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Effects of microalgae, with or without xylanase supplementation, on serum immunoglobulins, cecal short-chain fatty acids, microbial diversity, and metabolic pathways of broiler chickens

Pravin Mishra, Razib Das, Ajay Chaudhary, Birendra Mishra, and Rajesh Jha[1]

Department of Human Nutrition, Food and Animal Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, HI 96822, USA

ABSTRACT Modern broilers are highly susceptible to environmental and pathogenic threats, leading to gut disorders and poor nutrient utilization if not managed properly. Nutritional programming using several feedstuffs and coproducts to manage gut health has been studied. This study used microalgae as a functional compound and xylanase enzyme in broilers' diets as a strategy to manage gut health. A total of 162 one-day-old unsexed Cobb 500 broiler chicks were randomly assigned to 1 of the 3 dietary treatments: a) corn-soybean meal-based control diet (**CON**), b) 3% microalgae (**MAG**), and c) MAG with xylanase enzyme (**MAG +XYN**). The chicks were reared for 35 days (**d**) on a floor pen system maintaining standard environment conditions to evaluate the effects of microalgae, with or without xylanase supplementation, on serum immunoglobulins, cecal short-chain fatty acids (**SCFA**) production, cecal microbial diversity, and metabolic pathways. No significant differences were found for serum immunoglobulin and cecal SCFA among the treatment groups ($P > 0.05$). Relative microbial abundance at the genus level showed that MAG and MAG +XYN groups had a diverse microbial community on d 3 and d 35. However, no bacterial genus had a significant difference ($P > 0.05$) in their relative abundance on d 3, but 16 genera showed significant differences ($P < 0.05$) in their relative abundance among the dietary treatments on d 35. Most of these bacteria were SCFA-producing bacteria. Moreover, MAG and MAG+XYN-fed broilers had better responses than CON groups for metabolic pathways (D-mannose degradation, pectin degradation I and II, β-1-4-mannan degradation, tetrahydrofolate biosynthesis, glutathione biosynthesis, glutathione-peroxide redox reactions, lactate fermentation to propionate, acetate, and hydrogen, etc.) both on d 3 and d 35. The results suggest that using microalgae, with or without xylanase, had no statistical impact on serum immunoglobulins and cecal SCFA production in broilers. However, an improvement in the cecal microbial diversity and metabolic pathways, which are essential indicators of gut health and nutrient utilization, was observed. Most of the improved metabolic pathways were related to fiber utilization and oxidative stress reduction.

Key words: broiler, gut health, metabolic pathways, microalgae, xylanase

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INTRODUCTION

There is an increasing demand for animal-based protein to meet the nutritional needs of the ever-increasing global population. The protein obtained from broiler meat is one of the most promising animal-sourced proteins, considering its quality and affordability. To contribute to the supply of animal-based protein, fast-growing broiler strains have been developed for higher muscle yield in a short period. However, broiler's faster growth in a limited time results in unintended gastrointestinal disorders, including poor gut development, improper nutrient utilization, and dysbiosis. Poultry scientists are adopting several strategies to maintain high production and better gut health together, including specialized nutritional programming. The addition of antibiotics in poultry feed was a basic norm to support gut health and enhance production. However, the use of antibiotics as a growth promotor (**AGP**) has either been banned or strictly regulated in different parts of the world due to public health concerns (Jha et al., 2020). This changed situation has necessitated evaluating alternative feedstuffs and feed additives to maintain production without compromising gut health while

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[1]Corresponding author: rjha@hawaii.edu

replacing the use of AGPs. Several alternative feedstuffs and coproducts are found efficacious in improving gut health through gut eubiosis (Jha and Berrocoso, 2015), consequently improving the growth of chickens. Common feed additives used as alternatives to AGPs are probiotics, prebiotics, exogenous enzymes (mainly fiber degrading and phytase enzymes), organic acids, phytogenic compounds, and others (Yadav and Jha, 2019).

Similarly, alternative feedstuffs rich in fibers are also studied for their functional properties to improve broilers' gut health (Jha and Mishra, 2021). Studies have found that gut microbiota play a vital role in broiler production as they improve immune response and increase nutrient utilization and short-chain fatty acid (**SCFA**) production (Yadav and Jha, 2019). Colonization of the gut by healthy bacteria also maintains the structure of intestinal epithelial tissue (Kim et al., 2017). These bacteria ferment fiber and produce SCFA, eventually reducing gut inflammation and beneficially modifying gut microbiota. A study found that SCFA impacts the functions of neutrophils during inflammation and regulates immunity through the upregulation of cytokine-induced neutrophil chemoattractant $2\alpha\beta$ production (Akhtar et al., 2021). In addition, butyrate, a SCFA, provides energy to the enterocytes, promotes mucin production by binding to *GPR109A* in goblet cells, and improves mucosal lining (Liu et al., 2021b). Healthy intestinal epithelial tissue helps maintain intestinal integrity, resulting in better nutrient utilization, absorption, and protection from pathogens. Together with intestinal epithelium, microbes or their metabolites improve immunity (Okumura and Takeda, 2017). However, it is necessary to understand the relationship among different feeds, feed additives, and gut microbiota to make any nutritional recommendation for use in broiler feeding programs.

Microalgae are well known for their high content of protein, polysaccharides, lipids, and bioactive compounds (Milledge, 2011; Wolkers et al., 2011). It has been found to promote growth performance by improving antioxidant capacity and intestinal barrier function in broiler chickens (Liu et al., 2020; Chaudhary et al., 2023). Microalgae contain 53.85% carbohydrates, enriched with a higher percentage of different NSPs (Kusmiyati et al., 2020). So, supplementing a diet with microalgae could add up the availability of the prebiotic NSP within the gut. This increased availability of NSP may aid in fermentation, which leads to increased cecal microbial diversity and SCFA production. However, the presence of fiber content makes the diet less digestible because of the incapability of the endogenous enzymes of the broiler to degrade the fibers. Adding fiber-degrading enzymes like xylanase may degrade NSP and improve digestibility, hence nutrient utilization (Singh et al., 2019). No studies investigated the effect of NSPase on the liberation of sugar units and its consequences on gut health due to microalgae supplementation in broiler's diet. Previously, the effect of microalgae was investigated at higher inclusion levels (up to 20 g/kg) as an alternative protein source in chicken diets (Coudert et al., 2020). But, low doses (2.5−4 g/kg) were widely studied as feed additives to assess their functional values (Martins et al., 2021). However, only a limited number of studies examined the functional effects of microalgae. To the best of our knowledge, no study has evaluated the combined effects of microalgae and xylanase targeting the microbiota diversity and metabolic pathways in broilers.

Our recently published paper originated from the same study reported that microalgae as a functional compound, with or without xylanase, improved growth performance, organ development, and the ileal gene expression related to immunity, gut barrier, antioxidant, and nutrient transporter in broilers (Mishra et al., 2023). Therefore, we hypothesized that supplementing microalgae (*Arthrospira platensis*) with or without xylanase to the broiler's diet might improve immunoglobulin levels and other gut health parameters. This study aimed to evaluate the effects on serum immunoglobulins, cecal SCFA production, microbial diversity, and metabolic pathways in broiler chickens when fed with a diet supplemented with microalgae, with or without xylanase enzyme.

MATERIALS AND METHODS

This is a companion manuscript of our previously published manuscript (Mishra et al., 2023) from the study using the same animals and feeds. All research animal care procedures were approved by the Institutional Animal Care and Use Committee of the University of Hawaii, HI (Protocol #15-2274-7).

Experimental Design, Management, and Diet

A 35 days feeding trial with 162 one-day-old unsexed Cobb 500 chicks was conducted at the Small Animal Facility of the University of Hawaii at Manoa (**UH Manoa**). The birds were raised on a litter system, maintaining the standard environment (temperature: 18°C −24°C, humidity: 50 ± 5%) required for Cobb 500 broilers (Cobb-Vantress, 2021). They had ad libitum access to feed and water throughout the experiment. The photoperiod cycle was set at 23:1 h of light and dark. The chicks were randomly assigned to 1 of the 3 dietary treatment groups: a) corn-soybean meal diet (**CON**), b) 3% microalgae (*A. platensis*, **MAG**), and c) MAG with xylanase enzyme (**MAG+XYN**). Each dietary treatment group had 6 replicates with 9 chicks per replicate ($n = 54$ per group) at d 0. The mash diets were formulated in 2 phases, starter (d 0−21) and finisher (d 22−35), and met the nutritional requirements of Cobb 500 broilers (Cobb-Vantress, 2022). Microalgae were added at a dose of 30 g/kg as a premix, and xylanase enzyme was added on top of MAG diet at a dose of 1,600 BXU per kg (i.e., 100 g/ton). The feed ingredients, formulation, nutritional values of the diets, and enzymes used in this study were presented in our previously published manuscript (Mishra et al., 2023).

Sample Collection

On d 3 and d 35, randomly, 6 birds per treatment (1 bird per pen) were euthanized with CO_2, and samples were collected. Blood was collected on d 35 to separate serum for immunoglobulins analysis and stored at −20°C. Cecal digesta were collected for SCFA (on d 35) and DNA extraction for gut microbiota and metabolic pathway analysis (on d 3 and d 35). The collected cecal digesta were immediately snap-frozen into liquid nitrogen and then stored at −80°C until processed.

Serum Immunoglobulins Analysis

Serum immunoglobulins were analyzed by enzyme-linked immunosorbent assay (**ELISA**) using the commercial kit for Chicken IgA (Bethyl Laboratories, Montgomery, TX, Catalog No: E33-103) and Chicken IgG, also known as IgY (Bethyl Laboratories, Montgomery, TX, Catalog No: E33-104). The procedures were followed as described by the manufacturer with some modifications. Briefly, 10 standards with different concentrations (1,000, 333, 111, 37.04, 12.35, 4.12, 1.37, 0.456, 0.152, and 0 ng/mL) and samples diluted into 1:1,000 and 1:2,000 were prepared for IgA concentration determination. Similarly, for IgG, 12 standards (500, 166.67, 55.56, 18.52, 6.17, 2.06, 0.69, 0.23, 0.08, 0.03, 0.009, and 0 ng/mL) and samples diluted into 1:100,000 and 1:200,000 were analyzed. The plates were observed for concentration at a wavelength of 450 nm using a plate reader (BioTek Synergy LX multimode reader, BioTek Instruments, Winooski, VT).

Short-Chain Fatty Acids Analysis

Short-chain fatty acid analysis was performed using gas chromatography (**GC**, Trace 1300, Thermo Scientific, Waltham, MA) equipped with a flame ionization detector and an autosampler (AI 1310, Thermo Scientific, Waltham, MA). The sample preparation and run process was similar to those previously described by Singh et al. (2021a). Standard SCFA mix (Sigma-Aldrich, St. Louis, MO, Catalog No: CRM46975) was run in 7 different concentrations (0.1, 0.5, 1, 2, 4, 6, and 8 mM) for acetic, propionic, isobutyric, and butyric acids. This standard mix was considered an external standard, and 3.3 mM trimethyl acetic acid was considered an internal standard. Later, the standard SCFA mix was analyzed using GC to get a calibration curve for each external standard compared to the compound based on their response ratio to the internal standard. A chromatography data system (Chromeleon 7.2, Thermo Scientific, Waltham, MA) was used to handle and process the data. Data were processed based on the peaks observed after chromatography and compared to the peak area of specific SCFA against the calibration curve of their standard.

DNA Extraction and 16S rRNA Library Preparation

Genomic DNA were extracted from cecal digesta using a commercial kit, Qiagen for QIAamp fast DNA stool mini kit (Qiagen, Hilden Germany, Catalog Number: 51604), following manufacturer's guidelines. After that, DNA was quantified for their concentration using nanodrop (NanoDrop One, Thermo Scientific, Madison, WI) and processed at the Advanced Studies in Genomics, Proteomics, and Bioinformatics core facility of UH Manoa for Illumina MiSeq sequencing.

Library preparation was performed as described by Singh et al. (2021b) by targeting V3 to V4 variable regions of the 16S rRNA gene and appended with Illumina overhang adapters. The forward primer was S-d-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′), and the reverse primer was S-d-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′). The overhang adapters hanged with forward and reverse primer at 5′ were TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG (forward) and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG (reverse) (Singh et al., 2021a).

Microbiota Characterization

Microbiota characterization was performed using CLC Genomics Workbench 22.0.2 and CLC Genomic modules (Qiagen, Hilden, Germany). The samples were analyzed following the standard protocol as described in the Operational Taxonomic Unit (**OTU**) Clustering Step-by-step Tutorial (Qiagen, 2021). SILVA 16S SSU version 138.1 was used as a reference database (Quast et al., 2013). Different indexes like Chao1, Shannon entropy, and Simpson were used to calculate alpha diversity, while Bray Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac were used to calculate beta diversity. Alpha and beta diversity were visualized as box and whisker plots, and principal coordinate analysis plots, respectively. In addition, a nonparametric t test was performed to understand the differential abundance taxa and their effect size using Statistical Analysis of Metagenomic Profiles (**STAMP**) 2.1.3 (Parks et al., 2014).

Functional Pathways Determination

Analysis for functional metabolic pathways of cecal microbes was carried out following the user manual of the CLC Microbial Genomic Module (Qiagen, 2023). Filtered sequences obtained during microbiome characterization and MetaCyc pathway database 2022-05 were used to predict metabolic pathways. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (**PICRUSt2**) was used to perform functional inference for bacterial abundance. The obtained pathway table was then subjected to statistical analysis.

Statistical Analysis

For serum immunoglobulins and SCFA concentration data, a statistical analysis using one-way ANOVA followed by Tukey multiple comparison test was performed in JMP Pro, Version 17 (SAS Institute Inc., Cary, NC). For microbiome data, alpha diversity was analyzed using the Kruskal-Wallis H test followed by Mann-Whitney U test, and beta diversity using the Permutational multivariate analysis of variance followed by Bonferroni test in CLC genomic workbench 22.0.2. Microbial abundances and metabolic pathways were analyzed in STAMP 2.1.3 using a two-sided White's nonparametric t test with DP: bootstrap of 0.95. The significance level was set at $P < 0.05$, and the trend at $P < 0.1$.

RESULTS

Serum Immunoglobulins

The effects of microalgae, with or without xylanase supplementation, on broiler's serum immunoglobulins are presented in Figure 1. The values were not significantly different ($P > 0.05$); however, there was a numerical increment in the concentration of both IgA and IgG compared to the CON group. Similarly, microalgae with xylanase also had a numerical increment in the concentration of IgA but lower IgG concentration compared to the CON group.

Cecal Short-Chain Fatty Acids

Supplementation of microalgae, with or without xylanase, in diets did not show any significant changes among the treatment groups ($P > 0.05$). However, there was a substantial numerical increment in the concentration of acetate, propionate, isobutyrate, butyrate, and total SCFA when microalgae were added to the diet. However, the microalgae with xylanase numerically lowered the concentration compared to the CON group for all SCFA, except for isobutyrate (Figure 2).

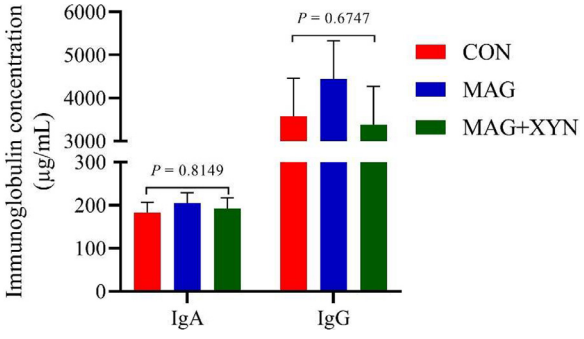

Figure 1. Effects of microalgae with or without xylanase supplementation on serum IgA and IgG. X-axis represents the name of immunoglobulins, and Y-axis represents their concentration in μg/mL. Results are expressed as the least square means of 6 replicate pens, and the error bar in the graph represents the pooled standard error of the mean (SEM) [IgA: immunoglobulin A; IgG: immunoglobulin G; CON: control; MAG: 3% microalgae; MAG+XYN: MAG with xylanase].

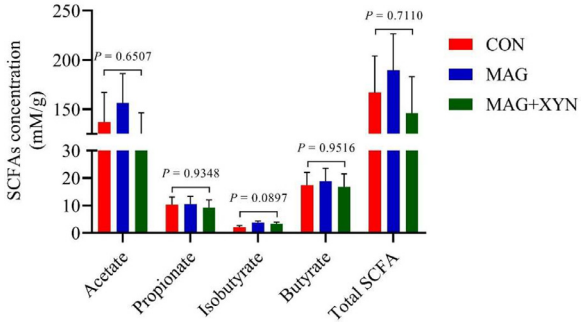

Figure 2. Effects of microalgae with or without xylanase supplementation on cecal SCFA production. X-axis represents the name of SCFA, and Y-axis represents their concentration in mM/g. Results are expressed as the least square means of 6 replicate pens, and the error bar in the graph represents the pooled standard error of the mean (SEM) [SCFA: short-chain fatty acids; CON: control; MAG: 3% microalgae; MAG+XYN: MAG with xylanase].

Cecal Microbiota Diversity

The relative diversity of the cecal microbiota is presented in Figure 3. On d 3, Proteobacteria was the most abundant bacteria, followed by Firmicutes. While on d 35, it was just the opposite; the most abundant bacteria were Firmicutes, followed by Proteobacteria. At the genus level, d 3 had a higher abundance of *Escherichia-Shigella,* followed by the torques group of *Ruminococcus* and uncultured genera from the family Lachnospiraceae. On d 35, *Eisenbergiella* was the most abundant bacteria, followed by *Escherichia-Shigella* and uncultured genera from the Lachnospiraceae family. Looking over the specific genera, *Eubacterium fissicatena* group, *Lactobacillus, Corynebacterium, Faecalibacterium*, and *Escherichia-Shigella* were significantly changed among the dietary groups (Figure 4).

Alpha Diversity

Alpha diversity of the cecal microbiota was determined based on 3 different indexes—Chao 1, Shannon entropy, and Simpson's index—on d 3 and d 35 (Figure 5). Chao 1 index, which is a nonparametric index, determines the richness of microbial species, including the rare species, and considers both singletons (observed once) and doubletons (observed twice). The result of this study showed no significant changes on d 3 ($P > 0.05$), but on d 35, MAG ($P = 0.05$) and MAG +XYN ($P = 0.04$) significantly increased the diversity of the cecal microbiota compared to the CON group. A higher microbial diversity on d 35 was in the MAG +XYN group, followed by MAG and CON groups. The study found no significant changes among the treatment groups ($P > 0.05$) in the Shannon entropy index on d 3 and d 35. Simpson's index determines the diversity of microbial species based on both the number of different species present (richness) and the relative abundances of each species (evenness). This index ranges from 0 to 1, where 0 means no diversity and 1 means highly diverse. This index also found no significant changes among the treatment groups.

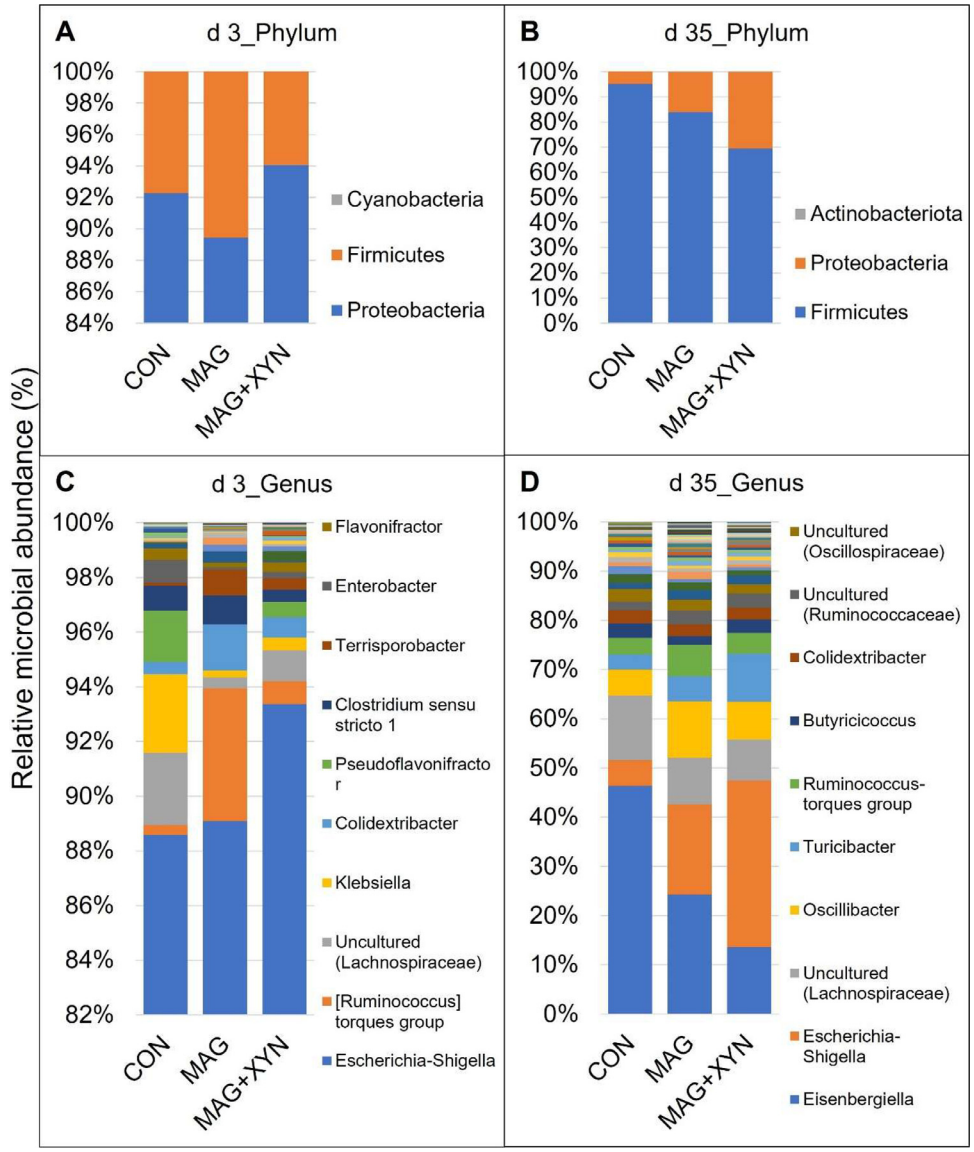

Figure 3. The stacked bar graph displays a relative abundance of the cecal microbial diversity of broiler chickens among different dietary treatments at the Phylum level (d 3: A and d 35: B) and at the genus level (d 3: C and d 35: D). The stacked bar represents all microbiota, while only the top 10 names were used for indexing [CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

Beta Diversity

Beta diversity of the cecal microbiota was determined based on 4 different indexes: Bray Curtis, Jaccard, Unweighted UniFrac, and Weighted UniFrac on d 3 and d 35 (Figures 6 and 7). On d 3, none of the indexes showed significant changes ($P > 0.05$) in diversity. However, on d 35, Weighed UniFrac, related to phylogenetic relatedness and relative abundance of their constituent taxa, showed significant changes ($P = 0.04$) in diversity among the diets. In addition, Bray Curtis, which measures the similarity or dissimilarity between 2 or more microbial communities or samples, showed a trend ($P = 0.097$).

Shared Amplicon Sequence Variants

An improved alternative to the traditional OTU method uses amplicon sequence variants (**ASV**) that differ from each other by a single nucleotide. A Venn diagram of shared ASV suggests that the number of ASV increased over the period, but the percentage of common ASV among all diets remained constant on d3 and d35. However, the percentage of unique ASV in each dietary group increased, and common ASV between the 2 groups decreased (Figure 8).

Intestinal Metabolic Pathway

White's nonparametric two-sided t test, a test for nonparametric data, was utilized to analyze the difference in the mean proportion of metabolic pathways between treatment groups (CON vs. MAG, CON vs. MAG +XYN, and MAG vs. MAG+XYN) and the result showed an improvement in cecal microbial metabolic pathways. Statistically significant pathways were mostly involved with fiber utilization and the reduction of oxidative stress (Figures 9 and 10).

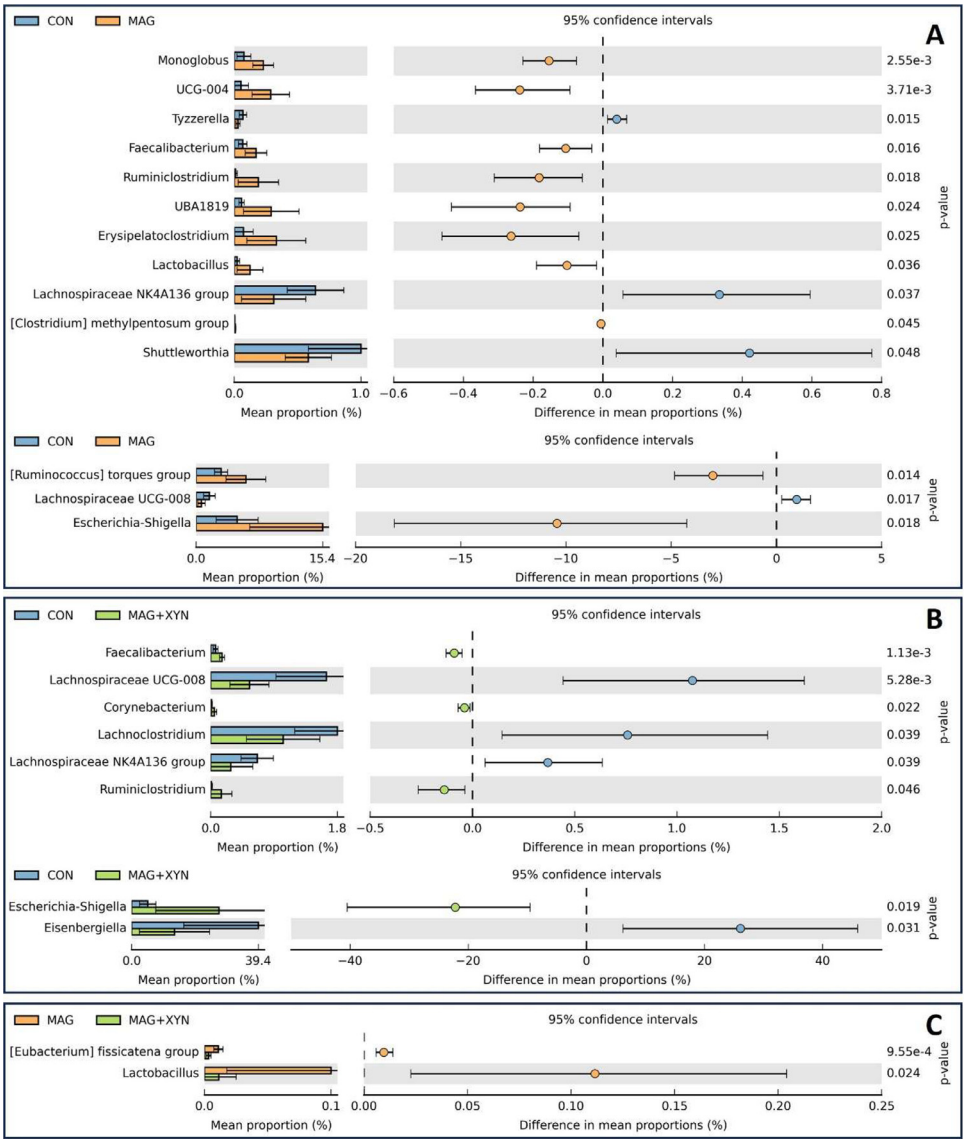

Figure 4. Extended error bar plot showing the difference in mean proportion (%) of cecal microbial community at genus level between (A) CON and MAG, (B) CON and MAG+XYN, and (C) MAG and MAG+XYN at d 35. The *P* value on the right was derived from White's nonparametric *t* test in the STAMP. [CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

DISCUSSION

The highly productive modern broilers are constantly under the threat of pathogens repressing final yield. Thus, the immune status of broiler chickens is one of the significant determinants of broiler production. The birds' immunity depends on several factors, including gut microbial composition and its associated interaction with the gut and diet. The SCFAs are precursors of energy. Additionally, these fermentation products strengthen intestinal epithelium and improve immunity. The study investigated the effects of microalgae, with or without xylanase supplementation, on serum immunoglobulins, cecal SCFA production, cecal microbial community, and the metabolic pathways of cecal microbiota in broiler chickens.

The results suggest that adding microalgae to the diet had no statistical impact on the production of serum immunoglobulins, IgA and IgG. The relationship between serum immunoglobulins and gut health is interconnected; however, a comprehensive mechanism has not yet been elucidated. Therefore, further inquiry is necessary to validate this association. Zeng et al. (2016) reported that microbiota in the gut induces the IgG response and helps to neutralize the antigens and prevent several infections, including *E. coli* and *Salmonella* infection. Microalgae, when added to a diet, improves immunity by increasing immunoglobulin concentration and phagocytic activity in the blood, as reported by Elshabrawy et al. (2022), but this study did not find any improvement in the immunity due to microalgae addition. However, a study by Khalilnia et al. (2023) reported that the broilers supplemented with a dose of 0.2 g/kg and 0.3 g/kg of *S. platensis* powder showed no significant differences in the immunoglobulin concentration, which was a similar finding to this study. Though the concentration of immunoglobulins were not significantly changed, the presence of different bioactive

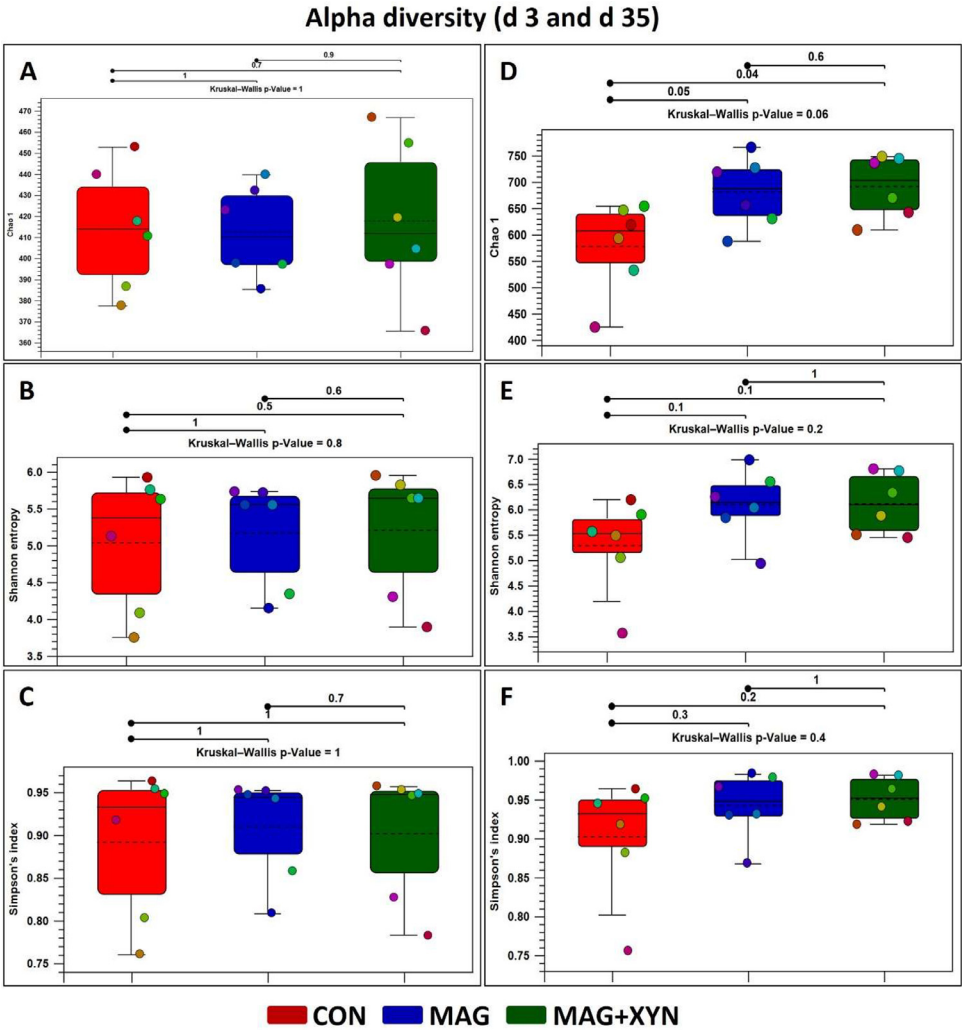

Figure 5. Box and whisker plot showing alpha diversity based on different indexes on d 3 (A: Chao1, B: Shannon entropy, and C: Simpson's index) and d 35 (D: Chao1, E: Shannon entropy, and F: Simpson's index). The middle dense line of the box and whisker plot represents the median, and the dotted line represents the mean, while the colored bubble represents individual value. The lower and upper hinges denote the first and third quartiles, respectively. The whiskers extended from the box show the highest and lowest values of the interquartile range. The statistical significance was determined by the nonparametric Kruskal-Wallis test, and the significance level was set at $P < 0.05$ [CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

compounds in microalgae, including β-carotene and vitamin B12 may have the potential to modulate different immune cells and their components.

In this study, there was no significant difference in SCFA concentration. SCFA are bacteria-derived fermentation metabolites and are defined based on the carbon number of fatty acids between 2 and 6. SCFA reduces intestinal pH, which promotes the growth of beneficial bacteria and suppresses the growth of pathogenic bacteria. Microalgae, due to their fibrous component, act like prebiotics which promote bacterial activity and helps in the production of SCFA (Jha et al., 2019; Jha and Mishra, 2021). Production of SCFA in the gut, mainly acetate, butyrate, and propionate, exhibits antimicrobial activity and prevents gut colonization by pathogenic bacteria (Zhang et al., 2020; Jha and Mishra, 2021). Short-chain fatty acids also reduce the load of *Clostridium perfringens*, and *E. coli* in the broiler's gut (Shang et al., 2018). In addition, SCFA also acts as a fuel for colonocytes via energy production through glycolysis and gluconeogenesis (Jha et al., 2019). No change in the SCFA concentration after xylanase addition might be due to the fiber-degrading properties of xylanase. Thus, nutrient utilization of microalgae was enhanced, so fermentation substrate was not readily available for microbial actions (Jha et al., 2010).

In the past few decades, there has been a tremendous increase in gut health-related studies. One of the major parameters of gut health study is the cecal microbiome. This study found a variation in the diversity of the microbial community among the treatments. The MAG or MAG with Xylanase supplementation showed 20 to 21% unique ASV in the treatment groups on d3 and d35 (Figure 8). Broiler performance is believed to be associated with the microbiota available in the gut (Dittoe et al., 2022). In the study, when comparing CON, MAG, and MAG+XYN groups, SCFA-producing bacteria (*Lactobacillus, Monoglobus*, torques group of *Ruminococcus, Faecalibacterium, Ruminiclostridium*) were significantly higher in abundance in microalgae added groups. This result is similar to the findings of a previous

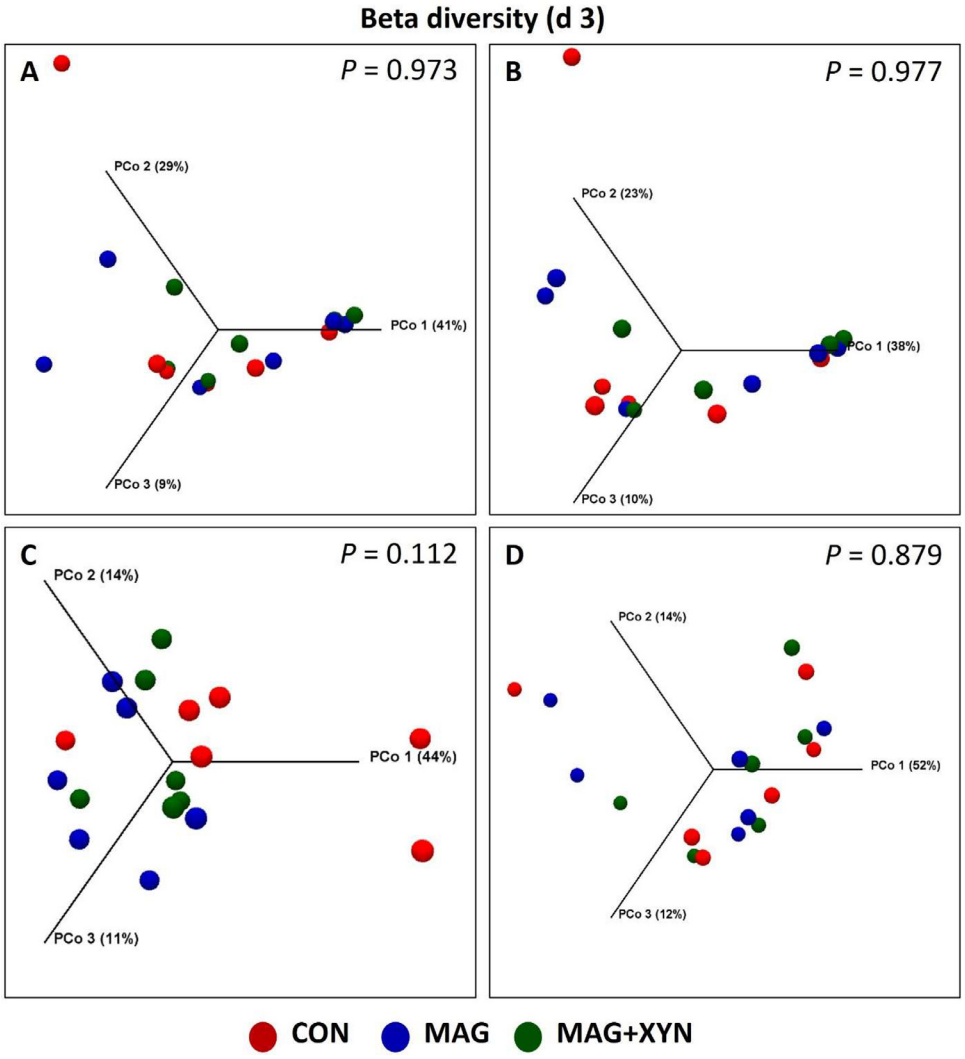

Figure 6. 3D presentation of principal coordinate (PCo) analysis shows the beta diversity of microbial community based on different indexes on d 3: (A) Bray Curtis, (B) Jaccard, (C) Unweighted UniFrac, and (D) Weighted UniFrac. The X- (PCo 1), Y- (PCo 2), and Z- (PCo 3) axis indicate the first, second, and third coordinates, respectively, and the values in parentheses show the percentages of the community variation explained. The color of the bubble displayed at the bottom indicates the dietary treatment groups [CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

study (Park et al., 2018). Although the changes were small, the MAG group has a significant increase in the methylpentosum group of *Clostridium,* which helps in the utilization of 2 methylpentoses (L-rhamnose and L-fucose) and 2 pentoses (L-xylose and D-arabinose) (Liu et al., 2021a). As discussed earlier, the presence of a higher number of beneficial bacteria might be due to the prebiotic properties of microalgae. A study by Zou et al. (2020) also reported that prebiotics promote the growth and activity of beneficial bacteria. The study found some of the beneficial bacteria (*Intestinomonas, Anaerosporobacter*), though not in higher abundance, were higher in MAG and MAG+XYN compared to the CON group. *Intestinomonas* helps in digestion, while *Anaerosporobacter* helps utilize a wide range of carbohydrates. *Anaerosporobacter* also helps in the production of several SCFAs. In addition, microalgae with xylanase enhance nutrient utilization by increasing different metabolic pathways (D-mannose degradation, pectin degradation, beta-1,4-mannan degradation, etc.). This study found a significant number of *Escherichia-shigella* despite having antibacterial properties in microalgae, contrary to the findings of previous studies (Abedin and Taha, 2008; Shang et al., 2018). However, rather than having several bacteria, a ratio of bacteria is important for optimum gut functionality. In addition, gut bacteria are good sources of neuromediators and hormones, including serotonin, catecholamine, melatonin, and histamine, which directly modulate intestine activity and indirectly extraintestinal organs like the brain, liver, and kidney (Park, 2018).

This study showed a significant increase in the metabolic pathways related to fiber utilization and oxidative stress reduction. A significant increase in the number of beneficial bacteria like *Monoglobus* spp. promotes metabolic pathways relevant to uronic acids, pentose sugar of hemicellulose/pectin, and fructose utilization (Kim et al., 2019), which was also found in this study. Microalgae, with or without xylanase supplementation, improved fiber utilization by improving metabolic pathways like β-(1,4)-mannan degradation, pectin degradation I, and pectin degradation II. Most of the bacteria

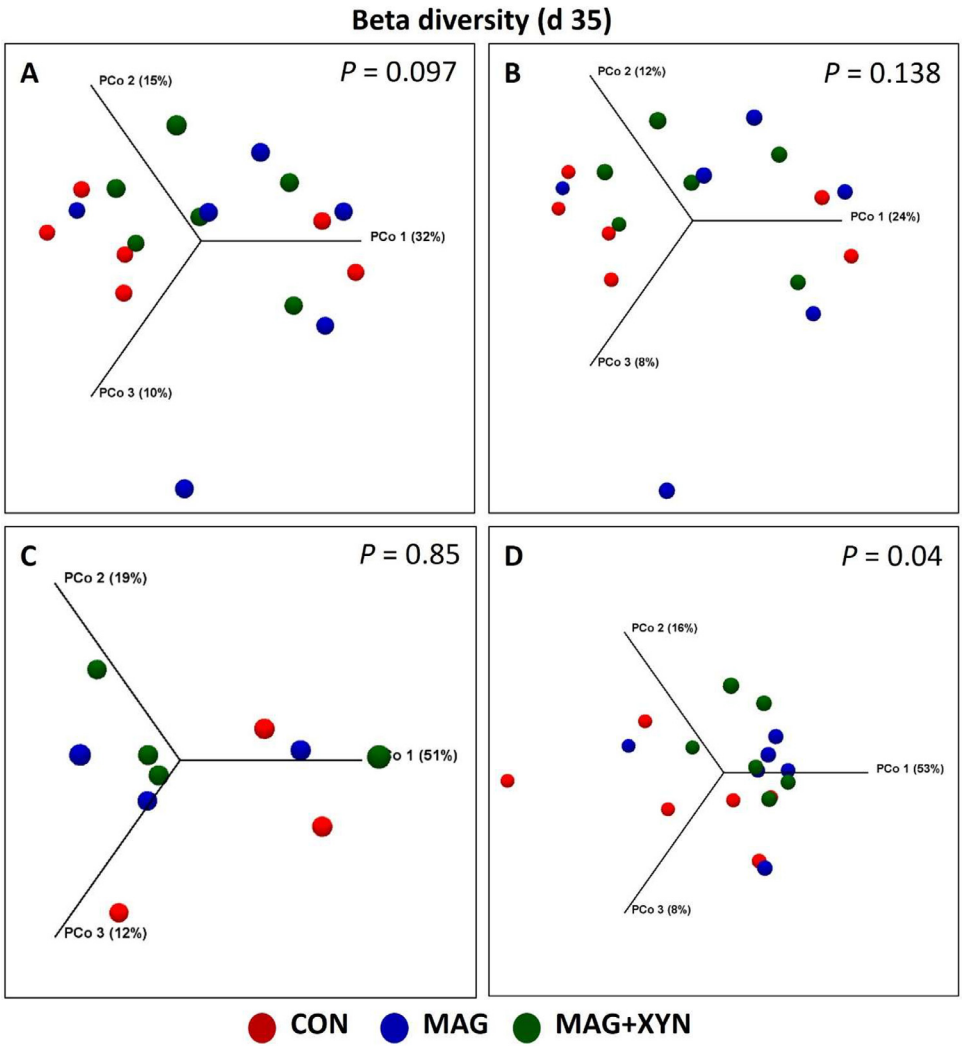

Figure 7. 3D presentation of principal coordinate (PCo) analysis shows the beta diversity of microbial community based on different indexes on d 35: (A) Bray Curtis, (B) Jaccard, (C) Unweighted UniFrac, and (D) Weighted UniFrac. The X- (PCo 1), Y- (PCo 2), and Z- (PCo 3) axis indicate the first, second, and third coordinates, respectively, and the values in parentheses show the percentages of the community variation explained. The colors of the bubble displayed at the bottom indicate the dietary treatment groups. [CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

found in the study were responsible for SCFA production by improving different metabolic pathways. Also possessing prebiotic character, fermentation-related pathways like lactate fermentation to propionate, acetate, and hydrogen were found significant. Significant improvement in ppGpp (guanosine 3′-diphosphate 5′-diphosphate) metabolism pathway in the MAG group suggests that this group had a better response toward nutrient or energy starvation and other environmental stress than the CON group. This study also found a

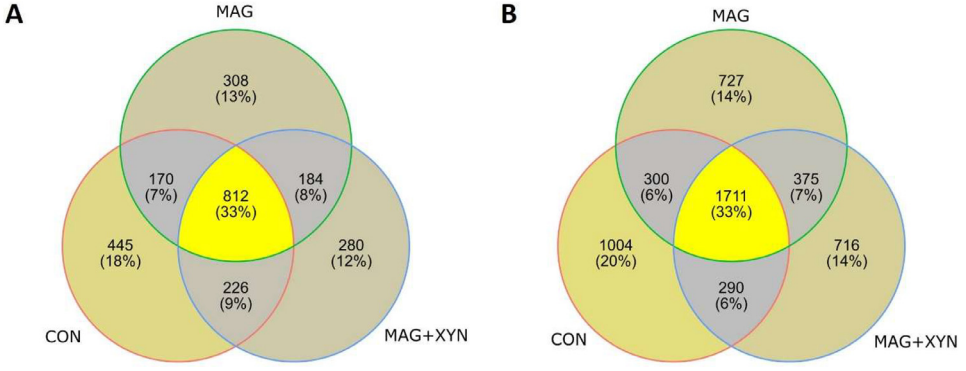

Figure 8. Venn diagram illustrating the observed overlap of ASV from each dietary treatment group along with the unique ASVs present in each dietary treatment group in the cecal microbiota on d 3 (A) and d 35 (B) [ASV: amplicon sequence variants; CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

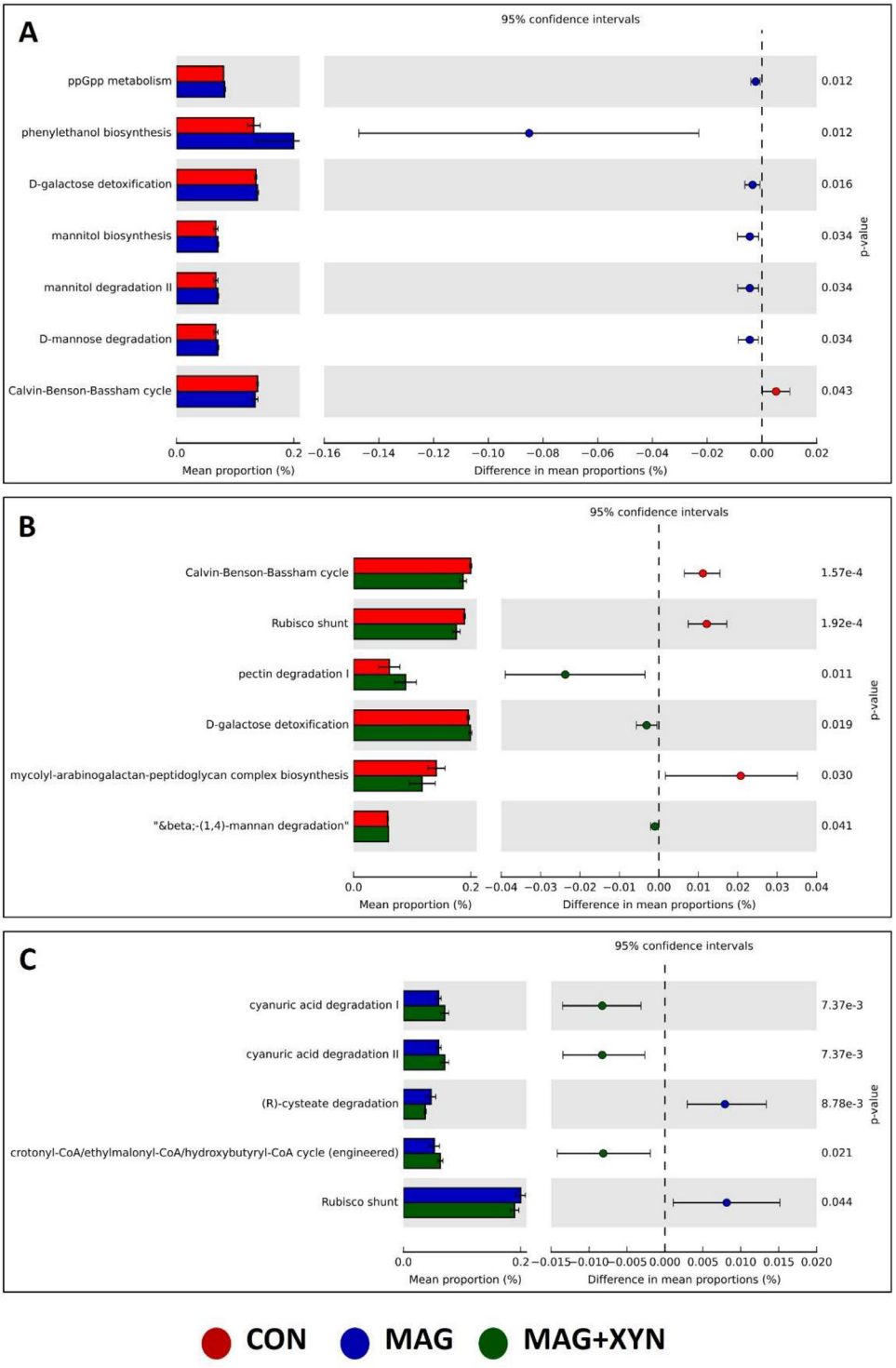

Figure 9. Extended error bar plot showing the difference in mean proportion (%) of metabolic pathways of cecal microbiota between (A) CON and MAG, (B) CON and MAG+XYN, and (C) MAG and MAG+XYN on d 3. The *P* value on the right was derived from White's nonparametric *t* test in the STAMP [CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

significant increase in the oxidative stress reduction pathways like glutathione biosynthesis and glutathione-peroxide redox reactions, which is due to the presence of bioactive compounds related to antioxidants like phycocyanin, β-carotene, flavonoids, and different vitamins and minerals (Kumar et al., 2022).

In conclusion, using microalgae has no direct impact on serum immunoglobulins (IgG and IgA) and cecal SCFA production. However, microalgae addition resulted in differences in mean proportion of several bacterial at genus level including *Lactobacillus, Monoglobus*, torques group of *Ruminococcus, Faecalibacterium, Ruminiclostridium* and metabolic pathways like D-mannose degradation, pectin degradation I and II, β-1-4-mannan degradation, tetrahydrofolate biosynthesis, glutathione biosynthesis, glutathione-peroxide redox reactions, lactate fermentation to propanoate, acetate, and hydrogen, etc., which are the indicators of improved gut

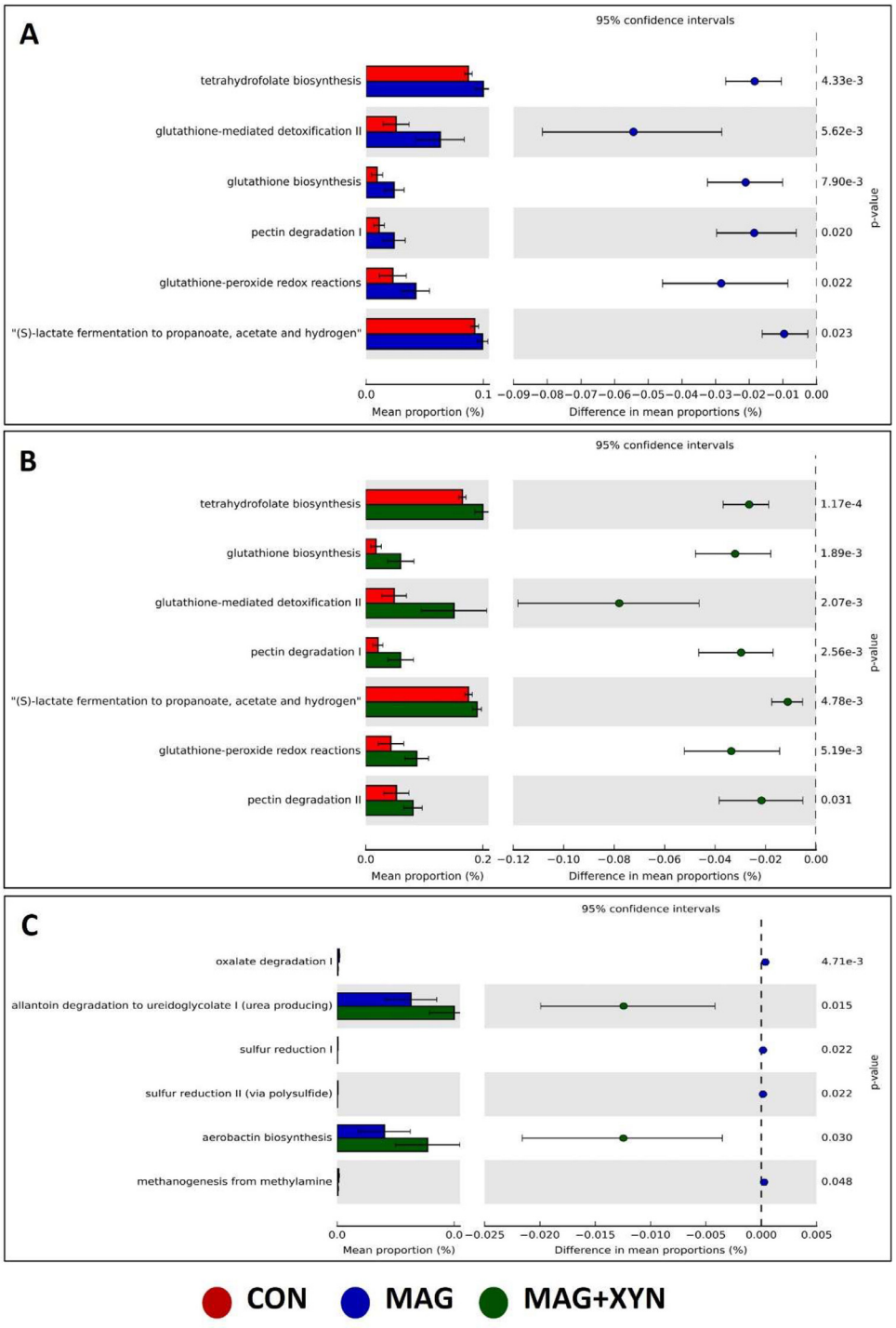

Figure 10. Extended error bar plot showing the difference in mean proportion (%) of metabolic pathways of cecal microbiota between (A) CON and MAG, (B) CON and MAG+XYN, and (C) MAG and MAG+XYN at d 35. The *P* value on the right was derived from White's nonparametric *t* test in the STAMP. [CON: control; MAG: 3% microalgae; and MAG+XYN: MAG with xylanase].

health and nutrient utilization. However, most of the bacteria found in the study were responsible for SCFA production and improvement of gut health status. This study was unable to find the reason for no significant changes in SCFA concentration. So, more studies are required to explain the working mechanism of microalgae with or without xylanase targeting immunoglobulins and SCFA. More changes in the microbial diversity and metabolic pathways in the study were observed in the microalgae-supplemented group rather than xylanase. So, further studies with different concentrations of xylanase are needed to understand the potential of xylanase addition to microalgae-supplemented broiler diet.

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DISCLOSURES

The authors declare no conflicts of interest.

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