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RESEARCH ARTICLE

Dose-dependent Role of Natural Betaine on Gut Morphometry, Cecal Microbiota, and Egg Production in Backyard Golden Misri Hens

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The study aimed to investigate gut health-based production performance of backyard Golden Misri laying hens upon supplementing natural betaine at 0 (Control), 0.34 (Low betaine), and 0.68g (High betaine) natural betaine/kg body weight. A total of 150 hens (25±1 weeks) were equally assigned to these groups, with 5 replicates each containing 10 birds. After 7h daily scavenging, birds were offered commercial concentrate and water containing respective betaine doses. Hens in both betaine treatments increased feed intake, and Low betaine improved egg production, egg mass, and eggshell strength relative to Control (P<0.05). The jejunal villus height: crypt depth ratio showed a higher tendency for Low betaine relative to High betaine (P=0.07). By employing 16S rRNA gene sequencing, both betaine treatments indicated a significant increase in microbial alpha diversity parameter Sobs (P=0.02) and a similar trend for Chao1 (P=0.06). The genus *Desulfovibrio* proliferated at both betaine doses (P=0.04), and *Parabacteroides* (P=0.02) and *Odoribacter* (P=0.09) only in the High betaine group in comparison to the Control. Low-dose natural betaine is more suitable for ensuring health of the early gut compartments and production status, while High dose has more pronounced effects in later parts of the gut like ceca. In conclusion, Betaine water containing 0.34g/kg body weight is suggested for backyard hens during captivity.

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INTRODUCTION

Backyard poultry is instrumental in supporting the rural agriculture economy and combating food security challenges, especially in developing and under-developed countries. While reared with less investment and low infrastructure, it adapts well to the local environmental conditions, thereby, providing additional support during challenging times as has been observed during COVID 19 pandemic (Kausar *et al*., 2024). Unlike commercial poultry, these birds are exposed to climate, health, and diet-related stressors including extreme ambient temperature, lack of bio-security, non-standardized diet, and inconsistent water quality (Singh *et al*., 2022; Kefale *et al*., 2023; Nayak *et al*., 2024). It may jeopardize gut health, including microbial population (Choct, 2009) and epithelial integrity, both of which play a critical role in gut digestive and absorptive processes, ultimately driving production status and product quality. Among poultry gut, jejunum provides the primary site for absorption (Sklan and Noy, 2023), while ceca harbors highest microbial population (Xu *et al*., 2016). In a highly coordinated way, cecal microbiota performs various useful functions like nutrient degradation, epithelial integrity, immune function, micro-nutrient biosynthesis, and pathogen exclusion (Shang *et al*., 2018; Hou *et al*., 2020). Ceca allow digesta to stay for a longer time than any other part of the gut, thereby giving bacteria sufficient time to extract nutrients from the material that is nearly wasted and will shortly pass by. Keeping in view the expected and unexpected threats to the gut health of backyard poultry, it is imperative to facilitate nutritional interventions.

Betaine - a tri-methylated zwitterion - has the potential to protect cecal bacteria and epithelium from the hazardous effects of unknown challenges under extensive rearing systems. This is due to the diverse chemical properties of betaine, especially in its natural form which is more beneficial for poultry birds than its synthetic form (Awad *et al*., 2022). Under normal conditions, bacteria degrade betaine to acetate, which is an important energy-providing a substance (Bose *et al*., 2019). During stressful conditions, it is up-taken by epithelial cells and gut bacteria to maintain their cell turgor, as betaine is a potent organic osmolyte (Ratriyanto and Mosenthin, 2018). Due to being a methyl donor, betaine aids in sparing methionine and choline for their basic functions which otherwise can be compromised due to the shift of these compounds to osmoregulatory functions (Mahmoudi *et al*., 2018; Park and Kim, 2019; Gregg *et al*., 2022). Natural betaine also acts as molecular chaperone by stabilizing the molecular structure, and repair the intestinal barrier functions in broilers which were impaired during stressful conditions (Liu *et al*., 2021). All this information translates into improved production performance in broiler birds as indicated by highest weight gain and feed conversion ratio observed upon feeding betaine as anti stressor (Asad *et al*., 2019). The beneficial effects of betaine on gut microbiota have already been well explained for microbiota and fermentation in earlier parts of the gut like the rumen of cattle (Mahmood *et al*., 2022a, b) and the small intestine of pig (Metzler-Zebeli *et al*., 2009). However, microbiota assessment in the last parts of the gut like the ceca of backyard poultry by employing 16S rRNA gene sequencing is lacking. 16S rRNA gene sequencing appears as most promising, accurate, and reliable tool than others for quantifying bacteria and defining their taxonomic relationships (Petti *et al*., 2005). Moreover, as betaine is highly degradable in the gut environment (Mahmood *et al*., 2020a), it is required to ascertain whether betaine reaches the cecum to benefit the cecal microbiota or is used/degraded in the upper gut parts. Therefore, a higher dose is also tested in this study. In short, this study addresses the problem of low production in backyard poultry. Therefore, we aimed to explore egg production, egg quality, jejunal villi characteristics, and diversity and composition of caecal microbiota in response to feeding natural betaine at 0.34 and 0.68g/kg body weight in backyard Golden Misri hens.

MATERIALS AND METHODS

The animal ethics statement: The trial was performed at the Free-Range Poultry Unit of the University of Veterinary and Animal Sciences (UVAS), Jhang campus from March-April 2024. The study was undertaken in compliance with the institutional guidelines of the Ethical Review Committee, office of research, innovation, and commercialization, UVAS, Lahore, Pakistan (No. DR/408).

Housing management, treatments, and study design: A total of 150 laying egg-laying hens of the Golden Misri breed (average weight 950±50g, age 25±1 weeks) were randomly divided into three groups: Control, Low betaine, and High betaine. Hens in each group were equally placed into 5 replicates with each replicate containing 10 hens in a 3×5×10 design. While reared for a total period of 9 weeks with the first 3 weeks of adaptation, all hens were daily provided 7h outdoor scavenging period from 09:00-16:00 in alfalfa fields. During the rest of the time, the birds were captivated into their respective pens and offered commercial concentrate layer feed (Table 1). A lighting schedule of 16h light:8h dark was followed throughout the trial. The birds had free access to drinking water in their pens. Hens in treatment groups Control, Low betaine, and High betaine received 0, 0.34, and 0.68g natural betaine/kg body weight, respectively, in drinking water at night. Natural betaine (Actibeet® 97 containing 97% natural betaine) was obtained from AGRANA Sales & Marketing GmbH, Vienna, Austria. Respective betaine doses were started after the first week of the trial.

Table 1: Nutrient composition of commercial layer feed

Nutrient (% Dry matter until mentioned)	Composition
Dry matter (%)	89
Metabolisable Energy (KCals/kg)	2609
Crude protein	16.97
Crude Fiber	3.07
Ether Extract	3.42
Calcium	4.4
Phosphorus	0.62
Lysine	1.1
Methionine	0.55
Tryptophan	0.18
Cystine	0.28
Methionine+Cystine	0.83

Sampling and measurements for production performance: During the last 6 weeks, eggs were collected daily at 07:00 in the morning. The data for water consumption (mL), feed consumption (g), average egg production (%), and average egg weight (g) were recorded daily from each replicate. Water consumption and feed intake were calculated by the difference in the respective amount offered and refused. The birds in Low betaine and High betaine ultimately consumed 0.32 and 0.65g/kg body weight of natural betaine, respectively. Egg weight was determined with the help of a digital weighing balance of 0.01g precision level. Egg mass (g/day) was calculated by multiplying the average egg production with average egg weight.

External and internal egg quality attributes were determined following the procedures described by Rath *et al*. (2015), Liu *et al*. (2017), and Novotny *et al*. (2022). On the last day of the trial, 3 eggs were randomly collected from each replicate (n=15 per individual treatment). Eggshell strength was measured by using an egg force reader (ORKA Food Technology, Bountiful, Utah, USA). Shortly after separating from the broken eggs, the shells were washed, dried, and subsequently weighed to determine eggshell weight (g). Subsequently, eggshell ratio (%) was calculated by multiplying the ratio of egg shell weight and egg weight with 100. The yolk color was judged based on the score obtained after matching the yolk with one of the 15 bands of the "1961, Roch Improved Yolk Color Fan" (Bulbul *et al*., 2014). The Yolk index was calculated by multiplying the ratio of yolk height and yolk width by 100, and Haugh unit (HU) by the formula HU=100 Log (H+7.57–1.7W37), where H is albumin height (mm) and W= weight of egg. The albumin height, yolk width, and yolk height were measured (mm) by using a Vernier caliper.

Sampling and measurements for histomorphometry: At the end of the trial, 2 hens from each replicate across all treatments were randomly slaughtered after providing 12-hour feed withdrawal period. After slaughter, 1cm proximal intestinal tissue samples from the jejunum (n=10/group) were cut in cross-section and subsequently fixed in freshly prepared 10% neutral buffered formalin (NBF) for histomorphometric study. Jejunal tissue samples were subjected to histology processing procedures mentioned in Bancroft and Gamble (2008) in a histopathology laboratory. Briefly, after overnight washing, tissue samples were dehydrated with a series of ascending concentrations of ethyl alcohol, then cleared in xylene and embedded in soft Paraffin wax. Paraffin-embedded tissue blocks were sectioned into 5µm thick slices using a manual rotatory microtome. The sections were made free paraffin-free and stained with standard hematoxylin and eosin stains. Images of tissue sections were taken with a trinocular microscope Mcx50 (Micros, Austria) equipped with a Canon Camera. Villus height (VH) and crypt depth (CD) were measured by using Image J software (version 1.54) and the VH:CD ratio was also calculated.

Sampling and analysis for cecal microbiota: For microbiota analysis, cecal digesta was collected from 5 hens across 4 replicates (randomly selected) of each treatment group. Soon after collection, cecal digesta was snap-frozen in liquid nitrogen and later preserved at -40°C for subsequent microbial analysis. At the time of microbial analysis, the cecal digesta across all 05 hens from the same replicate was pooled together. Extracted DNA from the pooled samples was subjected to 16s rRNA gene sequencing. The procedures described by Mahmood *et al.* (2020a) were followed. Paired end V3-V4 amplicon sequencing was performed through universal primer set 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) using Illumina MiSeq. Read quality was evaluated using reader software (v1.0), and low-quality reads with ambiguous bases were removed using cutadapt (v.2.6). Paired-end sequencing data were merged using FLASH software (v1.2.11), and the effective tags were obtained by removing chimera sequences. Tags were clustered to OTU (Operational Taxonomic Unit) by USEARCH (v7.0.1090) at 97% similarity. The taxonomic classification of the OTU against the Silva database (138 release) was performed using the RDP classifier (v2.2). The reads clustered into 950 common and 53 unique OTUs (Fig. 1). Among alpha diversity metrics Sobs (Species Observed), Chao1, Shannon and Simpson, ACE (abundance coverage estimator), and Good's Coverage were evaluated. Beta diversity based on the Bray-Curtis dissimilarity matrix was calculated in QIIME-2 (v1.80) and visualized via principal component analysis.

Statistical analysis: Data were analyzed under a completely randomized design (CRD) using the one-way Analysis of Variance (ANOVA) through SPSS (version 21). The reported mean values are represented by least square means ± pooled standard error of the mean (SEM). The significant means were separated by Tukey's HSD, and the $P \leq 0.05$ denotes the significance of an effect, and the tendency of the effects is manifested by $0.05 < P \leq 0.10$.

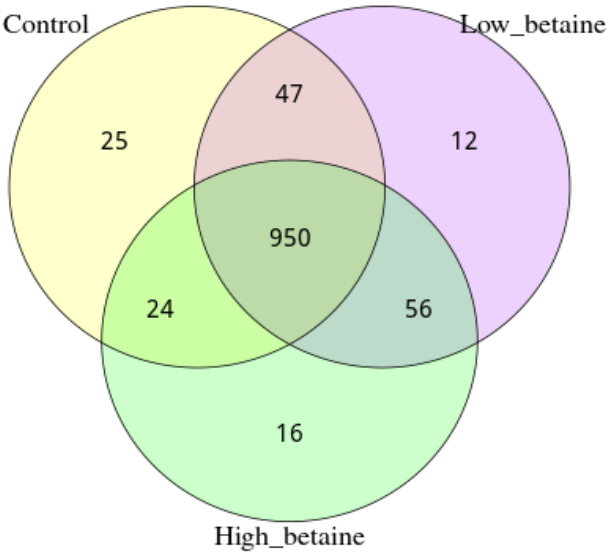

Fig. 1: Venn diagram to represent common and unique OTUs of backyard Golden Misri hens among treatments Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight; High Betaine: 0.68g betaine/kg body weight)

RESULTS

Production performance and egg quality characteristics: The data on feed intake, egg production, and egg quality as affected by treatments have been shown in Table 2. Hens in both the Low betaine and the High betaine groups exhibited significantly higher feed intake relative to hens in the Control group ($P<0.05$). Average egg production and egg mass were significantly elevated by hens in the Low betaine group than hens in the High betaine group and the Control group, respectively ($P<0.05$). No treatment effect was observed for the High betaine group regarding eggshell characteristics. However, a 14.15% reduction in egg ratio and a 7.28% increase in eggshell strength were found for Low betaine than Control ($P<0.05$). The yolk color index was significantly lowered only for the High betaine than both other groups ($P<0.05$), and no treatment effect was found for the yolk index. Despite no overall treatment effect on HU, eggs obtained from the Low betaine group significantly increased albumin width than those of both other groups ($P<0.05$).

Histomorphometric and histopathological characteristics of Jejunum: Jejunal histomorphometry analysis revealed that the treatments Low betaine and High betaine did not affect these parameters when compared with Control (Table 2) (Fig. 2). The villus height remained unaffected by treatments except for only a numerical decrease in case of Low betaine and numerical increase in case of High betaine group as compared to Control ($P>0.05$). The variation existed between betaine treatments for CD, which was significantly lower for the Low betaine than the High betaine ($P<0.05$). It resulted in an increasing trend of VH:CD for the Low betaine than the High betaine ($P<0.1$). Both of the betaine groups showed only numerical differences relative to Control group.

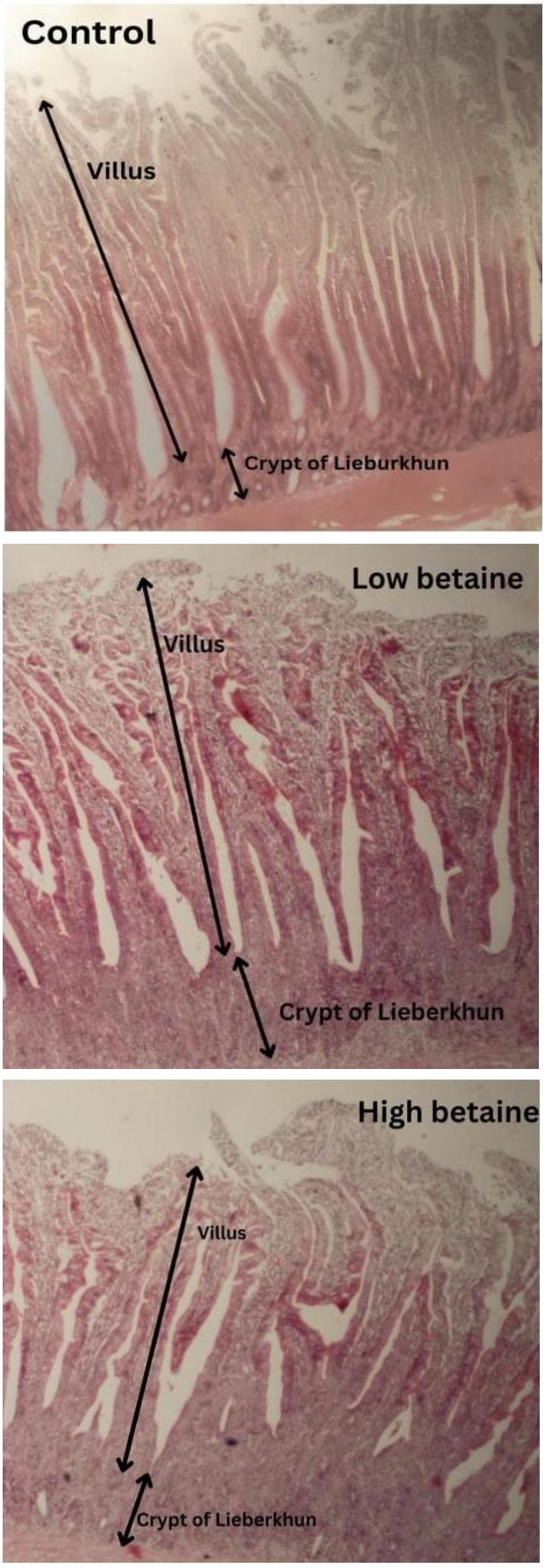

Fig. 2: Histomorophology of jejunal villi of backyard Golden Misri hens offered either Control (no betaine), Low betaine (0.34g betaine/kg body weight) or High betaine (0.68g betaine/kg body weight). Sections were stained with hematoxylin and eosin. The image acquisition phase was done with a 10x magnification objective.

Table 2: Production performance, egg quality, and jejunal histomorphometric characteristics of backyard Golden Misri hens as affected by treatments*

Parameter	Control	Low Betaine	High Betaine	SEM	P-value
Production and quality parameters					
Water intake (mL/d)	96.9	96.6	97.4	0.51	0.96
Feed Intake (g/d)	65.6^{b}	77.3^{a}	81.9^{a}	2.19	< 0.01
Egg Production (%)	37.0^{a}	45.8^{b}	39.7^{ab}	2.49	0.04
Egg mass (g/hen/d)	18.3^{b}	22.7^{a}	19.0^{ab}	1.27	0.03
Eggshell ratio (%)	13.4^{a}	11.5^{b}	12.2^{ab}	0.37	< 0.01
Eggshell strength (N)	37.0^{b}	45.8^{a}	39.7^{ab}	2.49	0.04
Yolk color index (%)	8.8^{a}	8.9^{a}	7.7^{b}	0.29	< 0.01
Yolk Index (%)	38.7	43.2	43.8	0.19	0.2
Albumin height (mm)	7.2	7.2	7.4	0.44	0.92
Albumin width (mm)	71.1^{b}	79.7^{a}	73.2^{b}	1.08	< 0.01
Haugh unit	87.9	89.7	90.9	2.45	0.23
Histomorphometric characteristics					
Villus height (µm)	760.5	740.6	771.0	52.53	0.66
Crypt Depth (µm)	175.2^{ab}	157.7^{b}	190.5^{a}	17.45	0.01
^{1}VH: CD	4.5^{xy}	4.8^{x}	4.2^{y}	0.57	0.07

*Treatments include Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight and High Betaine: 0.68g betaine/kg body weight. Mean values in a row with different superscripts (a-c) indicate significant difference (denoted by P≤0.05) from one another. Mean values in a row with different superscripts (x-z) indicate the tendency of difference (denoted by 0.05≤P≤0.1) from one another. SEM=Standard error of mean. 1Villus height: Crypt Depth.

Among jejunal histopathological changes, the sloughing of columnar epithelial cells lining the walls of the villi can be clearly seen at the apex of the villi. Conversely, in treated groups, intact columnar epithelial cells can be observed (Fig. 2). Bases of villi are seen attached with crypts in all treatment groups. Furthermore, no infiltration of inflammatory cells was observed.

Cecal microbiota

Alpha and Beta diversity: The alpha diversity indices of bacterial population Sobs and Chao1 were improved upon betaine addition (Table 3). Both Low betaine and High betaine significantly raised Sobs (Species Observed) at the rate of 5% in comparison to Control group (P=0.02). There was an increasing tendency for Chao 1 (p=0.06) relative to Control. Chao1 measures the minimum number of observed species taking into account, the singltones and doubletones. In comparison to Control group, Low betaine and High betaine indicated 4 and 6% higher values of Chao1, respectively. The other diversity indicators ACE and Shannon index showed numerical increments upon addition of betaine. The Simpson index and Good's coverage remained totally unaffected upon betaine addition (P>0.05).

Beta diversity which compares the diversity between samples, based on Bray Curtis distance showed no clear separation between groups indicating lower differences between bacterial composition of the treatment groups. As samples within each group were farther distanced, it indicates an intra-individual inherent variability of cecal microbiota (Fig. 3).

Core microbiota composition and differential analysis: Independent of the treatment effect, more than 90% of the bacteria in the cecal environment belonged to only two major phyla Bacteroidetes and Firmicutes. The other prominent phyla included Proteobacteria, Synergistetes, Spirochaetes, and others, in the order of their relative abundances (Fig. 4). Except for Actinobacteria, which

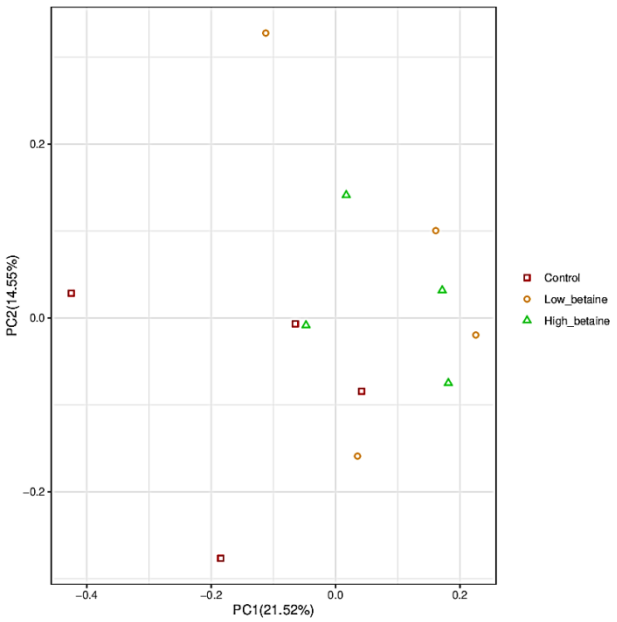

Fig. 3: Principal components analysis (PCA) for beta diversity of cecal bacteria of backyard Golden Misri hens as affected by betaine supplementation (Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight; High Betaine: 0.68g betaine/kg body weight).

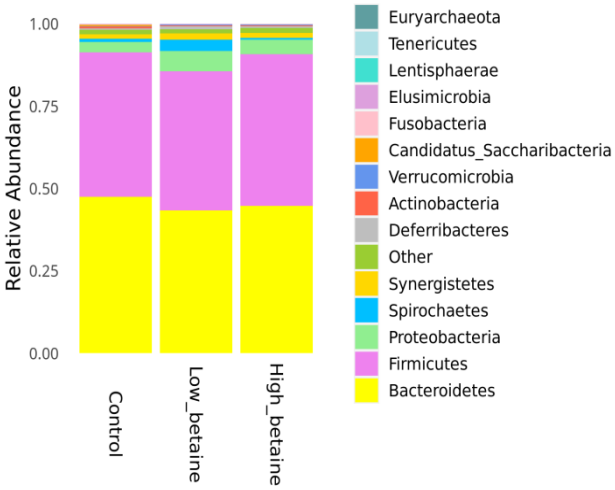

Fig. 4: Relative abundances of bacterial phyla of pooled DNA from cecal digesta contents of backyard Golden Misri hens as affected by betaine supplementation (Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight; High Betaine: 0.68g betaine/kg body weight). Only Actinobacteria showed a decreasing tendency: the tendency is manifested by 0.05≤P≤ 0.1.

Table 3: Alpha diversity measures of the bacterial community in ceca of backyard Golden Misri hens affected by treatments as determined using QIIME and 16S rRNA sequences*

Parameters	Control	Low betaine	High betaine	SEM	P-value
Sobs	775.2^{b}	813.0^{a}	815.5^{a}	9.55	0.02
Chao1	888.6^{y}	920.7^{x}	941.4^{x}	13.47	0.06
ACE	891	933	913	12.43	0.18
Shannon Index	4.9	4.9	5.0	0.05	0.25
Simpson Index	0.01	0.01	0.01	0.001	0.30
Good's Coverage	0.9	0.9	0.9	0.028	0.63

*Treatments include Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight and High Betaine: 0.68g betaine/kg body weight. Mean values in a row with different superscripts (a-c) indicate significant differences (denoted by P≤0.05) from one another. Mean values in a row with different superscripts (x-z) indicate the tendency of difference (denoted by 0.05≤P≤0.1) from one another. SEM=Standard error of mean.

indicated a decreasing tendency upon the addition of natural betaine at both levels (P=0.08), no change in relative abundance was observed at the phylum level (Supplementary Table S1).

At the genus level, the taxa *Desulfovibrio*, *Parabacteroides*, and *Odoribacter* responded to betaine addition (Table 4). The relative abundance of *Desulfovibrio* represented by its species *Desulfovibrio piger* (Fig. 5) (Supplementary Table S1) was significantly increased by both Low betaine and High betaine groups than Control (P=0.04). Only the High betaine group significantly increased the population of the genus *Parabacteroides* (P=0.02) and showed an increasing tendency for the genus *Odoribacter* (P=0.09) represented by its species *Odoribacter laneus*. Low betaine could not affect these taxa at both genus and species levels. At the species level, both betaine treatments significantly increased the relative abundance of *Bacteroides plebeius* at the expense of *Bacteroides salanitronis* (Fig. 5) (Supplementary Table S1) (P<0.05). No treatment effect was observed for the taxonomy of the major genera including *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Ruminococcus*, and *Paraprevotella* (P>0.05).

Table 4: Community structure in terms of relative abundance (%) of cecal bacteria at genus level in backyard Golden Misri hens as affected by treatments*

Parameter	Control	Low Betaine	High Betaine	SEM	P-value
Bacteroides	19.0	21.0	21.0	1.85	0.50
Faecalibacterium	9.6	7.5	8.2	1.06	0.40
Prevotella	5.6	1.9	2.6	1.64	0.29
Ruminococcus	2.9	2.2	2.1	0.73	0.69
Intestinimonas	1.9	2.0	2.2	0.35	0.89
Paraprevotella	1.9	1.7	2.7	0.60	0.50
Desulfovibrio	1.2^{b}	2.6^{a}	2.9^{a}	0.40	0.04
Megamonas	1.3	0.3	0.6	0.64	0.52
Pseudoflavonifractor	1.0	2.9	2.7	0.84	0.54
Cloacibacillus	1.2	.1.9	1.4	0.25	0.20
Parabacteroides	1.0^{b}	1.1^{b}	1.8^{a}	0.15	0.02
Clostridium_XlVb	1.1	0.6	0.7	0.49	0.76
Clostridium_IV	0.1	0.7	0.9	0.14	0.40
Oscillibacter	0.8	0.7	0.9	0.05	0.19
Clostridium_XlVa	0.7	0.7	0.9	0.14	0.40
Flavonifractor	0.7	1.2	1.2	0.23	0.34
Mucispirillum	0.5	0.9	0.5	0.28	0.56
Odoribacter	0.3^{y}	0.4^{xy}	0.8^{x}	0.13	0.09
Brachyspira	0.2	2.8	0.1	1.42	0.35
Escherichia	0.04	1.7	0.1	0.90	0.35
Other	48.9	47.3	45.7	1.89	0.62

*Treatments include Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight and High Betaine: 0.68g betaine/kg body weight. Mean values in a row with different superscripts (a-c) indicate significant difference (denoted by P≤0.05) from one another. Mean values in a row with different superscripts (x-z) indicate tendency of difference (denoted by 0.05≤P≤0.1) from one another. SEM=Standard error of mean.

DISCUSSION

The current study evaluated histological assessment, cecal microbiota, and production performance of backyard Golden Misri hens in response to feeding natural betaine at 0.34 and 0.68g natural betaine/kg body weight.

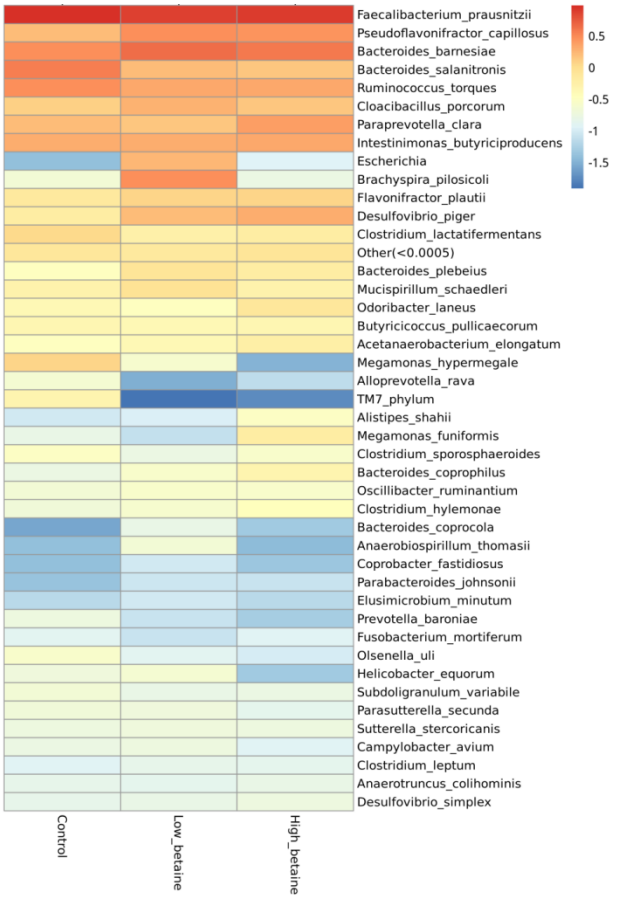

Fig. 5: Relative abundances of bacterial species of pooled DNA from cecal digesta contents of backyard Golden Misri hens as affected by betaine supplementation (Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight; High Betaine: 0.68g betaine/kg body weight). The color scheme represents different microbial abundances among treatments: Highest to lowest values are represented by dark red to dark blue color. Significance among treatments is denoted at P≤0.05 and it existed for *Bacteroides plebeius*, *Desulfovibrio piger*, and *Bacteroides salanitronis*, whereas, the tendency was marked as 0.05≤P≤0.1 and it existed for *Odoribacter laneus*.

Supplementary Table S1: Community structure in terms of relative abundance (%) of cecal bacteria at phylum and species level in backyard Golden Misri hens as affected by treatments*

Parameter	Control	Low Betaine	High Betaine	SEM	P-value
Phylum					
Bacteroidetes	47.1	43.0	43.2	2.6	0.56
Firmicutes	43.0	42.0	42.0	1.9	0.74
Proteobacteria	3.2	6.0	6.2	0.3	0.15
Spirochaetes	0.9	3.2	3.2	0.2	0.39
Synergistetes	1.2	2.0	2.0	0.2	0.19
Deferribacteres	0.5	0.9	0.9	0.2	0.56
Actinobacteria	0.4a	0.2b	0.2b	0.1	0.08
Verrucomicrobia	0.1	0.2	0.2	0.01	0.61
Candidatus_Saccharibacteria	0.05	0.01	0.01	0.05	0.37
Fusobacteria	0.1	0.08	0.08	0.01	0.88
Elusimicrobia	0.06	0.09	0.09	0.01	0.80
Lentisphaerae	0.03	0.02	0.02	0.001	0.11
Tenericutes	0.02	0.01	0.01	0.001	0.91
Others	3.3	2.2	1.9	0.3	0.84
Species					
Faecalibacterium prausnitzii	9.6	7.5	8.2	1.5	0.40
Bacteroides barnesiae	3.0	4.4	3.8	1.1	0.47
Pseudoflavonifractorcapillosus	1.6	2.9	2.7	0.3	0.54
Ruminococcus torques	2.9	2.1	2.1	0.8	0.69
Bacteroides salanitronis	3.8^{a}	1.6^{b}	1.4^{b}	0.6	0.01
Intestinimonas butyriciproducens	1.9	2.0	2.2	0.4	0.89
Paraprevotella clara	1.6	1.4	2.5	0.4	0.43
Cloacibacillus porcorum	1.2	1.9	1.4	0.2	0.20
Flavonifractor plautii	0.7	1.2	1.2	0.1	0.34
Desulfovibrio piger	0.6^{b}	1.6^{a}	2.0^{a}	0.01	0.01
Clostridium lactatifermentans	1.0	0.5	0.6	0.08	0.74
Mucispirillum schaedleri	0.5	0.9	0.5	0.2	0.56
Brachyspira pilosicoli	0.2	0.2	0.1	0.01	0.35
Bacteroides plebeius	0.3^{y}	0.8^{x}	0.6^{x}	0.001	0.07
Odoribacter laneus	0.4^{y}	0.3^{y}	0.8^{x}	0.001	0.09
Escherichia	0.04	0.1	0.1	0.001	0.35
Other	70.6	70.6	69.8	0.025	0.31

*Treatments include Control: 0g betaine/kg body weight; Low Betaine: 0.34g betaine/kg body weight and High Betaine: 0.68g betaine/kg body weight. Mean values in a row with different superscripts (a-c) indicate significant difference (denoted by P≤0.05) from one another. Mean values in a row with different superscripts (x-z) indicate tendency of difference (denoted by 0.05≤P≤0.1) from one another. SEM=Standard error of mean.

Efficacy of betaine at low dose in backyard poultry: In the current study, betaine-fed birds at low doses significantly out-competed the birds reared without dietary betaine in terms of laying performance, egg quality, and jejunal mucosa characteristics. This is evident from the higher egg production, egg mass, eggshell strength, albumin width, and VH:CD of the jejunum obtained with the low dose. Thus, backyard poultry responded to betaine in a similar way as commercial poultry which is reported by previous studies. Irrespective of the variation in type of poultry, rearing conditions, and level of betaine used, many researchers witness similar findings. For example, an elevated laying rate upon betaine supplementation was documented in the commercial layer (Omer *et al*., 2020) and broiler breeder hens (Rokade *et al*., 2020) upon dietary betaine addition. In another study by Du *et al*. (2025), addition of betaine at 3000 mg/kg increased laying rate at different stages of hens' life. Similarly, in replacement to dietary choline, betaine improved the egg mass of the commercial laying hens (Zaki *et al*., 2023). Shin *et al*. (2018) found 11% higher eggshell strength for the eggs of the laying hens receiving betaine in diet. Norouzian *et al*. (2018) documented higher VH:CD for jejunal mucosa in broiler and Awad *et al*. (2022) reported low CD upon betaine supplementation in both natural and synthetic forms. The best explanation seems to be that betaine promotes the secretion of triglycerides from the liver (Zhang *et al*., 2019) and enhances serum estradiol levels (Egbuniwe *et al*., 2021). Triglycerides are fundamental for yolk formation (Omer *et al*., 2020), while estradiol is important for producing yolk precursor substances and supporting reproductive functions (Mehlhorn *et al*., 2022). Since yolk formation is a crucial step in egg formation, betaine supplementation might have ultimately supported increased egg production. It is also in line with our results of histomorphometry and cecal microbiota. The higher VH:CD ratio, although only numerically in the current study, is a potential indicator of greater absorptive capacity of the gut (Silva *et al*., 2009; Hamedi *et al*., 2011). It indicates that a considerable part of a low dose of betaine has possibly been used for osmoregulatory functions of the jejunal enterocytes which is one of the primary functions of betaine. It might have reduced crypt activity to shift the energy towards production rather than cell proliferation and differentiation. In agreement, Al Sulaiman *et al*. (2024) reported a higher jejunal VH and VH:CD in Ross broilers upon betaine supplementation. It suggests that addition of betaine improves gut absorption rate which might have

contributed to elevated egg production potential. This is due to the fact that increased villus height indicates mature enterocytes and high absorptive capacity (Yu *et al*., 2024). As sloughing of epithelium was also reversed by Low betaine, it is speculated that betaine improved the intactness of jejunal villi for better absorption. The non-intact villi in Control group were might be related to scavenging activity threats in the absence of osmoprotective substance like betaine. In agreement, Song *et al*. (2021) documented higher intestinal barrier integrity as compared to birds which did not receive betaine. We found that adding betaine supported the growth and richness of cecal bacteria indicated by elevated alpha diversity parameters Sobs and Chao1. It is either attributed to the usage of betaine as an energy substrate or organic osmolyte by the bacterial population, in both cases facilitating the flow of nutrients to the body. Exclusive proliferation of *Desulfovibrio* points out that betaine is principally used as an energy substrate rather than osmolyte, as members of this genus are previously known for degrading betaine to acetate and trimethylamine (Bose *et al*., 2019). Acetate is the predominant short chain fatty acid used for energy provision to the birds after absorption (Sergeant *et al*., 2014). Thus, all these in-line explanations might have cumulatively contributed to the efficacy of the low dose supplementation of betaine, ultimately supporting the productivity of backyard poultry.

Efficacy of betaine at high dose in backyard poultry: We found that the effects of betaine on backyard poultry performance were not dose dependent except for cecal microbiota. The efficacy of high-dose varied in different gut compartments. Although, no major negative outcome was observed, nevertheless, a high dose of betaine could not benefit the level of the low dose and moderately reduced a few performance indicators in earlier parts of the gut. A numerically higher CD and lower VH: CD ratio was the concerning finding noticed for a high dose of betaine, for instance, a high dose in our study was equivalent to 6 times more than that used by Awad *et al*. (2022). The possible explanation for slightly deeper CD can be the inflammatory response (Haschek *et al*., 2010; Belote *et al*., 2023) due to very high betaine concentration. As VH remained unchanged, compromised absorption may not be the case for high dose. But deeper CD may indicate higher energy loss in maintaining gut integrity than shifting to production (Qaisrani *et al*., 2019; Shi *et al*., 2024), and it was evident by the lower effect of high dose than low dose on improvement in egg production. However, like Low betaine, High betaine also corrected the villi structure. The benefits of the high dose were observed in later parts of the gut i.e., ceca where it surpassed the effects of the low dose. In addition to benefiting bacterial populations similar to that of low dose, effects of high dose were more pronounced on bacterial profile. The genera *Odoribacter* and *Parabacteroides* exclusively thrived by high doses of betaine. Data regarding these taxa in poultry is lacking, however, Ephraim and Jewell (2020) reported a higher relative abundance of genus *Odoribacter* in dog feces on feeding betaine with fibrous diet. Similarly, Sun *et al*. (2019) found a higher abundance of *Parabacteroides* in the gut of mice offspring when betaine was supplemented maternally. The members of the genus *Odoribacter* are specialists in butyrate production which is responsible for strengthening colonocytes (Ephraim and Jewell, 2020), whereas, *Parabacteroides* play a role in fiber degradation (Cui *et al*., 2022). Reimer *et al*. (2021) reported their higher abundance in humans in response to enhancing dietary fiber. Moreover, Pardo *et al*. (2023) documented a positive relationship of dietary betaine with butyrate production, which might be due to the support provided by betaine to *Odoribacter* and similar butyrate specialist bacteria. In another study, Chen *et al*. (2023) found a correlation of cecal *Parabacteroides* in poultry with abdominal fat deposition. Thus, it can be stated with confidence that high betaine addition may have increased absorptive capacity and flow of nutrients in the ceca. But as it was not translated into a significant effect on production performance, it is obvious that the benefits of a high dose in ceca were outweighed by its negative impact in jejunum which is a major absorptive site than ceca (Proszkowiec-Weglarz, 2022). The slight negative effect of betaine at the jejunum coupled with a positive impact at the cecum suggests that the administered dose was higher than the optimum. However, by the time it reached the cecum, it remained at desirable level due to partial usage in earlier gut parts. Another change in microbial community at the specie level was observed exclusively with high doses where *Bacteroides plebeius* proliferated at the expense of *Bacteroides salanitronis*. Marcolla *et al*. (2023) stated these are part of the core microbiota of poultry. The comparative role of both these species is not yet clear in poultry, however, it can be interpreted that betaine may support the metabolic pathways which are more favorable for *Bacteroides plebeius* than *Bacteroides salanitronis* or the earlier may have a higher capacity to metabolize betaine.

The above discussion provides insight that a low dose of betaine is safer for the early gut compartments but may not be adequate to achieve a full modulatory effect on cecal microbiota. Despite of being available in a cecal environment, it vanishes earlier. Conversely, the higher dose provides sufficient support for cecal microbiota, as proved by its broader impact on microbial groups.

Conclusions: The taxonomy of microbial population in ceca of backyard poultry is similar to that of commercial poultry. Natural betaine has a dose-dependent modulatory effect on gut health. The dose of betaine is determined by the target purpose. Low betaine dose is more suitable for ensuring health of the early gut compartments ensuring integrity of histomorphological and histopathological integrity, while High dose has more pronounced effects in later parts of the gut like ceca and its commensal microbiota. In conclusion, natural betaine at a dose rate of 0.34g/kg body weight is suggested to be executed in backyard poultry operations to enhance the productivity of the backyard laying hens. Further research is required to validate these findings in other backyard poultry breeds to enhance the role of backyard poultry in the agricultural economy.

Supplementary materials: Supplementary Table S1. Community structure in terms of relative abundance of cecal bacteria at phylum and species level in backyard Golden Misri hens as affected by treatments:

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Authors contribution: MM, KR, AG, and SN conceived and designed the study. KR, MKY, MM, and AR executed the experiment and analyzed the egg quality and tissue samples. RN, EU, and AR analyzed the data. MM and KR wrote the original draft. All authors interpreted the data, critically revised the manuscript for important intellectual contents, and approved the final version. AG, KR, and RN curated data. SN, AG, AR, and EU played a role in visualization.

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