


Research Note: Antimicrobial resistance profile of *Enterococcus* spp. isolated from the eggshell of laying hens submitted to pharmacological treatment

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ABSTRACT The occurrence of antimicrobial resistance in microorganisms isolated from eggshells, especially *Enterococcus*, might serve as a parameter to assess the selection of bacteria due to the use of drugs in the diet of laying hens. In order to evaluate the frequency and the antimicrobial resistance profile of *Enterococcus* spp. isolated from the eggshells, 225 Hy-line laying hens were submitted to a 25-d long trial. The treatments were the following: hens fed ration without antimicrobials (control) and groups that received oxytetracycline (10 mg kg⁻¹), doxycycline (20 mg kg⁻¹), lincomycin (50 mg kg⁻¹), and enrofloxacin (10 mg kg⁻¹) in the ration for 5 d. Six replications were analyzed per treatment, composed of a pool of 5 eggs each. They were collected before treatment and on days 3, 6, 15, and 25, totaling 150 samples. Eggshells were submitted to

counts of *Enterococcus* spp., which were identified by proteomic analyses. Antimicrobial resistance was determined by the disk-diffusion test. It was observed that 97.3% (n = 146) of the samples were contaminated with *Enterococcus* spp. There were no differences ($P > 0.05$) in the bacterial counts between treatments on the same day of evaluation. *E. faecalis* and *E. faecium* were the most frequent on the eggshells of all treatments. Multiresistance to the four classes of antimicrobials was also verified in the isolated bacteria. A total of 83% of the *Enterococcus* isolates showed resistance to neomycin, which was not administered to the hens, demonstrating an environmental problem. Thus, feeding laying hens with diets added with antimicrobials induces drug resistance in *Enterococcus* spp., isolated from the eggshells.

Key words: eggshell, microbiological quality, multiresistance, *Enterococcus*, MALDI-TOF/MS

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INTRODUCTION

Antibiotics and chemotherapeutics have been used in laying poultry as therapeutic or prophylactic agents, and have become important tools for maintaining animal health and welfare standards. Residues of those drugs, which cause adverse health effects to human beings, can also lead to the selection of bacterial strains resistant to antimicrobials that can accumulate and spread in the environment. Resistant microorganisms reduce the options of effective drugs for the treatment of infections, in addition to being responsible for a possible transfer of this undesirable characteristic to susceptible bacteria, causing a unique health risk (Shang et al., 2018).

The World Organization for Animal Health (OIE) has recognized that bacterial resistance to antimicrobials is influenced by inappropriate use in human beings, animals, and plants, and has therefore, established recommendations concerning active surveillance and monitoring. In order to achieve those goals, sample collections in the production and retail environments in addition to conducting experimental trials are needed (Terrestrial Animal Health Code, 2019).

Microorganisms present on the eggshells generally come from the microbiota of the cloaca and feathers of the hens, but they also originate from the environment. Among them, the importance of *Enterococcus* is highlighted, since it can be found in the microbiota of hens and eggs and are generally classified as low pathogenic, but have a tendency to induce antimicrobial resistance (Shi et al., 2020).

The objectives of this study were to isolate, enumerate and identify *Enterococcus* spp. from the eggshells of laying hens fed with antimicrobials during and after the treatment and also to evaluate the drug resistance profile of the isolated bacteria.

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MATERIALS AND METHODS

Animal Experimentation

A total of 225 Hy-line W-36 strain, 90-wk-old, laying hens were used. They were distributed in 5 groups of 45 birds each, which were housed in production cages receiving water and feed ad libitum. The control group was fed ration without antimicrobials throughout the experimental period, while the other groups received feed containing oxytetracycline (10 mg kg⁻¹); doxycycline (20 mg kg⁻¹), lincomycin (50 mg kg⁻¹) and enrofloxacin (10 mg kg⁻¹) for 5 d and the experiment lasted for more 20 d. These drugs are widely used in raising of laying hens. On d 1, eggs were collected before feeding hens with drugs. Six replicates of a pool of 5 eggs each were collected from all treatments on d 1st, 3rd, 6th, 15th and 25th. The hens had never previously received any type of antimicrobial.

The eggs were collected in the morning, immediately after laying. They were individually identified and sent to the laboratory at room temperature (25°C).

This study was carried out according to the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The protocol was approved by the Ethics Committee on Animal Experimentation at the Universidade Federal de Minas Gerais, under the Protocol # 400/2015.

Microbiological Analyses

Eggs were rinsed with 225 mL of 1% saline solution and kept in this solution for 1 h, at room temperature. Then, the solution was incubated at 35 ± 1°C for 24 h and serial decimal dilutions (10⁻¹ to 10⁻⁷) were prepared in 0.1% saline solution. Soon after, 100 µL aliquots of the 10⁻⁵ to 10⁻⁷ dilutions were spread on Enterococcosel agar (Difco/BBL, MI). The plates were incubated at 35 ± 1°C for 24 h. Then, counts and selection of *Enterococcus* typical colonies (small, translucent with zones between brown-black and black) were performed (Teixeira et al., 2015). *Enterococcus faecalis* ATCC 29212 was used as a positive control.

Proteomic Analyses

Suspected colonies of *Enterococcus* spp. were submitted to proteomic identification using the Microflex MALDI-TOF/MS equipment (Bruker Daltonics, Bremen, Germany) with its corresponding database. Before the measurements, the equipment was calibrated with a bacterial test standard (*E. coli* DH5 alpha; Bruker Daltonic). The obtained spectra were analyzed by the MALDI Biotyper program (Bruker Daltonics) (Singhal et al., 2015).

Antimicrobial Resistance Profile

The antimicrobial resistance tests were performed using the disk-diffusion (CLSI, 2020). The resistance

profile was determined according to CLSI (2020). The cutoff points proposed by Charteris et al. (1998) were used for antimicrobials not described by the CLSI.

Antibiograms were performed with tetracycline (30 µg), doxycycline (30 µg), lincomycin (2 µg), enrofloxacin (5 µg), and neomycin (30 µg) disks (CEFAR, Farmaco Diagnostico Ltda., SP, Brazil, and DME, Specialized Microbiological Diagnostics, SP, Brazil). The results were classified as sensitive, intermediate or resistant (Charteris et al., 1998; CLSI, 2020). The test was performed in 6 repetitions, each one in duplicate.

Experimental Design

The experiment was conducted in a completely randomized design in subdivided plots, with the plots represented by the groups of hens fed or not with the antimicrobials and the subplots constituted by the days of egg collection. Six replicates per treatment were used, composed of a pool of 5 eggs each. The results of *Enterococcus* spp. counts were submitted to homoscedasticity analysis by the Shapiro-Wilk test and the medians were compared by the Kruskal Wallis and Friedman tests ($P < 0.05$). The means of the results of the antimicrobial resistance were compared by the Tukey test ($P < 0.001$).

RESULTS AND DISCUSSION

Enumeration of *Enterococcus* spp. on the eggshell

High counts of *Enterococcus* spp. on eggshells, 5.00 to 9.40 log₁₀ CFU mL⁻¹, were recorded. The bacterium was present in 146 (97.33%) samples. Differences ($P < 0.05$) were observed over the days on bacterial counts considering the same treatment. However, the addition of antimicrobials to the ration did not influence ($P > 0.05$) the values of the medians of this variable when comparing the results on the same collection day (Table 1).

Despite of the presence of *Enterococcus* on the eggshell, the influence of the antimicrobial addition in the diet of hens on the count of this bacterium and the drug resistance profile of the isolated species have not been the object of scientific research according to the literature consulted. Based on the results of this research (Table 1), the use of antimicrobials in the feed of the hens did not interfere in the count of *Enterococcus* spp. on the eggshell.

The counts of *Enterococcus* spp. found on d 1, when no hen received antimicrobial treatment, were lower ($P < 0.05$) than the counts recorded on d 3rd, 6th, 15th and 25th, which were similar to each other. From d 3 on, there seems to have been an adaptation of the eggshell microbiota to the new conditions of the diet.

The increase in *Enterococcus* counts on the eggshells of hens treated with antimicrobials compared to d 1, when they had not received ration added with drugs, indicates the existence of resistant microorganisms on that surface. Since these bacteria are present in the

Table 1. Median counts of *Enterococcus* spp. (\log_{10} CFU mL⁻¹) isolated from rinse of eggshell, collected on different days, from laying hens that were treated with antimicrobials in the ration.

Treatments	Control	Oxytetracycline (10 mg kg ⁻¹)	Doxycycline (20 mg kg ⁻¹)	Lincomycin (50 mg kg ⁻¹)	Enrofloxacin (10 mg kg ⁻¹)	
D 3	Maximum	7.73	8.01	9.00	7.70	7.99
	Median	7.2 A	6.89 A	7.62 A	7.07 A	7.0 A
	Minimum	8.30	8.40	8.40	8.03	8.31
D 6	Maximum	9.40	8.40	8.40	8.40	8.40
	Median	8.06 AB	8.4 B	8.40 AB	8.34 B	8.38 B
	Minimum	7.90	7.45	8.29	7.28	7.62
D 15	Maximum	9.05	8.84	8.40	8.54	8.60
	Median	8.41 B	8.11 B	8.96 B	8.16 AB	7.95 A
	Minimum	6.85	7.46	8.04	7.89	8.06
D 25	Maximum	8.86	8.52	8.72	8.26	8.66
	Median	8.3 AB	8.09 AB	8.28 AB	8.3 B	8.28 B
	Minimum	7.56	7.63	5.85	5.00	7.93
	Maximum	8.61	8.73	8.76	8.95	8.80
	Median	8.3 B	8.39 B	7.86 AB	6.91 A	8.42 B

Medians followed by distinct uppercase letters in the columns are significantly different ($P < 0.05$) by the Friedman test.

organism and breeding environment, they can represent a unique health problem.

Identification of *Enterococcus* spp.

E. avium, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. termitis*, *E. raffinosus*, and *E. gallinarum* were isolated and identified by MALDI-TOF/MS. *E. faecalis* and *E. faecium* were found in all treatments. *E. durans* was only detected in the control, while *E. termitis* and *E. raffinosus* were found in the treatments with oxytetracycline and lincomycin, respectively. According to the literature consulted, this is probably the first description of *E. termitis* on the shell of consumption eggs.

E. faecalis and *E. faecium* predominate in products of animal origin. This fact was also observed in the present study. The predominance of *E. faecalis* among the bacteria isolated from eggshells is related to its large quantities in the microbiota of the intestine and the cloaca of hens (Fertner et al., 2011).

Antimicrobial Resistance of *Enterococcus* spp.

Enterococcus resistant to the four classes of antimicrobials was detected. Regardless of the drug added to the diet, that the use of antimicrobials resulted in the selection of the resistant *Enterococcus* spp.

Considering individually the groups that received antimicrobials in the diet, there was an increase in the percent of resistant isolates to the same drug, from the first to the 25th d of experimentation. Before the beginning of the administration of drugs, high percent of resistant *Enterococcus* spp. was detected for all antimicrobials. After 3 d of treatment, 100% of resistant isolates were recorded for all antimicrobials, except lincomycin. The oscillation of results for oxytetracycline may be related to the use of the tetracycline disk. Even after ceasing the feeding of ration added with doxycycline, 100% of resistant isolates were detected up to the

end of the trial. High percent of lincomycin resistant isolates was verified throughout the experimental period. It was also verified that the values for enrofloxacin resistance showed an increasing linear behavior.

High percent of lincomycin (65.3%) and neomycin (94.6%) resistance were found in *Enterococcus* isolates. *E. faecalis* and *E. faecium*, which were the most predominantly isolated, also had the highest number of isolates resistant to antimicrobials. Of the 88 isolates of *E. faecalis*, 86 were resistant to neomycin, 73 to doxycycline and tetracycline, 69 to enrofloxacin and 70 to lincomycin. All the 24 isolates of *E. faecium* were resistant to neomycin (100%), 95.8% to enrofloxacin, 83.3% to doxycycline and tetracycline, while 58.3% were resistant to lincomycin. Only one isolate of *E. gallinarum* was sensitive to lincomycin, while all others were resistant to all tested drugs. *E. avium* and *E. casseliflavus* presented 91.7 and 63.6% of isolates sensitive to lincomycin, respectively. The frequencies of resistance to doxycycline and tetracycline were similar (82.3 and 83.0%, respectively), since they are drugs of the same class. Resistance to enrofloxacin in high percent may be associated with its indication as an antimicrobial of choice for the treatment of infections in poultry. Of all the antimicrobials tested, lincomycin, which in many countries can still be used in underdoses for the purpose of improving feeding efficiency and prophylaxis, showed the lowest resistance index. The inappropriate use of antimicrobials, for others purposes other than the treatment of infections, results in the selection of resistant microorganisms in food production environments.

Microorganisms isolated from the eggshells that showed resistance to two or more antimicrobials, belonging to different classes, were considered multidrug-resistant. The presence of *Enterococcus* isolates with this phenotype was observed in all treatments (Table 2). Of the 88 isolates of *E. faecalis*, 58 (65.9%) showed resistance to the 4 classes of antimicrobials, 14 (15.9%) to 3, 10 (11.4%) to 2, 5 (5.7%) to 1 and only 1 (1.1%) was sensitive to all tested antimicrobials. Of the 24 isolates of *E. faecium*, 14 (58.3%) were resistant to the 4 classes of antimicrobials, 7 (29.2%) to 3, 3 (8.3%) to 2 and only 1

Table 2. Numbers and percents (%) of *Enterococcus* spp., isolated from rinsed eggshells, which showed resistance to two or more antimicrobials (multidrug-resistant).

Species	Control	Oxytetracycline (10 mg kg ⁻¹)	Doxycycline (20 mg kg ⁻¹)	Lincomycin (50 mg kg ⁻¹)	Enrofloxacin (10 mg kg ⁻¹)	Number of multiresistant isolates	Total of isolates
<i>E. avium</i>	2 (22.22%)	1 (11.11%)	4 (44.44%)	-	2 (22.22%)	9 (75.00%)	12
<i>E. casseliflavus</i>	8 (72.72%)	1 (9.10%)	-	-	2 (18.20%)	11 (100.00%)	11
<i>E. durans</i>	1 (100.00%)	-	-	-	-	1 (100.00%)	1
<i>E. faecalis</i>	10 (12.20%)	18 (22.00%)	18 (22.00%)	23 (28.00%)	13 (15.90%)	82 (93.20%)	88
<i>E. faecium</i>	3 (13.04%)	6 (26.09%)	3 (13.04%)	4 (17.40%)	7 (30.43%)	23 (95.83%)	24
<i>E. hirae</i>	-	1 (25.00%)	2 (50.00%)	1 (25.00%)	-	4 (80.00%)	5
<i>E. termitis</i>	-	1 (100.00%)	-	-	-	1 (100.00%)	1
<i>E. raffinosus</i>	-	-	-	1 (100.00%)	-	1 (100.00%)	1
<i>E. gallinarum</i>	1 (25.00%)	-	1 (25.00%)	1 (25.00%)	1 (25.00%)	4 (100.00%)	4
Total	25 (18.38%)	28 (20.59%)	28 (20.59%)	30 (22.05%)	25 (18.38%)	136 (92.52%)	

(4.2%) was not multidrug-resistant, showing resistance only to neomycin. The contribution of multidrug resistance to *Enterococcus* spp., originally selected in the agricultural sector, for resistance in human pathogens is not well known. Since dosing regimens are less controlled in animal production than in human health, microorganisms of interest to veterinary medicine are often exposed to a lower concentration of antimicrobials, which is considered a risk factor for the selection of resistant bacteria (Kuile et al., 2016). According to Cheng et al. (2019), short treatments with higher doses, which do not cause unacceptable side effects, may be ideal for achieving therapeutic goals, minimizing the development of resistance.

The high multidrug resistance of *Enterococcus* spp. observed in this study can lead to a unique health problem, since they can be ingested by humans and the resistance may be transmitted to bacteria present in the gastrointestinal tract.

It was concluded that feeding laying hens with ration added with antimicrobials might induce drug resistance in *Enterococcus* spp. found on eggshell, revealing a production management problem that impact on animal health. Furthermore, *Enterococcus* drug resistance also may be related to environmental spread of resistant strains.

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DISCLOSURES

The authors have declared that no competing interests exist for the publication of the scientific article entitled “Antimicrobial resistance profile of *Enterococcus* spp. Isolated from the eggshell of laying hens submitted to pharmacological treatment”.

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