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Investigation of the effect of allithiamine-enriched feed on the poultry gut microbiome composition and resistome

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SUMMARY

Over the past 20–25 years, the poultry industry has evolved into a specific protein production system. However, the stress resulting from intensive rearing practices has led to numerous negative consequences, making the optimisation of livestock gut microbiome composition crucial for mitigating these effects. Advancements in modern molecular biology methods have brought attention to the impacts of nutrients on gut microbiota. In our study, we extensively investigated the changes induced by feed formulations rich in phytonutrients on the gastrointestinal microbiota of livestock using targeted 16S rRNA amplicon sequencing. Our objective is to examine how the developed feed prototype affects the composition of core microbiomes in raised poultry, community diversity, and the resilience of complex microbial networks. We seek correlations between biological livestock and environmental samples to identify which community constituents, in what proportions and occurrences, may play a role in the development of specific diseases. Based on our measurement results, it can be asserted that allithiamine positively modulated "beneficial" community constituents. Beyond the impact of allithiamine-enriched feed rich in phytonutrients, the composition of the microbial community in the poultry gastrointestinal tract is significantly influenced by the age of the birds. Furthermore, due to the presence of multi-drug-resistant pathogens in environmental samples from livestock facilities, appropriate transmission risk management measures are of paramount importance.

Keywords: *phytonutrient; 16S rRNA amplicon; microbiome; gastrointestinal tract*

INTRODUCTION

The poultry sector has been one of the most efficient protein-producing sectors over the last two decades, forming the basis of global protein production (Clavijo and Flórez, 2018). To do this, genetic and technological improvements have been made, resulting in a significant increase in nutrient utilization; therefore, growth has intensified and the breeding season has become shorter (Lan et al., 2005). This intensive growth rate can be associated with many pathological conditions, such as increased susceptibility to infections (Wideman et al., 2007). The gastrointestinal tract (GIT) microbiome plays an important role in the general health and functioning of livestock (Kers et al., 2018). The GIT microbiome plays a crucial role in reducing the negative effects of stress caused by intensive rearing, as it has a positive effect on the immune system and physiology of the gastrointestinal tract (Sommer and Bäckhed, 2013), and plays a role in detoxifying and producing certain compounds that determine productivity (Stanley et al., 2012). These positive effects are particularly important in the poultry industry because of the short breeding period in which the goal is to achieve efficient weight gain (Li et al., 2019). Increasing evidence suggests that changes in GIT microbiome composition may play a role in the development of metabolic disorders (Stanley et al., 2014). Diversity of the microbiome is one of the most important factors in determining resistance to pathogens. A higher microbiome diversity implies a healthier state of livestock, while a significant decrease in the complexity of the microbial community increases the susceptibility to colonization by various pathogens (Stanley et al., 2012). Therefore, selective manipulation of the composition and biological potential of the GIT helps to restore the equilibrium microbiome, which has a positive effect on animal health and immune status (Dibner and Richards, 2005). Thus, a balanced gut microbiome plays an important role in digestion and bile acid synthesis, and provides the host body with important vitamins and trace elements. Furthermore, through fermentation of indigestible carbohydrates and proteins, they produce energy and promote the formation and absorption of short-chain fatty acids (Yegani and Korver, 2008).

Plant-active ingredients are receiving increasing attention, particularly in developing countries, owing to their antibacterial and performance-enhancing effects (Sarangi et al., 2016). Complex plant extracts rich in bioactive compounds enhance the secretion of digestive enzymes and the absorption of nutrients, thereby increasing feed utilization in poultry stocks for intensive meat production (Stanley et al., 2014).

Owing to modern molecular biology methods, the effects of nutrients on GIT microbiota are receiving increasing attention, and certain phytonutrient-enriched feeds modulate these microbial community constituents to a significant extent. Based on our previous research we applied 16 S rRNA amplicon gene sequencing to assess the effects of phytonutrient-enriched feed on the bacterial composition of the GIT microbiome of poultry (Tolnai et al., 2021). These supplements are rich in plant-derived immunostimulants, such as phytochemicals, vitamins, and minerals (Siddiqui and Moghadasian, 2020). In this study, the modulating effect of sulfur-containing allithiamine extracted from garlic by extraction

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technique on the gastrointestinal tract microbiome composition of intensively reared poultry was investigated.

MATERIALS AND METHODS

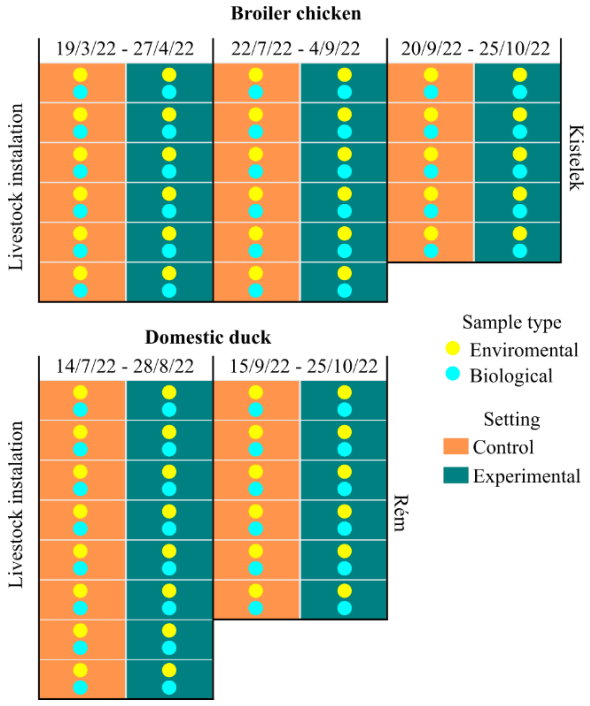
Feed additive

The garlic extract (allithiamine) used in this study was produced by researchers at the Institute of Food Technology of the University of Debrecen. The experimental group received a basal diet containing 0.5% allithiamine. Since the production of this active substance is not the subject of my research, I do not explain the detailed protocol of its production and use as a feed additive in my research.

Animals and sampling

The samples were collected from the Kistelek and Rém sites of Hungerit Zrt. Intensively reared poultry species (broiler chicken, domestic duck) were also examined. Three rearing periods were examined for broiler chicken and two for domestic duck stocks (*Figure 1*). Biological and environmental footbag samples were collected daily, transported on ice and stored at -80 °C upon return. Unification of the environmental and biological samples was performed weekly. The pooled samples consisted of seven individual samples. In the case of broiler chicken stocks, six pooled samples were formed in the first and second rearing periods, and five in the third rearing period. In the case of domestic duck stocks, eight pooled samples were formed in the first rearing period and six in the second rearing period.

Figure 1. **Livestock instalation and experiment settings in different poultry sites**



Sample homogenization

Bacteria present in feces and the environment, representing the microbial community of the gut flora and site, were extracted and explored from pooled (footbag) samples. Previously, cell suspensions were prepared based on an optimized methodology (Fidler et al., 2020), then microbes were separated from the sources of contamination by centrifugation with sterile phosphate-buffered saline. The supernatant was removed and the bacterial sediment was suspended in 3 ml of sterile phosphate-buffered saline in each case. For cell exploration, 1000 µL of cell suspension was used.

Cell exploration

In our experiments, a combination of mechanical and chemical lysis was performed to increase the efficiency of cell exploration. During mechanical lysis, a ceramic beading process (Power Fecal Bead) was used. Furthermore, MagNa Lyser (30 s 6000 RPM) was used to explore the cells. Inhibitex lysis buffer was used for chemical lysis. Power Fecal lysis bead was added to the 1000 µL suspension and centrifuged at 15000 g for 5 min. During chemical lysis, the supernatant was removed and 1000 µL of Inhibitex lysis buffer was added to the bacterial sediment. Then, the samples were vortexed and a MagNa Lyser was used. The samples were then incubated for 7 min at 800 RPM at 95 °C, centrifuged for 1 min at high speed, and the supernatant was used for isolation.

DNA extraction

We used a standardized kit-based DNA extraction technology. For DNA isolation from the bacterial cell suspension, a commercially available DNA cleaning kit, the QIAamp Fast DNA Stool Mini Kit (cat.n: 51604) was used according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany).

Quantitative and qualitative control of DNA isolates

Depending on the DNA extraction and purification and the characteristics of the sample, significant amounts of contaminants may remain that inhibit PCR function. The purity and quantity of DNA were determined photometrically using a Nanodrop ND-1000 spectrophotometer. Before PCR reactions, the amount of DNA was measured with a Qubit 2.0 fluorimeter using Qubit™ dsDNA HS Quantitation Assay Kit (cat.n: Q32851) according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). To determine the integrity of the DNA, automated microcapillary gel electrophoresis, 4200 TapeStation System (G2991BA), Genomic DNA ScreenTape (cat.n: 5067-5365) and Genomic DNA Reagents (cat.n:5067-5366) were used according to the manufacturer's instructions (Agilent Technologies, Santa Clara, USA).

16S rRNA gene-based metagenomics library creation

We created a 16S rRNA gene-based metagenomics library based on the Illumina library creation protocol (15044223 Rev. B.). The V3 and V4 variable regions

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of the 16S ribosomal RNA gene were specifically amplified. Universal primers were used for PCR reaction, followed by paired-end sequencing (Illumina MySec).

Bioinformatics analysis

We used Illumina BaseSpace software to demultiplex paired-end readings and create FASTQ files. Sequencing data were analyzed using the Quantitative Insight Into Microbial Ecology (QIIME2) program package. Multiple sequence matching was performed using the Mafft software, and the sequences were fitted to Silva reference databases. The Naıv Bayesian algorithm, trained on the Silva reference database, was used to classify the taxonomy. The phylogenetic tree was created using FastTree program. Alpha and beta diversity values were calculated using QIIME 2 software. For alpha diversity, we worked with Shannon and Chao-1 phylogenetic diversity data. To analyze the beta diversity, an unweighted Unifrac distance was calculated. Beta diversity matrices (PCoA) were created using the Emperor program. For alpha diversity, statistical evaluation was performed using the Kruskal-Wallis test, as the data did not show a normal distribution. To visualize the figures, we converted the biome files obtained during Qiime data analysis into TSV files and worked with them further. R and Phython programs were used to create the figures. Box plots were created using the R ggplot2 package, and the taxonomic heat-tree was created with the Metacoder R package. For the taxonomic heat tree, the differences were determined using the Wilcoxon rank-sum test. LEfSe analysis was performed using bioBakery programs developed by the Huttenhower Laboratory (Segata et al., 2011).

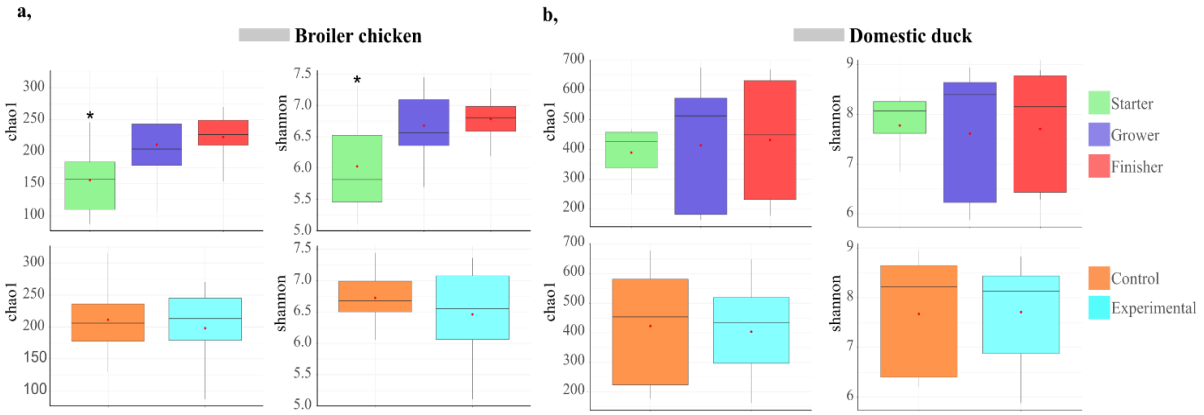
RESULTS AND DISCUSSION

Diversity is proportional to community viability. Diversity is a good measure of the value of a balanced, healthy microbiome. The more diverse a microbiome community, the more resilient it is. We examined two aspects of biodiversity: biodiversity and species balance.

The Chao1 index examines diversity, and the Shannon index considers balance (*Figure 2*). The alpha diversity of the broiler chicken flocks (*Figure 2/a*) showed that the alpha diversity rate increased significantly in each detection phase compared to the initial phase (Starter Chao1 = 155.5±59.9, grower Chao1 = 210.9±55.7 (p = 0.025), finisher Chao1 = 222.9±36.6 (p = 0.029). Starter Shannon = 6±0.8, grower Shannon = 6.7±0.5 (p = 0.013), finisher Shannon = 6.8±0.3 (p = 0.022)). In the case of domestic duck flocks (*Figure 2/b*), the degree of alpha diversity did not result in significant differences in either detection compared to the initial phase (starter). (Starter Chao1 = 389.3±91.9, grower Chao1 = 414.1±211.6, finisher Chao1 = 431.8±206.4. Starter Shannon = 7.7±0.7, grower Shannon = 7.6±1.3, finisher Shannon = 7.7±1.2).

Furthermore, the phytonutrient feed additive had no significant effect on poultry microbiome diversity (*Figure 2*).

Figure 2. **Effect of phytonutrient enriched feed and rearing phase on the alpha diversity of the gut microbiome of reared poultry**



The figure parts show the results for different poultry: (**a**) broiler chicken (**b**) domestic duck. Boxplots represent comparisons of an alpha diversity metrices (Chao-1 and Shannon diversity index) measured in different groups (control, experimental and starter, grower, finisher). The asterisked group is significantly different from every other group (p> 0.05*).

The feed used in different rearing phases had a significant influence on the intestinal diversity of broiler chicken and domestic duck flocks, with a large separation based on distance matrices (*Figure 3*).

The age of reared poultry is a factor with a significant influence, which, owing to the feed used in successive rearing phases, has a significant influence on the composition, ratio, and metabolic function of intestinal bacteria. Owing to the establishment of more stable bacterial taxa, the composition of the microbiome varied significantly during the different rearing phases of broiler chickens and domestic ducks (*Figure 4/a and Figure 4/b*).

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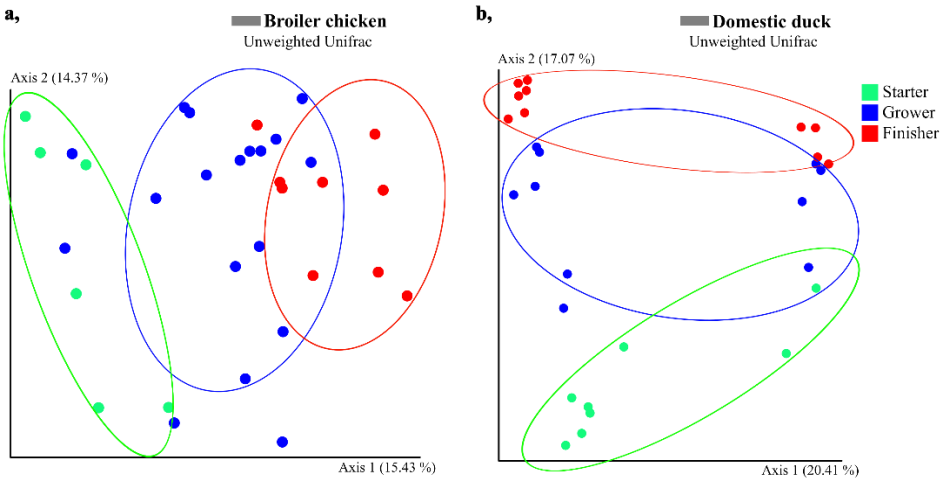
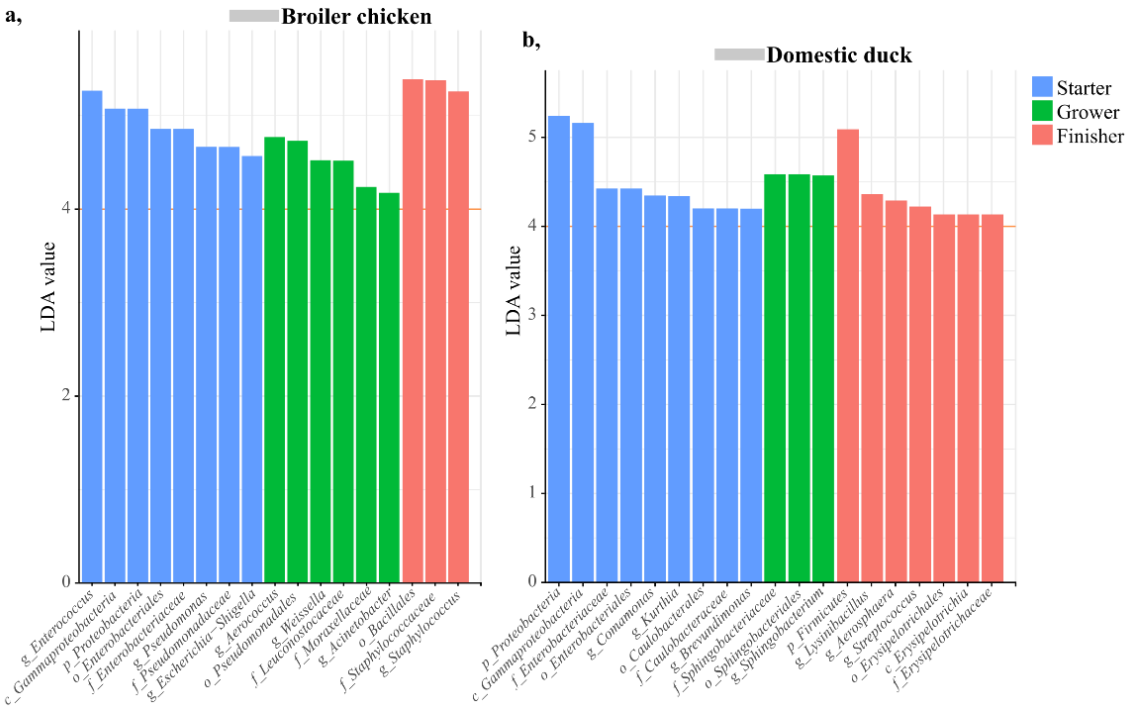
Figure 3. **Effect of rearing stages on the beta diversity of the intestinal microbiome of reared poultry**



The figure parts show the results for different poultry: (**a**) broiler chicken (**b**) domestic duck. Beta diversity distributions summarizing the differences in community composition caused by aging (starter, grower, finisher). Beta diversity relationships are summarized in two-dimensional scatterplots. Each point represents a sample, and distances between dots are representative of differences in microbiota compositions.

Figure 4. **Bacterial clades involved in significant taxonomic shifts by ageing**



The figure parts show the results for different poultry: (**a**) broiler chicken (**b**) domestic duck. Linear discriminant analysis (LDA) effect size (LEfSe) identifies bacterial clades involved in significant taxonomic shifts (LDA > 4 giving the significant difference between the rearing phases). Columns in blue represent the starter phase, columns in green represent the grower phase, and columns in red represent the finisher phase.

During the poultry starter phase, the proportions of *Gammaproteobacteria* (broiler chicken LDA = 5.07, domestic duck LDA = 5.16), *Proteobacteria* (broiler chicken LDA = 5.07, domestic duck LDA = 5.23), and *Enterobacteriales* (broiler chicken LDA = 4.85, domestic duck LDA = 4.42) were significant. In broiler chicken flocks (*Figure 4/a*), the proportion of *Aerococcus* (LDA = 4.76), *Pseudomonadales* (LDA = 4.72), *Weisella* (LDA = 4.52) and *Acinetobacter* (LDA = 4.17) was significant during the grower phase, while *Sphingobacterium* (LDA = 4.57) was significant in the domestic duck flocks (*Figure 4/b*). In broiler chicken flocks (*Figure 4/a*), the proportions of *Aerococcus* (LDA = 4.76), *Pseudomonadales* (LDA = 4.72),

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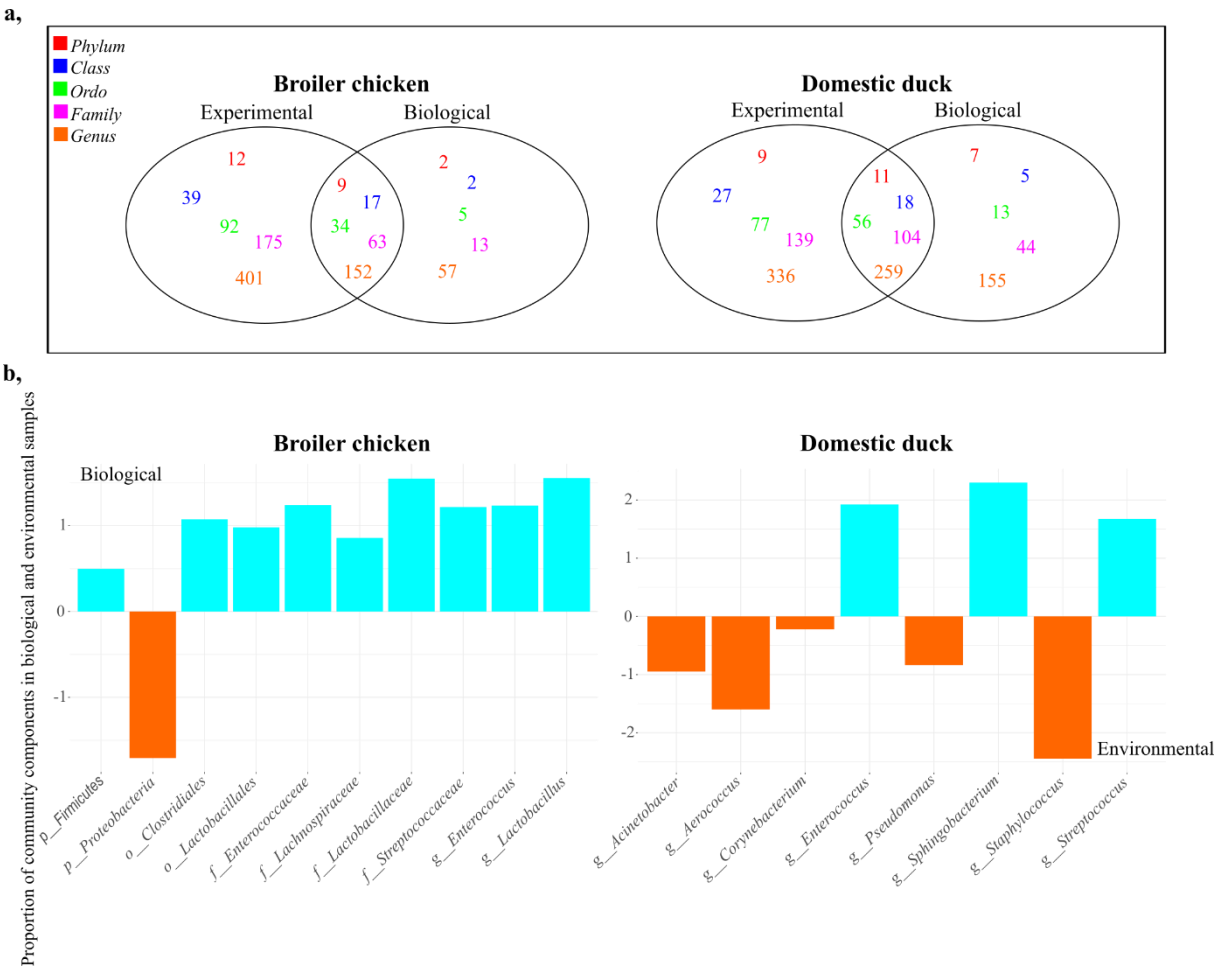
Weisella (LDA = 4.52) and *Acinetobacter* (LDA = 4.17) were significant during the grower phase, while the proportion of *Sphingobacterium* (LDA = 4.57) was significant in the domestic duck flocks (*Figure 4/b*). Furthermore, it can be stated that in the case of broiler chickens (*Figure 4/a*), despite their outstandingly high values, the number of community constituents present in significant proportion in the finisher phase was the lowest, including *Bacialles* (LDA = 5.38) and *Staphylococcus* (LDA = 5.25). For the domestic duck flocks (*Figure 4/b*), the number of community constituents present in significant proportions in the finisher phase was greater than, that of *Firmicutes* (LDA = 5.09) and *Streptococcus* (LDA = 4.22).

Figure 5/b shows the different proportions of the constituents of the core microbiome, with the highest frequency in the environmental and biological samples of poultry (domestic duck and broiler chicken), which were present in all samples without exception. Microbes in the environment, as potential pathogenic sources of infection, can cause significant health problems not only in animals, but also in humans. For example, *Staphylococcus* (log_2 = -2.46), which is present in a significant proportion in the environmental sample of domestic ducks (*Figure 5/b*), in relation to which it can be stated that *Staphylococcus aureus* is one of the multidrug-resistant pathogens causing the most problems, illnesses and deaths in intensive care units and healthcare institutions.

As shown in *Figure 5/a*, bacteria in the environment and gut microbiome of reared poultry (domestic ducks and broiler chickens) were examined at different taxonomic levels, as well as which taxis are present in both environmental and biological samples. Microbes in the environment play an important role in colonizing an animal's initial gut microbiome, as the numbers show, many taxa are found in both biological and environmental samples.

Figure 5. **Shared and unique community-forming bacteria present in environmental and biological samples**



(**a**) Venn diagram showing the number of OTUs at different taxonomic levels (phylum, class, order, family, genus) in environmental and biological samples and the intersection of the two. (**b**) Log_2 ratio of microbiome components present in environmental and biological samples. In the case of a positive value, the bacteria abundance is more in the biological sample, and in the case of a negative value more in the environmental sample.

Using taxonomic tree, the influence of feed additives applied to poultry flocks on taxonomy was investigated (*Figure 6*). Allithiamine-enriched feed had a positive effect on the proportion of *Acinetobacter*

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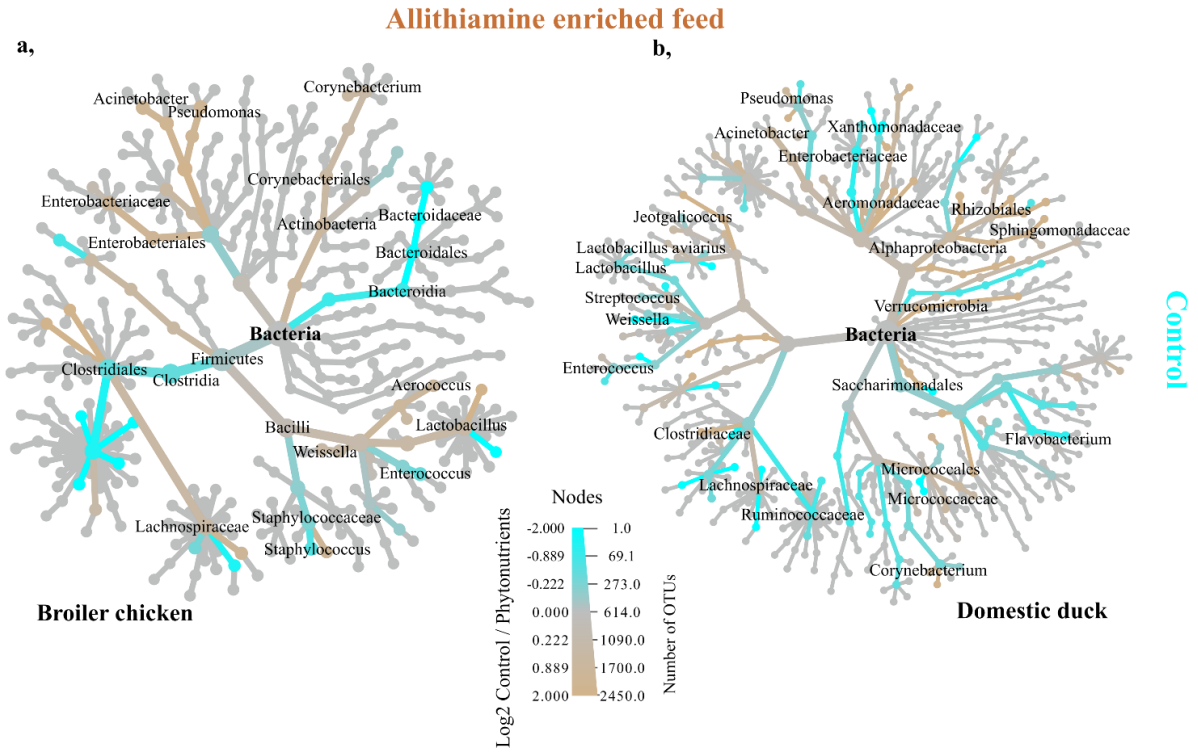
(broiler chicken $\log_2 = 0.79$, roast duck $\log_2 = 0.59$) in both poultry flocks (domestic duck and broiler chicken). The plant bioactive enriched feed, positively affected the ratio of *Actinobacteria* ($\log_2 = 0.79$), *Corynebacterium* ($\log_2 = 0.48$), *Lactobacillus* ($\log_2 = 0.49$), *Jeotgalicoccus* ($\log_2 = 1.22$), *Enterobacteriaceae* ($\log_2 = 1.31$), *Pseudomonas* ($\log_2 = 1.39$) for broiler chicken. Allithiamine-enriched feed had a positive effect on the ratio of *Alphaproteobacteria* ($\log_2 = 0.61$), *Sphingomonadaceae* ($\log_2 = 1.77$) and *Micrococcales* ($\log_2 = 0.58$) in domestic ducks.

Figure 6. **Microbiome composition shifts caused by phytonutrient feed additives**



The figure parts show the results for different poultry: (**a**) broiler chicken (**b**) domestic duck. Metacoder taxonomic heat-tree illustrates the difference in microbial phylotypes between control (without phytonutrient supplementation) and experimental (with phytonutrient supplementation) groups. Colored taxa represent the extents of $\log_2$ differences in taxon abundances: brown represents higher abundance in the phytonutrient-fed group, while blue means higher abundance in the control group. Nodes in the heat tree correspond to phylotypes, as indicated by node labels, while edges link phylotypes in accordance with the taxonomic hierarchy. Node size corresponds to the number of operational taxonomic units (OTUs) observed within a given phylotype.

CONCLUSIONS

Feed processing technology, feed components, and additives significantly affect the intestinal microbial community. Several pre-, pro-, and synbiotic-based preparations have been shown to restore the balance of the dysbiotic gut flora (Pandey et al., 2015). In contrast, phytonutrient treatment had no significant impact on community diversity (*Figure 2*), presumably because of the outstanding quality and composition of feed used in intensive production. Although we did not observe a significant difference in alpha diversity when using the feed additive, this does not mean that it did not modulate the "beneficial" community creators. In the case of broiler chicken flocks, the phythonutrient-enriched feed positively influenced the ratio of *Corynbacteriales* and *Lactobacilli* (*Figure 6*), which play an important role in lipid homeostasis and in preventing colonization of various pathogenic bacteria (Yegani and Korver, 2008), and contribute to increased absorption of nutrients by improving intestinal epithelial integrity (Kau et al., 2011).

Similar to other study results (Crhanova et al., 2011), we also found that the proportion of the composition of the microbial community in the gastrointestinal tract of poultry was significantly influenced by the age of poultry (*Figure 4*).

Several predictions related to transmission risks (Cheng et al., 2019) are also consistent with our results (*Figure 5/b*), indicating that it is important to establish appropriate precautions as antibiotic-resistant pathogens are a growing health problem worldwide (Singer et al., 2003). The WHO (World Health Organization) predicts that by 2050, antibiotic-resistant microbes will kill tens of millions of people each year (Mcallister et al., 2018). Therefore, in the future, we would like to investigate the effectiveness of service periods, site disinfection, and mapping of antibiotic resistance genes that may be found in the environment.

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