Functional amino acids supplementation for shrimp to support moulting: improved cuticle formation leads to higher growth performance

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Abstract

This study explores the benefits of functional amino acids (AA) in shrimp nutrition, expanding beyond the conventional classification of indispensable and dispensable AA. The effects of supplementing 1% poultry hydrolysate keratin-derived free amino acids (KFAA1%) on whiteleg shrimp (*Litopenaeus vannamei*) were evaluated over a 60-day feeding trial. Two diets were tested: a control diet and a KFAA1% diet, with five replicates per treatment (n=225 shrimp/treatment). Growth performance was assessed (65 shrimp/treatment), while exoskeleton structure and composition were analyzed (15 shrimp/treatment) during the early premoult stage (proecdysis). Exoskeleton formation after moulting was evaluated based on cuticle thickness and the number of layers measured using Scanning Electron Microscopy (SEM). The composition of mineral and elemental deposits in the exoskeleton was determined using. Energy Dispersive X-ray Spectroscopy (EDS). Overall, shrimp fed KFAA1% for 60 days showed a higher average daily gain and weight gain, resulting in higher final body weight and an improved feed conversion rate (-24.8%). Exoskeleton formation was enhanced, with increased chitin content (+19.4%), greater cuticle thickness (+39.7%), and a higher number of layers (+37.7%) in shrimp fed KFAA1%. Supplementing functional AA also increased calcium (+8.1%) and magnesium (+20.0%) in the exoskeleton while reducing sodium (-22.7%) and chloride (-52.2%). These results highlight the benefits of supplementing KFAA1% to support moulting and exoskeleton formation in shrimp during the grow-out stage. For precision nutrition, this study encourages a broader discussion on the roles of amino acids in physiology beyond traditional classifications as indispensable or dispensable.

Keywords Sustainability, Shrimp exoskeleton, Precision nutrition

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Introduction

Amino acids (AA) are essential nutrients for aquatic animals contributing to their growth, reproduction, and immune system health. Traditional AA nutrition has often relied on a binary classification of AA as essential or non-essential. However, recent studies have highlighted the importance of functional AA, which goes beyond this simple classification (Li *et al*., 2009; Pereira *et al*., 2017; Wu, 2009, 2014).

Functional AA are defined as those which participate and regulate key metabolic pathways to improve growth and health in mammals and fish. This AA group encompasses arginine, cysteine, glutamine, glutamate, glycine, leucine, proline, and tryptophan regardless of their designation as dispensable or indispensable (Wu, 2010, 2013; Wu *et al*., 2014). The functional amino acids concept in shrimp nutrition has been advanced considerably in recently (Xinyu Li *et al*., 2021).

In 2023, shrimp farming produced nearly 8 million tons, with an annual growth rate of 6–8% (Khoklang *et al*., 2024). As the global industry grows, a thorough understanding of shrimp nutrition, particularly through the optimization of functional amino acids (AAs) in their diet, is crucial for improving growth and overall resilience throughout outer barriers such as exoskeleton for more sustainable aquaculture practices.

The keratin, a major component of poultry feathers, constitutes a circular-economy ingredient with significant potential for aquafeed applications (Li *et al*., 2009). The keratin-derived free amino acids mix (KFAA) has emerged as a supplementation strategy to address amino acid challenge-oriented nutrition. KFAA consists of a water soluble and ready-to-be-absorbed source of 17 amino acids (Wangkahart *et al*., 2022; Wangkahart *et al.,* 2023; Khoklang *et al.,* 2024). Previous studies highlighted the effects of supplementing KFAA on higher disease resistance in whiteleg shrimp challenged with *Vibrio parahaemolyticus* (AHPND/EMS) and WSSV (Kersanté *et al*., 2021) and on feed attractability or improved growth performance (Le Reste *et al*., 2019).

This study aims to evaluate the effects of a keratin-derived free amino acids mix (KFAA) for whiteleg shrimp (*Litopenaeus vannamei*) to support moulting through better cuticle formation to improve growth performance. By applying the principles of precision nutrition, this research seeks to advance sustainable shrimp aquaculture practices.

Materials and methods

Feeding trial and experimental diets

The feeding trial was conducted in an indoor semi-recirculating system at the Department of Aquatic Science, Faculty of Science, Burapha University, Bangsaen, Chonburi, Thailand. A total of 450 whiteleg shrimp juveniles (*Litopenaeus vannamei*), with an initial body weight of 14.12g±0.35, were reared in rectangular plastic tanks of 250L (0.55m x 0.82m x 0.5m) at a stocking density of 45 shrimp/tank (100 ind/m^2) under a natural photoperiod. A

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5-day acclimation period was applied to adapt the shrimp to the experimental conditions. Water quality parameters were monitored twice a week, and the average values recorded were as follows: salinity of 15 ppt, temperature ranging from 28 to 30°C, pH 7.8 to 8.2, dissolved oxygen between 6 and 7 mg/L, and NH_3^+ <0.50 mg/L. Every two days, 10% of the water was replaced, and 50% of the water was replaced every six days.

The experimental design consisted of two diets and five replicates (n=225 shrimp/treatment), with shrimp fed for 60 days, four times a day (08:00, 12:00, 16:00, and 20:00) at 5% of body weight (Fig. 1). A control reference diet was formulated to meet the nutritional requirements of whiteleg shrimp (Table 1). The second diet differed from the control by including a 1% supplementation of a functional amino acid mix (KFAA1%), chosen based on a previous study (Le Reste *et al.,* 2019). The KFAA diet was prepared by top-coating the feed with free amino acids dissolved in distilled water (20 g DW/kg feed) and mixing it for 15 minutes. The control feed underwent the same process but was sprayed with distilled water only. Both feeds were air-dried at room temperature for 24 hours, then coated with cod liver oil (20 g/kg feed) and allowed to dry for another 24 hours at room temperature. The prepared feeds were stored in sterile bags and kept at 4°C for the duration of the trial.

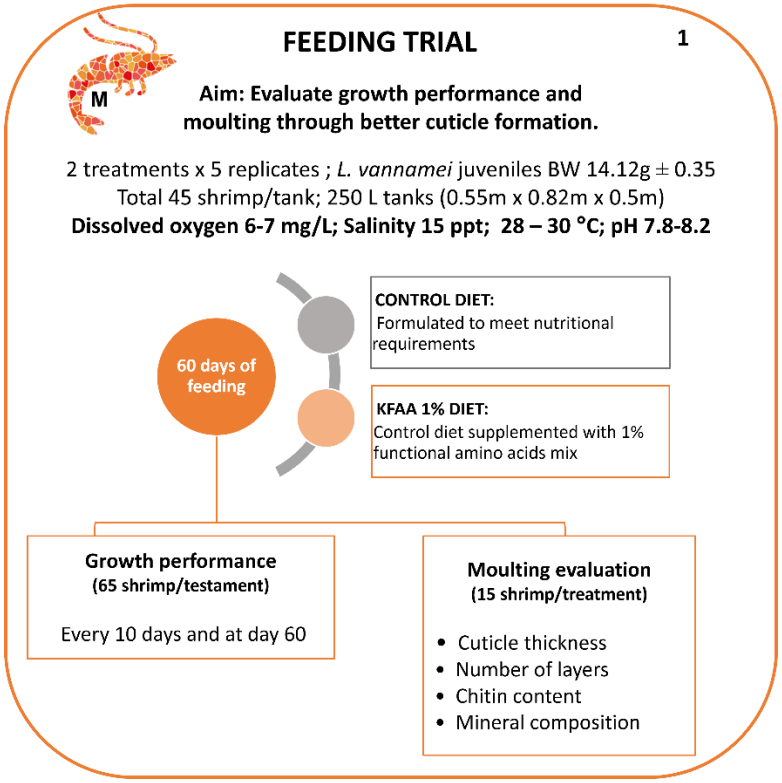


Figure 1: Schematic summary of the experimental design for the moulting trial.

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Table 1: Formulation (% dry matter) and chemical composition of experimental diets.

Ingredients	% dry matter
Wheat flour	24.8
Soybean meal	19.5
Fishmeal 60% Protein	15.8
Poultry meal 64% Protein	11.4
Rice bran	6.6
Shrimp by Product	4.8
Squid by product	3.5
Fermented soy bean	3.4
Lecithin	2.0
Fish hydrolysate	2.0
Squid liver oil	2.0
Squid liver paste	1.7
Corn protein concentrate	1.4
Wheat gluten	1.1
Chemical composition %	
Dry matter	91.2
Crude Protein	38.5
Moisture	8.8
Crude Fat	7.6
Crude Ash	5.2
Crude Fiber	2.9

KFAA is obtained through a biotechnological process that promotes extensive hydrolysis of poultry keratin, resulting in the complete denaturation of the protein chain to produce free amino acids. Originally developed for extracting cystine (Cys) and tyrosine (Tyr) for pharmaceutical and human nutrition applications, this industrial process also yields a mixture primarily composed of free amino acids and mineral salts, with a minimal content of small peptides. KFAA is a unique product particularly rich in free amino acids (FAA), with a characteristic amino acid profile shaped by the combination of raw materials, partial extraction, and purification steps involved in its production (Tables 2 and 3).

Table 2: Proximate composition of the KFAA (Kera-Stim®50).

Items	Value
Dry matter	98.4%
Total amino acids (CE 152/2009)	50.4%
Free amino acids (CE 152/2009)	47.3%
Crude ash	43.8%

Table 3: Amino acids profile of KFAA (Kera-Stim®50) with the proportion of each amino acid under free form.

Amino acid	Mean value (g/100g KFAA)	Free AA/total (%)
Serine	6.60	100
Proline	5.84	100
Glutamic acid	5.43	96
Glycine	4.48	98
Valine	4.07	73
Leucine	3.96	95
Aspartic acid	3.67	99
Arginine	3.40	94
Alanine	2.53	98
Phenylalanine	2.49	97
Threonine	2.48	100
Isoleucine	2.44	82
Cystine	1.04	71
Lysine	0.97	93
Histidine	0.32	89
Methionine	0.30	96

This product is manufactured by BCF Life Science, France (KERA STIM®50; https://www.bcf-lifesciences.com/en/market-segments/aquaculture/).

Sampling procedures

At the end of the 60-days feeding trial, samples were collected for growth performance and exoskeleton evaluations. A total of 13 shrimp per tank (n=65 shrimp per treatment) were used for growth performance evaluation. Additionally, 3 shrimp per tank (n=15 shrimp per treatment) were collected and dissected to obtain exoskeletons for structural and compositional analyses.

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The shrimp selected for exoskeleton composition and cuticle formation evaluations were standardized as those in the early premoult stage, proecdysis (D0), when shrimp begin preparing for moulting. This was determined according to the method described by Pratoomchat *et al.* (2002). Exoskeleton samples at the D0 premoult stage were identified under light microscopy as those exhibiting a straight epidermal line in the uropod (Fig. 2). Carapace as representing exoskeleton samples were then dried at 45°C for 30 hours before evaluation. All procedures used in this study were approved by the Ethics Committee of Animal Welfare of Burapha University, Bangsaen, Chonburi, Thailand.

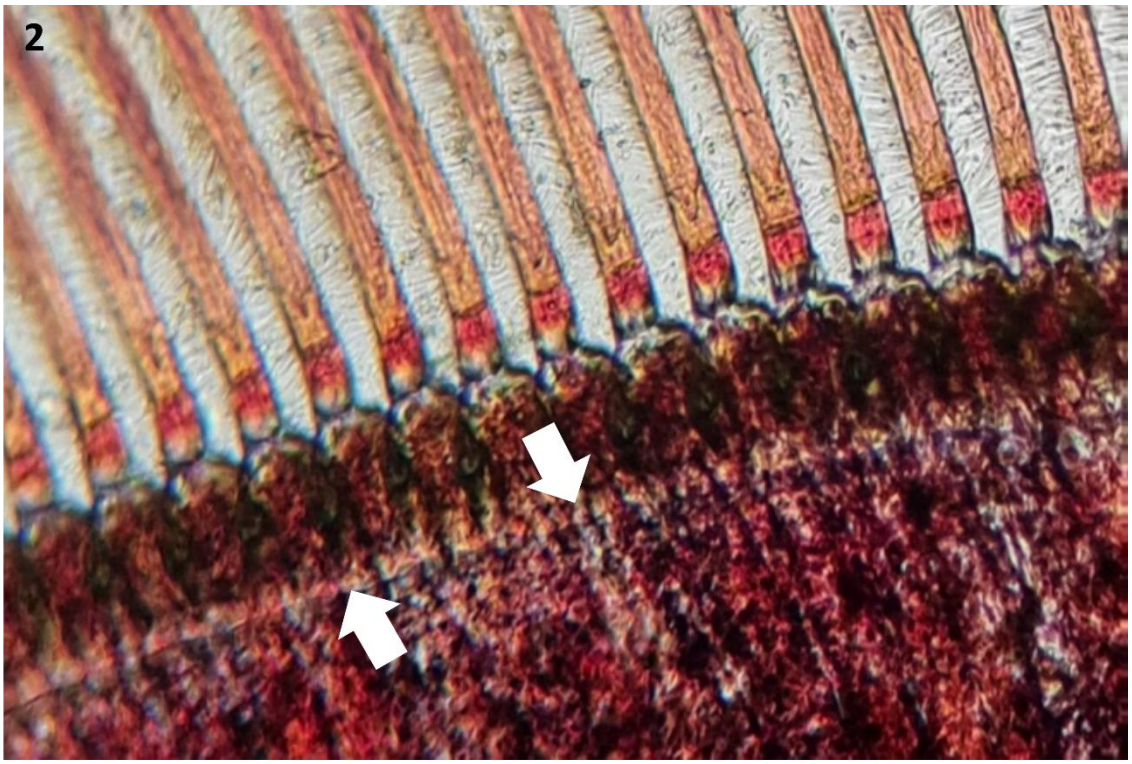


Figure 2: Photomicrography of the exoskeleton samples at D0 pre-moult stage identified under light microscopy as those showing a straight epidermal line in the uropod.

Growth performance and moulting frequency

Growth performance and moulting frequency were calculared as follows: a) Average daily growth (ADG, %)=*[(final weight – initial weight/initial weight) /x 100]*; b) Weight gain (WG, %)=*[(final weight – initial weight/initial weight) x 100]*; c) Length gain (LG, %)=*[(final length – initial length/initial length) /x 100]*; d) Specific growth rate for weight (SGRW, %)= *[(ln(final weight) – ln(initial weight)) / days]*; e) Specific growth rate for length (SGRL, %) = *[[(ln(final length) – ln(initial length)) / days] × 100]*; f) Size variation (SV, %) = *Convariace of length and weight*; g) Survival rate (SR, %) = *[(number of surviving shrimp / initial number of shrimp) × 100]*; h) Moulting frequency

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(MF, %) = *[(Number of molted shrimp × 100) / (days on feed × Number of shrimp)]*.

Exoskeleton formation: Minerals and elemental deposition, cuticle thickness and number of layers at Premoult D0

The formation of the exoskeleton after moulting was assessed by measuring cuticle thickness and the number of layers using Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-Ray Spectroscopy (EDS) (model LEO 1450 VP, Zeiss, Germany). The combination of SEM and EDS allows for the analysis of both the quantity of X-rays emitted and their energy, enabling the identification and quantification of chemical elements present at detectable concentrations in percentage by weight. Briefly, the anterior-lateral aspect of the carapace from three shrimps at the premoult D0 stage from each replicate was partially dissected exoskeleton samples measuring 2mm × 4mm were mounted on stubs with carbon tape and coated with a thin layer of gold in a sputter coater (SCD 050, Bal-Tec) for 2 min. at an accelerating voltage of 10 and 15 kV. SEM imaging was performed at varying magnifications to capture detailed morphological features. Cuticle thickness was determined by comparing the relative thickness of the sample to a scale bar and expressed in micrometers (μ), while the number of layers was counted at high magnification and expressed as a numerical value (n). EDS data were plotted as a graph with kV on the x-axis and peak intensity on the y-axis. The energy changes corresponding to peak locations on the x-axis were converted into atomic elements using specialized software to evaluating the composition of mineral and elemental deposits of the exoskeleton including calcium (Ca), magnesium (Mg), sodium (Na), chloride (Cl), manganese (Mn), copper (Cu), phosphorus (P), sulfur (S), and the elements oxygen (O) and carbon (C). The results are expressed as percentages of weight (Sakthivel *et al*., 2014).

Chitin content in exoskeleton at Premoult D0

The whole dried exoskeleton (~200 mg/sample) was used for analyses of chitin content according to Vigh and Dendinger (1982). Samples were sequentially decalcified in 2 mL of 2N HCl to extract "HCl-protein" (for mineralization). The samples were then incubated in 2 mL of water at 100°C for 4 h to remove "H_2O protein" (for hardening). NaOH-protein" (for hardening) was extracted in 2 mL of 2N NaOH at 100°C for 4h to obtain the residual protein. Each extract was centrifuged at 2,000 g for 20 min. The pellets were dried to constant weight and evaluated as chitin. The results are expressed as mg/g of exoskeleton dry weight.

Statistical analysis

All data were analyzed using SPSS software. One-way ANOVA was performed and, the model included fixed effects of diets on growth performance, moulting and exoskeletons evaluations.

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Normality and homogeneity of variance were evaluated. The significance level was set at $p<0.05$ and differences among means were compared by F-test. Results were reported as least square means with standard deviation mean (S.D).

Results

Growth performance

KFAA supplementation at 1% significantly improved growth performance (Table 4, Fig. 3). Shrimp fed KFAA for 60 days showed higher average daily gain, weight gain, and final body weight, along with a lower feed conversion ratio (FCR, $p<0.05$). The specific growth rate for weight was also higher in shrimp fed KFAA, while no differences were observed in the specific growth rate for length. No differences were found in survival or moulting frequency.

Table 4: Growth performance and moulting frequency of whiteleg shrimp fed functional amino acids mix (KFAA) at 1% for 60 days.

	Control	KFAA 1%	S.D.	*p*-value	Difference
Final body weight, FBW g	15.91[b]	20.04[a]	2.92	*	+26.0%
Average daily gain, ADG g/day	0.26[b]	0.33[a]	0.06	*	+26.9%
Body weight gain, WG %	112.7[b]	141.9[a]	4.2	*	+25.9%
Length gain, LG %	54.4	54.9	1.6	ns	/
Specific growth rate of weight, SGRW %	1.25[b]	1.47[a]	0.05	ns	+17.7%
Specific growth rate in length, SGRL %	0.71	0.73	0.06	ns	/
Feed conversion rate, FCR	2.34[a]	1.76[b]	0.25	*	-24.8%
Covariance of weight, CVW	14.2	16.9	4.2	ns	/
Covariance of length, CVL	5.3	6.0	1.7	ns	/
Survival rate, %	74.7	80.0	6.3	ns	/
Moulting frequency, MF %	5.2	5.4	0.4	ns	/

Whiteleg shrimp juveniles (*Litopenaeus vannamei*) initial body weight 14.12g±0.35.
Results obtained from One-way ANOVA and F-test. Different superscript letters within a row indicate significant differences $p<0.05$; ns, not significant $p>0.05$. Results are reported as least square means with

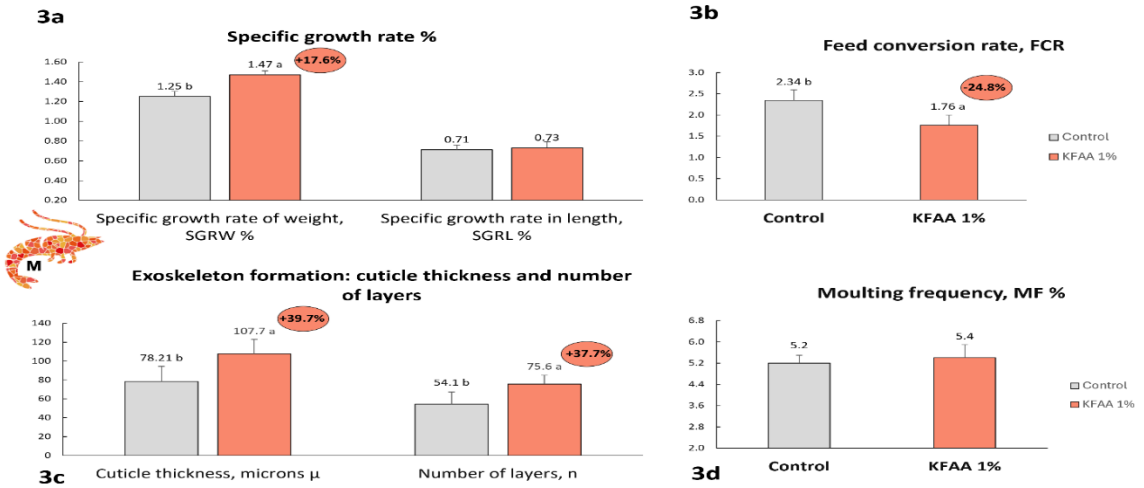


Figure 3: Growth performance and exoskeleton formation of the whiteleg shrimp fed diets supplemented at 1% of functional amino acids mix (KFAA) for 60 days. Whiteleg shrimp juveniles (*Litopenaeus vannamei*) initial body weight 14.12g±0.35. Results obtained from One-way ANOVA and F-test. Different superscript letters within a row indicate significant differences ($p<0.05$). Results were reported as least square means with standard deviation mean (S.D).

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Moulting evaluation: Cuticle thickness and number of layers at premoult D0-D1

The cuticle thickness and the number of layers of the D0 cuticle (Table 5) in the KFAA 1% group were significantly higher than those in the control group (p<0.05), +27.38% and 28.44% respectively. The cuticle architecture of the KFAA 1% and control groups is shown in Figure 4. Overall, the electro-microscopy shows a more organized and better-developed cuticle structure.

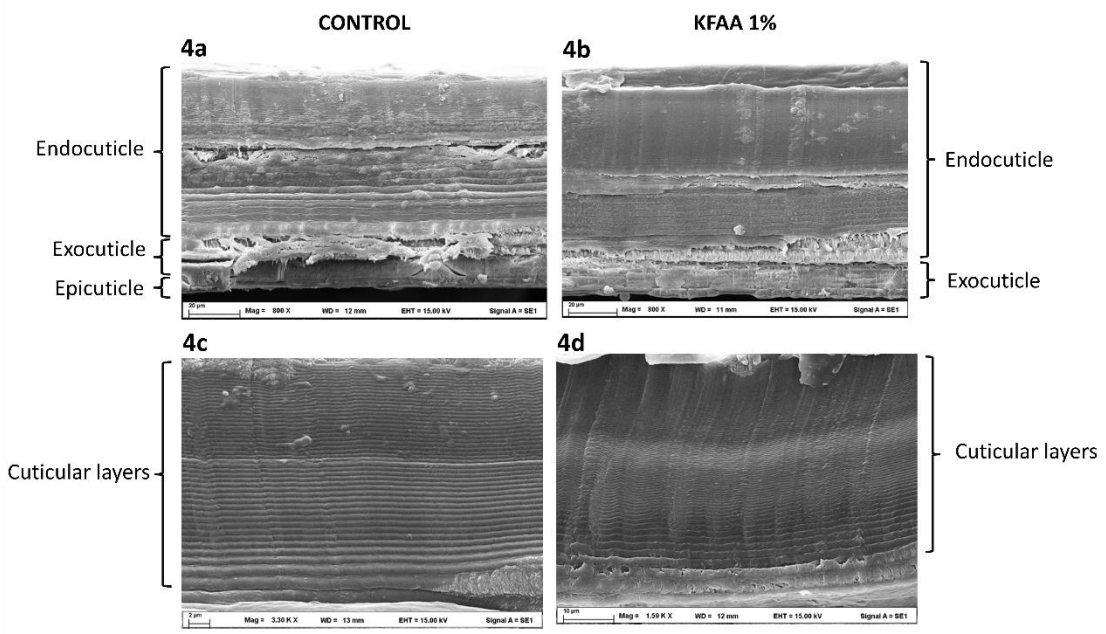


Figure 4: Exoskeleton cuticle architecture in high magnification obtained from electron microscopy and energy dispersive system of the whiteleg shrimp fed diets supplemented at 1% of functional amino acids mix (KFAA) for 60 days. (4a–4b) Note the endocuticle and exocuticle formations and thickness differences between the two treatments. (4c–4d) Note the number of layers differences between the two treatments.

Chitin content in exoskeleton at Premoult D0-D1

The chitin content in the KFAA 1% group was significantly higher +16.6% (p<0.05) compared to the control group (Table 5).

Minerals and elemental deposition in the exoskeleton at Premoult D0

The mineral and elemental deposition are shown in Table 6. The percentages of Ca and Mg in the cuticle of the KFAA 1% group were significantly higher than those in the control group (p<0.05), +7.5% and 16% respectively. On the other hand, the percentages of Na and Cl were significantly lower in shrimp fed the 1% KFAA 1% diet (p<0.05), -29.4% and 109.0% respectively. No significant differences were observed between groups for the other minerals nor elements P, S, Mn, Cu, P, C and O.

Discussion

The described feeding trial in whiteleg shrimp emphasizes the benefits of

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supplementing functional amino acids for better exoskeleton formation during molt events which lead to improved growth performance. Shrimp fed with 1% KFAA feed exhibited superior body weight and lower feed conversion ratio. The improved growth performance may be attributed to the role of KFAA in providing free key amino acids during the critical stages of the molt cycle.

The moulting cycle of shrimp can be described, taking into consideration the proximity to ecdysis (stage E), as follows: the preparation period (premoult, substages D0, D1, D2, D3, D4) and recovery (post-moult, substages A and B) intercalated by a 'moult-independent period' (intermoult, C) (Lemos and Phan, 2001). Premoult begins with a rise in moulting hormones, particularly 20-hydroxyecdysone, in the hemolymph. This dynamic phase represents up to 70% of the moulting process and consists of substages D0, D1, D2, D3, and D4. Throughout premoult, routine activities such as feeding and swimming continue alongside the physiological changes essential for successful moulting (Lemos and Weissman, 2021).

In an extensive review Lemos and Weissman (2021) highlighted the importance of many nutrients for moulting events. Protein can account for 12% of exuviae dry weight. This protein corresponds to a loss of amino acids after each ecdysis cycle. The amino acids content in exuviae is reported following an order of importance: glutamic acid, aspartic acid, glycine, alanine, proline, phenylalanine, arginine, valine, leucine and threonine (Lemos and Weissman, 2021). Changes have also been noticed in the body content varying with moulting stages as following: increasing trend for arginine, histidine, leucine and threonine; reduction for phenylalanine. Body content is higher during stages A and D compared to C for lysine and methionine while valine levels are higher in stage C than in stages A and D (Lemos and Weissman, 2021).

Shrimp fed with 1% KFAA showed significantly greater cuticle thickness and an increased number of layers compared to the control group. The KFAA supplement can provide most of the amino acids (Table 3.) indicated as the content of exuviae which explains the better cuticle formation results.

Regarding the changes in mineral profile in exoskeleton, this enhancement is likely due to improved precipitation of Ca and Mg carbonate crystals, which play an important role in cuticle formation. Elevated Ca and Mg levels in the cuticle further support this explanation. Additionally, higher chitin content in KFAA-fed shrimp suggests that KFAA promotes the formation of the chitin-protein complex, which interacts with inorganic components such as Ca and Mg.

Previous research has established that calcium carbonate is the predominant mineral in crustacean exoskeletons (Pratoomchat *et al*. 2002b, c). In *Penaeus indicus*, the protein content in the shell and flesh structure was found to be 32.5% and 41.3%, respectively (Ravichandran *et al*., 2009). Mineral composition analysis of *L. vannamei*

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carapaces revealed Ca and Mg concentrations of 72.73% and 1.8%, respectively (Sakthivel *et al*., 2014). Notably, control shrimp exhibited high Na and Cl levels in their cuticles, which may interfere with cuticle formation. Na^+ ions can compete with Ca^{2+} for binding sites, while Cl^- may reduce bicarbonate (HCO_3^-) availability, affecting carbonate deposition. These findings suggest that KFAA supplementation accelerates and optimizes cuticle formation, contributing to improved growth performance.

The chitin content in the exoskeleton of shrimp fed with 1% KFAA was significantly higher than in the control group. This suggests that KFAA supplementation plays a role in enhancing chitin synthesis, which is essential for cuticle development. Chitin forms a structural complex with proteins and inorganic minerals, particularly Ca and Mg, contributing to a more robust exoskeleton. The increase in chitin content supports the hypothesis that KFAA enhances the formation of a well-structured exoskeleton, which is essential for shrimp growth and survival.

The concentrations of Mg and Ca in the exoskeleton of *L. vannamei* significantly increased following KFAA supplementation, while Na levels significantly decreased. No significant differences were observed in P, S, Mn, Cu C and O levels. Previous studies have demonstrated that dietary glycine supplementation can increase Ca concentration in the whole body of shrimp (Xie *et al*., 2014).

Conclusions

This study encourages the discussion and wider understanding of amino acids and their role in physiology such as moulting demands above and beyond the traditional approaches as essential and dispensable. The supplementation of 1% KFAA in shrimp diets significantly improved growth performance. It also promoted the development of thicker cuticles, more layers, and increased chitin deposition, all contributing to better moulting and structural formation. Additionally, the higher deposition of essential minerals such as calcium and magnesium supported exoskeleton mineralization. These findings highlight the role of functional amino acids in shrimp precision nutrition as a tool to address their physiological needs.

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