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Free amino acids mix made of poultry keratin improves survival of whiteleg shrimp post larvae (*Litopenaeus vannamei*) challenged with acute hepatopancreatic necrosis disease and white spot syndrome virus

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Abstract
With their low molecular weight and high level of assimilation, mixes of free amino acids (MFAA) obtained from the extensive hydrolysis of poultry keratin are potential interesting candidates for aquaculture feeds. Two trials, I and II, were conducted to evaluate the effects of MFAA on whiteleg shrimp *Litopenaeus vannamei* post larvae (PL). Both trials included a growth phase and experimental challenges. In these two trials, PL were fed four diets (control; control+1% MFAA; control+5% MFAA; control+10% MFAA) respectively for 28 days (trial I) and 21 days (trial II) for growth phases. Following this growth phases, animals were either experimentally infected with white spot syndrome virus (WSSV group) or *Vibrio parahaemolyticus*, with a toxin gene-bearing plasmid responsible for acute hepatopancreatic necrosis disease (AHPND group), or mock infected (non-infected control) considering four diets treatments (control; control+1% MFAA; control+5% MFAA; control+10% MFAA) during 28 days for trials I and II. In these two trials, survival and biomass reached higher rates in WSSV infection groups, for PL fed with MFAA. For AHPND infection group in trial II, survival and biomass were also higher for PL fed with MFAA. Those results show the potential of MFAA to enhance shrimp PL performance and their application as shrimp feeding ingredients with functional benefits on animal survival in case of immune challenge.

Keywords Hydrolysates · Free amino acids · Functional ingredients · Aquafeed · Shrimp · Survival

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Introduction

World production of farmed shrimps reached almost 4 million tonnes in 2018, increased by 3 to 5% over 2018 (FAO 2019). Infectious diseases are considered as the main threat to shrimp farming expansion. Diseases such as acute hepatopancreatic necrosis disease (AHPND), of bacterial aetiology, and white spot syndrome virus (WSSV) viral disease, have induced billions of US$ of losses in the last decade (Shinn et al. 2018). The WSSV belongs to the genus *Whispovirus*, the only genus of the family Nimaviridae and is historically the most devastating pathogen affecting the shrimp industry since its apparition in 1992 (OIE 2019). The AHPND has emerged in 2010 and is sometimes referred to as early mortality syndrome (EMS). This disease is mainly caused by *Vibrio parahaemolyticus* strains that carry a plasmid that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB, although other *Vibrio* species that contain this plasmid have also been shown to produce AHPND in shrimps (Devadas et al. 2018).

Economic losses are recurring problems in shrimp farming, and disease management requires a global approach including parameters such as feed. Strategies based on biosecurity and pathogen management only are generally not successful. In aquaculture, farming practices combined to variations of environmental conditions can induce stress for the animals. These factors of stress generally linked to pollution, temperature and water quality (oxygen, salinity, pH, ammonia and its by-products), but also handling during pond transfers can affect immune system performances and generate development of opportunistic infections (Tendencia and Verreth 2011; Chen and He 2019).

Amino acids (AA) are essential in shrimp nutrition (NRC 2011). In formulator's toolbox, synthetic AA (Met, Lys) are generally applied in shrimp feed in order to reach shrimp essential AA requirements (Nunes et al. 2014). This practice is reinforced with the trend of novel diets development based on fishmeal replacement by plant proteins with less adapted amino acids profiles (Gatlin III et al. 2007; Suresh et al. 2011). In addition to their action in protein synthesis, AA play various functional roles in animal nutrition. Their effects have been underlined in different fields: attraction (Derby and Sorensen 2008), meat firmness (Østbye et al. 2018) and antioxidant defences (Wu 2010). In addition, AA can significantly improve the immune response in fish (Clark et al. 2020).

In a previous study, a mix of 17 free amino acids (MFAA), in soluble form and with 92% of the composition under free form, obtained from poultry keratin extensive hydrolysis, demonstrated its ability to act as feeding stimulants and improve shrimp growth performances in tank and pond conditions (Le Reste et al. 2019). However, according to our knowledge, potential action of MFAA to induce a better immune response in case of infectious bacteriological and viral challenges has not been investigated before this study.

Another study has recently showed that the low molecular weight (lower than 800 Dalton) of these MFAA is linked to a very high *in vivo* digestibility of 96.8% (Duperray et al. 2018, unpublished data). This makes MFAA an interesting candidate to feed shrimp larvae that are equipped with immature digestive tracks (Navarrete del Toro and García-Carreño 2019).

Feed supplementation can be one of the keys of success as it has a major impact on gut microbiota (Aasen et al. 2012) and can act as health booster of the immune system (Holt et al. 2020; Li et al. 2018). In addition, shrimp can be programmed by early nutritional stimulus (Lage et al. 2018), and the nutritional programming concept (Lucas 1998) shows that early nutritional events may have a persistent long-term effect either on metabolism and/or physiology (Patel and Srinivasan 2002; Patel et al. 2009). In this context, MFAA supplementation during early development stages could be an efficient feeding strategy. Supplying animal devoid of specific immune system such as shrimp could provide them with easily digestible useful metabolites.

Table 1 Proximate composition of the MFAA tested in experiments I and II

Items	Value
Dry matter	98.6%
Total amino acids (CE 152/2009)	88.7%
Free amino acids (CE 152/2009)	83.8%
Crude ashes	7.7%

The interest of hydrolysates in aquaculture is underlined in many papers (see review by Martínez-Alvarez et al. 2015). Extensive hydrolysis of poultry keratin leads to a complete denaturation of the protein chain to reach the state of free amino acids (FAA). This industrial process was initially developed to extract cystine and tyrosine for pharmaceutical and nutraceutical applications. It also generates a mix containing free AA, short peptides and mineral salts. This MFAA is particularly rich in FAA with a typical amino acids profile resulting from combination of raw material and partial extraction and purification steps of single amino acids (composition available in Table 1 and Table 2).

In order to evaluate the potential effect of this MFAA on whiteleg shrimp *Litopenaeus vannamei* performance, two consecutive trials have been recently conducted in Incabiotec/ Concepto Azul Research Centre located in Tumbes, Peru. Each trial was designed to alternate a growth phase with an infectious challenge with AHPND or WSSV.

Experiments

Material and methods of experiments I and II

Feed preparation and feeding protocol

Two locally produced commercial feed for shrimp post larvae (PL) (Nicovita Origin 0.5 for PL11-20 and Nicovita Origin 0.8 for PL21-1g, 45% crude protein and 10% lipid) were used as

Table 2 Amino acids contained in the MFAA with the proportion of each AA under free form

	Total amino acids	Proportion of free AA
Aspartic acid	6.87	99%
Threonine	4.55	100%
Serine	12.10	100%
Glutamic acid	10.33	96%
Glycine	7.84	98%
Alanine	4.64	98%
Valine	7.42	73%
Cystine	1.71	71%
Methionine	0.40	96%
Isoleucine	4.18	82%
Leucine	7.09	95%
Tyrosine	0.26	72%
Phenylalanine	2.33	97%
Lysine	1.80	93%
Histidine	0.58	89%
Arginine	5.69	94%
Proline	10.86	100%

a base feed (composition available in Table 3). The MFAA was obtained from BCF Life Sciences (Boisel, France). In order to identify a potential dose effect, three MFAA concentrations were tested: 10g/kg of feed, 50g/kg of feed and 100g/kg of feed (MFAA 10, MFAA 50 and MFAA 100, respectively) in comparison with a negative control feed. MFAA doses were selected in accordance with a previously published work (Le Reste et al. 2019).

The MFAA was mixed with a fixed quantity of water (3/5 of MFAA and 2/5 of water). This solution was homogenized and mixed by coating with the feed for 3 min in a mixer. The control feed followed the same process and was sprayed with water only. After 12-h drying at room temperature, each feeds were coated with a 2:1 mix of fish oil and soy lecithin sprayed at 30 g/kg. After an additional 12-h drying at room temperature, batches of feeds were stored in individual sterile bags and stored in a clean and cool stocking room (4°C) for the whole trial duration.

Animal husbandry and experimental protocol for the growing period

Shrimp PL were obtained from commercial broodstock (certified IHHNV, WSSV, NHP free) and were previously analysed by polymerase chain reaction (PCR) to confirm the absence of WSSV, infectious hypodermal and haematopoietic necrosis virus (IHHNV), necrotizing hepatopancreatitis (NHP) and AHPND (Dangtip et al. 2015; Tang et al. 2000; Nunan et al. 1998, 2008).

In addition, before the beginning of the trials, microbiological counting of *Vibrio* spp. and *Pseudomonas* spp. was performed in thiosulfate-citrate-bile salts-sucrose (TCBS) and cetrimide agar, respectively. The PCR analysis results in PL were negative for AHPND, IHHNV, NHP and WSSV. Bacterial counts indicate 320 cfu/PL by agar TCBS, and 0 cfu/PL by cetrimide agar were obtained.

Thirty-six glass tanks of 20-l capacity were filled each with 15 l of seawater (32ppt salinity). Each tank was stocked with 90 healthy *L. vannamei* post larvae, PL14 and PL21/22 (initial individual average weight of 0.005g and 0.028g, respectively, for trials I and II). All the glass tanks were randomly placed in the same room. Animals were maintained under natural photoperiod, and salinity was maintained at 32 ppt. Water was at room temperature.

The tanks were divided into four groups of nine tanks. Each of these groups was fed with one of the previously described diets, respectively, for a period of 28 and 21 days for trials I and II. The PL were hand-fed five times per day according to the biomass evolution and table feeding provided by the feed manufacturer. Survival rate and biomass evolution were checked weekly during this period.

Table 3 Proximate composition of feeds used in experiments I and II

Items	Control diet
Dry matter, %	> 90%
Crude protein, %	> 45%
Lipid, %	> 10%
Ash, %	< 10%
Fibre, %	< 2%

Animal husbandry and experimental protocol for the infectious challenge period

At the end of the growing period, 324 PL of each treatment were placed in 9 tanks of 15 l filled with the same brackish water described above. Animals were divided into three groups (non-infected control, AHPND group, WSSV group). The PL average initial body weights were respectively 0.26g and 0.091g for trials I and II at the beginning of infectious challenge phase.

All the glass tanks were randomly placed in the same room. Animals were maintained under natural photoperiod, and the salinity was maintained at 32 ppt.

AHPND and WSSV infections were processed by dipping, following standard infection protocols for these two pathogens: *Vibrio parahaemolyticus* 106 cfu/ml and adding of infected WSSV tissues at 20% of initial biomass (Devadas et al. 2018; Domínguez-Borbor et al. 2019).

Each of these groups was fed with one of the previously described diets, for a period of 28 days for trials I and II.

Survival rate and biomass evolution were followed weekly during this period to identify potential effects of MFAA in case of immune challenges. For biomass determination, all the larvae were harvested, gently dried through a net and weighted before individual count.

Water quality parameters

During growing and infection stages, ammonium, nitrite, nitrate and phosphate were checked and recorded twice a week during water renewal (50% of volume).

Water temperature, pH and dissolved oxygen were checked and recorded twice a day, at 8 AM and 6PM (Table 4).

Data statistical analysis

Data were subjected to ANOVA, and in case of significance ($P \leq 0.05$), a Duncan test was performed. Statistical analyses were made with the SPSS software.

Results experiments I and II

Results for the growing period

Water quality parameters measured during these two experiments (Table 4) were in conformity with known requirements *for L. vannamei* shrimps (Alday-Sanz 2010).

Survivals of the animals were above 81% and 87% for controls of trials I and II, respectively (Table 5). Survival was significantly improved of 8.08% for the animals fed

Table 4 Water quality parameters measured in the glass tanks used for experiments I and II

	T° (°C)	DO (ppm)	pH	pH	NH3 (ppm)
Trial I	Average	26.9	7.53	7.61	1.62
	Min	26.0	6.16	7.45	0.50
	Max	28.3	8.57	7.75	4.00
Trial II	Average	27.8	6.37	7.30	0.50
	Min	27.6	6.06	7.00	0.50
	Max	28.5	6.92	7.99	0.50

Table 5 Survival and biomass of *Litopenaeus vannamei* PL shrimp fed feeds containing respectively 10g/kg, 50g/kg and 100g/kg of the mix of free amino acids (MFAA) at the end of the growth phase in 18 experiments I and II

		Control	MFAA 10	MFAA 50	MFAA 100	*P*-value
Trial I	Survival (%)	80.99 ± 2.96[b]	81.11 ± 3.29[b]	87.53 ± 3.72[a]	79.63 ± 4.48[b]	0.000 VHS
	Base 100	100	100.15	108.08	98.32	
	Biomass (g)	19.16 ± 2.34[a]	19.89 ± 1.5[a]	21.45 ± 3.44[a]	12.72 ± 3.34[b]	0.000 VHS
	Base 100	100	103.81	111.95	66.39	
Trial II	Survival (%)	87.29 ± 1.68[b]	90.25 ± 1.9[a]	88.03 ± 1.55[ab]	88.4 ± 1.58[ab]	0.006 VHS
	Base 100	100	103.39	100.85	101.27	
	Biomass (g)	7.17 ± 0.61[b]	8.11 ± 0.76[a]	7.52 ± 1.13[ab]	6.43 ± 0.81[b]	0.002 VHS
	Base 100	100	113.11	104.88	89.68	

MFAA 50 in trial I and of 3.39% MFAA 10 in trial II. In this latest group, biomass increase was also significantly improved of 13.11% for the animals fed MFAA 10.

Results for the infectious challenge period

The AHPND- and WSSV-infected control groups showed a significant decrease of survival and biomass increase (Table 7 and Table 8), while these parameters remained steady in non-infected groups (Table 6). Unlike control group, moribund shrimps were observed few days after experimental challenges. Affected animals showed lethargy, decreased or absent feed consumption with clinical symptoms such as pale white atrophied hepatopancreas and reddish-whitish bodies for AHPND and WSSV groups, respectively. In WSSV-infected groups of trials I and II and in AHPND-infected group of trial II, animals fed with MFAA treatments showed significantly higher survival rates from the second week to the end of the infection phase (Figs. 1 and 2 and Tables 7 and 8), in comparison with the animals fed with the control diet without MFAA.

Discussion

The present results underline the potential of MFAA extracted from poultry keratin after extensive hydrolysis when applied on shrimp feed for early stages of development, particularly to face infectious challenges. During the first growing period, survival was significantly and positively influenced in the two experiments. Biomass increase was significantly improved in experiment II. The AHPND and WSSV challenged groups were followed during 4 weeks. Altogether the onset of symptoms and mortalities were in accordance with classic clinical signs observed in WSSV and AHPND diseased animals (OIE, 2019). Infected groups faced a strong

Table 6 Survival and biomass of non-infected group of *Litopenaeus vannamei* PL fed with the control feed at the end of the infectious challenge phase in experiments I and II

		Control
Trial I	Survival (%)	85.17 ± 1.62
	Biomass (g)	41.6 ± 8.22
Trial II	Survival (%)	73.15 ± 1.6
	Biomass (g)	12.82 ± 0.98

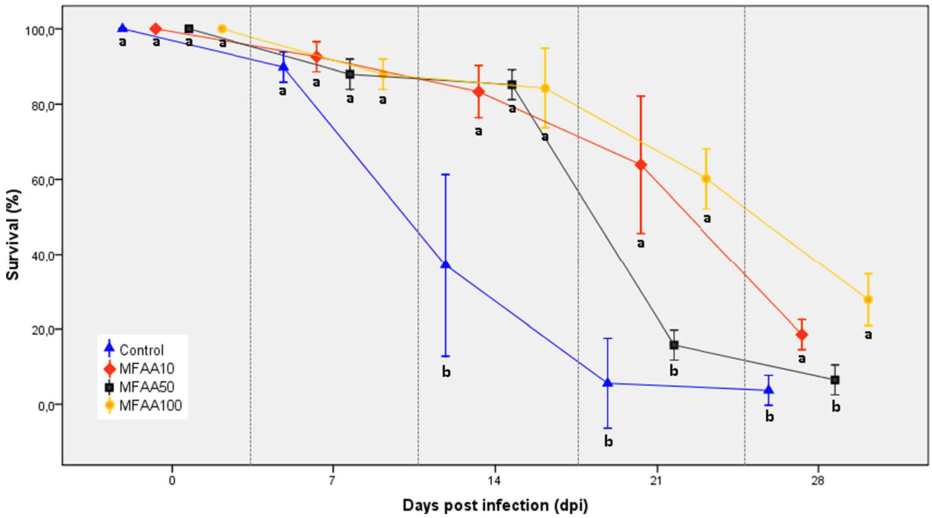

Fig. 1 Evolution of survival week by week after WSSV infection in trial I (95% CI of averages and Duncan test by week)

drop of the PL population in comparison with the non-infected control groups for the two experiments. In these challenging conditions, it is noteworthy that animal fed with feeds supplemented with different levels of MFAA showed a significantly higher survival and biomass increase in case of AHPND infection for trial II and WSSV infection for trials I and II.

MFAA applied on shrimp PL feed in present study is composed of both essential and non-essential amino acids for shrimp (NRC 2011). It appears that these two categories of amino acids can act by different specific actions on immune-health improvement status. Between non-essential amino acids, glutamine, present in high proportion in glutamic-acid form in evaluated MFAA

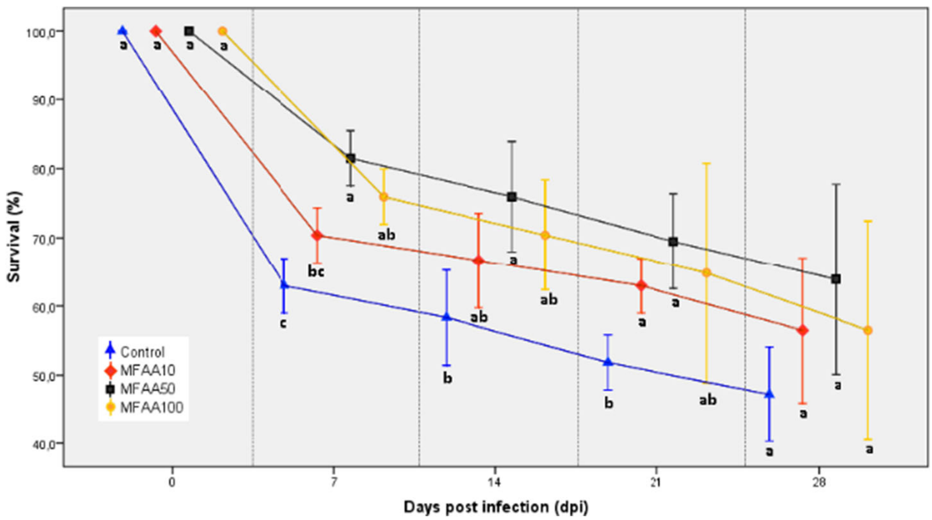

Fig. 2 Evolution of survival week by week after AHPND infection in trial II (95% CI of averages and Duncan test by week)

Table 7 Survival and biomass of AHPND-infected groups of *Litopenaeus vannamei* PL fed feeds containing, respectively 0, 10, 50 and 100g/kg of the mix of free amino acids (MFAA) at the end of the infectious challenge phase in experiments I and II

		Control	MFAA 10	MFAA 50	MFAA 100	*P*-value
Trial I	Survival (%)	59.23 ± 1.62^{b}	63.87 ± 4.79^{ab}	66.67 ± 5.55^{ab}	68.53 ± 5.77^{a}	0.167 NS
	Base 100	100	107.83	112.56	115.70	
	Biomass (g)	26.19 ± 3.95^{a}	24.51 ± 1.31^{a}	26.23 ± 3.72^{a}	27.85 ± 2.61^{a}	0.639 NS
	Base 100	100	93.59	100.15	106.34	
Trial II	Survival (%)	47.22 ± 2.78^{b}	56.48 ± 4.24^{a}	63.89 ± 5.56^{a}	56.48 ± 6.42^{ab}	0.037 S
	Base 100	100	119.61	135.30	119.61	
	Biomass (g)	7.19 ± 0.73^{b}	8.26 ± 0.47^{ab}	9.03 ± 0.57^{a}	8.12 ± 0.74^{ab}	0.048 S
	Base 100	100	114.88	125.59	112.93	

(11.6% of total AA), is one of the most abundant free AA in fish plasma and muscle and is crucial to the immune response in fish (Buentello and Gatlin 1999; Li et al. 2007). Dietary glutamine supplementation also enhances weight gain, feed intake and improve feed ratio, intestinal development and digestive enzyme activities in Jian carp (Lin and Zhou 2006).

Arginine is present in high proportion in evaluated MFAA (6.42% of total AA), and fish have particularly high requirements for dietary arginine because it is abundant in protein as a peptide bound AA (Mommsen et al. 2001). Arginine also generates growth and health effect for some fish species. For example, Buentello and Gatlin (2001) showed that the survival of channel catfish in response to challenge with *Edwardsiella ictaluri* critically depends upon dietary arginine levels.

Proline is also present in high proportion in evaluated MFAA (12.25% of total AA), and its supplementation improves amino acid constituent, anti-oxidative capacity, immune response and NH3 stress tolerance of juvenile *Litopenaeus vannamei* (Xie et al. 2015).

Lysine is present in smaller proportion (2.03% of total AA) in evaluated MFAA but considered as one of the most limiting amino acid in aquafeed formulations, and fishmeal replacement by plant protein sources particularly reinforce this point (Mai et al. 2006a). In addition, dietary lysine supplementation is effective in improvement of growth and enhancement of intestinal and hepatopancreatic enzyme activities of sub-adult grass carp and could promote the antioxidant defence in fish intestine (Li et al. 2014).

Phenylalanine can be converted to tyrosine that acts as a common precursor for important hormones and neurotransmitters. Dietary levels of phenylalanine and tyrosine could profoundly influence pigmentation development, feed intake, growth performance but also immunity and survival of fish and shrimp in natural environment (Chang et al. 2007; Yoo et al. 2000).

Table 8 Survival and biomass of WSSV infected groups of *Litopenaeus vannamei* PL fed feeds containing respectively 0, 10, 50 and 100g/kg of the mix of free amino acids (MFAA) at the end of the infectious challenge phase in experiments I and II

		Control	MFAA 10	MFAA 50	MFAA 100	*P*-value
Trial I	Survival (%)	3.73 ± 1.62^{d}	18.5 ± 1.56^{b}	6.5 ± 1.56^{c}	27.8 ± 2.8^{a}	0.000 VHS
	Base 100	100	495.98	174.26	745.31	
	Biomass (g)	0.65 ± 0.21^{c}	4.13 ± 1.01^{b}	0.82 ± 0.42^{c}	7.38 ± 0.47^{a}	0.000 VHS
	Base 100	100	635.38	126.15	1135.38	
Trial II	Survival (%)	46.3 ± 1.6^{b}	61.11 ± 5.56^{a}	57.41 ± 3.21^{a}	54.63 ± 4.24^{a}	0.036 S
	Base 100	100	131.99	124.00	117.99	
	Biomass (g)	5.93 ± 0.59^{bc}	7.41 ± 0.86^{ab}	8.08 ± 1.12^{a}	5.9 ± 0.46^{c}	0.054 NS
	Base 100	100	124.96	136.26	99.49	

MFFA have already demonstrate their ability to generate significant gains on zootechnical performances by positively influencing feed intake and growth when applied on shrimp feed at low incorporation rates (5 to 10g/kg). During the two studies presented in this synthesis, it appears that MFAA also give a positive influence on shrimp survival in case of bacteriological and viral challenges. Mechanisms of action are probably multiple as each amino acid acts at different levels of different metabolic pathways, perhaps in relation with the immune response. We can hypothesize that these actions could have synergetic effects. Regarding immune response improvement through nutritional approach and amino acids supplementation, it is probably preferable to have a holistic approach. In the present case, evaluated MFAA is composed of 17 amino acids with some of them already identified to generate positive actions on immune response of aqua species.

In evaluated MFAA, a more important bioavailability of free amino acids than native protein chains is directly in relation with a high level of assimilation (96.8% in vivo digestibility measured on cockerel) (Larbier Zuprizal and Chagneau 1991). This characteristic is particularly important for young animal with immature digestive tracts if we consider that a fast absorption after feed ingestion can contribute to improve general metabolic pathways (Rønnestad et al. 2003; Zambonino-Infante et al. 2008). Another point to take into account is also the highly soluble form of these amino acids mix in comparison with crystalline amino acids mainly used for feed supplementation and generally individually extracted at their isoelectric point, which corresponds to their lower water solubility. This particularity could also accelerate this MFAA assimilation by the animal.

To our knowledge, there is no previous available scientific work in shrimp nutrition underlining effect of a MFAA inducing a better immune response in case of bacteriological or viral challenge. In general, research studies mainly focus on single amino acids and their individual effects in feed supplementation. For example, methionine is usually the first limiting AA in formulations containing high levels of plant protein sources (Mai et al. 2006b).

More investigations are available in field of fish nutrition where amino acids role is studied in relation with the development of functional and environmentally oriented aquafeeds inducing specific requirements. Amino acids and their metabolites are important regulators of key metabolic pathways that are necessary for maintenance, growth, feed intake, nutrient utilization, immunity, behaviour, larval metamorphosis, reproduction as well as resistance to environmental stress and pathogenic organisms in various fishes (Li et al. 2009). Considering these several points, we can hypothesize that a combination of different amino acids available in free form provides a synergetic effect on immune response of juvenile aqua species.

In addition, a positive influence of MFAA on microbiota composition generating a better immune response should also be one interesting hypothesis to evaluate deeply in order to explain the better survival rates obtained with experimental treatments.

Conclusions and perspectives

Those two experiments have demonstrated that MFAA obtained from extensive poultry keratin hydrolysis have an interesting potential as feeding ingredient for shrimp feed with positive effect on *L. vannamei* PL survival in case of AHPND and WSSV infection challenges. The infection protocols generated a strong drop of the population excepted for treatments including MFAA supplementation at 10, 50 and 100 g/kg.

In a context of marine ingredients substitution, AA supplementation is commonly applied to reach nutritional requirements. Present results are opening new possibilities for AA utilization in aquafeed formulations. Further research is needed to better understand the mode of action of each AA, considered individually or in synergy.

In conclusion, MFAA obtained from extensive poultry keratin hydrolysis offers a sustainable valorisation of a non-digestible protein source converted into an efficient functional ingredient to improve survival of white shrimp, *L. vannamei*, in case of bacteriological and viral challenges.

Availability of data and material Data are available upon request to the corresponding author.

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Declarations

Ethics approval This work followed the ethical rules of Peru, where it was conducted.

Consent to participate All the people involved in this work gave their consent to participate.

Consent for publication All the people mentioned in this paper gave their consent to be co-author.

Competing interests The authors declare no competing interests.

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