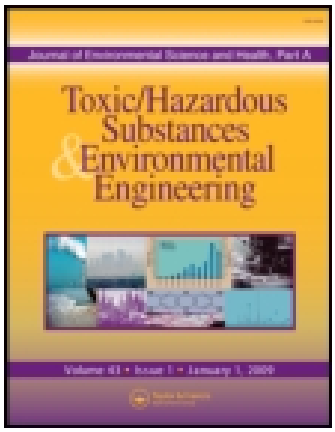


Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lesa20

# In vitro and In vivo antimicrobial activity of propolis on the microbiota from gastrointestinal tract of chickens

Miroslava Kačániová [a] , Katarína Rovná [b] , Henrieta Arpášová [c] , Juraj Čuboň [d] , Lukáš Hleba [a] , Jaroslav Pochop [a] , Simona Kunová [e] & Peter Haščík [d]

[a] Department of Microbiology, Faculty of Biotechnology and Food Sciences , Slovak University of Agriculture , Nitra , Slovak Republic

[b] Department of Green's Biotechnics, Horticulture and Landscape Engineering Faculty , Slovak University of Agriculture , Nitra , Slovak Republic

[c] Department of Poultry Science and Small Animal Husbandry, Faculty of Agrobiology and Food Resources , Slovak Agricultural University , Nitra , Slovak Republic

[d] Department of Animal Products Evaluation and Processing, Faculty of Biotechnology and Food Sciences , Slovak University of Agriculture , Nitra , Slovak Republic

[e] Department of Hygiene and Food Safety, Faculty of Biotechnology and Food Sciences , Slovak University of Agriculture , Nitra , Slovak Republic

Published online: 15 Jun 2012.

To cite this article: Miroslava Kačániová , Katarína Rovná , Henrieta Arpášová , Juraj Čuboň , Lukáš Hleba , Jaroslav Pochop , Simona Kunová & Peter Haščík (2012) In vitro and In vivo antimicrobial activity of propolis on the microbiota from gastrointestinal tract of chickens, Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 47:11, 1665-1671, DOI: 10.1080/10934529.2012.687248

To link to this article: http://dx.doi.org/10.1080/10934529.2012.687248

*Journal of Environmental Science and Health, Part A* (2012) **47**, 1665–1671

ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934529.2012.687248



# *In vitro* and *In vivo* antimicrobial activity of propolis on the microbiota from gastrointestinal tract of chickens

MIROSLAVA KAČÁNIOVÁ[1], KATARÍNA ROVNÁ[2], HENRIETA ARPÁŠOVÁ[3], JURAJ ČUBOŇ[4], LUKÁŠ HLEBA[1], JAROSLAV POCHOP[1], SIMONA KUNOVÁ[5] and PETER HAŠČÍK[4]

[1]*Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Nitra, Slovak Republic*
[2]*Department of Green's Biotechnics, Horticulture and Landscape Engineering Faculty, Slovak University of Agriculture, Nitra, Slovak Republic*
[3]*Department of Poultry Science and Small Animal Husbandry, Faculty of Agrobiology and Food Resources, Slovak Agricultural University, Nitra, Slovak Republic*
[4]*Department of Animal Products Evaluation and Processing, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Nitra, Slovak Republic*
[5]*Department of Hygiene and Food Safety, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Nitra, Slovak Republic*



The aim of this study was to examine the effect of propolis extracts on the microbial colonization of chicken gastrointestinal tract *in vivo*. The propolis was administered to both feed mixtures in various amounts except of the control group. The addition of 150 mg propolis to 1 kg of feed was included in the first experimental group, the addition of 450 mg.kg$^{-1}$ in the second experimental group, the addition of 600 mg.kg$^{-1}$ the third experimental group and 800 mg kg$^{-1}$ in the fourth one. The highest count of faecal enterococci was found in the third group (8.6 cfu.g$^{-1}$) where 600 mg of propolis to 1 kg was added to the feed mixture. The highest count of lactobacilli was detected in the fourth experimental group (8.83 cfu.g$^{-1}$) where was 800 mg of propolis added to 1 kg of feed mixture and number of *Enterobacteriaceae* genera count was found in control group (8.73 cfu.g$^{-1}$). With RTQ PCR detected species from the genus *Enterococcus* were: *E. avium, E. casseliflavus, E cecorum, E. faecalis, E. faecium, E. gallinarum, E. hirae* and *E. malodoratus* and from *genus Lactobacillus* were: *Lactobacillus crispatus*, *L. acidophilus* and *L. salivarius*. With MALDI TOF MS Biotyper from *Enterobacteriaceae* genera were identified *Citrobacter braakii*, *Raoultella ornithinolytica*, *Serratia fonticola*, *Escherichia coli* and *Klebsiella oxytoca*. Antimicrobial activities *In vitro* of six species of bacteria isolated from gastrointestinal tract of chickens were also tested. The best antimicrobial effect of *Citrobacter braakii* on ethanolic propolis extract in all concentrations were found.

**Keywords:** Propolis, chickens, intestinal microflora, antimicrobial effect, pathogens.

## Introduction

The investigation of natural products with antimicrobial activity has attracted the attention of many researchers, motivated mainly by the increasing bacterial resistance to traditional antimicrobial agents[1–2] and the side effects frequently observed after the use of antibiotics.[3–5]. Propolis has been considered a good candidate for an adjuvant in the treatment or prevention of many infectious diseases among those natural products. Propolis is relatively non-toxic[6–7] and displays a wide range of antimicrobial activity against a variety of bacteria, fungi, parasites, and virus.[8–11] In addition to an antimicrobial activity, other biological and pharmacological properties have also been demonstrated for propolis.[12–15]

Plants and plant extracts are effective mainly on the digestive system of animals. Their function either by wiping out the pathogenic microflora in the digestive system or increasing the concentration of microbial population in the digestive system that contributes to improved digestion and absorption of nutrients.[16] Animals, poultry in particular, are very sensitive to pathogenic bacteria such as *Escherichia coli, Salmonella* ssp*., Clostridium perfringens* and *Campylobacter sputorum*.

Antibiotics have been used as growth promoters in poultry rations stabilizing the intestinal microbial flora for long time and improving the growth performance by inhibiting some specific intestinal pathogens.[17] Some bioactive derived from fungus and higher plants have been reported to

Address for correspondence to Miroslava Kačániová, Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Tr. Andreja Hlinku 2, 949 76, Nitra, Slovak Republic; E-mail: miroslava.kacaniova@gmail.com
Received February 13, 2012.

kill pathogenic bacteria species in chickens while increasing beneficial bacterium species such as lactic acid bacteria and bifidobacteria. The stimulation of such beneficial bacteria generates an effective protection against pathogenic microorganisms and a balanced intestinal microflora.[18]

To determine the effects of propolis significant decrease in the numbers of pathogenic extracts on intestinal microbiology is accompanied by the increase in the characteristics in broilers due to the antibacterial amount of mucous IgA protecting the intestinal mucosa properties of these extracts against pathogenic microorganisms.[19]

The present literature shows that there is no information about growth promoting and antimicrobiological effects of animal-borne natural substance as propolis in broilers. The purpose of this study was to determine the effects of propolis extracts on intestinal microbiota in broilers due to its antibacterial properties of these extracts.



## Materials and methods

### *Animals*

In this experiment, quantitative counts of individual groups of microorganisms in caecum of 49-day-old chicken were investigated. The experiment was planned in accordance with animal welfare. A total of 1-day-old 600 broiler chickens (Hubbard JV) were used. We used 75 chickens' gastrointestinal tracts for microbiological analyses. The gastrointestinal tracts of chickens immediately after slaughter in sterile laboratory were collected to sterile Petri dishes.

Animals were kept in a thermoneutral hall (33°C on until 19°C at the end). Thermo-aggregates and experimental conditions with defined temperatures were installed in the closed hall and humidity was simulated by sensor. Fattening itself went on from 1 to 49 days of chicken age. One-day-old chickens of Hubbard JV breed were randomly distributed to 5 groups. Chickens were fed with complete feed mixture KKZ (Biofeed, a.s., Kollarovo, Slovak Republic) as follows: KKZ HYD-01 (powdery form) from day 1 of feeding till day 21 of feeding and KKZ HYD-02 (granular form) from day 22 till day 42. Ingredients and nutrient composition of diets are shown in Table 1. Feed and water were provided *ad libitum*.

### *Propolis samples*

Propolis samples were collected from Slovak republic. Hand collected propolis samples were kept dried in the dark until processing. Propolis samples were extracted for a week with 100 mL of 70% ethanol, at room temperature to obtain the extract.[20]

### *Dosing of feed additives*

Propolis was administered to both feed mixtures in various amounts in addition to the control group. The animals were divided into the following groups: Control group (CG) the feed mixture without the addition of propolis were used. We used feed mixture with the addition of 150 mg propolis per 1 kg in the first experimental group (EG1), feed mixture with the addition of 450 mg propolis per 1 kg in the second one (EG2), feed mixture with the addition of 600 mg propolis per 1 kg in the third one (EG3) and feed mixture with the addition of 800 mg propolis per 1 kg of feed in the fourth one (EG4).

**Table 1.** Diet composition of feed mixture KKZ HYD-01 and KKZ HYD-02.

| *Ingredient* | *KKZ HYD-01* | *KKZ HYD-02* |
|---|---|---|
| Dry matter ($g.kg^{-1}$) | 917.3 | 913.3 |
| Crude protein ($g.kg^{-1}$) | 211.3 | 199.7 |
| Fat ($g.kg^{-1}$) | 25.5 | 23.0 |
| Starch ($g.kg^{-1}$) | 413.0 | 434.8 |
| Total sugar ($g.kg^{-1}$) | 49.5 | 31.7 |
| ME (MJ) | 11.7 | 11.6 |
| Ca ($g.kg^{-1}$) | 12.1 | 8.2 |
| P ($g.kg^{-1}$) | 7.8 | 6.8 |

ME-Metabolized Energy, Ca-calcium, P-phosphorus.

### *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. Gelatinous nutritive substrate in Petri dishes was inoculated with 1 mL of chyme samples pour plate method 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. Enterococci were counted on Slanetz-Bartley agar and incubated at 37°C for 48–72 h, Lactobacilli were counted on MRS Lactobacillus agar and incubated at 37°C for 48–72 h and coliforms bacteria were counted on MacConkey agar and incubated at 37°C for 24–48 h. Isolated species, genera and groups of microorganisms and their fundamental identification were performed as per standard norms.[21]

## Bacterial strains and DNA extraction

For isolation of DNA growth colonies of bacteria that we had isolate of individual samples in pure culture were used. Gram-positive ($G^+$) bacteria were prepared in peptone water (peptone 10 g, NaCl 5.0 g, distilled water 1000.0 mL) before DNA isolation. Peptone and NaCl were dissolved in hot water, filtered and pH adjusted (with NaOH or HCl) to 7.2 to 7.8, as appropriate and then sterilized in an autoclave at 0.1 MPa for 20 min. GenElue TM Bacterial Genomic

**Table 2.** Characteristic of primers used for lactobacilli identification (Drisko et al.[22]).

| *Species* | *Primer* | *Sequence (5′–3′)* |
|---|---|---|
| *L. salivarius* | Lsal-1 | AATCGCTAAACTCATAACCT |
| | Lsal-2 | CACTCTCTTTGGCTAATCTT |
| *L. acidophilus* | Laci-1 | TGCAAAGTGGTAGCGTAAGC |
| | 23–10C | CCTTTCCCTCACGGTACTG |
| *L. crispatus* | Cri 16SI | GTAATGACGTTAGGAAAGCG |
| | CRI 16SII | ACTACCAGGGTATCTAATCC |

**Table 3.** Characteristics of primers used for enterococci identification (Desay et al.[23]).

| *Species* | *Primer* | *Sequence (5′–3′)* |
|---|---|---|
| *E. avium* | AV1 | GCTGCGATTGAAAATATCCG |
| | AV2 | AAGCCAATGATCGGTGTTTT |
| *E. caseliflavus* | CA1 | TCCTGAATTAGGTGAAAAAC |
| | CA2 | GCTAGTTTACCGTCTTTAACG |
| *E. cecorum* | CE1 | AAACATCATAAACCTATTTA |
| | CE2 | AATGGTGAATCTTGGTTCGCA |
| *E. gallinarum* | GA1 | TTACTTGCTGATTTGATTCG |
| | GA2 | TGAATTCTTCTTTGAATCAG |
| *E. faecium* | FL1 | TAGAGACATTGAATATGCC |
| | FL2 | TCGAAATGTGCTACAATC |
| *E. faecalis* | FM1 | ATCAAGTACAGTTAGTTCT |
| | FM2 | ACGATTCAAGCTAACTG |
| *E. hirae* | HI1 | CTTCTGATATGGATGCTGTC |
| | HI2 | TAATTCTTCCTTAAATGTTG |
| *E. malodoratus* | MA1 | GTAACGAACTTGAATGAAGT |
| | MA2 | TTGATCGCACCTGTTGGTTT |

DNA Kit (Sigma Aldrich, St. Louis, USA) were used for isolation:

*$G^+$ bacteria:* 1.5 mL of 24 h bacterial culture was centrifuged during 2 min/12,000 to 16,000 g. The supernatants were removed, the pellets were resuspensed in 200 $\mu$L lysis solution and incubated for 30 min/37°C, then 20 $\mu$L proteinase K was added and incubated for 30 min/55°C, then 200 $\mu$L of lysis solution C was added, about 15 s vortex mixed and incubated at 55°C for 10 min. Next 500 uL Column Preparation Solution was added to each Gen Miniprep Bindinb Colum and about 12,000 *g* was centrifuged for 1 min, then 200 $\mu$L of ethanol (95–100%) was added to the lysate and vortex mixed 5–10 s. The mixture was centrifuged at about 6500 *g* for 1 min. The eluates were removed, 500 $\mu$L washing buffer was added, then the combination was centrifuged at maximum speed with drying of membrane and then transferred to a new Eppendorf tube. An amount measuring 200 $\mu$L of elution solution was directly added to the center of the membrane, then centrifuged for 1 min. at 6500 *g*.

The types of primers designed by Drisko et al.[22], which were used for species identification of Lactobacilli, are in Table 2. The types of primers used, as designed by Desay et al.[23], and species identification of Enterococci used are found in Table 3.

### *Real-time PCR*

Data were collected during each elongation step. PCR products were detected by monitoring the increase in fluorescence of the reporter dye at each PCR cycle. Applied Biosystems software plots the normalized reporter signal, ΔRn, (reporter signal minus background) against the number of amplification cycles and also determines the threshold cycle (Ct) value i.e., the PCR cycle number at which fluorescence increases above a defined threshold level were used.

### *Fluorescence in situ Hybridization (FISH)*

The reliability of traditional methods of cultivation samples was evaluated by fluorescence *in situ* hybridization (FISH) for the control. *Lactobacillus* cells, isolated from gastrointestinal tract, were detected after hybridization of fluorescently labeled probe with bacterial cells. The fluorescence *in situ* Hybridization kit (Lactobacillus Cluster) of Ribo Technologies (Groningen, Netherlands) was used for detection of Lactobacilli, which samples can be evaluated within 24 h. The Lactobacilli after hybridization were evaluated with microscope FLIM (Fisher, Slovakia) after 24 hour.

### The biochemical and MALDI TOF MS Biotyper identification of *Enterobacteriaceae* genera

There was used the method on the Triple sugar iron agar (Biolife, Italy) for the basic biochemical identification of *Enterobacteriaceae* genera and ENTEROtest 24 (Pliva-Lachema, Brno), including TNW Lite 7.0 identification software (Pliva-Lachema, Brno) for more detailed biochemical identification. Preparation of identification plates of ENTEROtest 24 was done inside the Laminaire box (ADS Laminaire, Le Pre-Saint Gervais) to ensure the high sterility, less risk of contaminations from air and for precise results. Working procedure of ENTEROtest 24 is described in the manual of manufacturer. After biochemical identification of *Enterobacteriaceae* genera were used MALDI TOF MS Biotyper (Brucker, Germany) of Slovak Academy of Sciences in Kosice, Institute of Animal Physiology, as previously described.[24]

### Antimicrobial activity

The isolated propolis ethanolic extracts (70% ethanol) of samples were studied for their antimicrobial activity. *In vitro* antimicrobial studies were carried out by the dilution method, as previously described [25], measuring the MIC values in 96-hole plates against two $G^+$ strains (*Bacillus*

*cereus, Staphylococcus epidermis*) and four $G^-$ strains (*Citrobacter braakii*, *Escherichia coli, Klebsiela oxytoca, Serratia fonticola*). The bacterial strains were isolated and identified from gastrointestinal tract of chickens from our experiment. Stock solutions of the tested extracts and pure compounds were prepared at 10 $mg.mL^{-1}$, respectively.

Suspensions of the microorganisms were prepared to contain approximately $10^8$ $CFU.mL^{-1}$ and then 100 $\mu$L of these suspensions were inoculated in plates containing agar medium. Serial dilutions of the stock solutions in broth medium (100 $\mu$L of Müller-Hinton broth), were prepared in a microtitre plate (96-wells). Then 1 $\mu$L of the microbial suspension (the inoculum, in sterile distilled water) was added to each well. The growth conditions and the sterility of the medium were checked and the plates were incubated as referred to above for each strain. There were assayed various concentrations of the stock 512, 256, 128, 64, 32, 16, 8 $\mu g.mL^{-1}$ against the tested bacteria to measure the MIC values. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible microbial growth. Standard antibiotic chloramphenicol was used into control the sensitivity of the tested bacteria. There was used a pure solvent for each experiment and it was also applied as a blind control. The experiments were repeated three times, and the results were expressed as average values.[25]

### *Statistical analysis*

The statistical processing of the data obtained from number of microorganisms was implemented by means with Statgraphics 5 software. Experimental results of microorganisms' number were expressed as means, standard deviation (SD) and coefficient of variability (CV). A statistical analysis was performed with Student's *t*-test. Confidence limits were added at $P < 0.05$; $P < 0.01$; $P < 0.001$.

## Results and discussion

The application of bee propolis affected faecal enterococci of chickens, as can be seen in Table 4. There were found statistically significant differences ($P < 0.05$) between control and first experimental group in the trial with chickens after application of propolis. The number of enterococci count in the control group ranged from 7.12 to 7.52 log $CFU.g^{-1}$.

The number of enterococci ranged from 8.15 to 8.52 log $CFU.g^{-1}$ in the first group with 150 mg propolis per 1 kg of feed mixtures, from 8.19 to 8.61 log $CFU.g^{-1}$ in the second group, from 8.52 to 8.72 log $CFU.g^{-1}$ in the third one and from 8.25 to 8.92 log $CFU.g^{-1}$ in the fourth one. The highest count of faecal enterococci was found in the third group where 600 mg of propolis to 1 kg was added to feed mixture. The lower count of faecal enterococci was found in the control group.The lower gastrointestinal tract (GIT) of most animal species including poultry is normally populated by large numbers of microorganisms, and through various competitive niches and virulence capabilities, some are able to survive.

**Table 4.** Summary statistical values of *Enterococcus* spp. number in GIT (log $cfu.g^{-1}$).

| *Values/Groups* | *CG* | *EG1* | *EG2* | *EG3* | *EG4* |
|---|---|---|---|---|---|
| Number of chickens GIT samples | 15 | 15 | 15 | 15 | 15 |
| Average | 7.23 | 8.27 | 8.45 | 8.60 | 8.59 |
| Standard deviation | 0.10 | 0.12 | 0.11 | 0.06 | 0.18 |
| Coefficient of variation % | 1.37 | 1.50 | 1.33 | 0.75 | 2.07 |
| Minimum | 7.12 | 8.15 | 8.19 | 8.52 | 8.25 |
| Maximum | 7.52 | 8.52 | 8.61 | 8.72 | 8.92 |

CG-control group; EG1-propolis 150 $mg.kg^{-1}$; EG2- propolis 450 $mg.kg^{-1}$; EG3-propolis 600 $mg.kg^{-1}$; EG4-propolis 800 $mg.kg^{-1}$.

The capabilities of microorganisms associated with the mucosa of the GIT to with stand the flow rates of food material is essential for the development of protective mechanisms such as surface mucus colonization, deep mucus, development of specialized insertional structures, and crypt association by specific adhesions. Changes in the passage rates that are representative of dilution rates can alter the limiting nutrients and therefore could ultimately affect microflora composition in the GIT ecology.

Historically, the microbial composition of the GIT of avian species has not been extensively defined compared to what is known about microorganisms in ruminants.[26] There is the perception that the role of microorganisms in chickens is not as important as is the case for ruminants.[27] However, extensive strict anaerobic metabolism including methanogenesis fermentation occurs in birds fed a variety of diets.[28–31] The ceca are the major fermentation sites in the GIT of chickens and contain the largest number of bacteria.

Over 200 different bacteria have been isolated and characterized, and these bacteria are known to be influenced by various factors including diet, health, and age.[26]

We identified the species range of the genera *Enterococcus* in the intestinal tract of broilers using a real-time PCR method. There were collected overall 185 strains of enterococci from chickens but only 150 strains were identified. We detected species from the genus *Enterococcus*: *E. avium* (24.0%), *E. casseliflavus* (6.7%), *E cecorum* (8.0%), *E. faecalis* (12.0%), *E. faecium* (18.7%), *E. gallinarum* (15.3%), *E. hirae* (7.3%) and *E. malodoratus* (8.0%). There were the most frequent species of *E. avium E. faecium* and *E. gallinarum* in the experimental groups (caecal samples). We monitored representation of species *E. avium* in the individual isolates which captured RTQ PCR sensitivity ranged from 1 to $10^5$ $CFU.g^{-1}$ and identification of the species *E. casseliflavus* in each of isolates by RTQ PCR method. We found that the presence of these bacteria were in the range of 1 to $10^4$ $CFU.g^{-1}$.

**Table 5.** Summary statistical values of *Lactobacillus* spp. number in GIT (log cfu.g$^{-1}$).

| *Values/Groups* | *CG* | *EG1* | *EG2* | *EG3* | *EG4* |
|---|---|---|---|---|---|
| Number of chickens GIT samples | 15 | 15 | 15 | 15 | 15 |
| Average | 7.33 | 8.65 | 8.64 | 8.66 | 8.83 |
| Standard deviation | 0.31 | 0.20 | 0.19 | 0.20 | 0.14 |
| Coefficient of variation % | 4.27 | 2.41 | 2.21 | 2.35 | 1.55 |
| Minimum | 7.03 | 8.25 | 8.25 | 8.32 | 8.59 |
| Maximum | 7.95 | 8.99 | 8.95 | 8.96 | 9.01 |

CG-control group; EG1- propolis 150 mg.kg$^{-1}$; EG2- propolis 450 mg.kg$^{-1}$; EG3-propolis 600 mg.kg$^{-1}$; EG4-propolis 800 mg.kg$^{-1}$.

**Table 6.** Summary statistical values of *Enterobacteriaceae* genera number in GIT (log cfu.g$^{-1}$)

| *Values/Groups* | *CG* | *EG1* | *EG2* | *EG3* | *EG4* |
|---|---|---|---|---|---|
| Number of chickens GIT samples | 15 | 15 | 15 | 15 | 15 |
| Average | 8.73 | 7.33 | 7.23 | 7.15 | 7.13 |
| Standard deviation | 0.17 | 0.12 | 0.03 | 0.03 | 0.06 |
| Coefficient of variation % | 1.97 | 1.68 | 0.44 | 0.52 | 0.78 |
| Minimum | 8.48 | 7.21 | 7.18 | 7.11 | 7.01 |
| Maximum | 9.01 | 7.56 | 7.30 | 7.25 | 7.21 |

CG-control group; EG1- propolis 150 mg.kg$^{-1}$; EG2-propolis 450 mg.kg$^{-1}$; EG3-propolis 600 mg.kg$^{-1}$; EG4-propolis 800 mg.kg$^{-1}$.



There was detected *Enterococcus faecium* in all tested samples. The threshold value was 12.82 for *E. faecium* samples. The cycle treshold (Ct) value of positive samples was on average 33.44 whereby the lowest value of positive samples was found at 17.39 and the highest value was at 39.32. We detected strain of *E. gallinarum* in seven out of ten our samples. The threshold value was 822.72 by *E. gallinarum* samples. The Ct value of positive samples was on average 53.53 whereby the lowest value of positive samples was found at 29.16 and the highest value was at 57.61. We detected strain of *E. cecorum* in 6 out of 10 of our samples. The threshold value was 11.76 by *E. cecorum* samples. The Ct value of positive samples was on average 18.35, whereby the highest value was at 24.25 and the lowest value of positive samples was found at 11.16.

Thus, the PCR-based detection of bacteria depends on the efficiency of DNA extraction procedure used to prepare the template DNA. We detected strains of *E. faecalis* in all tested samples. The threshold value was 13.25 by *E. faecalis* samples. The Ct value of positive samples was on average 18.73, whereby the lowest value of positive samples was found at 13.83 and the highest value was at 27.68.

The results of the application of propolis influenced Lactobacilli number of chicken gastrointestinal tract are shown Table 5. There were no statistically significant differences in the trial with chickens after application of propolis. The lowest count was detected in the control experimental group. The highest count was detected in the fourth experimental group with 800 mg of propolis added to 1 kg of feed mixture.We used FISH method after classical method for enumeration of Lactobacilli. The number of *Lactobacillus* cells ranged from $10^2$ to $10^6$ CFU.g$^{-1}$. The higher number of Lactobacilli was found in the first group where pollen 200 mg per kilogram was used. Similar results of enumeration of Lactobacilli were found in the study Nováková et al.[32]

We used RTQ PCR for identification of individual species of Lactobacilli. We identified three species of genus *Lactobacillus*: *Lactobacillus crispatus*, *L. acidophilus* and *L. salivarius w*ith real-time polymerase chain reaction. Similar results of individual species of Lactobacilli were found in the study Nováková et al.[32]

There were collected total 53 strains of Lactobacilli from all groups of animals but only 50 strains were identified. Detected species from the genus *Lactobacillus* were: *Lactobacillus crispatus* (25%), *L. acidophilus* (35%) and *L. salivarius* (40%).

The number of *Enterobacteriaceae* genera after propolis application in chicken gastrointestinal tract are shown Table 6. There were found statistically significant differences ($P < 0.05$) between third and fourth experimental group in the trial with chickens after application of propolis. The number bacteria of *Enterobacteriaceae* genera ranged from 7.12 to 7.52 log CFU.g$^{-1}$ in the control group, from 8.15 to 8.52 log CFU.g$^{-1}$ in the first one, from 8.19 to 8.61 log CFU.g$^{-1}$ in the second group, from 8.52 to 8.72 log CFU.g$^{-1}$ in the third group and from 8.25 to 8.92 log CFU.g$^{-1}$ in the fourth one.

The highest count of *Enterobacteriaceae* genera was found in the control group. The lower count of *Enterobacteriaceae* genera was found in the fourth experimental group where was 800 mg of propolis added to 1 kg of feed mixture. We used MALDI TOF MS Biotyper for identification of *Enterobacteriaceae* genera and others microorganisms. There were identified from *Enterobacteriaceae* genera *Citrobacter braakii* (10%), *Raoultella ornithinolytica* (5%), *Serratia fonticola* (5%), *Escherichia coli* (35%) and *Klebsiella oxytoca* (10%). From non - *Enterobacteriaceae* species were identified *Pseudomonas oryzihabitans* (5%), *Staphylococcus warneri* (5%), *Acinetobacter lwoffi* (5%), *Acinetobacter genomospecies* 3 (10%), *Bacillus cereus* (5%), and *Staphylococcus epidermis* (5%). Rahmani et al.[33] were found in their study similar results as our results. Their results showed that the microflora content of the broiler ileum is significantly ($P < 0.05$) influenced by propolis dose dependently. Propolis or bee glue as a natural product contains different kinds of flavonoids, polyphenols and phenolic acids. Falvonoids and polyphenols are candidates that influence the bacterial growth by inhibiting metalloenzyme activity of microorganisms.

One-day-old male chicken (Ross 308) the number of 480 received the normal corn soybean-meal diet supplemented with 0 (control), 40, 80 and 120 mg.kg$^{-1}$ of oil extracted propolis (OEP) in a completely randomized design test

**Table 7.** Minimum inhibitory concentrations of propolis ethanolic (70% ethanol) extracts ($\mu$g.mL$^{-1}$).

| *Bacterial strains/ Concentration of propolis* | *512* | *256* | *128* | *64* | *32* | *16* | *8* |
|---|---|---|---|---|---|---|---|
| *G-positive bacteria* | | | | | | | |
| *Bacillus cereus* | − | − | − | − | + | + | + |
| *Staphylococcus epidermis* | − | − | − | + | + | + | + |
| *G-negative bacteria* | | | | | | | |
| *Citrobacter braakii* | + | + | + | + | + | + | + |
| *Escherichia coli* | − | + | + | + | + | + | + |
| *Klebsiela oxytoca* | − | − | + | + | + | + | + |
| *Serratia fonticola* | − | − | + | + | + | + | + |

(−) negative bacterial growth, (+) positive bacterial growth.

with 4 treatments and 4 replicates. The microbial population of chicken ileum increases with age, and at the first week *Lactobacillus* is predominant, but gradually the *E. coli* and *Clostridium* level increases in control group. Comparing the total population shows propolis significantly ($P < 0.05$) and dose dependently controls the microbial load, particularly the *E. coli* and clostridium rates. These findings confirmed our previous studies that propolis improves the performance and immunity in broilers.[33]

Pochop et al.[34] examined the effect of propolis extracts in chickens feed against colonization of GIT with *Salmonella* spp. by Step One real-time PCR. Their results indicate positive effect of propolis against colonization of GIT with *Salmonella* spp. in all experimental groups with propolis extraction on the feed.

The Step One real-time PCR assay is extremely useful for any laboratory equipped by real-time PCR. Thus, these results proved that the real-time PCR can be useful as a rapid diagnostic test for the direct detection of pathogens in food, without the need of enrichment steps.[35, 36]

The MICs of propolis samples ranged from 8 to 512 $\mu$g.mL$^{-1}$ (Table 7). The control sample (chloramphenicol) did not have any effect on the growth of bacteria (data not shown). Propolis is more active against $G^+$ bacteria than $G^-$, according to Koru et al.[37] Conversely, we found that MIC values of $G^+$ bacteria were lower than MIC values of $G^-$bacteria. We investigated the antibacterial activity of the natural propolis product because of increasing resistance to antibiotics may lead to the failure of therapy of gastrointestinal tract diseases. All tested bacterial strains were determined susceptible to this bee product, and the MIC values ranged from 8 to 512 $\mu$g.mL$^{-1}$, regardless of the propolis ethanolic extract of origin in which propolis were collected.

## Conclusion

The microbial diversity of the GI ecology plays an important role in the food animal industry and human medicine. In this relation propolis 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 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. The positive influence was followed in number of Lactobacilli and Enterococci in the experimental groups. Our results showed that propolis application doesn't influence different representation of strains of Lactobacilli and Enterococci. All tested Gram-positive and Gram-negative bacterial strains *in vitro* were determined susceptible to this bee product.

## Acknowledgments

This study was supported by Cultural and Educational Grant Agency no. 053 SPU-4/2011 and 013 SPU-4/2012.

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