Enterococcus Genus Identification Isolated from Gastrointestinal Tract of Chickens after Bees Products Application Using MALDI TOF MS Biotyper

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Abstract
The general objective of this study was to examine the effect of bee product on the Enterococci colonization of chickens. Bee products were administered to both feed mixtures in various amounts in addition to the control group. First experimental group was with propolis in feed mixture with the addition of 200 mg propolis per 1 kg of compound and second group was with pollen with the addition of 250 mg pollen per 1 kg of compound. In this experiment, quantitative counts of Enterococci in ceca of 49-day-old chicken (Ross 308) using classical and MALDI TOF MS Biotyper method were investigated. Counts of Enterococci on Slanetz-Bartley agar were monitored. *Enterococcus* cells, isolated from gastrointestinal tract, were detected using MALDI TOF MS Biotyper. Counts of CFU of Enterococci were compared in experimental and control treatments, respectively. The lowest count was detected in the control experimental group. The highest count was detected in the first experimental group where was 200 mg of propolis added to 1 kg of feed mixture. Using MALDI TOF MS Biotyper, we identified the species range of the genera *Enterococcus* in the intestinal tract of broiler. Detected species from the genus *Enterococcus* were: *E. avium, E. casseliflavus, E cecorum, E. faecalis, E. faecium, E. gallinarum, E. hirae* and *E. malodoratus*. In the experimental groups (caecal samples) were most frequent species of *E. avium E. faecium* and *E. gallinarum*.

Keywords: bee's products, chickens, Enterococci, MALDI TOF MS Biotyper

1. Introduction

The history of the Enterococci began when Thiercelin [1] first used the term to indicate the intestina origin of a Gram-positive diplococcus. The new genus *Enterococcus* was proposed by Thiercelin and Jouhaud [2]. Later on, Andrewes and Horder [3] renamed Thiercelin's "entérocoque'' as *Streptococcus faecalis*. It was assumed that the strain, isolated from a patient with endocarditis, originated from the human intestine.

Organisms belonging to the genus *Enterococcus* are ubiquitous in nature, being found in very diverse environmental habitats as well as animal, bird, and invertebrate hosts. Currently five groupings, comprising sixteen different species are recognized. Clinically, the majority of human infections associated with Enterococci are due to *E. faecalis* and to a lesser extent *E. faecium*. Other

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enterococcal species are less frequently, if at all, isolated clinically [4].

Studies on chicken caecal microflora, by both culture-based [5] and culture-independent [6-8] methods, have indicated that this environment is dominated by obligate anaerobes, but a diverse range of species have been detected. The traditional culture-based methods of assessing mammalian gastrointestinal tract community structure are extremely laborious, and it has been estimated that only 10–60% of total bacteria from this environment are able to be cultured [5]. Nonculture methods for assessing gut microbial ecology [9], such as the construction and analysis of 16S rDNA clone libraries [6-8], for example, have been instrumental in the discovery of new intestinal bacterial groups. Identification and classification of microorganisms can now be achieved using protein 'fingerprints' measured by MALDI-TOF mass spectrometry. The characteristic protein expression patterns of microorganisms, such as bacteria, yeasts and fungi, can be analyzed with the new MALDI BioTyper™ system from Bruker Daltonics.

Propolis, or bee glue, is a resinous material collected by worker bees from the leaf buds of numerous tree species. Once collected, this material is enriched with salivary and enzymatic secretions and is used by bees to cover hive walls, fill cracks or gaps and embalm killed invader insects [10]. Propolis presents a lot of biological and pharmacological properties, such as immunomodulatory, antitumor, antiinflammatory, antioxidant, antibacterial, antiviral, antifungal, antiparasite activities [11-13].

Pollen is a fine, powder-like material produced by flowering plants and gathered by bees considered as a valuable special food with varied enhancing effects on health. This beehive product also has several useful pharmacological properties, such as antibiotic, antineoplastic, antidiarrhoeatic and with nutritional composition, antioxidant and antiradical activity. Pollen contains nutritional compounds like carbohydrates, proteins, amino acids, lipids, vitamins, minerals and traces of micronutrients. In addition, pollen contains significant amounts of polyphenolic substances, mainly flavonoids [14].

The purpose of this study was to compare the *Enterococcus* species in gut microflora of broiler chickens in control group against experimental group were it was applied bee products. In this study for enumeration of Enterococci were using classical method and for identification of Enterococci MALDI TOF MS Biotyper.

2. Materials and methods

In this experiment, quantitative counts of Enterococci in caecum of 49-day-old chicken were investigated. The trial was carried out on an experimental basis of the Department of Poultry and Small Farm Animals at Slovak Agricultural University in Nitra. The experiment was realized in three-etage cage from the company SALMET. Cage technology has been divided into 3 parts: each cage (11 pcs chicken), i.e. one group of experiments (3 cages), i.e. a total of 33 chickens. Each cage had parameters 70x100 cm.

Experiment of monitoring the impact of propolis an pollen in the form of the extracts applied as a feed additive through the feed mixture were realized in half-operating conditions in the experimental operation. Fattening itself went on from 1 to 49 days of chicken age. One-day-old chickens of Ross 308 breed were randomly distributed to 3 groups. Chickens were fed *ad libitum* with standard mixture in two phases of feeding:

HYD-01 starter (powder mixture) Norm-type within 21 days of feeding

HYD-02 growth (powder mixture) Norm-type from 21st day of feeding to the end of feeding (42 days)

Bee products were extracted with ethanol (80%), under reflux condenser at 80°C during one hour. After chilling the mixture was centrifuged and supernatant was evaporated in the vacuum rotary evaporator at temperatures 40-45°C. The evaporation residue was dissolved. Residues of bee products were applied to feed mixture.

Dosing of feed additives

Bee products were administered to both feed mixtures in various amounts in addition to the control group.

Control group: the feed mixture without the addition of bee products.

1st Experimental group: feed mixture with the addition of 200 mg propolis to 1 kg of compound,

2nd Experimental group: feed mixture with the addition of 250 mg pollen to 1 kg of compound.

Plate diluting method

Determination of CFU counts: Plate diluting method was applied for quantitative CFU counts determination of respective groups of microorganisms in 1 g of substrate.
Agar in Petri dishes was inoculated with 1 ml of faecal chyme samples pour plate method on surface (faecal Enterococci) in three replications. Homogenized samples of faecal chyme (chyme was taken to sterile Petri dishes) were prepared in advance by sequential diluting based on decimal dilution system application. Counts of Enterococci on Slanetz-Bartley agar were monitored. Isolated species, genera and groups of microorganisms and their fundamental identification signs [15].

MALDI TOF MS Biotyper identification

The bacterial diagnostics and possible clonal relatedness of the studied isolates was determined by Maldi-tof analysis. A sufficient number of stable mass signals of major housekeeping proteins, mainly ribosomal proteins, can be used for bacterial species identification, and for estimation of the similarities between protein spectra of the same species (bacterial fingerprint). Bacterial extracts for mass spectrometry measurements were prepared as recommended by the manufacturer of the MS instrument. For MALDI TOF MS Biotyper analysis, one colony was spotted onto a ground steel target (Bruker Daltonik GmbH, Leipzig, Germany) and air dried for 15 min. Each sample spot was overlaid with 2 µl of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid), and again air dried for 15 min. To identify microorganisms, the raw spectra obtained for each isolate were imported into BioTyper software, version 2.0 (Bruker Daltonik GmbH, Leipzig, Germany), and analyzed without any user intervention [16].

3. Results and discussion

The microbial populations in the gastrointestinal tracts of poultry play a key role in normal digestive processes and in maintaining animal health. Disease and stress induced changes in the physicochemical environment in the gastrointestinal tract, or simple changes in feed management practices can significantly influence the microbial populations and their effects on animal performance and health. In the last five decades, increased knowledge of the factors that influence the activities of microorganisms in the alimentary tract has helped to define the critical role of these symbiotic organisms [17].
The application of bee pollen influenced faecal Enterococci of chickens showed table 1. In the trial with chickens after application of bee pollen, no statistically significant differences were found. The number of Enterococci count in the control group ranged from 6.78 to 6.95 log $CFU.g^{-1}$. In the first group with 200 mg of propolis per 1 kg of feed mixtures Enterococci number ranged from 8.25 to 8.99 log $CFU.g^{-1}$ and in second group with 250 mg of pollen per 1 kg of feed mixtures Enterococci number ranged from 7.50 to 9.40 log $CFU.g^{-1}$. The highest count of feacal Enterococci was found in the group where 200 mg of propolis to 1 kg was added to feed mixture. The lower count of faecal Enterococci was found in the control group.
SB agar also known as M-enterococcus agar has been widely used for the isolation, cultivation and enumeration of Enterococci from water, sewage and faeces, in combination with the membrane filter method. Samples can be directly plated onto the medium in order to detect and enumerace faecal streptococci [18, 19].
Using MALDI TOF MS Biotyper method, we identified the species range of the genera *Enterococcus* in the intestinal tract of broilers. Detected species from the genus *Enterococcus* were: *E. avium, E. casseliflavus, E cecorum, E. faecalis, E. faecium, E. gallinarum, E. hirae* and *E. malodoratus*. In the experimental groups (caecal samples) were most frequent species of *E. avium E. faecium* and *E. gallinarum*. In the all experimental groups were found all *Entercoccus* species. Table 2 shows the percentage of Enterococci in gastrointestinal tract of chickens. The percentages of isolates are in number of chickens in the group (15 chickens in the group).

Table 1. Summary statistical values for *E*nterococcus spp. in log cfu.g^{-1}

Values/Groups	C	P1	P2
Average	6.87	8.65	8.60
Standard deviation	0.09	0.20	0.70
Coefficient of variation (%)	1.24	2.41	2.81
Minimum	6.78	8.25	7.50
Maximum	6.95	8.99	9.40

Legend: C-control group; P1-experimental group with 200 mg of propolis; P2-experimental group with 250 mg of pollen

Table 2. *Enterococcus* species isolates from chickens GIT isolated in %

***Enterococcus* species**	C	P1	P2
E. avium	25	23	22
E. casseliflavus	7	5	8
E. cecorum	3	5	7
E. faecalis	5	5	6
E. faecium	25	24	26
E. gallinarum	25	24	22
E. hirae	4	8	4
E. mlodoratus	6	6	5

Legend: C-control group; P1-experimental group with 200 mg of propolis; P2-experimental group with 250 mg of pollen

In the study of King et al. [20] *Enterococcus faecalis* was the most frequently observed protease-secreting bacterial species having been isolated from 28 of 82 chickens (34%), followed by *Enterococcus gallinarum* 26 of 82 (32%), and *Proteus mirabilis* 20 of 82 (24%).

Enterococci are commonly found in poultry environments and are considered normal microflora of the intestinal tract of poultry [21].

In study Nováková and Kačániová [22] were focused the major species isolate of *Enterococcus* genus in various GIT segments of broilers. The four enterococcal species: *E. faecium, E. faecalis, E. gallinarum* and *E. cecorum* were isolated from broilers on the end of feeding. The PCR method using *sodA* gene which catalyzes the dismutation of superoxide shoved that in some cases distinct bands were not evident for *E. cecorum* and *E. gallinarum*. On the other hand, a distinct DNA band for *E. faecium and E. faecalis* was observed. These organisms covered 100% of the total isolated Enterococci. *E. gallinarum* was the most frequently identified *Enterococcus* spp. (87.5%) especially in ceaces. They find similar isolated species as in our study.

4. Conclusions

In this relation propolis and pollen as a natural additive might be a candidate for controlling the microbial content of broilers GIT instead of probiotics or antibiotics, but further researches are needs to evaluate propolis and pollen fractions in this relation. In conclusion, the results of this experiment show that the majority of microbiological parameters, *in vivo,* may be positively influenced by supplementation of propolis and pollen. The positive influence was followed in number of Enterococci in the experimental groups. Our results showed that propolis ad pollen application doesn't influence different representation of strains of Enterococci.

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