

## SPECIES DISTRIBUTION AND ANTIMICROBIAL SUSCEPTIBILITY OF ENTEROCOCCI ISOLATED FROM BROILERS INFECTED EXPERIMENTALLY WITH *EIMERIA* SPP AND FED WITH DIETS CONTAINING DIFFERENT SUPPLEMENTS

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### ABSTRACT

Resistant bacteria in animal can be spread to environment and to humans. Poultry feed and infections caused by *Eimeria* spp. are important factors in determining the intestinal microbial communities. The aim of this study was to verify the prevalence of species and antimicrobial susceptibility of *Enterococcus* isolated from broilers fed with different supplements and infected experimentally with *Eimeria* spp. Broilers were divided in eight groups, fed with diets supplemented with a combination of antimicrobial, ionophore-coccidiostatics, probiotic, essential oil. At 14 days old all birds, except the control, received a solution containing oocysts of *Eimeria* spp. Samples of cloacal swabs from broilers were collected. A total of 240 *Enterococcus* sp. strains were isolated, confirmed genus by PCR, classified as species, tested for antimicrobial susceptibility and screened by PCR for the presence of *tet(L)*, *tet(M)* and *erm(B)* genes. The overall distribution of species isolated from fecal samples was *E. faecalis* (40%), followed by *E. casseliflavus/E. gallinarum* (10.8%), *E. mundtii* (10.8%), *E. faecium* (10.8%), *E. columbae* (5.8%) and *E. gallinarum* (4.2%). Changes in the composition or frequency of *Enterococcus* species were observed in all dietary supplementation. Antimicrobial susceptibility tests showed resistance phenotypes a range of antibiotics, especially used in humans such as, streptomycin, penicillin, rifampicin and vancomycin. There was no correlation between different supplementation for broilers and antimicrobial resistance and the presence of *tet(M)*, *tet(L)* and *erm(B)* genes. Dietary supplementation had effect on the *Enterococcus* sp. colonization, but did not have significant effect on the phenotype and genotype of antimicrobial resistance in enterococci.

**Key words:** *Enterococcus* sp.; broilers feed; antimicrobial resistance; resistance genes.

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## INTRODUCTION

The poultry industry worldwide has increased over the last 40 years. In 2009, world chicken meat production was 91.9 million tonnes (ton), and Brazil, United States and Europe are the world's largest exporters (12). Modern technologies of poultry production use massive doses of antimicrobial as therapeutics and for growth promotion, during all stages of production (23). The use of antimicrobial as growth promoter in animal feed has caused an increase in resistant bacteria isolated from animal (36). Almost all poultry dietary contain of 2 to 40 g/ton of any type of antimicrobial agent. In 2006, the European Union banned several antimicrobial growth promoters, however in Brazil, some growth promoters are used to feed commercial poultry and broilers chickens (43). The demand of products free of antibiotics and chemotherapy has led to the exploration of new growth promoters, such as probiotics, prebiotics, and essential oils. In broiler nutrition, probiotic species belonging to genera *Lactobacillus* and *Enterococcus* are utilized to modulate intestinal microbial communities and pathogen inhibition (34). On the other hand, studies testing the addition of essential oils in the diet of broiler chickens have demonstrated *in vitro* antimicrobial, anti-inflammatory, antioxidant and anticoccidiostatic activity of many plants (29, 43). Hume et al. (21) observed that essential oil supplemented in the diets avoided the drastic shifts after a mixed challenge of *Eimeria* spp. and the microbial communities were better modulated than monensin.

The feeding is an important factor in determining the intestinal microbial communities. Commensal enteric bacteria from chickens are frequently objects of researches, for supervision of animal products for human consumption and marketing. Enterococci are gram-positive bacteria widely distributed in the environment, inhabiting mainly the gastrointestinal tract of humans and animals (33). Furthermore, enterococci are opportunistic pathogens commonly associated with immunocompromised patients and nosocomial

infection (15). *Enterococcus* sp. resistant to several antimicrobials were isolated from clinical specimens and environment (5,8,15,18,37). These microorganisms are intrinsically resistant against many antimicrobial agents commonly prescribed for Gram-positive cocci and exhibit resistance to a wide variety of other antimicrobials, by acquisition of resistance genes via transposons or plasmids, such as, tetracycline and macrolides (25). Resistance to tetracycline are mediated by two major groups, the first includes the *tet(M)*, *tet(O)* and *tet(S)* genes that encoding ribosomal protection proteins, and the second is represented by *tet(L)* and *tet(K)* genes encodes tetracycline efflux pumps (3). One of the most widespread mechanism of resistance to macrolides is mediated by methylation of a specific adenine residue in 23S rRNA and is associated with the *erm(B)* gene (29).

In Brazil, few studies have investigated the presence of enterococci resistant in poultry (28, 43, 44, 47). The aim of this study was to verify the effect of diets containing different supplements and the presence or not of *Eimeria* spp. on the specific colonization and antimicrobial susceptibility of *Enterococcus* sp. in the gastrointestinal tract of broilers.

## MATERIAL AND METHODS

### Animals

The study was conducted using a total of 250 day-old male Cobb 500. The broilers were housed indoors and distributed in a completely randomized design divided into eight treatment groups with five replicates each (12 birds/box). Broilers were fed with a diet based on corn-soybean meal, vegetable oil, minerals and vitamins and supplemented with a combination of ionophore-coccidiostatics (monensin) at 90g/ton, probiotic (*Enterococcus faecium* 3,5 x 10<sup>10</sup> UFC/g) at 35g/ton, essential oil (extracted of thyme and clove) at 100g/ton and/or antimicrobial (bacitracin methylene disalicylate 11%) at 200g/ton. At 14 days old, all birds, expect

the control, received a solution containing  $5 \times 10^4$  and  $1 \times 10^4$  oocysts/chicken of *Eimeria maxima* and *Eimeria acervulina*, respectively. The broilers were maintained until 28 days old at the Departamento de Zootecnia, Universidade Federal do Rio Grande do Sul. The nutritional requirements used are as indicated in Rostagno *et al.* (40).

#### Isolation and confirmation of enterococci genus

Five cloacal swabs samples from each group were collected and it was avoided environmental contamination (Table 1). Each swab was inserted into Cary-Blair medium and sent to the laboratory. The samples were inoculated in Broth Azide (Himedia, Mumbai, India) media for 24 hours at 35° C. One hundred microliters of broth were inoculated on agar plates with Brain Heart Infusion agar (BHI) (Himedia, Mumbai, India) supplemented with 6.5% NaCl and incubated as described above. Phenotypic criteria such as size/volume, shape, color, hemolytic profile, Gram staining, catalase production, and esculin hydrolysis tests were used to separate the enterococci and the non-enterococcal strains. Enterococci genus was confirmed by polymerase chain reaction (PCR) technique. DNA extraction and amplification using *tuf* gene followed the protocols described by Ke *et al.* (27) and Riboldi *et al.* (37). Thirty enterococci were randomly select from each group.

#### Phenotype characterization of enterococci resistant

The phenotype characterization was determined according to the protocol determined by Facklam *et al.* (12). *Enterococcus faecalis* ATCC 51299 and *Staphylococcus aureus* (ATCC 25923) were used as control strains.

#### PCR-RFLP of 16S ribosomal DNA to confirm the *Enterococcus gallinarum* and *Enterococcus casseliflavus*

Isolates biochemically classified as *E. gallinarum* or *E. casseliflavus* were confirmed by PCR-RFLP using primers specific region of 16S ribosomal DNA, as described by

Medeiros *et al.* (32). Extraction of genomic DNA followed the method of Riboldi *et al.* (37). The PCR product of 661 bp amplified was submitted to digestion with the restriction enzyme *Hinf*I (Jena Bioscience GmbH, Germany) following the manufacturer instructions.

#### Antimicrobial susceptible testing

All isolates were subjected to antimicrobial susceptibility assay by disk diffusion method, as recommended by the Clinical and Laboratory Standards Institute (6). The following antimicrobials were tested: ampicillin (AMP 10 µg), penicillin (PEN 10 U), erythromycin (ERI 15 µg), streptomycin (STR 10 mg), chloramphenicol (CLO 30 µg), ciprofloxacin (CIP 5 µg), nitrofurantoin (NIT 300 µg), vancomycin (VAN 30 µg), rifampicin (RIF 5 µg) and tetracycline (TET 30 µg). As positive and negative controls strains *E. faecalis* ATCC 51299 and *E. faecium* ATCC 53519 were used, respectively.

#### Detection of *tet(M)*, *tet(L)* and *erm(B)* genes:

All strains were tested for the presence of *tet(M)*, *tet(L)* and *erm(B)* genes by PCR. *Enterococcus* sp. genomic DNA was extracted by the boiling method as described by Riboldi *et al.* (37). The primer of *tet(M)*, *tet(L)* and *erm(B)* genes and PCR amplifications were performed as described by Frazzon *et al.* (15).

#### Statistical analysis

The experimental data was submitted to analysis of variance using the Program SASM-AGRI for Statistical Significant Differences at  $P < 0.05$  and  $P < 0.01$  (2).

## RESULTS AND DISCUSSION

#### Identification of enterococci isolates

A total of 240 *Enterococcus* were isolated and confirmed the genus through PCR. Table 1 shows the overall distribution of species identified in fecal samples from broilers. Sixteen

different species were identified and *E. faecalis* was the most prevalent specie (40%), followed by *E. casseliflavus/E. gallinarum* (10.8%), *E. mundtii* (10.8%), *E. faecium* (10.8%), *E. columbae* (5.8%) and *E. gallinarum* (4.2%). Other species identified were *E. pseudoavium*, *E. saccharolyticus*, *E. avium*, *E. hirae*, *E. durans*, *E. cecorum*, *E. sulfureus*, *E. asini*, *E. malodoratus* and *E. raffinosus* characterizing 10% of the isolates. Sixteen isolates (6.6%) could not be identified to the species level, and were classified as *Enterococcus* sp. The species identified in the current study were also reported by Hwang *et al.* (23), Fracalanza *et al.* (14), Debnam *et al.* (9) for

broilers and chicken meat. Enterococci are part of the gastrointestinal microbiota of chickens (17). In chickens, the presence of *Enterococcus* species varies with age of birds. In young birds the most prevalent species are *E. faecalis* and *E. faecium*, with the maturity of the birds there is a decline in *E. faecium*, followed by *E. faecalis*, allowing the growth of other species (10). The prevalence of *E. casseliflavus/E. gallinarum* was relatively higher in the present study compared to other countries (9, 23). Meanwhile, some studies conducted in Brazil have been observed high prevalence of these species from samples of chicken meat (15, 14).

**Table 1.** Groups, supplements and distribution of *Enterococcus* species from broilers feed with different dietary supplemented.

Group <sup>diets</sup>	Number of isolates (%)						
	<i>E. faecalis</i>	<i>E.casseliflavus/ E.gallinarum</i>	<i>E. mundtii</i>	<i>E. faecium</i>	<i>E. gallinarum</i>	<i>E. columbae</i>	Other species <sup>2</sup>
1 <sup>a</sup>	15 (50)	4 (13.3)	1 (3.3)	0	3 (10)	0	7 (23.3)
2 <sup>b</sup>	18 (60)	0	0	2 (7)	0	5 (16.6)	5 (16.6)
3 <sup>c</sup>	17 (56.6)	3 (10)	5 (16.6)	0	0	1 (3)	4 (13.3)
4 <sup>d</sup>	11 (36.6)	3 (10)	4 (13.3)	5 (17)	3 (10)	0	4 (13.3)
5 <sup>e</sup>	5 (16.6)	7 (23)	4 (13.3)	5 (17)	1 (3.3)	3 (10)	5 (16.6)
6 <sup>f</sup>	7 (23)	5 (16.6)	7 (23)	1 (3.3)	2 (6.6)	1 (3.3)	7 (23.3)
7 <sup>g</sup>	13 (43.3)	3 (10)	1 (3.3)	5 (17)	1 (3.3)	3 (10)	4 (13.3)
8 <sup>h</sup>	10 (33.3)	1 (3.3)	4 (13.3)	8 (26.6)	2(6.6)	1 (3.3)	4 (13.3)
Total	96 (40)	26 (10.8)	26 (10.8)	26 (10.8)	10 (4.2)	14 (5.8)	40 (16.6)

Diets: a) unmedicated infected control; b) ionophore coccidiostatic infected group; c) probiotic infected group; d) ionophore coccidiostatic and probiotic infected group; e) essential oil infected group; f) ionophore coccidiostatic and essential oil infected group; g) growth promoter infected group; h) unmedicated uninfected control <sup>2</sup>*E. pseudoavium*, *E. saccharolyticus*, *E. avium*, *E. hirae*, *E. durans*, *E. cecorum*, *E. sulfureus*, *E. asini*, *E. malodoratus* and *E. raffinosus* and *Enterococcus* spp.

Of the 39 isolates classified as *E. casseliflavus* and *E. gallinarum* and tested by PCR-RFLP, only two isolates did not showed DNA fragments pattern expected to *E. gallinarum* and *E. casseliflavus*, and were reclassified as *Enterococcus* sp. The correct identification of these species is very important to distinguish between intrinsic and acquired resistance to vancomycin.

#### Species distribution according to dietary supplement employed

Changes in the composition or frequency of *Enterococcus* species were noticed (Table 1). The species distribution

between all groups was comparable with the control. The unmedicated, uninfected control (group 8) the most prevalent species were *E. faecalis* (33.3%), *E. faecium* (26.6%), *E.mundtii* (13.3%), *E. gallinarum* (6.6%), *E. casseliflavus/E. gallinarum* (3.3%), *E. columbae* (3.3%), and *Enterococcus* sp. (13.6%). Changes in the composition of *Enterococcus* species were detected in groups 1, 2, 3 and 4. In group 1 (unmedicated, infected control) no *E. faecium*, *E. columbae* and *E.mundtii* were isolated. One reason for this change in the species frequency should be due the intestinal dysbacteriosis caused by *Eimeria* spp. Fukata *et al.* (16) reported that chickens infected with *Eimeria tenella* showed a change in the

composition of the intestinal microbiota, compared to uninfected chickens. In group 2 (ionophore-coccidiostatic, infected) *E. casseliflavus/E.gallinarum*, *E. gallinarum*, and *E. mundtii* were isolated. Monensin is a polyether antibacterial of the ionophore group, and demonstrate an anticoccidial action against *E. tenella*, *E. acervulina*, *E. mivati*, *E. brunetti*, *E. maxima*, *E. necatrix* in chickens. Since monensin is antibacterial agent, it may have an effect against some enterococcus species (41). Debnam *et al.* (10) verified that antibiotic virginiamycin had a negative effect of some enterococcus species. In swabs from group 3 (probiotic, infected) no *E. faecium* and *E. gallinarum* were isolated and from group 4 (ionophore-coccidiostatic and probiotic, infected) *E. columbae* was detected, but *E. faecium* and *E. gallinarum* were observed. The diarrhea caused by *Eimeria* spp. in the broilers from group 3 may have been impaired the colonization of the gut by *E. faecium* (probiotic) and eliminated the probiotic and, may possibly be the reason why this species was not detected in group 3. The association ionophore-coccidiostatic and probiotic appeared to have a positive effect on this species. The reduction in the prevalence of some species detected in groups 3 and 4 should be caused by the presence of probiotics in the diet of broilers, since probiotic microorganisms has the ability to produce inhibitory substances (antagonize other microorganism) and also competitive capacity (hydrophobic cell wall, that facilitate adhesion to the epithelium intestinal) (44). Changes in the frequency of *Enterococcus* species were perceived in groups 5, 6 and 7. In groups 5 (essential oil, infected) and 6 (ionophore-coccidiostatic and essential oil, infected) an increase of 20% and 13% in the frequency of *E. casseliflavus/E.gallinarum*, respectively, was observed. In the group 6, the highest prevalence of *E. pseudoavium* (20%) was identified, showing that the association ionophore-coccidiostatic and essential oil have a positive effect on this specie. Alterations in the frequency of some enterococcus species observed in groups 5 and 6 should be caused by essential oil. Antibiotic and

anticoccidial activity of plants oil against *Eimeria* spp. are well known (29, 43). In the broilers receiving antibiotic and were infected with *Eimeria* spp. (group 7), variations in the frequency of *Enterococcus* species were noted. The antibiotic in the supplement, should selected the most adapted organisms inside the gut of broilers. Leme *et al.* (28) have demonstrated that broilers fed with feed supplemented with avoparcin as growth promoter showed an increase of *E. faecium* when compared with control group.

#### **Antimicrobial susceptibility according to dietary supplement used**

Table 2 shows the antibiotic susceptibility of *Enterococcus* species isolated from cloacae of poultries. The prevalence of antimicrobial resistance between all groups was compared, and an increase or decreases in frequency of resistance as observed. Absence of susceptibility to at least one of the antimicrobials tested was detected, except to the antimicrobial ampicillin. *Enterococcus* sp. ampicillin-susceptible has been related in isolated from chickens and food (38, 44).

Resistance to tetracycline was verified in all groups (56.7%-100%). Although tetracycline is an antimicrobial not approved by the European Union as a food supplement for chickens, therapeutic and prophylactic use in veterinary is common, and *Enterococcus* resistant to tetracycline are frequently found in broilers, animal feed, poultry production environment and chicken carcasses (7,15,20). The higher percentage of erythromycin resistant enterococcus was noticed in groups 1, 3, 4, 5 and 7 (53-70%). Hwang *et al.* (23) observed same prevalence of erythromycin resistance in enterococcus isolated from fecal samples of chicken in Korea. The erythromycin resistant enterococcus is very interesting, since this drug is the choice for treatment of several infection and also enterococcal infection in penicillin-allergic patients.

An increase in the percentage of *Enterococcus* sp. resistant to penicillin was observed in groups 1 3, 4, and 7. The prevalence of penicillin-resistant enterococcus are presented

here was highest, contrasting to the results reported by Aarestrup *et al.* (1) and Hayes *et al.* (20), where low levels of penicillin-resistant enterococcus isolated from chickens were detected, in spite of the fact that San Martín *et al.* (42) have found a high percentage of enterococci resistant to penicillin in broilers samples. Penicillin-resistant enterococci isolate from poultries is important, for the reason that the animals can serve as an important reservoir of resistant bacteria that can be spread to humans through the food chain and also by the reason that penicillin is the basis of therapy of enterococcal infection (36). A lower prevalence of erythromycin-resistant enterococci (7%) was perceived in group 2. However, Jacob *et al.* (24) had reported that ionophore-coccidiostatics (monensin) in pigs increased prevalence of erythromycin-resistant enterococci.

The difference observed between the previous study and results showed here, may be caused by the lower concentration of erythromycin (8 mg) used by Jacob *et al.* The interactions ionophore-coccidiostatics and probiotic (group 4) and ionophore-coccidiostatics and essential oil (group 6) increased the prevalence of *Enterococcus* sp. resistant to chloramphenicol. The presence of enterococci resistant to chloramphenicol is undesirable, because the use of this drug is prohibited in Brazil (44). However, chloramphenicol-resistant enterococci have already been observed in isolates from broilers (20, 42). Among the 240 enterococcus isolated from broilers, 183 (76.7%) were resistant to 2 or more antimicrobial tested. Multi drug resistant *Enterococcus* sp. has been isolated from broilers and chicken meat (14, 15, 40).

**Table 2.** Frequency of antimicrobial resistance patterns in *Enterococcus* sp. isolated from cloacal swabs of broilers feed with different dietary supplemented

Group	Resistance % to <sup>a</sup>									
	AMP	PEN	VAN	CIP	ST	RIF	CLO	NIT	TET	ERI
1*	0	67.7	0	0	3.3	23.3	0	0	56.7	50
2*	0	40	10	6.6	3.3	33.3	0	0	100	6.6
3*	0	56.7	3.3	16.6	16.6	60	0	0	100	53.3
4*	0	60	6.6	3.3	46.7	26.7	13.3	0	90	70
5*	0	33.3	3.3	0	43.3	20	0	6.6	100	53.3
6*	0	33.3	0	6.6	13.3	20	6.6	3.3	87	26.7
7*	0	50	6.6	0	23.3	63.3	3.3	0	93	53.3
8	0	30	3.3	6.6	6.6	56.7	0	3.3	97	10

<sup>a</sup>Antibiotics: ampicillin (AMP), penicillin (PEN), vancomycin (VAN), ciprofloxacin (CIP), streptomycin (ST), rifampicin (RIF), chloramphenicol (CLO), nitrofurantoin (NIT), tetracycline (TET), erythromycin (ERI). \* Broilers inoculated with *Eimeria* spp.

An interesting observation of this study was vancomycin-resistant enterococcus (VRE). In Brazil, few studies have been focused on VRE in broilers. Souza *et al.* (44) detected a high of the VRE isolated from commercial broilers, however Xavier *et al.* (46-47) have not detected VRE in cloacal swabs collected from poultry. The differences observed in these studies could be due to several factors, including stage of production, which samples were taken.

Dietary supplementations did not influence the resistance profile in enterococcus isolated from broilers. Some explanations to this should be: i) resistant bacteria may persist on production equipments or in other parts of the production environment that are difficult to decontaminates (7, 19); ii) there is the possibility the resident birds, small rodents or insects work as carries of resistant bacteria (35) and also iii) the poultry feed and feed ingredients could be the source of

resistant enterococcus (7).

#### Frequency of *tet(M)*, *tet(L)* and *erm(B)* genes.

All strains were tested for the presence of *tet(M)*, *tet(L)* and *erm(B)* genes by PCR. The *tet(M)* was present in 62%, *tet(L)* in 3.8 % and *tet(M)* and *tet(L)* in 23.3% of isolates. The prevalence of these genes is consistent with other previous studies in poultry and chicken meat (1, 2, 12). Five strains susceptible to tetracycline carried, at least one of the *tet* genes. This can be attributed to the lack of expression of the resistance gene, as described by Martineau *et al.* (31) and Martel *et al.* (30), and Frazzon *et al.* (15). In our study, 94 % and 30% of tetracycline resistance strains showed the presence of *tet(M)* and *tet(L)* genes, respectively. Huys *et al.* (22) observed in *Enterococcus* sp. tetracycline resistance strains isolated from food a prevalence of 95% *tet(M)* and 35% *tet(L)* genes.

The *erm(B)* gene was detected in 39.6 % of all isolates and 97.9% of erythromycin resistant present the *erm(B)* gene. This gene is frequently observed in enterococci of animal origin and is reported to be the most common gene for resistance to macrolides (1, 24). Evidence of dissemination of erythromycin resistance has been shown in *Enterococcus* sp. isolated from pigs, chickens and humans (11). Furthermore, eighty four isolates positive for *tet(M)* also contained *erm(B)* gene. In most cases, *tet(M)* is carried by conjugative transposons such as Tn916/Tn1545 and in this plasmids *tet(M)* is associated with *erm(B)* (5, 39).

Dietary supplementation did not have significant effect on the presence of *tet(M)*, *tet(L)* and *erm(B)* genes. Jacob *et al.* (24), also were reported that steam-flaked corn diet with wet distillers grains for cattle, did not affect the presence of either *tet(M)* or *erm(B)* genes, in fecal samples.

To our knowledge, this is the first study to report the distribution and antimicrobial resistance in *Enterococcus* sp. in relation to dietary supplementation for broilers in Brazil. Dietary supplementation had effect on the *Enterococcus* sp.

colonization, but did not have significant effect on the phenotype and genotype of antimicrobial resistance in enterococci. The presence of resistant enterococci in feces of broilers is very important, for the reason that these bacteria can spread to environment and to humans, through the food chain or dropping or aerosol particles, which can be inhaled by healthy individuals.

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