DETEKCIA A IDENTIFIKÁCIA DRUHU *LACTOBACILLUS* V GASTROINTESTINALNOM TRAKTE KURČIAT ZA POUŽITIA METÓD FISH A PCR

DETECTION AND IDENTIFICATION OF *LACTOBACILLUS* SPECIES IN GASTROINTESTINAL TRACT OF CHICKENS BY FISH AND PCR ANALYSIS

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ABSTRACT

The aim of the present study was a rapid detection and identification of *Lactobacillus* sp. in gastrointestinal tract of chickens by fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR) analysis. As a biological material broiler chickens Hybro were used. They were fattening by the combined probiotic preparation (1st and 2nd experimental group) for elimination of pathogens and better utilization of feed. The 100 % of isolated strains covered *Lactobacillus salivarius* and *Lactobacillus acidophilus*. For quantification of bacterial count was used FISH method. The statistical evaluation showed significant differences ($P \leq 0.001$) between control group and first experimental group in the number of *Lactobacillus sp.* counts. The same differences were found between control group and second experimental group. In the number of *Lactobacillus sp.* counts were found statistical significant differences ($P \leq 0.05$) between first and second experimental group.

Key words: *Lactobacillus* sp., chicken, identification, fluorescence *in situ* hybridization, polymerase chain reaction

INTRODUCTION

The gastrointestinal microbiota is known to affect the health and growth of host animals. In healthy chicken, the composition of intestinal microbiota remains stable.

The microbial community of the gastrointestinal tract ultimately reflects the coevolution of microorganisms with their animal host and the diet adopted by the host. Changes in the composition of the animal's microflora can have beneficial or detrimental effects on health, growth, and maturation of the animal host, as is evident from the beneficial effects of rearing food animals on feeds containing antibiotics **(Lu et al., 2003)**. However, this stability may be disrupted by various factors such as pathogen invasion, antibiotic administration and environmental stress (e.g., overcrowding, poor feeding, extremely high or low temperature, transportation). Young animals under stressful conditions suffer from changes in the composition and activity of the gut microbiota **(Lan et al., 2004).**

A number of factors contribute to the colonization and continued presence of bacteria within the digestive tract of animals. These factors have been extensively reviewed by **van der Wielen et al. (2002)**. Included among these factors are: (1) gastric acidity; (2) bile salts; (3) peristalsis; (4) digestive enzymes; (5) immune response; and (6) indigenous micro-organisms and the antibacterial compounds which they produce. The first four factors are dependent on the phenotype of the host and may not be practically controllable variables. The immune response in the gastrointestinal (GI) tract is not easily modulated. The factors involving indigenous micro-organisms and their metabolites are dependent on the normal flora of the GI tract. Attempts to control the microbiological flora in the chicken GI tract must take into account all of the above factors in order to maintain the desirable organisms and to eliminate human enteropathogens.

The native intestinal lactic acid bacteria (LAB), including *Lactobacillus* sp., are candidates because of their ability to colonize the GI tract, to produce bacteriocins, hydrogen peroxide, reuterin and certain organic acids, and to deconjugate bile acids and salts to yield the more inhibitory free bile acids **(Juven 2001)**.

Lactobacillus sp. are normal inhabitants of the intestinal tract, especially of poultry. The genus *Lactobacillus* contains a diverse assemblage of gram-positive, catalase-negative, nonsporulating, rod-shaped organisms and includes more than 25 species **(Heilig et al., 2002)**. Within a few hours after hatching, various bacteria including faecal streptococci, enterobacteria and clostridia may be found randomly scattered through the alimentary tract, but within a few days the lactobacilli become established **(Tannock, 2004)**. An association between lactobacilli and the epithelial lining of the chicken crop is established within a few days after hatching and persists throughout the life of the chicken **(Rada and Marounek, 2005)** Lactobacilli also predominate more distally in the small intestine and were shown by **Zhu and Joergert (2003)** to adhere to the columnar epithelial cells. Lactobacilli are the only group of organisms reported as generally present in numbers exceeding 10 000 per gram of intestinal contents in the small intestine of chickens at 2-6 weeks of age. Both heterofermentative and homofermentative lactobacilli have been isolated from commercial and experimental batches of chicks. However, the heterofermentative strains (e.g. *Lactobacillus fermenturn* and *Lact. reuteri)* occur more frequently in the very young chick, while the homofermentative strains (e.g. *Lact. acidophilus)* develop more slowly but often predominate after several days **(Zhu et al., 2002)**. The following species of lactobacilli have been isolated from the chicken GI tract: *Lact. salivarius*, *Lact. acidophilus* and *Lact. fermentuml Lact. reuteri* **(Tannock, 2005).**

Studies on the composition of the intestinal microbiota of chickens date back to 1901. Additional studies were carried out in the 1940s, but not until the 1970s were comprehensive surveys attempted by culturing as many of the intestinal bacteria as possible. These studies were technically difficult and extremely time consuming because strict anaerobic conditions had to be maintained, and numerous biochemical differentiation tests had to be carried out. Using such methods, only an estimated 10 to 60% of the bacteria in the cecum could be cultivated **(Zhu and Joergert, 2003).**

To circumvent some of the problems associated with culture-based surveys, culture-independent molecular approaches have been used to study the composition of the cecal microbiota of chickens **(Gong et al., 2002; Zhu et al., 2002)**. These PCR-based approaches are powerful tools to provide an overview of the microbial diversity present in a particular sample, but they can also be biased by cellular rDNAcopy number **(Smirnov et al., 2005)**, DNA extraction methods, primer selection **(Drisko, 2005)**, PCR amplification **(Speksnijder et al., 2001)**, and cloning strategy and efficiency **(Lu et al., 2003)**.

The percentage distribution of 16S rRNA gene sequences generated by PCR from DNA extracted from mixed environmental samples cannot be used to infer the quantitative distribution of species in the microbial community from which the DNA was extracted or to predict the contribution of these species to the activity of the whole microbial community **(Sghir et al., 2000)**. Therefore, fluorescent in situ hybridization has been used as an alternative means to quantify the abundance and determine the distribution of bacterial groups in natural communities **(Harmsen et al., 2000)**.

The aim of the present study was detected and identified *Lactobacillus* sp. in gastrointestinal tract (GIT) of chickens by fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR) analysis.

MATERIAL AND METHODS

As biological material were used one day old chickens of meat type Hybro. They were treated for 35 days by the probiotic preparation based on *Lactobacillus delbrueckii ssp. bulgaricus* LAT187, *Lactobacillus helvetius* LAT179, *Lactobacillus acidophilus* LAT180, *Lactobacillus delbrueckii ssp.lactis* LAT182, *Streptococcus termophilus* LAT205, *Enterococcus faecium* E-

253 (1st and 2nd experimental group). The control group contained feedstuff without addition of probiotic.

Sample collection. The birds were killed by cervical dislocation, and GI tracts were removed, placed in sterile plastic bags, and immediately immersed in ice for transport to the laboratory. Four types of samples (crop, gizzard, duodeum, ceacum) were prepared for microbial cultivation and for in situ hybridization studies.

Microbiology. Chyme contents were streaked directly onto MRS Agar (Biomark, Pune, IND) and incubated overnight at 37 °C. After icubation, colonies formed on the respective media were carefully observed, counted and picked up for Gram staining. At least ten *Lactobacillus* sp. colonies were selected from each sample and incubated (overnight at 37 °C) in Peptone water. A 2 ml of solution was used for isolation of bacterial DNA.

DNA isolation. DNA was extracted with GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, USA).

PCR analysis. Genus primers designed by **Drisko et al. (2005)** were used in each reaction to confirm the genus of lactobacilli. Two PCR master mixes consisting of different primer set were prepared (Table 1). Master mix 1 was *Lactobacillus salivarius*; Master mix 2 was *Lactobacillus acidophilus*.

Table 1 Primer used for PCR

Bacterial species	Primer	Orientation	Primer sequence
Lactobacillus salivarius	Lsal-1	F	5`- AATCGCTAAACTCATAACCT-3`
	Lsal-2	R	5`- CACTCTCTTTGGCTAATCTT-3`
Lactobacillus acidophilus	Laci-1	F	5`- TGCAAAGTGGTAGCGTAAGC-3`
	23-10C	R	5`- CCTTTCCCTCACGGTACTG-3`

F-forward primer; R-rewerse primer

The base master mix consisted of 10x Restorase reaction buffer, 0,05 U.μl^{-1} Restorase DNA Polymerase (Sigma-Aldrich, St. Louis, USA), H_2O (redistilled) in addition to final volume and 1 μl (2 μM) each genus primer. PCRs were performed in a final volume of 50 μl consisting of 1 – 2 ng. μl^{-1} DNA template. Each PCR product was amplified according to the following conditions: initial denaturation at 95°C for 3 min, followed by PCR cycles of denaturation at 94 °C for 30sec, annealing and temperatures specified in Table 2 for 60 sec and extension at 72 °C for 1 min. Each reaction concluded with a 10 min final extension at 72 °C. The number of amplification cycles and the expected amplified product size are presented in Table 2.

PCR products were electrophoresed on a 2% Tris-acetate-EDTA agarose gel and stained in GoldView ™.

Table2 Conditions for amplification of PCR products

Species	Annealing temperature	No. cycles	Product size (bp)
L. salivarius	60 °C	35	411
L. acidophilus	68 °C	45	210

FISH. For FISH analysis was used Fluorescence *in-situ* Hybridization Lactobacillus Cluster Kit (Ribo Technologies, Groningen, NL). The slides were stored at 4 °C at dark until visualized using a Nikon ECLIPSE E800 microscope

RESULTS AND DISCUSSION

The diversity of members of the *Lactobacillus* genus was investigated by using primers designed by **Drisko et al. (2005)** on bacterial DNA isolated from various parts of chicken gastrointestinal tract. In our study we were focused to isolate the major species *Lactobacillus* genus, especially *Lactobacillus salivarius* and *Lactobacillus acidophilus.* These organisms covered 100% of the total isolated lactobacilli. Our result we have to compare with some others authors **(Drisko, 2005; Lu et al., 2003, Tannock et al., 2004)**. **Lan et al. (2004)** state that, these bacteria are formed during the development of indigenous microbiota in chicken intestine, thus, in the control chicken intestine there may also be present *L. agilis* or *L. salivarius*, which are referred to as indigenous strains. Generally, the relative abundance and prevalence of *L .agilis* decrease with age and could not be detected in 54-day-old chickens. In contrast, *L. salivarius* maintained certain population with a high prevalence in the jejunum of the probiotic old-age chickens.

For quantification (Figure1) of bacterial count was used fluorescent in situ hybridization method. Cells of *Lactobacillus* sp. were targeted with fluorescently labelled probes. Examples of fluorescent images are shown in Figure 2. The images were viewed and analyzed with LUCIA 5 (Laboratory Universal Computer Image Analysis) software. For each content at least ten microscope fields were enumerated. The average count of cells ranged from 10^6 to 10^8. The count of *Lactobacillus* sp. ranged from 0.07 $\log.g^{-1}$ to 2.28 $\log.g^{-1}$ in control group. In the first experimental group the average count ranged from 0.25 $\log.g^{-1}$ to 4.20 $\log.g^{-1}$. In the second experimental group ranged bacterial count from 0.16 $\log.g^{-1}$ to 3.90 $\log.g^{-1}$.

The lowest counts of bacterial cells were determined in gizzards of chickens and the highest percentage occurred in caeces (especially in 1st experimental group).

Our result we have to compare with some others authors **(Zhu et al., 2003; Gérard et al., 2008; Nava et al., 2009)**. They are very similar in isolated species.

Average count of *Lactobacillus* sp. in GIT of broiler chickens

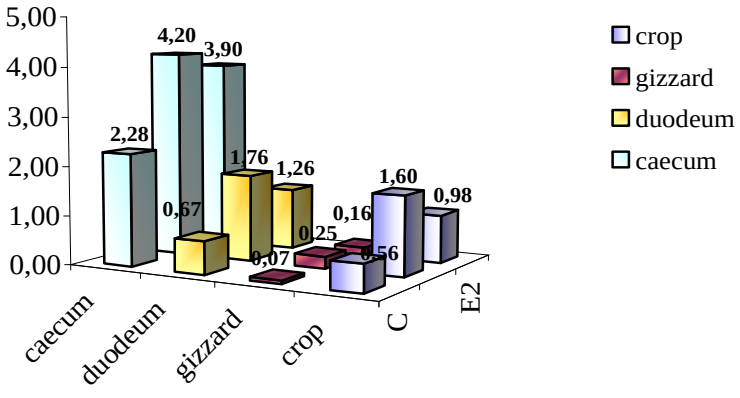
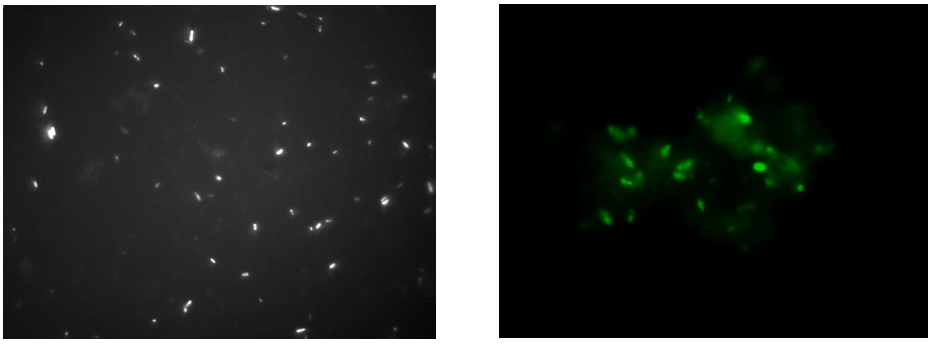


Figure 1 Average count of *Lactobacillus* sp. in GIT of broiler chickens analysed by the fluorescent in situ hybridization method



A **B**

Figure 2 Examples of the fluorescent in situ hybridization images of bacteria from caecum (A) and crop (B) of 35-days-old chicken

CONCLUSION

In production of health poultry we have to know relationship between the indigenous intestinal microflora supported by the probiotic strains and pathogens. The molecular techniques for the more accurate identification of intestinal microflora help define the function of commensally bacteria in the GIT. The previous report compares two base techniques for rapid identification of bacterial population. Future advances in probe development such as the design of probes that are specific perhaps to the species level will allow the location and enumeration of bacterial species directly in chyme samples.

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