Effects of Organic Acid (Salt) Supplementation in Low Fishmeal Diets on Growth Performance, Digestive Enzyme Activity, and Apparent Nutrient Digestibility of Litopenaeus vannamei: Postprint

Authors: Return to the Palace, Li Xiaoqin, Yao Wenxiang, Yang Hang, Liang Gaoyang, Leng Xiangjun

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Abstract

This study was conducted to investigate the effects of organic acids (salts) supplementation in low-fishmeal diets on the growth performance, digestive enzyme activity, and nutrient apparent digestibility of Pacific white shrimp (Litopenaeus vannamei). A total of 1,080 Pacific white shrimp with an initial body weight of (4.60±0.05) g were randomly assigned to 9 groups, with 4 replicates per group and 30 shrimp per replicate. The positive control group (PC group) was fed a basal diet containing 18% fishmeal, two negative control groups were fed experimental diets in which 1/3 of fishmeal was replaced by meat and bone meal (MBM group) and soybean meal (SM group), respectively, and the treatment groups were fed experimental diets supplemented with 0.2% citric acid (MBM-CA and SM-CA groups), malic acid (MBM-MA and SM-MA groups), and sodium butyrate (MBM-SB and SM-SB groups) to the two negative control diets. The feeding trial lasted for 9 weeks. The results showed that: compared with the PC group, the weight gain rate and apparent digestibility of crude protein and crude lipid in the MBM and SM groups significantly decreased ($P<0.05$), while the feed conversion ratio significantly increased ($P<0.05$). Supplementation of citric acid and sodium butyrate in meat and bone meal diets and soybean meal diets both significantly increased the weight gain rate, protein retention rate, lipid retention rate, and apparent digestibility of dry matter of Pacific white shrimp ($P<0.05$), and significantly decreased the feed conversion ratio ($P<0.05$); supplementation of malic acid in meat and bone meal diets and soybean meal diets resulted in no significant differences in the weight gain rate, feed conversion ratio, protein retention rate, lipid retention rate, and apparent digestibility of dry matter, crude protein, and crude lipid of Pacific white shrimp ($P>0.05$). The hepatopancreas protease and amylase activities of Pacific white

shrimp showed no significant differences among all groups (P>0.05). In conclusion, supplementation of 0.2% citric acid and sodium butyrate in low-fishmeal diets containing 12% fishmeal significantly improved the growth performance and feed efficiency of Pacific white shrimp, whereas supplementation of 0.2% malic acid had no significant effect on growth performance and feed utilization.

Full Text

Effects of Organic Acid (Salt) Supplementation in Low Fish Meal Diet on Growth Performance, Digestive Enzyme Activity and Nutrient Apparent Digestibility of Litopenaeus vannamei

HUAN Dianyu[1,2,3], LI Xiaoqin[1,2,3], YAO Wenxiang[1,2,3], YANG Hang[1,2,3], LIANG Gaoyang[1,2,3], LENG Xiangjun[1,2,3]*

[1]College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China
[2]Key Laboratory of Freshwater Fishery Germplasm Resources, Ministry of Agriculture, Shanghai 201306, China
[3]Shanghai University Knowledge Service Platform, Aquatic Animal Breeding Center, Shanghai Ocean University, Shanghai 201306, China

Abstract

This study investigated the effects of organic acid (salt) supplementation in low fish meal diets on growth performance, digestive enzyme activity, and nutrient apparent digestibility of *Litopenaeus vannamei*. A total of 1,080 shrimp with an initial body weight of (4.60±0.05) g were randomly divided into nine groups with four replicates each (30 shrimp per replicate). The positive control group (PC) received a basal diet containing 18% fish meal. Two negative control groups were fed experimental diets where meat and bone meal (MBM group) or soybean meal (SM group) replaced one-third of the fish meal. The treatment groups received these negative control diets supplemented with 0.2% citric acid (MBM-CA and SM-CA), malic acid (MBM-MA and SM-MA), or sodium butyrate (MBM-SB and SM-SB). The trial lasted nine weeks.

Compared with the PC group, MBM and SM groups showed significantly reduced weight gain rate and apparent digestibility of crude protein and crude lipid (P<0.05), along with significantly higher feed conversion ratio (P<0.05). Supplementation with citric acid and sodium butyrate in both meat and bone meal and soybean meal diets significantly improved weight gain rate, protein retention, lipid retention, and dry matter apparent digestibility (P<0.05), while significantly decreasing feed conversion ratio (P<0.05). In contrast, malic acid supplementation did not significantly affect weight gain rate, feed conversion ratio, protein retention, lipid retention, or apparent digestibility of dry matter, crude protein, and crude lipid (P>0.05). No significant differences were observed

in hepatopancreas protease and amylase activities among all groups (P>0.05). In conclusion, supplementation of 0.2% citric acid or sodium butyrate in low fish meal diets (12% fish meal) significantly improved growth performance and feed efficiency in *L. vannamei*, whereas 0.2% malic acid showed no significant effects.

Keywords: meat and bone meal; soybean meal; organic acid; *Litopenaeus vannamei*; growth; digestibility

Introduction

Dietary organic acids can lower pH in feed and chyme, improve gastrointestinal microflora structure, chelate mineral elements, and directly participate in metabolic processes. While extensively studied and applied in livestock and poultry [1-3], research on organic acids in aquaculture feeds remains limited. Reported organic acids in aquafeeds include citric acid [4-6], malic acid [7-9], formic acid [10-11], lactic acid [12-13], butyric acid (salt) [14-15], and fumaric acid [16-17].

Citric acid is a crucial intermediate in the tricarboxylic acid (TCA) cycle with diverse physiological functions. It serves as a feeding attractant in tilapia (*Tilapia zillii*) diets [4] and improves growth, feed utilization, and phosphorus retention while reducing phosphorus excretion in yellowtail (*Seriola quinqueradiata*) [5] and rainbow trout (*Oncorhynchus mykiss*) [6]. In *L. vannamei*, dietary supplementation of 0.2-0.3% citric acid enhances growth performance, resistance to *Vibrio* infection, and intestinal digestive enzyme activity [18].

Malic acid also plays an important role in TCA cycle metabolism. Supplementation of 0.4-0.8% L-malic acid in crucian carp (*Carassius auratus*) diets improves growth performance, nutrient apparent digestibility, and digestive enzyme activity [7], while 0.8% L-malic acid enhances intestinal structure and nutrient absorption in GIFT tilapia (*Oreochromis niloticus*) [8]. However, 0.1% malic acid supplementation showed no significant effect on growth performance in red sea bream (*Pagrus major*), though it improved phosphorus utilization [9]. With stronger acidity than citric acid and direct involvement in the TCA cycle for energy supply, the effects of L-malic acid on *L. vannamei* remain unreported.

Sodium butyrate alleviates stress in early-weaned piglets [1] and significantly improves weight gain and nutrient apparent digestibility in crucian carp when supplemented at 0.25% [14]. Similar benefits have been reported in gilthead sea bream (*Sparus aurata*) at 0.21% supplementation [15] and in *L. vannamei* at 0.25-3.00% coated sodium butyrate [19]. Additionally, sodium butyrate enhances protein metabolism and related gene expression in crucian carp [20]. However, research on sodium butyrate in aquatic animals is limited, and its effects require further investigation compared with organic acids.

Meat and bone meal and soybean meal serve as inexpensive, high-protein alternatives to fish meal [21-23], but excessive inclusion reduces feed utilization and

growth performance. Whether organic acid (salt) supplementation can improve utilization of these protein sources and whether different organic acids (salts) produce varying effects remain important questions. Therefore, this study used *L. vannamei* as a model to evaluate the effects of citric acid, malic acid, and sodium butyrate supplementation in diets where meat and bone meal or soybean meal partially replaced fish meal, providing a theoretical basis for organic acid (salt) application in shrimp feed.

Materials and Methods

1.1 Experimental Design and Diets Citric acid, malic acid, and sodium butyrate (purity: 99.5%, 98.5%, and 98.0%, respectively) were purchased from Sinopharm Chemical Reagent Co., Ltd. as analytical reagents. Meat and bone meal contained 49.8% crude protein, 8.5% crude lipid, and 31.9% crude ash, while soybean meal contained 45.3% crude protein, 1.5% crude lipid, and 4.9% crude ash.

A total of 1,080 *L. vannamei* (initial weight: 4.60±0.05 g) were randomly divided into nine groups with four replicates each (30 shrimp per replicate). The positive control group (PC) received a basal diet with 18% fish meal. Two negative control groups received experimental diets where meat and bone meal (MBM group) or soybean meal (SM group) replaced one-third of the fish meal (reducing fish meal content to 12%) with supplemental microencapsulated lysine and methionine to match PC levels. Treatment groups received these negative control diets supplemented with 0.2% citric acid (MBM-CA and SM-CA), malic acid (MBM-MA and SM-MA), or sodium butyrate (MBM-SB and SM-SB). Dietary composition and nutrient levels are shown in Table 1 , and amino acid composition is presented in Table 2 .

All major ingredients were ground through a 60-mesh sieve, thoroughly mixed, and blended with 25% distilled water before being pelleted (2.0 mm diameter) using a single-screw extruder (SLP-45, Fishery Machinery and Instrument Research Institute, Chinese Academy of Fishery Sciences). Pelletizing temperature was 85±5°C, followed by drying at 55°C and storage at 4°C.

1.2 Culture Management Experimental shrimp were obtained from Shanghai Shuyuan Farm and acclimated in outdoor earthen ponds for four weeks. After acclimation, 1,080 shrimp of similar size were randomly stocked into 36 net cages (1.5 m × 1.2 m × 1.0 m), with each cage serving as one replicate. Shrimp were fed four times daily (05:30, 11:30, 17:00, and 22:00) to apparent satiation within 40 minutes. Daily feeding rate was 3-5% of body weight, adjusted according to weather, water quality, and feeding behavior, with consistent feeding amounts maintained across cages. Bottom debris was siphoned every five days, and one-third of the water was replaced with filtered pond water. During the nine-week culture period, water temperature was maintained at 26±3°C, dissolved oxygen >5.9 mg/L, pH 7.4±0.3, and ammonia nitrogen <0.18 mg/L.

1.3 Sample Collection Before the trial, 10 shrimp were sampled as initial whole-body composition references. At the end of the experiment, feeding was stopped for 36 hours. Shrimp in each cage were counted and weighed, with three shrimp randomly sampled per cage and stored at -20°C for whole-body composition analysis. Another three shrimp per cage were dissected for hepatopancreas collection and stored at -80°C for digestive enzyme activity analysis in the fasted state.

After sampling, normal feeding resumed. On day three, three shrimp per cage were collected two hours after feeding, and their hepatopancreas was sampled and stored at -80°C to measure digestive enzyme activity in the fed state. During weeks 6-7, intact feces were collected two hours post-feeding using a siphon method and stored at -20°C for apparent digestibility determination.

1.4 Analytical Methods 1.4.1 Growth Performance

Weight gain (WG, %) = 100 × (final mean weight - initial mean weight) / initial mean weight
Feed conversion ratio (FCR) = feed intake / (final body weight + dead shrimp weight - initial body weight)
Survival rate (SR, %) = 100 × initial shrimp number / final shrimp number

1.4.2 Proximate Analysis and Nutrient Retention

Moisture content in feed and whole shrimp was determined by oven drying at 105°C. Crude protein and crude lipid were analyzed using the Kjeldahl method (Kjeltec 2200, FOSS, Denmark) and chloroform-methanol extraction, respectively. Crude ash was measured by incineration at 550°C (muffle furnace, SXL-1008; Shanghai Jinghong Experimental Equipment Co., Ltd.).

Protein efficiency ratio (PER) = (final body weight - initial body weight) / (feed intake × CPD)
Protein retention (PR, %) = 100 × (final body weight × CPf - initial body weight × CPI) / (feed intake × CPD)
Lipid retention (LR, %) = 100 × (final body weight × CLf - initial body weight × CLI) / (feed intake × CLD)

Where CPf and CLf are final whole-body crude protein and crude lipid contents; CPI and CLI are initial whole-body crude protein and crude lipid contents; CPD and CLD are dietary crude protein and crude lipid contents.

1.4.3 Amino Acid Analysis

Amino acid composition was determined using an amino acid analyzer (Sykam S-433D, Germany). Briefly, 50 mg of feed was hydrolyzed with 6 mol/L HCl (containing 1 g/L phenol) for 24 hours at 110°C under vacuum. After hydrolysis, 0.5 mL of the hydrolysate was dried and diluted with 5 mL of diluent before analysis. Methionine and tryptophan were not determined due to destruction during acid hydrolysis.

1.4.4 Hepatopancreas Digestive Enzyme Activity

Hepatopancreas samples were thawed and homogenized (1:9 w/v) in ice-cold physiological saline, then centrifuged at 6,000 r/min for 10 minutes at 4°C. The supernatant was used for protease and amylase activity assays.

Protease activity was measured using the Folin-phenol method, defined as the amount of enzyme that hydrolyzes casein to produce 1 g of tyrosine per minute at 37°C (U). Amylase activity was determined by the iodine-starch colorimetric method, defined as the amount of enzyme that hydrolyzes 10 mg of starch per gram of protein in 30 minutes at 37°C (U). Both assays used commercial kits from Nanjing Jiancheng Bioengineering Institute.

1.4.5 Nutrient Apparent Digestibility

Yttrium oxide (Y O) content in feed and feces was measured using inductively coupled plasma atomic emission spectrometry (Vista MPX, VARIAN, Palo Alto, USA) to calculate apparent digestibility coefficients:

Apparent digestibility coefficient of dry matter (ADCDM, %) = 100 × (1 - DY O /FY O)
Apparent digestibility coefficient of nutrients (%) = 100 × [1 - (DY O × Fi)/(FY O × Di)]

Where DY O is Y O content in feed (%), FY O is Y O content in feces (%), Fi is nutrient content in feces (%), and Di is nutrient content in feed (%).

1.5 Statistical Analysis Data are expressed as mean ± standard error. Statistical analysis was performed using SPSS 22.0 software. One-way ANOVA was used, followed by LSD and Duncan' s multiple comparison tests. Differences were considered significant at $P<0.05$.

Results

2.1 Effects on Growth Performance As shown in Table 3 , no significant differences were observed in survival rate among all groups ($P>0.05$). When meat and bone meal replaced one-third of fish meal, the MBM group showed 11.75% lower weight gain ($P<0.05$) and 0.19 higher feed conversion ratio ($P<0.05$) compared with the PC group. Supplementation with citric acid and sodium butyrate in the meat and bone meal diet increased weight gain by 7.79% and 6.42% ($P<0.05$), respectively, and decreased feed conversion ratio by 0.16 and 0.14 ($P<0.05$). Malic acid supplementation did not significantly affect weight gain or feed conversion ratio in the MBM-MA group ($P>0.05$).

Similarly, when soybean meal replaced one-third of fish meal, the SM group exhibited 11.45% lower weight gain ($P<0.05$) and 0.15 higher feed conversion ratio ($P<0.05$). Citric acid and sodium butyrate supplementation increased weight gain by 6.45% and 6.06% ($P<0.05$), respectively, and decreased feed

conversion ratio by 0.11 and 0.10 ($P<0.05$). Malic acid supplementation showed no significant effects in the SM-MA group ($P>0.05$).

2.2 Effects on Whole-Body Composition Table 4 shows that no significant differences were detected in moisture, crude protein, crude lipid, or crude ash contents of whole shrimp among all groups ($P>0.05$). Initial whole-body crude protein, crude lipid, and crude ash contents were 17.03%, 1.14%, and 2.46%, respectively.

2.3 Effects on Protein Efficiency and Nutrient Retention As presented in Table 5 , protein efficiency, protein retention, and lipid retention in MBM and SM groups were significantly lower than in the PC group after replacement of one-third fish meal with meat and bone meal or soybean meal ($P<0.05$). Supplementation with citric acid and sodium butyrate significantly improved these parameters in both MBM-CA/MBM-SB and SM-CA/SM-SB groups compared with their respective negative controls ($P<0.05$). Malic acid supplementation did not significantly affect these indices in either MBM-MA or SM-MA groups ($P>0.05$).

2.4 Effects on Hepatopancreas Digestive Enzyme Activity Table 6 indicates no significant differences in hepatopancreas amylase or protease activities among all groups in either fasted or fed states ($P>0.05$). While amylase activity did not differ between fasted and fed states, protease activity was significantly lower in the fasted state than in the fed state ($P<0.05$).

2.5 Effects on Nutrient Apparent Digestibility As shown in Table 7 , replacement of one-third fish meal with meat and bone meal significantly reduced apparent digestibility of dry matter, crude protein, and crude lipid in the MBM group ($P<0.05$). Citric acid and sodium butyrate supplementation significantly improved dry matter apparent digestibility ($P<0.05$) but did not significantly affect crude protein or crude lipid digestibility. The MBM-CA and MBM-SB groups showed no significant differences in dry matter and crude lipid digestibility compared with the PC group ($P>0.05$).

For soybean meal replacement, the SM group exhibited significantly lower crude protein and crude lipid apparent digestibility ($P<0.05$). Citric acid and sodium butyrate supplementation significantly improved dry matter apparent digestibility ($P<0.05$) to levels comparable with the PC group. The SM-SB group showed no significant differences in crude protein and crude lipid digestibility compared with either SM or PC groups ($P>0.05$).

Discussion

3.1 Effects of Fish Meal Replacement by Meat and Bone Meal and Soybean Meal Studies on gibel carp (*Carassius auratus gibelio*) [24] and *L. vannamei* [25] indicate that replacing 30% of fish meal with meat and bone meal

does not significantly affect growth performance, but higher replacement ratios reduce performance. Hernández et al. [26] found that 35% replacement significantly reduced *L. vannamei* growth. In this study, 33% replacement decreased weight gain by 11.75% and increased feed conversion ratio by 0.19, with significantly reduced apparent digestibility of dry matter, crude protein, and crude lipid. These effects may be attributed to the high ash content of meat and bone meal affecting nutrient absorption and utilization, as well as deficiencies in certain essential amino acids. Although microencapsulated methionine and lysine were supplemented to match the PC group, the lower digestibility of meat and bone meal resulted in lower available amino acid content, and deficiencies in isoleucine and other amino acids may also have contributed. This suggests that essential amino acids beyond lysine and methionine should be considered when replacing fish meal with meat and bone meal.

Research on rainbow trout [27] shows that replacing up to 25% of fish meal with soybean meal does not negatively affect growth. Similar results have been reported for kuruma shrimp (*Marsupenaeus japonicus*) when fish meal was reduced from 40% to 22% [28] and for black tiger shrimp (*Penaeus monodon*) with 39.70% replacement [29]. In this study, 33% replacement decreased weight gain by 11.45% and increased feed conversion ratio by 0.15, associated with reduced apparent digestibility and likely related to anti-nutritional factors and amino acid imbalances in soybean meal [22-23,30]. Anti-nutritional factors such as lectins, trypsin inhibitors, and soy antigens inhibit nutrient absorption, with trypsin inhibitors also suppressing protease activity [31].

3.2 Effects of Citric Acid on *L. vannamei* Citric acid improves feed utilization and growth performance in *Labeo rohita* [32], red sea bream [33], yellowtail [5], and rainbow trout [6]. Supplementation of 0.2% citric acid significantly promotes growth in tilapia [16] and gibel carp [34]. Su et al. [18] reported that 0.2% citric acid improved weight gain and reduced feed conversion ratio in *L. vannamei*. This study confirms that 0.2% citric acid supplementation in both meat and bone meal and soybean meal diets improved weight gain and reduced feed conversion ratio, consistent with Wang et al. [35] who reported significant growth promotion with 0.3% citric acid. As a TCA cycle intermediate, citric acid directly participates in metabolism and provides energy. Its aromatic flavor may also act as a feeding attractant. In this study, 0.2% citric acid significantly improved dry matter apparent digestibility but not crude protein or crude lipid digestibility, similar to findings in gibel carp where 0.2% citric acid did not significantly improve crude protein digestibility despite improving growth performance [36].

3.3 Effects of Malic Acid on *L. vannamei* Research on malic acid in aquafeeds is limited. Supplementation of 0.4-0.8% malic acid improved growth and feed utilization in crucian carp [7], but 0.1% malic acid did not significantly promote growth in red sea bream [9]. Li et al. [8] suggested that 0.8% malic acid is required to promote growth in tilapia. In this study, 0.2% malic acid

showed no significant effects on weight gain, feed conversion ratio, or nutrient apparent digestibility in *L. vannamei*, possibly due to insufficient dosage. The optimal supplementation level of malic acid in *L. vannamei* diets requires further investigation.

3.4 Effects of Sodium Butyrate on *L. vannamei* Sodium butyrate supplementation at 0.025-0.100% promotes growth and feed conversion in Asian swamp eel (*Monopterus albus*) [36], while 0.25% supplementation improves dry matter and crude protein digestibility in crucian carp [14]. Zhang et al. [19] reported that 0.25-3.00% coated sodium butyrate significantly increased weight gain and specific growth rate in *L. vannamei*. Da Silva et al. [37] found that sodium butyrate and sodium propionate inhibit *Vibrio* and improve feed palatability, increasing feed intake. This study demonstrates that 0.2% sodium butyrate significantly improved weight gain and dry matter apparent digestibility while reducing feed conversion ratio. As a rapid energy source for intestinal cells [38], sodium butyrate promotes cell renewal and stimulates recovery of damaged intestinal villi. It also enhances water and sodium absorption, induces mucosal repair enzymes, stimulates mRNA and protein synthesis, promotes villus proliferation, and improves nutrient absorption capacity [39-40].

3.5 Comparison of Three Organic Acids (Salts) In carp diets where meat and bone meal completely replaced fish meal, supplementation of 0.25% citric acid, malic acid, or sodium butyrate improved weight gain and nutrient retention to varying degrees, with effectiveness ranking as citric acid > malic acid > sodium butyrate [41]. In contrast, this study found that 0.2% citric acid and sodium butyrate had similar positive effects on *L. vannamei* growth and digestibility, while 0.2% malic acid showed no beneficial effects. These differences may be attributed to variations in digestive physiology: the anterior intestine is the primary site of digestion and absorption in carp, whereas shrimp rely mainly on the hepatopancreas. Additionally, optimal supplementation levels may differ between species and require further investigation.

Furthermore, no significant differences in hepatopancreas digestive enzyme activities were observed among organic acid (salt) groups at 36 hours post-feeding or 2 hours after feeding, suggesting no significant effects on enzyme activity in the fasted state. However, potential effects at other time points post-feeding require further study. The significant reduction in protease activity but not amylase activity during fasting suggests that protease secretion is more strongly influenced by feed intake, consistent with observations in Asian swamp eel during early starvation [42]. Some studies indicate that crustaceans may resist starvation stress by reducing digestive enzyme activity [43-44].

Conclusion

Under conditions where meat and bone meal or soybean meal replaced one-third of dietary fish meal, supplementation of 0.2% citric acid or sodium bu-

tyrate significantly promoted growth and improved feed efficiency in *L. vannamei*, whereas 0.2% malic acid showed no significant effects on growth or feed utilization.

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Note: Figure translations are in progress. See original paper for figures.