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# Diversity of the taxonomic structure present in the litter of broiler chickens under industrial conditions

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**A B S T R A C T**

The purpose of the current work was to determine the microbial composition of litter generated during the industrial rearing of broiler chickens. This is necessary in order to achieve an understanding of the influence of various microorganisms on the processing of waste products for absorption into the soil without harming and polluting the environment, for example, with bound phytate ions. The object of the study was litter sampled from under broiler chickens of different ages and parent flocks. During the study, the following groups of microorganisms were identified: peptococci (family *Peptococcaceae*); actinomycetes (except for the family *Pasteurellaceae*) from the order *Actinomycetales*; actinobacillus (family *Pasteurellaceae*); lactobacilli and enterococci (genera *Lactobacillus* and *Enterococcus*, respectively); bacteroides (phylum *Bacteroidetes*); ruminococci (genus *Ruminococcus*); eubacteria (family *Eubacteriaceae*) and enterobacteria (family *Enterobacteriaceae*). Quantitative indicators of colonies of various taxonomic groups were determined, and it was shown that these indicators changed with increasing period of litter use. Furthermore, the study revealed that the microbial composition of the litter changed over time. As the litter age increased, there was an increase in the abundance of certain bacteria, such as *Peptococci* and *Actinobacillus*, which are known to be involved in the decomposition of organic material.

**Keywords:** microbial composition, litter generated, broiler chickens, microorganisms, litter microbiome

**И З В О Д**

Сврха овог рада је била да се утврди микробни састав простирке настале током индустријског узгоја бројлерских пилића. Ово је неопходно да би се постигло разумевање утицаја различитих микроорганизама на прераду отпадних производа за апсорпцију у земљиште без штетног утицаја на животну средину и њеног загађивања, на пример, везаним фитат јонима. Предмет проучавања били су узорци простирке пилића бројлера различите старости и родитељских јата. Током истраживања идентификоване су следеће групе микроорганизама: пептококе (фамилија *Peptococcaceae*); актиномицете из реда *Actinomycetales* (осим породице *Pasteurellaceae*); фамилија *Pasteurellaceae*; лактобацили и ентерококе (род *Lactobacillus* и *Enterococcus*); бактероиди (филум *Bacteroidetes*); род *Ruminococcus*; еубактерије (фамилија *Eubacteriaceae*) и ентеробактерије (фамилија *Enterobacteriaceae*). Утврђени су квантитативни показатељи колонија различитих таксономских група и показано је да се ови показатељи мењају са повећањем периода коришћења простирке. Штавише, студија је открила да се микробни састав простирке временом мењао. Како се старост простирке повећавала, дошло је до повећања обиља одређених бактерија, као што су *Peptococci* и *Actinobacillus*, за које се зна да учествују у разградњи органског материјала.

**Кључне речи**: микробни састав, створена простирка, бројлерски пилићи, микроорганизми, микробиом простирке

## 1. Introduction

At present, the need of the country's population for high-grade animal protein is satisfied to a large extent by poultry products (Buyarov et al., 2017). Intensive production associated with the cultivation of broiler chickens faces many problems: the high cost of feed, its quality, the effect of feed composition on the growth and development of birds, various diseases of birds, primarily infectious diseases, which damage production (Gotovskii and Shindila, 2020). The microbial factor is of particular importance in conditions of intensive production and increasing stocking density when growing broiler chickens. In the current conditions also relevant is the study of the microbial composition of the air of industrial premises and litter for broiler chickens, since the content of bioaerosols containing pathogenic microorganisms in the air of poultry houses can be a risk factor for environmental pollution, which threatens the health of animals and humans (Fisinin et al., 2018). The microbial composition of the litter, which serves as a source of air pollution, has been studied to a lesser extent than air pollution. The bacterial biodiversity associated with air and the respiratory tract is also understudied (Ivulic et al, 2022). The microbial composition of the respiratory tract, intestines, indoor air and litter is a complex issue that requires constant monitoring, since these factors can cause a decrease in the productivity of broiler chickens in industrial production. Growing conditions (cage or floor) and modifications of feed additives can affect the microflora and change the immune status of birds. Thus, it has been shown that floor growing strengthens the immunity of broiler chickens in comparison with cell rearing (Joerger et al., 2020). The influence of feed modification (adding xinalase and yeast fermentate), aimed at improving productive qualities, on the intestinal microbiota (Elshaimaa et al., 2022) and the addition of acid additives to the litter of broiler chickens increase the content of *E. coli* from the intestines of chickens (Johnson et al., 2021). The addition of probiotics to feed has a beneficial effect on the microflora and modulates the immune system (Cox and Dalloul, 2015). The contamination of the litter with microorganisms is a source of microbial air pollution and, as a result, a factor that determines the incidence of diseases in broiler chickens and affects the yield of products. It is also of interest to study the dynamics of changes in microbial diversity in the process of growing broiler chickens, since this also affects the incidence of diseases and safety of chickens (Fisinin et al., 2018; Gotovskii and Shindila, 2020). On the other hand, the determination of the taxonomic affiliation of microorganisms living in the litter will make it possible to identify groups that are most actively able to process the litter, which will facilitate its disposal.

The purpose of this work was to study the taxonomic diversity of the litter microbiome of broiler chickens under industrial production conditions. The main hypothesis is that certain groups of microorganisms, such as *Peptococcal* and *Actinomycetes*, are able to persist in litter throughout its decomposition period, since various microorganisms of the microbiome, as an element of a complex system, can have a significant impact on the ecological state of this system (Mihailovič S. A. et al., 2019). Studies have demonstrated the composition of the microbiome and the influence of various factors on it, but in this region there have been no studies of changes in its composition over time, and since litter decomposition occurs in a closed system where no new nutrients enter, the balance between microorganisms may play a key role in this process. With the discovery of groups that stably persist in litter as decomposition progresses, it will be possible to begin the development of biotechnological additives and methods for interacting with the microbiome to accelerate litter recycling.

## 2. Materials and methods

### *2.1. Materials*

The studies were carried out in 2021–2022, at the Department of Ecology and Genetics of the Tyumen State University and at “PRODO Tyumensky Broiler JSC”.

The object of the study was litter sampled from under broiler chickens of different ages and parent flocks provided by the enterprise (Table 1).

The diet for broiler chickens consisted of fodder wheat, fodder corn, soybean and sunflower meal, monocalcium phosphate, limestone flour, vitamin and mineral premixes and additives, amino acids and enzymes. At the beginning of growing, broiler chickens received compound feeds, containing crude protein 22.5%, and fiber 3.6%; at the final stage, crude protein was no more than 20% and fiber up to 4.5%. The diet of the parent stock included: wheat, oats, fodder corn, soybean and sunflower meal, wheat bran, sunflower oil, vitamin and mineral premixes and additives, ground limestone, shells, monocalcium phosphate, amino acids, enzymes, adsorbents. In feed for laying hens, crude protein was no more than 14%.

**Table 1.**
Average values and heterosis for seed yield (kg $ha^{-1}$)

| Samples | Duration of keeping chickens on the litter (“age” of the litter) |
|---|---|
| Control | Manure |
| Sample 1 | 1-day litter |
| Sample 2 | 15-day litter |
| Sample 3 | 37-day litter |
| Sample 4 | 114-day litter |
| Sample 5 | 413-day litter |

### *2.1. Methods*

The microbial composition was determined according to standard methods (Avdović et al., 2023). Before inoculation, each of the samples was diluted with distilled water: in 100 (100x) for further inoculation (Akebe et al., 2015). Sowing was carried out on standard PPS nutrient media. Media composition: 2% glucose, 1% peptone, 0.5% yeast extract, 2% agar. Sowing was carried out by the lawn method in Petri dishes. Cultivation was carried out in a "Binder" thermostat at a temperature of 37°C. Counts were made after 24 hours, then after 7 days of cultivation. Colony counts were performed using a Scan 100 Interscience colony counter. To study the dynamics of changes in the abundance of bacteria colonies capable of decomposing cellulose (actinomycetes and bacteroids) under aerobic conditions and playing an important role in litter processing, Winogradsky's medium was used (Gusev and Mineeva, 1992; Shlegel, 1987; Asonov, 1989).

To determine the taxonomic affiliation of microorganisms found in the litter, standard methods based on specific staining were used: Gram staining, Ziehl-Neelsen staining, staining according to Romanovsky-Giemsa.

Determination of microorganisms was carried out at a microscope magnification of 1000 times (approx. 10 x vol. 100). Morphological features (appearance, shape, structural features) were determined based on specific staining.

To confirm the taxonomic affiliation, species-specific primers for 16S rDNA were selected and used: Fusobacteria (forward- AACGCTGACAGAATGCTTAA, reverse- AACTCTCGTGGTGTGACGGG), Euryarchaeota (forward- GGCCCTACGGGGCGCAGCA, reverse- TTTGACGGGCGGTGTGTGC), Tenericutes (forward- GGGTGAGTAACA, reverse- CACGAGCTGACGACAACCATGCACCA), Firmicutes (forward- TACGTGCCAGCAGCCGCGGTAA, reverse- GGGACTTAACCCAACATCTCAC), Proteobacteria (forward- CGTGCCAGCAGCCGCGGTAA, reverse- AACCCAACATCTCACGACAC), Bacteroidota (forward- GACTCCTACGGGAGGCAGCA, reverse- CCAGGGTATCTAATCCTGTT), Actinobacteria (forward- ACGGCCCAGACTCCTACGGG, reverse- GGGTTGCGCTCGTTGCGGGA).

Statistical data processing was carried out according to standard methods using the Statistica software package (StatSoft, USA). Samples were compared using the t-test (Student's t-test).

## 3. Results and discussions

Studies of the microbial composition of the litter from broiler chickens and laying hens made it possible to identify changes in the number of colonies of microorganisms cultured on a standard medium (Table 2). In control and experiment 1 on the 7th day, an increase in the number of colonies was found in comparison with a period of 24 hours of cultivation. There was no significant increase in the number of colonies in the litter in experiments 2, 3 and 4 on the 7th day. A significant increase in the number of colonies of microorganisms was noted in the litter from laying hens (experiment 5) by 7 days. The duration of litter operation led to a sharp increase in the number of microorganisms by 15 and 37 days (experiments 2 and 3, respectively) in comparison with a one-day litter (experiment 1). In 114-day-old litter from the parent flock of broiler chickens, the number of colonies decreased by 30% after 24 hours of cultivation, and by 28% after 7 days of cultivation.

The lower number of microorganisms in the litter from laying hens (experiment 5) in comparison with the control and the other experimental options is obviously associated with the absence of those groups of microorganisms that cause rapid growth, reproduction and colony formation. Of the eight taxonomic groups presented in experiment 4 and experiment 5, only seven were found, and enterobacteria were absent (Table 3).

**Table 2.**
Total number of colonies in the studied litter samples

| Samples | Average number of colonies ($x \pm m_x$) | |
|---|---|---|
| | in 24 hours | in 7 days |
| Control | 61.5 ± 2.89 | 77.0 ± 3.01¥ |
| Sample 1 | 7.0 ± 0.28* | 59.5 ± 2.5*¥ |
| Sample 2 | 100.0 ± 3.3*• | 100.0 ± 1.73*• |
| Sample 3 | 100.0 ± 2.1*•⊗ | 100.0 ± 1.58*•⊗ |
| Sample 4 | 70.0 ± 2.9*•⊗◊ | 72.0 ± 2.92*•⊗◊ |
| Sample 5 | 30.5 ± 1.13*•⊗◊ | 49.0 ± 2.12*•⊗◊¥ |

Note: the number of repetitions in each variant of the experiment is 5; dilution 100 times.
* – the differences between control and experiments are statistically significant ($P < 0.001$);
• – the differences between experiment 1 and experiments 2, 3, 4, 5 are statistically significant ($P < 0.001$);
⊗ – the differences between experiment 2 and experiments 3, 4, 5 are statistically significant ($P < 0.001$);
◊ – the differences between experiment 3 and experiments 4, 5 are statistically significant ($P < 0.001$).
¥ – the differences between 24 hours and 7 days are statistically significant ($P < 0.01$)

When determining the taxonomic diversity, the following microorganisms that represent the normal microflora of the intestines of birds were identified: bacteriodes, involved in the fermentation of carbohydrates, utilization of proteins and biotransformation of bile acids; ruminococci, which are part of the normal microflora; eubacteria, as obligate representatives of the gastrointestinal tract; lactobacilli and enterobacteria, fermenting sugars with the formation of lactic acid and other end products; conditionally pathogenic microorganisms, which live in the gastrointestinal tract of birds, on the skin, in the respiratory tract (peptococcal), and microorganisms that process the litter material: actinomycetes, which decompose complex substrates (Table 3).

**Table 3.**
Taxonomic diversity of microorganisms in litter of different ages

| Groups of microorganisms | Control | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
|---|---|---|---|---|---|---|
| 1. Peptococcal sp. *Peptococcaceae* | + | + | + | + | + | + |
| 2. Actinomycetes (excluding *sp. Pasteurellaceae*) order *Actinomycetales* | + | + | + | + | + | + |
| 3. Actinobacillus *sp. Pasteurellaceae* | + | + | + | + | + | + |
| 4. Lactobacilli and enterococci *Lactobacillus* spp. *Enterococcus* spp. | + | + | + | + | + | + |
| 5. Bacteroides phylum *Bacteroidetes* | + | + | + | + | + | + |
| 6. Ruminococcus *sp. Ruminococcus* | + | + | + | + | + | + |
| 7. Eubacteria *sp. Eubacteriaceae* | + | + | + | + | + | + |
| 8. Enterobacteriaceae *sp. Enterobacteriaceae* | – | – | + | + | – | – |

Note: + presence of this group

The most complete taxonomic groups of microorganisms were represented in 15 and 37-day bedding (experiments 2, 3). In 114-day-old litter (experiment 4) and in litter samples from laying hens (experiment 5), seven groups out of eight found in other variants were found when inoculated on a standard medium (Table 3). Enterobacteriaceae were absent. In samples of litter (control) and 1 daily litter (experiment 1), enterobacteria were also not detected when seeded on a standard medium.

The determination of the specific proportion of different taxonomic groups (by the number of colonies in % of the total number of colonies) showed that the predominant group in the litter from broiler chickens were eubacteria (from 24.6% in experiment 2 to 28.0% in experiment 3 and 21.2% in experiment 4) (Figure 1). A similar picture is also typical for the second largest taxonomic group of ruminococci: there was a slight increase in the specific content in experiment 2 and a decrease in experiment 4. The proportion of bacteroids in litter samples from broiler chickens (experiments 2, 3) remained stable and sharply decreased in experiment 4. The shares of the other taxonomic groups, with the exception of peptococcal bacteria, fluctuated insignificantly in the litter of different ages (experiments 2, 3, 4). The number of colonies of peptococcal bacteria increased with the age of the litter (from 15.1% in experiment 2 and 12.2% in experiment 3 to 24.1% in experiment 4) (Fig. 1). The ratio of different groups of microorganisms in experiment 5 (litter from laying hens) differed markedly from the other options: lactobacilli and enterococci (30.0%) and peptococcal bacteria (23.3%) accounted for a larger proportion. In litter samples (control) and one-day litter (experiment 1), eubacteria were predominant.

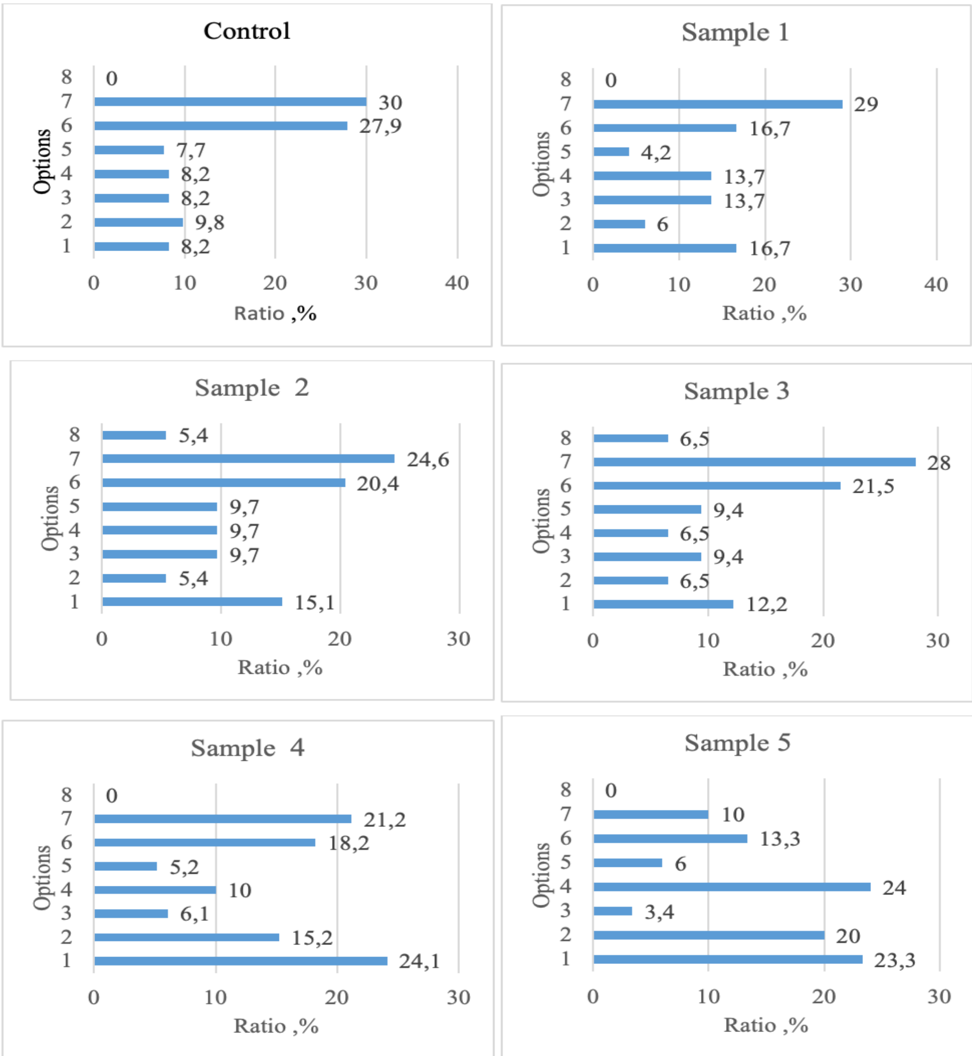


**Figure 1**. The ratio of different groups of microorganisms in the litter of different ages: 1) peptococcal; 2) actinomycetes (excluding sp. *Pasteurellaceae*); 3) actinobacilli; 4) lactobacilli and enterococci; 5) bacteroids; 6) ruminococci; 7) eubacteria; 8) enterobacteria

The use of Winogradsky's medium (a nutrient medium for detecting the ability of bacteria to decompose fiber under aerobic conditions) for the cultivation of microorganisms living in the litter created favorable conditions for the reproduction of actinomycetes and bacteroids (Table 4). The dynamics of changes in the number of colonies with an increase in the life of the litter indicateda more than 3-fold significant increase in comparison with the control and experiments 1, 2, 3. The result is comparable to the results of the studies by Fisinin at al. (2019) of the microflora of the intestinal tract since chicken droppings are the main source of the microbiome in the litter after its sterilization. The largest number of colonies of actinomycetes (22.1%) was noted in the litter from the parent stock. This may be due to the duration of the use of the litter (in experiment 4 – 114 days; in experiment 5 – 413 days), its composition and the activity of actinomycetes, which actively multiply under conditions of complete supply of the substrate. It is known that actinomycetes decompose complex substrates and participate in the decomposition of humic substances, the concentration of which increases towards the late life of the litter. The increase in the number of bacteroids, which are representatives of the normal intestinal microflora of birds, was not so significant in experiments 3, 4 and 5 in comparison with actinomycetes (Table 4).

**Table 4.**
Change in the number of colonies of actinomycetes and bacteroides in litter samples from broiler chickens and laying hens

| Samples | Average (over replicates) number of colonies (%) | |
|---|---|---|
| | Actinomycetes (excluding *sp. Pasteurellaceae*) *(*ordo *Actinomycetales)* | Bacteroides phylum *Bacteroidetes* |
| Control | 5.0 ± 0.6 | 5.9 ± 0.4 |
| Sample 1 | 2.6 ± 0.4 | 3.0 ± 0.3 |
| Sample 2 | 3.0 ± 0.6 | 7.6 ± 0.6 |
| Sample 3 | 4.0 ± 0.4 | 11.6 ± 0.5*• |
| Sample 4 | 15.2 ± 0.4*•⊗◊ | 9.9 ± 0.5*• |
| Sample 5 | 22.1 ± 1.2*•⊗◊ | 10.2 ± 0.7*• |
| Total colonies, pcs. | 157 | 146 |

Note: the number of repetitions in each variant of the experiment is 5;
* – the differences between control and experiments are statistically significant ($P < 0.001$);
• – the differences between experiment 1 and experiments 2,3,4,5 are statistically significant ($P < 0.001$);
⊗ – the differences between experiment 2 and experiments: 3,4,5 are statistically significant ($P < 0.001$);
◊ – the differences between experiment 3 and experiments: 4, 5 are statistically significant ($P < 0.001$).

## 4. Conclusions

A study of the taxonomic diversity of microorganisms present in the litter from broiler chickens and laying hens showed that, in general, the gastrointestinal tract of birds was inhabited by bacterioids, ruminococci, eubacteria, lactobacilli and enterobacteria, and peptococcal bacteria. They are representatives of the normal microflora of birds. With an increase in the life of the litter from 1 day to 413 days, the number of actinomycetes decomposing complex substrates increased. The maximum number of colonies of microorganisms, noted in experiments 2 and 3, was obviously associated with the emergence and reproduction of a group of enterobacteria, which was absent in the other experimental variants (Table 3). The pattern of changes in the ratio of different groups of microorganisms in the litter was not regular (Fig. 1). An increase in the number of actinomycetes responsible for the processing of complex organic substrates and peptococcal, conditionally pathogenic microorganisms living in the gastrointestinal tract of birds, on the skin, and in the respiratory tract was noted with the life of the litter. Also, a sharp decline in groups such as eubacteria and enterobacteria was noticed, and was caused by unfavorable conditions for them in an environment such as litter and the fact that, over time, substances on which they were able to feed were pumped in.

Thus, the study showed that the microflora of the litter from broiler chickens under industrial production conditions mainly included the representatives of the normal microflora of the gastrointestinal tract of birds and actinomycetes. The most active part in the processing and decomposition of complex substrates of the litter was played by actinomycetes, the number of which increased with the increase in the period of use of the litter.

Actinomycetes have good potential for biotechnological manipulations to control the process of litter decomposition, since they are only conditionally pathogenic and, in the process of life activity, they create threads that penetrate the thickness of the litter, giving access to the largest part of the material.

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