# Antibiotic resistance in *Enterococcus* isolates from poultry swabs in Slovakia

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**Summary**

The role of susceptibility or resistance to antimicrobial agents in enterococci is important for the food industry. Eighty two isolates (18 *Enterococcus faecium,* 26 *E. faecalis*, *35 E. casseliflavus* and 3 *E. gallinarum* ) were taken from cloacal swabs of poultry and from the water intended for livestock breeding during the years 2004–2006. The isolates were evaluated for susceptibility to antimicrobial agents: ampicillin, gentamicin, streptomycin, erythromycin, tetracycline, and vancomycin determined by the disk diffusion test and E-test. All isolates were found to be susceptible to ampicillin and vancomycin. Resistance rates to gentamicin, streptomycin, tetracycline and erythromycin were 1%, 4%, 40% and 4%, respectively. One isolate of *E. faecium* was resistant to gentamicin and erythromycin. Two and six isolates of this species were resistant to streptomycin and tetracycline, respectively. One isolate of *E. faecalis* was resistant to streptomycin and erythromycin. High level resistance to tetracycline was observed in *E. faecalis* (46%) and *E. casseliflavus* (37%) isolates. One isolate of *E. casseliflavus* was resistant to erythromycin. *E. gallinarum* isolates showed a low level of resistance to gentamicin, streptomycin, tetracycline and erythromycin (0–2%). This research suggests that enterococci of animal and environmental origin may play a potential role as a reservoir of resistance to antimicrobial agents.

**Keywords:**

*Enterococcus*; probiotics; resistance; vancomycin; gentamicin

Despite constantly increasing the knowledge about its properties, the position of *Enterococcus* in the food industry and agriculture is still problematic. The safety of the use of probiotics and production strains has been studied in detail. Every isolate used in the food industry goes through a very strict examination of virulence factors, presence of resistance genes and conjugative transfer ability [1]. Given the fact that the *vanA* operon transfer to a probiotic strain was successfully achieved under experimental conditions [2], it is impossible to guarantee the absence of the change of the harmless phenotype to the pathogenic one in the host body. The exchange of the genetic material of *Enterococcus* localized on plasmids occurs mainly via bacterial conjugation and may be accompanied by the transfer of resistance determinants against glycopeptides to bacteria from the genus *Staphylococcus* [3]. This extrabreed character of transfer represents a very dangerous property of *Enterococcus*.

Enterococci constitute a significant part of the autochtonous microflora of the gastrointestinal tract (GIT) of mammals [4]. Their inherent resistance against a large group of antimicrobial substances and the ability to live and grow in unfavourable conditions, give enterococci an advantage in the evolutionary struggle to survive.

## *Enterococcus* in food

### Meat

Presence of enterococci in the digestive tract of animals supports a presumption of meat contamination during the slaughter process in abattoirs [4]. The resistance of enterococci to pasteurization temperatures (they belong to the most thermotolerant microorganisms among non-sporulating bacteria) may cause the problem of damage to heat-treated products [5]. The ability to adapt to worsening conditions (high or low temperature, extreme pH) facilitates their survival in raw products (meat, milk) [6].

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Cheese

Enterococci are naturally present and grow in many kinds of cheese - mainly in traditionally-produced products in the south of Europe where the primary material is raw or pasteurized goats', ewes' or cows' milk. High counts of enterococci are most often a result of insufficient hygiene conditions during the production, and cause the deterioration of sensory properties of certain kinds of cheeses [7]. On the other hand, in many cases they play an important role during maturation and the development of aroma. The dominant presence of enterococci in many cheeses during maturing is caused by their wide range of temperatures suitable for the growth of their cultures, and the high tolerance to salt and acids [8]. The proteolytic activity of enterococci, in particular, casein hydrolysis is important for cheese maturing. This activity is higher compared to other bacteria of milk fermentation. Because of this property, enterococci are used as starting cultures during green feed conservation and ensilage processing [5].

**Enterocines – bacteriocins of *Enterococcus* spp.**

Certain strains of *Enterococcus* spp. have the ability to produce enterocines, which may play a prominent role in the process of fermentation [6]. Enterocines have the ability to protect the fermented products against damage. This feature is important since natural ways of conservation could replace the commonly used chemical approach. [9, 10]. Enterocines are often active against other enterococci and against *Clostridium* spp., *Staphylococcus aureus*, *Listeria monocytogenes*. The antilisteria activity is associated with the phylogenetic relatedness of *Enterococcus* and *Listeria*. Strains producing bacteriocins can be applied as "antilisterial protectives" in the dairy industry, mainly in certain types of soft cheeses [9].

**_Enterococcus_ used in the form of probiotics**

Probiotics represent pure or mixed cultures of live microorganisms, after being administered to human or animals, positively influence the host organism by ameliorating properties of intestinal microflora [11]. Positive effects on the organism are the maintenance or renewal of the normal intestinal flora, function of the growth stimulator in feed and amelioration of the nutritional value of food from feeders [12]. Therapeutic applications of probiotics as an alternative for antimicrobial substances in livestock breeding at least partially reduce the growth, spread and transfer of resistant bacterial strains through the food chain [4]. The control of the usage of probiotic feeding mixtures is regulated by 94/40/EEC directive of the European Commission with a list of indications for the permitted usage of probiotics [13]. The probiotic strain *E. faecium* SF68 was studied in detail from the human and veterinary usage perspectives [5]. It is an intestinal comensal with a short lag phase and generation period of approximately 20 min. It is moderately resistant to antimicrobial substances and it has an inhibitory effect in vitro on *E. coli, Salmonella* spp., *Shigella* spp. and *Enterobacter* spp. It is resistant to low pH values and insensitive to

**Tab. 1**. Summary of resistance mechanisms in *Enterococcus* spp.

| Antibiotic | Action mechanism of antibiotic | Resistance mechanism |
|---|---|---|
| β-lactams | – inhibition of enzymes of synthesis of cell wall (PBPs) | – appearance of low affinity PBPs<br>– increased synthesis and structural changes of low affinity PBPs<br>– synthesis of LD-transpeptidase instead of DD-transpeptidase ("bypass")<br>– synthesis of β-lactamase |
| aminoglycosides | – bond to 16S rRNA of 30S sub-unit<br>– proteosynthesis inhibition | – aminoglycoside-modifying enzymes<br>– mutation in gene for 30S ribosomal sub-unit |
| glycopeptides | – disturbance of synthesis of cell wall, bond to peptidoglycane precursors causes inhibition of transglykolisation reaction | – effect modification of targeted place<br>– glycopeptid dependence ("nonsense" mutation in *ddl* gene ) |
| macrolides | – bond to 23S rRNA (V domain) and 50S sub-unit<br>– proteosynthesis inhibition | – adenin-$N^6$-methyltranspherase (methylation of adenine residues 23S rRNA)<br>– efflux |
| tetracyclines | – affinity decrease of binding place on 30S subunit for aminoacyl-tRNA, impaired proteosynthesis | – efflux<br>– structural change of binding place<br>– unknown mechanism |

bile acid salts. Individuals show a high tolerance to this strain without any side effects [11].

In veterinary use, the probiotic products are used for prevention, treatment of intestinal illness by the creation of physiological homeostasis as well as a growth stimulator. *E. faecium* SF68 was used as an additive to dried feeding mixtures for dogs with a subsequent significant increase in animal immune system functions [14]. A positive effect was recorded with the *E. faecium* SF68 strain during the treatment of *E. coli* massive diarrhoea [4].

### *Enterococcus* and antimicrobial resistance

Inherent and acquired mechanisms of resistance against therapeutically used substances create a unique picture of enterococci as initiators of serious infection disseases [15, 16]. When talking about the resistance of *Enterococcus*, there exists the so-called effect of cross-resistance that is created, in addition to other factors, as a consequence of the usage of structural analogues of antibiotics in the form of growth stimulators. Aarestrup et al. [17] state that more that half of the world's consumption of antimicrobial substances is connected with livestock breeding where the substances are used not only for therapy and the prevention of antibacterial infections, but as growth stimulators as well [18]. Avoparcin is an example of a growth promotor similar to vancomycin in its chemical structure. The chemotherapeutic is used for the treatment of serious enterococcal infections. It was the application of avoparcin in the past that led to the creation and spread of vancomycin-resistant enterococci via the food chain [19–21].

Aminoglycoside antibiotics have an important place in the treatment of enterococcal infections. Bactericidal effects, suitable pharmacokinetic properties and synergistic effects with β-lactams and glycopeptides are reasons that determine their frequent prescription in the antibiotic treatment of serious illnesses. However, the increase in the resistance to aminoglycosides is related to their frequent application in agriculture [22]

A brief description of some resistance mechanisms occurring in *Enterococcus* spp. strains is listed in Tab. 1 [23].

An important point is that multi-resistant bacteria in the intestinal tract may become a source of resistance genes for other microorganisms [18]. This is the reason why the food chain monitoring for multi-resistant bacteria with a potential for the subsequent transport to humans should become an inevitable part of the human health protection. In this relation, the presented work deals with the species spectrum and susceptibility or resistance to clinically important antimicrobial agents in poultry and water enterococci.

## MATERIAL AND METHODS

### *Enterococcus* isolates and reference materials

Isolates for microbiological examination were taken from cloacal swabs of 3-weeks old chicken and from the environment (water for livestock breeding) during 2004–2006. Samples originated from the tributary area of State Veterinary and Food Institute, Dolný Kubín, Slovakia (districts Dolný Kubín, Prievidza, Liptovský Mikuláš, Žilina, Považská Bystrica, Martin, Nové Mesto nad Váhom, Poprad). Suspect colonies of *Enterococcus* spp. were cultivated on the selective medium containing bile, aesculin and sodium azide (Slanetz Bartley agar, HiMedia, Mumbai, India) and on a non-selective medium containing 5% washed erythrocytes (Columbia agar; Oxoid, Basingstoke, United Kingdom). Culures were incubated at (37 ± 1) °C for (48 ± 2) h. Presumptive colonies were selected from gram-positive cocci without a hemolytic zone and with negative catalase and oxidase tests. Isolates also produced PYR enzyme (PYRolidonylarylamidase; detected by Mikro-La-Test, Pliva-Lachema, Brno, Czech Republic).

### Strain identification

Strain identification of *Enterococcus* isolates was carried out using EN-COCCUS phenotype micro-tests (Pliva-Lachema) and API20 Strep (bioMérieux, Marcy l'Etoile, France). All isolates were plated on Columbia agar and cultivated for 24 h at (37 ± 1) °C. A cell suspension was prepared in 0.85% NaCl from a pure 24 h culture with a cell density corresponding to degree 2 on the McFarland scale ($6 \times 10^8$ CFU·ml$^{-1}$). An appropriate test platform was inoculated by the suspension according to the manufacturer's instructions. The numerical code acquired by the substrate colour change of the platform was evaluated by the TNW Pr.6.5 Analytic Profile Index computer system (Pliva-Lachema). In the case of aberrant isolates, additional identification was done using the API20 Strep. The numerical code acquired by the substrate colour change of platform was evaluated by the API Analytic Profile Index computer system (bioMérieux).

Preliminary results from the conventional phenotypic identification were verified by the PCR method with primers targeting the *ddl* gene coding D-alanine-D-alanine ligase of *E. faecalis* and *E. faecium* strains [24]. This PCR was combined with

**Tab. 2.** Antimicrobial substances used and disk diffusion method interpretation.

| Antibiotic | Content in disk [$\mu$g] | Results interpretation [mm] | | |
|---|---|---|---|---|
| | | Isolate is resistant | Isolate is medium sensitive | Isolate is sensitive |
| ampicilin | 10 | < 16 | – | > 17 |
| gentamicin | 120 HLRG | < 6 | 7–9 | > 10 |
| streptomycin | 300 HLRS | < 6 | 7–9 | > 10 |
| erythromycin | 15 | < 13 | 14–22 | > 23 |
| tetracycline | 30 | < 14 | 15–18 | > 19 |
| vancomycin | 30 | < 14 | 15–16 | > 17 |

HLRG – isolate high-level resistant against gentamicin, HLRS – isolate high-level resistant against streptomycin.

primers of *vanC1* and *vanC2/C3* genes that are responsible for inherent enterococcal resistance and, at the same time, they are so-called identification markers for *E. gallinarum* (*vanC1*) and *E.casseliflavus* / *flavescens* (*vanC2/C3*) strains.

**Phenotype profile analysis of isolates' sensitivity to chosen groups of antimicrobial agents**

An antimicrobial profile of tested isolates of *Enterococcus* spp. with exactly defined antimicrobial substances was evaluated using the qualitative disk diffusion method. Based on the results of the disk diffusion method in isolates resistant to tested antibiotics, minimal inhibitory concentration (MIC) was determined using the E-test (bioMérieux). Both methods were carried out in accordance with the CLSI document M31-A3 [25]. The tested antimicrobial agents were ampicillin (AMP), gentamicin (GEN), streptomycin (STR), vankomycin (VAN), erythromycin (ERY), tetracycline (TET).

The disc diffusion method was carried out in a way that a Petri dish with the prepared Muller-Hinton agar (Oxoid) was inoculated with a bacterial suspension with a cell density corresponding to degree 0.5 on the McFarland scale ($1.5 \times 10^8$ CFU·ml$^{-1}$), which was prepared by diluting the overnight bacterial culture in 0.85% NaCl. A volume of 50 $\mu$l of such adjusted suspension was inoculated on a pre-dried cultivation medium and spread equally over the entire surface using a sterile cotton swab. After the absorption was completed, disks containing an antibiotic were applied by a sterile injection needle on the surface. The diameter of the inhibition zones of growth using a slide ruler after a 24h cultivation at a temperature of (37 ± 1) °C was measured, which was subsequently evaluated according to criteria in CLSI document M31-A3 [25]. A spectrum of 6 antimicrobial substances was chosen based on publications about resistance incidence in livestock breeds (Tab. 2).

**Tab. 3.** Antimicrobial substances used in E-test.

| Antibiotic | Concentration range [$\mu$g·ml$^{-1}$] | Marginal value for resistance assessment [$\mu$g·ml$^{-1}$] |
|---|---|---|
| gentamicin | 0.016–256 | HLRG > 512 |
| streptomycin | 0.064–1 024 | HLRS > 1 024 |
| erythromycin | 0.016–256 | > 8 |
| tetracycline | 0.016–256 | > 16 |
| vancomycin | 0.016–256 | > 32 |

HLRG – high-level resistance to gentamicin, HLRS – high-level resistance to streptomycin.

Determination of the MIC for resistant isolates was done by E-test (bioMérieux). Plates of Muller-Hinton agar were inoculated with a bacterial suspension with a cell density corresponding to degree 0.5 on the McFarland scale ($1.5 \times 10^8$ CFU·ml$^{-1}$), which was prepared by diluting the overnight bacterial culture in 0.85% NaCl. A volume of 50 $\mu$l of such adjusted suspension was inoculated to a pre-dried cultivation medium and was spread equally over the entire surface using the sterile cotton swab. After the absorption was completed, a plastic stripe with an exponential gradient of the antimicrobial substance concentrations was applied on the surface after the suspension absorption. The MIC level was assessed after a 24 h cultivation of the inoculated platforms at a temperature of (37 ± 1) °C in the place where was the admissible zone of growth inhibition – the ellipse traversing the stripe edge. The MIC value was evaluated in accordance with CLSI document M31-A3 instructions (Tab. 3) [25].

## RESULTS

The collection consisted of 82 *Enterococcus* spp. isolates. All isolates went through strain identification carried out using EN-COCCUS and API20 Strep microtests. The results of the conventional phenotype identification were verified using

PCR methods. Based on the results, 82 *Enterococcus* spp. isolates were classified into four species as follows: 18 *Enterococcus faecium*, 26 *E. faecalis*, 3 *E. gallinarum* and 35 *E. casseliflavus*. Among the tested isolates, 1 isolate showed a high level of resistance to GEN, 3 isolates had a high level of resistance to STR, 0 isolates were resistant to VAN, 33 isolates were resistant to TET and 3 isolates were resistant to ERY, as determined by the qualitative disk diffusion method. Results obtained are presented in Fig. 1.

MIC was determined by E-test in isolates resistant to the tested antimicrobials. The MIC value ranges in the tested resistant isolates are presented in Tab. 4.

Predominant strains causing infections in humans are the *E. faecalis* and *E. faecium* [16, 26]. Fig. 2 presents the prevalence of the resistance of these species to the tested antimicrobial substances. Out of 26 tested *E. faecalis* isolates, 0 isolates (0%) were resistant to AMP, 0 isolates (0%) had a high level of resistance to GEN, 1 isolate (3.8%) was high-level resistant to STR, 0 isolates (0%) were resistant to VAN, 12 isolates (46%) were resistant to TET and 1 isolate (3.8%) was resistant to ERY. Out of 18 tested *E. faecium* isolates, 0 isolates (0%) were resistant to AMP, 1 isolate (5.6%) had a high level of resistance to GEN, 2 isolates (11.1%) had a high-level of resistance to STR, 0 isolates (0%) were resistant to VAN, 6 isolates were resistant to TET (33.3%) and 1 isolate (5.6%) was resistant to ERY.

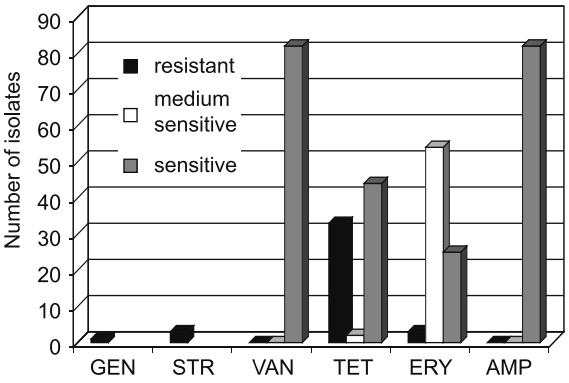


**Fig. 1.** Antimicrobial profiles of tested isolates determined by the disk diffusion method.

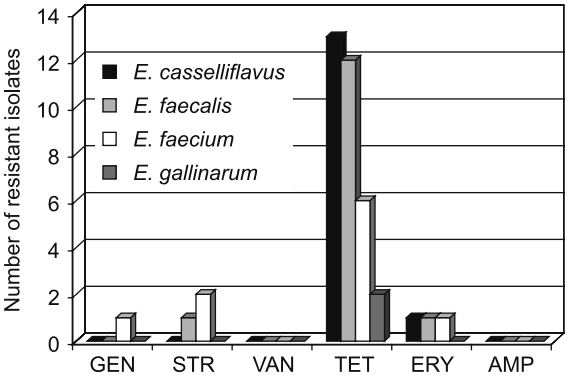


**Fig. 2.** Antimicrobial profiles of tested isolates in dependence on species.

**Tab. 4.** MIC ranges of antibiotics against the tested isolates.

| Antibiotic | Concentration range [$\mu g \cdot ml^{-1}$] |
|---|---|
| gentamicin | > 256 |
| streptomycin | > 1 024 |
| erythromycin | ≥ 8 |
| tetracycline | 16–94 |

## DISCUSSION AND CONCLUSION

Evaluating the position of *Enterococcus* in the food chain is very difficult. Enterococci not only belong to the natural intestinal microflora of humans and animals, but many strains can be found in soil, water, plants and often they are isolated from food. Enterococci are presented as an important part of microflora of many cheeses, mainly during their maturation, where their numbers reach up to $10^7$–$10^8$ CFU·$g^{-1}$ [26, 27]. However, other authors [1, 2] state that *Enterococcus* may be a source of resistance genes - for example to vancomycin - with the ability of inter-species transfer via conjugative plasmids [28–31].

The analysed collection of 82 *Enterococcus* spp. was tested for an occurrence of resistance to important antimicrobial agents. Resistance to AMP and VAN was not observed in any of the tested isolates on the fenotype level and represents 100% sensitivity. The therapy of serious enterococcal infections, i.e. patients allergic to β-lactams, requires treatment with glycopeptide antibiotics or, in the case of endocarditis and meningitis, a combination of AMP and GEN is suitable [32]. Thus, the low resistance prevalence of enterococcal isolates to AMP and VAN is an important and positive component of human health protection [33]. The resistance of enterococcal isolates to aminoglycosides accounted for a 1. 2% high-level resistance to GEN. This resistance may be due to the presence of the bifunctional enzyme Aac(6')–Ie-Aph(2")-Ia that leads to the elimination of all aminoglycosides except for STR and spectinomicin from therapy [34]. Good sensitivity to GEN, and the absence of resistance genes to VAN in tested enterococci of

animal and environmental origin, correlates with data obtained in neighbouring countries [35–37] and is probably a reflection of the low consumption of these antimicrobial substances in livestock breeding in Slovakia. The results obtained are confirmed by the recent level of incidences of resistance to therapeutics used in human or veterinary medicine in enterococcal isolates originating from traditional ewes' cheese [28].

The high-level resistance to STR was recorded in 3 isolates (3.7%; 1 *E. faecalis* isolate, 2 *E. faecium* isolates) and is probably connected with its frequent application in livestock breeding [38, 39]. Aph(3') – aminoglycosid-O-phosphotransferase and Ant(6') – aminoglycosid-O-nucleotidyltransferase enzymes were detected in high-level resistant isolates with a value of MIC > 1024 $\mu g \cdot ml^{-1}$ [40, 41]. Bacterial strains producing Aph(3') enzyme are resistant to kanamycin, although GEN keeps its bacterial efficiency [40], Ant(6') enzyme modifies antimicrobial substances – STR manifesting a high value of MIC > 2000 $\mu g \cdot ml^{-1}$ [41]. The prevalence of resistance to STR in isolates acquired from cloacal swabs of poultry was higher compared to isolates from ewes' cheese where resistance was not observed ($n = 0$) [28].

Thanks to their relatively low toxicity, macrolide and tetracycline antibiotics are often used for the treatment not only of enterococcal but staphylococal and streptococal infections in humans as well as in animals [36]. An effective range of resistance genes by conjugative transfer occurs because of this selective pressure [42]. The resistance level of the tested isolates to tetracyclines accounted for 40.2% with MIC ranging from 16 $\mu g \cdot ml^{-1}$ to 96 $\mu g \cdot ml^{-1}$, which corresponds with data obtained by KROČKO et al. [43], who determined tetracycline resistance in white meat (poultry) in up to 56% isolates.

The resistance level to ERY was found to be considerably lower and accounted for only 3.7%, compared to data from BELICOVÁ et al. [28], who published figures as high as 36% *E. faecium* resistant isolates and 22% *E. faecalis* originating from traditional ewes' cheese or KROČKO et al. [43], 15% of resistant isolates from white meat (poultry). However, many studies acknowledge the mutual correlation between resistance to ERY and VAN, because resistance genes to these antibiotics are often localized on the same mobile element [44]. It is interesting to compare the results obtained in this study, where isolates came from animals designed for human consumption, with results obtained by KROČKO et al. [43], who studied the prevalence of antimicrobial resistance of *Enterococcus* in raw white meat, pork and beef meat. He came up with some very surprising results; up to 15% of tested isolates being found resistant to VAN, 15% to ERY, 27% to AMP, 25% to GEN and 56% to TET. The published results demonstrate that even processing did not decrease the quantity of resistant isolates; on the contrary, it is possible that in some cases a secondary contamination occured. This might have been caused by the fact that enterococci are ubiquitous microorganisms, and so the food chain is a vector of resistance gene transfer to the human population. Taken together, from the perspective of human health, enterococci of animal and environmental origin may be classified as a potential source of resistance genes and this fact should not be forgotten in the complex evaluation of enterococci. On the other hand, significant benefit of probiotics including enterococci is well documented [45, 46]. Moreover, recently HOSSEINI et al. [47] published data about heat-stable bacteriocin producing *E. faecium* strains isolated from non-fermented animal foods and assumed their potential use as common ingredients in food.

Based on all the information attained, it is possible to conclude that prior to summarizing positive and negative effects of these bacteria, it is probably necessary to carry out epidemiological analyses dealing with the food chain and the circulation of resistance determinats in enterococci between animal and humans.

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