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BACTERIA IN CHICKEN GASTROINTESTINAL TRACT DETECTED BY REAL TIME PCR AFTER BEE POLLEN APPLICATION IN CHICKENS DIET

Miroslava Kačániová[1], Peter Haščík[2], Jana Petrová[1], Lukáš Hleba[1], Martin Mellen[3]*

Address(es): prof. Ing. Miroslava Kačániová, PhD.,
[1]Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.
[2]Department of Animal Products Evaluation and Processing, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Slovak Republic.
[3]Hydina Slovensko (Poultry Slovakia), Nová Ľubovňa, 065 11 Nova Ľubovňa.

*Corresponding author: Miroslava.Kacaniova@uniag.sk

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ABSTRACT

The aim of this study was to examine the effect of pollen on the microbial colonization of chicken gastrointestinal tract. The pollen was administered to both feed mixtures in various amounts except of the control group. The addition of 50 mg pollen to 1 kg of feed was included in the first experimental group, the addition of 100 mg.kg^{-1} in the second experimental group, the addition of 200 mg.kg^{-1} the third experimental group (EG), 300 mg kg^{-1} in the fourth EG and 400 mg kg^{-1} in fifth EG. The highest count of faecal enterococci was found in the fourth experimental group (EG) where 300 mg of pollen to 1 kg was added to feed mixture. The lower count of faecal enterococci was found in the first experimental group where 50 mg of pollen to 1 kg was added to feed mixture. The highest count of lactobacilli was found in the second and fifth experimental group where 100 g and 400 g of pollen to 1 kg were added to feed mixture. The lower count of lactobacilli was found in the first experimental group where 50 g of pollen to 1 kg was added to feed mixture.

Keywords: pollen, gastrointestinal tract, bacteria, real time PCR, chickens

INTRODUCTION

Modern intensive poultry production has achieved phenomenal gains in the efficient and economical production of high quality and safe chicken meat and eggs as a part of strategy how to contribute to the health and wellbeing of the customers (**Horská *et al.*, 2013**). The use of feed additives has been an important part of achieving this success. Common feed additives used in poultry diets include antimicrobials and antioxidants. Acidifiers are widely used in the food and feed industry as a feed additive because of their strong antibacterial action (**Hashemi *et al.*, 2012**).
Intestinal bacteria play an important role in pathogenesis of intestinal diseases since they are believed to protect against colonization of the intestine by pathogens and to stimulate the immune response of chickens (**Mead, 2000**). Extensive studies of the culturable bacterial flora of chickens have been performed in animals intensively reared (**Rolfe, 2000; Gong *et al.*, 2002; Jiangrang *et al.*, 2003**). The main bacteria in cecum are obligate anaerobes, while *Lactobacillus, Enterococcus* and *Streptococcus* are prevalent in ileum (**Jiangrang *et al.* 2003**).
Bee-pollen is a hive derived product of great commercial interest owing to its high nutritional quality and can be considered as potential source of energy and proteins for human consumption (**Kroyer and Hegedus, 2001; Campos et al., 2003**). Pollen is a fine, powder-like material produced by flowering plants and gathered by worker bees. Bees use pollen as their nutritional source of proteins (25-30%), carbohydrates (30-55%), lipids, including fatty acids and sterols (1-20%), vitamins and minerals. Bee pollen and beebread (stored pollen) are consumed for api-therapeutical purposes for their nutritional and medicinal properties. In its composition it presents valuable nutrients such as free aminoacids, minerals and oligo-elements and for this reason it is used in the human diets providing a well-being sensation and contributing to functional and harmonious balance of the body (**Stanciu *et al.*, 2009**). These components were also rich in carotenoids, flavonoids, phytosterols, polyphenols and other healthy compounds (**Moreira *et al.*, 2008**). A number of the fatty acids such as decanoic (capric), dodecanoic (lauric), myristic, linoleic and linolenic acids have antimicrobial properties (**Feldlaufer *et al.*, 1993**).
Different bacterial species respond differently to fatty acids. **Ababouch *et al.* (1992)** found linolenic and dodecanoic acids were most inhibitory to spores of *Bacillus cereus*, whilst linoleic and myristic acids were less so. Decanoic, palmitic and stearic acids showed only partial inhibition. **Petschow *et al.* (1996)** showed that dodecanoic acid was the only satured free fatty acid with bactericidal activity against *Helicobacter pyroli*, the bacteria associated with stomach ulcers in humans. Without knowing the fatty acid composition of pollen, **Petschow *et al.* (1996)** observed that their bee-collected pollen sample was inhibitory to *Staphylococcus aureus, Bacillus anthracis, Escherichia coli* and two strains of *Salmonella, B. cereus* was not inhibited.
The objective of our study was to follow effect of different doses of pollen in chicken's diet on abundance of bacteria in GIT (gastrointestinal tract).

MATERIAL 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 100 broiler chickens (Hubbard JV) were used. Fattening itself went on from1 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. Feed and water were provided *ad libitum*.

Chicken feed additives

Pollen dose was administered to both feed mixtures (HYD-01, HYD-02) in various doses: Control group: compound without the addition of pollen,
1st experimental group: pollen at a dose of 50 mg.kg^{-1},
2nd experimental group: pollen at a dose of 100 mg.kg^{-1},
3rd experimental group: pollen at a dose of 200 mg.kg^{-1},
4th experimental group: pollen at a dose of 300 mg.kg^{-1},
5th experimental group: pollen at a dose of 400 mg.kg^{-1}.

DNA Extraction

As a pre-preparation step for the Step One real-time PCR, DNA extraction was performed using DNA extraction method: PrepSEQ Rapid Spin Sample Preparation Kit (Applied Biosystems, USA). Sample of 750 µL was loaded onto

the spin column and microcentrifuged for 3 minutes at maximum speed. Supernatant was discarded and to the pellet was added 50 µL of Lysis Buffer. Samples were incubated for 10 minutes at 95°C.

Primers and Real-time PCR

The molecular diagnosis of *Enterococcus avium, E. caseliflavus, E. cecorum, E. gallinarum, E. hirae, E. malodoratus, Lactobacillus salivarium, L. acidophilus, L. crispatus* were focused on the presence of primers: *Enterococcus avium*, *E. caseliflavus*), *E. cecorum*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, *Lactobacillus crispatus*, *L. salivarium*, *L. acidophilus* genes.
Reaction mixture: 15 µl SYBR Green PCR, Master Mix, 1.5µl of primers (F + R), 3µl samples after isolation,10.5µl of deionized water.

The PCR primers and reaction conditions were as follows:

Enterococcus avium: primer AV1 5´-GCTGCGATTGAAAAATATCCG-3´ and AV2 5´-AAGCCAATGATCGGTGTTTTT-3´.
E. caseliflavus: primer CA1 5´-TCCTGAATTAGGTGAAAAAAC-3´ and CA2 5´-GCTAGTTTACCGTCTTTAACG-3´.
E. cecorum: primer CE1 5´-AAACATCATAAAACCTATTTA-3´ and CE2 5´-AATGGTGAATCTTGGTTCGCA-3´.
E. gallinarum: primer GA1 5´-TTACTTGCTGATTTTGATTCG-3´ and GA2 5´-TGAATTCTTCTTTGAAATCAG-3´.
E. hirae: primer HI1 5´-CTTTCTGATATGGATGCTGTC-3´ and HI2 5´-TAAATTCTTCCTTAAATGTTG-3´.
E. malodoratus: primer MA1 5´-GTAACGAACTTGAATGAAGTG-3´ and MA2 5´-TTGATCGCACCTGTTGGTTTT-3´.
L. crispatus: primer Cri 12SI 5´-GTAATGACGTTAGGAAAGCG-3´ and Cri 12SII 5´-ACTACCAGGGTATCTAATCC-3´.
Lactobacillus salivarium: primer Lsal-1 5´-AATCGCTAAACTCATAACCT-3´ and Lsal-2 5´-CACTCTCTTTGGCTAATCTT-3´.
L. acidophilus: primer *Laci*-1 5´-TGCAAAGTGGTAGCGTAAGC -3´ and 23-10C 5´-CCTTTCCCTCACGGTACTG-3´(**Drisko et al. 2005, Desay et al. 2001**).

The reaction consists of starting denaturation (95°C, 10 min) and 40 cycles of denaturation (95°C, 15 sec), annealing and elongation (60°C, 1 min).
Data were collected during each elongation step. PCR products were detected by monitoring of the increase in fluorescence of the reporter dye at each PCR cycle. TaqMan® probes labeled with both a fluorophore and a quencher dye were used in real-time PCR assays to detect amplification of specific DNA targets. FAM™, which has an emission of 520 nm, has become the most commonly used fluorophore for single plex qPCR reactions. TAMRA™ will efficiently quench the fluorescence of FAM™, until the probe hybridizes to the target and is cleaved by the 5'exonuclease activity of the polymerase. We used three fluorophore detection chemistries that include FAM™ and VIC® dye-labeled TaqMan® MGB probe-based assays, VIC® and TAMRA™ dye-labeled probe-based assays and ROX™ as passive reference dye. PCR products were detected by monitoring of 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.

RESULTS AND DISCUSSION

Bee pollen could be therefore used as a potential feed additive with prebiotic activity to the poultry diet (**Kačániová *et al.*, 2013**). The highest count of faecal enterococci was found in the fourth experimental group (EG) where 35 g of pollen to 1 kg was added to feed mixture as shows table 1. The lower count of faecal enterococci was found in the first experimental group where 5 g of pollen to 1 kg was added to feed mixture. We identified the species range of the genera *Enterococcus* in the intestinal tract of broilers using a real-time PCR method. We detected species from the genus *Enterococcus*: *E. avium, E. casseliflavus, E. cecorum, E. gallinarum, E. hirae* and *E. malodoratus*. There were the most frequent species of *E. avium* and *E. cecorum* in control group, second, third, fourth and fifth experimental groups and *E. malodoratus* in control group, second and fourth EG.

Table 1 Abundance of bacteria in gastrointestinal tract

Species	Control	EG1	EG2	EG3	EG4	EG5
E. avium	+	-	+	+	+	+
E. casseliflavus	-	-	-	-	-	-
E. cecorum	-	-	-	-	+	+
E. gallinarum	-	-	-	-	-	-
E. hirae	-	-	-	+	-	-
E. malodoratus	+	-	+	-	+	-

(+) presence, (-) absence

The highest count of lactobacilli was found in the second and fifth experimental group where 100 mg and 400 mg of pollen to 1 kg was added to feed mixture as shows table 2. The lower count of lactobacilli was found in the first experimental group where 50 mg of pollen to 1 kg was added to feed mixture. We identified the species range of the genera *Lactobacillus* in the intestinal tract of broilers using a real-time PCR method. We detected species from the genus *Lactobacillus*: *L. salivarium, L. acidophilus and L. crispatus*. There were the most frequent species of *L. crispatus* in the control group, third, fourth and fifth experimental groups and *L. acidophilus* and *L. crispatus* second EG. The similar results with MALDI TOF MS Biotyper identification of bacterial strains in gastrointestinal tract of chickens after pollen application in their feed were found in the study **Kačániová *et al.* (2013)**. In this study were identified the following genera: *Escherichia coli*, *Proteus mirabilis*, *Klebsiella oxytoca*, as well as *Lactobacillus acidophilus*, *L. crispatus*, *L. fermentum* and *L. salivarius* from the lactobacilli group and *Enterococcus avium*, *E. casseliflavus*, *E. cecorum*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae* and *E. malodoratus* from the enterococci group.

Table 2 Abundance of bacteria in gastrointestinal tract

	Control	EG1	EG2	EG3	EG4	EG5
L. crispatus	+	-	-	+	+	+
L. salivarium	-	-	+	-	-	+
L. acidophilus	-	-	+	+	-	-

(+) presence, (-) absence

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 (**Dunkley *et al.*, 2009**). There is the perception that the role of microorganisms in chickens is not as important as is the case for ruminants (**Jozefiak et al., 2004**). However, extensive strict anaerobic metabolism including methanogenesis fermentation occurs in birds fed a variety of diets (**Saengkerdsub *et al.*, 2006**). The ceca are the major fermentation sites in the GIT of chickens and contain the largest number of bacteria.

CONCLUSION

The microbial diversity of the gastrointestinal ecology plays an important role in the food animal industry and human medicine. Our results indicated that the Step One real-time PCR assay developed in this study could sensitively detect Lactobacilli and Enterococci. The positive influence was followed in number of lactobacilli and enterococci in the experimental groups. Our results showed that pollen application doesn't influence different representation of strains of lactobacilli and enterococci.

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