

***Lactobacillus* Genus Identification Isolated from Gastrointestinal Tract of Chickens after Bee Products Application Using FISH and RTQ PCR Methods**

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Abstract

The general objective of this study was to examine the effect of bee products on the lactobacilli 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 400 mg propolis per 1 kg of compound and second group was with pollen in feed mixture with the addition of 450 mg pollen per 1 kg of compound. In this experiment, quantitative counts of lactobacilli in ceca of 49-day-old chicken (Ross 308) using classical and fluorescence *in situ* hybridization (FISH) method were investigated. Counts of lactobacilli on Man, Rogosa and Sharpe (MRS) agar were monitored. To check the reliability of traditional methods of cultivation samples were evaluated by fluorescence *in situ* hybridization (FISH). *Lactobacillus* cells, isolated from gastrointestinal tract, were detected after hybridization of fluorescently labeled probe with bacterial cells. Counts of colony forming units (CFU) of lactobacilli 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 third experimental group where was 450 mg of pollen added to 1 kg of feed mixture. Using Real-time polymerase chain method (PCR), we identified the species range of the genera *Lactobacillus* in the intestinal tract of broiler. Detected species from the genus *Lactobacillus* were *L. crispatus*, *L. salivarius* and *L. acidophilus*.

Keywords: bee products, chickens, FISH, lactobacilli, RTQ PCR

1. Introduction

Identification of *Lactobacillus* strains for use as delivery vectors, competitive exclusion agents, or probiotics is complicated by the difficulty in selecting truly autochthonous strains capable of reliably and consistently colonizing the chicken

gastrointestinal tract (GIT) upon subsequent inoculation.

Traditionally, strain selection for *in vivo* applications has involved several *in vitro* characterization assays, including assays of aggregation, coaggregation, cell wall hydrophobicity, acid tolerance, bile salt tolerance, adhesion to epithelial cell lines, and antimicrobial activity [1-4]. While these assays can be used to reduce the number of strains examined, they may

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also bias the selection of strains and could potentially overlook strains which may be competitive or have other desirable characteristics *in vivo*. One of the limitations of *in vivo* screening of lactobacilli is the need for reliable high-throughput screening techniques to identify and track persistent strains. Recently, our group reported the application of enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) to simultaneously type large numbers of *Lactobacillus* isolates from the chicken GIT to the species and strain level [5, 6].

Propolis (Bee glue) is a complex resinous hive product and mixture of wax, sugars and plant exudates collected by bees from certain plant sources. More than 300 constituents have been identified in different propolis samples. In general, propolis composition is directly related to that of bud exudates collected by bees from various trees poplar, birch, beech, horse chestnut, alder and various conifers. The ethanolic extract of propolis has some activities such as antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, hepatoprotective, immunostimulating and cytostatic [7].

One of the most widely used natural supplements is the bee pollen because it contains most of the essential nutritional elements needed for growth and development in humans and animals [8, 9], and it could also promote the early development of the digestive system, and therefore is a potentially beneficial food supplement [10-12].

Therefore, the present study investigated the effect of 80% ethanolic extract of bee products samples collected from different regions of Slovakia on performance of Ross (308) broiler chickens to lactobacilli in the control and two experimental groups. To check the reliability of traditional methods of cultivation samples were evaluated by fluorescence *in situ* hybridization (FISH). Using Real-time PCR method, we identified the species range of the genera *Lactobacillus* in the intestinal tract of broiler.

2. Materials and methods

In this experiment, quantitative counts of lactobacilli in ceca 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-stage 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 bee products in the form of the extract applied as a feed additive through the feed mixture was 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 6 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 1 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. Residue of bee products was applied to feed mixture.

Dosing of feed additives

Propolis and pollen was 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 400 mg propolis per 1 kg of compound,

2nd Experimental group: feed mixture with the addition of 450 mg pollen per 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.

Gelatinous nutritive substrate in Petri dishes was inoculated with 1 ml of chyme samples pour plate method (*Lactobacillus sp.*) 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 lactobacilli

on MRS agar were monitored. Isolated species, genera and groups of microorganisms and their fundamental identification signs were identified by Holt et al. [13].

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. Before DNA isolation of Gram-positive bacteria was prepared in peptone water of following composition: peptone 10 g, NaCl 5.0 g, distilled water 1000.0 ml. Peptone and NaCl in hot water were dissolved, filtered and pH adjusted to 7.2 to 7.8, as appropriate and then sterilized in an autoclave at 0.1 MPa for 20 minutes. For isolation GenElue™ Bacterial Genomic DNA Kit (Sigma Aldrich, St. Louis, USA) were used:

G⁺ bacteria: 1.5 ml of 24 hours bacterial culture was centrifuged 2 min/12.000 to 16.000 g. The supernatant were removed, the pellet was

dissolved in 200 µl lysis solution and 30 min/37°C were incubated, 20 µl proteinase K were added and 30 min/55°C were incubated, then 200 µl of lysis solution C were added, about 15 s vortex mixed and at 55°C incubated for 10 min. We added 500 µl Column Prep. Solution to each GenMiniprep Bindinb Colum, about 12000 g centrifuged for 1 min 200 µl of ethanol (95-100%) were added in the lysate and vortex mixed for 5-10 sec. Then about 6500 g centrifuged for 1 min. The eluate were removed, 500 µl washing buffer were added, then centrifuged at maximum speed unless drying of membrane and then transferred to a new Eppendorf tube, 200 µl of elution solution directly to the center of the membrane were added, then centrifuged for 1 min. at 6500 g.

The types of used primers designed by Drisko et al. [14], which were used for species identification of lactobacilli are showed in Table 1.

Table 1 Primers used for lactobacilli identification

species	primer	sequence (5'- 3')
<i>L. salivarius</i>	Lsal-1	AATCGCTAAACTCATAACCT
	Lsal-2	CACTCTCTTTGGCTAATCTT
<i>L. acidophilus</i>	Laci-1	TGCAAAGTGGTAGCGTAAGC
	23-10C	CCTTCCCTCACGGTACTG
<i>L. crispatus</i>	Cri 16SI	GTAATGACGTTAGGAAAGCG
	CRI 16SII	ACTACCAGGGTATCTAATCC

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. Components of PCR reactions are showed in Table 2.

Table 2 Components of PCR reaction

Components/concentration	Quantity [20 µl]
Master mix (1x)	10.00
primer F (400nM)	0.20
primer R (400nM)	0.20
sample	2.00
PCR water	7.60

Florescence *in situ* Hybridization

To check the reliability of traditional methods of cultivation samples were evaluated by fluorescence *in situ* hybridization (FISH). *Lactobacillus* cells, isolated from gastrointestinal tract, were detected after hybridization of fluorescently labeled probe with bacterial cells. For detection of lactobacilli was used Fluorescence *in-situ* Hybridization kit (Lactobacillus Cluster) of Ribo Technologies (Groningen, Netherlands), which samples can be evaluated within 24 hours. After 24 hour the lactobacilli after hybridization were evaluated with microscope FLIM (Fisher, Slovakia). The basic statistical values and P value, we evaluated by STATGRAPHIC software.

3. Results and discussion

The application of propolis and pollen influenced to lactobacilli number of chickens are showed in

table 3. In the trial with chickens after application of propolis and pollen, no statistically significant differences were found. The lowest count was detected in the control experimental group. The highest count was detected in the third experimental group where was 450 mg of pollen added to 1 kg of feed mixture.

It was also around this time that the *Lactobacillus* spp. was established in low concentrations. The mechanism for these changes in bacteria has not been defined. Lactobacilli are predominant in the caecal contents in the healthy chickens and may be their presence is considered clinical for maintaining the ecological balance of the caecal microflora [15].

Table 3. Summary statistical values for *Lactobacillus* spp. in log cfu.g⁻¹

Values/Groups	K	P1	P2
Average	7.12	8.70	8.75
Standard deviation	0.38	0.20	0.24
Coeff. of variation (%)	5.31	2.30	2.61
Minimum	6.89	8.51	8.35
Maximum	7.56	8.91	8.92

Thus population of bacteria within the microflora of the caecum appears to undergo significant changes fluctuation in number before a dynamic equilibrium is established between the species (14-21days). The demonstration of the clinical symptoms in the infected birds highly correlated with decreased concentration of Lactobacilli and Bifidobacteria and reverse-the number of aerobic and anaerobic bacteria returned to normal levels in correlation with clinical resolution of the disease. It is known that Lactobacilli and Bifidobacteria [16] protect against potentially harmful bacteria such as *Salmonella*. Therefore, an increase in the number of these strains will improve the status of microbial ecology in the chicken's gut making it less sensitive to colonization by pathogens. A practical example of this hypothesis can be seen from studies on the therapeutic possibilities of supplementing diets with these bacterial species. The use of native gut microflora and competitive exclusion culture [17], which have been contained these bacterial species, partially protect against *Salmonella gallinarum* and it was recommended in geographic areas where poultry production is adversely affected by fowl typhoid newly hatched chicks to be treated with such bacterial cultures.

After classical method for enumeration of lactobacilli we used FISH (fig. 1). The number of *Lactobacillus* cells ranged from 10² to 10⁶. The higher number of lactobacilli was found in the third group where pollen 450 mg per kilogram was used. Similar results were found in the study Nováková et al. [18].

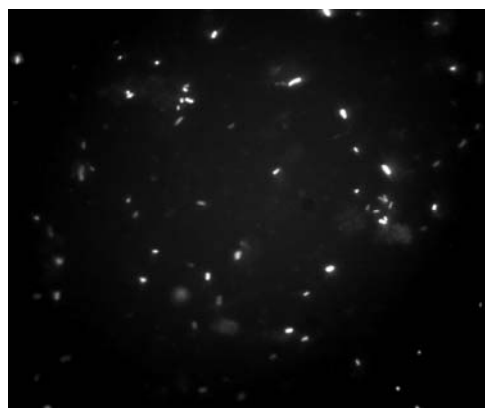


Figure 1. *Lactobacillus* sp. in GIT of chickens (FISH)

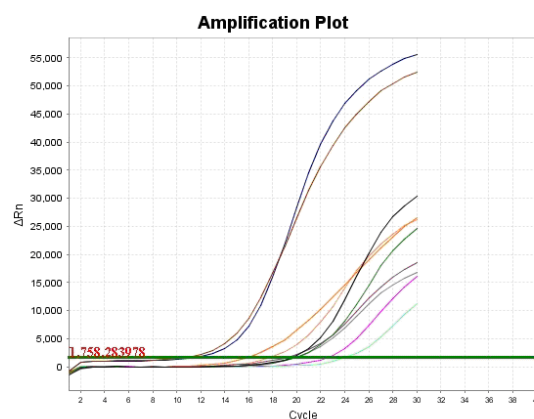


Figure 2 Evaluation of RTQ PCR in cells of *Lactobacillus crispatus*

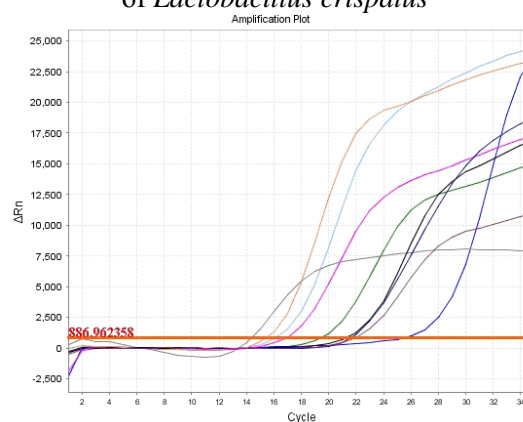


Figure 3 Evaluation of RTQ PCR in cells of *Lactobacillus acidophilus*

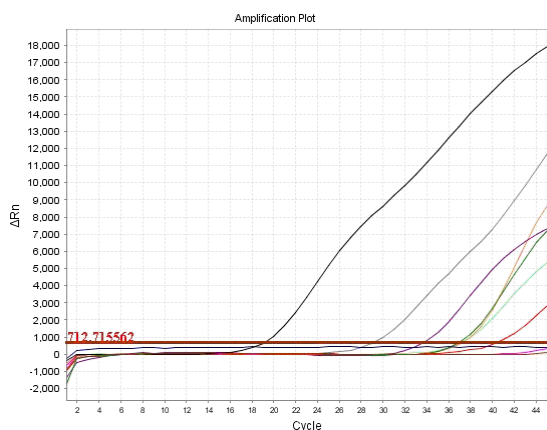


Figure 4 Evaluation of RTQ PCR in cells of *Lactobacillus salivarius*

For identification of individual species of lactobacilli we used RTQ PCR. With real time polymerase chain reaction we identified three species of genus *Lactobacillus*: *Lactobacillus crispatus* (fig. 2), *L. acidophilus* (fig. 3) and *L. salivarius* (fig. 4). Similar results were found in the study Nováková et al. [18].

4. Conclusions

This study has revealed that lactobacilli are autochthonous residents in chickens, where they predominate in the distal GIT. The most commonly identified *Lactobacillus* species are *Lactobacillus crispatus*, *Lactobacillus acidophilus* and *Lactobacillus salivarius*. A detailed understanding of the relationship between these bacteria and their host under different dietary and environmental conditions will facilitate the development of lactobacilli for various applications directed toward increasing broiler production efficiency and improving chicken health.

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