



Insights of *Nauphoeta cinerea*, as a protein source for replacing fish meal and soybean meal in tropical gar, *Atractosteus tropicus*, and Nile tilapia, *Oreochromis niloticus*, diets

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ABSTRACT - This study aims to investigate the proximate composition, heavy metal contents, antimicrobial activity, and *in vitro* digestibility of *Nauphoeta cinerea* (Nc) as a replacement for fish meal (FM) and soybean meal (SBM) in diets for a carnivorous (tropical gar, *Atractosteus tropicus*) and an omnivorous (tilapia, *Oreochromis niloticus*) fish. *Nauphoeta cinerea* were fed a formulated diet based on fruits, vegetables, beef liver, and grains. They had an acceptable level of protein (46.50 g 100 g⁻¹) and high contents of lipids (14.49 g 100 g⁻¹) compared with FM and SBM. Amino acid and free fatty acid (FFA) profiles more closely resembled previously reported levels in insect meals than those of FM and SBM. The mineral content of Nc was similar to that of both FM and SBM. The heavy metal contents of Nc were within standard permissible limits established for FM and SBM. *Nauphoeta cinerea* showed a chitin content of 4.20±0.08% (percentage of glucosamine), equivalent to 42.02±0.08 µg of glucosamine (chitin) per mg of insect meal. The antimicrobial assay showed that Nc did not inhibit the growth of bacterial species with probiotic properties commonly present in fish intestinal microbiota. The *in vitro* digestibility of Nc performed better in tilapia than in tropical gar during the acid stage. During the alkaline stage, the degree of hydrolysis (DH) and relative digestibility (RD) of Nc were higher than that of FM and SBM. *Nauphoeta cinerea* is nutritionally feasible (in terms of proximal composition), digestible (in terms of *in vitro* digestibility), safe (in terms of heavy metals contents), and biologically functional (in terms of antimicrobial activity) as a replacement for FM and SBM in the diets of omnivorous and carnivorous fish.

Keywords: *Atractosteus tropicus*, insect meals, *in vitro* digestibility, *Oreochromis niloticus*, quality assessment

1. Introduction

Aquaculture feeds are crucial to sustain industrial growth and the increasing demand for seafood worldwide (Hua et al., 2019). Commercial feeds for aquaculture are traditionally formulated with fish (FM) and soybean (SBM) meal (Henry et al., 2015) as primary protein sources. However, there is a growing trend towards using alternative feed ingredients to achieve sustainable aquaculture goals. Insect meals are gaining significant attention as novel protein sources in aquaculture diets (Govorushko, 2019). Insects play a crucial role in the nutritional physiology of aquatic organisms. For example, insect meals provide high-quality nutrients essential for fish growth and development. They possess balanced amino acid profiles, essential fatty acids, vitamins, and minerals. Furthermore, insect cultivation has a significantly lower environmental impact compared with traditional terrestrial-derived ingredients. Finally, aquaculture diets formulated with insect meals can be economically viable due to their efficient conversion rates (Freccia et al., 2020).

Assessing the sustainability of insect meals as alternative protein sources requires several complementary types of information. The proximal composition must be characterized to determine their feasibility as FM and SBM replacers (Alfiko et al., 2022). Hierarchical clustering analysis (HCA) may be used to assess similarities among different protein candidates (Barroso et al., 2014). The toxicological content (e.g., heavy metals) of non-essential elements with toxic properties (Henry et al., 2015) must be assessed. Different chitin levels are present in insect meals. Chitin is a long-chain polymer of N-N-acetylglucosamine, an amide derivative of glucose that, at specific concentrations, may interfere with the correct digestive physiology of fish (Kvasnička et al., 2023). Hence, when supplementing insect meals in aquaculture diets, concentration levels of chitin must be assessed. The antimicrobial activity must be tested to determine whether the target insect meal inhibits the growth of probiotic bacteria or yeast naturally occurring in fish intestinal microbiota (Sibinga et al., 2023). *In vivo* (ADC – apparent digestibility coefficients) / *in vitro* protein digestibility must be quantified to understand how feeds or food are processed or digested (Ferrer Llagostera et al., 2019). Studies on *in vitro* digestibility of insect meals in Nile tilapia (*Oreochromis niloticus*) has been reported recently (Moyano et al., 2015; Irungu et al., 2018). On the other hand, previously conducted studies on *in vitro* digestibility of tropical gar (*Atractosteus tropicus*) have been focused on the use of other traditionally utilized ingredients in aquaculture (Guerrero-Zárate et al., 2024) but not in insect-derived ingredients.

Nauphoeta cinerea (Blaberidae) (Nc) is a cockroach that is mainly cultured as live feed for amphibians and reptiles, and it may represent a good candidate for aquaculture. In southeastern Mexico, regional environmental attributes, including warm temperature, adequate relative humidity, and soil and water availability, are favorable for the controlled mass production of Nc (personal communication). Additionally, this insect species was reported as a nutritional source of protein enrichment for human (Oliveira et al., 2017) and fish (Freccia et al., 2016; Freccia et al., 2020; Tubin et al., 2023) consumption. For example, Tubin et al. (2023) reported using this species in tilapia cultured in a biofloc technology system. The authors concluded that Nc can be included in up to 10% of diets for tilapia raised in biofloc systems without significant productivity losses.

In addition, tropical aquaculture in the southeast of Mexico is based on the exploitation of Nile tilapia (an omnivorous species) and tropical gar (a carnivorous species). The last one has been deeply studied (Guerrero-Zárate et al., 2019; Jiménez-Martínez et al., 2020; Méndez-Pérez et al., 2020; Sepúlveda-Quiroz et al., 2021; De la Cruz-Marín et al., 2023), evidencing that digestive enzyme activities of tropical gar exhibit regular physiology typical of carnivorous fish species. There is a lack of published information regarding the use of insect meals in tropical gar. However, a vast bibliography can be found related to the use of dietary insects in Nile tilapia diets (Henry et al., 2015; Tubin, 2020; Govorushko, 2019; Nogales-Mérida et al., 2019).

For these reasons, the present study aimed to investigate proximate composition, heavy metal contents, antimicrobial activity versus benefit bacteria naturally occurring in the fish gut, and *in vitro* digestibility of Nc as SBM and FM replacer in diets for a carnivorous (*Atractosteus tropicus*) and an omnivorous (*Oreochromis niloticus*) fish.

2. Material and methods

This study was conducted at the Juárez Autonomous University of Tabasco, Academic Division of Biological Science, Laboratory of Physiology of Aquatic Resources in Villahermosa, Tabasco, Mexico. *Nauphoeta cinerea*, SBM, and FM were subjected to proximal composition analysis, amino acid and fatty acid profiles, mineral and heavy metal contents (including an HCA), and an antimicrobial assay.

2.1. Rearing and processing of Nc

Organ sampling of animals was carried out following the guidelines of NOM-062-ZOO-1999 (Mexican official regulations) to manage animals in the laboratory for experimental use. *Nauphoeta cinerea* was massively produced in Villahermosa, Tabasco, Mexico (17°59'12.8" N, 92°55'49.01" W). The husbandry conditions of Nc are described below. The diet for Nc in this study was based on dry fruits, vegetables, dry cow liver, and grains. Cockroaches (mix of sexes) were kept in plastic 100-L containers at all stages. The top of the containers was coated with Vaseline to prevent escapes. Lids of the containers had ~60-70% of their surface removed and were replaced with a 400-µm plastic mesh to allow ventilation and avoid contamination from native insects. Each container was provided with an arrangement of egg cartons vertically oriented to increase surface area and maximize production. Food and water were provided *ad libitum*. Plastic containers were maintained in a well-ventilated area. Temperature and humidity (30±2 °C and 65-75%, respectively) were measured with a digital thermometer - hygrometer (ThermPro TP50™, Shenzhen, China). The photoperiod was 12:12 (light:dark). Harvesting occurred every 45 days. Egg cartons were shaken off on strainers of different sizes to separate individuals per size. Cockroaches were harvested as close as the latest instar before adulthood to minimize chitin generation in the wings. However, older adults were euthanized as well. Frozen insects were dried for 9 h at 55 °C (San-Son convection oven) and then ground in a kitchen blender (CGOLDENWALL, China). The Nc meal was refrigerated until analysis.

2.2. Reference meals

Commercial FM and SBM were purchased from the domestic providers: Proteínas marinas y agropecuarias (PROTMAGRO) S.A. de C.V. (Guadalajara, Jalisco, México) and Agropecuaria Tabasco (Villahermosa, Tabasco, México), respectively.

2.3. Geographical location

All laboratory analyses were conducted in Villahermosa, Tabasco, Mexico (17°59'12.8" N, 92°55'49.01" W).

2.4. Meal quality analyses

Ash (RSU LABSUPPLY™, Monterrey, NL, Mexico), crude protein (Foss Analytics, Kjeltac 8400™, CDMX, México), crude lipid (Foss Analytics, ST 243™, CDMX, México), crude fiber (Fibertec 8000™, CDMX, México), and phosphorous (Hendress+Hauser, Liquiline System CA80TP™, Madrid, Spain) were determined using AOAC (1999) techniques. Nitrogen-free extract (NFE) was calculated as $NFE = 100 - (\text{ash} + \text{crude protein} + \text{crude lipid} + \text{crude fiber})$. Gross energy (GE) was calculated according to Gatlin (2010) as $GE \text{ (kcal kg}^{-1}\text{)} = [\text{crude protein} \times 5.65 + \text{lipid} \times 9.4 + NFE \times 4.15] \times 10$. The amino acid profile was assessed through hydrolysis of the sample with 6 N HCl for 2 h at 110 °C, followed by a sequence of filtration, derivatization, and separation using gas chromatography (Agilent 8890 GC System Gas Chromatograph™, Santa Clara, CA, USA) (Takeuchi, 1988). The free fatty acids (FFA) profile was determined through C6 gas chromatography under regulation NMX-F-490-1999-NORMEX (Agilent 8860 System Gas Chromatograph™ Santa Clara, CA, USA). The mineral profile and heavy

metal content were analyzed through inductively coupled plasma (ICP) – atomic emission spectrometry (ICP–AES) using an optical induction spectrophotometer (OIS) (AVIO 200 PerkinElmer, MA, USA).

2.5. Chitin content determination

Chitin was quantified in Nc meal by the method described by Lehmann and White (1975) and Bensebaa et al. (2015), with slight modifications. Briefly, triplicate samples of Nc meal in a solution of sodium dodecyl sulfate (SDS 3% wt/vol) were heated (100 °C) for 15 min; then chitin was converted into chitosan through alkaline digestion (14 M KOH) at high temperature (130 °C). Chitosan was then depolymerized with NaNO₂ (10%) and KHSO₄ (10%), a process that releases the amine residues from the glucosamine forming a soluble aldehyde in a reaction with HNO₂, followed by a reaction with 3-methyl-2-benzothiazolinone-hydrazone (MBTH) and Fe⁺³, causing the development of a blue coloration. The absorbance was measured spectrophotometrically (Model 840-208200, Genesys 10S UV-Vis, Madison, Wisconsin, USA) at 650 nm. A standard curve of glucosamine solutions of different concentrations (50–500 µg) expresses the chitin content as glucosamine equivalents: µg glucosamine per mg of Nc meal.

2.6. Hierarchical clustering analysis (HCA)

This analysis was conducted to determine similarities in the nutritional composition among the meals. For amino acid and FFA profiles, we also included the lower and higher limits of previously reported insect meals (hereafter abbreviated L–H). FM, SBM, Nc, and L–H were sorted into three dendrograms based on the proportion of AAs, the proportion of FFA, and mineral content. Degrees of association/similarity among data were identified by mean and average-linkage and Euclidian distance. Clusters were initially built with DataTab[®] and confirmed with Statistica TIBCO Data Science Work Bench (version 14.0.1.25) (Palo Alto, CA, USA).

2.7. Antimicrobial assay of Nc

The agar well and disk diffusion methods evaluated the antimicrobial activity (Balouiri et al., 2016) of Nc against the five probiotic properties of microorganisms (initially isolated from fish and chicken and laboratory-stocked). The bacterial strains *Debaryomyces hansenii*, *Bacillus subtilis*, *Lactococcus lactis*, *Lactobacillus rhamnosus*, and *Pediococcus acidilactici* were reactivated and incubated (ThermoFisher Scientific Heratherm™, 194 I 230 V, Waltham, MA, USA) at 37 °C for 24 h. The cultures were adjusted to an optical density (ThermoFisher Scientific Spectrophotometer UV-Vis Genesys™, Waltham, MA, USA) of 0.08–0.09 at a wavelength of 600 nm, which corresponds to an approximate concentration of 1×10⁸ CFU/mL in saline solution (0.85%). The bacterial suspension (3.5 mL) was taken and added to a flask with 31.5 mL Muller-Hinton agar (Difco, BD, NJ, USA) at an approximate temperature of 40–45 °C (ThermoFisher Scientific Heratherm™, 194 I 230 V, Waltham, MA, USA), suspended, gently homogenized, and poured into square plates of 12 × 12 cm, when the agar gels, 6 mm diameter perforations were made and 100 µL of Nc 1% (wt/vol) is added to each well. The plates were stored at 4 °C for 4 h to facilitate diffusion and incubated at 37 °C for 24 h (ThermoFisher Scientific Heratherm™, 194 I 230 V, Waltham, MA, USA). Chloramphenicol (30 µg/well) was used as a positive control, and sterile water was used as a negative control. The assays were performed in triplicate, and the antimicrobial activity was recorded by measuring the diameter of the zone of inhibition.

2.8. Experimental fish

Juvenile tropical gar and Nile tilapia were obtained in Villahermosa, Tabasco, Mexico. Before the feeding experiment, their health status was checked by visual external and internal observation, according to indications proposed by Johansen et al. (2006). In brief, a fish stock (tilapia and tropical gar) was kept in quarantine. Fish were usually fed and isolated in a recirculating system. Water was treated with UV light (to avoid presence of pathogens in the rearing water). Physical parameters of

water were monitored and maintained stable (day/light period, noise and vibrations, density of fish). One day before actual sampling, five fish were slaughtered to observe internal organs visually to ensure fish stocks were free of pathogens and disease.

After quarantine and fish health status check period, 20 experimental organisms were kept in 300-L tanks (Nile tilapia and tropical gar, separately) of a recirculating system at 28 °C (Boyu Precision Thermometer BT-02™, Boyu, CDMX, México), oxygen (6.82 ± 0.5 mg/L), ammonia (0.68 ± 0.5 mg/L), nitrite (0.68 ± 0.3 mg/L), pH (6.45 ± 0.1), and 12:12 light:dark photoperiod. Individuals were fed a commercial diet (Silver cup, 45% protein, and 16% lipids) three times daily for three weeks before sampling.

2.9. Multi-enzyme extracts

Five specimens (22.48 ± 5.27 and 17.40 ± 4.36 g, tropical gar and tilapia, respectively) from each species were dissected at cold temperature using an ice plate. The stomachs and intestines were isolated. Each organ was weighed with an analytical balance (CGoldenwall, digital balance 5000 g 100 V™, CDMX, México) to the closest milligram (Table 1). The stomach and intestinal tissues were homogenized separately with a homogenizer (Ultra Turrax™ Ika T18 Basic, Shirley, NY, USA) at a proportion of 1:5 (tissue:distilled water). Homogenates were centrifuged at 14,000 rpm (4 °C for 30 min) (Eppendorf 5810–R. F45–3011™ CT, USA). The supernatant was recovered and kept at –20 °C until use.

Table 1 - Whole body, stomach, intestines weights (g), and proteolytic enzyme activity (acid and alkaline proteases) of the species used (tropical gar, *Atractosteus tropicus*–carnivorous model– and tilapia, *Oreochromis niloticus*–omnivorous model–) for the *in vitro* assay (values are mean $\pm$ standard deviation; n = 3)

Item	Tropical gar (<i>Atractosteus tropicus</i>)	Tilapia (<i>Oreochromis niloticus</i>)
Weight		
Whole body	22.48 ± 5.27	17.40 ± 4.36
Stomach	0.04 ± 0.02	0.18 ± 0.05
Intestine	0.35 ± 0.09	0.92 ± 0.21
Acid proteases		
U mL ⁻¹	10.31 ± 0.73	11.50 ± 1.40
U mg protein ⁻¹	0.39 ± 0.03	2.07 ± 0.25
U mg tissue ⁻¹	54.05 ± 3.83	12.79 ± 1.56
Alkaline proteases		
U mL ⁻¹	7.95 ± 1.53	11.55 ± 1.54
U mg protein ⁻¹	2.78 ± 0.54	10.68 ± 1.43
U g tissue ⁻¹	4.55 ± 0.88	2.50 ± 0.33

2.10. Stomach and intestine protease-specific activity

A modified hemoglobin 1% protocol after Walter (1984) and Anson (1938) was employed to evaluate the quantity of units of enzymes from stomach homogenates. To assess the activity of multi-enzyme extracts from intestinal homogenates, casein Hammarsten (Sigma–Aldrich, E0789, Darmstadt, Germany) at 1% was used (Kunitz, 1947; Walter, 1984). Absorbance was measured at 280 nm in a spectrophotometer (cphnano, Jenway 6405 UV™/visible, Farum, Denmark). One centimeter of light passage was defined as one unit of activity of the catalyzed enzyme amount by releasing 1 µg of tyrosine per minute. Acid and alkaline proteolytic activities were calculated compared to a blank. The values obtained were used to calculate the volume of multi-enzyme extracts for both acid and alkaline hydrolysis of FM, SBM, and Nc.

Soluble protein was determined by Bradford (1976) method using albumin as a control. All reactions were conducted in triplicate. Acid and alkaline protease activities of tropical gar (used as a carnivorous model) and tilapia (used as an omnivorous model) were calculated using the following formulae:

$$U \text{ mL}^{-1} = [\text{ABS (280 nm)} \times \text{Final reaction (mL)}] / [\text{MEC of tyrosine} \times \text{Time (min)} \times \text{Volume (mL)}] \times \text{Dilution factor}$$

in which ABS = absorbance and MEC = molar extinction coefficient.

$$U \text{ mg protein}^{-1} = [\text{Intestine or stomach weight (mg)} \times U \text{ mL}^{-1}] / 100$$

$$U \text{ g tissue}^{-1} = [U \text{ mL}^{-1} / [1000 \times \text{Tissue weight (g)}]] / \text{Dilution factor}$$

2.11. *In vitro* assay

The degree of hydrolysis (DH) of FM, SBM, and Nc when exposed to homogenates from the stomach (acid phase) and intestine (alkaline phase) of tropical gar (Table S1) and tilapia (Table S2) were assessed in triplicate. A pH Stat (Titrino, Metrohm 718™, Herisau, Switzerland) was employed according to Dimes and Haard (1994), after Saunders et al. (1973). Assays of acid and alkaline digestions were conducted with a final volume of 5 mL of reaction mixture. Target meals were diluted in distilled water to a concentration of 8 mg of protein mL⁻¹ for acid digestion with pH = 2 adjusted with HCl 1N. Alkaline digestion was carried out at pH = 7.5, adjusted with NaOH 1N. Hemoglobin and Hammerstein casein were used as references for acid and alkaline digestions, respectively. Acid and alkaline digestions lasted 900 and 2700 s, respectively.

Degrees of hydrolysis were calculated as:

$$\text{DH (acid phase)} = [(\text{consumption (mL) of HCl} \times 0.1) \times [(\alpha\text{-NH}_2 \text{ dissociation constant})] \times [\text{protein content in reaction sample}]$$

$$\text{DH (alkaline phase)} = [(\text{consumption (mL) of NaOH} \times 0.1) \times [(\alpha\text{-COOH dissociation constant})] \times [\text{protein content in reaction sample}]$$

Relative digestibility (RD) of each ingredient during acid and alkaline stages were calculated based on hemoglobin (for acid stage) and casein (for alkaline stage) DH values. These calculations were conducted using the protein content (%) and protein weight (mg) of FM, SBM, and Nc (Table S3).

2.12. Statistical analysis

The DH and RD were tested for normality (Kolmogorov-Smirnov test) and variance homogeneity (Levene's test) before ANOVA. Duncan's multiple comparison tests were employed to assess differences among meals. Data were subjected to a sigma-restricted parameterization and effective hypothesis decomposition method to discriminate similar data. Statistical analyses were carried out in Statistica TIBCO Data Science Work Bench (version 14.0.1.25) (Palo Alto, CA, USA) considering a significance level of 0.05. This software was also used to confirm HCA analyses.

3. Results

3.1. Proximate composition

The crude protein content of Nc was similar to that of SBM and lower than FM. The crude lipid of Nc was higher than that of FM and SBM. Nitrogen-free extract was higher in SBM than in Nc and FM. In contrast, crude fiber was substantially higher in Nc than in SBM and FM. The ash content of FM was higher than that of SBM and Nc. The phosphorous content was higher in FM than in SBM and Nc. The gross energy was similar among FM, SMB, and Nc (Table 2).

Table 2 - Proximate composition of studied meals: A) traditionally used protein sources in aquaculture: fish meal (sardine) (FM) and soybean meal (SBM); and B) alternative protein meal derived from lobster cockroach *Nauphoeta cinerea* (Nc)

		Composition (g 100 g ⁻¹)							Gross energy (kJ kg ⁻¹)
		Moisture	Crude protein	Crude lipid	Ash	Crude fiber	Nitrogen free extract	Phosphorus	
A	FM	10.00	67.00	6.00	23.00	1.00	3.00	28.45	18.70
	SBM	12.64	44.71	1.76	5.85	4.34	43.34	6.43	18.70
B	Nc	8.07	46.50	14.49	5.48	15.88	17.65	5.43	19.73

3.2. Amino acid and FFA profile

Compared with FM and SBM, Nc had lower arginine, histidine, isoleucine, lysine, phenylalanine, valine, aspartic acid, glycine, proline, and serine levels. In contrast, it had higher levels of leucine, threonine, alanine, and glutamic acid. Arginine, histidine, isoleucine, lysine, valine, aspartic acid, proline, serine, and tyrosine levels of Nc were lower than L-H. Leucine, phenylalanine, threonine, alanine, glutamic acid, and glycine levels were observed within the range of L-H. The $\Sigma\text{EAA}:\Sigma\text{Non-EAA}$ ratio of Nc was within the range of L-H (Table 3).

Palmitic, stearic, oleic, and linoleic acids, as well as the total saturated fatty acids (ΣSFA), mono-unsaturated fatty acids (ΣMUFA), and poly-unsaturated fatty acids (ΣPUFA), were all higher in Nc than in FM and SBM. The gamma-linolenic acid content of Nc was within the level range in FM and SBM. Palmitic and oleic acids, ΣSFA and ΣMUFA were higher in Nc than in L-H. Meanwhile, the linoleic and gamma-linolenic acids and ΣPUFA values of Nc were within the range of L-H (Table 4).

Table 3 - Laboratory analysis of the amino acid composition (g 100 g⁻¹) of three meals: A) traditionally used protein sources in aquaculture: fish meal (sardine) (FM) and soybean meal (SBM); B) alternative protein meal derived from lobster cockroach, *Nauphoeta cinerea* (Nc); and C) previously reported ranges in different insect meals [L (lowest) – H (highest)]

Amino acid	A		B	C ¹	
	FM	SBM	Nc	L	H
Essencial amino acids (EAA)					
Arginine	2.15	1.80	1.75	1.94	6.06
Histidine	1.71	1.66	1.00	1.07	3.01
Isoleucine	5.21	4.29	1.04	1.06	3.05
Leucine	2.43	1.69	3.36	2.18	6.42
Lysine	3.91	4.64	0.56	1.68	5.00
Phenylalanine	5.23	2.14	1.70	1.32	3.82
Threonine	1.93	1.43	2.32	0.88	3.32
Valine	6.35	4.71	1.00	1.91	5.02
ΣEAA	28.91	22.37	12.74		
Non-essential amino acids (non-EAA)					
Alanine	3.53	1.37	5.60	1.51	7.14
Aspartic acid	5.80	6.37	0.02	0.64	6.47
Glutamic acid	6.52	6.61	8.37	0.00	8.44
Glycine	4.47	3.50	2.10	1.03	5.91
Proline	2.84	1.68	0.15	1.56	5.55
Serine	2.24	3.58	0.07	0.91	3.68
Tyrosine	2.64	0.82	1.11	1.27	4.44
$\Sigma\text{Non-EAA}$	28.05	23.92	17.41		
$\Sigma\text{EAA}:\Sigma\text{Non-EAA}$ ratio	1.03	0.94	0.73	0.73	1.95

ΣEAA - total essential amino acids; $\Sigma\text{non-EAA}$ - total non-essential amino acids.

¹ Nogales-Mérida et al. (2019).

Table 4 - Laboratory analysis of the free fatty acid (FFA) composition (g 100 g⁻¹ of dry matter) of three meals: A) Traditionally used protein sources in aquaculture: fish meal (sardine) [FM] and soybean meal [SBM]; B) Alternative protein meal derived from lobster cockroach, *Nauphoeta cinerea* [Nc]; and C) previously reported ranges in different insect meals [L (lowest) – H (highest)]

Free fatty acid	A		B	C ¹	
	FM	SBM	Nc	L	H
Palmitic acid	14.50	1.90	41.50	0.36	38.01
Stearic acid	4.40	0.60	9.60	1.10	8.54
Oleic acid	5.90	2.90	67.00	0.10	57.63
Linoleic acid	1.40	8.00	19.40	2.82	41.39
Gamma-linolenic acid	0.40	1.00	0.60	0.00	7.50
ΣSFA	18.90	2.60	51.20	1.46	46.55
ΣMUFA	5.90	2.90	67.00	0.10	57.63
ΣPUFA	1.90	9.10	20.00	2.82	48.89

ΣSFA - total saturated fatty acids; ΣMUFA - total monounsaturated fatty acids; ΣPUFA - total polyunsaturated fatty acids.

¹ Nogales-Mérida et al. (2019).

3.3. Mineral profile and heavy metal content

Nauphoeta cinerea showed high potassium, copper, and zinc levels compared to those of FM and SBM. In contrast, a selenium deficiency was detected in Nc. The heavy metal contents of Nc were within the permissible levels for FM and SBM according to Food and Agriculture Organization (FAO, 2003) and Codex Alimentarius (2019), respectively, except for lead, which was slightly elevated (Table 5).

Table 5 - Mineral profile (macro-minerals and trace minerals) and contents of heavy metals (non-essential for metabolic activities and exhibit toxic properties) of studied meals: traditionally used protein sources in aquaculture: fish meal (sardine) [FM] and soybean meal [SBM] and alternative protein meal derived from lobster cockroach, *Nauphoeta cinerea* [Nc] (fed fruits, vegetables, cow liver and grains)

	Previously reported		Maximum regulated permissible levels		Analyzed at laboratory		
	FM ¹	SBM ¹	FM ²	SBM ³	FM	SBM	Nc
Macrominerals (g 100 g ⁻¹)							
Calcium	3.73	0.03					0.24
Sodium	1.10	0.04					0.50
Potassium	0.90	0.31					2.02
Magnesium	0.24	0.13					0.15
Phosphorous	2.43	0.28					0.69
Sulfur	0.54	0.08					0.22
Trace minerals (mg kg ⁻¹)							
Copper	9.03	10.00					43.07
Iron	220.00	48.00					57.30
Manganese	9.50	15.80					9.64
Selenium	1.36	0.20					0.00
Zinc	103.00	17.00					210.20
Heavy metals (mg kg ⁻¹)							
Lead			0.40	0.10	1.00	0.07	0.90
Cadmium			0.50	0.10	0.25	0.08	0.20
Arsenic			4.17	0.35	1.00	0.30	0.70
Mercury			0.50	0.10	0.10	0.05	0.05

¹ NRC (2011).

² FAO (2003).

³ Codex Alimentarius (2019).

3.4. Chitin content

Nauphoeta cinerea showed a chitin content of $4.20 \pm 0.08\%$ (percentage of glucosamine) equivalent to $42.02 \pm 0.08 \mu\text{g}$ of glucosamine (chitin) per mg of insect meal.

3.5. Hierarchical clustering analysis

The HCA of the amino acid profiles (Figure 1a) generated three clusters: FM and SBM, the highest content of L-H, and Nc and the lowest content of L-H. In the HCA of FFA profiles (Figure 1b), three clusters were generated: FM, SBM and the lowest content of L-H, and Nc and the highest content of L-H. The mineral content of FM, SBM, and Nc showed two clusters with high similarities among studied meals (Figure 2).

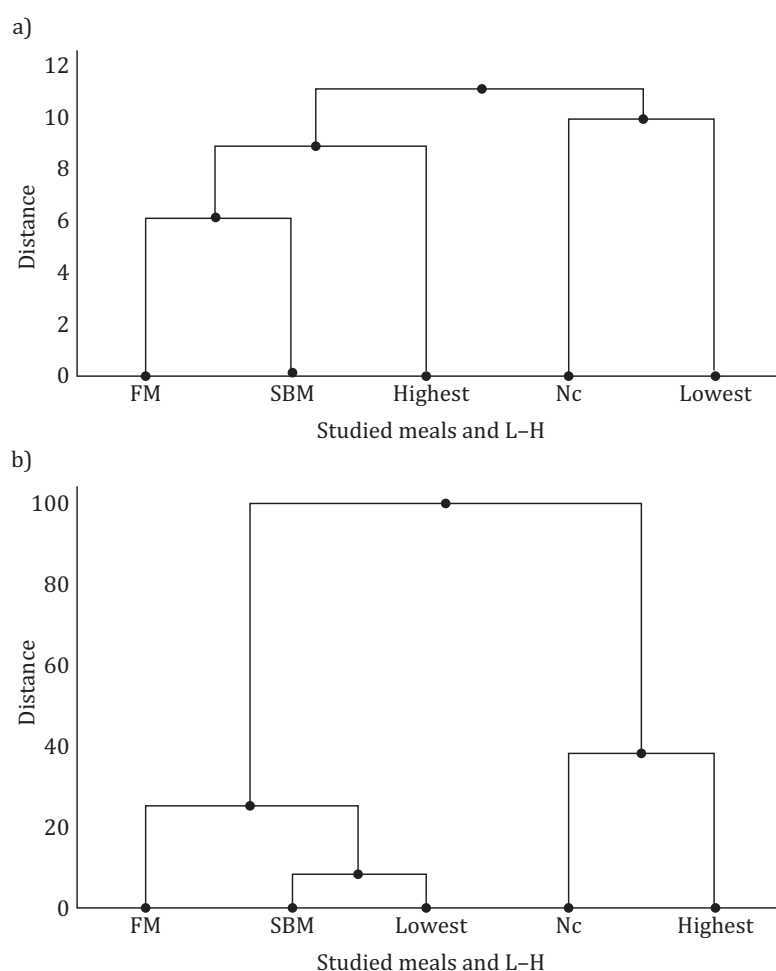


Figure 1 - Hierarchical clustering analysis of the similarity in amino acid (a) and free fatty acid (b) profiles of *Nauphoeta cinerea* (Nc), fish meal (FM), soybean meal (SBM), and L-H (lowest-highest ranges of previously reported contents in insects).

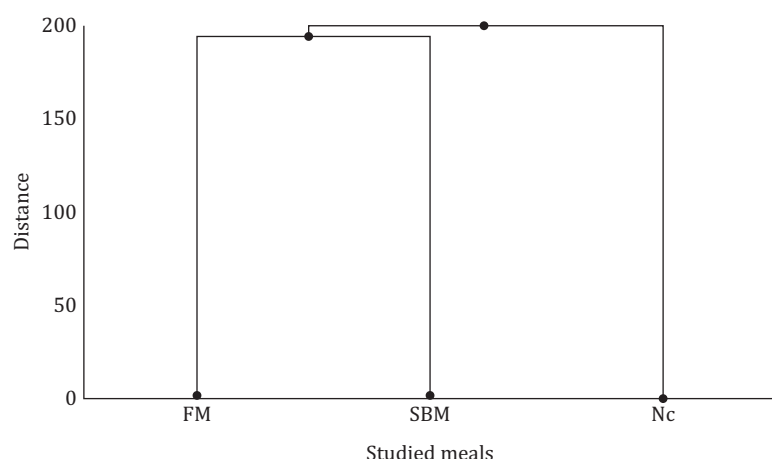


Figure 2 - Hierarchical clustering analysis of mineral content similarity among fish meal (FM), soybean meal (SBM), and *Nauphoeta cinerea* (Nc).

3.6. Antimicrobial assay

The antimicrobial activity assays showed that Nc does not inhibit the growth of probiotic bacteria or yeasts evaluated by the well diffusion method.

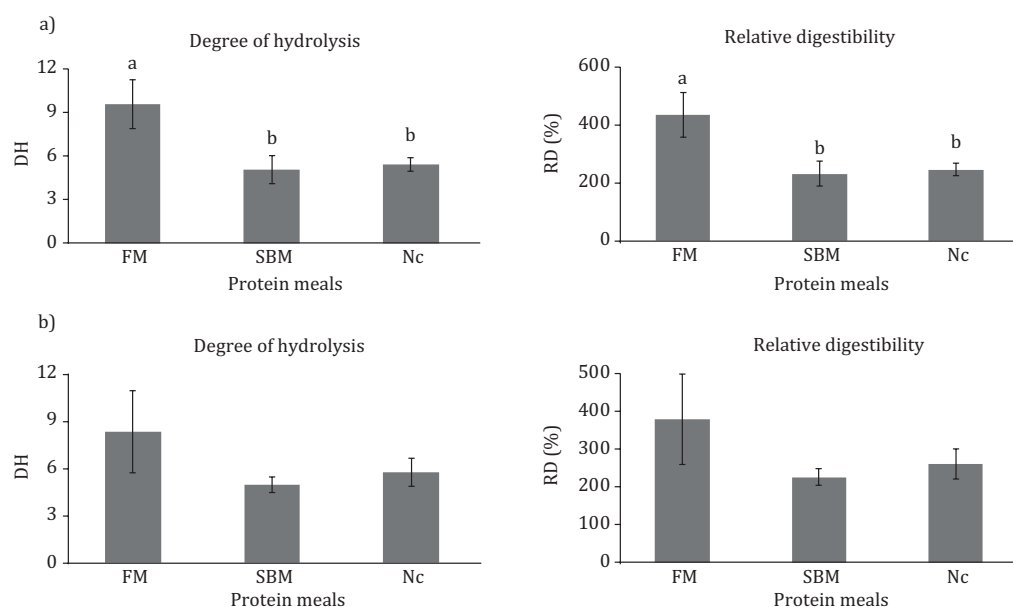
3.7. *In vitro* digestibility

The DH and RD for tropical gar were significantly ($P < 0.05$) lower in SBM and Nc than in FM, with no significant difference between SBM and Nc (Figure 3a). In the case of tilapia, there were no significant differences in DH or RD among FM, SBM, and Nc (Figure 3b). For the alkaline stage in tropical gar and tilapia, the DH and RD of Nc were significantly higher than of FM and SBM (Figures 4a and 4b) (Tables S1, S2, S4, and S5).

4. Discussion

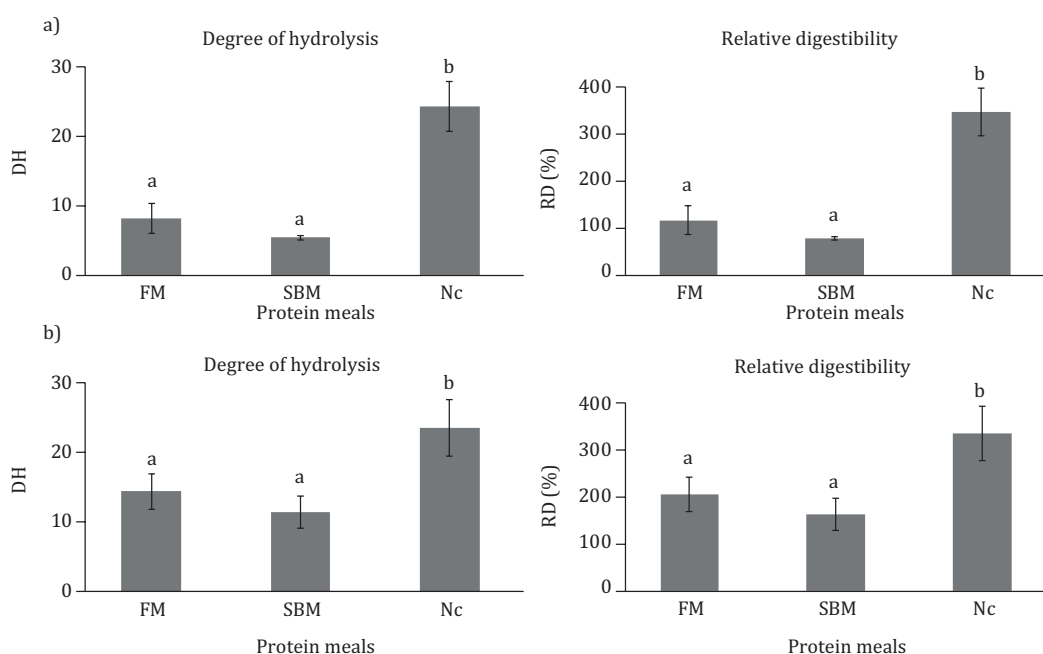
4.1. Proximal composition

The nutritional content of insects may vary depending on the feed they are fed and the developmental stage of the animal (Nogales-Mérida et al., 2019). Crude protein ranges from 40 to 63 g 100 g⁻¹ in most investigated insect species previously reported in scientific literature and produced in industry (Hua et al., 2019). In the present study, Nc fed dried fruits, vegetables, cow liver, and grains showed 46.50 g 100 g⁻¹ of crude protein. A previous study with Nc found up to 59.79 g 100 g⁻¹ of protein content (Oliveira et al., 2017). The protein content in insect meals varies according to the developmental stage of the insects. Silva et al. (2019) reported protein levels in Nc as 50 g 100 g⁻¹ in nymphaea, 62.2 g 100 g⁻¹ in nymphaea-adult, and 68.5 g 100 g⁻¹ in adults. In the present study, Nc was harvested mainly during the nymphaea stage, which may explain the lower protein level observed. Proteins in Nc, as in most insects, may keep strong linkages (in the exoskeleton or cuticle, specifically, in the wigs: cuticular proteins composed of amino acids and chitinase-like proteins), increasing insect size but not protein content (Arakane and Muthukrishnan, 2010). This may explain the lower protein levels of Nc in the present study compared with those previously reported.



Values are mean $\pm$ standard deviation (n = 3). Different letters indicate significant (P < 0.05) differences.

Figure 3 - *In vitro* digestibility (degree of hydrolysis [DH], analyzed through pH STAT, and relative digestibility [%] [RD], calculated from reference ingredient (hemoglobin) value) of fish meal (sardine) [FM], soybean meal [SBM], and lobster cockroach (*Nauphoeta cinerea* [Nc]) during the acid stage in (a) tropical gar (*Atractosteus tropicus*), used as a carnivorous model, and (b) tilapia (*Oreochromis niloticus*), used as an omnivorous model.



Values are mean $\pm$ standard deviation (n = 3). Different subscript letters indicate significant (P < 0.05) differences.

Figure 4 - *In vitro* digestibility (degree of hydrolysis [DH] and relative digestibility [%] [RD]) of fish meal (sardine) [FM], soybean meal [SBM], and lobster cockroach (*Nauphoeta cinerea* [Nc]) during the alkaline stage in (a) tropical gar (*Atractosteus tropicus*), used as a carnivorous model, and (b) tilapia (*Oreochromis niloticus*), used as an omnivorous model.

Like proteins, whole-body lipid content varies widely as a function of the developmental stage. In addition, lipid variation in insects is taxonomy-related. Alfiko et al. (2022) reported 25.70 g 100 g⁻¹ for silkworm pupae meal, 26.00 g 100 g⁻¹ for black soldier fly larvae, 18.90 g 100 g⁻¹ for housefly maggot meal, 36.1 g 100 g⁻¹ for yellow mealworm, and 8.5 g 100 g⁻¹ for lesser mealworm. The present study found a crude lipid level of 14.49 g 100 g⁻¹, higher than FM and SBM. However, it is lower than the 23.29 g 100 g⁻¹ reported previously in Nc by Oliveira et al. (2017). This variation may be attributable to the diet received during rearing. The Nc from Oliveira et al. (2017) were provided with a traditional diet mainly composed of dried fruit and vegetables enriched with oilseeds, while the diet of the Nc in the current study contained dried fruit and vegetables and was enriched with cow liver.

Ash levels in Nc in the current study are comparable with those reported by Barroso et al. (2014) in various insects. Thus, the ash content of Nc is within the ranges of other insect species used as protein ingredients for aquaculture diets. Nitrogen-free extract and energy content in insect bodies are essential parameters for assessing their potential as an ingredient of diets for aquatic organisms (Plouhinec et al., 2023). The NFE and energy contents of Nc were within the standard ranges of other insect species used as protein sources in fish diets in previous studies (Barroso et al., 2014; Basto et al., 2020; Tubin et al., 2020).

Fiber is the indigestible carbohydrate fraction of an insect body (Liu et al., 2017). In fish, dietary fiber (at certain levels) plays a vital role in digestible processes, gut health, and overall nutritional balance. Understanding the fiber content in insect meals is fundamental to assessing the suitability of insects as protein sources in aquaculture diets (Makkar et al., 2014). Although fiber content is a limiting nutrient in raw materials for fish diets, fiber levels of Nc are similar to those of plant ingredients typically used in aquaculture (NRC, 2011). The phosphorous content of insect meals varies according to several factors, including the insect species, diet consumed, and rearing conditions. Although this nutrient is essential to formulate aquaculture diets to support optimal growth and health of culture species, phosphorous also has ecological implications, since releasing high levels of it into the area surrounding the rearing environment causes water pollution (Petkova, 2019). In the present study, phosphorous accounted for 5.43 g 100 g⁻¹ of the composition of Nc, which is similar to the values found in environmentally friendly meals (of plant origin) and lower than in FM (Makkar et al., 2014).

4.2. Amino acid and FFA profiles

Because protein is the most expensive component in fish feed, it is important to accurately assess its AA composition (Conceição et al., 2003). Although the AA profile is already known for various insects commonly investigated and utilized in the aquaculture industry (Barroso et al., 2014), the AA profile of Nc is not entirely understood. Although some AA of Nc are present in low levels (compared with those of FM and SBM), most have levels located within ranges of L–H. In general, the AA content of Nc is similar to other insect species previously published by Finke (2002) and Finke (2007), who analyzed the AA content of *Acheta domesticus* harvested as a nymph (the same life stage at which Nc mainly was used to produce our experimental insect meal). The $\Sigma\text{EAA}:\Sigma\text{Non-EAA}$ ratio evaluates whether the AA profile of any raw material provides an adequately balanced AA profile. Where the closest $\Sigma\text{EAA}:\Sigma\text{Non-EAA}$ ratio is 1.00, the most well-balanced is the meal (Hua et al., 2019). In the present study, these ratios were 1.03, 0.94, 0.73, and 0.73–1.95 for FM, SBM, Nc, and L–H, respectively. This indicates that although FM and SBM present the optimal AA balance, Nc has an acceptable ratio within those of L–H. According to HCA, Nc showed a higher AA similarity with the lowest L–H value. Despite this feature, it is possible to formulate aquaculture diets with Nc with the proper use of different meals to compensate for the low AA levels (Alfiko et al., 2022).

Free fatty acids are a crucial component of insect meals and play an important role in their nutritional composition. It is well known that insects are a rich source of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and of a lesser degree, poly-unsaturated fatty acids (PUFA) (Nogales-Mérida et al., 2019). *Nauphoeta cinerea* showed high levels of SFA similar to those previously reported in different species in Nogales-Mérida et al. (2019). In the present study, the MUFA measured

was oleic acid, with similar levels to *Tenebrio molitor* (Nogales-Mérida et al., 2019). Linoleic and gamma-linolenic acids (PUFA) levels of Nc are comparable to levels reported in different insect species by Hossain et al. (2023). Regarding similarity, the FFA profile of Nc more closely resembles the highest levels of L-H than FM, SMB, or the lowest levels of L-H. Fatty acids levels of Nc showed similar contents to other insect species that contain high levels of fats, like *Musca domestica* (larvae), *A. domesticus* (adult), and *T. molitor* (Hossain et al., 2023).

4.3. Mineral profile and heavy metal contents

The mineral content of Nc is not well known. Although it showed similarities to those of FM and SBM, it has higher contents of some minerals than those contained in mentioned meals. Assessing the minerals contained in insect meals is fundamental because they may affect the bioaccessibility rate of the meal itself and the bioavailability of other nutrients, like vitamins, which affect the growth and health of fish (Kosečková et al., 2022). Heavy metals are minerals not essential for metabolic activities and exhibit toxic properties. Insects can accumulate heavy metals from their surroundings, including the feed they consume and the environment they inhabit (Fernández-Cassi et al., 2018). In the present study, we measured the contents of lead, cadmium, arsenic, and mercury in Nc, all of which had values below the maximum regulated permissible levels in FM (FAO, 2003) and SBM (Codex Alimentarius, 2019). This indicates that Nc is a safe protein source regarding heavy metal content. In our study, Nc had a lead level of 0.90 mg kg^{-1} , consistent with reports by Diener et al. (2015), who found $0.6\text{--}0.9 \text{ mg kg}^{-1}$ in black soldier fly prepupae.

In contrast, other studies have reported higher levels ($0.55\text{--}2.68 \text{ mg kg}^{-1}$) in black soldier fly larvae (Tschirner and Simon, 2015). Cadmium has been reported in yellow mealworms, superworms, grasshoppers, locusts, termites, and black soldier fly larvae (Mlček et al., 2017; Lange and Nakamura, 2021). In the present research, cadmium showed a content of 0.20 mg kg^{-1} in Nc. Previous reports have observed 1.68 , 0.94 , and 0.50 mg kg^{-1} in larvae, pupae, and adults of the black soldier fly, respectively (Gao et al., 2019).

In insects, arsenic has been observed in several concentrations: 0.19 mg kg^{-1} in house flies, 0.009 mg kg^{-1} in blue bottle flies, and 0.73 mg kg^{-1} in blow flies (Truzzi et al., 2019). In the present research, Nc showed a concentration of 0.70 mg kg^{-1} of arsenic. According to European Union and Chinese limits on toxic elements in feeds, the recommended level of arsenic in feedstuff of animal origin is no more than 2 mg kg^{-1} , suggesting that the arsenic content in Nc is safe (Malematja et al., 2023).

Depending on its concentration, mercury is considered a highly toxic metal (Rehman et al., 2021). The origin of mercury accumulation in insects may be natural or come from agricultural products used as substrates or feedstuffs (Adamse et al., 2017). Mercury content in insects has been previously reported by Truzzi et al. (2019), who observed mercury concentrations of $0.010\text{--}0.032 \text{ mg kg}^{-1}$ in black soldier fly pre-pupae. Other authors have reported higher levels of this heavy metal in Chinese mealworms (0.03 and 0.08 mg kg^{-1}) (Yang et al., 2022). The present study observed a 0.05 mg kg^{-1} concentration of mercury in Nc, indicating that this insect meal is suitable for feedstuff in aquaculture diets.

4.4. Chitin content

Emerging insect meals are valuable feedstuffs for aquaculture because of their nutritional profiles. However, different chitin content levels in this kind of meals represent a limiting factor for their inclusion in aquaculture diets (Kvasnička et al., 2023), due to its potential negative effects in the digestive physiology of fish (Abro et al., 2014). However, tolerance to dietary chitin in insect meals varies among fish species. Furthermore, chitin content will also vary according to the insect source (Nogales-Mérida et al., 2019). In the present study, the chitin content of Nc (4.2%) is lower than that of previously reported (8.7%) (Reilly et al., 2022) for this insect species. Variations of chitin content is attained to different factors: insect species, life stage, and molt cycle (Soetemans et al., 2020), among

the most representatives. Variations of chitin contents in the same insect species have been previously reported. Black soldier fly larvae meal, recorded a variation of chitin content from 2.86 to 7.0% of the dry weight (Marono et al., 2015; Renna et al., 2017; Sprangers et al., 2017; Meneguz et al., 2018; Perez-Velazquez et al., 2024).

4.5. Antimicrobial activity

The ingredients used in aquaculture diets can significantly impact the composition and activity of the microbiota of fish intestines. It has been widely demonstrated that insect meals promote or inhibit the growth or maintenance of bacteria in fish gut by interfering with microbial functions (Gaudioso et al., 2021; Li et al., 2022). In the present study, however, we found that Nc possesses no sign of antimicrobial activity against five species of bacteria that naturally occur in the fish gut (*Debaryomyces*, *Bacillus*, *Lactococcus*, *Lactobacillus*, and *Pediococcus*). Based on this assay, Nc is a safe protein meal for aquaculture diets in terms of antimicrobial activity.

4.6. *In vitro* digestibility

The *in vitro* digestion assay is a valuable tool for determining the nutritional quality and performance of feed ingredients, especially those derived from insects. This method is less time-consuming and cheaper than the ADC method (Silva et al., 2022). The *in vitro* approach reproduces the physiological conditions of the digestive tract of the target species to provide insight into how feeds or food are processed (Moyano et al., 2015). In insects, *in vitro* assessments have been performed mainly for *Hermetia illucens* and *Tenebrio molitor* (Irungu et al., 2018; Kovitvadhi et al., 2019; Kiiru et al., 2020; Silva et al., 2022). Most insect species used as protein sources for aquaculture diets lack these studies. In the present study, we evaluated the DH and RD of Nc compared to those of FM and SBM during the acid and alkaline stages of digestion for tropical gar and tilapia using an *in vitro* assay. During the acid stage, for the tropical gar, FM had significantly higher DH and RD than Nc or SBM, while for tilapia, the DH and RD were similar among all the meals tested. Omnivorous species like tilapia have a high capacity to hydrolyze a wide range of proteins during acid conditions (Santos et al., 2016). For example, Cuenca-Soria et al. (2013) reported that the omnivorous species *Mayaherus urophthalmus* possesses a high capacity to hydrolyze a wide range of ingredients in the stomach, while in tropical gar (a carnivorous fish), acid proteases are more adapted to hydrolyze specific types of proteins such as FM (Frías-Quintana et al., 2010). During the alkaline stage in both fish species, Nc had higher DH and RD than FM and SBM. This may be explained by the fact that most of the digestion of protein ingredients in carnivorous and omnivorous fish species occurs in the stomach (Jonás et al., 1983), where most of the antinutrients are broken down and digested. Hence, by the time they reach the alkaline conditions of the intestine, they efficiently finish the digestion process, resulting in higher DH and DG (Cuenca-Soria et al., 2013) of Nc in the present study. It is well known that chitin is one of the main antinutrients in insect-derived meals (Kvasnička et al., 2023). In general, insect chitin is primarily considered fiber because its structure is similar to cellulose. Chitin is a polysaccharide of glucosamine and N-acetylglucosamine, which both contain nitrogen atoms (Nogales-Mérida et al., 2019).

Most fish species' natural diets regularly include insects. Evolutionarily, fish have developed a chitinolytic activity provided by chitinase during acid digestion (stomach) and chitobiase during alkaline digestion (intestine) (Abro et al., 2014). The presence of these two enzymes and the low level of chitin found in Nc meal may explain why the digestion (DH and RD) of Nc was comparable or better (during acid and alkaline stages, respectively) than the other meals tested in a carnivorous (tropical gar) and an omnivorous (tilapia) model fish species.

5. Conclusions

When formulating diets, the AA deficiencies of Nc can be compensated by supplementing with individual complementary additives. The mineral profiles, heavy metal contents, and antimicrobial

activities of Nc indicate that they are not an issue for replacing FM and SBM. Low chitin contents found in Nc demonstrated that this may not be a limiting factor for Nc meal supplementation in aquaculture diets. Furthermore, Nc showed acceptable DH and RD in tropical gar and tilapia during *in vitro* digestibility assay. Based on the findings of the present study, Nc is a suitable candidate for replacing FM and SBM in aquaculture diets.

Supplementary material

The supplementary material of this article can be found online at: https://www.rbz.org.br/wp-content/uploads/articles_xml/1806-9290-rbz-55-e20240032/1806-9290-rbz-55-e20240032-suppl01.pdf

Data availability

All information of present study is available in this document. Additional data is available upon request to the corresponding author.

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Conflict of interest

The authors declare no conflict of interest.

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