

Research Article

Methionine sources and *Bacillus amyloliquefaciens* CECT 5940 effects on growth, body composition, and nutrient metabolism of *Penaeus vannamei* fed reduced fishmeal diets

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ABSTRACT. An eight-week feeding trial investigated the effects of fishmeal (FM) replacement by soybean meal (SBM) and poultry by-product meal (PBM) in diets supplemented with two methionine sources (DL-Met and AQUAVI®) and their combinations with ECOBIOL® (*Bacillus amyloliquefaciens* CECT 5940) on growth performance, body composition, and nutrient metabolism of *Penaeus vannamei*. Four hundred shrimp (0.30 ± 0.04 g) randomly distributed into 20 tanks (20 shrimp tank⁻¹) were fed with five experimental diets (four repetitions per diet). A control diet (CD; 20% FM) and four diets with 50% FM replacement supplemented with different methionine sources and ECOBIOL® combinations: D1 (0.13% DL-MET), D2 (0.06% AQUAVI®), D3 (0.13% DL-MET plus 0.10% ECOBIOL®), and D4 (0.06% AQUAVI® plus 0.10% ECOBIOL®). Shrimp fed D2 and D4 had better growth performance, nutrient utilization, and body composition. Shrimp fed D3 also had good growth performance but higher lipid body composition. Shrimp fed D1 had the worst growth performance, nutrient utilization, and body composition values. Nutrient metabolism-related genes were affected by dietary treatments. Protein synthesis-related genes decreased mostly in shrimp fed D1 and D2 while increased mostly in shrimp fed D3 and D4, lipolysis-related genes had a better transcriptional response in shrimp fed D4 and lipogenesis-related genes were mostly downregulated in shrimp fed D2 while were mostly upregulated in shrimp fed D3 and D4. Results suggested that FM could be partially replaced with SBM and PBM in shrimp feeds supplemented with 0.06% AQUAVI® alone or combined with 0.10% ECOBIOL® without adversely affecting the growth and nutrient metabolism of *P. vannamei*.

Keywords: *Penaeus vannamei*; shrimp nutrition; fishmeal replacement; nutrient metabolism

INTRODUCTION

The Pacific white shrimp *Penaeus vannamei* is the most representative species in shrimp farming, and the production reached almost 11.2 million tons in 2022, accounting for about 52% of the global shrimp production (FAO 2022). Fishmeal (FM) is a major protein source in commercial shrimp feed (Cho & Kim 2011). The use of aquatic feeds has increased FM demand due to the rapid development of the world's aquaculture. However, FM supply is limited by resource depletion and overfishing, resulting in an increasing price (Yu et al. 2022). Therefore, it is required research into alternative protein sources more profitable and contributes to aquaculture sustainability (Song et al. 2022).

Previous studies focused on shrimp growth reported that FM can be partially substituted by various animal and vegetable sources (Yao et al. 2022). Due to the high protein content, soybean meal (SBM) is the major vegetal source used in aquafeed. Still, anti-nutritional factors, poor digestibility, and an imbalance of essential amino acids (EAA) affect growth and nutrient absorption (Zhang et al. 2020). Poultry by-product meal (PBM) has been used as the animal protein source in FM-reduced shrimp feed. Nevertheless, methionine and lysine deficiency could affect the growth and welfare of aquatic organisms (Chaklader et al. 2020).

Methionine is a functional amino acid because, in addition, to participating in protein synthesis also participates in immunity, reproduction, and nutrient metabolism (Wang et al. 2021), hence is considered a limiting amino acid in FM alternative protein sources as SBM and PBM; then, a reduced methionine availability could impair the feed utilization, nutrient metabolism, energy allocation, and reduced shrimp growth (Nguyen et al. 2019). Therefore, low FM feeds require methionine supplementation to reduce the adverse effects on the metabolism and growth of aquatic organisms (Zhou et al. 2021). Also, it has been reported that probiotics supplementation could help to alleviate the negative impacts on the health and growth of shrimp-fed reduced FM diets due to their capacity for improving feed utilization, enzymatic digestion, and nutrient absorption (Tao et al. 2022). Among probiotics, *Bacillus* bacteria such as *B. amyloliquefaciens* are widely used in aquaculture because they have antibacterial activity and can produce digestive exoenzymes that improve nutrient metabolism and, consequently, the growth performance of aquatic organisms (Silva et al. 2015). Hence, adding methionine sources and probiotics in low FM aquafeeds could guarantee an essential nutrient supply, improve

growth performance, preserve the diet's physico-chemical composition, and maintain the aquatic environment's quality (Bai et al. 2015). Due to the above, the main purpose of this study was to evaluate the effects of replacing FM with SBM and PBM in diets supplemented with DL-Met (two methionine isomers racemic mixture: D-Met and L-Met), AQUAVI® (four methionine stereoisomers mixture: LD-Met-Met, DL-Met-Met, LL-Met-Met and DD-Met-Met), ECOBIOL® (*B. amyloliquefaciens* CECT 5940) and their combinations on growth performance, body composition, and nutrient metabolism of *P. vannamei*.

MATERIALS AND METHODS

Diet preparation

Table 1 shows the experimental diet formulation and proximate composition; the shrimp's nutritional requirements were achieved according to recommendations (NRC 2011). The control diet (CD) had 200 g kg⁻¹ FM without supplementation. Reduced FM diets (D1-D4), where SBM and PBM replaced FM at 50%, were supplemented with two different methionine sources (DL-MET and AQUAVI®), *B. amyloliquefaciens* CECT 5940 (ECOBIOL®), and their combinations. D1: 0.13% DL-MET, D2: 0.06% AQUAVI®, D3: 0.13% DL-MET plus 0.10% ECOBIOL®, and D4: 0.06% AQUAVI® plus 0.10% ECOBIOL®.

Shrimp and feeding trial

Juvenile *P. vannamei* was purchased from a shrimp farm in Sonora, Mexico. The organisms were acclimated for one week to experimental conditions and fed with a commercial feed. Before the feeding trial, 400 healthy shrimp (0.30 ± 0.04 g) were randomly distributed to 20 circular tanks (150-L volume) at 20 shrimp per tank density. Four replicate tanks were randomly assigned for each dietary treatment. Shrimp were fed to apparent satiation with an initial ration of 12% of their biomass divided into three rations during the day (8:00, 13:00, and 16:00 h) for 56 days, adjusted daily depending on residual feed presence or absence. During the feeding trial, 30% of water was changed daily to maintain water quality parameters: temperature (27.84-28.36°C), salinity (36.98-37.07), dissolved oxygen (4.00-5.17 mg L⁻¹), pH (7.41-7.84). The experimental units were exposed to a normal photoperiod cycle (12:12 h light: dark). The uneaten feed, feces, molts, and dead shrimp were removed daily.

Sample collection and shrimp body composition

The shrimp were weighed and counted per tank after a feeding trial and a 24-h fasting period. Three shrimp

Table 1. Ingredients composition (g kg⁻¹ dry weight) and the experimental diets' proximate analysis (%). 1,6: Alimar S.A. de C.V. (Cd. Obregón, Sonora, México); 2: COLPAC (Navojoa, Sonora, México); 3,7-11: ARY Agroindustrial S.A. de C.V. (Cd. Obregón, Sonora, México); 4: MUNSA Molinos S.A. de C.V. (Cd. Obregón, Sonora, México); 5: COLPAC (Navojoa, Sonora, México); 12-17: EVONIK México S.A. de C.V. (Tlalpan, CDMX, México).

Ingredients (g kg ⁻¹ dry weight)	CD	D1	D2	D3	D4
	20% FM	0.13% DL-MET	0.06% AQUAVI	0.13% DL-MET 0.10% ECOBIOL®	0.06% AQUAVI 0.10% ECOBIOL®
Fishmeal ¹	200.0	100.0	100.0	100.0	100.0
Soybean meal ²	277.1	319.3	316.1	319.7	316.2
Poultry meal ³	40.0	80.0	80.0	80.0	80.0
Wheat flour ⁴	426.9	434.5	438.1	433.1	437
Soy lecithin ⁵	35.0	35.0	35.0	35.0	35.0
Fish oil ⁶	15.0	15.0	15.0	15.0	15.0
Pellet binder ⁷	3.0	3.0	3.0	3.0	3.0
Calcium propionate ⁸	1.0	1.0	1.0	1.0	1.0
Minerals ⁹	1.0	1.0	1.0	1.0	1.0
Vitamins ¹⁰	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium phosphate ¹¹	0.0	2.7	2.7	2.7	2.7
DL-Methionine ¹²	0.0	1.3	0.0	1.3	0.0
AQUAVI® Met-Met ¹³	0.0	0.0	0.6	0.0	0.6
Biolys 77 ¹⁴	0.0	4.9	5.0	4.9	5.0
L-Threonine ¹⁵	0.0	1.2	1.3	1.2	1.3
L-Tryptophan ¹⁶	0.0	0.1	0.2	0.1	0.2
ECOBIOL® ¹⁷	0.0	0.0	0.0	1.0	1.0
Total (g)	1000.0	1000.0	1000.0	1000.0	1000.0
Nutritional composition (%)					
Dry matter	90.4	90.4	90.4	90.4	90.4
Crude protein	35.0	32.9	32.8	32.9	32.8
Crude lipid	8.53	8.51	8.51	8.51	8.51
Ash	5.16	4.86	4.86	4.86	4.86
Methionine	0.7	0.7	0.7	0.70	0.7
Energy (kcal kg ⁻¹)	4.54	4.50	4.50	4.50	4.50

were randomly sampled from each replicate to analyze whole-body crude protein, crude lipid, and ash. Also, hepatopancreas of three shrimp from each tank were collected and stored at -80°C for RNA extraction.

According to standard protocols, shrimp body composition was determined in triplicate (AOAC 2000). Briefly, moisture was analyzed by shrimp oven-drying at 105°C to a constant weight, and the micro Kjeldahl method was used to determine nitrogen levels. Crude protein was calculated by multiplying by 6.25, crude lipid was estimated by ether extract, and ash content was analyzed by combustion at 550°C.

Transcriptional response of nutrient metabolism-related genes

Total RNA was isolated from shrimp hepatopancreas using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were checked with a NanoDrop 2000 spectrophotometer (Thermo Fisher

Scientific, Wilmington, DE, USA), ensuring an A260:A280 ratio between 1.8 and 2.2. RNA was treated with RNA-free DNase (Promega®, Madison, WI, USA), and 500 ng was used for cDNA synthesis according to ImProm-II™ Reverse Transcription System (Promega®) and oligo d(T)20 (T4OLIGO, Irapuato, GTO, MEX). The cDNA was diluted with 80 µL of ultrapure water, and 5 µL was used as the template for the real-time quantitative PCR (qPCR) reaction.

Transcriptional response was analyzed from nutrient metabolism-related genes and β-actin as a reference gene (Table 2). The qPCR reactions were made in triplicate for a final volume of 15 µL following the instructions of GoTaq® Flexi DNA Polymerase (Promega®) with 0.2 µM of each primer (T4OLIGO), 0.0125 µM of EvaGreen® 20X (Biotium, Fremont, CA, USA) and 5 µL of cDNA. The qPCR was conducted on the CFX Connect™ Real-Time PCR detection system (BioRad, Hercules, CA, USA), and amplification condi-

Table 2. Primer pairs sequences used for transcriptional response of nutrient metabolism-related genes by qPCR. *tor*: target of rapamycin; *RagA*: RAS-related GTP-binding protein A; *RagC*: RAS-related GTP-binding protein C; *s6k*: ribosomal protein 6 kinase; *4e-bp*: eukaryotic initiation factor 4 binding protein; *fabp*: fatty acid binding protein; *cpt1*: carnitine palmitoyl transferase 1; *ampka*: AMP-activated protein kinase subunit alpha; *srebp*: sterol regulatory element-binding protein-1; *fas*: fatty acid synthase; *6pgd*: 6-phosphogluconate dehydrogenase; *acc*: acetyl-COA carboxylase; 1: primers for gene expression related to protein synthesis (Lu et al. 2021); 2: primer for β -actin expression and gene expression related to lipolysis and lipogenesis (Duan et al. 2022).

Gen	Primer F (5'→3')	Primer R (5'→3')	Access number
Protein synthesis-related genes ¹			
<i>tor</i>	CTGCTTTGAAGTTGCCATGA	CAAAGGCTTCAAGCATAGCC	XM_027372359.1
<i>RagA</i>	CTTGGGAAATTTGGTCCTGA	TGCCTCTAAGCAGCTTTGGTT	XM_027377090.1
<i>RagC</i>	GGCATGGAACAGATTCACCT	TGGAGAGCACATCAAACAGG	XM_027357730.1
<i>s6k</i>	GCAAGAGGAAGAGACGCCATA	CCGCCCTTGCCCAAAACCT	XM_027368997.1
<i>4e-bp</i>	ATGTCTGCTTCGCCCCGTCGCTCGCC	GGTTCTTGGGTGGGCTCTT	XM_027367939.1
Lipolysis-related genes ²			
<i>fabp</i>	CGCTAAGCCCCTGCTGGAAGT	CTCCTCGCCGAGCTTGATGGT	KF471026
<i>cpt1</i>	GCTGTATCGTGATGCAATGAATGGC	AGGCTGCTGGGATGTTGATAATGTC	XM_027373671
<i>ampka</i>	CCACCTGCTCCTCCTACTCCATC	GTAGTCCACGATCACCCTCAATGC	KP272116
Lipogenesis-related genes ²			
<i>srebp</i>	AGATGGCTGAGATGTTGGTAATGGC	CCCTTGTTGGCTCTTCTCTTTGC	MG770374
<i>acc</i>	CGGCAGACAACATCCATAACCACAG	GCAACCAGCGAGAGCAGTAACC	XM_027360190
<i>6pgd</i>	CATGTGGAGAGGAGGCTGCATTATC	GAGGAGGTTGTTGAGTTGAGGGTTC	XM_027353839
<i>fas</i>	TGCTACTGTGCCTGTTGTGTATGC	CCACCAGAACCTGCGTGAATGAG	HM595630
Reference gene ²			
β -actin	CGAGGTATCCTCACCTGAA	GTCATCTTCTCGCGGTTAGC	AF300705

tions were initial denaturation at 95°C for 10 min, followed by 40 denaturation cycles at 95°C for 15 s, annealing for 30 s, and extension at 60°C for 1 min. The dissociation curve (60-95°C) was analyzed at a temperature transition rate of 0.5°C s⁻¹ for each primer pair. The levels of gene-relative expressions were calculated according to the 2^{- $\Delta\Delta$ CT} equation (Livak & Schmittgen 2001). Data from relative gene expression were transformed with log₁₀+1 to achieve normal distribution.

Calculations

The growth performance, feed utilization, and nutrient retention were calculated as follows:

Final weight (FW) = (Σ final individual weight) / final number of shrimps

Weight gain (WG) = final weight - initial weight

Survival rate = 100 × (final number of shrimps / initial number of shrimps)

Final biomass (FB) = final weight × final number of shrimps

Feed conversion ratio (FCR) = feed intake / final biomass

Feed efficiency ratio (FER) = 100 × (final biomass / feed intake)

Protein efficiency ratio (PER) = weight gain / (feed intake × protein percent)

Lipid efficiency ratio (LER) = weight gain / (feed intake × lipid percent)

Protein retention efficiency (PRE) = protein gain / (feed intake × protein percent)

Lipid retention efficiency (LRE) = lipid gain / (feed intake × protein percent)

Statistical analysis

Growth, nutrient retention data, body composition, and transcriptional response were presented as mean ± standard error (SE) and subjected to one-way variance analysis (ANOVA). If any significance was observed, Tukey's test was performed for means comparison. Statistical analysis was performed with Statgraphics Centurion XVI. Significance was set at 95% probability levels.

RESULTS

Growth, feed utilization, and body composition

Growth, nutrient retention, and body composition data are shown (Table 3). The growth performance and survival rate (more than 75%) did not differ significantly ($P > 0.05$) among diet treatments, but shrimp fed D4 had higher survival and growth values (FW, WG, and FB) than shrimp fed D1. There were no significant differences ($P > 0.05$) in feed utilization values (FCR, FER, PER, and LER), but according to FCR and FER values, the shrimp fed D2 and D4 had better feeding efficiency. Nutrient retention (PRE and LRE) differed significantly ($P < 0.05$) between diet treatments. Concerning PRE, shrimp fed D1 had the lowest protein retention value, while shrimp fed D2 had the highest value. LRE was increased in all reduced FM diets, but lipid retention significantly increased in shrimp fed D1 and D3. Significant differences ($P < 0.05$) were observed in shrimp body composition (moisture, protein, and lipid content), except for ash content. The lowest moisture and protein contents were observed in shrimp fed D2-D4, while the highest lipid contents were observed in shrimp fed D1 and D3.

Transcriptional response of nutrient metabolism-related genes

The transcriptional response of nutrient metabolism-related genes in the hepatopancreas of shrimp fed a reduced FM diet was determined compared with the CD. Protein synthesis-related gene expression is presented (Fig. 1). The *tor* gene expression increased ($P < 0.05$) in shrimp fed D3 while decreased ($P < 0.05$) in shrimp fed D1, D2, and D4. The *RagA* gene expression was not different in shrimp fed D1 and D2 but increased ($P < 0.05$) in shrimp fed D3 and D4. The *RagC* gene expression decreased ($P < 0.05$) in shrimp fed D1 and D2 while it increased ($P < 0.05$) in shrimp fed D3, but without significant differences in shrimp fed D4. No differences were observed in *s6k* gene expression of shrimp fed D1, D2, and D4 but increased ($P < 0.05$) in shrimp fed D3. The *4e-bp* gene expression increased ($P < 0.05$) in shrimp fed D3 and D4 while decreased ($P < 0.05$) in shrimp fed D2, but without significant differences in shrimp fed D1.

Lipolysis-related gene expression is shown (Fig. 2). The *fabp* gene expression increased ($P < 0.05$) in all dietary treatments. The *cpt1* gene expression increased ($P < 0.05$) in shrimp fed D4, while no differences were observed in shrimp fed D1, D2, and D3. The *ampka* gene expression increased ($P < 0.05$) in shrimp fed D1,

while no differences were observed in the other dietary treatments.

Lipogenesis-related gene expression is shown (Fig. 3). The *srebp* gene expression increased ($P < 0.05$) in shrimp fed D3 and D4 while decreased ($P < 0.05$) in shrimp fed D2, but without difference in shrimp fed D1. The *fas* gene expression increased ($P < 0.05$) in shrimp fed D1, D3, and D4, while no difference was observed in shrimp fed D2. The *6pgd* gene expression decreased ($P < 0.05$) in shrimp fed D1, D2, and D4, while it increased ($P < 0.05$) in shrimp fed D3. The *acc* gene expression was not different in shrimp fed D1 but decreased ($P < 0.05$) in shrimp fed D2 and increased ($P < 0.05$) in shrimp fed D3 and D4.

DISCUSSION

Previously, many studies have evaluated the supplementation of DL-MET and AQUAVI® as methionine sources in reduced FM diets on growth and feed utilization of *P. vannamei* under different farming systems, suggesting various supplementation levels in a range of 0.06-0.30 DL-MET and 0.15-1.7% for AQUAVI® for better growth performance and feeding efficiency (Façanha et al. 2016, Xie et al. 2017, Nunes et al. 2019, Wang et al. 2019, Ji et al. 2021, Lu et al. 2021). Probiotic effects of ECOBIOL® have also been studied in the water of biofloc system for farmed white shrimp, suggesting a dissolution of 2.8×10^3 CFU mL⁻¹ (Llario et al. 2019) and 9.48×10^4 CFU mL⁻¹ (Llario et al. 2020) for better growth performance and feeding efficiency. However, the effects of supplements in reduced FM diets have not been reported in white shrimp. Methionine levels in shrimp feeds are recommended according to shrimp species, methionine source, growth stage, culture conditions, and dietary protein level. However, there is limited information about the optimal dietary methionine levels to support maximum shrimp growth performance fed reduced FM diets (Nunes & Masagounder 2022). Therefore, this study evaluated the effect of FM replacement by SBM and PBM in diets supplemented with 0.13% of DL-MET, 0.06% of AQUAVI®, and their combinations with 0.1% ECOBIOL® (equivalent to 1×10^9 CFU g⁻¹ *B. amyloliquefaciens* CECT 5940) on growth performance, feed utilization, nutrient retention and body composition of juvenile shrimp cultured in hyper intensive conditions.

Shrimp growth performance and feed utilization were within the allowed ranges in previous reports. Except for shrimp fed D1, the shrimp fed reduced FM diets had higher survival rates and growth performance

Table 3. Effects of experimental diets on growth performance, nutrient retention, and body composition of *Penaeus vannamei*. IW: initial weight; FW: final weight; WG: weight gain; SR: survival rate; FB: final biomass; FCR: feed conversion ratio; FER: feed efficiency ratio; PER: protein efficiency ratio; LER: lipid efficiency ratio; PRE: protein retention efficiency; LRE: lipid retention efficiency. Values are mean \pm standard error of the mean of three replicates, and values in the same row with different letters are significantly different ($P < 0.05$). CD: 200 g kg⁻¹ FM; D1: 0.13% DL-MET; D2: 0.06% AQUAVI®; D3: 0.13% DL-MET plus 0.10% ECOBIOL® and D5: 0.06% AQUAVI® plus 0.10% ECOBIOL®.

	Diets				
	CD	D1	D2	D3	D4
Growth performance					
IW [g]	0.28 \pm 0.01	0.31 \pm 0.02	0.29 \pm 0.02	0.28 \pm 0.02	0.32 \pm 0.01
FW [g]	5.00 \pm 0.10 ^{ab}	4.60 \pm 0.38 ^a	5.34 \pm 0.26 ^{ab}	5.28 \pm 0.10 ^{ab}	5.98 \pm 0.21 ^b
WG [g]	4.72 \pm 0.11 ^{ab}	4.29 \pm 0.40 ^a	5.05 \pm 0.25 ^{ab}	5.00 \pm 0.09 ^{ab}	5.66 \pm 0.22 ^b
SR [%]	77.50 \pm 5.95	73.75 \pm 6.57	86.25 \pm 7.74	83.75 \pm 6.57	91.25 \pm 3.75
FB [g]	77.64 \pm 6.78 ^{ab}	67.98 \pm 8.42 ^a	93.21 \pm 12.10 ^{ab}	88.26 \pm 6.64 ^{ab}	108.94 \pm 4.39 ^b
FCR	1.87 \pm 0.02	1.94 \pm 0.16	1.75 \pm 0.09	1.86 \pm 0.06	1.75 \pm 0.04
FER (%)	53.36 \pm 0.65	52.66 \pm 3.90	57.62 \pm 2.99	53.97 \pm 1.72	57.26 \pm 1.7
PER	1.74 \pm 0.07	1.88 \pm 0.07	1.86 \pm 0.11	1.78 \pm 0.11	1.76 \pm 0.09
LER	7.13 \pm 0.28	7.28 \pm 0.28	7.23 \pm 0.43	6.92 \pm 0.42	6.81 \pm 0.37
PRE	68.0 \pm 1.54 ^b	57.4 \pm 2.36 ^a	94.9 \pm 1.19 ^d	87.6 \pm 0.55 ^c	89.3 \pm 1.54 ^{cd}
LRE	21.3 \pm 0.76 ^a	29.9 \pm 1.30 ^b	27.1 \pm 0.99 ^{ab}	37.6 \pm 0.92 ^c	27.5 \pm 2.24 ^{ab}
Shrimp body composition (wet weight %)					
Moisture	74.0 \pm 0.55 ^b	73.8 \pm 0.48 ^b	66.5 \pm 0.49 ^a	66.4 \pm 0.90 ^a	65.0 \pm 0.26 ^a
Protein	71.8 \pm 1.01 ^a	70.9 \pm 1.50 ^a	77.6 \pm 0.67 ^b	77.6 \pm 0.34 ^b	74.1 \pm 0.89 ^{ab}
Lipid	4.9 \pm 0.12 ^a	6.8 \pm 0.21 ^b	5.3 \pm 0.15 ^a	7.3 \pm 0.15 ^b	5.3 \pm 0.33 ^a
Ash	7.4 \pm 0.38	6.5 \pm 0.85	8.3 \pm 0.72	7.7 \pm 0.30	7.1 \pm 0.02

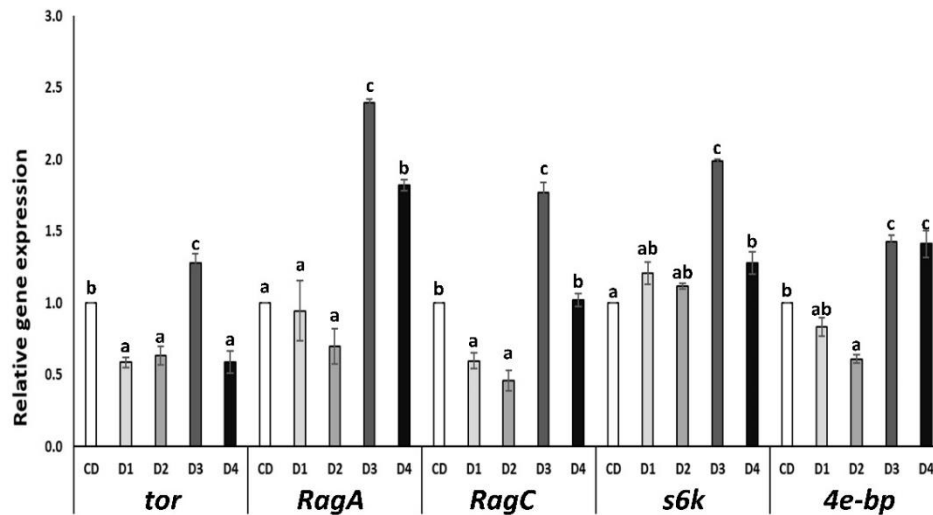


Figure 1. Transcriptional response of protein synthesis-related genes. *tor*: target of rapamycin; *RagA*: RAS-related GTP-binding protein A; *RagC*: RAS-related GTP-binding protein C; *s6k*: Ribosomal protein 6 kinase; *4e-bp*: eukaryotic initiation factor 4 binding protein; CD: 200 g kg⁻¹ FM; D1: 0.13% DL-MET; D2: 0.06% AQUAVI®; D3: 0.13% DL-MET plus 0.10% ECOBIOL® and D5: 0.06% AQUAVI® plus 0.10% ECOBIOL®. Data are presented as mean \pm standard error. Values with different letters are significantly different ($P < 0.05$).

values than shrimp fed CD. A comparison between methionine sources (DL-MET and AQUAVI) on growth and feed utilization of shrimp fed reduced FM diets showed that shrimp fed D1 (0.13% DL-Met) had

the worst growth and feed utilization values than shrimp fed D2 (0.06% AQUAVI®). Further, the comparison between combinations of methionine sources and probiotics highlighted that shrimp fed D4

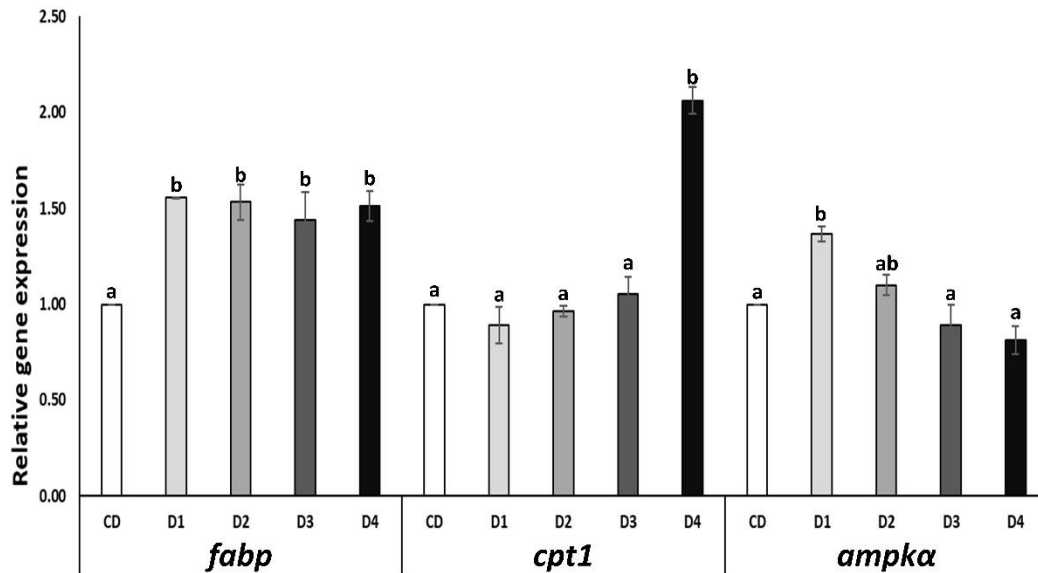


Figure 2. Transcriptional response of lipolysis-related genes. *fabp*: fatty acid binding protein; *cpt1*: carnitine palmitoyl transferase 1; *ampka*: AMP-activated protein kinase subunit alpha; CD: 200 g kg⁻¹ FM; D1: 0.13% DL-MET; D2: 0.06% AQUAVI®; D3: 0.13% DL-MET plus 0.10% ECOBIOL® and D5: 0.06% AQUAVI® plus 0.10% ECOBIOL®. Data are presented as mean ± standard error. Values with different letters are significantly different ($P < 0.05$).

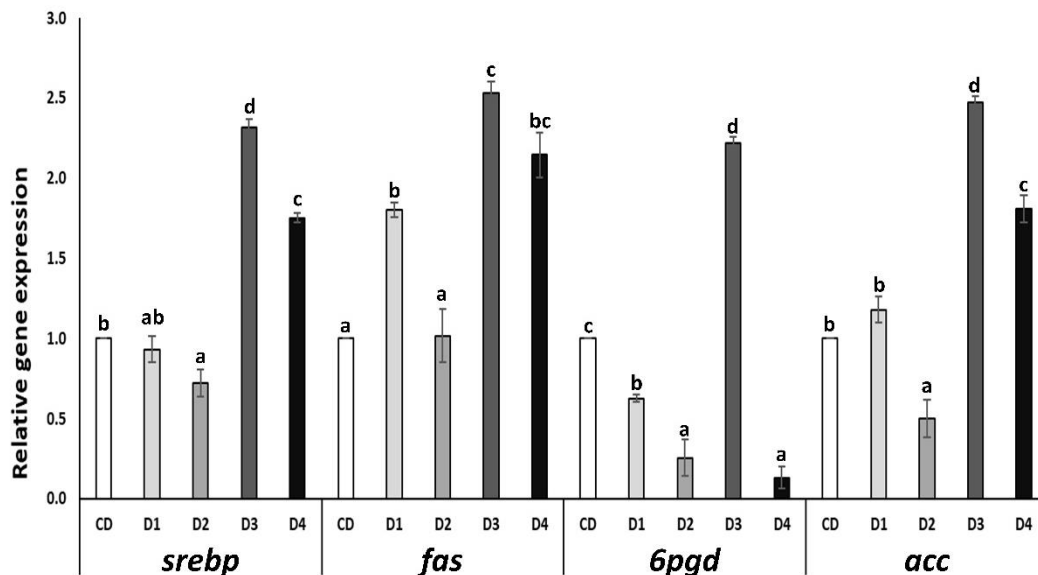


Figure 3. Transcriptional response of lipogenesis-related genes. *srebp*: sterol regulatory element-binding protein-1; *fas*: fatty acid synthase; *bpgd*: 6-phosphogluconate dehydrogenase; *acc*: acetyl-CoA carboxylase; CD: 200 g kg⁻¹ FM; D1: 0.13% DL-MET; D2: 0.06% AQUAVI®; D3: 0.13% DL-MET plus 0.10% ECOBIOL® and D5: 0.06% AQUAVI® plus 0.10% ECOBIOL®. Data are presented as mean ± standard error. Values with different letters are significantly different ($P < 0.05$).

(0.06% AQUAVI® plus 0.1% ECOBIOL®) had the best growth and feed utilization values than shrimp D3 (0.13% DL-Met plus 0.1% ECOBIOL®). On the other hand, nutrient transfer efficiency and nutritional value can be determined by proximal composition analysis

(Silva et al. 2015). In this study, the nutrient retention values (PRE and LRE) and shrimp body composition were significantly affected by dietary treatments. The protein contents were by PRE values. The highest values were observed in shrimp fed D2, D3, and D4.

Lipid contents were also consistent with LRE values; the lowest values were observed in shrimp fed CD, D2, and D4. These results were in agreement with previous reports that suggested the AQUAVI[®] incorporation in reduced FM diets improved feed utilization, nutrient retention, and consequently, the growth of *P. vannamei* (Xie et al. 2017, Niu et al. 2018, Ji et al. 2021). However, when shrimp fed D3 (DL-Met combined with *B. amyloliquefaciens*), the growth performance also increased compared to shrimp fed D1 (reduced fishmeal diet containing only DL-Met). Still, a possible inconvenience with this dietary treatment could be the high lipid deposition, which is not desirable for quality shrimp production.

In contrast, a shrimp's lean growth is characterized by protein accumulation (Chuphal et al. 2021). The high protein retention values may be due to the shrimp stage because juvenile shrimp use nutrients more efficiently for rapid growth than adult shrimp (Niu et al. 2008). In addition, the shrimp's digestive system is activated in the initial stages. The methionine and probiotic use could synergistically improve growth, protein retention, and shrimp body composition (Novriadi et al. 2023).

Since methionine is a limiting functional amino acid in reduced FM diets, this study investigated the effects of it supplemented with DL-MET, AQUAVI[®] and their combinations with ECOBIOL[®] on the transcriptional response of nutrient metabolism-related genes. Shrimp growth depends on protein deposition, and the target of the rapamycin (TOR) signaling pathway influences nutrient sensing and protein metabolism of aquatic organisms and mammals (Cao et al. 2021, Chen et al. 2022, Mu et al. 2023). TOR is a highly conserved serine/threonine kinase protein (Li et al. 2023) which is activated by binding *Rag* proteins (*RagA* and *RagC*) with Raptor (Lu et al. 2021, Chen et al. 2022). Protein synthesis is regulated by *tor* through the downstream targets ribosome protein S6 kinase (*s6k*) and the eukaryotic translation initiation factor 4E-binding protein (*4e-bp*), while our results indicated that protein synthesis-related genes (*tor*, *RagA*, *RagC*, *s6k*, and *4e-bp*) were significantly affected by dietary treatments, a low transcriptional response was mostly observed in shrimp fed D1 and D2. In contrast, a high transcriptional response was mostly observed in shrimp fed D3 and D4. A previous study suggested that optimal AQUAVI[®] supplementation enhanced the transcriptional response of protein synthesis-related genes (Lu et al. 2021). Unlike the present results, the 24-h fasting period may have affected the transcriptional response of protein synthesis-related genes. Protein synthesis can

be decreased by a brief fast, particularly when functional amino acids are not present (Wang et al. 2021). Besides reacting to nutrients and functional amino acids, the TOR signaling pathway could respond to other stimuli, such as probiotic metabolites presence (Zhang et al. 2023). For example, it has been reported that *Bacillus subtilis* produces amino acids to stimulate protein synthesis in ruminal tissue explant by altering the gene expression related to the TOR signaling pathway (Wang et al. 2022). *B. amyloliquefaciens* is a methionine producer (Ajogwu et al. 2020), which could contribute to maintaining the essential amino acid balance and enhancing the protein synthesis-related genes, as observed in shrimp fed D3 and D4.

Some reports suggest a close connection between dietary methionine and lipid metabolism (Ji et al. 2022), and methionine deficiency inhibits lipogenesis while stimulating lipolysis and induces energy consumption (Hu et al. 2023). The transcriptional response of genes related to lipolysis and lipogenesis was studied to investigate how reduced FM diets supplemented with DL-MET, AQUAVI[®], and their combinations with ECOBIOL[®] affect lipid metabolism. The enzymes related to lipolysis are fatty acid binding-protein (*fabp*) related to fatty acid transport from lipid membrane to oxidation site, carnitine palmitoyl transferase 1 (*cpt1*) related to mitochondrial beta-oxidation, and AMP-activated protein kinase subunit alpha (*ampka*) regulates biological energy metabolism (Liu et al. 2020, Chen et al. 2022). The lipogenic enzyme includes sterol regulatory element-binding protein-1 (*sreb1*) related to cholesterol synthesis, fatty acid synthase (*fas*) participates in fatty acid biosynthesis, acetyl-CoA carboxylase (*acc*) as the main enzyme in the fatty synthesis process, and 6-phosphogluconate dehydrogenase (*6pgd*) involved in NADPH production for fatty acid biosynthesis (Zheng et al. 2013, Liu et al. 2020). In this study, the transcriptional response of lipolysis-related genes indicated that *fabp* was upregulated in all shrimp groups, *cpt1* was upregulated in shrimp fed D4, and *ampka* was upregulated in shrimp fed D1. These results could suggest that shrimp fed D4 improved lipid utilization by fatty acid beta-oxidation, particularly increasing lipolysis over other energetic pathways.

In contrast, shrimp fed D1 requires the induction of ATP production for lipid metabolism but could be necessary to reduce the energy used for growth. It has been proposed that methionine deficiency activates *ampka* to stimulate energy metabolism then mitochondrial function is enhanced for lipid metabolism (Wang et al. 2020), as was observed in shrimp fed D1.

The transcriptional response of lipogenesis-related genes was mostly downregulated in shrimp fed D2. Moreover, lipogenesis-related genes were upregulated in shrimp fed D3 and D4, with low gene expression values in shrimp fed D4. Nevertheless, the increase could be attributed to FM replacement or probiotic function.

On the one hand, increased FM substitution by other protein sources leads to increased lipogenic enzymes associated with higher lipid retention and body lipid accumulation (El-Naby et al. 2023), as was observed in shrimp-fed reduced fishmeal diets. On the other hand, *B. amyloliquefaciens* can produce methionine (Ajogwu et al. 2020) and consequently increase the methionine levels in shrimp hepatopancreas. This phenomenon was reported in yellowhead catfish (*Pelteobagrus fulvidraco*) when methionine excess caused hepatic lipid accumulation while the lipogenesis-related genes were upregulated (Song et al. 2018).

The above results could also be due to the physicochemical properties of methionine sources; since AQUAVI® has high bioavailability in comparison with DL-MET, this could lead to a more methionine-efficient use and a better growth performance (Xie et al. 2017, Niu et al. 2018, Guo et al. 2020). The above may also be a reason for the survival rate because methionine is a beneficial additive for health and immunity in reduced FM diets (Noor et al. 2021). Even a previous report suggested that shrimp fed reduced FM diets without the methionine addition (used as negative control) increased the stress indexes and decreased the immune response in comparison with shrimp fed positive CD (20% FM) and reduced FM diets supplemented with methionine (Ji et al. 2021). In addition to the *B. amyloliquefaciens* CECT 5940 beneficial properties like antimicrobial activity and digestive enzyme production that increase nutrient utilization (Gharib-Naseri et al. 2020), it has been documented that it is a methionine producer (Ajogwu et al. 2020) which could contribute to maintain the essential amino acid balance and to improve the metabolism of the nutrient, positively influencing shrimp growth performance. Due to the limited information on optimal dietary methionine levels and its high cost (US\$ 5.0 kg of feed⁻¹), the present results could be useful for developing feeds for aquaculture economic efficiency (Nunes & Masagounder 2022).

In conclusion and considering the values of growth performance, body composition, and nutrient metabolism, the 50% FM replacement by SBM and PBM in diets supplemented with 0.06% AQUAVI® and 0.06% AQUAVI® plus 0.1% ECOBIOL® could be feasible without affecting quality production of shrimp cultured

in hyper intensive conditions. In a reduced FM diet, methionine and probiotic supplementation could promote white shrimp's growth and protein synthesis by improving TOR pathway signaling and supplying energy through lipogenesis and lipolysis balance. However, further research is required to investigate graduated levels of methionine sources and synergistic effects of amino acids and probiotics in reduced FM diets on farmed shrimp's growth, health, and nutrient metabolism.

ACKNOWLEDGMENTS

Arevalo-Sainz, K., and Fraijo-Valenzuela, A. thank Consejo Nacional de Humanidades, Ciencia y Tecnología (CONAHCYT) for the doctoral scholarship. This work was supported by Coordinación de Gestión y Apoyo a los Cuerpos Académicos del Instituto Tecnológico de Sonora (PROFAPI 2023-098), Evonik's aqua R&D group, and CONAHCYT through the Cátedras CONACYT Program (Project N°1037).

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Received: October 5, 2023; Accepted: February 16, 2024