cintas *et al.,* 1997, 1998), and the antibiotic resistance determinants of tetracycline (*tet*K and *tet*M) and erythromycin (*erm*A, *erm*B and *erm*C) (Strommenger *et al.,* 2003) because those for tetracycline and erythromycin are the most frequent resistances observed in enterococci isolated from dogs and cats (Poeta *et al.,* 2005, 2006; Ossiprandi *et al.,* 2008; Jackson *et al.,* 2009).

RESULTS

Isolation and identification of bacterial strains

Enterococci were demonstrated and the preliminary identification of the isolates to species level by biochemical tests was confirmed by PCR results in all 115 samples from dogs and cats. The most frequently isolated species were *Enterococcus faecium* (42; 36.5%) and *Enterococcus faecalis* (36; 31.3%), followed by *Enterococcus casseliflavus* (15; 13%), *Enterococcus durans* (12; 10.4%), *Enterococcus gallinarum* (6; 5.2%), *Enterococcus hirae* (3; 2.6%), *Enterococcus avium* (1; 0.9%).

Antimicrobial susceptibility testing

All 115 isolates displayed antibiotic resistances. In particular, 32 strains (27.8%) showed from

TABLE 2 - *Distribution (%) of minimum inhibitory concentration among Enterococcus strains (n=115).*

	MIC (μg mL^{-1})													*Break Point*
	≤0.5	*1*	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	*1024*	*2048*	
Penicillin (30.4%)	15.7%	25.2%	20.9%	5.2%	2.6%	**7.8%**	**0**	**0**	**22.6%**	**0**				>8
Ampicillin (56.5%)	5.2%	13%	10.4%	9.5%	5.2%	**13%**	**20%**	**23.5%**	**0**	**0**				>8
Amoxicillin (39.1%)	25.2%	30.4%	5.2%	0	0	**39.1%**	**0**	**0**	**0**	**0**				>8
Piperacillin (31.2%)	13%	17.4%	13%	22.6%	2.6%	**0**	**7.8%**	**5.2%**	**7.8%**	**10.4%**				>8
Streptomycin (18.2%)					0	0	7.8%	7.8%	13%	2.6 %	50.4%	**13%**	**5.2%**	>512
Gentamicin (40.8%)	2.6%	2.6%	0	5.2%	22.6%	0	13%	0	13%	**13%**	**14.8%**	**0**	**13%**	>128
Chloramphenicol (61.8%)	0	0	0	5.2%	33%	0	**14.8%**	**35.7%**	**11.3%**	**0**				>16
Imipenem (56.4%)	9.5%	15.7%	5.2%	13%	0	**10.4%**	**2.6%**	**2.6%**	**1.7%**	**39.1%**				>8
Tetracycline (97.4%)	0	2.6%	0	0	0	**2.6%**	**25.2%**	**23.5%**	**46.1%**	**0**				>4
Erythromy-cin (81.7%)	5.2%	2.6%	2.6%	7.8%	**2.6%**	**5.2%**	**35.6%**	**2.6%**	**23.5%**	**12.2%**				>4
Lincomycin (0)	64.3%	35.7%	0	0	**0**	**0**	**0**	**0**	**0**	**0**				≥8
Rifampicin (60.8%)	13%	5.2%	20.9%	**25.2%**	**7.8%**	**18.3%**	**5.2%**	**0**	**2.6%**	**1.7%**				≥4
Nitrofurantoin (49.6%)	0	0	1.7%	2.6%	0	2.6%	5.2%	38.3%	**38.3%**	**11.3%**				>64
Ciprofloxacin (7.8%)	63.5%	15.6%	13%	**0**	**0**	**0**	**2.6%**	**0**	**0**	**5.2%**				≥4
Teicoplanin (0)	100%	0	0	0	0	**0**	**0**	**0**	**0**	**0**				>2
Vancomycin (0)	5.2%	46%	48.7%	**0**	**0**	**0**	**0**	**0**	**0**	**0**				>4
Tigecycline (0)	100%	0	0	0	0									>0,5

The numbers in bold show the percentage of strains resistant to each of the antibiotics tested.

2 to 4 antibiotic-resistances, 68 (59.1%) from 5 to 9, and 15 (13%) more than 9. The highest percentage of resistance (Table 2) was observed for tetracycline (97.5%), erythromycin (81.7%), chloramphenicol (61.8%) and rifampicin (60.8%). Enterococci were also resistant to ampicillin (56.5%), imipenem (56.4%), nitrofurantoin (49.6%), gentamicin (40.8%). A lower rate of resistance was found for amoxicillin, piperacillin and penicillin (39,1%, 31,2% and 30.4%, respectively) and only 21 strains (18.2%), were resistant to streptomycin and 9 (7.8%) to ciprofloxacin. While the relatively low percentage of quinolone-resistant isolates is in agreement with the results by Poeta *et al.,* (2006) reporting 8% of ciprofloxacin resistance, the resistance to high level of aminoglycosides (gentamicin, streptomycin) was detected in our isolates with a frequency higher than enterococci from pets in the EU studies of Poeta *et al.,* (2006) and Damborg *et al.,* (2008).

At last all enterococci were susceptible to lincomycin, teicoplanin and tigecycline, and with the exception of *E. casseliflavus* and *E. gallinarum* species that showed a characteristic intrinsic resistance, they were also susceptible to vancomycin. This result is in agreement with the studies on healthy and sick pets (dogs and cats) reported from Portugal (Rodrigues *et al.,* 2002; Delgado *et al.,* 2007), Italy (Ossiprandi *et al.,* 2008), the USA (Jackson *et al.,* 2009), Spain (Lopez *et al.,* 2013), Japan (Kataoka *et al.,* 2013), and Korea (Chun *et al.,* 2014). In our study, erythromycin and tetracycline resistances were observed more frequently than the other antimicrobials and similar results were reported by Poeta *et al.* (2005, 2006), Ossiprandi *et al.* (2008) and Jackson *et al.* (2009). As tetracycline and erythromycin are used for the treatment of a variety of infections in dogs and cats (urinary tract infections, periodontitis, upper respiratory tract infections, conjunctiva) (Guardabassi *et al.,* 2004; Escher *et al.,* 2012), their use could be the cause of a selective pressure for the resistance phenotype (Jackson *et al.,* 2009). Figure 1a shows the two predominant patterns of resistance (erythromycin and tetracycline) examined by PCR for the presence of genes encoding antibiotic resistance. Of the two types of *tet* genes investigated (*tet*K and *tet*M) only *tet*M have been identified. Of the three types of *erm* genes investigated (*erm*A, *erm*B and *erm*C) only *erm*A and *erm*B have been detected and *erm*B gene was the most represented. This result is in agreement with studies of enterococci from animal and human origin (Jensen *et al.,* 1999; Schmitz *et al.,* 2000; Mlynarczyk *et al.,* 2010). The finding that a significant rate of tetracycline resistant isolates shows co-resistance to erythromycin suggests that the selection of tetracycline resistant genotypes may provide a suitable molecular basis for the further selection of multiple resistances (Huys *et al.,* 2004).

Hemolysis, gelatinase, casein hydrolysis assays and PCR detection of virulence factor genes

β-hemolytic activity was absent in all enterococci, as also reported by Gulhan *et al.* (2006), while α-hemolysis was observed in 73 isolates

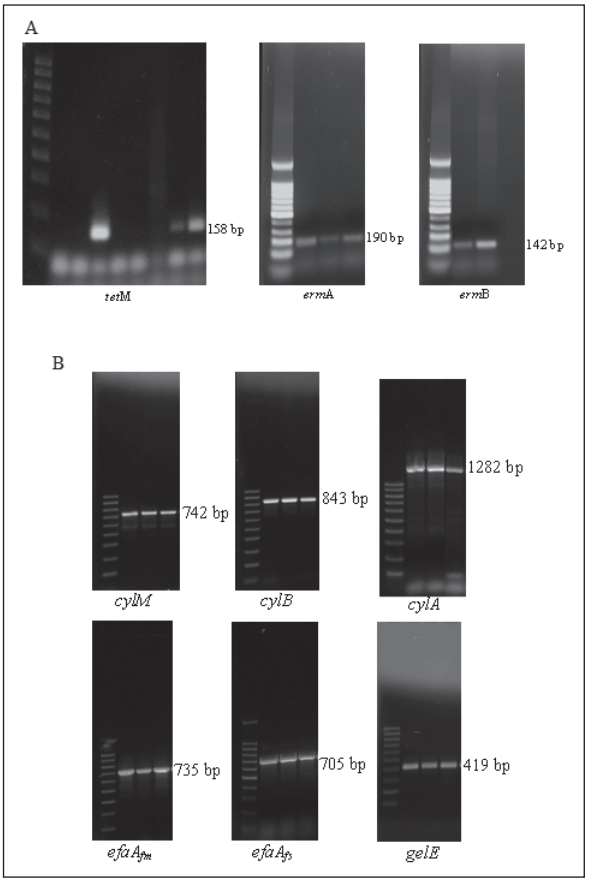

FIGURE 1 a, b - *Examples of detection of resistance (a) and virulence factor (b) genes in isolated enterococci by PCR reactions.*

(63.5%) (Table 3). Despite the absence of β-hemolysis, 15 enterococci (13%) gave positive results for the cytolysin production genes (*cylA*, *cylB*, *cylM*) (Table 4, Figure 1b). The lack of β-hemolytic activity may be explained by an incomplete hemolysin gene set, low levels or down-regulation of gene expression or an inactive gene product (Eaton and Gasson, 2001). Nevertheless, the molecular screening of *cyl* genes is necessary in non-hemolytic strains to evaluate their potential pathogenicity, since environmental factors may be involved in the control of cytolysin expression (Semedo *et al.,* 2003). Similarly, many enterococci carrying the *gel* gene were unable to hydrolyze gelatin suggesting either the existence of silent genes or the dependence of *gel* gene expression on environmental and culture conditions (Lopes *et al.,* 2006; Gomes *et al.,* 2008). Actually, thirty of the isolates (26,1%) produced gelatinase, while PCR analysis for the gelatinase gene *gelE* gave positive results for 72 enterococci (62.6%). In particular, *gelE* gene was present in all isolated species with the exception of *E. gallinarum* and *E. hirae*. This result is in agreement with studies that reported finding the *gelE* gene in *Enterococcus* species different from *E. faecalis* and *E. faecium* (Poeta *et al.,* 2006; Brtkova *et al.,* 2011).

Production of the adhesin-like *E. faecalis* and *E. faecium* endocarditis antigens (EfaAfs and EfaAfm respectively) is considered a potential virulence determinant. In our study, the *efaAfs* and *efaAfm* genes were only found in *E. faecalis* and *E. faecium* (33% and 50% of the isolates respectively). However, Semedo *et al.* (2003) and Barbosa *et al.* (2010) obtained negative amplifications for the *efaAfs* in *E. faecalis* and found *E.*

TABLE 3 - *Hemolysis, gelatinase and casein hydrolysis producers (%) among the 115 isolated strains.*

	α-hemolysis (%)	*β-hemolysis (%)*	*γ-hemolysis (%)*	*Gelatinase (%)*	*Casein hydrolysis (%)*
E. faecium (n=42)	27 (64.2)	0	15 (35.7)	3 (7.1)	36 (85,7)
E. faecalis (n=36)	21 (58.2)	0	15 (41.7)	15 (41.7)	36 (100)
E. casseliflavus (n =15)	6 (40)	0	9 (60)	9 (60)	9 (60)
E. durans (n=12)	9 (75)	0	3 (25)	3 (25)	12 (100)
E. gallinarum (n=6)	6 (100)	0	0	0	3 (50)
E. avium (n=3)	3 (100)	0	0	0	3 (100)
E. hirae (n=1)	1 (100)	0	0	0	1 (100)
Total (n=115)	73 (63.48)	0	42 (36.52)	30 (26.08)	100 (86.96)

TABLE 4 - *Distribution of virulence genes (%) found in the 115 isolated strains.*

	cylA (%)	*cylB (%)*	*cylM (%)*	*agg (%)*	*gelE (%)*	*esp (%)*	*efaAfs (%)*	*efaAfm (%)*	*ent A (%)*	*entP (%)*	*entL50 (%)*
E. faecium (n=42)	0	0	3 (7.1)	0	24 (57.1)	0	0	21 (50)	12 (28.5)	12 (28.5)	0
E. faecalis (n=36)	3 (8.3)	3 (8.3)	3 (8.3)	0	30 (80.3)	0	12 (33.3)	0	3 (8.3)	0	3 (8.3)
E. casseliflavus (n=15)	0	3 (20)	0	0	9 (60)	0	0	0	0	6 (40)	0
E. durans (n=12)	0	0	0	0	6 (50)	0	0	0	0	0	0
E. gallinarum (n=6)	0	0	0	0	0	0	0	0	3 (50)	0	0
E. avium (n=3)	0	0	0	0	3 (100)	0	0	0	0	0	0
E. hirae (n=1)	0	0	0	0	0	0	0	0	0	0	0
Total (n=115)	3 (2.6)	6 (5.2)	6 (5.2)	0	72 (62.6)	0	12 (10.4)	21 (18.2)	18 (15.6)	18 (15.6)	3 (2.6)

faecalis and other species isolated from different samples with *efaAfm*.
Aggregation substance (Agg) is a pheromone inducible surface protein that promotes mating aggregate formation during bacterial conjugation (Clewell, 1993). The enterococci *agg* positive may act as a source for horizontal transfer to other enterococci in the gut. This virulence factor could mediate the invasion and translocation through intact colonic mucosa (Giridhara Upadhyaya *et al.,* 2010) and this may be the reason aggregation substance is more common among why blood and endocarditis isolates (Coque *et al.,* 1995). In our strains, the gene for aggregation substance was not found, a reassuring result that suggests a reduced capability of the strains to transfer antibiotic resistance and virulence traits by mating.

Bacteriocin-like substances evaluation and PCR amplification of enterocin genes
One hundred and three of the 115 enterococci (89.6%), showed antibacterial activity against at least two indicators (Table 5). The prevailing type of antagonism (75 producers equal to 77.1%), was directed against *Listeria* genus while *L. acidophilus* ATCC 13651 and *S. aureus* ATCC 6538 were inhibited respectively by 21 (18.3%) and 9 (5.2%) strains. Conversely, *E. coli* ATCC 25922 was not inhibited. The antibacterial activity was not significantly related to the isolated species even if *E. faecalis* exhibited the best inhibitory action. For bacteriocin genes tested (*entA*, *entL50* and *entP*) (Table 4), 39 isolates (33.9%) gave positive results. Enterocin A gene was found in 12 *E. faecium* (28.6%), 3 *E. faecalis* (8.3%) and 3 *E. gallinarum* (50%), enterocin L50 gene in 3 *E. faecalis* (8.3%), enterocin P gene in 12 *E. faecium* (28.6%) and 6 *E. casseliflavus* strains (40%). The gap detected in our study between the antibacterial activity and the occurrence of bacteriocin genes suggests that other types of bacteriocins or other inhibitory substances may have affected the outcome. Our results are in agreement with Brandão *et al.* (2010) studies on enterococci in which the gene encoding EntP (*entP*) was the most represented in all tested ecosystems (humans, pets and wild animals) and the structural genes of EntA (*entA*) and EntL50 (*entL50A* and *entL50B*) were the next most frequently detected. The results related to the bacteriocin-like substances production and to the enterocin genes also show no important differences compared to those obtained from enterococci isolated in different environmental niches (Sabia *et al.,* 2008). Actually there is no preferential ecological environment for the isolation of bacteriocin-producing enterococci. This fact may be explained by the capability of enterococci to acquire and exchange genetic information by conjugation.

TABLE 5 - *Bacteriocin-like substance producers (%) among the 115 isolated enterococci.*

Indicators % BLS producers	-	+	++
Listeria monocytogenes NCTC 05105	38.5%	54%	7.5%
Listeria monocytogenes NCTC 10888	36%	61.5%	2.5%
Listeria monocytogenes NCTC 10890	69%	18%	13%
Listeria innocua ATCC BAA-680	36%	61.5%	2.5%
Listeria innocua NCTC 11288	79.5%	20.5%	0%
Listeria innocua ATCC 33090	90%	7.5%	2.5%
Enterococcus faecalis ATCC 29212	28%	56%	16%
Lactobacillus acidophilus ATCC 13651	82%	18%	0%
Staphylococcus aureus ATCC 6538	92%	8%	0%
Escherichia coli ATCC 25922	100%	0%	0%

-, no zone of inhibition; +, 5 mm <zone <10 mm; ++, 10 mm<zone<15 mm.

DISCUSSION

This work investigated antibiotic resistances and virulence traits, and the production of bacteriocin-like substances in enterococci isolated from faeces of healthy dogs and cats. No important differences between strains isolated from the two types of companion animals were observed, and we collected and processed our data considering dogs and cats a unique reservoir of potentially pathogenic enterococci. In all samples we confirmed the presence of enterococci, with the predominance of *E. faecium* and *E. faecalis*, species endowed with multidrug resistance and virulence traits. Our results are in agreement with other authors (Rodrigues *et al.,* 2002; Poeta *et al.,* 2006; Ossiprandi *et al.,*

2008; Jackson *et al.,* 2009) and confirm that *E. faecium* and *E. faecalis* are the most frequently isolated species from faeces of dogs and cats. Multi-resistant bacteria colonizing the intestinal tract of healthy pets can reach a human host and exchange their resistance genes with bacteria resident in the human host; these bacteria are a cause of concern especially when the microorganisms carry resistance genes of clinical relevance and if associated with transferable genetic elements (Guardabassi *et al.,* 2004). PFGE data in a recent work by Chang *et al*. (2014) strongly indicated the possibility for cross-transmission of antibiotic-resistant *Enterococcus* clones among veterinary hospital-associated environments, such as dog patients, their owners, veterinary staff, and hospital environments.

In agreement with previous studies (Poeta *et al.,* 2005, 2006; Ossiprandi *et al.,* 2008; Jackson *et al.,* 2009), erythromycin and tetracycline are the two predominant patterns of resistance observed in our isolates. This result is confirmed by the presence of *tet*M, *erm*A and *erm*B genes the most common genes encoding tetracycline and erythromycin resistance in enterococci (Jensen *et al.,* 1999; Schmitz *et al.,* 2000; Mlynarczyk *et al.,* 2010). The co-localization on Tn1545 of tetracycline and erythromycin resistances (*erm*B and *tet*M) has previously been reported in these bacteria (De Leener *et al.,* 2004). Consequently, acquired resistance to tetracycline was frequently found in enterococci carrying the *erm*B gene (Cauwerts *et al.,* 2007). The use of tetracyclines may thus co-select for resistance to macrolides, which may be important as an alternative therapy for enterococcal infections (Cauwerts *et al.,* 2007). In a way it is comforting to know that no resistance was observed against vancomycin, a glycopeptide considered to be the last resort drug in the treatment of enterococcal infections. Some authors (Lopez *et al.,* 2013) claim the selective pressure for maintenance of VRE with acquired mechanisms is less strong more than one decade after the avoparcin ban on animal growth promotion in the EU. This fact could justify the absence of vancomycin resistance among our isolates.

The other biological traits (hemolytic, gelatinase and caseinase activities), in addition to the ability to produce bacteriocin-like substances, and the presence of virulence genes (*gelE*, *efaAfm*, *efaAfs*, cytolysin and enterocin genes), may emphasize the pathogenicity of the isolates. In some cases, our strains possessed silent virulence genes also, and it is now known that environmental signals may play a role in gene expression, influencing the switch to pathogenicity. Nevertheless, none of the detected biological characters should be considered a definitive marker of pathogenicity. All of them could contribute to the virulence potential of enterococci, either for the presence of additional virulence factors or for a decrease in the host of the immunity response (Marra *et al.,* 2007). In conclusion, from our results, pets can be considered a reservoir of potentially pathogenic enterococci endowed with antimicrobial resistances and virulence factors. Therefore we cannot exclude the possibility that enterococci isolated from dogs and cats may be responsible for opportunistic infections in humans, particularly among high-risk owners.

REFERENCES

Aymerich T., Holo H., Håvarstein L.S., Hugas M., Garriga M., Nes, IF. (1996). Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Appl. Environ. Microb.* **62**, 1676-1682.

Barbosa J., Gibbs P.A., Teixeira P. (2010). Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. *Food Control.* **21**, 651-656.

Brandão A., Almeida T., Muñoz-Atienza E., Torres C., Igrejas G., Hernández P.E., et al. (2010). Antimicrobial activity and occurrence of bacteriocin structural genes in *Enterococcus* spp. of human and animal origin isolated in Portugal. *Arch. Microbiol.* **192**, 927-936.

Brtkova A., Revallova M., Bujdakova E. **(**2011). Dissemination of virulence factors and antimicrobial resistance in faecal enterococci from poultry. *Curr. Nutr. Food Sci.* **7**, 137-143.

Cauwerts K., Decostere A., De Graef E.M., Haesebrouck F., Pasmans F. (2007). High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the *erm*(B) gene. *Avian Pathol.* **36**, 395-399.

Chung Y.S., Kwon K.H., Shin S., Kim J.H., Park Y.H., Yoon J. W. (2014). Characterization of veterinary

hospital-associated isolates of *Enterococcus* species in Korea. *J. Microbiol. Biotechnol.* **24**, 386-393.

Cintas L.M., Casaus P., Håvarstein L.S., Hernández P.E., Nes I.F. (1997). Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* with a broad antimicrobial spectrum. *Appl. Environ. Microb.* **63**, 4321-4330.

Cintas L.M., Casaus P., Holo H., Hernández P.E., Nes I.F., Håvarstein L.S. (1998). Enterocins L50 A and L50 B, two novel bacteriocins from *E. faecium* L50, are related to staphylococcal hemolysins. *J. Bacteriol.* **180**, 1988-1994.

Clewell D.B. (1993). Bacterial sex pheromone-induced plasmid transfer. *Cell.* **73**, 9-12.

Clinical and Laboratory Standards Institute (CLSI), 2011. Performance standards for antimicrobial susceptibility testing; Table *Enterococcus* spp. M02 and M07 (M100-S21).

Coque T.M., Patterson J.E., Steckelberg J.M., Murray B.E. (1995). Incidence of hemolysin, gelatinase and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community base persons. *J. Infect. Dis.* **171**, 1223-1229.

Damborg P., Sørensen A.H., Guardabassi L. (2008). Monitoring of antimicrobial resistance in healthy dogs: first report of canine ampicillin-resistant *Enterococcus faecium* clonal complex 17. *Vet. Microbiol.* **132**, 190-196.

De Leener E., Martel A., Decostere A., Haesebrouck F. **(**2004). Distribution of the *erm* (B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. *Microb. Drug Resist.* **10**, 341-345.

de Niederhäusern S., Bondi M., Messi P., Iseppi R., Sabia C., Manicardi G., et al. (2011). Vancomycin-resistance transferability from VanA enterococci to *Staphylococcus aureus*. *Curr. Microbiol.* **62**, 1363-1367.

de Niederhäusern S., Sabia C., Messi P., Guerrieri E., Manicardi G., Bondi M. (2004). Glycopeptide-resistance transferability from vancomycin-resistant enterococci of human and animal source to *Listeria* spp. *Lett. Appl. Microbiol.* **39**, 483-489.

Delgado M., Neto I., Correia J.H., Pomba C. (2007). Antimicrobial resistance and evaluation of susceptibility testing among pathogenic enterococci isolated from dogs and cats. *Int. J. Antimicrob. Agents.* **30**, 98-100.

Descheemaeker P.R., Chapelle S., Devriese L.A., Butaye P., Vandamme P., Goossens H. (1999). Comparison of glycopeptides-resistant *Enterococcus faecium* isolates and glycopeptide resistance genes of human and animal origins. *Antimicrob. Agents Chemother.* **43**, 2032-2037.

Dutka-Malen S., Leclercq R., Coutant V., Duval J., Courvalin P. (1990). Phenotypic and genotypic heterogeneity of glycopeptides resistance determinants in gram-positive bacteria. *Antimicrob. Agents Chemother.* **34**, 1875-1879.

Dutka-Malen S., Evers S., Courvalin P. (1995). Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* **33**, 24-27.

Eaton T.J., Gasson M.J. (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microb.* **67**, 1628-1635.

Escher M., Vanni M., Intorre L., Caprioli A., Tognetti R., Scavia G. (2011). Use of antimicrobials in companion animal practice: a retrospective study in a veterinary teaching hospital in Italy. *J. Antimicrob. Chemother.* **66**, 920-927.

European Committee on Antimicrobial Susceptibility Testing (EUCAST) **(**2011). http://www.eucast.org. Accessed 5 September 2011.

Giridhara Upadhyaya P.M., Umapathy B.L., Ravikumar K.L. (2010). Comparative study for the presence of enterococcal virulence factors gelatinase, hemolysin and biofilm among clinical and commensal isolates of Enterococcus faecalis. *J. Lab. Physicians.* **2**, 100-104.

Gomes B.C., Esteves C.T., Palazzo I.C., Darini A.L., Felis G.E., Sechi L.A., et al. (2008). Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. *Food Microbiol.* **25**, 668-675.

Guardabassi L., Schwarz S., Lloyd D.H. (2004). Pet animals as reservoirs of antimicrobial-resistant bacteria. *J. Antimicrob. Chemother.* **54**, 321-332.

Gulhan T., Aksakal A., Ekin H., Savasan S., Boynukara B. (2006). Virulence factors of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from humans and pets. Turk. *J. Vet. Anim. Sci.* **30**, 477-482.

Huys G., D'Haene K., Collard J.M., Swings, J. (2004). Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. *Appl. Environ. Microb.* **70**, 1555-1562.

Jackson C.R., Fedorka-Cray P.J., Davis J.A., Barrett J.B., Frye J.G. (2009). Prevalence, species distribution and antimicrobial resistance of enterococci isolated from dogs and cats in United States. *J. Appl. Microbiol.* **107**, 1269-1278.

Jahan M., Holley R.A. (2013). Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25°C and 37°C. *Int. J. Food Microbiol.* **170**, 65-69.

Jensen L.B., Frimodt-Moller N., Aarestrup F.M. (1999). Presence of *erm* gene classes in gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiol. Lett.* **170**, 151-158.

Kalmokoff M.L., Daley E., Austin J.W., Farber J.M.

(1999). Bacteriocin-like inhibitory activities among various species of Listeria. *Int. J. Food Microbiol.* **50**, 191-201.

Kataoka Y., Ito C., Kawashima A., Ishii M., Yamashiro S., Harada K., et al. (2013). Identification and antimicrobial susceptibility of enterococci isolated from dogs and cats subjected to differing antibiotic pressures. *J. Vet. Med. Sci.* **75**, 749-753.

Kekessy D.A., Piguet J.D. (1970). New method for detecting bacteriocin production. *J. Appl. Microbiol.* **20**, 282-283.

Knijff E., Dellaglio F., Lombardi A., Andrighetto C., Torriani, S. (2001). Rapid identification of *Enterococcus durans* and *Enterococcus hirae* by PCR with primers targeted to the *ddl* genes. *J. Microbiol. Methods*. **47**, 35-40.

Laverde Gomez J.A., Hendrickx A.P., Willems R.J., Top J., Sava I., Huebner J., et al. (2011). Intra- and interspecies genomic transfer of the *Enterococcus faecalis* pathogenicity island. PLoS One **6**, e16720.

Leclercq R., Derlot E., Weber M., Duval J., Courvalin P. (1989). Transferable vancomycin and teicoplanin resistance in *Enterococcus faecium*. Antimicrob. *Agents Chemother.* **33**, 10-15.

Lopes M.F., Simões A.P., Tenreiro R., Marques J.J., Crespo M.T. (2006). Activity and expression of a virulence factor, gelatinase, in dairy enterococci Int. *J. Food Microbiol.* **112**, 208-214.

Lopez M., Tenorio C., Torres C. (2013). Study of vancomycin resistance in faecal enterococci from healthy humans and dogs in Spain a decade after the avoparcin ban in Europe. *Zoonoses Public Health.* **60**, 160-167.

Marra A., Dib-Hajj F., Lamb L., Kaczmarek F., Shang W.C., Beckius G., et al. (2007). Enterococcal virulence determinants may be involved in resistance to clinical therapy. *Diagn. Microbiol. Infect. Dis.* **58**, 59-65.

Mlynarczyk B., Mlynarczyk A., Kmera-Muszynska M., Majewski S., Mlynarczyk G. (2010). Mechanisms of resistance to antimicrobial drugs in pathogenic Gram-positive cocci. *Mini Rev. Med. Chem.* **10**, 928-937.

Noble W.C., Virani Z., Cree R.G. (1992). Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **72**, 195-198.

Ossiprandi M.C., Bottarelli E., Cattabiani F., Bianchi E. (2008). Susceptibility to vancomycin and other antibiotics of 165 *Enterococcus* strains isolated from dogs in Italy. Comp. *Immunol. Microbiol. Infect. Dis.* **31**, 1-9.

Phillips I., Casewell M., Cox T., De Groot B., Friis C., Jones R., et al. (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother.* **53**, 28-52.

Poeta P., Costa D., Rodrigues J., Torres, C. (2005). Study of faecal colonisation by *van*A-containing *Enterococcus* strains in healthy humans, pets, poultry, and wild animals in Portugal. *J. Antimicrob. Chemother.* **55**, 278-280.

Poeta P., Costa D., Rodrigues J., Torres C. (2006). Antimicrobial resistance and the mechanisms implicated in faecal enterococci from healthy humans, poultry and pets in Portugal. *Int. J. Antimicrob. Agents.* **27**, 131-137.

Rodrigues J., Poeta P., Martins A., Costa D. (2002). The importance of pets as reservoirs of resistant *Enterococcus* strains with special reference to vancomycin. *J. Vet. Med. B Infect. Dis. Vet. Public Health.* **49**, 278-280.

Sabia C., Niederhausern S., Guerrieri E., Messi P., Anacarso I., Manicardi G., et al. (2008). Detection of bacteriocin production and virulence traits in vancomycin-resistant enterococci of different sources. *J. Appl. Microbiol.* **104**, 970-979.

Schmitz F.J., Sadurki R., Krav A., Boos M., Geisel R., Kohrer K., et al. (2000). Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. *J. Antimicrob. Chemother.* **45**, 891-894.

Schwarz S., Kehrenberg C., Walsh T.R. (2001). Use of antimicrobial agents in veterinary medicine and food animal production. *Int. J. Antimicrob. Agents.* **17**, 431-437.

Semedo T., Almeida S.M., Martins P., Lopes M.F.S., Figueiredo Marques J.J., Tenreiro R., et al. (2003). Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the cyl operon in enterococci. *J. Clin. Microbiol.* **41**, 2569-2576.

Strommenger B., Kettlitz C., Werner G., Witte W. (2003). Multiplex PCR Assay for Simultaneous Detection of Nine Clinically Relevant Antibiotic Resistance Genes in *Staphylococcus aureus*. *J. Clin. Microbiol.* **41**, 4089-4094.

Sundsjord A., Simonsen G.S., Courvalin P. (2001). Human infections caused by glycopeptide-resistant *Enterococcus* spp.: are they a zoonosis? *Clin. Microbiol. Infect.* **7,** 16-33.

Werner G., Coque T,M., Franz C.M., Grohmann E., Hegstad K., Jensen L., et al., (20013). Antibiotic resistant enterococci-tales of a drug resistance gene trafficker. *Int. J. Med. Microbiol.* **303**, 360-379.