Xenobiotics

Soil, Food and Human Health Interactions

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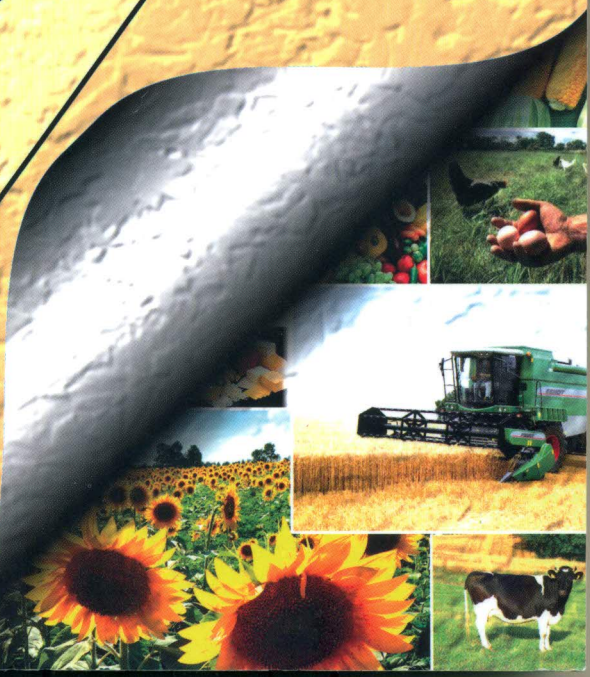

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Contents

Lactobacillus genus identification isolated from gastrointestinal tract of chickens after application of propolis using FISH and RTQ PCR methods

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Abstract

The general objective of this study was to examine the effect of propolis on the lactobacilli colonization of chickens. Propolis was 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, second group was with propolis in feed mixture with the addition of 300 mg propolis per 1 kg of compound and third group was with propolis in feed mixture with the addition of 400 mg propolis per 1 kg of compound.

In this experiment, quantitative counts of lactobacilli in cca of 49-day-old chicken (Ross 308) using classical and FISH method were investigated. Counts of lactobacilli on 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 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 first experimental group where was 200 mg of propolis added to 1 kg of feed mixture. Statistical significant differences ($P \leq 0.05$) were not found between groups. Using Real-time PCR method, 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: lactobacilli, chickens, propolis, FISH, RTQ PCR

Introduction

The ecology of the chicken gastrointestinal tract (GIT) has been studied in depth using both culture-dependent (Bjerrum et al., 2006) and -independent methods (Knarreborg et al., 2002; Apajalahti et al., 2004; Bjerrum et al., 2006). These studies have revealed that lactobacilli are autochthonous residents in chickens, where they predominate in the proximal GIT and are present but less abundant within the distal GIT (Stephenson et al., 2010). The most commonly identified *Lactobacillus* species are *Lactobacillus crispatus*, *Lactobacillus reuteri* and *Lactobacillus salivarius* (Guan et al., 2003; Bjerrum et al., 2006; Dumonceaux et al., 2006; Abbas Hilmi et al., 2007; Gong et al., 2007). 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.

Lactobacilli are excellent candidates for alternative control methods due to their autochthonous nature and dominance of the upper GIT microbiota, particularly within the small intestine where NE occurs. Their potential utility in the control of NE has been demonstrated, with several strains of *Lactobacillus* showing some efficacy as probiotics to decrease *C. perfringens* carriage within the small intestine of chickens (Decroos et al., 2004; La Ragione et al., 2004; Smulikowska et al., 2005; Gérard et al., 2008; Kizerwetter-wida and Binek, 2009). Lactobacilli are also excellent candidates as mucosal delivery vectors designed to express bioactive peptides *in situ* to reduce colonization by *C. perfringens*. The use of lactobacilli and other lactic acid bacteria (LAB) as live delivery vectors for therapeutic proteins has recently been reviewed (Wells and Mercenier; 2008; Berlec and Trukelj, 2009), but few studies have been conducted in chickens (Sieo et al., 2006; Yu et al., 2007, 2008). 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 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 [Shin et al., 2002; Koenen et al., 2004; Kizerwetter-wida and Binek, 2005; Taheri et al., 2009]. While these assays can be used to reduce the number of strains examined, they may 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 (Stephenson et al., 2009).

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 (Shalmany and Shivazad, 2006).

Therefore, the present study investigated the effect of 80% ethanolic extract of propolis samples collected from different regions of Slovakia on performance of Ross (308) broiler chickens to lactobacilli in the control and three 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.

Material and Methods

In this experiment, quantitative counts of individual groups of microorganisms in cca 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 the parameters of 70x100 cm.

Experiment of monitoring the impact of propolis 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)

Propolis was 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. 1st experiment

Control group: the feed mixture without the addition of propolis.

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

2nd Experimental group: feed mixture with the addition of 300 mg propolis per 1 kg of compound,

3rd Experimental group: feed mixture with the addition of 400 mg propolis 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 (Holt et al., 1994).

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 TM 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 ul 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 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. (2005), which were used for species identification of lactobacilli showed Table 1.

Table 1. Characteristic of primers used for lactobacilli identification

species	primer	sequence (5'- 3')
L. salivarius	Lsal-1	AATCGCTAAACTCATAACCT
	Lsal-2	CACTCTCTTTGGCTAATCTT
L. cidophilus	Laci-1	TGCAAAGTGGTAGCGTAAGC
	23-10C	CCTTTCCCTCACGGTACTG
L. crispatus	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 showed Table 2.

Table 2. Components of PCR reaction

Components	Quantity [20 µl]
Master mix	10.00
primer F	0.20
primer R	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.

Results and Discussion

The application of propolis influenced Lactobacilli number of chickens showed table 3. In the trial with chickens after application of propolis, no statistically significant differences were found. 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.

It was also around this time that the *Lactobacillus* spp. and Bifidobacteria were established in low concentrations. The mechanism for these changes in bacteria has not been defined. Lactobacilli and Bifidobacteria 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 (Kokosharov, 2001).

Table 3. Summary statistical values for *Lactobacillus* spp.

Values/Groups	K	P1	P2	P3
Average	7.12	8.70	8.48	8.40
Standard deviation	0.38	0.20	0.22	0.25
Coeff. of variation (%)	5.31	2.30	2.61	2.99
Minimum	6.89	8.51	8.23	8.25
Maximum	7.56	8.91	8.65	8.69

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 (Robertfroid et al., 1998) 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 (Nisbet et al., 1995), 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.

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

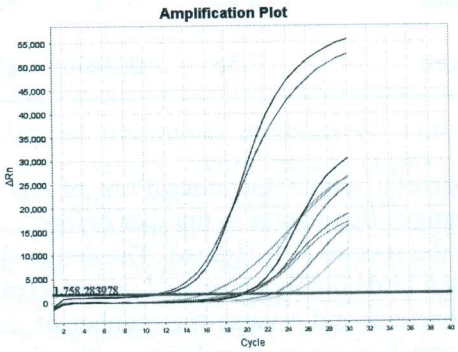

Figure 2. Evaluation of RTQ PCR in cells of *Lactobacillus crispatus*

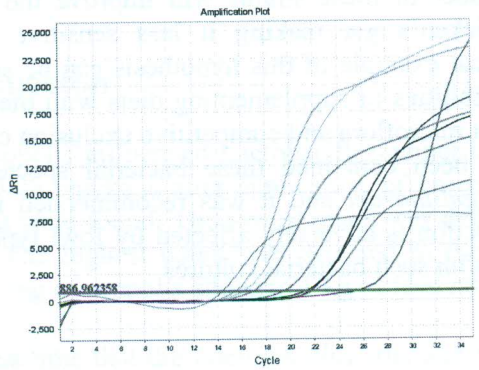

Figure 3. Evaluation of RTQ PCR in cells of of *Lactobacillus acidophilus*

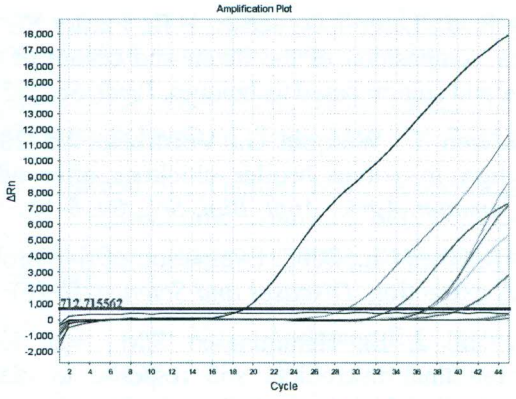

Figure 4. Evaluation of RTQ PCR in cells of *Lactobacillus salivarius*

After classical method for enumeration of lactobacilli we used FISH (fig. 1). The number of *Lactobacillus* cells ranged from 10^2 to 10^6. The higher number of lactobacilli was found in the first group where pollen 200 mg per kilogram was used. Similar results were found in the study Nováková et al., (2010).

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., (2010).

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