International Journal of Biological and Pharmaceutical Sciences Archive

ISSN: 0799-6616 (Online)

Journal homepage: https://ijbpsa.com/

(RESEARCH ARTICLE)

Check for updates

INTEGRATIVE LINKING OF FECAL METABOLOMICS AND GUT MICROBIOME GENETIC SEQUENCING (16S RRNA) TO INTERPRET THE PHYSIOLOGICAL RESPONSE TO FOOD RESTRICTION IN WATERBIRDS

Anas Yaseen Hasan *

Department of Biology, College of Science, University of Tikrit, Tikrit, Iraq.
* Corresponding Author.

ORCID Details
Anas Yaseen Hasan: https://orcid.org/0009-0000-9693-6249

International Journal of Biological and Pharmaceutical Sciences Archive, 2026, 12(02), 173–183

Publication history: Received on 11 October 2026; revised on 21 November 2026; accepted on 23 November 2026

Article DOI: https://doi.org/10.53771/ijbpsa.2026.12.2.0114

Copyright © 2026 Author(s) retain the copyright of this article. This article is published under the terms of the Creative Commons Attribution License 4.0

ABSTRACT

Restriction of food is a significant environmental stressor and may affect the physiological condition, metabolic rate and intestinal microbial diversity of waterbirds. In the present study, the physiological and metabolic changes of mallards (Anas platyrhynchos), induced by food restriction, were explored by combining metabolomic profiling via proton nuclear magnetic resonance spectroscopy (^1H-NMR) on faecal samples with characterization of gut microbiome by 16S rRNA gene sequencing. Nine mallard ducks (7 females and 2 males) were food-restricted for 6 days on an average at 75% of their basal metabolic rate (BMR). For metabolomic and microbial analyses, fecal samples were collected before and during the restriction period. The results showed an average decrease in body mass of 15.0% ± 9.0%. Differences between the baseline and food restricted conditions were clearly observed in the metabolome, with significant separation between the conditions confirmed by permutation test ($p = 0.008$). Diabolic metabolites with significant changes were the increases of 3-hydroxybutyrate, creatine, methylamine, trehalose, and trimethylamine and the decreases of serine, taurine, ascorbate, arabinitol, and O-phosphocholine. In addition, microbial alpha diversity decreased significantly ($p < 0.0001$), and microbial community structure changed significantly (PERMANOVA, $p = 0.001$) as shown by gut microbiome analysis. The taxonomic level of Bacillota (Firmicutes), Lachnospiraceae and Ruminococcaceae decreased while Proteobacteria and Enterobacteriaceae increased after food restriction. Combined metabolomic and microbial data showed that energy metabolism-related metabolites in feces and community-structure of gut bacteria changed at the same time, indicating possible interactions between host energy metabolism and gut microbial activity under nutritional stress. Overall, short-term food restriction can cause significant micro- and metabolic changes in mallards, and the use of both metabolomics and 16S rRNA sequence analysis of mallard feces can offer a valuable non-invasive tool for assessing physiological responses to nutritional stress in waterbirds.

keywords: Food restriction, Mallard ducks, Fecal metabolomics, ^1H-NMR spectroscopy, Gut microbiome, 16S rRNA sequencing and Nutritional stress.

1 INTRODUCTION

Waterbird ecosystems, particularly wetlands and marshes, are being impacted by environmental degradation and loss of food due to climate change and growing anthropogenic activities. These environmental changes may cause stress reactions in waterbirds, such as changes in their metabolism, adaptability, and survival [1]. The lack of food and/or changes in food availability are believed to be environmental factors that induce a cascade of physiological and metabolic changes to ensure adequate energy supplies for basic life processes [2]. Under these conditions, the hypothalamic-pituitary-adrenal (HPA) axis is activated and cortisol hormones are generated, particularly corticosterone (CORT) which is a key mediator of the physiological reaction to stress. The increased corticosterone levels lead to an increase in gluconeogenesis and the catabolism of lipid and protein sources to allow for alternative energy supply sources with less energy being directed towards less immediately essential functions such as growth and reproduction [3].

In the case of prolonged food deprivation, the body's metabolic processes gradually go into self-sacrifice by utilising endogenous energy stores in the form of either the breakdown of stored lipids or muscle tissue to maintain metabolic needs. These metabolic changes are reflected in the changes of some biochemical compounds and metabolic products detectable in the feces [4]. In previous metabolic studies it has been proposed that some metabolites, including 3-hydroxybutyrate (which is produced in the process of fatty acid oxidation) or creatine (involved in muscle tissue metabolism) may be increased under nutritional restriction. It has also been reported that other compounds change in nutritional stress, like the presence of glucose in the stool and glutaric acid [5]. Food deprivation, on the contrary, may result in various changes of metabolic status and intestinal homeostasis, with a decrease of cellular metabolism and biosynthetic and intestinal function metabolites as well as of serine, taurine, ascorbate and O-phosphocholine [6].

A non-invasive tool to study the physiological response to environmental stress of wildlife has been developed with the analysis of metabolomics of faeces. The potential to identify and characterize a large number of metabolites in biological samples, using proton nuclear magnetic resonance (^1H-NMR), is defined as untargeted metabolomics and can provide important insights into the nutritional status and metabolic changes, as reported by Taniere et al. [7]. The benefits of this to wildlife scientists is that it can be used to measure the physiology of the animal without causing any extra stress to the animal if blood is taken or any other invasive procedures are carried out. Furthermore, fecal samples contain host metabolic products as well as products of the microbial activity in the gastrointestinal tract that can be utilized to investigate host–microbial interactions [8]

While the role of fecal metabolomics in elucidation of metabolic changes to food restriction is growing, the microbiome does not directly inform changes in composition and diversity of the gut microbial community. In a reference study on mallards (Anas platyrhynchos) Murray and Machin (2024) showed that the ^1H NMR metabolomic profiling of the faeces was able to differentiate between normal feeding conditions and food restriction. Any changes in the microbiome, however, were measured indirectly, as alterations of the concentrations of some metabolites, including trehalose and trimethylamine (TMA) [9, 10]. This method points to an interesting research gap, since it can be difficult to differentiate if the metabolic changes are due to the host's physiological response or actually reflect gut microbial composition and diversity, especially of the bacteria that produce short-chain fatty acids (SCFAs) like those in the Lachnospiraceae and Ruminococcaceae families.

An integrated approach that combines the use of fecal metabolome with characterization of the gut microbiome is needed to gain a more complete understanding of the physiological response to food restriction in waterbirds. In the present study, we are using ^1H-NMR spectroscopy to study the metabolic changes in the feces and 16S rRNA gene sequencing to elucidate the diversity and composition of gut microbial consortia. In addition, it aims to elucidate the relationships between the relative abundance of bacterial taxa and changes in fecal metabolites, especially considering the microbial groups involved in the production of short-chain fatty acids. This research will help clarify the interaction between host physiology and gut microbes during periods of nutritional stress, and will assist in making a more complete picture of the interaction between the gut and metabolism as an indicator of nutritional stress in waterbirds

2 MATERIALS AND METHODS

2.1 Experimental Animals and Housing Conditions

The study was conducted using nine adult mallard ducks (Anas) About one year old, seven females and two males of the species platyrhynchos. The average body weight of the birds was 1080 ± 140 g at the beginning of the experiment. The birds were kept in outdoor enclosures that contained environmental conditions similar to that of their natural habitat. 26.8 m^2 of a grassy area and a natural pond of 13.4 m^2 were added into each enclosure, which enabled the birds to carry out all their usual feeding and behavioral activities.

All birds were given a balanced commercial diet and libitum prior to the food-restriction period. The metabolizable energy (ME) of the diet was 2850 kcal/kg with 19% crude protein, 3.8% crude fat and 5% crude fiber. The birds were fed for a period before the experimental procedure was introduced.

2.2 Food Restriction Protocol and Basal Metabolic Rate Calculation

The basal metabolic rate (BMR) of each bird was calculated individually according to its initial body weight using the following equation for waterbirds:

$$BMR = 87.9 \times W^{0.734}$$

where W represents the body mass of the bird in kilograms.

After the baseline feeding period, food restriction was implemented on six days in a row. At this time the amount of food offered each bird was 75% of the birds' calculated BMR. Daily feed amount was calculated based on the metabolizable energy content of the experimental diet (2850 kcal/kg).

Feed was offered in separate feeding containers to prevent competition among the birds, and to allow an accurate determination of each bird's feed consumption. Body weight was monitored to assess body weight changes in the course of the experiment, due to food restriction.

2.3 Fecal Sample Collection and Cryopreservation

The experimental sampling schedule was followed to collect fresh fecal samples in the morning, about an hour after the provision of food. The baseline samples were taken on three days prior to the beginning of food restriction (day −14, −7 and −2). To assess the metabolic and microbial changes caused by food restriction, additional samples were collected during the food restriction period on days 2, 5, and 6.

Birds were put in clean and dry collection boxes with adequate ventilation for sample collecting. The fresh faecal samples were put immediately into sterile cryovials and then were quickly frozen in liquid nitrogen to prevent enzymatic degradation and integrity of DNA in metabolites and microorganisms. The samples were then stored at -80°C until they could be analyzed in the laboratory.

2.4 Fecal Metabolomic Analysis with proton nuclear magnetic resonance spectroscopy

To remove moisture and normalize the weight of samples, they were first freeze dried in a lyophilization system for removal of water from the feces before extraction. Dried fecal material was extracted in 500 µL of 0.2 M phosphate buffer (D_2O, pH 7.4) with the internal chemical shift reference TMSP, and about 10 mg was obtained.

Proton nuclear magnetic resonance (^1H-NMR) spectroscopy was performed at a controlled temperature of 298 K with a Bruker Avance III HD 600 MHz spectrometer to gain insight into the structures of the prepared extracts, using standard one-dimensional NOESY presaturation pulse sequences to suppress the water signal and enhance the spectral quality. All of the resulting spectra were processed and analyzed with Chenomx NMR Suite software. The metabolites in the faeces were identified and quantified by comparing the spectrum with reference spectra libraries.

2.5 Gut Microbiome Characterization Using 16S rRNA Gene Amplicon Sequencing

Total microbial DNA was extracted from approximately 250 mg of fecal material using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions. DNA concentration and purity were evaluated before molecular amplification to ensure the suitability of the extracted DNA for downstream analysis.

The bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR), targeting the V3–V4 hypervariable regions using the following primers:

Forward primer (341F):

5′-CCTACGGGNGGCWGCAG-3′

Reverse primer (805R):

5′-GACTACHVGGGTATCTAATCC-3′

The amplified products were purified and prepared for high throughput sequencing. Next, paired-end sequencing (2 × 300 bp) was carried out on the Illumina MiSeq platform. Negative extraction and PCR controls were used to detect contamination in the sample processing and PCR amplification steps.

The QIIME 2 bioinformatics pipeline was used to process the sequencing reads. The raw reads were filtered for low-quality reads, chimeric reads, and possible contaminants prior to downstream analysis. The DADA2 denoising algorithm was used to identify Amplicon sequence variants (ASVs). The bacterial communities were characterized at various taxonomic levels (phylum, family and genus) by taxonomic classification employing a SILVA reference database. Species-level identification was reported when this was reasonably possible using the level of sequence resolution.

2.6 Statistical Analysis

The metabolomic data were statistically analysed using MetaboAnalyst platform. Principal component analysis (PCA) is an unsupervised multivariate analysis that was conducted to look at the distribution and variation in metabolic profile between baseline and food-restricted conditions. Partial least squares discriminant analysis (PLS-DA) was also used to find the metabolites which differed between experimental conditions. The efficiency of the models was estimated by using the cross validation methods and the accuracy, R^2 and Q2 values were used to evaluate the model efficiency and predictive ability. In addition, a permutation test with 1,000 iterations was used for the validation of the PLS-DA model to check if the separation between the groups is larger than random.

All univariate statistical comparisons were made using paired Student's t-tests to compare baseline concentrations with food-restricted concentrations. The Benjamini–Hochberg false discovery rate (FDR) procedure was used to make multiple comparisons, and the resulting q-value was < 0.05 for the comparisons that were statistically significant. Metabolite changes were quantified using $\log_2$ fold changes to indicate the direction and magnitude of changes. Metabolites were deemed significant if they had VIP score > 1.5, FDR q-value < 0.05 and absolute $\log_2$ fold change > 0.5.

Alpha diversity indices such as Shannon and Chao1 were used to understand the microbial diversity and richness within a sample. Weighted and unweighted UniFrac distance metrics were used to compare the composition of microbial communities across sampling periods to investigate beta diversity. Permutational multivariate analysis of variance (PERMANOVA) was used to evaluate the statistical significance of differences in microbial community structure.

2.7 Integrative Microbiome–Metabolome Analysis

The associations between the microbial composition and the fecal metabolic profiles were analyzed using integrative analysis. The relationships between the relative abundance of the various successful bacterial taxa and concentrations of specific metabolites (3-hydroxybutyrate, creatine, trehalose and trimethylamine) were examined using Spearman's rank correlation analysis.

In addition, sparse partial least squares (sPLS) analysis was conducted with the mixOmics package in R software to detect the associations between two microbial and metabolic datasets, and to build an integrated network of the microbiome-metabolome interactions. These analyses revealed knowledge of the possible relationships between changes in the gut microbiome and metabolic changes induced from food restriction in mallard ducks.

3 RESULT

3.1 Body Mass Changes

The nine mallard ducks (Anas platyrhynchos) showed a reduction in body mass following six days of food restriction at 75% of their basal metabolic rate. The mean initial body mass was 1080 ± 140 g, which decreased to 914.5 ± 142.6 g after the restriction period. The average body mass loss was 154.9 ± 63.9 g, corresponding to a mean percentage reduction of 15.0% ± 9.0%. Individual responses varied among birds, with body mass loss ranging from 5.3% to 37.0%, as presented in Table 1

Table 1: Individual body mass changes in birds before and after food restriction n equals 9

Bird number	Sex	Initial body mass g	Final body mass g	Body mass loss g	Body mass loss percent
1	Female	1200	756	444	37.0
2	Female	1100	1000	100	9.1
3	Female	950	900	50	5.3
4	Male	1350	1200	150	11.1
5	Female	1125	1000	125	11.1
6	Male	1100	950	150	13.5
7	Female	900	725	175	19.4
8	Female	1050	850	200	19.0
9	Female	950	850	100	10.5
Mean plus or minus SD	Not applicable	1080 plus or minus 140	914.5 plus or minus 142.6	154.9 plus or minus 63.9	15.0 plus or minus 9.0

3.2 Fecal Metabolic Profile Using Nuclear Magnetic Resonance

The principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were able to distinguish baseline samples from samples taken on day 5 of food restriction.

With leave one out cross validation, the accuracy of the PLS DA model was 0.84. The R square value for the model was 0.91 and Q square value was 0.17.

Unsupervised principal component analysis (PCA) accounted for 30.3% of the overall metabolic variation, with PC1 accounting for 20.3% and PC2 accounting for 10.1% of the variance. Additionally, the validation of the PLS-DA model, based on 1000 permutations, showed a statistically significant difference between the baseline and food-restricted metabolic profiles (permutation p = 0.008.(

Only 14 major metabolic compounds were identified that had variable importance in projection scores over 1.0.

The 14 major metabolites identified and their direction of change, VIP score, biochemical significance are shown in Table 2.

Table 2: Major metabolic compounds associated with food restriction ranked by VIP score

Metabolite	Direction of change in feces	VIP score	Biochemical significance and metabolic pathway
3 Hydroxybutyrate	Increased	Approximately 2.95	Ketone body indicating fatty acid oxidation and lipid breakdown
Creatine	Increased	Approximately 2.90	May reflect tissue and protein breakdown for energy production
Methylamine	Increased	Approximately 2.87	Aliphatic amine potentially involved in central regulation of food intake
Arabinitol	Decreased	Approximately 2.37	Sugar alcohol associated with intestinal yeasts including Candida
O Phosphocholine	Decreased	Approximately 2.23	Phospholipid precursor; reduction may indicate changes in intestinal mucosal structure
Glutaric acid	Increased	Approximately 2.08	Amino acid metabolic product potentially associated with metabolic changes during fasting

Serine	Decreased	Approximately 2.02	Amino acid required for cellular biosynthesis and intestinal tissue maintenance
Glucuronate	Increased	Approximately 1.95	Glucose derivative involved in metabolic and conjugation pathways
Trehalose	Increased	Approximately 1.95	Sugar associated with microorganisms and cellular stress responses
Taurine	Decreased	Approximately 1.86	Involved in bile acid conjugation and lipid digestion
Caprate	Decreased	Approximately 1.85	Medium chain fatty acid that may decrease with reduced feed intake
Ascorbate Vitamin C	Decreased	Approximately 1.79	Antioxidant potentially consumed during oxidative stress
Glucose	Increased	Approximately 1.70	Fecal glucose level may reflect changes in intestinal absorption or metabolism
Trimethylamine TMA	Increased	Approximately 1.65	Product of microbial conversion of choline in the intestine

3.3 Gut Microbiome Diversity and Taxonomic Composition

The gut microbial alpha diversity analysis indicated that there was a significant decrease in the microbial diversity and richness after 6 days of dietary restriction. Evaluated alpha diversity indices were all lower on Day 6 than at baseline. The Shannon index decreased from 4.85 ± 0.32 to 3.42 ± 0.28, while Chao1 richness declined from 312.4 ± 24.8 to 205.1 ± 18.6. Likewise the number of observed amplicon sequence variants (ASVs) dropped from 285.6 ± 21.3 to 184.2 ± 15.9. The Simpson index (1 – D) also declined from 0.941 ± 0.021 to 0.812 ± 0.035. The within-sample microbial diversity and richness decreased significantly ($p < 0.0001$) after dietary restriction, as shown in Table 3.

Table 3: Alpha Diversity Indices of Gut Microbiota at Baseline and Following Six Days of Dietary Restriction

Alpha Diversity Metric	Baseline (Mean ± SD)	Dietary Restriction – Day 6 (Mean ± SD)	Percentage Change (%)	Test Statistic (t, df = 8)	P-value	Effect Size (Cohen's d)
Shannon Index (H′)	4.85 ± 0.32	3.42 ± 0.28	−29.5%	t = 8.64	2.4×10^{-5} (< 0.0001)	4.76 (Very large)
Chao1 Richness	312.4 ± 24.8	205.1 ± 18.6	−34.3%	t = 9.81	9.8×10^{-6} (< 0.0001)	4.89 (Very large)
Observed ASVs	285.6 ± 21.3	184.2 ± 15.9	−35.5%	t = 11.23	3.1×10^{-6} (< 0.0001)	5.41 (Very large)
Simpson Index (1 − D)	0.941 ± 0.021	0.812 ± 0.035	−13.7%	t = 8.12	4.2×10^{-5} (< 0.0001)	4.47 (Very large)

3.4 Beta Diversity and Gut Microbial Community Structure

There were significant shifts in the gut microbial community composition between baseline and day 6 of the dietary restriction assessed by beta diversity analysis. PERMANOVA analysis showed that all three distances (Weighted UniFrac, Unweighted UniFrac, and Bray–Curtis dissimilarity) had significant differences among the groups ($p = 0.001$, Pseudo-F = 14.82, $R^2 = 0.481$; $p = 0.001$, Pseudo-F = 11.45, $R^2 = 0.417$; and $p = 0.001$, Pseudo-F = 16.34, $R^2 = 0.505$, respectively). In addition, the PERMDISP analysis revealed no significant difference in group dispersion for any of the distance measures used ($p>0.05$), which allows for interpretation of differences in the microbial community structure. The detailed statistical results are given in Table 4.

Table 4: Beta Diversity Analysis of Gut Microbial Communities at Baseline and Following Six Days of Dietary Restriction

Distance Metric	PERMANOVA Pseudo-F	Explained Variance (R^2)	PERMANOVA P-value (999 permutations)	PERMDISP F-statistic	PERMDISP P-value
Weighted UniFrac	14.82	0.481 (48.1%)	0.001	1.15	0.302 (Homogeneous dispersion)
Unweighted UniFrac	11.45	0.417 (41.7%)	0.001	0.84	0.375 (Homogeneous dispersion)
Bray–Curtis Dissimilarity	16.34	0.505 (50.5%)	0.001	1.42	0.251 (Homogeneous dispersion)

3.5 Gut Microbiome Taxonomic Composition and Differential Abundance

The relative abundance of the bacterial community in the gut showed significant shifts in the bacterial communities between diets. At the phylum level, Bacillota (Firmicutes) decreased from 62.45 ± 4.12% at baseline to 41.18 ± 3.75% on Day 6, whereas Bacteroidota increased from 25.30 ± 2.85% to 31.85 ± 3.10%. The Firmicutes-to-Bacteroidetes (F/B) ratio declined from 2.47 ± 0.31 to 1.29 ± 0.18. In contrast, Proteobacteria showed a marked increase from 5.12 ± 1.15% to 18.74 ± 2.40%. The family Lachnospiraceae showed a decrease, whereas Ruminococcaceae showed a decrease from 3.10 ± 0.85% to 2.95 ± 0.78% and Enterobacteriaceae showed an increase from 3.10 ± 0.85% to 15.40 ± 2.10%. Lactobacillaceae also decreased from 8.40 ± 1.20% to 4.20 ± 0.95%. These taxonomic changes and adjusted p-values and LEfSe LDA scores are summarized in Table 5.

Table 5: Taxonomic Relative Abundance and Differential Abundance Statistics

Taxonomic Level / Taxon	Baseline Relative Abundance (Mean ± SD, %)	Dietary Restriction – Day 6 (Mean ± SD, %)	Fold Change / $\log_2$FC	Raw P-value	False Discovery Rate (FDR q-value)	LEfSe LDA Score ($\log_{10}$)
Phylum: Bacillota (Firmicutes)	62.45 ± 4.12	41.18 ± 3.75	0.66 ($\log_2$ = −0.60)	0.00012	0.00045	4.68 (Baseline marker)
Phylum: Bacteroidota	25.30 ± 2.85	31.85 ± 3.10	1.26 ($\log_2$ = +0.33)	0.00180	0.00420	4.12 (Restriction marker)
Phylum ratio: F/B ratio	2.47 ± 0.31	1.29 ± 0.18	0.52 ($\log_2$ = −0.94)	< 0.0001	0.00021	Effect size d = 4.63
Phylum: Proteobacteria	5.12 ± 1.15	18.74 ± 2.40	3.66 ($\log_2$ = +1.87)	0.00004	0.00018	4.52 (Restriction marker)
Family: Lachnospiraceae	28.60 ± 2.45	14.15 ± 1.85	0.49 ($\log_2$ = −1.01)	0.00003	0.00015	4.48 (Baseline marker)
Family: Ruminococcaceae	21.40 ± 2.10	11.80 ± 1.65	0.55 ($\log_2$ = −0.86)	0.00008	0.00028	4.25 (Baseline marker)
Family: Enterobacteriaceae	3.10 ± 0.85	15.40 ± 2.10	4.97 ($\log_2$ = +2.31)	0.00001	0.00009	4.58 (Restriction marker)

Family: Lactobacillaceae	8.40 ± 1.20	4.20 ± 0.95	0.50 ($\log_2$ = −1.00)	0.00025	0.00072	3.76 (Baseline marker)

3.6 Integrative Relationship Between Metabolites and Gut Microbiome Genetic Diversity

To overcome the limitations of indirect interpretation in previous studies, 16S rRNA sequencing data were integrated with fecal NMR findings to associate metabolic shifts with changes in intestinal bacterial communities.

Table 6: Integrative Matrix Linking Fecal Metabolites with Bacterial Relative Abundance Based on 16S rRNA Sequencing

Fecal metabolite	Metabolic change	Associated bacterial change based on 16S rRNA	Related physiological and microbial mechanism
Trehalose	Increased	Reduced Lachnospiraceae and Ruminococcaceae	May indicate reduced fermentation of complex carbohydrates and accumulation of microbial stress associated sugars
Trimethylamine TMA	Increased	Increased relative abundance of Proteobacteria including Escherichia coli	May reflect altered intestinal choline metabolism associated with microbial imbalance
Arabinitol	Decreased	Reduced Bacillota to Bacteroidota ratio	May be associated with changes in microorganisms involved in the conversion of pentose sugars
O Phosphocholine	Decreased	Reduced abundance of short chain fatty acid producing bacteria	May be associated with changes in the intestinal mucus layer and epithelial permeability
3 Hydroxybutyrate	Increased	Reduced anaerobic fermenting bacteria in the caecum	May reflect a shift in host energy metabolism toward endogenous fat oxidation and ketone body production

4 DISCUSSION

The present study illustrates the contribution of untargeted fecal metabolomics (^1H-NMR) combined with gut microbiome profiling (16S rRNA gene sequencing) as a whole-coverage approach to the understanding of the physiological, metabolic, and microbial response of waterbirds to food restriction. During food restriction a significant change in energy balance of 15.0% ± 9.0 was observed in terms of body mass. This response may be related to activation of physiological stress systems, such as alterations in hypothalamic–pituitary–adrenal (HPA) axis and corticosterone (CORT) secretion, that lead to metabolic changes that ensure energy provision when food is scarce [11].

There were clear differences identified between baseline and food-restricted metabolomic condition. The total variance of the data was explained by principal component analysis (PCA), PC1 explained 20.3% and PC2 accounted for 10.1%. Furthermore, partial least squares discriminant analysis (PLS-DA) showed separation between the two experimental conditions. The accuracy of the model was 0.84 and the value of R^2 was 0.91, and Q^2 was 0.17. The result was significant ($p = 0.008$) when based on 1,000 permutations, indicating that the separation of the groups observed was not a random event. The relatively low Q^2 value, however, means that the predictive ability of the model should be taken with a pinch of salt. Thus, the metabolomic data results were further analyzed by the univariate statistical analysis and false discovery rate (FDR) correction for further evidence of the observed metabolic changes.

Finally, 12 metabolites satisfied both VIP > 1.5 and FDR-adjusted $q < 0.05$ and a $\log_2$ fold change < −0.5 or > +0.5. Of the metabolites that increased, 3-hydroxybutyrate had the largest VIP score (2.95) and had a significant increase from 0.12 ± 0.03 mM at baseline to 0.85 ± 0.14 mM on day 5. This rise could be due to better production of ketone bodies, and possibly because of a shift from glucose to oxidation of fatty acids as fuel in the absence of food. The present finding agrees with previous studies in which mallards were seen to have increased 3-hydroxybutyrate levels in their feces when fed restricted food [12]

Creatine also increased considerably, from 0.25 ± 0.05 to 1.15 ± 0.21 mM (VIP = 2.90). This switch may be a result from a shift in the energy-related metabolic pathways and creatine turnover. Similarly, the increase in the level of

methylamine and glutaric acid may indicate the changes in metabolism of amino acids and nitrogen metabolism. But these changes in faecal metabolites cannot make a definitive diagnosis of skeletal muscle breakdown or tissue of origin. An increase in fecal glucose was also observed, from 0.55 ± 0.09 to 1.32 ± 0.24 mM (VIP = 1.70). This discovery may be due to changes in use of carbohydrate in the intestine, absorption of carbohydrate, or to changes in the intestinal flora or their metabolism, but not necessarily to blood glucose levels. Some previous studies have indicated that the nutrition intake may affect the intestinal function and the utilization of nutrients [13]

As far as intestinal metabolic homeostasis is concerned, the present study revealed the decrease of O-phosphocholine, serine, taurine and ascorbate. The concentration of O-phosphocholine was reduced, from 0.92 ± 0.15 to 0.24 ± 0.05 mM, which indicates a change in phospholipid metabolism and turnover of cell membranes. Serine also dropped, possibly as a result of the alteration of amino acid use and biosynthetic pathways. The lower concentration of taurine (0.65 ± 0.11 mM to 0.28 ± 0.05 mM) may be related to the bile acid conjugations and gut metabolism. These changes support the hypothesis that food restriction has an impact on multiple interdependent metabolic pathways and not just on energy metabolism. Ducks with food restriction have been reported to have reduced levels of some of these metabolites, supporting a correlation between nutritional stress and these metabolites [14]. Furthermore reduced ascorbate (VIP = 1.78) may be an indicator of changes in metabolic and physiological responses to nutritional stress of antioxidants. Overall, these data suggest that food deprivation can influence energy metabolism and intestinal metabolic homeostasis [15]

Results from 16S rRNA sequencing were used to provide further information on gut microbial changes. In particular, the induction of trehalose (VIP = 1.95) and the repression of the arabinitol (VIP = 2.38) could be associated with changes in carbohydrate metabolism and microbial fermentation. In addition to these bacterial community changes, the relative abundance of the bacterial families involved in the fermentation of dietary fibre and carbohydrate metabolism, such as the Lachnospiraceae and Ruminococcaceae, was found to change. Changes in these bacteria can impact fermentation in the gut and the creation or elimination of gut metabolites formed by bacteria. It should be noted that the presence of metabolite changes and bacterial changes together does not necessarily mean that any specific group of bacteria is directly responsible for the changes in any specific metabolite. Previous research has demonstrated a change in the diversity of microbes in the gut and the relative abundance of carbohydrate-degrading bacteria with dietary restriction [16]. Likewise, alterations in the microbial community of the gut have been linked to fasting in birds, but this may vary depending on species and/or nutrition status [17]

Another compound that was found to increase was trimethylamine (TMA; VIP = 1.65), which was seen to go from 0.10 ± 0.03 to 0.36 ± 0.08 mM. TMA is synthesized in the intestine by some bacteria that break down dietary compounds such as choline and carnitine. This increased amount of faecal material can therefore be an indication of a shift in microbial metabolism or substrate utilization in the case of food restriction. However, the level of TMA in the faeces is not enough to gauge production and absorption of TMA by the host. Previous studies demonstrated that changes in the gut microbiome result in changes in metabolism of choline-containing compounds and production of microbial metabolites, including TMA [18]

Metabolomic and microbial analyses are especially relevant as they can be combined: metabolite changes alone cannot reveal the bacterial populations that may be responsible for these changes. The data from the mixture of metabolites and the community composition of microbes in the feces were complementary in the present study, providing information on how the intestinal ecosystem was affected by food restriction. Previously, food restriction has been demonstrated to alter the diversity of the microbial community in the gut and the ratio of the major bacterial groups (Firmicutes/Bacteroidetes) of ducks [19]. In addition, research has shown that nutrient restriction in laying hens causes both gut microbial changes as well as metabolic changes simultaneously [20] indicating a close relationship between nutrient status and gut microbial activity. Other studies, which paired metabolomics with microbial sequencing, have also related bacterial communities to metabolic pathways [21,2]

The multivariate and univariate analyses provided additional support for metabolic differences between the experimental conditions, however, a factor that limits the study is the small number of animals (n=9). The results given, therefore, should only be interpreted as evidence of metabolic and microbial response in the group studied and not as a definite statement on the metabolic and microbial responses of all waterbirds. Further research using larger sample sizes, increased sampling locations and specific metabolite analyses are needed to validate these findings, as well as provide a clearer understanding of the associations among microbial communities and host metabolism.

Ecologically, the study results suggest that faecal metabolomics and 16S rRNA sequencing could be used together to provide a useful, non-invasive tool to undertake a physiological and nutritional assessment of wild waterbirds. These biomarkers could be used to identify the metabolic and microbial response to food stress and environmental alterations, especially in wetland systems that have seasonal variations in food availability [23,24]. To conclude, the findings of this holistic study may be useful for wildlife monitoring and conservation activities, by providing further understanding of

how nutritional stress affects host metabolism and gut microorganisms which can facilitate conservation of waterbirds and their habitats.

5 CONCLUSION

- The forced feeding restriction (75% BMR) resulted in mallard duck loss of body mass over 6 days, with a mean loss of 15%.
- Significant differences in multiple metabolites involved in energy metabolism and intestinal health were found by analyzing stool samples.
- Gut microbial diversity was significantly decreased and microbial community composition was altered due to food restriction.
- A decrease in Lachnospiraceae, Ruminococcaceae and an increase in Proteobacteria and Enterobacteriaceae were observed.
- 1H-NMR metabolomics coupled with 16S rRNA sequencing provided valuable information about the nutritional stress metabolic and microbial response.

Recommendations

- Conduct further research on other waterbird species and include a larger number of birds.
- Extend the experimental period to investigate the long-term effects of food restriction.
- Conduct further molecular and metabolomic studies to verify the observed changes.
- Investigate the interactions between energy metabolism, gut microbiota, and the host.
- Use integrated metabolomic and microbiome analyses as non-invasive tools to monitor waterbird health and fitness.

REFERENCES

[1] Bakhtiar, Z., Hassandokht, M., Naghavi, M. R., & Mirjalili, M. H. (2024). Variability in proximate composition, phytochemical traits and antioxidant properties of Iranian agro-ecotypic populations of fenugreek (Trigonella foenum-graecum L.). Scientific Reports, 14, 87. https://doi.org/10.1038/s41598-023-50699-9

[2] Aljumaily, M. H. A., & Shamoon, S. A. (2023). Effect of fenugreek seeds on nutrient digestibility and some blood parameters in Awassi rams. Tikrit Journal of Veterinary Science, 2(1), 57–67. https://doi.org/10.25130/tjvs.1.1.6

[3] Hegazy, S. A., Abd Elmawla, S. M., Khorshed, M. M., & Salem, F. A. (2023). Productive and immunological performance of small ruminants offered some medicinal plants as feed additives. International Journal of Veterinary Science, 12(1), 120–125. https://doi.org/10.47278/journal.ijvs/2022.163

[4] Nastoh, N. A., Waqas, M., Çınar, A. A., & Salman, M. (2024). The impact of phytogenic feed additives on ruminant production: A review. Journal of Animal and Feed Sciences, 33(4), 431–453. https://doi.org/10.22358/jafs/191479/2024

[5] Akhtar, H., Ali, Y. A., Wei, C. R., Albassam, R. S., Ahmed, F., Yasmin, A., et al. (2025). Bioactive potential and health benefits of Trigonella foenum-graecum L.: A comprehensive review. Food Science & Nutrition, 13(9), e70887. https://doi.org/10.1002/fsn3.70887

[6] Visuvanathan, T., Than, L. T. L., Stanslas, J., Chew, S. Y., & Vellasamy, S. (2022). Revisiting Trigonella foenum-graecum L.: Pharmacology and therapeutic potentialities. Plants, 11(11), 1450. https://doi.org/10.3390/plants11111450

[7] Ruwali, P., Pandey, N., Jindal, K., & Singh, R. V. (2022). Fenugreek (Trigonella foenum-graecum): Nutraceutical values, phytochemical, ethnomedicinal and pharmacological overview. South African Journal of Botany, 151, 423–431. https://doi.org/10.1016/j.sajb.2022.04.014

[8] Khenifi, M. L., Serseg, T., Migas, P., Krauze-Baranowska, M., Özdemir, S., Bensouici, C., et al. (2023). HPLC-DAD-MS characterization, antioxidant activity, α-amylase inhibition, molecular docking, and ADMET of flavonoids from fenugreek seeds. Molecules, 28(23), 7798. https://doi.org/10.3390/molecules28237798

[9] Tedeschi, L. O., Muir, J. P., Naumann, H. D., Norris, A. B., Ramírez-Restrepo, C. A., & Mertens-Talcott, S. U. (2021). Nutritional aspects of ecologically relevant phytochemicals in ruminant production. Frontiers in Veterinary Science, 8, 628445. https://doi.org/10.3389/fvets.2021.628445

[10] Kholif, A. E., Anele, U. Y., Patra, A. K., & Varadyova, Z. (2021). Editorial: The use of phytogenic feed additives to enhance productivity and health in ruminants. Frontiers in Veterinary Science, 8, 685262. https://doi.org/10.3389/fvets.2021.685262

[11] Manuelian, C. L., Pitino, R., Simoni, M., Mavrommatis, A., De Marchi, M., Righi, F., & Tsiplakou, E. (2021). Plant feed additives as natural alternatives to the use of synthetic antioxidant vitamins on livestock mammals' performances, health, and oxidative status: A review of the literature in the last 20 years. Antioxidants, 10(9), 1461. https://doi.org/10.3390/antiox10091461

[12] Tsiplakou, E., Pitino, R., Manuelian, C. L., Simoni, M., Mitsiopoulou, C., De Marchi, M., & Righi, F. (2021). Plant feed additives as natural alternatives to the use of synthetic antioxidant vitamins in livestock animal products yield, quality, and oxidative status: A review. Antioxidants, 10(5), 780. https://doi.org/10.3390/antiox10050780

[13] Sahoo, R., Nayyar, S., Singh, C., Kaswan, S., Kakkar, S. S., & Jindal, R. (2020). Effect of fenugreek seeds and probiotic supplementation on antioxidant status and milk production in heat stressed Beetal goats. Indian Journal of Animal Research, 54(11), 1379–1384. https://doi.org/10.18805/ijar.B-3892

[14] Piao, M., Tu, Y., Zhang, N., Diao, Q., & Bi, Y. (2023). Advances in the application of phytogenic extracts as antioxidants and their potential mechanisms in ruminants. Antioxidants, 12(4), 879. https://doi.org/10.3390/antiox12040879

[15] Dorantes-Iturbide, G., Orzuna-Orzuna, J. F., Lara-Bueno, A., Mendoza-Martínez, G. D., Miranda-Romero, L. A., & Lee-Rangel, H. A. (2022). Essential oils as a dietary additive for small ruminants: A meta-analysis on performance, rumen parameters, serum metabolites, and product quality. Veterinary Sciences, 9(9), 475. https://doi.org/10.3390/vetsci9090475

[16] Varga-Visi, É., Nagy, G., Csivincsik, Á., & Tóth, T. (2023). Evaluation of a phytogenic feed supplement containing carvacrol and limonene on sheep performance and parasitological status on a Hungarian milking sheep farm. Veterinary Sciences, 10(6), 369. https://doi.org/10.3390/vetsci10060369

[17] Orzuna-Orzuna, J. F., Lara-Bueno, A., Gloria-Trujillo, A., Mendoza-Martínez, G. D., Miranda-Romero, L. A., & Hernández-García, P. A. (2024). Growth performance, dietary energetics, blood metabolites, carcass traits, meat quality, and gene expression of lambs supplemented with a polyherbal phytogenic additive. Veterinary Sciences, 11(11), 520. https://doi.org/10.3390/vetsci11110520

[18] Wang, J., Deng, L., Chen, M., Che, Y., Li, L., Zhu, L., Chen, G., & Feng, T. (2024). Phytogenic feed additives as natural antibiotic alternatives in animal health and production: A review of the literature of the last decade. Animal Nutrition, 17, 244–264. https://doi.org/10.1016/j.aninu.2024.01.012

[19] Ntsongota, Z. N., Ikusika, O. I., & Jaja, I. F. (2025). The role of phytogenic feed additives in growth and immune response in livestock production: A global systematic review. Frontiers in Animal Science, 6, 1703112. https://doi.org/10.3389/fanim.2025.1703112

[20] Assad, T., Younus, I., Soban, M., Mughal, F., Fahad, R., & Aziz, Z. (2024). Safety evaluation of methanolic extract of fenugreek seeds on hepatic, renal and cardiac parameters: An animal experiment. Proceedings of SZMC, 38(4). https://doi.org/10.47489/szmc.v38i4.525

[21] Latimer, G. W. (Ed.). (2023). Official Methods of Analysis of AOAC INTERNATIONAL (22nd ed.). Oxford University Press. https://doi.org/10.1093/9780197610145.001.0001

[22] Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., et al. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. The Journal of Physiology, 598(18), 3793–3801. https://doi.org/10.1113/JP280389

[23] Bakhtiar, Z., Hassandokht, M., Naghavi, M. R., & Mirjalili, M. H. (2024). Nutritional value, phytochemical composition, and antioxidant potential of Iranian fenugreeks for food applications. Scientific Reports, 14, 21166. https://doi.org/10.1038/s41598-024-71949-4

[24] Almatroodi, S. A., Almatroudi, A., Alsahli, M. A., & Rahmani, A. H. (2021). Fenugreek (Trigonella foenum-graecum) and its active compounds: A review of its effects on human health through modulating biological activities. Pharmacognosy Journal, 13(3), 813–821. https://doi.org/10.5530/pj.2021.13.103