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Unraveling changes in the duck microbiome and inflammatory processes due to allithiamine-enriched feed

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SUMMARY

The gastrointestinal tract of poultry harbors a diverse and intricate microbiome that plays a crucial role in nutrient digestion and absorption, immune system development, and enhances resistance against pathogens. Maintaining a healthy state and proper production is fundamentally determined by the symbiosis between the host and microbes. Due to genetic and technological improvements, intensive growth rate can be associated with many pathological conditions, such as increased susceptibility to infections. Intestinal inflammation in poultry industries detrimentally affects productivity by hindering nutrient absorption and the efficient allocation of nutrients for growth. The host releases different biomarkers in response to inflammation. Hence, there is an utmost interest of reliable, precise, sensitive and robust biomarkers to evaluate both the gastrointestinal health status and inflammation in poultry. The aim of this study was to determine how the developed feed prototype (allithiamine) affects the community diversity in raised duck, and the relationship between gut microbiome composition and inflammatory factor as calprotectin, using targeted 16S rRNA gene amplicon sequencing and Chicken Calprotectin ELISA Kit.

Keywords: *phytonutrient; 16S rRNA gene amplicon sequencing; microbiome; gastrointestinal tract; inflammation factor*

Table 1. **List of Abbreviation**

Abbreviation	Definition
GIT	Gastrointestinal track
IBD	Inflammatory bowel disease
PBS	Phosphate buffered saline
SCFAs	Short-chain fatty acids

INTRODUCTION

Maintaining intestinal health has utmost importance for the overall welfare of both animals and humans. In livestock, the consumption of feed and the effective absorption of nutrients largely depend on the health condition of the GIT (Morgan, 2017). This significance is particularly pronounced in poultry, where decades of dedicated selection aimed at bolstering daily weight gain and optimizing feed conversion ratios have given rise to breeds characterized by remarkably elevated feed consumption (Dal Pont et al., 2021). However, it's worth noting that this heightened feed intake, coupled with specific feed components, can impose notable strain on the digestive system. Overconsumption of feed, as well as specific feed components may put noticeable stress on the digestive system (Zou et al., 2018). Beyond a certain threshold, even in the lack of any specific pathogens, it can harm the health of the gastrointestinal tract, resulting in diminished functionality (malabsorption/diarrhea) (Nagalingam and Lynch, 2012). Three distinct yet interconnected mechanisms have been implicated, each of which has received considerable attention in current research: dysbiosis, mucosal barrier dysfunction, and inflammation. Therefore, there is a need for reliable, precise, sensitive, and resilient biomarkers to evaluate the gastrointestinal health and inflammation in poultry.

Calprotectin is a protein complex found in the cytoplasm of certain white blood cells, specifically neutrophils and macrophages (Sonawane and Nimse, 2017). Calprotectin is released by neutrophils and other immune cells in response to an inflammation. It plays a role in the host's defense against infection and contributes to the regulation of immune responses. Calprotectin is also involved in the binding and transport of divalent cations, such as calcium and zinc, which can affect various cellular processes (Røseth et al., 1992). Elevated levels of calprotectin in blood samples are often associated with inflammation in the intestines, making it a useful marker for diagnosing and monitoring conditions like IBD. The measurement of calprotectin levels in blood samples can help healthcare providers assess the severity of inflammation and guide treatment decisions (Morgan, 2017). In the poultry industry, chronic low-grade intestinal inflammation adversely affects productivity by hindering nutrient absorption and the efficient allocation of nutrients for growth (Dal Pont et al., 2021). Elevated calprotectin level is measured in poultry with inflammatory disease according to previous results (Dal Pont et al., 2021).

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Managing inflammation using individual nutrients and phytonutrients is receiving increasing attention, owing to their antibacterial and immune system-stimulating effects (Poles et al., 2021; Sarangi et al., 2016). In addition, certain phytonutrient-enriched feeds modulate the GIT microbial community constituents to a significant extent (Tolnai et al., 2021). These supplements are rich in plant-derived immunostimulants, such as phytochemicals, vitamins, and minerals (Siddiqui and Moghadasian, 2020). Allithiamine is a natural fat-soluble thiamine derivative that has been identified in Hungarian red peppers (*Capsicum annuum*), in addition to garlic (*Allium sativum*) (Biro et al., 2018). Several studies have suggested that sulfur-containing compounds of the Allium species may have beneficial effects on human health (Bayan et al., 2014). Additionally, the effect of allithiamine was examined in streptozotocin-induced diabetic mice exhibiting neuropathy. These findings indicate that allithiamine treatment, akin to benfothiamine treatment, enhanced neuropathic pain sensation in diabetic mice (Biro et al., 2018). From this perspective, the study of the GIT microbiota development and levels of calprotectin in blood, as the effects of phytonutrient-enriched feed has enormous potential and importance.

The objective of this study was to investigate the impact of the developed feed-additive prototype (allithiamine) on the diversity of the gut microbiota in raised ducks, as well as to explore the correlation between gut microbiome composition and the inflammatory factor such as calprotectin. We believe that the findings of this study provide fundamental knowledge in the field of animal microbiome research, and may potentially inform future strategies to rear healthier birds.

MATERIALS AND METHODS

Phytonutrient enriched feed

The allithiamine utilized in this study was developed by researchers at the Institute of Food Technology, University of Debrecen. Owing to the efficiency of the financial and manufacturing processes and the optimal nutrient requirements of the ducks, the experimental group received a basic diet containing 0.5% althiamine.

Birds, management and sampling programme

The samples were collected from the Rém sites of Hungerit Zrt. Intensively reared ducks (cherry valley) were examined. Three to three rearing periods were examined for domestic duck stocks. Blood samples were collected at 17, 32 and 40 days of age of ducks. In parallel with the blood samples, biological-footbag (stool) samples were also collected. Blood samples collected at different ages are unique, while stool samples for them are unificationed (collected from poultry barn). Due to the hygienic criteria of the livestock farm and poultry barn, the maintenance of a sterile environment (pathogens outside the farm), the collection of stool samples were carried out with textile footbags that fit the footwear. During sampling, poultry barns were carefully walked around to ensure homogeneous sampling. The biological-footbag (stool) samples were stored in individually marked lockable bags. The number of repetitions at different ages (17, 32 and 40 days of age) of ducks, as sample type (blood sample and biological-footbag (stool) sample), was 3. Biological-footbag (stool) and blood samples were transported on ice and stored at -80 °C upon return.

Sample preparation and mechanical cell lyses

Bacterial cell suspensions were made from pooled (biological-footbag) samples and homogenized with 40–40 ml of sterile PBS (biosera, Cholet, France) for 30 minutes by innova 40 (Incubator Shaker Series) at 350 RPM. The samples were centrifuged for 5 min at 500 RCF. Supernatants were collected and washing steps were repeated 2 times. To obtain the bacterial cells, the supernatants were centrifuged for 20 min at 13.000 RCF. The supernatants were discarded and the bacterial pellets were resuspended in 3 ml of sterile PBS buffer. Mechanical cell lyses were made to achieve the bacterial DNA. 1 ml aliquots of PBS were added to PowerBead Tubes (Qiagen, Hilden, Germany) and MagNa lyser Instrument (Roche Applied Sciences; Penzberg, Germany) was used for grinding for 30 seconds at 5000 RPM. After the mechanical cell lyses the samples were centrifuged at 16.000 RCF for 1 minute, and then the supernatants (~900 µL) were transferred into a new sterile Eppendorf tube. Previously, cell suspensions were prepared based on an optimized methodology (Fidler et al., 2020).

DNA extraction and calprotectin measurement

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). DNA concentrations were measured fluorometrically utilizing the Qubit® Fluorometric Quantitation system with a dsDNA assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) on a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). DNA purity was assessed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) by measuring the absorbance at wavelengths of 260 and 280 nm. DNA integrity (shearing/fragmentation) was determined automatically on the 4200 TapeStation System, (G2991AA, Agilent Technologies; Santa Clara, California, United States) by using Genomic DNA ScreenTapes (5067–5365) and Agilent Genomic DNA reagents (5067–5366). After processing, purified DNA samples were stored at -20 °C.

For calprotectin measurement from the blood plasma of ducks a commercially available calprotectin kit, the Chicken Calprotectin ELISA Kit was used according to the manufacturer's instructions

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(FineTest). During the process, the calprotectin was measured from 100 µL blood plasma.

16 S rRNA gene metagenomic library preparation and MiSeq sequencing

Standard library preparation was performed as detailed by our previous work (Fidler et al., 2020). Briefly, all samples were normalized to 5ng/µL with PCR grade water. A two-step PCR was performed following the Illumina (San Diego, California, United States) 16S Metagenomic Sequencing Library Preparation protocol (15044223 Rev. B). The V3 and V4 hypervariable regions of bacterial 16S rDNA were sequenced with Illumina MiSeq benchtop sequencer generating single amplicons of ~460 bp with a minimum overlap of ~50 bp in the middle by using the universal primer set: 341F-5' CCTACGGGNGGCWGCA 3'and 785R-5' GACTACHVGGGTATCTAATCC 3' primers flanked by Illumina overhang adapter sequences (forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AG 3', reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG 3'). Finally, 5 µl of pooled 4nM DNA library was prepared for sequencing on Illumina MiSeq platform. Paired-end (PE, 2x300 nt) sequencing with a 5% PhiX spike-in control (PhiX Control Kit v3 - FC-110-3001) was performed with MiSeq Regent Kit v3 - 600 cycle (MS-102-3003) following manufacturer's protocols (Illumina, Inc., San Diego, CA, USA). The automatic, built-in data analysis and the de-multiplexing of the raw reads were performed using the MiSeq Reporter (MSR) v3.0 software (BaseSpace).

Sequencing read preparation for downstream analyses

For demultiplexing the paired end reads an integrated software of the Illumina MiSeq sequencing machine was used. According to "Atacama Soil microbiome" tutorial, FastQ files were imported into Qiime 2 (ver 2019.7) pipeline (https://qiime2.org). Cutadapt Software (integrated to Qiime 2 pipeline) was used to check and trim the remained adapter sequences (CTGTCTCTTATACACATCT) from the 3' end of reads. DADA2 software was applied for quality trimming.

Biodiversity analyses

Alpha and beta diversity values were calculated using QIIME 2 software. For alpha diversity, we worked with Shannon 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.

Data visualisation

For data preparation, QIIME 2 pipeline was used. Heatmaps were generated in R studio (ver4.2.2). Boxplot plots were constructed using GraphPad Prism statistical software.

RESULTS

Alpha diversity index (Shannon's diversity) was created to compare the differences between the different time points (17 days, 32 days, 40 days) of the duck's development and the effect of phytonutrient-enriched feed additives (experimental) (*Figure 1/a, 1/b*). Based on our results, distinctive differences in GIT microbiome richness and diversity among the experimental group were not observed (*Figure 1/b*) (control Shannon = 7.61 ± 1.20, experimental Shannon = 7.80 ± 1.08). We also discovered that the microbiome diversity in the GIT of ducks was not significantly affected by age (*Figure 1/a*) (Control day 17 Shannon = 7.19 ± 1.21, Control 32 day Shannon = 7.87 ± 1.29, Control day 40 Shannon = 7.92 ± 1.42, Experimental day 17 Shannon = 7.73 ± 0.95, Experimental 32 day Shannon = 7.80 ± 1.39, Experimental day 40 Shannon = 7.90 ± 1.39).

The principle coordinate analyses (PcoA), UniFrac unweighted dissimilarity was generated to demonstrate the differences between samples taken from different time points and different treatments. In *Figure 1/d* each point represents the GIT microbial profile of the duck with colour representing the different time points (blue: 7 days, red: 32 days, orange: 40 days). PCoA resulted in three cluster groups with different spatial ordinations. Different profiles were delineated in the case of different time points. However, in *Figure 1/c*, different dietary supplementation did not exert statistical differences on beta diversity distances (blue: control, red: experimental).

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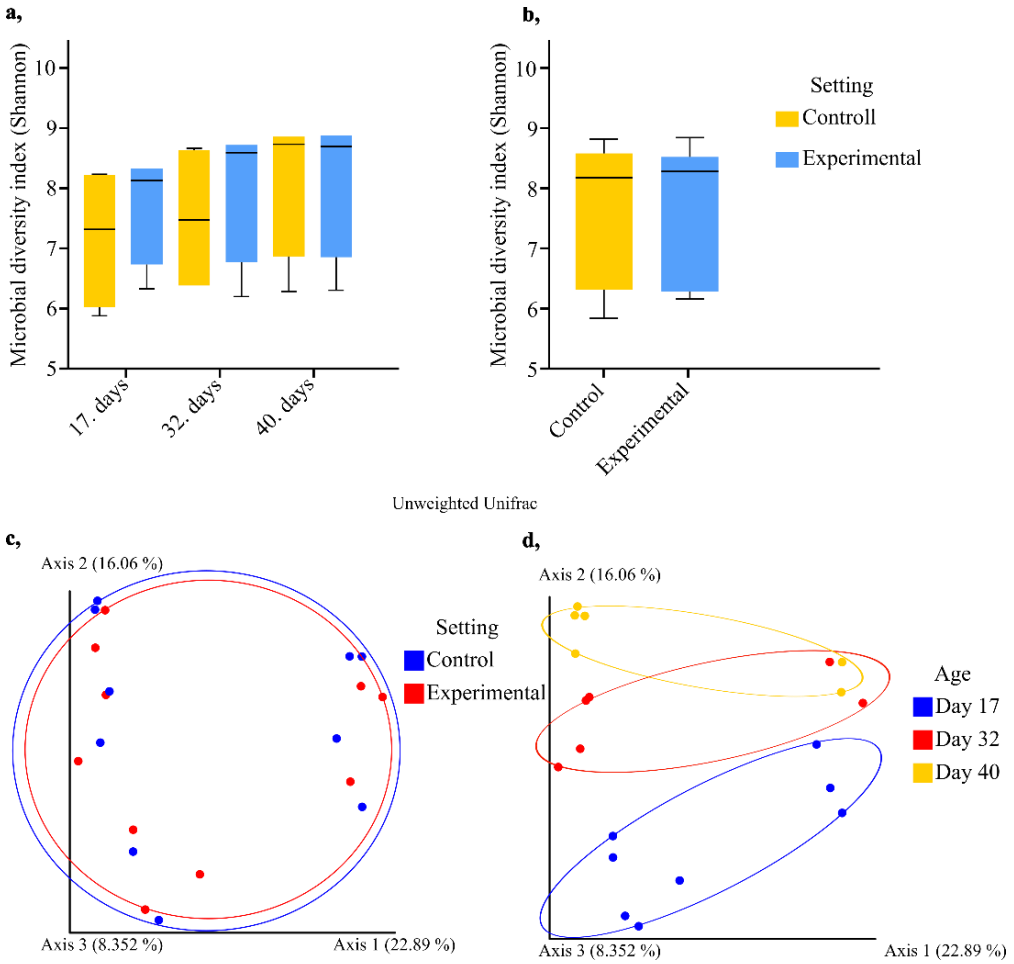
Figure 1. **Effect of allithiamine at different time points on the microbial diversity indexes**



Alpha diversity (1/a, 1/b) indices were created to compare the differences between experimental groups. Boxplots representing alpha diversity metrics of richness and evenness (Shannon) for samples from domestic ducks grouped according to age (days) and dietary supplementation (allithiamine). Each box represents the diversity metrics for a different group, and colour-coded according to diet; blue: control group, red: experimental group (allithiamine). Beta diversity (1/c, 1/d) represents the effect of dietary supplementation (experimental, control) and domestic duck development (day 17, day 32, day 40) on two-dimensional scatterplots. Each point represents a sample, and distances between dots are representative of differences in microbiota compositions. Sample distances were calculated based on qualitative (unweighted UniFrac) dissimilarity-based statistics.

We also examined the bacterial composition of GIT in livestock at phylum and genus levels (*Figure 2/a, 2/b*). We have selected the most generous community creators who represent domestic ducks well.

The details are shown in *Figure 2/a* species of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were dominant in all the segments of age, constituting about 87.84 to 89.34% of the microbiome at 17, 32 and 40 days. Furthermore, the average abundance of Firmicutes (39.59%) in all the segments was the highest (*Figure 2/a*).

By considering the thirty-seven most abundant genera, we found significant alterations in taxonomic data during different developmental stages (17, 32, and 40 days) of the feeding period when comparing the phytonutrient-treated ducks (experimental) to the non-supplemented group (control). Heatmap shows the distortions in the relative abundance data normalized to that of control birds (*Figure 2/b*). Among different time points, we observed remarkable increases in some of the important SCFA producer's quantity due to plant bioactive enriched feed additives. In phytonutrient-treated starter phase birds (17 days), remarkable increases were shown in *Bacteroides* and *Subdoligranulum*. During the grower phase (32 days), phytonutrient-enriched feed increased *Lactobacillus*, and the finisher phase (40 days) also increased *Faecalibacterium*. Furthermore, *Figure 2/b* in phytonutrient-treated finisher (40 days) phase birds remarkable increases were shown in most genera.

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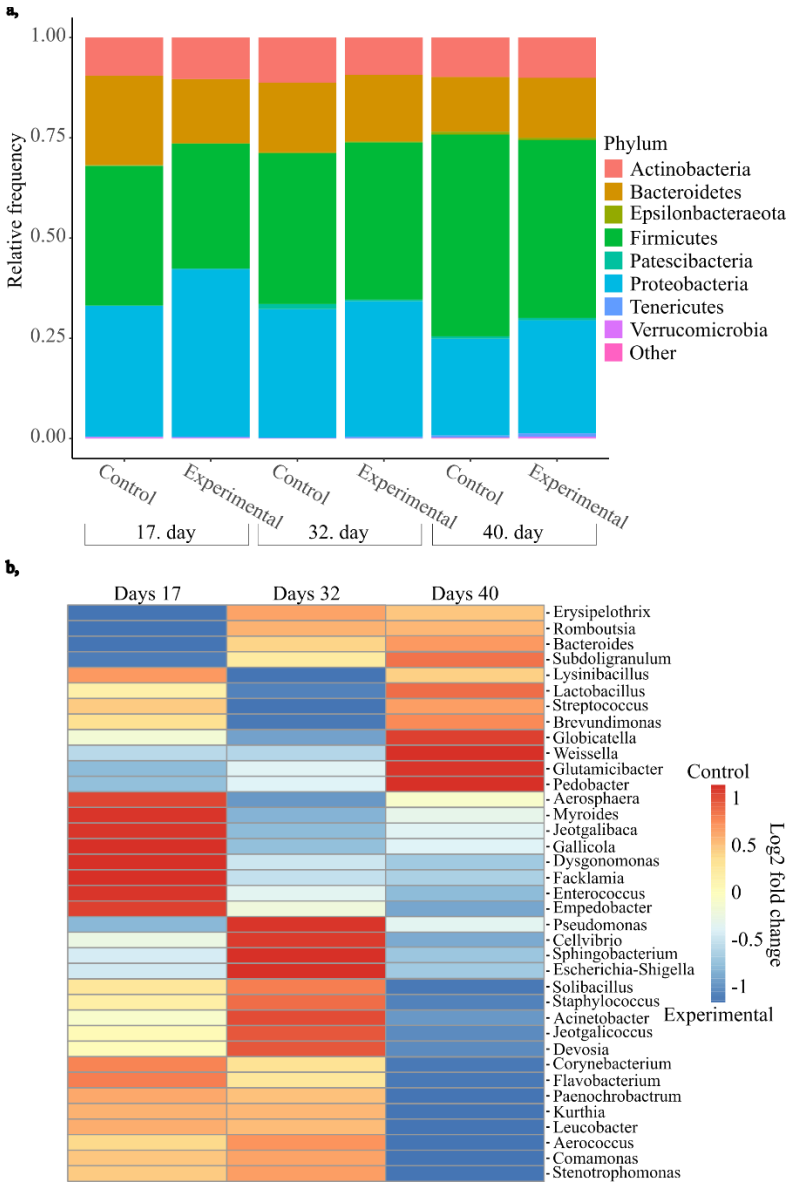
Figure 2. **Age and treatment-induced changes in bacterial composition**



Stack bar plots represent the diet and duck development-induced distortions in the main phyla (a). Heatmap shows the extent of the estimated differences with the normalized log2 fold change of the specified genus abundances. The blue scale represents the dominance of the genus due to dietary supplementation, log2 (control group/experimental group) > 0, whereas the red scale represents values of increases in favour of negative controls.

A significant difference was found (*Figure 3/a*) between phytonutrient-treated (allithiamine) duck's blood calprotectin level and basal diet duck's blood calprotectin level. In *Figure 3/a*, when examining the starter phase birds (17 days), calprotectin levels significantly decreased in phytonutrient-treated birds (Control day 17 = 52.62 ± 12.29, Experimental day 17 = 20.70 ± 12.17 ($P \leq 0.05$)). Also in the phytonutrient-treated grower and finisher (32, 40 days) phase duck's calprotectin levels decreases were shown in comparison to the control, however, these differences were not significantly influenced by the diet (Control day 32 = 30.61 ± 22.28, Experimental day 32 = 20.60 ± 18.33, Control day 40 = 35.24 ± 18.21, Experimental day 40 = 28.04 ± 16.16).

We managed to unravel alterations induced by age (17, 32, 40 days) and treatment (control, experimental) for 9 genera in the intestinal microbiota of domestic duck, finding remarkable correlations with calprotectin level (*Figure 3/b, 3/c*). A change in the strength and direction of correlations was obtained. In this study, out of the 9 genera, there were 5 (*Bacteroides*, *Faecalibacterium*, *Blautia*, *Bifidobacterium*, *Lactobacillus*) which belong to the SCFAs producers and there were 4 (*Escherichia-Shigella*, *Erysipelothrix*, *Staphylococcus*, *Enterococcus*) which belongs to pathogens. We estimated (*Figure 3/b*) that during the first phase (17 days) of the basal diet (control) moderate negative associations were detected with elevated calprotectin levels in case of the most of the SCAFs

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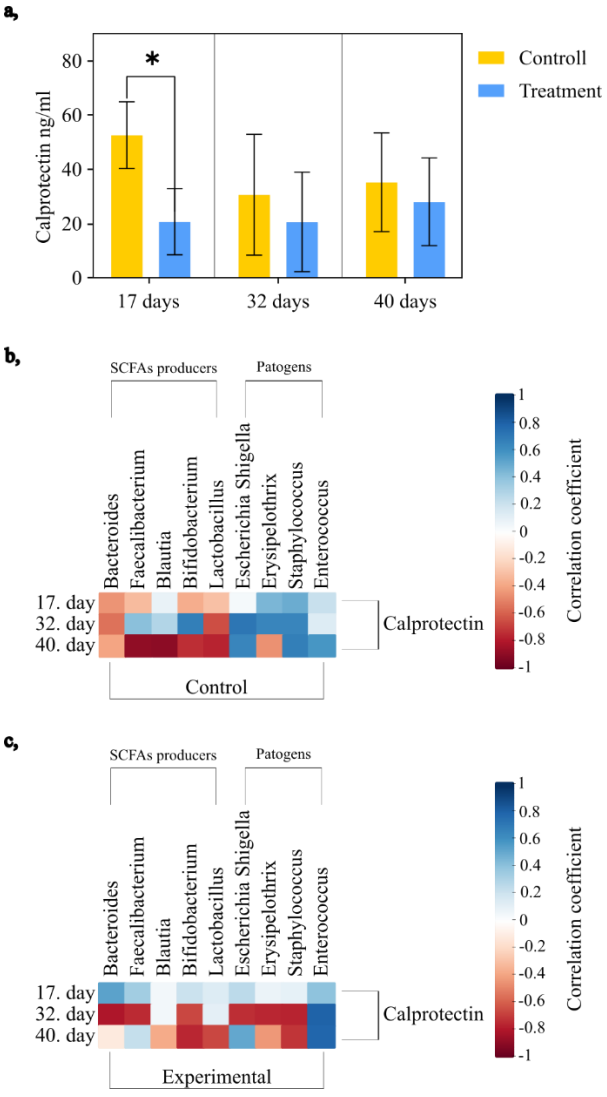
producers (*Bacteroides* = -0.45, *Faecalibacterium* = -0.32, *Bifidobacterium* = -0.37, *Lactobacillus* = -0.30) and moderate positive associations were detected in case of pathogens (*Escherichia-Shigella* = 0,04, *Erysipelothrix* = 0.45, *Staphylococcus* = 0.49, *Enterococcus* = 0.22). Interestingly, regarding the effect of phytonutrients on the correlation values of these genus (*Figure 3/c*) during first phase (17 days), we found moderate positive associations with decreased calprotectin level in case of the most of the SCAFs producers (*Bacteroides* = 0.53, *Faecalibacterium* = 0.34, *Bifidobacterium* = 0.22, *Lactobacillus* = 0.15) and nearly neutral in case of pathogens (*Erysipelothrix* = 0.08, *Staphylococcus* = 0.10).

Figure 3. **Correlation between microbiome composition and the inflammatory calprotectin level**



Bar plots represent the changing of the calprotectin levels caused by ageing and diet in the duck (a). Asterisks indicate statistical significance: *, $P \leq 0.05$. Sperman correlation was calculated to assess the correlation between the microbiome composition and calprotectin levels in control (b) and experimental groups (c).

DISCUSSION

Microbiome diversity is one of the most critical factors for fostering resistance against invading pathogens. Greater diversity in the microbial community results in a healthier host organism, whereas a notable decrease in complexity is linked to various diseases and elevated vulnerability to pathogen colonization (Ducatelle et al., 2015). Therefore, it is important to investigate the effects of different agents on diversity indices. Similarly, Zhu et al. (2020) reported, we also found that the diversity of the composition of the microbial community in the gastrointestinal tract of ducks was not significantly influenced by age (*Figure 1/a*).

Similar to our results (*Figure 2/a*), previous reports also have shown that the microbial population of GIT is most heavily populated by Firmicutes, Bacteroidetes, and Proteobacteria phyla (Zhu et al., 2020). In the gut microbiome, an increase in the amount of the Firmicutes is associated with increased absorption of nutrients (Lozupone et al., 2012), the more Firmicutes, the better the animal's feed utilization capacity, and its increased amount is also related to weight gain (Tolnai et al., 2021). The phyla Bacteroidetes plays a more significant role in carbohydrate metabolism, primarily involved in starch and glucan breakdown (Huang et al., 2021).

Our result (*Figure 2/b*), in agreement with other studies, the composition of SCFAs producers was strongly influenced by age and phytonutrient treatment (Tolnai et al., 2021). Bacteria in the gut microbiome produce energy during fermentation of indigestible carbohydrates and promote the production and absorption of SCFAs. This process allows the host to utilize the energy released from non-digestible carbohydrates and proteins in the upper parts of the GIT. The SCFAs are saturated open-chain monocarboxylic acids with carbon chains shorter than six carbon atoms (Morrison and Pretson, 2016). The most important of these fatty acids are acetate (acetic acid), propionate (propionic acid) and butyrate (butyric acid) (Hamer et al., 2008). Acetate can be produced by a wide range of bacterial species produced by most enteric bacteria, such as *Lactobacillus spp*. (Feng et al., 2018). The pathways of propionate and butyrate synthesis are conserved and substrate-specific can only be produced by certain bacteria, such as *Bacteroides spp*., in addition, *Subdoligranulum spp*. is also one of the most important butyrate-producing bacteria (Louis and Flint, 2017). Furthermore, ducks are the second most important avian species providing eggs and meat for human consumption. Ducklings demonstrate a growth rate surpassing that of other poultry species, with their body weight typically increasing by an average of 55 times within an 8-week period (Biesiada-Drzazga et al., 2017). Achieving a certain weight in ducks depends not only on intensive system variables (genetic and environmental factor, diet), but to a very great extent on the microbiome composition and the diversity, which can determine the production results, including health and the economic efficiency of

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rearing. The balanced gut microbiome is characterized by a diverse community that performs many health-influencing functions, helping to prevent the colonization of pathogenic bacteria by forming a physical barrier. Furthermore, it enhances the efficiency of the intestinal immune system, and protects the integrity of the intestinal mucosa (Shi et al., 2017). A well-functioning gut microbiota plays an important role in digestion, and bile acid synthesis, and provides the host body with important vitamins and trace elements, its metabolic activity is comparable to the function of an organ. (Deleu et al., 2021). They produce energy through fermentation of indigestible carbohydrates and proteins and promote the formation and absorption of SCFAs. In *Figure 2/b*, notable increases were observed in most genera of phytonutrient-treated finisher phase birds (40 days), which should be highlighted because of the development of a diverse community, ensuring a balanced gut microbiome.

However, a consequence of intensive livestock farming, animals are exposed to increased stress, which can be induced by many factors during their growth and development. These stress factors affect various processes in the host, such as the behavior and immune, cardiovascular, and gastrointestinal systems. Some can also occur during development, such as increased susceptibility to infections or changes in gastrointestinal development (Shiraishi et al., 2011). Furthermore, it also affects hormone balance (De Castro Junior and Silva, 2020). As a result of stress-induced molecular mechanisms, feed intake and weight gain of animals can be significantly reduced, enteric diseases may occur, and mortality increases in severe cases (Oke et al., 2021). In addition, during inflammatory diseases caused by stress, immune cells release cytosolic proteins, which can be detected in the blood and serve as predictors of inflammation (Sands, 2015). Also during our experiment (*Figure 3/a)*, the control stock had a significant increase in the amount of this inflammation-predicting protein (calprotectin). Calprotectin is produced during inflammatory processes, exhibits antimicrobial and anti-proliferative functions, and plays a regulatory role in inflammation (Zali et al., 2007).

Higher microbial diversity is generally associated with a healthier host status, whereas a deficiency in diversity within microbial community structures has been correlated with gastrointestinal disorders (Mohd Shaufi et al., 2015). Moreover, an imbalance in gut microbiome composition and a notable reduction in GIT diversity often results in the depletion of beneficial bacteria and concurrent increases in pathogenic bacteria (Kumar et al., 2019), which frequently leads to inflammation and increases in calprotectin levels (D'Amico et al., 2021). In the first phase of basal diet (control group, 17 days), presumably due to lack of beneficial effect of the active substance (allithiamine), a decrease in beneficial bacteria (SCFAs producers) and simultaneous growth of pathogenic bacteria occurred during an increase in calprotectin levels (*Figure 3/b*). Phytonutrients are compounds of natural origin that are responsible for the health and integrity of plants. The phytonutrients potential therapeutic use stems for their antioxidant, anti-inflammatory and antimicrobial properties (Gessner et al., 2017). Furthermore, positively influence antioxidant status and immune function by binding free radicals and inhibiting increased secretion of inflammatory cytokines (Tedeschi et al., 2021) and stimulating the quantity of beneficial SCFAs producer bacteria (Tolnai et al., 2021), which results also related to our results (*Figure 3/a, 3/b, 3/c*).

CONCLUSIONS

In intensive farming, animals are exposed to increased stress, which can be induced by many factors during their growth and development. These stress factors have an impact on many processes in the body, including the ability to influence the behavior, and immune, cardiovascular and gastrointestinal systems. The frequency of infectious diseases is high, and the production of inflammatory cytokines also increases. Taking all these factors into account, it is of paramount importance to minimize the negative effects arising from the husbandry technology occurring during large-scale livestock husbandry and to reduce the resulting inflammatory diseases in livestock. However, advancements in modern molecular biology methods have highlighted the influence of nutrients on gut microbiota, and certain phytonutrient-enriched feeds have been shown to have beneficial effects on the health and immune status of animals by restoring the balance of the microbiome.

In this study, we extensively examined the effects of feed formulations rich in phytonutrients (allithiamine) on the gastrointestinal microbiota and inflammatory factor such as calprotectin, using targeted 16S rRNA gene amplicon sequencing and the Chicken Calprotectin ELISA Kit. Our aim was to investigate the impact of the developed feed prototype on microbiome composition, community diversity, and secretion of inflammatory cytokines in domestically raised ducks.

A significant difference was found in the case of domestic ducks blood calprotectin levels due to the bioactive plant additive (allithiamine), when comparing phytonutrient-treated duck (experimental) to non-supplemented group (control) the calprotectin level significantly decreased in phytonutrient-treated birds. Furthermore, among different time points, we observed remarkable increases in some of important SCFA producer's quantity due to plant bioactive enriched feed additive (allithiamine). Important to underline the effect of phytonutrients on the correlation values of the highlighted genus, we found moderate positive associations with decreased calprotectin levels in case of the most of the SCAFs producers, and nearly neutral in the case of pathogens.

In this approach, reducing the inflammation, modulating the beneficial community composition (SCFAs producer) and decrease the susceptibility to colonization by various pathogens used through the application of feed additives rich in phytonutrients not

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only embodies a sustainable and environmentally conscious approach (including health and the economic efficiency of rearing) but it also contributes to the production of safe and high-quality duck meat for consumers.

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