Antibiotic Resistance of *Enterococcus faecalis* Isolated from Gastrointestinal Tract of Broiler Chickens after Propolis and Bee Pollen Addition

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Abstract
One of the safety aspects of antimicrobials (propolis and bee pollen) use in poultry farms as growth promoters is higher susceptibility of gastrointestinal microorganisms to antibiotics used for human treatment. Enterococci belong to the normal microbiota of gastrointestinal tract of chickens and are widely distributed in nature, but they are not generally recognized as safe (GRAS). Poultry enterococci carrying antimicrobial resistance genes may not only transfer these genes to other, possibly pathogenic, bacteria in the chicken gut, but upon transfer to zoonotic bacteria they also may pose a human health hazard. Antibiotic resistance prevalence of *E. faecalis* isolates found in the crop was subsequently found in the ileum and caecum within each group of broiler chickens. No resistance of *E. faecalis* isolates was found against vancomycin and teicoplanin. Intermediate resistance to erythromycin in the most *E. faecalis* isolates from gastrointestinal tract of broiler chickens with propolis supplement in their diet was eliminated. Our results suggest the probability of synergism effect of propolis and also bee pollen with some tested antibiotics against *E. faecalis* isolates.

Keywords: antibiotic resistance, bee pollen, broiler chickens, gastrointestinal tract, *Enterococcus faecalis*, propolis

1. Introduction

For replace of banned antibiotics used as growth promoters in broiler chicken nutrition, the numerous alternative supplements were tested. Propolis, a natural antibiotic and bee pollen belong to the group of naturally occurring substances of animal and plant origin with antioxidant and antimicrobial activity [1-3]. Propolis is a natural brownish-green resinous product collected by honeybees. It had been used in folk medicine to cure infections [4]. Bee gathered pollen is considered as a valuable functional food with varied enhancing effects in health [5]. The main antimicrobial action has been attributed to their several phenolic and flavonoids compounds with antioxidant activity [6, 7].

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Enterococci are part of the normal microbiota belonging to the gastrointestinal and genitourinary tract of humans and animals [8]. Their ubiquitous nature and resistance to adverse environmental conditions account for their ability to colonise different habitats and underlie their potential to easily spread through the food chain [9]. Enterococci belong to the lactic acid bacteria (LAB) group and are widely distributed in nature, but they are not generally recognized as safe (GRAS) [10, 11].
The use of broad spectrum antibiotics creates a selective pressure on the bacterial flora, thus increasing the emergence of multiresistant bacteria, which results in a vicious circle of treatments and emergence of new antibiotic resistant bacteria [12]. A major issue of concern is the transfer of antibiotic resistance from enterococci to more virulent pathogens such as multiple-drug-resistant staphylococci [13]. An

increasing number of enterococci isolated from food production have development resistance to various therapeutic antibiotics, including vancomycin [14 - 16]. From this point of view, the statement by Garg and Mital (1991) [17] "a food should be free not only from disease-producing organisms, but also from those that have the potential of causing disease"—seems legitimate.
Poultry enterococci carrying antimicrobial resistance genes may not only transfer these genes to other, possibly pathogenic bacteria in the chicken gut, but upon transfer to zoonotic bacteria they also may pose a human health hazard. Furthermore, these enterococci may be transferred, directly or indirectly, to man, where they might be able to cause disease or further disperse their antimicrobial resistance genes among the gastrointestinal bacterial community [18]. However, recently many investigators have speculated that commensal bacteria including lactic acid bacteria (LAB) may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens [19].
The objective of this study was to determine antibiotic resistance of *E. faecalis* isolated from the gastrointestinal tract of broiler chickens, which were fed with feed mixture enriched by supplement of natural growth promoters and antimicrobials – bee pollen and propolis. One of the safety aspects of antimicrobials (propolis and bee pollen) use in poultry farms as growth promoters is higher susceptibility of gastrointestinal microorganisms to antibiotics used for human treatment.

2. Materials and methods

The tested broiler chickens of hybrid combination Ross308 were fed by same complete feed mixture HYD-01 (starter – until to 21st day of age) and HYD-02 (growth – from 22nd day to 42nd day of age). The experiment included 300 one day-old chickens, which were divided into 5 groups: control (1C), experimental with pollen supplement in amount of 15 g. kg^{-1} (2nd group) and 45 g. kg^{-1} (3rd group). Broiler chickens of 4th and 5th groups were fed with propolis ethanol extract supplement in amount of 400 mg.kg^{-1}, 800 mg.kg^{-1}, respectively. The feed complete mixture HYD-01 and HYD-02 was prepared without antibiotic preparations and coccidiostatics.

Sample collection

After fattening, broilers were processed by stunning, bleeding, scalding, and picking. For microbial examination (30 samples) were whole crops, ileum and caecum aseptically removed from five carcasses per group (10 from control group) and were placed in separate sterile plastic bags. The chyme of each parts of gastrointestinal tract was weighed and properly homogenized in ten-fold amount of saline. Serial dilutions were made also in saline and cultured on selective diagnostic Slanetz–Bartley agar at temperature 37±1°C for 48±2 h (Biokar Diagnostic, France).

Genus identification of enterococci

Typical colonies of enterococci were transferred to bile esculin azide agar (Biokar Diagnostic) for species identification. Based on positive growth (esculin hydrolysis), the following tests were carried out for presumptive identification of the isolates: microscopic characteristic of colonies (conformation, motility, cleanness of cultures), Gram staining, production of catalase and pyrolidonyl arylamidase (PYRAtest, Lachema, Czech Republic), and pigmentation.

Isolation of enterococci genomic DNA

The isolation of genomic enterococci DNA from the overnight cultures at 37°C was prepared. The bacteria were transferred to 200µl of Tris-HCl and EDTA (100mM Tris-HCl pH 8.0 and 10mM EDTA) and washed twice in the same solution. After centrifugation the bacterial pellet were incubated at 37°C with intermitted shaking in conditions with 300µl of lysis buffer (0.1M NaCl, 0.02M Tris HCl, 0.001M EDTA, 1.2 % Trition X-100) and lysozyme solution (concentration 20 mg/ml) for 2 hours. The 5 µl proteinase K solution (Fermentas, Germany) and to remove RNA, 4 µl of Rnase-A solution (Fermentas) were added to the mix. The contents were mixed thoroughly and incubated at 58°C for 2 hours. After incubation the mixtures were centrifuged and genomic DNA in the supernatant (collected in sterile 1.5 ml tube) was precipitated out with izopropanol. The final

pellet was dissolved in 50 µl of Tris-HCl and EDTA (100mM Tris-HCl pH 8.0 and 10mM EDTA).

Species identification of E. faecalis by PCR method

The PCR method for enterococci species identification was performed using specific primers: (941 bp) F:5´ATCAAGTACAGTT AGTCTTTATTAG3´ R:5´ACGATTCAAAGCT AACTGAATCA GT3´ One microliter of DNA was added to a mixture containing 2.5 µl of 10 x PCR buffer (Fermentas), 0.5 µl of each 10 mM deoxynucleoside triphosphate (Fermentas), 2.0 µl 25 mM $MgCl_2$ (Fermantas), 0.25µl 5 U of Dream*Taq* polymerase (Fermentas) and 0.5 µl of each 10 pmol primers (IDT, USA). Samples were incubated for 3 min at 95ºC to denature the target DNA and were maintained 30 cycles of 95ºC for 30 s, 54ºC for 40 s and 72ºC for 60 s. The samples were then incubated at 72ºC for 10 min for a final extension and were maintained at 4 ºC until they were tested. Gels were stained with GelRed (Biotium, USA) and visualized in UV light. Isolates producing an amplicon band of the appropriate size by agarose gel (1.5 %) electrophoresis were considered positive for species identification. The reference strain *E. faecalis* (CCM 4224) was used.

Antimicrobial susceptibility tests

Inoculum was prepared by suspending of growth colonies from Plate count agar and the suspension was adjusted to equal a 0.5 McFarland standard according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI) [20]. Susceptibilities to antimicrobial agents were tested using the disk diffusion method with according to the CLSI requirements, using the following antimicrobial disks: vancomycin (VAN) 30 µg/disk, gentamicin (GEN) 120 µg/disk, erythromycin (ERY) 15 µg/disk, teicoplanine (TEI) 30 µg/disk, ampicillin (AMP) 10 µg/disk (Oxoid). The isolates were classified as susceptible, intermediate resistant or resistant.

3. Results and discussion

Of 54 isolates of *E. faecalis*, 13 were isolated from broilers of control group, 20 from broilers with propolis supplement addition and 21 from broilers with bee pollen supplement addition. No resistance of *E. faecalis* isolates was found against vancomycin and teicoplanin. Kilic et al. (2005) [21] reported that propolis could be used for the resistant strain-infection treatments such as methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci infections as an alternative therapy. In accordance to our results (Figure 1) Brtková and Bujdáková (2009) [22] in *E. faecalis* isolates from random chicken cloacal swabs in Slovakia not found resistance to vancomycin, but also determined the resistance to erythromycin.

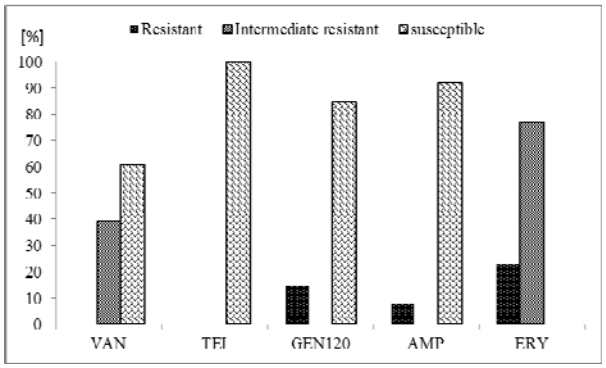

Figure 1. Antimicrobial resistance profiles of *Enterococcus faecalis* isolated from broiler chickens GIT of control group (n=13)
VAN–vancomycin, TEI–teicoplanin, GEN120–gentamicin, AMP–ampicillin, ER –erythromycin

Resistance against gentamicin and ampicillin was very rare in broiler chicken enterococci of 4th, 5th (propolis supplement) (Figure 2) and control group, whereas in 2nd and 3rd group (bee pollen supplement) (figure 3) these types of resistance were not detected. Erythromycin resistance of *E. faecalis* was at the same level regardless of group origin.

There were no significant differences (P>0.05) in antibiotic resistance prevalence among strains from experimental groups of broiler chickens which were fed with different amount of tested supplements. Also origin of isolates (crop, ileum, caecum) within each tested groups had no effect on antibiotic resistance profiles of isolates.

It means that antibiotic resistance prevalence found in the crop was subsequently found in the ileum and caecum within each group of broiler chickens.

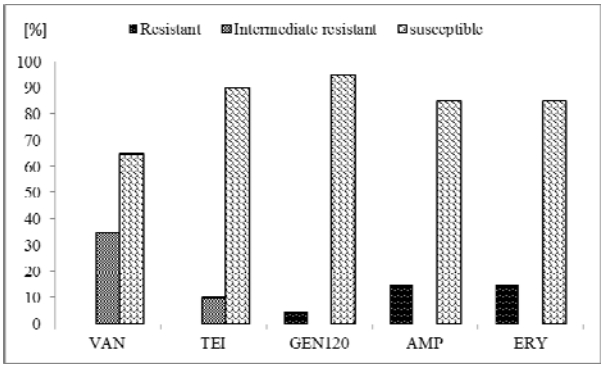

Figure 2. Antimicrobial resistance profiles of *Enterococcus faecalis* isolated from broiler chickens GIT fed by nutrition with propolis supplement (n=20) VAN–vancomycin, TEI–teicoplanin, GEN120–gentamicin, AMP–ampicillin, ERY–erythromycin.

Intermediate erythromycin resistance was occurred significantly ($P < 0.01$) more often among isolates from control, 2nd and 3rd group (bee pollen) than in isolates from 4th and 5th groups (propolis).

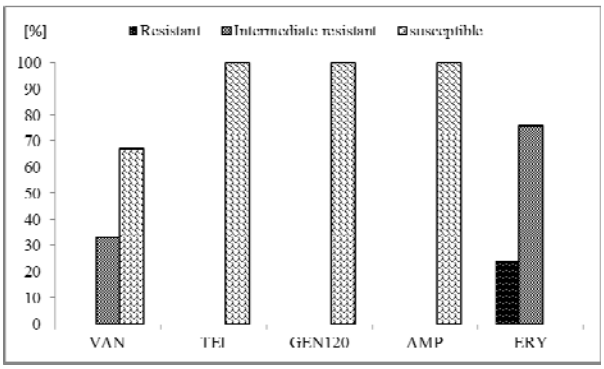

Figure 3. Antimicrobial resistance profiles of *Enterococcus faecalis* isolated from broiler chickens GIT fed by nutrition with bee pollen supplement (n=21) VAN–vancomycin, TEI–teicoplanin, GEN120–gentamicin, AMP–ampicillin, ERY–erythromycin.

The synergistic action of propolis with antimicrobial drugs *in vitro* was assayed mainly against *Staphylococcus aureus* [23] and *Salmonella typhi* [24, 25]. Scazzocchio et al. (2006) [26] found *in vitro*, that adding of propolis ethanolic extract to antibacterial tested drugs, it drastically increased the antimicrobial effect of ampicillin, gentamicin and streptomycin, moderately the one of chloramphenicol, ceftriaxon and vancomycin, while there was no effect with erythromycin. Also Stepanović et al. (2003) [27] found increased synergistic action of propolis with ampicillin against bacteria. However, they found that antimicrobial potential of propolis alone was less effective against *E. faecalis*.

Lotfy (2006) [28] stated that the bacteria types that are resistant to antibiotics were sensitive to propolis and propolis was effective against *S. aureus* ve *S. epidermis* bacteria in chickens under *in vitro* conditions.

4. Conclusions

It can be concluded that susceptibility of *E. faecalis* isolated from gastrointestinal tract of broiler chickens against vancomycin and teicoplanin were not affected by propolis and bee pollen nutrition supplement in comparison with control group. However, resistance to ampicilin and gentamicin were decreased after bee pollen addition. Also the intermediate resistance to erythromycin in the most *E. faecalis* isolates from gastrointestinal tract of broiler chickens with propolis supplement in their diet was eliminated. Our results suggest the probability of synergism effect of propolis and also bee pollen with some tested antibiotics against *E. faecalis* isolates.

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References

1. Tosi, A.E., Re' E., Ortega, M.E. et al., Food preservative based on propolis: bacteriostatic activity of propolis polyphenols and flavonoids upon Escherichia coli, Food Chemistry, 2007, 104, 1025-1029.
2. Erkmen, O., Özcan, M.M., Antimicrobial effect of Turkish propolis, pollen, and Laurel on spoilage and pathogenic food-related microorganisms, Journal of Medicinal Food, 2008, 11, 587-592.
3. Talas, Z.S., Gulhan, M.F., Effects of various propolis concentrations on biochemical and haematological parameters of rainbow trout. Ecotoxicology and Environmental Safety, 2009, 72, 1994-1998.

4. Santos, F.A., Bastos, E.A., Uzeda, M., Carvalho, M.A., Farias, L.M., Moreira, E.S., Braga, F.C. Antibacterial activity of Brazilian propolis and fractions against oral anaerobic bacteria, Journal of Ethopharmacology, 2002, 80, 1-7.
5. Bogdanov, S., Quality and standards of pollen and beeswax, Apiacta, 2004, 38, 334-341
6. Carpes, S. T., Begnini, R., Matias de Alencar, S., Masson, M. L. Study of preparations of bee pollen extracts, antioxidant and antibacterial activity, Cięncia e Agrotecnologia., 2007, 31, 1818-1825.
7. Koru, O., Toksoy, F., Acikel, C.H., Tunca, Y.M., Baysallar, M., Guclu, A.U., Akca, E., Tuylu, A.O., Sorkun, K., Tanyuksel, M., In vitro antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens, Anaerobe, 2007, 13, 140-145.
8. Murray, B. E. The life and times of the *Enterococcus*. Clinical Microbiology Reviews, 1990, 3, 46-65.
9. Fracalanzza, S.A.P., Scheidegger, E.M.D., Santos, P.F., Cardarelli-Leite, P., Teixeira, L.M., Antimicrobial resistance profiles of enterococci isolated from poultry meat and pasteurized milk in Rio de Janeiro, Brazil, Memórias do Instituto Oswaldo Cruz, 2007, 102, 853-859.
10. Tsai, C.C., Liu, T.H., Chen, M.H., Tsai, C.C., Tsen, H.Y., Toxicity evaluation for an *Enterococcus faecium* strain TM39 in vitro and in vivo, Food and Chemical Toxicology, 2004, 42, 1601-1609.
11. Gomes, B.C., Esteves, C.T., Palazzo, I.C.V., Darini, A.L.C., Felis, G. E., Sechi, L.A., Franco, B.D.G M., De Martinis, E.C.P., Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods, Food Microbiology, 2008, 25, 668-675.
12. Schjørring, S., Krogfelt, K. A., Assessment of Bacterial Antibiotic Resistance Transfer in the Gut, International Journal of Microbiology, 2011, 2011, 10
13. Morrison, D., Woodford, N., Cookson B., Enterococci as emerging pathogens of humans, Society for Applied Bacteriology Symposium 1997, 26, 89-99.
14. Giraffa, G., Domenico, C., Neviani, E. Enterococci isolated from dairy products: a review of risks and potential technological use, Journal of Food Protection, 1997, 60, 732-738.
15. Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E., Enterococci at the crossroads of food safety? International Journal of Food Microbiology, 1999, 47, 1-24.
16. Rahkila, R., Johansson, P., Säde, E., Björkroth, J., Identification of enterococci from broiler products and a broiler processing plant and description of *Enterococcus viikkiensis* sp. nov., Applied Environmental Microbiology, 2011, 77, 1196-1203.
17. Garg, S. K., Mital, B. K., Enterococci in milk and milk products, Critical Reviews in Microbiology, 1991, 18, 15-45.
18. Cauwerts, K., Decostere, A., De Graef, E.M., Haesebrouck, F., Pasmans F., High prevalence of tetracycline resistance in *Enterococcus* isolates from broilers carrying the erm(B) gene, Avian Pathology, 2007, 36, 395-399.
19. Mathur, S., Singh, R., Antibiotic resistance in food lactic acid bacteria, International Journal of Food Microbiology, 2005, 105, 281-295.
20. CLSI, Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement M100-S21, 2011, 31, 84-87.
21. Kilic, A., Baysallar, M., Besirbellioglu, B., Bekir, S., Sorkun, K., Tanyuksel, M., *In vitro* antimicrobiol activity of propolis against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*, Annals of Microbiology, 2005, 55, 113-117.
22. Brtková, A., Bujdáková, H., Antibiotic resistance in *Enterococcus* isolates from poultry swabs in Slovakia, Journal of Food and Nutrition Research, 2009, 48, 121-128.
23. Fernandes, F., Balestrin, E.C., Betoni, J.E.C., Orsi, R.O., Cunha, M.L.R.S., Montelli, A.C., Propolis: anti-Staphylococcus aureus activity and synergism with antimicrobial drugs, Memórias do Instituto Oswaldo Cruz, 2005, 100, 563-566.
24. Orsi, R.O., Sforcin, J.M., Funari, S.R. C., Fernandes, A., Bankova, V., Synergistic effect of propolis and antibiotics on the *Salmonella typhi*, Brazilian Journal of Microbiology, 2006, 37, 108-112.
25. Orsi, R.O., Fernandes, A., Bankova, V., Sforcin, J. M., The effects of Brazilian and Bulgarian propolis in vitro against *Salmonella Typhi* and their synergism with antibiotics acting on the ribosome, Natural Product Research: Formerly Natural Product Letters, 2012, 26, 430-437.
26. Scazzocchio, F., D'Auria, F. D. Alessandrini, D., Pantanella, F., Multifactorial aspects of antimicrobial activity of propolis, Microbiological Research, 2006, 161, 327-333.
27. Stepanović, S., Antić, N., Dakić, I., Vlahović, M. Š. In vitro antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs, Microbiological Research., 2003, 158, 353-357.
28. Lotfy, M., Biologiacal activity of bee propolis in health and disease, Asian Pacific Journal of Cancer Prevention, 2006, 7, 22-31.