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GUT MICROBIOTA OF BROILER CHICKENS INFLUENCED BY PROBIOTICS AND ANTIBIOTICS AS REVEALED BY T-RFLP AND RT-PCR

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Abstract

In poultry, and birds in general, the gut is not only the first line of the defense from exogenous pathogens, but also the biggest immunity organ. The microflora inhabiting blind gut plays multifunctional role in the maintenance of homeostasis in macroorganism, being involved in different processes including fodder digestion. Probiotic, antibiotic, prebiotic, symbiotic preparations, enzymes, etc., affecting directly or ndirectly the gut microflora, can thus increase daily weigh growth and viability in chickens, and the slaughter yield. They also improve a digestibility of fodder nutrients, quality and sanitary condition of the birds. However, the influence of these agents on gut microflora in chickens is still not cleared up. Recent molecular approaches allow studying microbial biodiversity without traditional limitations we are faced with when applying microbiological techniques. By T-RFLP and RT-PCR, the composition and growth dynamics of gut microflora were examined in the Cobb 500 broiler chickens fed with probiotic Cellobacterin-T and antibiotic Stafak-110 as feed additives to the complete diet. By these methods, the probiotic and antibiotic additives were shown to contribute to the development of microbiota. In particular they increased the total number of bacteria, what is more, the normal gastrointestinal flora, i.e. cellulolytic bacteria, lactobacilli, bifidobacteria, bacilli, selenomonas species, increased, while the unwanted enterobateria and actinimycetes decreased. In case we used the probiotics in poultry diet when compared to antibiotics, in the studied parts of the intestinal of broiler chickens there were more cellulolytic bacteria, and in the blind gut the number of lactobacilli, bifidobacteria and bacilli was 10-100 times higher, while clostridia and transit bacteria number decreased. Numerous investigations affirmed the effectiveness of probiotics in poultry basing on zootechnical parameters, however, only molecular identification of the gut microbiota members by means of T-RFLP-PCR and RT-PCR analysis allows detecting and attributing their taxonomic groups influenced by a specific feed additive for further justification of its rational use.

Keywords: broiler chickens, digestive tract, duodenum, blind gut, microflora, pathogenic microflora, T-RFLP, RT-PCR.

An advanced genetic research, breeding technologies and feeding together with effective veterinary control provide for high poultry meat production in the world (1). Genetically determined potential for growth and effective feed conversion in modern poultry crosses makes it possible to reach 2.2-2.3 kg weight of 38-day old chicken at 1.45-1.50 kg fodder consumed per 1 kg weight gain (2). The weight increases 3-4 times during the chicken's first week of life, at that the gut is even more intensely growing. As the higher the productivity, the more stress sensitive poultry is, it often results in health deterioration, and therefore many difficulties are arising at breeding (3).

Numerous researches show the role of normal microflora involved in the control of cardiovascular, endocrine and nervous system functions, hematopoesis, immune reactivity, synthesis of antibiotic substances, proteins, enzymes, hormones, vitamins, etc., and also in preventing infection and unlimited patho-

gens' proliferation in the host's body (4-6). Influencing fodder digestibility and the digestive tract development in poultry, the microflora affects significantly on the nutrients absorption, nutritional demands, physiological status and growth (7).

Few days before and after hatching are crucial for chicks' development and survival because of metabolic and physiological changes occurred as the dry fodder consumption starts instead of yolk utilization. At that the gut are to develop intensely providing for the effective use of fodder nutrients. In case of improper gut microbiota formation during first days of life the chicks are dramatically influenced by sanitary parameters of feeds, water and farm conditions (8).

Gut is commonly known to be not only the first defense line against exogenous pathogen colonization of a host macroorganism cells and tissues, but the biggest immune organ in the body. Any changes in gut morphology influence nutrient absorption and activate secretion and excretion, leading to diarrhea and a decreased disease resistance and productivity in general (9). Compared to other parts of the poultry digestive tract the blind gut is the most populated by microorganisms with their total number reaching 10^{10}-10^{11} per 1 g content (10). In various ways, including feed digestion, the blind gut microflora diversely provides homeostasis of the macroorganism.

Colonization resistance is a most important feature of normal microflora whereby it performs the defensive role. Adverse conditions destroy the established microbial community, and change the adhesiveness and colonization resistance of the indigenous flora leading to disbiosis (disbacteriosis) (11). As a result the uncharacteristic microorgatisms colonize the gut and the bacterial strains different from their obligate forms can prevail. At disbacteriosis more than 50 % of *Escherichia coli* cells possess hemolytic activity and produce hyaluronidase indicating an increased virulence. Besides, a multidrug resistance is characteristic for bacteria isolated at disbacteriosis. Moreover, there are a decreased number of gut lacto- and bifidobacteria essential in performing different functions such as protection of the gut mucosa from penetration of pathogenic and opportunistic bacteria into the blood, production of antibiotic-like substances and organic acids, vitamin B group synthesis, and activation of immune response (12-14).

The diet for high-producing poultry is specially balanced to provide the highest growth rate over a short time. However, an increased level of nutrients could cause a misbalance in gut microbiota (15). Commonly used antibiotic feed additives, the acidifiers and other chemicals influence the gut microbiota. Besides, expansion and granulation of compound feeds also can inactivate many bacteria (16).

Bio-industry recently produces a variety of preparations, namely probiotics, antibiotics, prebiotics, symbiotics, enzymes, etc., which influence the poultry gut mictobiota directly or indirectly, resulting in a greater daily weight gain and chickens' survival, the higher weight at slaughter, and also in better digestibility of the feed nutrients and improved quality and sanitary condition of the birds. However, in fact the effect of these preparations on gut microflora in chickens is not completely clarified.

Antibiotics as feed additives are used to control animal health and achieve economic benefit, but as they lead to the occurrence of antibiotic resistant pathogenic bacteria, that in turn can affect people health, currently they are not fed to poultry and pigs in the European countries, particularly in Sweden since 1986 and in EU since 2006 (18). Because of improper antibiotic use the helpful gut flora decreased in number, moreover, each 2-3 years new antibiotic resistant strain are isolated. The development of each next antibiotic formula takes more time, but the effect is not obvious as often the intestinal motor disturbance occurs (19). Thus the replacement of antibiotics in feed is attractive

and stimulates the search for alternative ways.

Probiotic microorganisms are widely used in EU since 2006 due to forbiddance of feed antibiotics and some other antibiotic substances. Probiotic preparations usually contain living bacteria normally inhabiting the gastrointestinal tract, such as lactobacteria, bifidibacteria, spore forming microorganisms, yeasts and some fungi (20). Probiotics are used in poultry to treat and prevent the gastrointestinal infections, to stimulate a non-specific immunity, to correct the disbacteriotic symptoms caused by an abrupt change of a compound feed composition or violation of the diet, as well as stresses under the translocations, and also they are applied to replace antibiotics in the compound feeds (21).

Because of large selection of the manufactured compound feeds and feed additives for poultry farming, their effects on gastrointestinal bacteria should be reasonably compared by precise, accurate and low-costing attributing (22).

The data on composition and the role of gastrointestinal microbial community were mostly obtained using methods of classical microbiology. However, they are significantly limited and having disadvantages. Particularly counting bacteria is incorrect in case the colonies are derived from bacterial agglomeration but not an individual cell (23). Besides, gastrointestinal bacteria were shown to be mostly not routinely cultivated on growth media (they are called uncultivated bacteria) (24). Uncultivated state is noted to be a global problem topical in soil, plant microbiology, medicine and veterinary medicine. Cell wall-less bacteria are very special but widely spread phenomenon not based on the principles of classical microbiology. Because of bacterial transformation into uncultivated L-forms the pathogens are hard to detect, and false negative results usually occur (25).

Modern molecular methods allow studying diversity of microorganisms without limitations in cultivation. T-RFLP-PCR analysis based on bacterial DNA restriction fragment amplification is considered the most advanced (26) as a good tool to evaluate the total number and the relative species abundance, and to attribute taxonomically all bacteria of the microbial ecosystem. Molecular methods enable a large-scale and detailed comparison of development and changes in the microbial community (27).

In Russia a modified molecular technique is recently used to study the poultry gastrointestinal bacteria (28). The developed protocol enables their prompt and accurate identification and quantitation, early pathogen detection and estimation of the feed additives' effects on the gut microbiota.

Herein, we studied the effects of antibiotic and probiotic feed additives on gastrointestinal microbiota and its qualitative and quantitative changes in growing broiler chicks as detected by T-RFLP—RT-PCR analysis.

Technique. During the experiment held in 2013 the Cobb 500 cross broiler chickens were kept in the vivarium at battery cage system R-15 from a day of age until day 35. Day-old baby chicks were grouped upon the weight by 70 chicks per group and 35 chicks per cage. All chicks were fed compound feed on full ration basis according to the poultry breeding company's recommendations:

	0-5 days	6-15 days	15-36 days
Wheat, %	60,40	59,96	67,11
Soybean meal, %	23,03	19,43	11,05
Sunflower meak, %	6,00	7,20	7,31
Fish meal, %	4,00	2,50	0
Meat and bone meal, %	0	2,00	5,00
Soybean oil, %	3,11	5,33	6,00
Limestone, %	0,87	1,01	0,19
Mono calcium phosphate, %	1,00	0,70	0
Tricalcium phosphate, %	0	0,37	1,84
Vitamin and mineral premix, %	1,50	1,50	1,50

In the control group I chicks were fed balanced feed (the basic ration,

BR) with 4.0, 4.5 and 5.0 % animal protein at 0-5 days, 6-15 days and from 15 days of age, respectively. For feeding the chicks from the groups II and III the antibiotic Stafac 110 (Phibro Animal Health Corporation, USA) or probiotic Cellobacterin-T (Biotrof Ltd, Russia) at 180 g per ton and 1 kg per ton, respectively, were mixed with feed. In each group 6 chicks were arbitrarily slaughtered at days 1, 7, 14, 21 and 35 for sampling. For more reliable results we selected the chicks with filled crop, and a day old chicks were surveyed 24 hours after feeding. The contents of the duodenum and the blind gut were aseptically sampled into Eppendorf tubes and frozen at –20 °C until transportation at –18 °C.

T-RFLP and RT-PCR were performed as reported (29, 30) after the approbation (31).

To extract DNA for T-RFLP the Genomic DNA Purification Kit (Fermentas, Lithuania) was used. A 200 μg sample in 1.5 ml Eppendorf tube with 400 μl lysing reagent was vortexed on a CVP-2 (BioSan, Latvia) and incubated in a thermoshaker TS-100 (Biosan, Latvia) at 65 °C for 30 minutes. After the precipitation solution was added the mixture was vortexed on V-32 (BioSan, Larvia) and centrifuged at 14000 rpm for 10 minutes (Beckman Coulter centrifuge, USA). The precipitate was vortexed with 100 μl NaCl solution and 300 μl 96 % ethanol and centrifuged as hereinabove. The final precipitates were thermostaed at 45 °C until complete drying. After 100 μl sterile water addition the samples were vortexed. The obtained DNA preparations were stored at –20 °C. To control DNA purification, the preparations were separated electroforetically in 1 % agarose gel (Bio-Rad, USA) with TAE (Tris-acetate-EDTA) buffer (Fermentas, USA) at 50 V for 1 hour using Gene Ruler molecular marker (Fermentas, USA). The gels were viewed with a UV transilluminator (New England BioGroup, USA).

For T-RFLP-PCR we used 10 pM primers, 10 units of Taq DNA Polymerase, 10× Taq DNA Polymerase buffer, 2.5 mM $MgCl_2$, 2.5 mM dNTPs (Fermentas, USA), 10 ng template DNA. To amplify 16S-rRNA bacterial gene, the specific 5′-end fluorescently labeled oligos, the 63f, 1087r, were chosen. A negative control with no added DNA and a positive control containing successfully amplified DNA were used. PCR was carried out in a iCycler (Bio-Rad, USA) according to the following protocol: initial denaturation at 95 °C for 3 minutes; 34 cycles of denaturation, annealing and elongation at 95 ° for 30 seconds, 42-60 °C for 30-60 seconds, and 72 °C for 1 minute, respectively; and final elongation at 72 °C for 10 minutes. The amplicon patterns were analized electrophoretcally (Bio-Rad, USA) as described hereinabove.

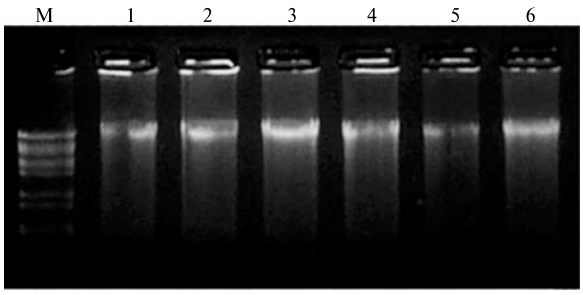

Fig. 1. Typical electrophoretic patterns of DNAs used in T-RFLP—RT-PCR analysis of poultry gut microbial community: M — molecular marker Gene Ruler (Fermentas, USA), 1-6 — DNA samples (1 % agarose gel, TAE buffer, 50 V, 1 hour).

DNA extraction from the gel slices was performed using Genomic DNA Purification Kit (Fermentas, Lithuania) according to the manufacturer's instructions. The obtained DNA precipitates were solubilized in 15 μl deionized water. For restriction a special kit («Fermentas», USA) was used with 1.5 μl restriction buffer and 10 units of Hae III, Hha I and Msp I endonucleases in the final volume brought to 15 μl, and heat termination at 72 °C for 20 minutes was applied.

Capillary electrophoresis was performed on a CEQ8000 DNA sequenser (Beckman Coulter, USA) with 0.2 μl of DNA Size Standard Kit-600 marker (Beckman Coulter, USA) added to each DNA probe to estimate fragment lengths. Based on a T-RFLP-PCR product fluorescence ranged from 10 to 220 thousand units and a control 600 bp fragment length, the data matrices were created in MS Excel and bacteria were phylogenetically attributed using FragSort program (http://mica.ibest.uidaho.edu). An accurate taxonomic attribution was obtained from the data for all three endonucleases, the Hae III, Hha I and Msp I, and a percentage of each bacterial group in microbial community was calculated.

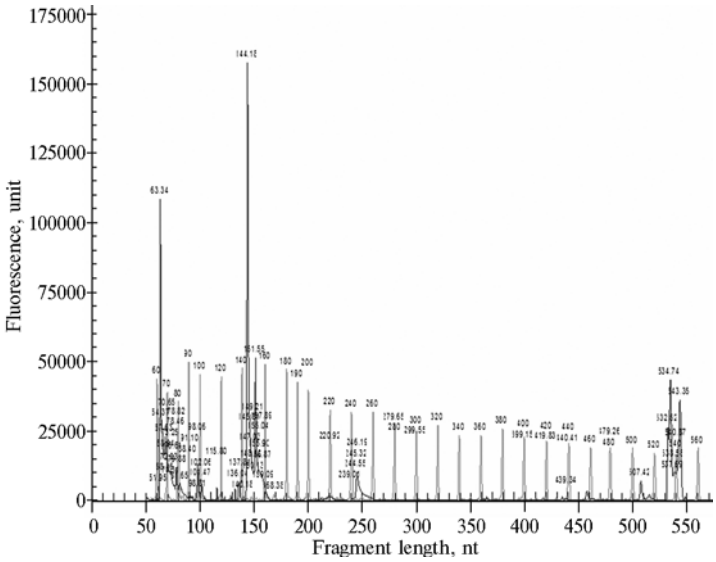

Fig. 2. A T-RFLP-PCR pattern for poultry gut bacterial DNA (gray peaks are the markers, and black peaks indicate DNA fragments in the sample).

In RT-PCR for a bacterial group quantitation the DNA extraction, fragmentation control and 16S-rRNA gene amplification were the same as described hereinabove (iCycler, Bio-Rad, USA), using DNA of a tested microorganism in a serial concentrations as four positive controls instead of a single one. A real-time calibration curves were developed to estimate the genome content in the probe. Results were processed mathematically using MS Excel.

Results. DNA gel electrophoresis indicated an admissible fragmentation (Fig. 1), and there were specific T-RFLP-PCR patterns obtained for DNA from gut bacteria (Fig. 2).

Stafac 110 possesses a bacteriostatic effect on most gram positive bacteria and some gram negative ones, being bactericidal at high concentrations. Its active substance, the virginiamycin, is not absorbed in the gastrointestinal tract and is not subjected to digestion, thus its concentration and antibiotic activity in the gut remain high for a long time. There is no virginiamycin accumulation in organs and tissues, thus it is excreted unchanged with feces (32). Probiotic Cellobacterin-T contains a microbial association of the animal rumen, being a multi active preparation (33). It contains cellulolytic bacteria and lactobacilli, being both enzymatic and prophylactic. Bacteria of this association produce xylanases, pectinase and β-glucanase, able to hydrolyze cellulose in feed, therefore the preparation is added to fodder with high cellulose content used at animal and poultry farming.

A total bacterial number were higher in case the antibiotic or probiotic preparation was used in the group II and group III compared to control (Table 1). More apparent differences were observed in 1-14 day-old chicks. Particularly in day-old chicks the total bacterial number in duodenum and blind gut was 100 and 10 times higher compared to control, respectively, that evidenced a more rapid microbial colonization important for gut development. At 7 and 14 days of age in the groups II and III the number of bacteria in the duodenum and the

blind guts was 10-fold as compared to that in control, moreover, in 14 day-old chicks in the blind guts it was up to 100 time higher compared to that in poultry fed antibiotics. It could be a result of a changed diet and increased content of animal protein. By day 21 the total bacterial number did not differ, and in 35-day-old chicks it was almost the same, except group II poultry demonstrated 10 times increased bacterial number in the duodenum.

1. Dynamics of total bacterial number (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
I (control)	Duodenum	9.6×10^{7}	2.6×10^{8}	2.1×10^{8}	1.5×10^{9}	2.2×10^{9}
	Blind gut	7.0×10^{9}	1.6×10^{10}	2.2×10^{10}	2.8×10^{10}	8.3×10^{10}
II (antibiotic Stafac 110)	Duodenum	1.9×10^{10}	6.4×10^{9}	1.3×10^{9}	1.5×10^{9}	7.1×10^{9}
	Blind gut	2.5×10^{10}	1.4×10^{11}	2.4×10^{9}	3.1×10^{10}	6.3×10^{10}
III (Probiotic Cellobacterin-T)	Duodenum	4.0×10^{10}	1.1×10^{8}	1.5×10^{9}	2.3×10^{9}	7.0×10^{10}
	Blind gut	1.4×10^{10}	1.2×10^{11}	1.3×10^{11}	2.6×10^{10}	8.3×10^{10}

Among normal gut microbe community the following cellulolytic bacteria are essential: *Bacteroidacea*, *Lachnospiracea*, *Ruminococcacea*, *Thermoanaerobacteriacea*, *Clostridiacea* (34) because of absence of the birds' own enzymes for degradation of celluloses and other non-starch polysaccharides.

The highest percentage of cellulolytic bacteria was calculated for blind gut of broilers fed the antibiotic and probiotic additives (Table 2).

2. Dynamics of total number of normal microflora (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
		Cellulolytic bacteria				
		Cellulolytics				
I (control)	Duodenum	1.9×10^{7}	1.6×10^{7}	1.1×10^{7}	2.4×10^{8}	5.8×10^{6}
	Blind gut	7.0×10^{5}	1.9×10^{9}	3.6×10^{9}	9.4×10^{9}	8.7×10^{9}
II (antibiotic Stafac 110)	Duodenum	1.1×10^{9}	6.8×10^{8}	$< 1.0\times10^{5}$	9.3×10^{7}	1.6×10^{8}
	Blind gut	5.2×10^{8}	3.1×10^{10}	6.2×10^{8}	8.0×10^{8}	1.6×10^{10}
III (Probiotic Cellobacterin-T)	Duodenum	1.7×10^{9}	1.4×10^{7}	3.6×10^{7}	3.3×10^{8}	8.3×10^{9}
	Blind gut	4.6×10^{8}	2.5×10^{10}	3.1×10^{10}	8.0×10^{9}	1.6×10^{10}
		Bacteroidaceae				
I (control)	Duodenum	7.3×10^{6}	1.6×10^{7}	7.0×10^{6}	8.0×10^{8}	9.8×10^{5}
	Blind gut	7.0×10^{5}	6.1×10^{8}	1.5×10^{8}	5.1×10^{8}	8.0×10^{6}
II (antibiotic Stafac 110)	Duodenum	4.2×10^{7}	1.4×10^{7}	$< 1.0\times10^{5}$	5.5×10^{7}	1.4×10^{7}
	Blind gut	8.8×10^{7}	1.4×10^{10}	4.0×10^{7}	5.0×10^{9}	3.6×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	1.1×10^{9}	1.2×10^{7}	1.9×10^{7}	2.3×10^{8}	3.6×10^{9}
	Blind gut	1.9×10^{8}	1.3×10^{10}	7.8×10^{9}	1.5×10^{9}	2.4×10^{9}
		Clostridiaceae				
I (control)	Duodenum	1.9×10^{6}	3.4×10^{6}	2.0×10^{4}	3.3×10^{7}	3.0×10^{6}
	Blind gut	4.5×10^{9}	4.3×10^{9}	6.7×10^{9}	2.8×10^{8}	1.9×10^{9}
II (antibiotic Stafac 110)	Duodenum	3.4×10^{7}	6.0×10^{5}	$< 1.0\times10^{5}$	$< 1.0\times10^{5}$	3.3×10^{7}
	Blind gut	7.5×10^{9}	5.8×10^{9}	2.4×10^{7}	2.0×10^{9}	4.0×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	1.9×10^{8}	4.2×10^{5}	1.4×10^{7}	2.1×10^{7}	5.0×10^{6}
	Blind gut	2.3×10^{9}	1.2×10^{10}	5.3×10^{9}	1.6×10^{8}	5.5×10^{6}
		Lactobacilli				
		Lactobacillaceae				
I (control)	Duodenum	5.8×10^{6}	1.7×10^{8}	1.6×10^{8}	9.1×10^{7}	1.3×10^{8}
	Blind gut	1.8×10^{7}	4.0×10^{9}	1.2×10^{9}	3.7×10^{9}	1.7×10^{9}
II (antibiotic Stafac 110)	Duodenum	1.5×10^{10}	2.0×10^{9}	1.2×10^{9}	8.4×10^{8}	1.4×10^{9}
	Blind gut	5.5×10^{8}	1.0×10^{10}	9.6×10^{7}	8.0×10^{8}	3.0×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	3.1×10^{10}	4.6×10^{7}	8.9×10^{8}	8.7×10^{8}	3.1×10^{9}
	Blind gut	3.7×10^{8}	2.7×10^{11}	4.4×10^{9}	4.0×10^{9}	1.7×10^{10}
		Bifidobacteriaceae				
I (control)	Duodenum	1.9×10^{6}	3.0×10^{6}	2.0×10^{4}	6.0×10^{7}	2.0×10^{4}
	Blind gut	7.0×10^{5}	2.0×10^{6}	2.4×10^{7}	9.0×10^{7}	8.0×10^{6}
II (antibiotic Stafac 110)	Duodenum	2.0×10^{6}	6.0×10^{5}	$< 1.0\times10^{5}$	1.5×10^{7}	4.3×10^{8}
	Blind gut	3.0×10^{6}	6.6×10^{8}	4.8×10^{6}	3.0×10^{6}	6.2×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	3.2×10^{8}	2.1×10^{6}	3.0×10^{7}	6.7×10^{7}	2.1×10^{9}
	Blind gut	1.0×10^{6}	1.8×10^{8}	1.0×10^{7}	1.4×10^{8}	6.8×10^{8}

Table 2 (continued)

		Helpful bacilli and selenomonas				
		Bacillaceae				
I (control)	Duodenum	1.6×10^{7}	1.3×10^{7}	7.5×10^{6}	5.0×10^{8}	2.0×10^{7}
	Blind gut	7.0×10^{5}	6.2×10^{8}	3.4×10^{8}	6.0×10^{8}	8.0×10^{6}
II (antibiotic Stafac 110)	Duodenum	1.9×10^{8}	4.6×10^{7}	1.0×10^{5}	1.2×10^{8}	3.3×10^{7}
	Blind gut	6.0×10^{8}	9.8×10^{9}	3.4×10^{7}	7.4×10^{8}	3.0×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	1.7×10^{9}	1.7×10^{7}	3.5×10^{7}	5.3×10^{8}	5.5×10^{8}
	Blind gut	6.5×10^{8}	8.4×10^{9}	1.0×10^{9}	1.2×10^{9}	1.1×10^{10}
		Veillonellaceae				
I (control)	Duodenum	1.5×10^{5}	2.0×10^{4}	2.0×10^{4}	1.0×10^{5}	2.0×10^{5}
	Blind gut	7.0×10^{5}	3.8×10^{8}	2.2×10^{9}	1.1×10^{9}	4.1×10^{8}
II (antibiotic Stafac 110)	Duodenum	9.0×10^{7}	5.1×10^{7}	1.0×10^{5}	1.0×10^{5}	1.9×10^{7}
	Blind gut	3.0×10^{6}	1.6×10^{10}	1.5×10^{8}	3.0×10^{9}	4.0×10^{8}
III (Probiotic Cellobacterin-T)	Duodenum	4.0×10^{6}	6.8×10^{5}	2.2×10^{6}	1.3×10^{7}	6.2×10^{8}
	Blind gut	1.0×10^{6}	5.5×10^{9}	3.5×10^{10}	4.7×10^{9}	6.8×10^{8}

In blind gut of control day-old chicks the celullolytic bacteria number was not more than 7×10^{5}, being in the group II and III almost 1000 times higher. In duodenum it was 100 times higher on average compared to control. In 7 day-old chicks in blind gut this parameter was about 10 times higher compared to control, and in duodenum of the chicks fed the antibiotic additive it was about 10 times higher if compared to other chicks. In 14 day-old chicks in both duodenum and blind gut cellulolytic bacteria were the most numerous when probiotic additive was used, and the smallest number was found in case the antibiotics was used. These indexes are very important as they reflect changes in the poultry diet. In 21 day-old chicks no significant differences in cellulolytics were found, except group II in which their number significantly decreased, probably due to their decreased number in 14 day-old poultry. At 35 days of age the cellulolytics were the most numerous in chicks fed the probiotic additive, and the smallest number was observed in the control chicks.

Special attention should be paid to *Bacteroidaceae*m which can also ferment starch feed components (35). During the experiment their number was the highest in poultry fed the probiotic additive and the smallest in control. Also clostridia are considered normal for bacterial community in poultry due to cellulose fermentation (36), but some pathogenic clostridia species can cause gastroenteritis and intoxication. The clostridia number varied significantly, nevertheless in blind gut of chicks fed probiotic preparation this index was generally lower.

Among lactobacilli *Lactobacillaceae* and *Bifidobacteriaceae* were identified (see Table 2).

Lactobacillaceae are helpful due to antimicrobial activity against pathogenic microorganisms (37). Their number was the highest in the gastrointestinal tract of broilers from group II and group III, and the smallest one in control poultry, being at that 10-100 times higher in the blind gut of the chicks from group III compared to group II.

Bifidobacteriaceae from poultry gut also possesses antimicrobial activity against pathogens (38). They were the most numerous in the group II and group III with the smallest number found in control. In the blind gut of broilers from the group III they were 10-100 times higher than in group II.

Also helpful *Bacillaceae* with antimicrobial activity and the *Veillonellaceae* able to degrade organic acids (39) were identified (see Table 2).

The largest bacilli populations were found in groups II and III compared to control, being at that 10-100 times higher in the blind gut of the chicks from group III compared to group II. The most *Veillonellaceae* were also observed in poultry fed the additives if compared to control chicks.

Enterobacteriaceae, including salmonellas, colibacteria, proteus, etc., are undesirable as often cause gastroenteritis (40). Their number was the lowest in

control and in broilers fed the probiotic additive, being however the highest in the blind gut (Table 3).

3. Dynamics of total numbers of enterobacteria and actimomycetes (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
		Enterobacteriacea				
I (control)	Duodenum	2.7×10^{6}	3.5×10^{6}	9.2×10^{5}	1.1×10^{8}	4.2×10^{6}
	Blind gut	7.0×10^{5}	4.8×10^{8}	6.3×10^{8}	7.0×10^{9}	3.3×10^{9}
II (antibiotic Stafac 110)	Duodenum	6.1×10^{8}	7.7×10^{8}	7.5×10^{6}	8.7×10^{7}	5.5×10^{8}
	Blind gut	4.4×10^{8}	3.5×10^{9}	7.2×10^{8}	1.5×10^{9}	2.1×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	4.0×10^{6}	5.6×10^{6}	1.4×10^{7}	3.8×10^{7}	2.0×10^{7}
	Blind gut	1.6×10^{9}	4.6×10^{9}	1.1×10^{9}	2.0×10^{9}	5.2×10^{7}
		Actinomyces				
I (control)	Duodenum	3.5×10^{5}	2.0×10^{4}	2.0×10^{4}	1.4×10^{7}	1.1×10^{6}
	Blind gut	1.5×10^{7}	5.7×10^{8}	3.8×10^{9}	1.6×10^{9}	3.0×10^{10}
II (antibiotic Stafac 110)	Duodenum	5.1×10^{7}	1.8×10^{7}	1.0×10^{5}	1.0×10^{5}	7.1×10^{7}
	Blind gut	4.3×10^{9}	1.4×10^{10}	5.7×10^{8}	1.6×10^{9}	5.7×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	8.0×10^{8}	2.4×10^{5}	5.1×10^{6}	3.2×10^{7}	2.8×10^{7}
	Blind gut	1.5×10^{9}	1.1×10^{10}	8.6×10^{9}	2.0×10^{9}	6.9×10^{9}

Actinomyces are also undesirable in poultry gut as they cause actinomycosis (41). They were the least in number in the gastrointestinal tract of control broilers from day 1 to day 14, and in blind gut of the chicks fed the additives from day 14 to day 35.

Pathogenic microflora was not found in broiler chicks as the number of staphylococci, fusobacteria, pasteurella and campylobacter was not noticeable and did not differ significantly among the chicks' groups.

Besides, in all chicks the transit microorganisms were detected which occur in the feed and are not important for fermentation. In the blind gut of 1 day-old chicks fed the probiotic additive their number was much lower compared to gut of other broilers. It probably indicates earlier gut colonization by helpful microflora in case the probiotic additive fed. From day 7 to day 21 the number of transit microorganisms increased possibly due to BR change (Table 4).

4. Dynamics of total numbers of transit microflora (genomes/g) **in the gastrointestinal tract** (GIT) **content of Cobb 500 broiler chicks fed antibiotic and probiotic feed additives estimated by T-RFLP-RT-PCR analysis**

Group	GIT	1 day	7 days	14 days	21 days	35 days
I (control)	Duodenum	2.6×10^{5}	4.6×10^{6}	2.0×10^{4}	1.0×10^{5}	2.0×10^{5}
	Blind gut	2.5×10^{9}	1.7×10^{9}	2.0×10^{6}	2.8×10^{8}	2.6×10^{8}
II (antibiotic Stafac 110)	Duodenum	2.0×10^{6}	9.0×10^{7}	1.0×10^{5}	9.4×10^{6}	2.6×10^{8}
	Blind gut	7.5×10^{9}	1.1×10^{10}	2.0×10^{5}	8.4×10^{8}	2.3×10^{9}
III (Probiotic Cellobacterin-T)	Duodenum	7.2×10^{7}	2.2×10^{6}	3.0×10^{7}	4.2×10^{7}	1.9×10^{7}
	Blind gut	1.9×10^{8}	1.2×10^{10}	2.2×10^{9}	1.1×10^{9}	2.2×10^{7}

Thus, T-RFLP—RT-PCR analysis of gut microbial community in growing chicks showed that in case the antibiotic or probiotic feed additives are used the total bacterial number increases, mainly due to helpful cellulolytic bacteria, lactobacilli, bifidobacteria, bacilli, *Veillonellaceae*, while the percentage of undesirable enterobacteria and actinomyces decreases. In case of the probiotic additive, if compared to the antibiotic, the gastrointestinal microbial community was enriched with cellulolytic bacteria, and in the blind gut there was 10-100 times more lactobacteria, bifidobacteria, bacilli, while clostridia and transit microorganisms decrease in number. A numerous research based on the production parameters confirmed the benefit of probiotic additives in poultry. However the molecular identification of gut microflora such as T-RFLP—RT-PCR analysis provides reliable data on changes caused in different gut microbial groups by different preparations to make a decision on their proper use.

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