

## Metagenomic Bacterial Community Profiles of Chicken Embryo Gastrointestinal Tract by Using T-RFLP Analysis

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**Abstract**—Thirty microbial phylotypes of microorganisms were found in the gastrointestinal tract of chicken belonging to the Hajseks White breed, and 38 phylotypes were found in the gastrointestinal tract of chicken belonging to the Hajseks Brown breed. The microbiome of the gastrointestinal tract of the chicken embryos of the Hajseks White breed was dominated by the typical representatives of avian intestinal microflora—bacteria of the family Enterobacteriaceae (47.3%), orders Actinomycetales (13.6%) and Bifidobacteriales (20.6%), and the family Lachnospiraceae (eleven%). The microbiome of the gastrointestinal tract of the chicken embryos of the Hajseks Brown breed was dominated by the pathogenic bacteria of the order Rickettsiales (94.8%). The metagenome of gastrointestinal tract of both breeds also contained a small number of genes of unidentified bacteria.

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It is conventionally believed that the gastrointestinal tract of avian embryos is sterile [1–3] and the microbiocenosis of the digestive system of chicks is formed after hatching as a result of contact with the environment [4–6]. However, the results of a number of studies, which were obtained by both the classical microbiological methods [7, 8] and real-time PCR [9] show that microorganisms can colonize the gastrointestinal tract of birds at a stage of embryonic development inside the egg.

The issue of studying the composition of microorganisms in the gastrointestinal tract of chicken embryos remains debatable, because the published papers devoted to this problem are scanty and focus primarily on studying the microorganisms that are pathogenic for humans.

Until the 1990s, the studies of microorganisms in different ecosystems were limited to studying cultured strains on artificial nutrient media. The understanding of the composition of microorganisms has significantly broadened due to the development of metagenomic methods of study of microorganisms, an important feature of which is that it is not necessary to culture microorganisms [10]. This point is fundamental in understanding the existing biodiversity, because

99% of microorganisms in the biosphere may play an important ecological role but cannot be cultured on artificial media [6]. In the available literature, we have found no information on the composition of the microbiome of the gastrointestinal tract of chicken embryos obtained by metagenomic analysis.

The aim of this study was to determine the composition and structure of microbial communities in the gastrointestinal tract of chicken embryos in the incubation period using terminal restriction fragment length polymorphism (T-RFLP).

This study was performed with two mixed-average samples of the GIR contents of three chicken embryos of the Hajseks White breed and three chicken embryos of the Hajseks Brown breed on day 16 of the incubation period. The eggs were incubated at the hatchery of the All-Russia Research and Technology Institute of Poultry.

Samples were obtained and prepared for analysis in the molecular genetics laboratory of the BIOTROPH+ Ltd. with a strict observance of sterility in accordance with the requirements [11].

The composition of the microbial community of the gastrointestinal tract of chicken embryos was studied by T-RFLP analysis. Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Fermentas, Lithuania) according to the manufacturer's recommendations. DNA amplification was performed in a Verity DNA thermocycler (Life Technologies, United States) using the bacterial primers 63F (CAGGCCTAACACATGCAAGTC) labeled at

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the 5' end (WellRed D4 fluorophore) and 1492R (TACGGHTACCTTGTTACGACTT).

The fluorescently labeled amplicons of the 16S rRNA gene were purified by the standard method [12]. Restriction of 16S rRNA amplicons (30–50 ng) was performed using restriction endonucleases *Hae*III, *Hha*I, and *Msp*I according to the manufacturer's recommendations (Fermentas). Restriction products were analyzed using a CEQ 8000 DNA sequencer (Beckman Coulter, United States) according to the manufacturer's recommendations. The CEQ 8000 instrument error did not exceed 5%.

The affiliation of bacteria to a particular phylogenetic group was determined using the Fragment Sorter software (<http://www.oardc.ohiostate.edu/trflpfragsort/index.php>).

The results of studies by T-RFLP-analysis showed that the microbiocenosis of the mixed-average samples of the gastrointestinal tract contents of the chicken embryo of both breeds was characterized by a fairly rich taxonomic diversity. For example, the microflora of the gastrointestinal tract of chicken embryos of the Hajseks White and Hajseks Brown breeds contained 30 and 38, respectively, phylotypes of various microorganisms (Table, Fig. 1).

The structure of metagenomic communities of chicken embryos of the two breeds differed in the taxonomic composition. The microbiocenosis of the gastrointestinal tract of the chicken embryos of the Hajseks White breed was dominated by the typical representatives of avian intestinal microflora—bacteria of the family Enterobacteriaceae (47.3%), the majority of which (46.9%) was represented by the bacteria belonging to the species *Escherichia coli*. The microbiocenosis of the gastrointestinal tract of the chicken embryos of the Hajseks White breed also contained other members of the autochthonous microorganisms of the gastrointestinal tract that were identified earlier in hatched chicks and adult birds [13]—representatives of the order Actinomycetales (13.6%) and Bifidobacteriales (20.6%) and the family Lachnospiraceae (11%).

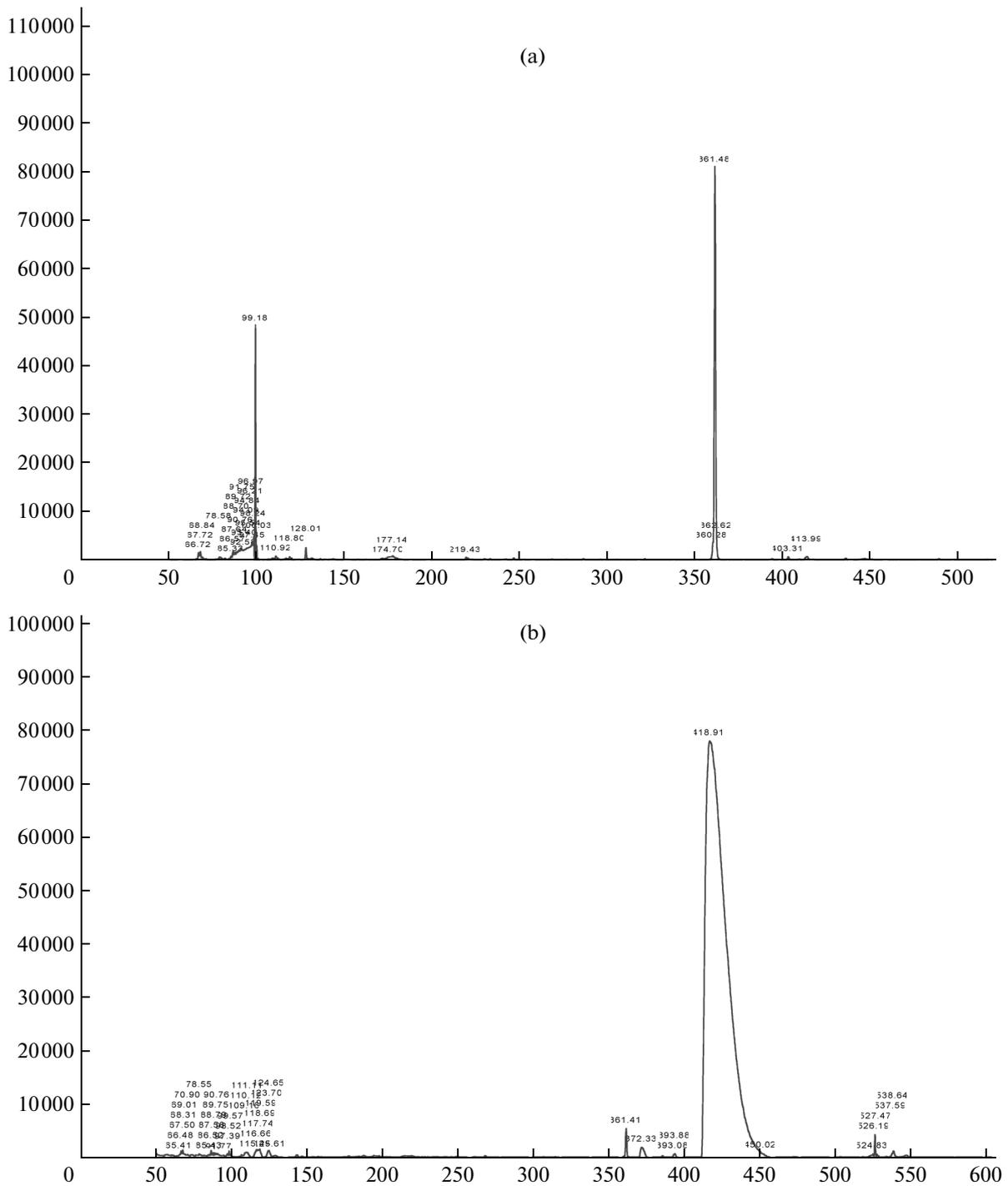
Amylolytic and proteolytic bacteria of the order Actinomycetales and cellulolytic microorganisms of the family Lachnospiraceae, which inhabit the gastrointestinal tract of hatched chicks and adult birds, play a significant role in metabolism, being actively involved in the fermentation of feed protein, starches, and polysaccharides. Representatives of the order Bifidobacteriales, producing organic acids and bacteriocins in the gastrointestinal tract of birds, ensure the microbiootope resistance against colonization by pathogenic microorganisms and synthesize nutrients [1].

Thus, the colonization of the gastrointestinal tract by the bacteria that ensure the adaptation of birds to the external environment takes place already at the stage of embryonic development. The metagenomic community of the gastrointestinal tract of the chicken

**Table 1.** Composition of the microbial community in the gastrointestinal tract of chicken embryos

Microbial taxon	Number of bacteria, %	
	Hajseks White breed	Hajseks Brown breed
Type Firmicutes	10.0	2.1
Order Lactobacillales	—	0.44
<i>Lactobacillus</i> sp.	—	0.16
<i>Enterococcus</i> sp.	—	0.04
<i>Tetragenococcus</i> sp.	—	0.25
Order Bacillales	8.9	1.7
<i>Alicyclobacillus</i> sp.	0.5	0.19
<i>Brevibacillus</i> sp.	—	0.14
<i>Bacillus</i> sp.	6.8	1.08
<i>Paenibacillus</i> sp.	1.2	0.28
<i>Staphylococcus</i> sp.	0.41	—
Family Lachnospiraceae	1.1	—
Type Actinobacteria	34.2	0.5
Order Actinomycetales	13.6	0.31
Order Bifidobacteriales	20.6	0.19
Type Proteobacteria	49.3	0.91
Family Enterobacteriaceae	47.3	0.15
<i>Klebsiella</i> sp.	—	0.08
<i>Salmonella</i> sp.	—	0.07
<i>Morganella</i> sp.	0.36	—
<i>Escherichia coli</i>	46.9	—
Order Burkholderiales	2.0	0.76
<i>Burkholderia</i> sp.	0.94	0.16
Order Burkholderiaceae	—	0.05
<i>Bordetella</i> sp.	1.1	—
<i>Pseudomonas</i> sp.	—	0.55
Type Bacteroidetes	—	0.49
<i>Bacteroidetes</i> sp.	—	0.08
Nonculturable representatives	—	0.41
Type Proteobacteria	0.3	94.8
Nonculturable representatives of the order Rickettsiales	—	94.8
<i>Caulobacter</i> sp.	—	0.04
<i>Brevundimonas</i> sp.	0.3	—
Unidentified bacteria	6.2	1.2
Total number of phylotypes	30	38

(—) – not determined.



**Fig. 1.** T-RFLP profiles of the bacterial community of the gastrointestinal tract of chicken embryos of (a) Hajseks White and (b) Hajseks Brown breeds. The abscissa axis shows the restriction fragment size (bp), and the ordinate axis shows the fluorescence intensity (arb. units).

embryos of the Hajseks White breed contained no typical representatives of the autochthonous avian symbiotic intestinal microflora—facultative anaerobic bacteria of the order Lactobacillales. In the gastrointestinal tract of the chicken embryos of the Hajseks Brown

breed, these microorganisms were found in an amount of 0.44%. As active acid producers, these microorganisms competitively displace the pathogenic microflora from the gastrointestinal tract of hatched chicks and adult birds [1]. Among the representatives of the order

Lactobacillales, the microorganisms of the genus *Lactobacillus* sp. accounted for 0.16%; genus *Tetragenococcus* sp., 0.25%; and genus *Enterococcus* sp., 0.04%.

In addition, the gastrointestinal tract of chicken embryos of both breeds contained a certain number of genotypes of unidentified bacteria. The metagenomic community of the gastrointestinal tract of chicken embryos of the Hajseks Brown breed was dominated by the bacteria of the order Rickettsiales (94.8%)—pathogens transmitted by representatives of the type Arthropoda.

In addition to the representatives of the order Rickettsiales, a number of other dangerous pathogens were found in the gastrointestinal tract of chick embryos—bacteria of the genera *Staphylococcus* sp., *Pseudomonas* sp., *Bordetella* sp., *Salmonella* sp., *Klebsiella* sp., etc.

It should be noted that bacteria *Staphylococcus* sp., *Pseudomonas* sp., and *E. coli*, found in the gastrointestinal tract of embryos during the incubation period, can cause omphalitis (umbilical-yolk infection)—a dangerous disease that is the major cause of death of chicks in the period from hatching to postnatal day 14 [14]. Bacteria of the genus *Bordetella* are pathogens of the respiratory tract of birds, mostly immature chicks [15]. Probably, a prerequisite to the occurrence of the described diseases is an unfavorable composition of the gastrointestinal tract microflora, which entails an increase in the number of the aforementioned pathogens at the stage of embryonic development of birds.

Our results agree with the data obtained by Babaca [8], who studied 3000 samples of the contents of the gastrointestinal tract of chicken embryos obtained from the hatcheries of three poultry plants using the conventional microbiological inoculation. Experiments were performed to determine the causes of the mass death of chicken embryos. It was found that the range of microorganisms that can cause the death of embryos is diverse: a large proportion was accounted for by bacteria *E. coli* (18.28%), *Staphylococcus* sp. (14.10%), *Pseudomonas* sp. (11.75%), and *Klebsiella* sp. (9.4%).

In addition, it was shown that the microflora of the hen plays a key role in the formation of the pathogenic microorganisms of the gastrointestinal tract of the embryo [9]. Using real-time PCR, it was shown that the content of these microorganisms in the gastrointestinal tract of embryos produced by the hens that were artificially infected with *Campylobacter coli* was 4.35–5.65 thousand cells per gram body weight.

Thus, the new microbiocological system of birds (the contents of the gastrointestinal tract together with the inhabiting microflora) is formed already at the stage of embryonic development. Apparently, the structure of the microbiotope of the gastrointestinal tract of the embryo is formed under the influence of the hen microflora due to vertical transfer via bacterial translocation. Another probable source of microflora colonizing the gastrointestinal tract of chicken

embryos may be the pores in the egg shell. The microorganisms present in the gastrointestinal tract of the embryo are the foundation that determines the formation of the starting intestinal biocenosis of hatched chicks.

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