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Antimicrobial Susceptibility of Enterococcal Species Isolated from Italian Dogs

Maria Cristina Ossiprandi and Laura Zerbini

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Abstract

Monitoring planes of the occurrence of antimicrobial resistance among bacteria isolated from both animals and humans should be considered essential and strategic for preserving not only human health but also animal welfare (well-being). Moreover, the use of antimicrobial in companion animals (pets) received little attention and is not currently regulated in comparison with what happens in livestock; for this reason, the prevalence of antibiotic resistance in 165 different *Enterococcus* strains isolated from dogs (subjected to previous antibiotic treatment(s) or not) was determined. For each strain, the minimum inhibitory concentration (MIC) against 9 different antibiotics was assessed. While all isolated strains were susceptible to vancomycin, high resistance frequency toward erythromycin, rifampicin, enrofloxacin, and tetracycline was detected. *Enterococcus faecium* strains isolated from the previously treated dogs demonstrated more resistance to tetracycline compared to the control ones. Although canine enterococci showed a high degree of antibiotic resistance, they were susceptible to vancomycin, and for this reason, the hypothetical contamination of vancomycin-resistant enterococcal strains in dogs is still considered to be minimal in Italy.

Keywords: *Enterococcus*, antimicrobial susceptibility, dogs

1. Introduction

Multidrug resistance is an emerging problem in human pathogens, including zoonotic pathogens [1, 2]. Antimicrobial agents are routinely used to treat and prevent diseases in human and veterinary practices. The overuse and misuse of antibiotics provides tremendous selection, perhaps contributing spread of resistant clones, and acquisition of resistance determinants from resistant bacteria [3].

The problem of antimicrobial resistance has been declared to be one of the top concerns of the US Centers for Disease Control (CDC) [4].

In the United States, the annual healthcare cost associated with the treatment of antibiotic-resistant infections exceeds $4 billion/year [5].

This economic burden is associated with increased severity of illness due to treatment failure and long-term hospitalization. Longer hospital stays caused increased healthcare costs and more exposure to antibiotics. This has increased the severity of illness, and mortality rate is also high.

Inappropriate use of antibiotics for therapeutic and prophylactic purposes is considered a significant contributor to the emergence of antibiotic resistance in zoonotic pathogens [6] such as MRSA (methicillin-resistant *Staphylococcus aureus*), VRE (vancomycin-resistant enterococci), and extended-spectrum β-lactamase-producing *Escherichia coli*.

Commensal bacteria have become reservoirs of antibiotic resistance genes [7]. Studies [8–10] revealed a high frequency of antibiotic resistance among the fecal microbiota in humans. Further, commensals can act as a source of horizontal transfer of resistance genes to pathogens. Similarly, clonal spread [11, 12] and the transfer of resistant genes from animal bacteria to human bacteria [12] is a concern associated with antimicrobial resistance among commensal bacteria.

Resistance gene transfer between commensals and pathogens depends on several factors such as total number of donors and recipients, nutrition, selective pressure, and transfer mechanisms. The gut gene pool is large, harboring diverse population of microbes and thus providing a suitable environment for antibiotic resistance gene transfer [7]. The level of resistance among gut commensals such as *Enterococcus* spp. is considered a good indicator of antibiotic resistance [13].

A major factor associated with the dissemination of resistant determinants is selection pressure exerted by the use of antibiotics, selecting resistant bacteria by killing the susceptible ones. The removal of selection pressure will not eliminate the resistance genes from this bacterial population [14]. This increase in the fitness cost in the absence of any antibiotic selection pressure allows rapid spread of antimicrobial-resistant strains by replacing the susceptible ones [15].

Besides selective pressure by the antibiotics, there are other factors, such as "stress in animal," that can play a role in the prevalence of resistant bacteria in the gut [16–18]. All bacteria including commensals obligate, or opportunistic pathogens within the host are subjected to stressful conditions. For example, enteric bacteria have to overcome the effects of gastric acid (with varying pH depending on the diet of the individual), bile and organic acids, competing gut commensals (for binding the receptor sites and for nutrition), and host immune responses. Animals subjected to stressors such as infection, transportation, and change in the environment can release stress hormones via the enteric nervous system. Evidence indicates that these stress hormones enhance the bacterial growth and the expression of virulence determinants in enteric pathogens [19, 20] and affect intestinal functions such as decreasing gastric acidity [21].

During the recent decades, enterococci have gained considerable attention among public health officials because of their increasing antimicrobial resistance and as important nosocomial pathogens.

Enterococci are a part of the normal microbial flora in the gastrointestinal tracts of humans, animals, and birds. The major enterococcal species include *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus durans*. Enterococci do not cause illness in healthy humans or animals. However, they have recently been recognized as opportunistic nosocomial pathogens that cause infections of the urinary tract (UTI) and central nervous system and lead to endocarditis and bacteremia. In addition, enterococci can rapidly acquire antimicrobial resistance through mutations or acquisition of plasmids and transposons that contain foreign genetic material, including vancomycin resistance genes [22].

In recent years, the appearance of vancomycin-resistant enterococci (VRE) has caused serious problems both in humans and in veterinary medicine [23].

Vancomycin is an antibiotic of last resort in the treatment of Gram-positive bacterial infections including enterococcal infections. The emergence of vancomycin-resistant enterococcal strains and the risk of transmission of resistance genes to the susceptible bacteria pose a serious risk to public health [24]. The presence of VRE in clinical patients results in a 20% increase in treatment failure, and mortality is also increased from 27 to 52% [25, 26].

The contribution of enterococci to the problem of antimicrobial resistance is associated with its ability to pass the resistance determinants to other bacteria of the same species or different species by the process of conjugation. Thus, resistance gene transfer to pathogenic species and emergence of new type of resistance is a serious concern associated with these bacteria. Genome sequences have revealed that one-fourth of the total genome of *E. faecalis* V583 is composed of mobile genetic elements [27]. About three to five co-resident plasmids are commonly found in clinical isolates [28, 29].

VanA, *VanB*, and *VanC* clusters determine enterococcal resistance toward glycopeptides, but the genotype *VanA* corresponds to the prevailing in terms of importance under epidemiological point of view. In fact, *VanA* genotype represents the predominant resistant one characterized by the ability to obtain inducible resistance to both teicoplanin and vancomycin. The *VanB* cluster determines inducible resistance to various levels of vancomycin, and the strains carrying it show susceptibility toward teicoplanin due to the fact that this antibiotic does not act as an inducer. The *VanC* genotype supports resistance to chromosomally encoded glycopeptide and constitutively/naturally expressed resistance to low levels of vancomycin but susceptibility toward teicoplanin. Intrinsic resistance has been recognized for *E. gallinarum*, *E. flavescens*, and *E. casseliflavus*. *E. faecium* strains resistant to vancomycin (VRE) have been isolated from different animal species (in particular from pigs, chicken, and cattle) as well as from meat derived from them. Various epidemiological studies suggest that animals can carry VRE in their intestinal microbiota and be the source of VRE infection in human (according to a classical zoonotic cycle). In fact, these VRE strains of animal origin can determine colonization of human guts expressing their pathogenicity by transferring their resistance genes to other human intestinal bacteria [23].

Cohabitation between household pets and humans creates advantageous conditions for transferring bacteria not only through direct contact such as by licking, petting, handling, and physical injuries but also through the intervention of domestic environment by food contamination as well as furnishing and so on.

Children represent the category most at risk because of their behavioral habits: close physical contact with dogs and cats but with environment eventually contaminated by the pets themselves (such as floor, toys, and carpets). It is important to remember that horizontal resistance gene transfer may occur in the opposite direction to bacterial transmission. In fact, sometimes, human bacteria that transmitted to pets can acquire resistance genes from animal microbiota and can be selected as a consequence of antimicrobial treatment occurred in these animals. Anyway, even in the case of human-to-household animal transmission, pets contribute to amplify and propagate acquired resistant bacteria through fecal shedding both in environment and in humans [30].

While there are several studies confirming the presence of VRE strains in livestock, few reports focus on the VRE colonization in household animals although VRE have been isolated from canine [31, 32] and feline gut [32] and direct contact with such animal species was considered as frequent infection source for humans [33].

A relatively high occurrence (7–23%) of VRE, mainly *E. faecium* in dogs living in urban areas, has also been reported in Europe [34].

Regular monitoring of the level of resistance in pathogens and in indicator bacteria of the normal flora, such as fecal *E. coli* and enterococci, between both humans and animals has been recommended [35, 36]. This monitoring activity is fundamental [37], allowing to match the prevalence and evolution of resistance profiles and possibly to identify resistant bacteria transferring from animals to humans and vice versa.

Thus, the aim of this study was, on the one hand, to determine the phenotypic resistance patterns in gastrointestinal enterococci in dog (with particular attention to vancomycin) and, on the other hand, to investigate whether enterococci belonging to the normal gut show more resistance in dogs that have been treated with antimicrobial therapy compared with non-treated ones.

2. Materials and methods

Ninety-nine dogs aged more than 6 months, randomly selected among those treated at the Didactic Veterinary Hospital of the Department of Veterinary Sciences in Parma (northern Italy), were collected from rectal swabs during the years 2005 and 2006.

The pets included in this research are dogs living in households located in Parma and its province. They followed a diet based on commercial products and were periodically vaccinated and treated for parasites.

In particular, fifty-six dogs had received, at least, one antimicrobial treatment over the six months preceding the survey, while the last treatment must have been made at least fourteen days before the collection of the samples.

As a whole, the dogs received 111 therapy cycles (having several subjects received two or more treatments). The formulation corresponding to amoxicillin–clavulanic acid corresponded to the most frequent (26.1%) antibiotics administered, while cephalosporins corresponded to approximately 20% of all the administered treatments; enrofloxacin and doxycycline accounted for about 15%.

The remaining 43 control dogs received no antimicrobial treatment since birth or during the preceding 12 months.

2.1. Bacteriological investigation

Rectal samples were, suitably, processed two hours after the collection. First, they were diluted in nutritive broth and kept at a temperature of 60°C in water bath; then, the samples were incubated in nutrient broth, opportunely enriched with NaCl 6.5%, and inoculated both on KF streptococcus agar (Difco) and on kanamycin aesculin azide agar base (Oxoid). After 24 and 48 h of incubation at 36°C, respectively, the suspicious colonies were subjected to biochemical characterization [38]. After conducting this initial screening, which led to the identification of a preliminary biochemical profile, the strains were identified contextually by the Rapid ID 32 Strept System and/or by the API 20 Strep System (both from bioMérieux).

After the identification, only a single strain for species belonging to the same dog has been introduced in the research (in those situations in which the same species had been isolated several times in the same subject).

2.2. Susceptibility assay

The minimum inhibitory concentration (MIC) values were obtained using microdilution test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [39].

In this study, the following nine antibiotics were tested: amoxicillin, ampicillin, ciprofloxacin, enrofloxacin, erythromycin, ofloxacin, rifampicin, tetracycline, and vancomycin. In order to reach final concentrations ranging between 64 and 0.0312 μg/ml, each antibiotic was twofold-diluted.

MIC breakpoint was always set on the basis of CLSI guidelines [39].

The isolate was considered "resistant" in the case in which its MIC was equal or greater than the values (expressed in μg/ml) reported for each antibiotic tested: amoxicillin, 16; ampicillin, 16; ciprofloxacin, 2; enrofloxacin, 1; erythromycin, 1; ofloxacin, 4; rifampicin, 2; tetracycline, 8; and vancomycin 8.

The type strain used to devise the identification scheme and to verify the quality control was *E. faecalis* ATCC 29212.

3. Results

The epidemiological study highlighted the presence of *Enterococcus* spp. strains in each fecal sample analyzed. During the identification phase, it was found the isolation of more than one species of *Enterococcus* in the same dog.

This situation allowed to isolate 165 strains from 99 fecal specimens subjected to analysis. In particular, the following species were identified: *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, and *E. hirae*.
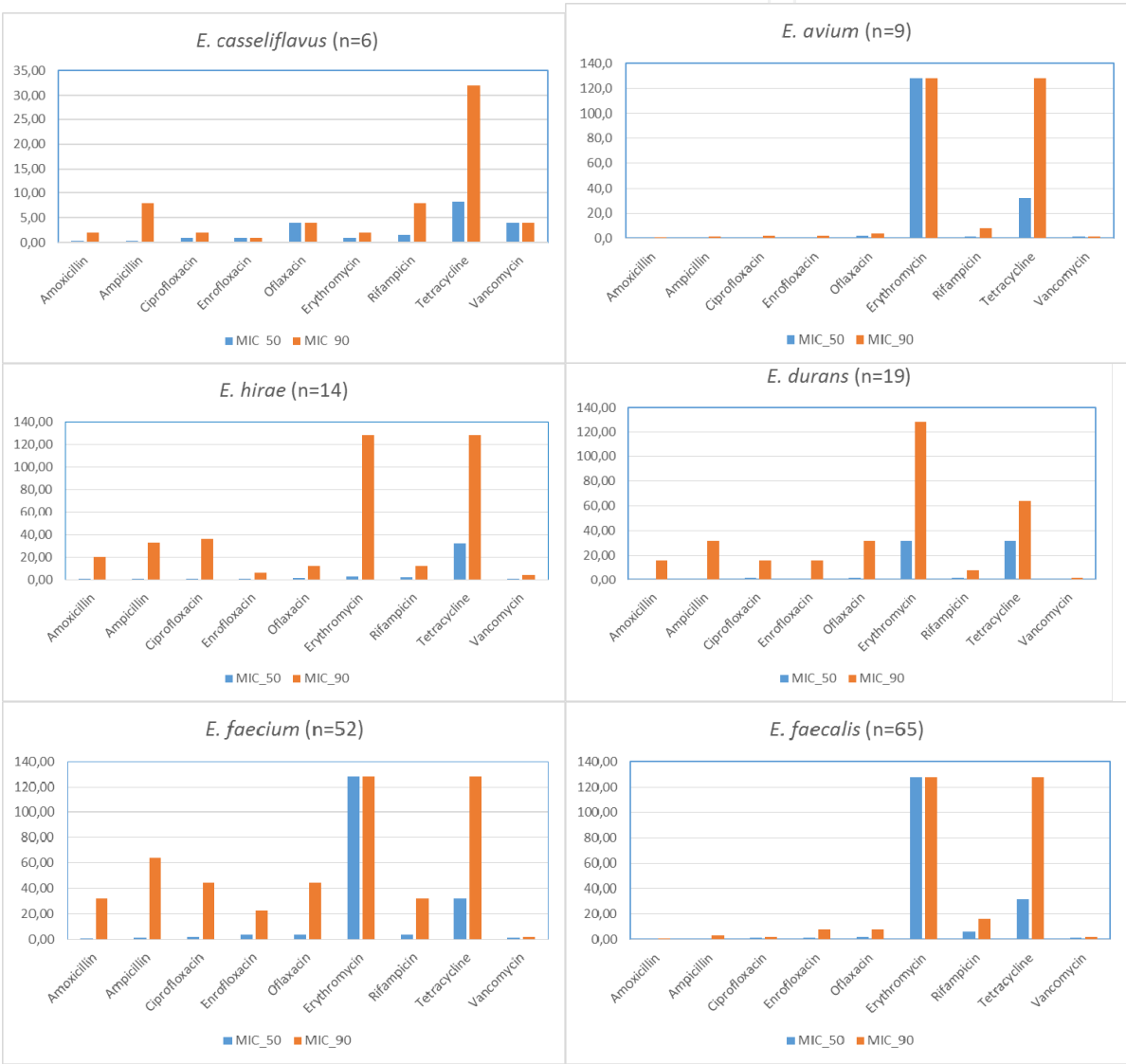

Figure 1. Results of susceptibility tests, for each species, based on MIC_{50} and MIC_{90} values [40]

Enterococcus faecalis corresponds to the prevalent species: 65 strains corresponding to 39.4% (95% CI: 32–34%), followed by *E. faecium* with 52 strains corresponding to 31.5% (95% CI: 25–39%). Together, the two above-mentioned species correspond to 70.9% of all the isolates.

Moreover, the other species isolated were 11.5% *E. durans*, 8.5% *E. hirae*, 5.5% *E. avium*, and 3.6% *E. casseliflavus*.

Results of susceptibility tests are presented in Figure 1, in which, for each species, the MIC_{50} and MIC_{90} values are summarized. These latter values represent the lowest concentration of an antimicrobial agent resulting in growth inhibition of 50% and 90% of the tested strains, respectively.
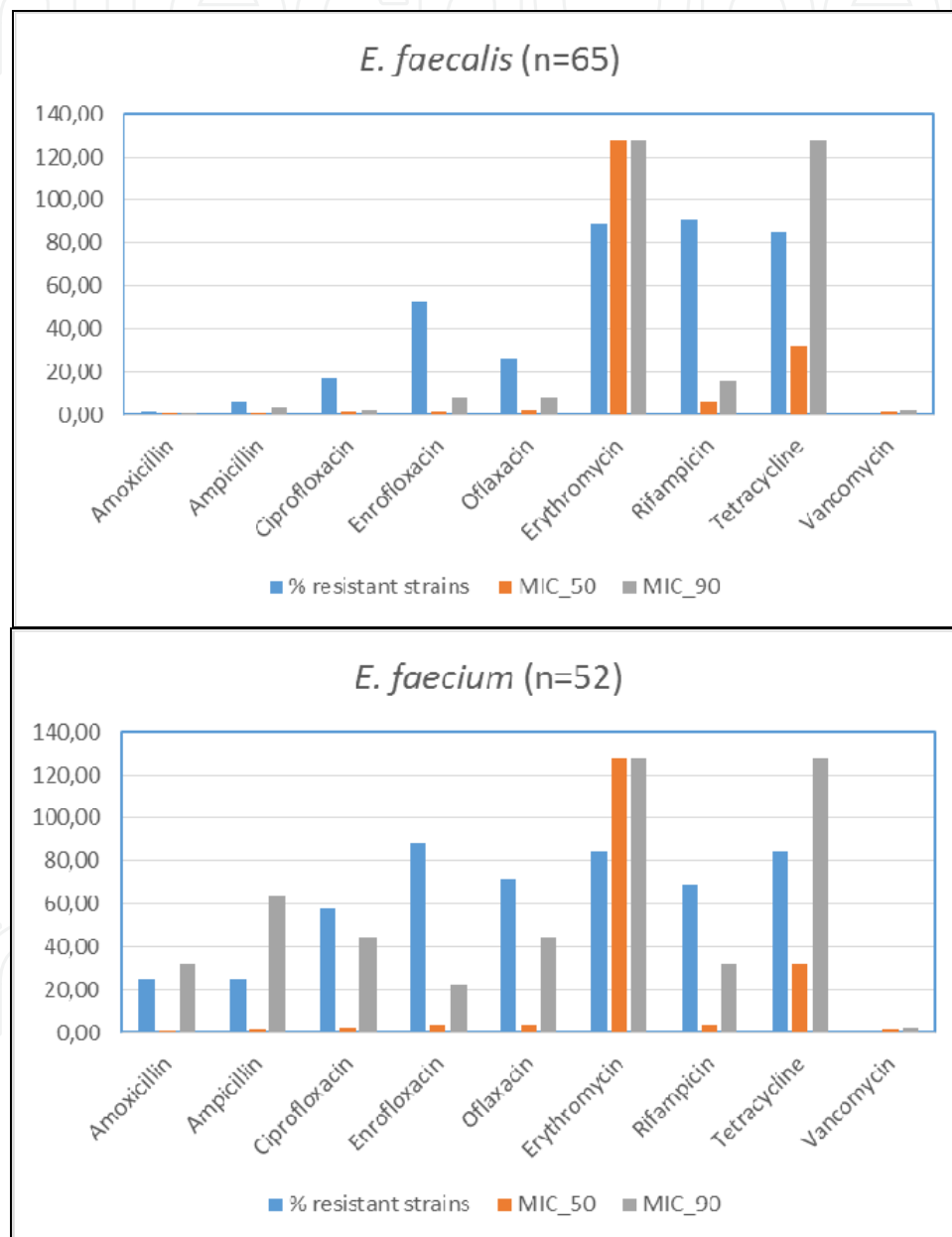

Figure 2. Cumulative percentages of multiresistance in *E. faecalis* and *E. faecium* [40]

As previously underlined, no vancomycin-resistant *Enterococcus* strains were identified and isolated; in fact, the MIC_{90} value concerning vancomycin for the two most representative

Enterococcus species above-mentioned (*faecalis* and *faecium*) was quantified as equal to 2 μg/ml. With regard to beta-lactam antibiotics, both amoxicillin and ampicillin demonstrated full action and effectiveness, representing the most effective antibiotics among the ones tested.
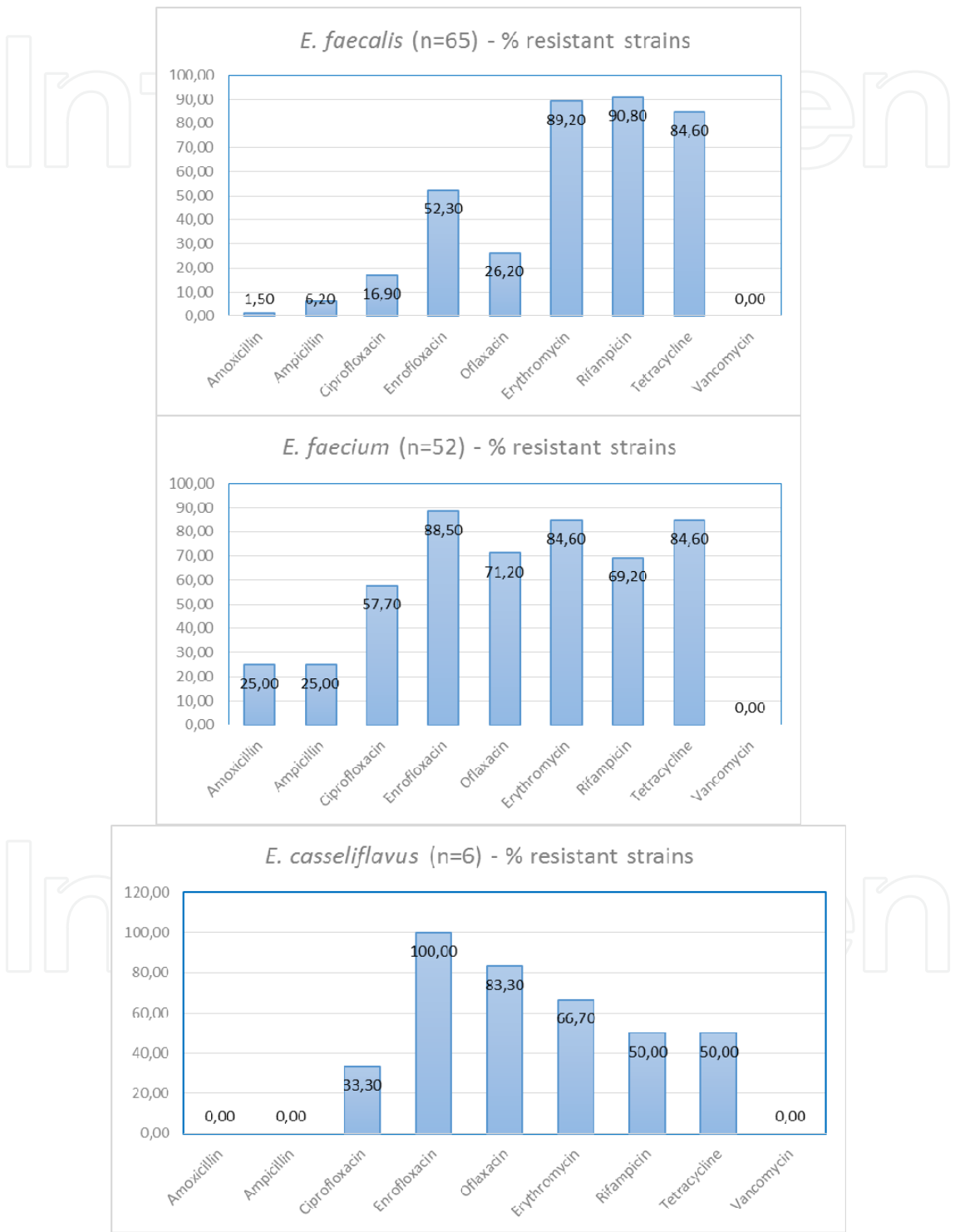
Figure 3. Resistance profile of *E. faecalis*, *E. faecium*, and *E. casseliflavus* [40]

Most strains belonging to *E. faecium* species showed quinolone resistance, while 100% of resistance to enrofloxacin was detected in *E. casseliflavus* and a high frequency of resistance (52.3%) in *E. faecalis* strains [40] (Figures 2 and 3).

The level of resistance to rifampicin, erythromycin, and tetracycline was high or very high, generally with more than 50% of strains resistant. When comparing the frequency of resistance between *E. faecalis* and *E. faecium*, we found that strains belonging to the latter species were significantly (*P* < 0.05) more resistant to the following antibiotics: amoxicillin, ampicillin, ciprofloxacin, enrofloxacin, and ofloxacin.

In Table 1, the percentages of *E. faecalis* and *E. faecium* resistant strains were reported on the basis of disaggregate data. Concerning the 117 strains evaluated, 67 (57.3%) correspond to the ones originated from dogs subjected to antibiotic treatment, while 50 isolates (42.7%) correspond to the ones from control dogs [40].

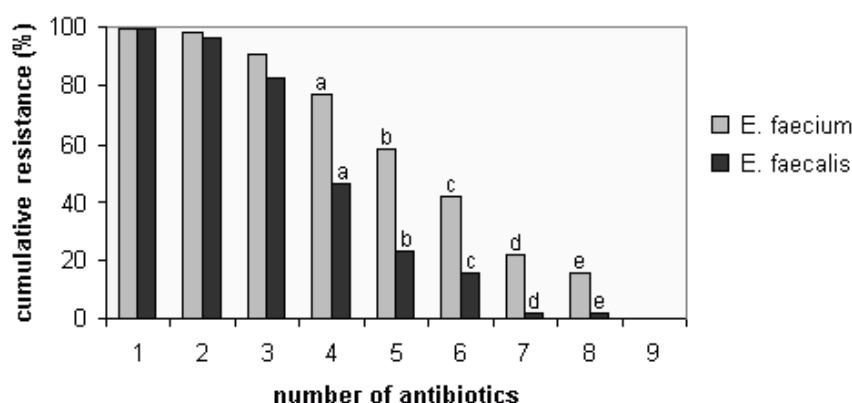| Antibiotics	*E. faecalis*		*E. faecium*	
	Treated dogs' strains (n = 36)	Control dogs' strains (n = 29)	Treated dogs' strains (n = 31)	Control dogs' strains (n = 21)
Amoxicillin	2.8	0.0	29.0	19.0
Ampicillin	5.6	6.9	29.0	19.0
Ciprofloxacin	13.9	24.1	67.7	42.9
Enrofloxacin	55.6	48.3	90.3	85.7
Ofloxacin	25.0	27.6	77.4	61.9
Erythromycin	94.4	79.3	90.3	81.0
Rifampicin	88.9	89.7	61.3	81.0
Tetracycline	88.9	75.9	93.5 [a]	71.4 [a]
Vancomycin	0.0	0.0	0.0	0.0

[a]*P* = 0.05. All other comparisons are not statistically significant.

Table 1. Percentages of *E. faecalis* and *E. faecium* resistant strains isolated from dogs treated and not treated with antibiotics [40]

The statistical analysis, comparing the resistance frequency in strains isolated from treated dogs and from control ones, showed a significant difference toward tetracycline (*P* = 0.005) in *E. faecium* case, with 93.5% of resistant strains isolated from treated dogs versus 71.4% from non-treated dogs. All the other comparisons were not statistically significant (*P* > 0.05).

In Figure 4, aggregate rates of multiresistance found in *E. faecalis* and in *E. faecium* are presented: it shows the cumulative percentage of strains that were resistant to one or more antibiotics tested. Over 80% of the strains belonging to both the previously mentioned species were

resistant up to three antibiotics, while *E. faecalis* and *E. faecium* were, respectively, 15.4% and 41.6% resistant up to six antibiotics. Ten of the 52 *E. faecium* strains (15.4%) were resistant to all the tested antibiotics, excluding vancomycin. Multiresistance was significantly more frequent in *E. faecium* than in *E. faecalis* species [40].

Note: Same letters indicate significantly different values ($P < 0.05$).

Figure 4. Cumulative rates of multiresistance in *E. faecalis* and *E. faecium* [40]

4. Discussion

The antibiotic resistance in bacteria, especially multidrug resistance (MDR) originating in household animals, represents a major health problem. The close contact established between pets, the dogs in this specific case, in situations of domestic coexistence clearly amplifies the possibility of bacteria transferring.

Enterococci as commensal bacteria possess natural gene transfer mechanisms and may, treacherously, spread multiple resistances. Therefore, it becomes crucial to first identify and then characterize the strains isolated from household animals [41].

Our results confirm that enterococci are constantly present in the intestine of the dog. The predominant species was shown to be *E. faecalis*, and this is in accord with De Graef et al. [42], who studied the fecal flora of dogs living in Belgium, and with Kataoka et al. [22], who analyzed fecal samples of dogs and cats.

On the contrary, Cinquepalmi et al. [34] found in southern Italy (Bari) 61.6% of *E. faecium* (45/73), 23.3% of *E. gallinarum* (17/73), and 5.5% of *E. casseliflavus* (4/73). Other species isolated (*E. raffinosus*, *E. avium*, and *E. durans*) accounted for 0.027% of the samples. *E. faecalis* was identified only in one specimen.

Studying *Enterococcus* spp. is particularly important because of their innate ability to express resistance to several antibiotics.

The research has demonstrated how *E. faecium* resistance profile versus amoxicillin, ampicillin, ciprofloxacin, enrofloxacin, and ofloxacin was significantly higher than *E. faecalis* one. This situation is confirmed by the data presented by The Surveillance Network (TSN) Database–USA [43], which shows an alarming increase in ampicillin resistance expressed by human *E. faecium* isolates.

Conversely, we found only one *E. faecalis* amoxicillin-resistant strain in the 52 strains tested; thus substantially confirming findings of De Graef et al. [44], who observed no ampicillin resistance among strains isolated from dogs.

The high resistance to erythromycin has already been observed in *E. faecalis* isolated from dogs [22], and it is probably associated with the methylation of the ribosomal target site of these antibiotics [45].

We found no vancomycin-resistant strains in the 165 samples examined, which is consistent with a number of studies on enterococci from dogs and cats [22, 34]. On the basis of this, it can be estimated that the prevalence of vancomycin-resistant strains in dog enterococcal population is <0.018 ($P = 0.05$).

Anyway, other European studies highlighted a relatively high VRE strain prevalence (mainly *E. faecium*) ranging from 7 to 23% in canine population living in contact with livestock, as well as in dogs living in urban areas. In Spain, Torres research group conducted a study on healthy animals demonstrating a higher VRE strain prevalence in household animals (23%) in comparison with swine strains (4%).

VRE occurrence has also been reported in the United States and New Zealand, countries in which the VRE presence has not been, anyhow, documented in food animals.

Dogs' VRE isolates largely contain the *VanA* resistance gene cluster and express multi resistance toward other antimicrobial categories such as tetracycline [*tet(M)* gene], macrolides [*ern(B)* gene], and aminoglycosides [*aac(6′)-aph(2′)* genes]. Therefore, even if vancomycin is generally not employed in pet veterinary practice, VRE have been considered co-selected by using such antibiotics [30].

In our study, antibiotic administration cannot be considered associated with an acquired antibiotic resistance increasing in the isolated strains analyzed, apart from tetracycline with reference to *E. faecium*. This result might be because the treatments based on tetracyclines of all our samples were carried out resorting to the use of doxycycline, a molecule that, contrary to what happens with the other tetracycline, owns a prevalently fecal excretion. This specific condition exposes the bacterial flora of the gut environment to a selective pressure for resistance.

Household dogs have long been recognized to be a potential source of zoonotic pathogens for human harboring them at intestinal level, and consequently, they have been shown to pose a significant sanitary risk for people. Humans are exposed to these pathogens through direct or indirect contact with infected dogs or their own feces, and they may also become infected after thoughtless ingestion of a zoonotic agent.

More neglected, but in any case not less important, is the fact that domestic dogs can act as the reservoir of antimicrobial-resistant agents; moreover, infections in humans and dog are often treated using similar antibiotics [30, 46].

Both the capability of non-human-origin antibiotic-resistant enterococci (e.g., sewage, raw meat, and animal feces) to colonize people and their ability to transfer resistance to human enterococci are actually not entirely known. In fact, although some researches have failed to demonstrate a relationship between antibiotic-resistant enterococci (glycopeptides included) isolated from humans and those isolated from non-human sources, some other studies have described a specific genetic relationship between *Enterococcus* strains isolated from humans and from animals (including dogs) [34].

Our study data confirmed that multiresistant enterococci (in particular, *E. faecium*) are also present in dogs even if they have never been subjected to antibiotic treatment. This result suggests that resistance transferring from dog to man should not be taken lightly.

The resistance monitoring in enterococci, which circulate between domestic animals, humans, and possibly other organisms present in the environment, and the demonstrations of similarities between resistance genes and their localization in dog and human genome could reveal many secrets of this phenomenon [44].

5. Conclusions

There are few studies that deal with the presence of microorganisms pathogenic to humans in dog feces and that address the role of these ones as a reservoir of multidrug-resistant (MDR) bacteria such as *Enterococcus*. Our study has demonstrated that in the city of Parma, northern Italy, MDR *Enterococcus* spp. were found.

Starting from the consideration that antibiotic-resistance-encoding genes can be transferred between bacteria and that actually the contact between pets and people owning domestic animals is closer than in the past, but also on the basis of our collected data, it is possible to suggest that contamination with dog feces carrying MDR microorganisms could represent a real problem for environmental and public health.

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