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ANTIBIOTICS RESISTANCE IN *Enterococcus* ISOLATES FROM POULTRY WASTE

Abstract: The aim of the study was to evaluate the drug resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolated from different types of poultry waste. The study material consisted of feather samples (duck, turkey, chicken), sludge and centrifuge sediment, originating from three poultry farms. The study was conducted in two stages; isolation and identification of *Enterococcus* bacteria from the waste and evaluation of their drug resistance using Kirby-Bauer method. Contamination of the poultry waste with *Enterococcus* isolates included *E. faecium* species (79 %) and *E. faecalis* (21 %). The most contaminated were sludge and sediment from the centrifuge as well as chicken feathers, irrespective of the place and time of sampling. Tested isolates showed multiple resistance and similar reaction to all antibiotics used in the study and *E. faecalis* strain was more resistant. *Enterococcus* isolates showed the highest resistance to streptogramins, carbapenems, fluoroquinones, aminoglycosides and penicillins, and the lowest for nitrofurantions and phenicols.

Keywords: poultry waste, bacterial resistance, *Enterococcus faecium*, *Enterococcus faecalis*

Introduction

Natural environment is the home of commensal organisms such as *Enterococcus* bacteria. They are a component of the autochthonous microflora of the gastrointestinal tract of birds, humans and various species of mammals, but also the reproductive system and skin flora. *Enterococcus* are gram-positive cocci that often occur in pairs (diplococci) or short chains, catalase-negative and non-spore forming, facultative anaerobic bacteria. They belong to opportunistic, potentially pathogenic microorganisms, which means that they can cause an infection (localized or generalized) outside the physiological site of living, especially with reduced host immunity [1-3].

Of the 45 known *Enterococcus* species derived from different bird species, the most commonly isolated are: *E. faecalis*, *E. faecium*, *E. hirae*, *E. cecorum*, *E. durans*, *E. avium*, *E. casseliflavus*, *E. gallinarum*, *E. raffinosus*, and *E. columbae* [4, 5].

The two most common species responsible for enterococcal infections in humans are *E. faecalis* and *E. faecium*, that can be the source of urinary tract, liver infections, endocarditis and septicemia. Treatment of diseases caused by this group includes the use of antibiotics such as: ampicillin, amoxicillin, nitrofurienin, vancomycin, fosfomycin,

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ciprofloxacin [6]. Antibiotics are also widely used in veterinary as therapeutic agents or growth promoters [7, 8].

Caron et al. [9] and Brown et al. [10] showed that *Enterococcus* are naturally resistant to cephalosporins, aminoglycosides, clindamycin, tetracyclines, erythromycin and intermediately susceptible to penicillin, ampicillin and glycopeptides.

While *E. faecalis* and *E. faecium* isolated from humans, broilers and pigs were resistance to chloramphenicol, macrolides, kanamycin, streptomycin, tetracycline and vancomycin [11]. Vancomycin-resistant enterococci (VRE) are of particular concern due to a lack of reliable bactericidal therapeutic options [12].

Uncontrolled usage of antimicrobial agents is recognized as the most important factor that favours the development and can be spread of resistant microorganisms to humans via food or water chain, and also by routes such as environmental contamination by poultry waste and direct contact with. The antimicrobial resistance of *Enterococcus* varies depending on the geographical site, national and local antimicrobial usage politics, and usage intensity of antibiotics [13, 14].

This bacteria as natural microbiota of the human and animal intestine, they are exposed to the selective action of antibiotics. The development of antibiotic-resistant bacteria in any country is of global importance because after their initial selection and local dissemination, resistant bacteria can be transferred across international borders by human travellers, animal and insect vectors, agricultural products, and surface water. The observed resistance becomes troubling as most of the organisms involved are of medical and public health importance [15]. The resistance of enterococci to most antimicrobials was more prevalent in China than in European or other Asian countries [16]. *Enterococcus* resistance to aminoglycosides, lincosamides, macrolides, nitrofurans, penicillins, quinolones, streptogramins, and tetracyclines has been reported from food animals in the United States [17]. Widespread use of antibiotics leads to selection of resistant bacteria at the same pace as new drugs are introduced into market.

The spread of antibiotic resistance in the environment depends on the presence and transfer of resistance elements among microorganisms, on the genetic mutations that result, and on the selection pressure to retain these genes within the population [18, 19].

The *Enterococcus* bacteria are characterized by a high potential for genetic material exchange [20]. Processes of accepting genes or fragments occur both in the natural environment and in human and animal organisms. However, many studies have shown that resistance genes are a component of many bacteria genome, whether or not they have been selectively treated with antibiotics [18].

The World Health Organization (WHO) document *Antimicrobial Resistance: Global Report on Surveillance* is the most serious warning that the post-antibiotic era is not just a distant apocalyptic picture but a real threat to the world in the 21st century [21]. The report highlights serious shortcomings in the global surveillance of the spread of microbial resistance to antibiotics and the urgent need to strengthen the cooperation and the need for immediate action within the global strategy to counteract this. The WHO strategy seeks to harmonize efforts to prevent and spread antibiotic-resistant microorganisms in the medical, veterinary, animal husbandry and food production sectors, and to strengthen the cooperation between national and international networks monitoring the antibiotic resistance of key bacterial pathogens as well as coordinating the local, regional and global surveillance.

The aim of the study was to evaluate the drug resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolated from different types of poultry waste.

Material and methods

The study material consisted of 4 feather samples (duck - 1, turkey - 1, chicken - 2), sludge (2 samples) and centrifuge sediment (2 samples), originating from three poultry farms of different sizes (B1, P2 and D3), located in the West Pomeranian Voivodeship. Samples were taken in four replicates. Samples have been collected since November 2015 to April 2016. The study was conducted in two stages. The first step involved the isolation of *Enterococcus* bacteria from the waste and the second was the evaluation of their drug resistance.

Stage I - Isolation of *Enterococcus* genus bacteria from poultry waste

Collected waste samples allowed for preparing the 20 g aliquots, suspending in 180 cm^3 of physiological solution and shaking for 20 minutes and then leaving to stand until the solid phase to fall down [22]. From the initial solution, successive tenfold dilutions were made (10-2-10-8) and propagated in a liquid selective medium containing sodium azide and bromocresol purple (APB - BTL, Poland) [23]. Samples were incubated at 37 °C for 48 hours. Colour change of the substrate from purple to yellow was considered a positive result. The assay was performed in triplicate.

Table 1

Antibiotics used in the research

No.	Group of antibiotics	Antibiotic	Symbol
1	Aminoglycosides	Gentamicin 30	CN30
2		Kanamycin 30	K30
3		Streptomycin 300	S300
4	Glycopeptides	Vankomycin 30	VA30
5		Teicoplanin 30	TEC30
6	Oxazolinones	Linezolid 10	LND10
7	Miscellaneous	Cotrimoxazole 25	SXT25
8		Rifampicin 5	RA5
9	Macrolides	Erythromycin 15	E15
10	Fosfomycins	Fosfomycin / Trometamol 200	FOS200
11	Nitrofurantions	Nitrofurantion 100	F100
12		Nitrofurantion 300	F300
13	Tetracyclines	Tigecycline 15	TGC15
14		Doxycycline 30	DO30
15		Tetracycline 30	T30
16	Penicillins	Ampicillin 10	AM10
17		Penicillin G 10	P10
18	Streptogramins	Chinopristin/synercid	SYN15
19	Phenicols	Chloramphenicol 30	C30
20	Fluoroquinolones	Ciprofloxacin 5	CIP5
21		Levofloxacin 5	LVX5
22		Norfloxacin 10	NOR10
23	Carbapenems	Imipenem 10	IMP10

Positive samples were subject to the reduced inoculation on solidified Slanetz - Bartley medium with sodium azide and TTC (BioMaxima, Poland) which is recommended for the detection of *Enterococcus* bacteria [24]. The culture plates were incubated at 37 °C for 48-72 hours. The characteristic reddish colonies of bacteria have shown the presence of *Enterococcus*. To isolate pure cultures, single and well demarcated colonies were screened on TSA medium. Chromogenic differentiating medium ChromID® VRE (BioMaxima, Poland) was applied to rapidly detect *Enterococcus faecium* (purple colonies) and *E. faecalis* (blue-green colonies) with acquired resistance to vancomycin.

Stage II - Evaluation of the antibiotic resistance of the obtained *Enterococcus* isolate

The 24-hours of *Enterococcus* sp. cultures, that were incubated at 37 °C on tryptose soybean medium (TSA - BTL, Poland), were used to evaluate antibiotic susceptibility. Bacteria were suspended in PBS solution (0.8 dm^3 H_2O, 8 g NaCl, 0.2 g KCl, 1.44 g, Na_2HPO_4, 0.24 g KH_2PO_4) achieving the inoculum of $2 \cdot 10^8$ $CFU \cdot cm^{-3}$ density.

Antibiotic susceptibility was assessed using Kirby-Bauer method in Mueller-Hinton medium (Biomaxima, Poland) using discs saturated with antibiotics (BTL, Poland) (Table 1).

The isolation resistance profile was established on the basis of the EUCAST recommendation [25]. Antibiotic resistance was established for isolates that exhibited resistance phenotype to at least three substances belonging to different groups. The results were statistically evaluated in the Statistica 12 Software.

Results

A total of 100 *Enterococcus* strains were isolated from samples of waste from three poultry farms (B1, P2, D3) located in the West Pomeranian Voivodeship, from November 2015 to April 2016.

The *E. faecium* species was dominant in all types of waste, which accounted for 79 % of isolates, and almost four times less frequently *E. faecalis* was isolated. Sludge and sediment from the centrifuge and chicken feathers were the most contaminated with these bacteria (Table 2).

Table 2

Number of *Enterococcus* isolated from poultry waste

No.	Type of waste	Number of isolates	
		E. faecium	***E. faecalis***
1.	Duck feathers	6	4
2.	Turkey feathers	9	4
3.	Chicken feathers	12	8
4.	Slime	26	4
5.	Precipitate after centrifugation	26	4
Amount		79	21

The number of poultry isolates obtained from poultry waste was dependent on both the sampling date and the type of poultry processing plant. The largest number of *Enterococcus* was isolated from poultry waste samples in the spring (7.04.2016), which accounted for 50 % of the total and the smallest in November (20 %). In turn, the most *Enterococcus* - contaminated were wastes from plant D3, from which 80 % of the isolates of these bacteria originated (Fig. 1).

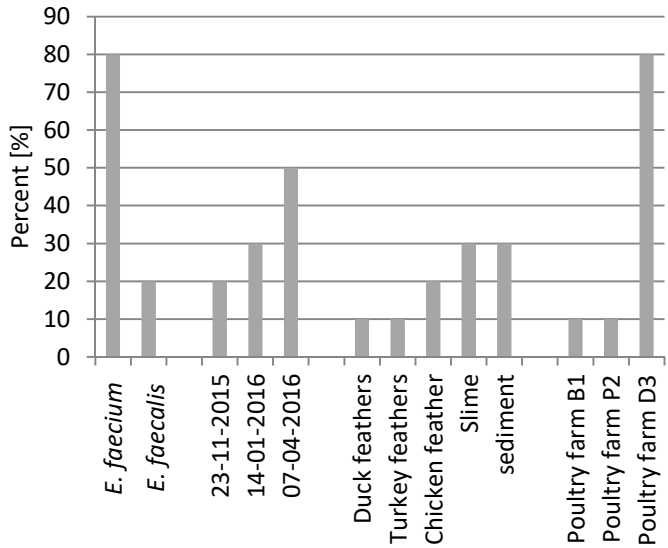


Fig. 1. Frequency of *Enterococcus* occurrence in poultry waste, depending on the time of sampling and type of processing plant

Enterococcus isolates, depending on the type of waste, date and place of sampling, showed varying susceptibility to tested antibiotics. More than 50 % of the strains showed resistance to 19 out of 23 antibiotics used.

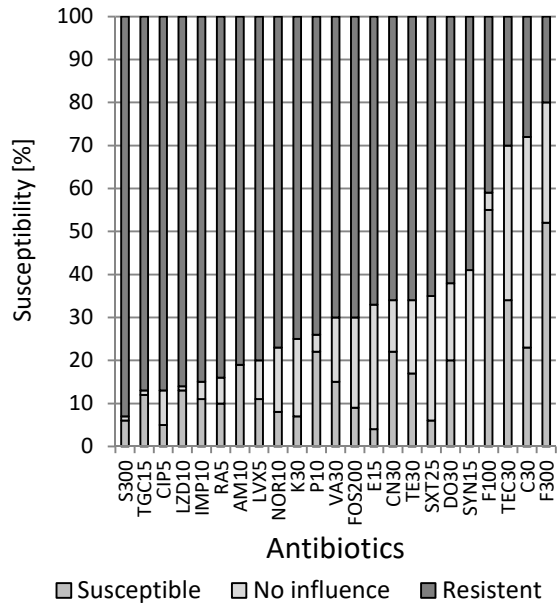
The largest resistance of isolates (from 90 to 95 %) was recorded towards streptomycin 300 (S300), tigecycline 15 (TGC15), and ciprofloxacin 5 (CIP5), while slightly lower (about 80 %) towards linezolid 10 (LZD10), imipenem 10 (IMP10), ampicillin (AM10), levofloxacin 5 (LVX5), norfloxacin 10 (NOR10), and rifampicin 5 (RA5). Obtained results indicate the presence of multiple resistance among *Enterococcus* isolates.

In contrast, the incidence of antibiotics-susceptible isolates was significantly lower, and only for nitrofurantion, regardless of its dose (F300, F100), it reached 50 %, and for another two (teicoplanin 30 (TEC30) and chloramphenicol (C30)), it ranged from 20 % to 30 %. Approximately 50 % of the isolates did not react with chloramphenicol (C30) and dalfopristin (SYN15) (Fig. 2a).

Taking into account the division of antibiotics into groups according to the active substance, resistance of *Enterococcus* isolates to all tested groups was demonstrated. Over 80 % of isolates showed resistance to streptogramins, carbapenems and fluoroquinolones, and only 30 % to nitrofurantions and phenicols. However, it should be emphasized that for the latter two groups of antibiotics, the reaction of the other strains varied; 50 % of isolates were susceptible to nitrofurantions, and 50 % did not react to phenicols. On the other hand, isolates showed either resistance (60 %) or no response (40 %) to chinopristin (Fig. 2b).

The largest number of resistant *Enterococcus* isolates (about 80 %) were found for 5 groups of antibiotics such as streptogramins, carbapenems, fluoroquinones, aminoglycosides and penicillins, and the least for nitrofurantions and phenicols.

a)



b)

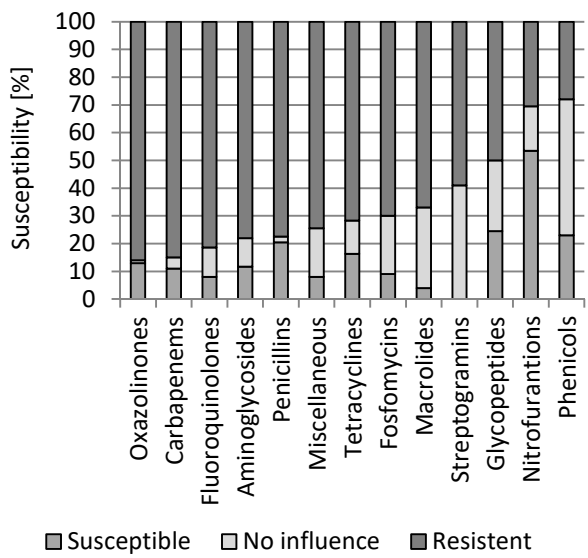


Fig. 2. Susceptibility of *Enterococcus* isolates to tested: a) antibiotics: S300 - streptomycin 300, TGC15 - tigecycline 15, CIP5 - ciprofloxacin 5, LZD10 - linezolid 10, IMP10 - imipenem 10, RA5 - rifampicin 5, AM10 - ampicillin, LVX5 - levofloxacin 5, NOR10 - norfloxacin 10, K30 - kanamycin 30, P10 - penicillin 10, V30 - vancomycin 30, FOS200 - fosfomycin, E15 - erythromycin 15, CN30 - gentamicin, T30 - tetracycline 30, SXT25 - cotrimaxazole, DO30 - doxycycline, SYN15 - chinopristin, F100 - nitrofurantion 100, TEC30 - teicoplanin 30, C30 - chloramphenicol 30, F300 - nitrofurantion 300; b) groups of antibiotics

The analysis of different types of *Enterococcus* isolates susceptibility distribution towards antibiotics confirms the distinctness of the test substances (Fig. 3).

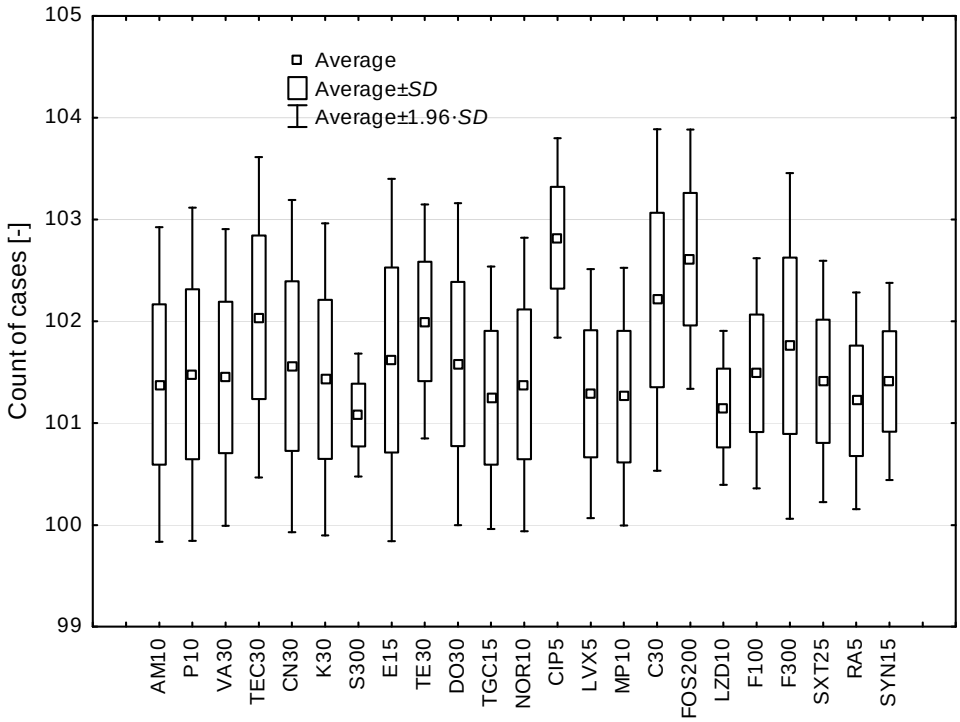


Fig. 3. Characteristics of established distributions of different types of *Enterococcus* isolates susceptibility for antibiotics (designations as in Figure 2a; variables scaling: resistant = 101, no effect = 102, susceptible = 103)

Enterococcus isolates, regardless of the type of poultry waste, from which they were isolated, have shown a high resistance to antibiotics reaching from 49 % for turkey feathers to 78 % for sludge. Furthermore, turkey feathers isolates were characterized by the highest susceptibility to antibiotics (30 %) (Fig. 4a). Based on the results, it was found that 65-70 % of isolated antibiotics-resistant *Enterococcus* strains originated from the poultry plants B1 and D3, while much lower number (49 %) from plant P2 (Fig. 4b). It was also observed that the sampling date influenced on the isolate susceptibility. The largest proportion of resistant strains was recorded in spring, whereas lower (about 60 %) in winter (Fig. 4c). Tested isolates, both from *E. faecium* and *E. faecalis* genus, have shown similar reaction pattern to all antibiotics applied in the research. Percentage of resistant isolates ranged from 67 to 71 %. In the case of *E. faecium*, slightly higher susceptibility and neutral activity to test antibiotics was observed as compared to *E. faecalis* (Fig. 4b).

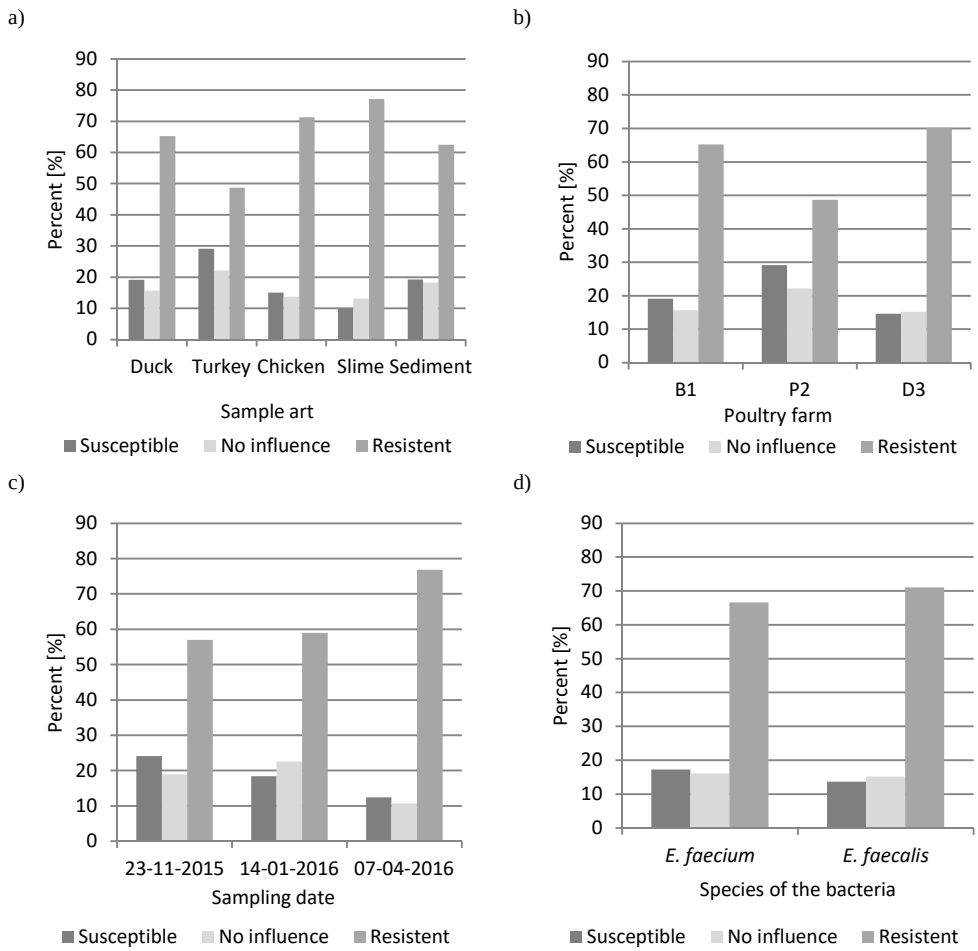


Fig. 4. Susceptibility of *Enterococcus* isolates to test antibiotics depending on the poultry: a) waste type, b) processing plant (B1, B2, P3), c) sampling date, d) *Enterococcus faecium* and *Enterococcus faecalis*

Results of this study showed different effects of the antibiotics used on *Enterococcus* isolates. The drug resistance of isolates was dependent on the species, type of waste, sampling time and location of the poultry plant. The majority of tested strains showed drug resistance, and only a few were susceptible or neutral to antibiotics.

Isolates showed resistance to most antibiotics, but some, e.g. nitrofuration, can be used to eliminate the contamination with *Enterococcus* bacteria.

Discussion

In recent years, there has been an increase in the importance of enterococci in the pathology of birds, especially poultry. Issues related to the infection of chicken broilers and laying hens by enterococci are not fully understood and are controversial. In the pathology

of birds, the most important is *Enterococcus faecalis*, then *E. faecium*, *E. hirae*, *E. cecorum* and *E. durans* [26-28], and only *E. faecalis* and *E. faecium* were found in the poultry waste tested.

In the treatment of bacterial infections, antibiotics as a panacea for everything are commonly used. Abused and inappropriately given, they cause the formation of the so-called bacterial resistance. Depending on the environment, from which bacteria are isolated, they may show a different susceptibility to antibiotics. Our research has shown that *Enterococcus* isolates, irrespective of the type of poultry waste, they were isolated from, showed varying resistance to antibiotics ranging from 49 % (turkey) to 78 % (sludge).

Enterococcus bacteria are naturally resistant to a number of antibiotics that are often used in therapy. Enterococci are naturally resistant to low levels of β-lactams, aminoglycosides, clindamycins, cephalosporins, lincosamides and nalidixic acid [29, 30]. These microorganisms can acquire and transfer the resistance to high levels of aminoglycosides, glycopeptides, quinolones, macrolides, tetracyclines, chloramphenicol and streptogramin to other species by means of mobile genetic elements (plasmids, transposons) [6, 9, 29, 31, 32].

Enterococci show the highest resistance to β-lactam antibiotics, all generations of cephalosporins and sulfonamides, and lower towards aminoglycosides, lincosamides and quinolones [33].

Vancomycin-resistant enterococcus (VRE) are the largest threat, because they are non-susceptible to the available antibiotics.

The lack of effective methods to prevent and control colonization with these strains is highlighted by, among others [34, 35]. In contrast, Yurdakul et al. [36] argue that the increase in enterococcal resistance to vancomycin is associated with the widespread use, another glycopeptide antibiotic, avoparcin, in Europe as a feed additive. This problem does not occur in the US, where avoparcin was not used.

Antibiotics-resistant bacteria evolve more rapidly, which often leads to the emergence of bacteria resistant to even several antibiotics at the same time [37, 38]. Our study has shown that 80% of *Entrococcus* strains are resistant to 5 groups of antibiotics - streptogramins, carbapenems, fluoroquinones, aminoglycosides and penicillins, indicating the phenomenon of multi-resistance.

The major problem with bacterial antibiotic resistance is that genes associated with the lack of susceptibility to antibiotics are localized on cellular elements (plasmids, transposons, bacteriophages and integrons) that can travel between bacteria of the same species, but also between non-phylogenetic bacteria [38, 39]. The processes of accepting genes or their fragments occur both in the natural environment and in the organisms of humans and animals. However, studies show that resistance genes are a component of the genome of many bacteria, whether or not they have been selectively treated by antibiotics and can be transmitted by horizontal gene transfer (HGT). Another mechanism that leads to rapid changes in bacterial genomes is transformation. In this process, competent bacteria can extract DNA fragments with antibiotic resistance genes from the environment [40].

Resistance of *Enterococcus* to penicillin (beta-lactam antibiotic group), according to literature data, is from 0 to 100 % and for ampicillin from 0 to 82 % [18]. Our studies revealed high resistance of *Enterococcus* isolates (90-95 %) to streptomycin 300 (S300), tigecycline 15 (TGC15), ciprofloxacin 5 (CIP5) and lower (about 80 %) to linezolid 10

(LZD10), ampicillin (AM10), levofloxacin 5 (LVX5), norfloxacin 10 (NOR10) and rifampicin 5 (RA5).

Many authors attribute the blame for the increasing resistance of enterococci to antibiotics by their overuse in animals, because in farms where antibiotics were used, there was significantly higher level of resistance to organisms isolated from animals as well as to humans [36, 41, 42]. In addition, various chemical compounds contaminating the environment [43] can be used by microorganisms as a source of nutrients and promote their multiplication.

Conclusion

1. Contamination of the poultry waste with *Enterococcus* isolates included *E. faecium* species - 79 % and *E. faecalis* - 21 %. The most contaminated with these bacteria were sludge and sediment from the centrifuge as well as chicken feathers, irrespective of the place and time of sampling.
2. Tested isolates of *Enterococcus* showed multiple resistance and similar reaction to all antibiotics used in the study and *E. faecalis* strain was more resistant.
3. *Enterococcus* isolates (about 80 %) showed the highest resistance to 5 groups of antibiotics - streptogramins, carbapenems, fluoroquinones, aminoglycosides and penicillins, and the lowest for nitrofurantions and phenicols.
4. The cluster analysis confirms the distinctness of antibiotic action to tested isolates. A separate group in this respect are nitrofurantions. Of the other groups, the phenotypic distinctness is shown by phenicols and glycopeptides. Other antibiotic groups differ in their activity to a lesser degree.
5. The most effective action in reducing the development of *Enterococcus* was revealed by nitrofurantions (50 %), phenicols and glycopeptides (20-25 %).

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