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ENHANCED ANALYSIS OF GROWTH, HISTOLOGY, AND METABOLOMICS PROFILES IN *PENAEUS VANNAMEI* FED DISTILLERS DRIED GRAINS WITH SOLUBLES SUPPLEMENTED WITH BUTYRIC ACID

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Abstract

This study thoroughly analyzes the growth, histology, and metabolomics profiles of *Penaeus vannamei* shrimp when fed a diet high in distillers dried grains with solubles (DDGS) supplemented with butyric acid (BA). The limited research on BA supplementation with high levels of DDGS makes this study particularly valuable, as its antimicrobial properties and improved nutrient absorption are crucial for promoting shrimp health. A total of 720 shrimp were divided into six groups, each receiving varying levels of BA supplementation: 0%, 0.5%, 1%, 1.5%, 2%, and 2.5%. The experiment lasted 56 days, with three replicates for each group. The results showed significant increases in final weight (FW), weight gain rate (WGR), and specific growth rate (SGR) with higher levels of dietary BA ($P<0.05$). Additionally, dietary BA led to a notable decrease in feed conversion ratio (FCR), with the most significant impact observed in the 1.5% BA group ($P<0.05$). Histological analysis of the hepatopancreas revealed that shrimp fed diets containing 1.5% and 2% BA displayed significantly higher B-cell numbers with a well-defined structure than the other groups. Metabolomics analysis showed distinct quadrants in the PCA score plots for the control and BA groups, indicating a clear separation in their metabolic profiles. Integration analysis of differential metabolites and genes further revealed the upregulation of functions related to ABC transporters, amino acid biosynthesis, and metabolism in shrimp supplemented with BA. Our findings suggest that the optimal BA requirement for supplementation in the DDGS diet for *P. vannamei* is estimated to be 1.6%. This information will be valuable for farmers during feed formulation and aquaculture growth in general.

Key words: butyric acid, growth, histology, *P. vannamei*, metabolomics

The production levels of the global aquaculture industry reached a new high in 2020, reaching 122.6 million tons with a first sale value of USD 265 billion (Tran et al., 2023). Findings reveal that aquaculture must persistently expand and vary its offerings to meet the surging demand for aquatic species. Out of the approximately 2500 shrimp species, only 343 are considered commercially important (Nunes et al., 2022). In recent years, production levels for the *P. vannamei* shrimp species have skyrocketed, making it one of the most cultivated varieties globally (Ray et al., 2021 b; McLean et al., 2020). Extensive research supports the cultivation of this species because it has rapid growth, disease resistance, high stocking density tolerance, and low dietary protein requirements (Bae et al., 2020). However, intensive farming methods can lead to intestinal problems and bacterial infections in these shrimp. Therefore, providing them with a balanced diet is crucial to ensure their optimal growth and well-being.

Protein is a crucial component of aquafeeds and the most costly feed ingredient. Fishmeal and soybean meal (SBM) have long been relied upon as the top protein sources within the aquafeed sector (Nagappan et al., 2021; Mugwanya, 2023). Excessive fishing of wild stocks, combined with rising consumer demand for fish and fish products, has resulted in a shortage of fishmeal that is driving up market prices (Ahmad et al., 2020). Research has suggested that plant-based protein diets, particularly those incorporating distillers dried grains with solubles (DDGS), are more cost-effective and less antinutritive (Hu et al., 2020; Gyan et al., 2021; Li et al., 2022). DDGS offers a highly utilizable source of phosphorus (Sándor et al., 2021). The quality of animal products can be enhanced by incorporating beneficial ingredients such as carotenoids and yeast (Ray et al., 2021 a). The immune-stimulating properties of yeast and the components found in DDGS may bolster animals' ability to combat infections (Allam et al., 2020; Yohana et al., 2024 a). However, it is important to note that DDGS can contain up to 500 g/kg of total carbohydrates by dry weight, with 341–420 g/kg being non-starch carbohydrates like indigestible fiber (Suehs and Gatlin, 2022). Shrimp diets may face challenges in incorporating DDGS due to its low levels of lysine and methionine

(Zhang et al., 2009). Additionally, DDGS is susceptible to mycotoxin contamination despite its potential as a feed ingredient (Khatibi et al., 2014; Oplatowska-Stachowiak et al., 2015; Abudabos et al., 2017; Mohammadi Shad et al., 2021). Mycotoxins pose a serious safety risk in DDGS, as they can harm animals and lead to chronic illnesses. Research has indicated that incorporating a high amount of DDGS in shrimp diets can have detrimental effects on shrimp growth and overall health. The elevated fiber content in DDGS can lead to digestive issues in shrimp, resulting in decreased feed intake and nutrient absorption. This can ultimately lead to stunted growth and higher mortality rates among shrimp populations. Additionally, the high levels of phytates in DDGS can bind to essential minerals in the shrimp's diet, rendering them inaccessible for absorption and causing nutrient deficiencies that further impact shrimp health. As a solution, there is a growing interest in the use of various feed additives, such as BA, in animal diets to mitigate the negative effects associated with plant protein diets. These additives aim to enhance growth performance, improve nutrient digestibility, and increase metabolizable energy content in shrimp diets (Jang et al., 2022).

Butyric acid (BA) plays a vital role in providing energy to the intestinal epithelial cells. It is a promising feed supplement because it balances gut microbiota, regulates immunity, and enhances animal growth. These benefits ultimately contribute to the overall health of aquatic animals (Górka et al., 2018; Sun et al., 2024). Additionally, BA has been shown to reduce mammalian intestinal inflammation and promote wound healing post intestinal surgery (Monier et al., 2022; Yohana et al., 2024 b). Its antimicrobial properties and improved nutrient absorption are key factors in its health-promoting effects (Rairat et al., 2023).

Despite the significant research on the effects of BA on shrimp diets, there is a lack of studies on incorporating BA into the high inclusion of DDGS diet for Pacific shrimp to remove the adverse effects caused by plant diet. The main focus of this study is to investigate the effects of adding BA to a high-DDGS diet on the growth, histological characteristics, and metabolomics profiles of *P. vannamei*.

Methodology

Ethics statement

The Institutional Animal Care and Use Committee (IACUC) of Guangdong Ocean University's esteemed College of Aquatic Sciences has diligently reviewed and approved this research project. The study was formally approved on April 2, 2024, and assigned the code GDOU-IACUC-2024-A2013. We have implemented various measures to minimize shrimp stress during our research. These include reducing the duration and frequency of handling, and providing a comfortable environment for the shrimp with adequate space, optimal water quality, and appropriate temperature. Additionally, we regularly monitor and assess the welfare of the shrimp throughout the research process to ensure their well-being.

Experimental diet

Table 1 thoroughly summarizes the main feed ingredients used in creating the diet and estimates the trial diet composition. The study incorporated six varying levels of BA supplementation, denoted as 0%, 0.5%, 1%, 1.5%, 2%, and 2.5%, respectively. The experimental diets were expertly prepared following the rigorous guidelines of the AOAC (2000) protocol. After a detailed procedure, the components were carefully dried, ground in a hammer mill (H-28 from South China University of Technology in Guangzhou, China), and sifted through an 80 mm mesh sieve. Afterward, the combination of macro and micro ingredients was meticulously mixed for ten minutes with a V-mixer (M256, South China University of Technology, Guangzhou, China). The mixture was infused with lecithin, soybean oil, fish oil, and a quarter of the volume in water. To form 2 mm and 4 mm pellets, the mixture underwent additional processing with the trial micro-feed extruder (F-26 II) at South China University of Technology in China. After being vacuum sealed, the pellets were dried in an oven at 60°C until reaching a moisture content of roughly 10% and subsequently preserved at –20°C.

Experimental animal

The study was conducted at the advanced Marine Biological Research Base of Guangdong Ocean University. Postlarvae of *P. vannamei* were provided by Hatchery Zhanjiang Allied Pacific Aquaculture Co. Ltd., a commercial farm. A total of 2000 shrimp occupied concrete ponds for 3 weeks. At this time, the shrimp were nourished with a premium commercial diet containing 10% crude fat and 45% crude protein. The shrimp weighed in at 0.19 g, to begin with. After adapting to their new environment, the 720 shrimp were evenly spread out across 18 plastic tanks (0.3 m^3), each holding 40 shrimp. For 56 days, the shrimp were meticulously tended to and given four daily feedings, adjusted according to their growth rate. The feeding amount was 10% of their body weight to guarantee adequate nutrition. Half the seawater was refreshed daily to preserve the tank's water purity. Dissolved oxygen levels were monitored and kept between 5.6 and 6.5 mg/l, salinity levels between 32‰ and 35‰, pH levels between 7.9 and 8.1, and water temperature between 28°C and 30°C, which were tested by a multi-parameter water quality detector (PTF-00lB, WBD Biotechnology Co., Ltd., China) throughout the experiment. Daily observations were made to document and record any atypical behavior displayed by the shrimp to ensure precision in the results.

Collection of samples

The shrimp were purposely withheld food for a day before the completion of the feeding experiment. All the shrimps were meticulously counted, measured, and weighed precisely to calculate the WG, SGR, and FCR for every tank. Following dissection, samples of the intestines and hepatopancreas were extracted and stored in liquid nitrogen in Eppendorf tubes at –80°C for future analysis. Also, for histological analysis, three shrimp per tank were picked randomly, and their hepatopancreases were removed and stored in Bouin's solution.

Table 1. The experimental diets formula and proximate composition (% dry matter)

Ingredients	T1 (0%)	T2 (0.5%)	T3 (1%)	T4 (1.5%)	T5 (2%)	T6 (2.5%)
Fish meal[a]	15	15	15	15	15	15
Soybean meal[a]	8	8	8	8	8	8
DDGS[a]	17.5	17.5	17.5	17.5	17.5	17.5
Beer yeast[a]	6	6	6	6	6	6
Wheat gluten[a]	15.5	15.5	15.5	15.5	15.5	15.5
Gluten flour[a]	23	23	23	23	23	23
Shrimp shell powder[a]	6	6	6	6	6	6
Fish oil + soybean oil[a]	1.8	1.8	1.8	1.8	1.8	1.8
Lecithin[a]	1.5	1.5	1.5	1.5	1.5	1.5
Calcium dihydrogen phosphate[b]	1.5	1.5	1.5	1.5	1.5	1.5
Vitamin premix[c]	0.7	0.7	0.7	0.7	0.7	0.7
Mineral premix[c]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin C[d]	0.04	0.04	0.04	0.04	0.04	0.04
Choline chloride[b]	0.03	0.03	0.03	0.03	0.03	0.03
Lysine[d]	0.0744	0.0744	0.0744	0.0744	0.0744	0.0744
Arginine[d]	0.4228	0.4228	0.4228	0.4228	0.4228	0.4228
Methionine[d]	0.3302	0.3302	0.3302	0.3302	0.3302	0.3302
Threonine[d]	0.0244	0.0244	0.0244	0.0244	0.0244	0.0244
Butyric acid	0.0	0.5	1.0	1.5	2.0	2.5
Microcrystalline cellulose[d]	10.552	10.052	9.552	9.052	8.552	8.052
Proximal composition (%)						
moisture	7.01	7.24	7.51	7.66	7.84	7.57
crude protein	38.62	38.65	38.68	38.80	38.77	38.73
crude lipid	8.86	8.69	8.67	8.52	8.72	9.14
ash	5.83	5.80	5.97	6.20	5.97	6.03

[a]The feed materials were provided by Zhanjiang Haibao Feed Factory in Zhanjiang, Guangdong, China.

[b]Mineral Element Premix (g/kg): ferric citrate 13.71 g, zinc sulfate monohydrate 28.28 g, seven hydrated magnesium sulfate 0.12 g, one hydrated manganese sulfate 12.43 g, five hydrated copper sulfate 19.84 g, six hydrated cobalt chloride 4.07 g, potassium iodide 0.03 g, potassium chloride 15.325 g, sodium selenite 0.02 g, cellulose 906.18 g. Bought from Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China

[c]Vitamin Premix (g/kg premix): thiamine 25.50 g; riboflavin 25.0 g; vitamin B_6 50.0 g; Vitamin B_{12} 0.1 g; vitamin K 5.0 g; vitamin E 99.0 g; cellulose 10.0 g, vitamin D 50 g; nicotinic acid 101.0 g; d-pantothenate ca. 61.0 g; biotin 25.0 g; folate 6.25 g; inositol 153.06 g.

[d]The amino acid was supplied by the renowned Shantou ACIXilong Chemical Factory located in Guangdong, China.

Analysis of growth performance

$$WG = [(FW - IW)/IW] \times 100$$
$$SGR = [100 \times (Ln\ FW - Ln\ IW)/FD]$$
$$FCR = FC\ (DW)/BWG$$

Abbreviations: *WG* for weight gain, *FW* for final weight, *IW* for initial weight, *SGR* for specific growth rate, *FD* for feeding days, *FCR* for feed conversion ratio, *FC* for feed consumed, *DW* for dry weight, and *BWG* for body weight gain.

HE stain for histopathological analysis

The 10% buffered formaldehyde solution was used to preserve the hepatopancreas of each treatment group for histological examination. After being sliced into 5 mm cassettes, the samples were washed, treated with alcohol to remove moisture, and encased in paraffin. The tissue samples underwent staining using hematoxylin and eosin (H&E). The Olympus BX51 microscope (Tokyo, Japan, serial number 9K18395) was utilized to analyze the tissue samples. The primary focus was to ascertain the presence of B, R, E, and F cells within the samples. One of the key criteria for analyzing hepatopancreatic cells was to examine any changes in cell morphology in shrimp that were supplemented with BA

Sample preparation for metabolite analysis

Before any analysis, the small pieces of hepatopancreas tissue samples were meticulously dissected and washed with phosphate-buffered saline. The samples were frozen in liquid nitrogen to preserve their integrity and kept at –80°C. Following thawing, the tissue samples were finely pulverized in ultra-pure water and combined with 300 milliliters of methanol within an electro-polish (EP) tube. The samples were then treated with ultrasonic radiation to remove any remaining proteins and incubated at 20°C. After spinning at 13,000 rpm and 4°C for 15 minutes, the resulting liquid was gathered and moved to a glass vial for studying with LC/MS. A new liquid chromatography method was also included in the process. Each sample was used in 20 µL for quality control. In comparison, 180 µL of the liquid was saved for further analysis using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS).

LC-MS analysis

The cutting-edge method utilized in this study, LC-MS analysis, was ultra-high-performance liquid chromatography (UHPLC). The mobile phase formulation consisted of acetonitrile, a pH 9.75 water solution containing 25 mM NH_4OAC and 25 mM NH_4OH, and an ultraperformance liquid chromatography ethylene bridged, hybrid (UPLC BEH) amide column coupled to a TripleTOFTM 5600 mm. The method utilized a gradient elution process at a 0.5 mL/min flow rate. The gradient began at 15% B and increased to 35% B over the first five minutes. It then remained at 37% B for the following six minutes, 79% B for the subsequent three minutes, 95% B for the next four minutes, and finally returned to 5% B for the final thirty minutes. The injection volume was 3 μL. Using a triple time of flight (TOF) mass spectrometer, the LC/MS analysis was conducted alongside information-dependent (IDA) MS/MS spectrometry. Images were produced using mass spectrometry (MS/MS) spectrum following completion of scan surveying procedures with specific pre-selected parameters. However, the methodology or evaluation did not detail the results of mass spectrometry (MS). At a collision energy of 30 V, twelve product ions were formed through the fragmentation of each precursor ion.

Analysis of metabolomics

Principal component analysis (PCA) and orthogonal projection to latent structure discriminant analysis (OPLS-DA) were conducted using the R platform's OPLS package (Version 1.6.2) for multivariate statistical analysis. Three key analytical indices were employed: R^2X, R^2Y, and Q2. R^2X and R^2Y represent the variance explained by the X and Y matrices, respectively, while Q^2 assesses the model's predictive capacity. A Q^2 value above 0.5 indicated a valid model, while a value exceeding 0.9 signified a robust model. Alignment authentication was crucial to ensure the reliability of the OPLS-DA models. The R^2 and Q^2 values of the new models were graphically represented as straight lines with blue and red dots indicating the model's values adjacent to the Y replacement. The variables important to prediction (VIP) analysis were utilized to identify differential metabolites in biological replicates based on their alignment with the R^2 and Q^2 values of the specific model. The selection criteria included folding factor (FC) > 3, P<0.01, and VIP > 1, along with the integration of variance multiples, VIP value, and t-test P-value from the OPLS-DA model. Furthermore, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was utilized to explore the enrichment of metabolic pathways associated with different metabolites. Data normalization using the R heatmap package and the Z-score method allowed for visualizing metabolite intensity areas in a heatmap clustering. Additionally, the R package GO plot 1.0.2 facilitated further research and the creation of visually appealing chord diagrams.

Statistical analysis

We used SPSS Statistics (version 22, SPSS Inc., Chicago, IL, USA) to analyze the data. The initial step involved a joint hypothesis test to determine all standard deviations (SEM). Then, the data standardness and equality of variance were examined using IBM SPSS version 22 (USA). Statistical analysis using Duncan multiple-comparison tests revealed notable discrepancies (P<0.05) between the treatment groups. The post hoc tests were conducted for pairwise comparisons among BA levels. Orthogonal polynomial contrasts were employed to distinguish between the linear and quadratic effects. Mean values and standard deviation (SD) were displayed, and any statistically significant differences were indicated by P<0.05.

Results

Growth performance

Table 2 displays the growth performance of *P. vannamei* shrimp after eight weeks of consuming different experimental diets. The results indicate that incorporating BA into DDGS significantly enhanced the shrimps' FBW, AWG, and SGR. 1.5% had the highest weight gain of all treatment groups. Additionally, the FCR notably decreased (P<0.05) with adding BA to the diet.

Table 2. Effects of supplementing BA with high inclusion of DDGS on growth of juvenile *L. vannamei*

Items	BA (0%)	BA (0.1%)	BA (1%)	BA (1.5%)	BA (2%)	BA (2.5%)	Linear Adj R^2	Quadratic Adj R^2
IW	0.19±0.00	0.19±0.00	0.19±0.00	0.19±0.00	0.19±0.00	0.19±0.00		
FW	11.93±0.42 a	12.2±0.67 a	12.96±0.09 a	14.37±0.34 b	13.06±0.05 a	11.91±0.55 a	–0.062	0.25
WG	455.5±35.85 a	466.±8.73 ab	505.3±13.67 cd	523.4±14.15 d	489.±8.16 bc	457.83±5.05 ab	–0.033	0.586
SGR	2.46±0.3 a	2.48±0.06 a	2.55±0.01 ab	2.65±0.04 b	2.55±0.00 ab	2.46±0.05 a	–0.029	0.394
FCR	1.32±0.02 d	1.29±0.02 cd	1.19±0.03 ab	1.15±0.03 a	1.22±0.02 cd	1.31±0.01 d	–0.03	0.617

Means ± SD represent the values (n=3). Values with the same letter in a row are not significantly different from each other (P>0.05). Abbreviations used are weight gain = WG, initial weight = IW, survival rate = SR, final weight = FW, feed conversion ratio = FCR, and specific growth rate = SGR.

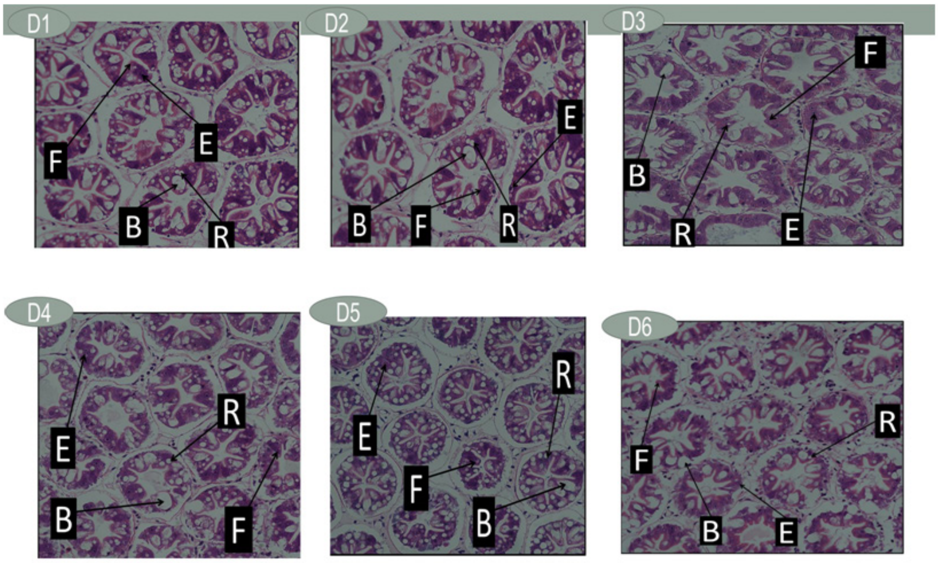

Figure 1. The hepatopancreas morphology of *P. vannamei* fed a diet with high inclusion of DDGS and supplemented with BA. Each letter in the figure corresponds to a specific type of cell: E for embryonalzellen cells, R for restzellen cells, F for fibrillenzellen cells, and B for blasenzellen cells

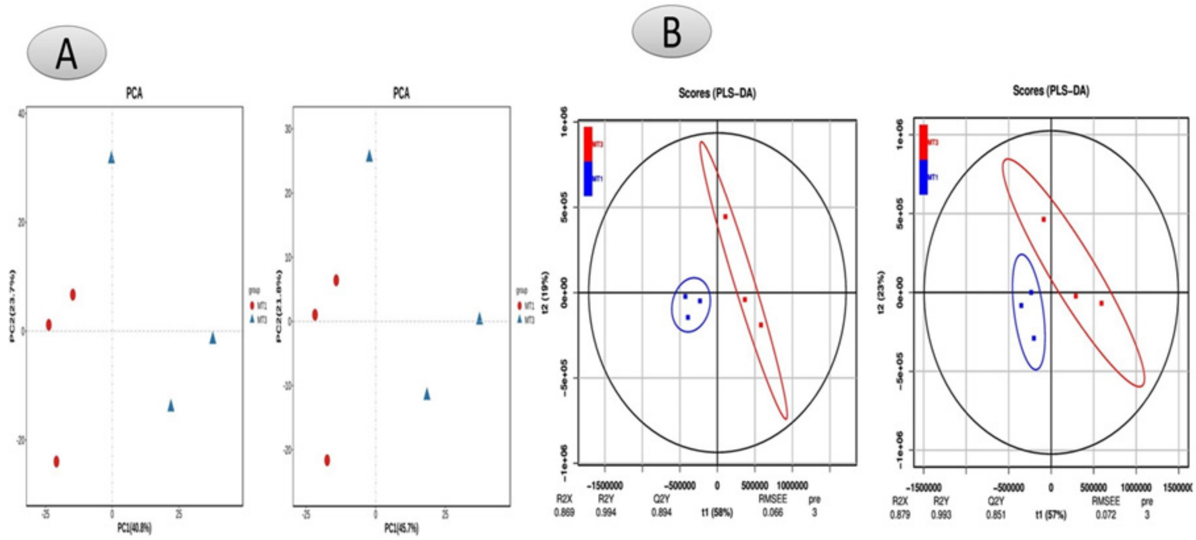

Figure 2. The multivariate analysis compared the control group with shrimp fed a diet high in DDGS and supplemented with BA. The PCA score diagram shows samples in positive and negative ion modes 3A, while the OPLS-DA score diagram focuses on positive and negative ion modes 3B

The impact of dietary DDGS diet on juvenile Pacific shrimp hepatopancreas histopathology

The data presented in Figure 1 indicates that shrimp fed with BA experienced a significant increase in their B and R cell counts. This suggests an enhancement in their secretory and absorptive capabilities. Furthermore, the hepatopancreas tubule arrangement in diet 5 exhibited a noticeable improvement compared to the control group. The tubules were closer together and more organized, indicating a potential adaptation to the dietary composition.

Multivariate analysis of shrimp fed DDGS supplemented with BA

Using PCA, distinct clusters representing the two groups were identified, as shown in Figure 2 A. Separating the control and BA groups in separate quadrants illustrates the discernible difference in their metabolic profiles. Furthermore, the OPLS-DA score plot shown in Figure 2 B effectively distinguished the treatment groups, emphasizing significant differences in the diets of shrimp-fed animals supplemented with BA compared to the control group. The evaluation parameters of the OPLS-DA models confirmed the distinct separation of the groups, with samples falling within the Hotelling's T2 ellipse. The model displayed $R^2X = 0.869$, $R^2Y = 0.994$, and $Q^2 = 0.894$ in the positive ion mode, and $R^2X = 0.879$, $R^2Y = 0.993$, and $Q^2 = 0.851$ in the negative ion mode.

Pathway analysis of differential metabolites in *P. vannamei*

Based on the top 20 KEGG enrichment analyses in Figure 3 A, we identified differentially expressed genes

(DEGs) involved in important biological processes. These processes include nicotinate and nicotinamide metabolism; mineral absorption; phenylalanine, tyrosine, and tryptophan biosynthesis; lysine biosynthesis; secondary bile acid biosynthesis; bile secretion; caffeine metabolism; primary bile acid biosynthesis; glycerophospholipid metabolism; biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid; tryptophan metabolism; arginine, and proline metabolism; choline metabolism in cancer; African trypanosomiasis; cholesterol metabolism; cholinergic synapse; glycine, serine, and threonine metabolism; Pertussis; ABC transporters; and D-amino acid metabolism. Based on the positive ln(P) and impact values, we further characterized the involvement of glycine, serine, and threonine metabolism; arginine and proline metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; phenylalanine metabolism; nicotinate, and nicotinamide metabolism; glycerophospholipid metabolism; tryptophan metabolism; and aminoacyl-tRNA biosynthesis as shown in Figure 3 B. Conversely, the negative ion mode significantly enriched primary bile acid biosynthesis and purine metabolism. Figure 4 displays a heatmap visualization of differentially expressed metabolites. The colors range from blue (indicating low expression) to red (indicating high expression), representing the varying gene expression levels in the samples. This heatmap clearly shows the different gene expression patterns in the control group compared to those treated with BA. The data indicates that genes with similar expression patterns may have a shared function or be part of a common metabolic pathway that helps clams adapt to their diet. Interestingly, metabolites like prostaglandin D2, acetyl-L-carnitine, stearoyl ethanolamide, and choline showed a significant increase in expression in shrimp fed BA.

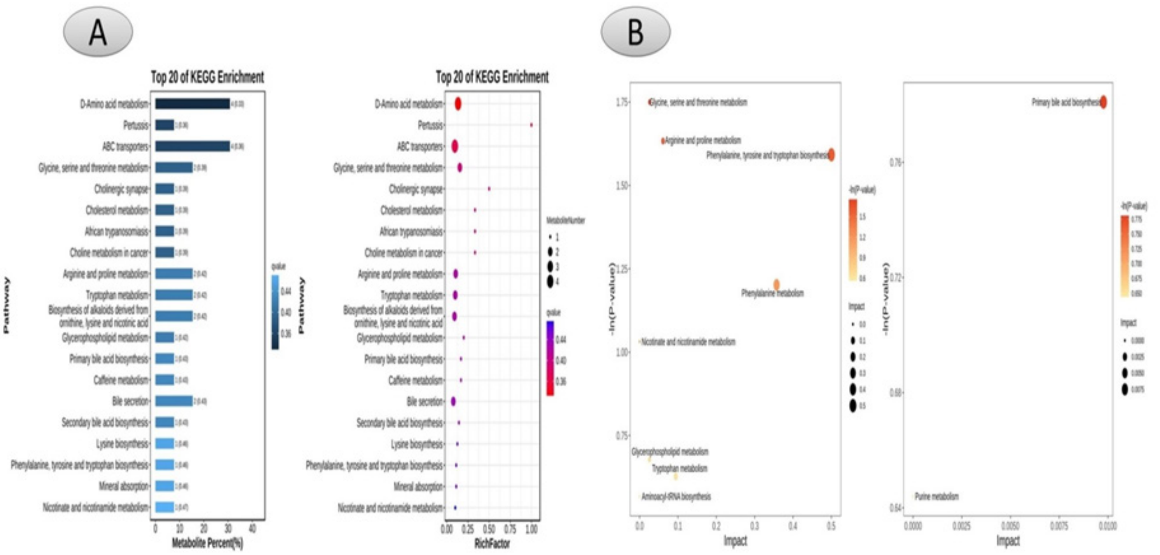

Figure 3. The analysis of metabolites in response to dietary BA supplementation is a key focus in the DDGS diet. The top 20 KEGG terms showed significant enrichment in the analysis of genes expressed differently in 4A. In 4B, the metabolic pathways of the different metabolites are displayed. Larger sizes in the figure indicate significant pathway enrichment and high pathway impact values

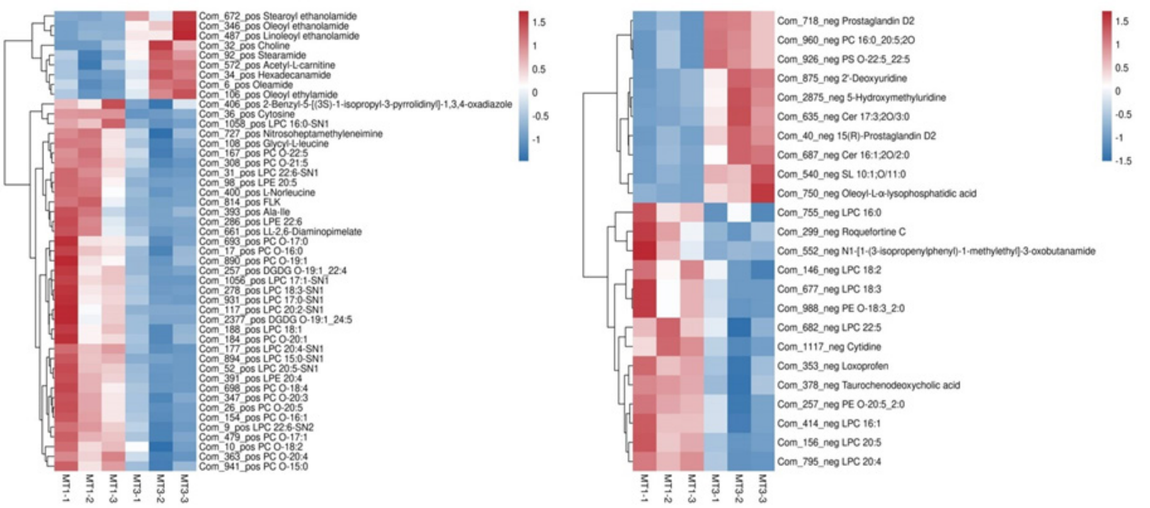

Figure 4. The heat map shows a hierarchical clustering analysis of the significantly different metabolites in reaction to dietary DDGS supplemented with BA in positive and negative ion modes

Discussion

Proper feed intake is essential for promoting aquatic animals' rapid and robust growth. Research has indicated that incorporating a high level of plant protein into shrimp diets can increase mortality rates (Yohana et al., 2024 b). To address this issue, one potential solution is introducing feed additives to aquafeeds' plant protein components. While traditional methods of assessing new feed formulations typically involve analyzing digestibility and composition, these approaches may not comprehensively understand how feeds affect shrimp metabolites. Metabolomic research, originally developed for human nutrition, has recently been adapted for livestock and aquaculture species (Yohana et al., 2023 a). In our study, we explored the impact of supplementing BA in DDGS to mitigate the negative effects of a plant-based diet. Instead of using BA directly, BA salts are commonly integrated into aquafeeds due to their stability and palatability. These additives can be easily blended into animal feed without coating (Zhao et al., 2023). Research has shown that commercial diets containing 2% sodium butyrate and sodium propionate can enhance the feed intake and attractiveness of *P. vannamei* (da Silva et al., 2013). Furthermore, supplements containing fumarate, butyrate, and succinate have been proven to enhance the survival, feed intake, growth, and nutrient retention efficiency of juvenile *P. monodon* (Emerenciano et al., 2022). Research has shown that providing P. vannamei with polyhydroxybutyrate (PHB) can improve WG and SGR (Kiran et al., 2020). Similarly, diets supplemented with 5 g/kg of BA have improved growth and overall health in *L. calcarifer* juveniles (Aalamifar et al., 2020). In another study, a juvenile yellow drum fed a diet high in soybean oil supplemented with 0.20% tributyrin exhibited improved growth performance and changes in muscle fatty acid composition (Zhu et al., 2020). Volatiana et al. (2020) found that incorporating TB into diets at levels between 0.05% to 0.2% led to improved growth performance. Similarly, Liu et al. (2019) discovered that adding SB at a concentration of 0.2% to diets high in SBM enhanced the growth performance of turbot fish by reducing leukocyte infiltration and increasing absorptive surface area, thereby mitigating enteropathy. In another study, Monier et al. (2022) demonstrated that incorporating nutritional nanosized sodium butyrate (NSB) at concentrations of 1.0 to 2.5 mg/kg of diet improved the performance of Nile tilapia without affecting their body composition. During the current research, adding BA to DDGS led to a notable improvement in shrimp growth and feed efficiency. Notably, the treatment group T4 (1.5%) exhibited the highest weight gain among all groups, and the feed conversion ratio improved significantly with the addition of BA to the diet. Regression analysis indicated that the optimal amount of BA supplementation in the DDGS diet for P. vannamei shrimp was 1.6%. These findings highlight the significance of dietary interventions in improving the growth performance of aquatic species.

The hepatopancreas plays a vital role in crustaceans, serving as an organ for digestion, food storage, secretion, and detoxification (Ray et al., 2020: Chen et al., 2023). Understanding the histological changes in shrimp resulting from dietary sources requires a deep dive into the histology of the hepatopancreas. The hepatopancreas comprises hepatopancreatic tubules that house various cell types, including E-, B-, R-, and F-cells, each with specialized functions in the breakdown and absorption of nutrients (Ray et al., 2020). B-cells break down and absorb nutrients filtered through the body's system to the shrimp's hepatopancreas (Abd El-Naby et al., 2024). They have a complex web of microvilli that expand the surface area in contact with the epithelial layer, facilitating effective nutrient absorption (Yohana et al., 2023 b). Research has shown that disruptions in the detoxification processes of the hepatopancreas can occur with a decrease in R-cells and an increase in vacuolated cells. For instance, studies have demonstrated that feeding juvenile yellow drums a diet high in soybean oil and supplementing them with 0.20% tributyrin can enhance their intestinal histology and structure (Zhu et al., 2020). When the organic acid blend (OAB) was incorporated into shrimp diets, there was a noticeable improvement in the histopathology of the hepatopancreas, with an increase in R-cells and B-cells and a decrease in damage. Previous research by Robles et al. (2013) has shown that adding pure sodium butyrate at a concentration of 0.21% to fish diets can enhance villi development and increase the absorptive surface area in sea bream (*Sparus aurata*). Our current study revealed a significant increase in B- and R-cell counts in shrimp fed with BA, enhancing their secretion and absorption capabilities. Furthermore, there was a clear improvement in the arrangement of the hepatopancreas tubules in diet 5 compared to the control group. The more organized and closely spaced tubules suggest that they may have adapted to the composition of the diet.

Studying metabolite profiles in fish and crustaceans has used metabolomics, a powerful method for analyzing metabolite changes and metabolic pathways in response to different diets (Petrick and Shomron, 2022). The field of nutrition research has experienced a significant transformation due to LC-MS-based metabolomics, known for its exceptional sensitivity, speed, and efficiency in nutrient characterization (Song et al., 2023). A deeper understanding of nutrition, feed quality, and health tracking can be obtained through metabolomics tissue analysis and feeding experiments. In this study, the KGG pathway demonstrates that the addition of BA to DDGS diets can effectively maintain cellular balance by regulating multiple pathways, including crucial functions such as D-amino acid metabolism, the ABC transporter, glycerophospholipid metabolism, aminoacyl-tRNA biosynthesis, and bile acids secretion. Amino acids are vital for body proteins and play a key role in supporting processes related to protein turnover (Yue et al., 2022). Notably, the incorporation of BA also significantly influenced several amino acid biosynthesis pathways, including arginine and proline metabolism, phenylalanine, tyrosine, lysine, and tryptophan biosynthesis, as well as metabolic pathways like glycine, serine, and threonine metabolism, and glycine metabolism. Proline significantly impacts various cellular processes, including protein synthesis, anti-oxidative reactions, and immune responses (Xie et al., 2015; Shao et al., 2023). The role of threonine in fish nutrition is vital, as it acts as the primary limiting amino acid in low-protein diets. It involves various physiological and biochemical processes, such as immune system function and growth (Alhasaniah, 2023). Research by Dong et al. (2017) has shown that a deficiency in threonine can decrease a fish's resistance to intestinal lysozyme, acid phosphatase, enteritis, and certain anti-inflammatory factors, ultimately impacting the growth and development of juvenile *C. idella*. Furthermore, studies by Yu et al. (2024) have demonstrated that a threonine deficiency can lead to reduced immune system function, antioxidant capacity, and growth inhibition in juvenile blunt-snout bream. Proline, an important amino acid, is crucial for collagen formation, which is essential for maintaining healthy connective tissues, as well as participating in protein synthesis and boosting antioxidative defenses (Ren et al., 2020 a; Wang et al., 2022; Xie et al., 2015; Shao et al., 2023). Studies have demonstrated that arginine, a functional amino acid type, can boost aquatic animals' immune system (Duan et al., 2023). Arginine is a phosphagen in crustaceans and is crucial in promoting optimal shrimp growth (Zhou et al., 2012). Previous research by Li et al. (2023 b) has indicated that arginine can improve muscle firmness and shear force, thereby enhancing the flesh quality of grass carp (*Ctenopharyngodon idella*). Similarly, studies on sea bream fish suggest that butyrate can stimulate growth by increasing the availability of amino acids (Robles et al., 2013). Derived from phenylalanine, tyrosine is an aromatic amino acid commonly utilized as a precursor for essential hormones and neurotransmitters, including triiodothyronine, epinephrine, nor-epinephrine, dopamine, and melanin (Yang et al., 2018). Phenylalanine, the sole precursor of tyrosine, is also an aromatic amino acid crucial for normal growth and metabolic functions (Zehra and Khan, 2014). Once phenylalanine enters the body, it can undergo one of three processes: conversion to tyrosine, incorporation into cellular proteins, or transformation into phenylpyruvic acid (Vockley et al., 2014). Research by Huang et al. (2020) has shown that phenylalanine can enhance lysozyme expression in *Danio rerio*, aiding in removing resistant *Vibrio alginolyticus*. Therefore, maintaining adequate levels of phenylalanine and tyrosine is vital for the survival, feed intake, growth performance, immunity, and overall physiological processes of aquatic animals (Li et al., 2023 b).

Furthermore, ABC transporter proteins not only assist in maintaining lipid homeostasis within cells by facilitating the export and storage of lipids from adipocytes but also play a role in regulating inflammation (Ma et al., 2024). These proteins are responsible for transporting lipophilic compounds such as steroid hormones and capturing the energy produced by ATP (Zhao and Xu, 2022). According to Xu et al. (2024), glycerophospholipids are a type of phospholipid compound known for their ability to lower serum cholesterol, promote lipid metabolism, prevent fatty liver, and improve circulation. These compounds are crucial components of cellular membranes, playing a key role in maintaining membrane structure and function (Shija et al., 2024). The BA group exhibited significantly higher glycerophospholipid metabolism, consistent with previous research findings (Wang et al., 2022). Research has shown that wounding can significantly impact the metabolic pathway of aminoacyl-tRNA biosynthesis. Albaladejo-Riad et al. (2023) explain that enzymes involved in this process play a vital role in binding amino acids to tRNAs, contributing to various processes essential for healing, such as transcription, inflammation, translation, splicing, cell proliferation, and apoptosis. Additionally, the study found that dietary factors also influence aminoacyl-tRNA biosynthesis. Bile acids are essential for the communication between the liver and the gut, serving as endogenous molecules synthesized from cholesterol. They are believed to act as versatile mediators, regulating various physiological processes and facilitating the absorption of nutrients (Su et al., 2021). The amphipathic structure of bile acids allows them to form micelles that aid in the digestion of fats, cholesterol, and lipid-soluble vitamins, enhancing overall nutrient absorption (Bao et al., 2024). Additionally, bile acids can function as hormones or signaling molecules by activating nuclear hormone receptors, leading to a range of effects. Recent research suggests bile acids may influence immune response and antioxidant defense regulation (Li et al., 2023 a). The heatmap analysis uncovered a significant discovery regarding the metabolite levels in shrimp fed BA compared to the control group. Specifically, metabolites such as prostaglandins, choline, and acetyl L-carnitine were significantly upregulated. Arachidonic acid serves as the precursor for prostaglandins (Ren et al., 2020 b; Kong and Yu, 2022) and is a key component of polyunsaturated fatty acids (Kudełka et al., 2021; Yang et al., 2022). Prostaglandins play a crucial role as arachidonic acid metabolites in animals' immune systems and antioxidant defense mechanisms (Xie et al., 2020; Yohana et al., 2024 b). These bioactive lipids can modulate T cell phenotype and function, leading to a spectrum of pro- and anti-inflammatory effects. This highlights the intricate ways prostaglandins operate, influencing immune cell activation and response regulation. The immune system is vital for maintaining internal equilibrium and safeguarding against external infections. Upregulated prostaglandins also play a key role in controlling the production of inflammatory cytokines, which serve as signaling molecules in the immune response (Luo et al., 2024). However, dysregulation of prostaglandin production can result in chronic inflammation and autoimmune diseases. Choline-based lipids are vital in cell structure upkeep and lipid transportation processes

(Lu et al., 2023). L-carnitine acts as a transporter of acyl and acetyl groups in the mitochondria. L-carnitine is a vital nutrient with multifunctional roles in aquatic organisms, essential for fatty acid metabolism (Mirrafiei et al., 2024). A crucial aspect of its purpose is the breakdown of fatty acids in the mitochondrial β-oxidation pathway, resulting in the production of energy and supporting lipid metabolism (Raghuvaran et al., 2023).

Conclusion

This study compared the growth performance, histopathology, and metabolomics profiles of shrimp that consumed a control diet to those that were given high levels of DDGS as the main protein source with BA supplementation. Shrimp supplemented with BA exhibited significantly higher absolute growth rates and a more significant number of B and R cells. Additionally, their tubules were more closely packed and organized, suggesting an adaptation to the dietary composition compared to the control group. Further analysis of key metabolic pathways revealed that shrimp supplemented with BA had a greater capacity for ABC transporter, phenylalanine and tyrosine metabolism, arginine and proline metabolism, and prostaglandins D2 compared to the control group. The optimal level of BA inclusion in high DDGS diets was 1.6%, while in a previous study with high corn gluten meal inclusion, the optimal level was 1.25%. This indicates that the BA needed in a plant protein diet depends on the specific plant protein used. Exploring the potential benefits of adding BA to feed formulas in continuous shrimp farming, it is advised that one comprehends its mechanisms and advantages better, mainly when plant-based proteins are used.

Authors' contribution

Qihui Yang oversaw funding, project supervision, and concept development. Beiping Tan provided supervision. Alatwinusa Yohana Mpwaga and Ray Watson Gyan were in charge of conceptualizing, designing, conducting experiments, and writing the manuscript. Shiyu Kou also edited the manuscript.

Declaration of conflict of interest

According to the authors, there were no conflicting interests in this study.

Availability of data

Data will be given when asked for.

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