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Original Article

Bacteria and fungi analyses of fish diets with grasshopper and cockroach meals: the potential replacement of fishmeal in fish diets

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Abstract
The need to further ascertain the quality of fishmeal with biological indicators has poised this study to determine the biological characterisation of formulated diets with supplemented insect protein (variegated grasshopper and American cockroach). The diets (A–F) were prepared with local ingredients (A, 100% grasshopper meal; B, 100% fishmeal; C, 1:1 grasshopper:fishmeal; D, 1:1 cockroach:fishmeal; E, 100% cockroach meal; F, commercial diet). Samples of prepared diets ready to be stored for use were subjected to bacteria and fungi test. Diet F recorded the highest ($4.60\pm1.10\times10^2$ cfu g^{-1}) total viable bacteria count. The highest ($3.00\pm0.05\times10^2$ cfu g^{-1}) fungi count was recorded in diet A. Six probiotics bacteria were isolated from the diets. *Lactobacillus delbrueckii* subsp. *bulgaricus* was only isolated in grasshopper containing diets; as *Pediococcus pentosaceus*, *Bifidobacterium longum*, *Mycobacterium marinum*, *Bacillus subtilisand*, *Lactococcocus lactis* were only isolated in diets F, E, D, A and B respectively. Two pathogenic bacteria isolated were *Streptocci pyogenes* and *Staphylococcus aureus*. *Aspergillus flavus* and *Penicillum* sp. are the two species of fungi isolated from diet A, and diets C and E respectively. The diets with insect proteins were rich in probiotic bacteria than other diets may be considered to replace fishmeal in fish diet.

Keywords: fish nutrition; insect protein; microbial analyses; probiotic bacteria; pathogenic organisms

1 | INTRODUCTION

The increasing use of locally produced fish feeds in developing countries in order to sustain and maintain aquaculture practice as a result of the high cost of foreign feeds is on the increase (Charo-Karisa and Gichuri 2010). The rationale behind the high cost of foreign feed is the use of fish and its oil as the protein source thereby putting more pressure on fish availability for other animals and human. Fish farming has grown by more than 200% in recent decades as a result of consumer demand which has create a great challenge in the fishery industry (FAO 2017). In order to mitigate further depletion of fish and reduce its competitiveness with other animals, studies are underway to replace fishmeal in fish diet with insect protein as insect proteins were reported to have required amino acid profiles to suitably replace fishmeal.

Insects are naturally part of fish diets in their natural environments and are one of the promising and used alternate proteins to substitute fishmeal in fish diet (Henry *et al.* 2015; Nogales-Merida *et al.* 2019). The choice of acclaimed insect pests (grasshopper and cockroach) in this study may be of help to convert its 'nuisance' status to wealth. The variegated grasshopper (*Zonocerus variegatus*) are one of the edible grasshoppers and are among the major pests of crops worldwide (Henry *et al.* 2015). Studies have shown their nutritional composition and their suitability as meal (Ssepuuya *et al.* 2017; Babayi *et al.* 2018; Ibarra-Herrera *et al.* 2020). Similarly studies have been reported on their bacteria and fungi associates (Ng'ang'a *et al.* 2018; Muratore *et al.* 2020). Cockroaches, on the other hand are primitive and highly successful winged insects (Jeffery *et al.* 2012). They are resilient insects and most species can endure prolonged starvation and dehydration (Guzman and Vilcinskas 2020). The American cockroach (*Periplaneta americana*) is one of the most dominant species in households in Nigeria. Their nutritional profile has been reported earlier (Boate and Suotonye 2020). In addition, their bacteria and fungi associates were also reported (Guzman and Vilcinskas 2020; Khodabandeh *et al.* 2020).

Insect proteins are not the only important ingredients in fish diets, presences of carbohydrate, minerals, and vitamins are also important. Most of the aforementioned are mostly of plant origin and thus, they can also be sources of microbial infestation in fish diets. The presences of microbial organisms in fish feeds may be dependent on the environment or the feed ingredients (Zmyslowska and Lewandowska 1999). Thus, formulated fish feeds are expected to be free from pathogenic and opportunistic pathogens, however, its richness in appropriate probiotics will contribute positive to the fish yield and its health as well as the consumers. Researchers have reported bacteria and fungi in commercial and locally prepared fish feeds (Olayiwola and Adedokun 2015; Marijani *et al.* 2019; Namulawa *et al.* 2020).

Therefore, the microbial quality of fish feeds is of considerably importance as it may reveal the presences of either probiotics, pathogenic or both organisms that may have either direct or indirect effects on the fish health or the consumers. Thereof, this study was conducted to determine the bacteria and fungi diversity in fish feeds prepared with supplemented insect proteins to test the variability and the potential use of the diets in fish farming.

2 | METHODOLOGY

2.1 Sample collection and preparation

In August 2017, the feed ingredients (maize flour, cassava flour, grasshopper; harvested from natural environment) and vitalytes were obtained from local markets whereas brown seaweed and cockroaches were sourced between latitude 6°25′10.47″N and 5°52′9.95″N and longitude 3°25′9.94″E and 4°52′9.95″E located at Ajegule-Enu-Ama Beach, Ilaje Local Government Area of Ondo State, Nigeria.

Cockroaches were caught manually by hand using sterile entomological forceps mostly at nights. They were frozen at 0°C for 30 minutes, air-dried in sterilized section in the laboratory for 15 days. The dried cockroaches were ground into powder and used for the feed formulation at appropriate percentage. The research study was conducted in the Department of Biological Sciences, Ibrahim Badamasi Babangida University, Lapai, Niger State, Nigeria. The prepared feed were labelled A to F (A, 100% grasshopper meal; B, 100% fishmeal; C, 1:1 grasshopper: Fishmeal; D, 1:1 cockroach: fishmeal; E, 100% cockroach meal; F, commercial diet [Blue Crown feed, Crown Flour Mill Ltd]).

From the experimental fish feeds, 300 to 500 g of each prepared feed were subsampled consisting of one-third of each sample and grinded for the analyses. From the grinded samples, 5 g was measured and the samples were diluted 10^{-1} and 10^{-2} in 50 ml of sterile distilled water. The slurry was centrifuged at 1500 rpm for 15 minutes. Serial dilution of the sample was dissolved, 1 g in 9 ml of distilled water up to 10^{-5} dilution factor. The incubation of the sample was done using two media (nutrient agar (NA) – L:S-Biotech and DeMan Rogosa and sharpe agar (MRS – Lactobacillus MRSAgar) – TM Media –Titan Biotech Ltd). The bacteria isolates colonies were identified by standard bacteriological procedures. Each representative colony was characterised by its macroscopic morphology; gram stain and other biochemical tests (Cheesbrough 2002).

For fungi isolation, it was plated on sterile potato dextrose agar (PDA) (Accumix(R)-Tulip Diagnostics (P) Ltd). Amended with tetracycline (100 mg kg^{-1}) to prevent incubation of bacteria and inoculated at 28 ± 2°C for 5 days before sub-culturing and fungi isolates identified (Adamu *et al.* 2018). Pure culture of the different fungal species were isolated and morphologically characterised following standard procedures (Willoughby 1994). The isolates were macroscopically studied when each colony was stained with 0.05% trypan blue in Lacto-phenol.

2.2 Data analysis

Data were analysed in Microsoft Excel 2013.

3 | RESULTS

The total viable counts for bacteria and fungi are presented in Table 1. The fungi was only recorded in diets A, C and E with the highest value recorded in diet A. Whereas, total viable bacteria recorded in all diets with the highest value ($4.60 \pm 1.10\times10^{2}$ cfu g^{-1}) in Diet F and the lowest value in diet D ($2.05 \pm 0.45\times10^{2}$ cfu g^{-1}).

TABLE 1 Total viable bacteria and fungi counts from formulated fish diets with supplemented insect protein (mean ± standard deviation).

Diets	Total viable counts (×10^2 cfu g^{-1})	
	Bacteria	Fungi
A	3.65 ± 0.15	3.00 ± 0.05
B	2.35 ± 0.35	-
C	3.25 ± 0.75	1.70 ± 0.05
D	2.05 ± 0.45	-
E	3.05 ± 1.45	2.25 ± 0.25
F	4.60 ± 1.10	-

A, 100% grasshopper meal; B, 100% fishmeal; C, 1:1 (grasshopper: fishmeal); D, 1:1 (cockroach: fishmeal); E, 100% cockroach meal; F, commercial diet. -, absent

Six probiotics bacteria were isolated from the diets. *Lactobacillus delbrueckii* subsp. *bulgaricus* was only isolated in grasshopper containing diets; as *Pediococcus pentosaceus*, *Bifidobacterium longum*, *Mycobacterium marinum*, *Bacillus subtilis* and *Lactococcus lactis* were only isolated in diets F, E, D, A and B respectively. The two pathogenic/opportunist bacteria isolated were *Streptoccoci pyogenes* and *Staphylococcus aureus* as presented in Table 2. Two species of fungi were isolated (*Aspergillus flavus* and *Penicillum* sp). *Aspergillus flavus* was isolated from diet A; whilst *Penicillum* sp. was isolated from diet C and E as presented in Table 3.

TABLE 2 Characterisation of bacteria isolates identified in formulated fish diets with supplemented insect protein.

Diet	Media	Appearance	Gram reaction	Shape	Catalase	Coagulase	Urease	Citrate	Oxidase	Indole	Glucose	Lactose	Sucrose	Probable organism
A	NA	Colourless colonies	+	Rod	+	-	-	+	+	-	A/G	A	A	*Bacillus subtilis*
	MRS	Creamy colonies	+	Rod	-	-	-	-	-	-	A	A	-	*Lactobacillus delbrueckii* subsp. *bulgaricus*
B	NA	Colourless colonies	-	Cocci	-	+	-	+	-	-	A	A	A	*Streptococcipyogenes*
	MRS	Brownish colony	+	Cocci	-	-	-	-	-	-	A	A	A	*Lactococcus lactis*
C	NA	Yellow to orange colonies	+	Cocci	+	+	-	+	-	-	A/G	A	A	*Staphylococcus aureus*
	MRS	Colourless colonies	+	Rod	-	-	-	-	-	-	A	A	-	*Lactobacillus delbrueckii* subsp. *bulgaricus*
D	NA	Yellow to orange colonies	+	Cocci	+	+	-	+	-	-	A/G	A	A	*Staphylococcus aureus*
	MRS	Creamy and dried colonies	-	Appendages	+	-	+	+	-	+	A/G	A/G	A/G	*Mycobacterium marinum*
E	NA	Yellow to orange colonies	+	Cocci	+	+	-	+	-	-	A/G	A	A	*Staphylococcus aureus*
	MRS	White slimy colonies	+	Rod	-	-	+	+	-	-	A/G	A/G	A	*Bifidobacterium longum*
F	NA	Yellow to orange colonies	+	Cocci	+	+	-	+	-	-	A/G	A	A	*Staphylococcus aureus*
	NA	Colourless colonies	-	Cocci	-	+	-	+	-	-	A	A	A	*Streptococcipyogenes*
	MRS	Grey white colony	+	cocci	-	-	+	+	-	-	A	A	A/G	*Pediococcus pentosaceus*

A, 100% grasshopper meal; B, 100% fishmeal; C, 1:1 grasshopper : fishmeal; D, 1:1 cockroach : fishmeal; E, 100% cockroach meal; F, commercial diet; -, negative; +, positive; NA, nutrient agar; MRS, lactobacillus MRS agar

4 | DISCUSSION

Feed ingredients health are very important in enhancing the safety and quality of fish feed (Xiaoying *et al.* 2014), thus this study ensures that the feed ingredients used are in good and safety condition. Microbial screening was not conducted on individual feed ingredients in this study to ascertain the position of Marijani *et al.* (2019) that plant ingredients in diets present the best natural substrate for fungi growth. Olayiwola and Adedokun (2015) reported the isolations of *Bacillus* spp., *Staphyloccocus* spp. and *Streptoccocus* spp. from their works on commercial feeds. However, in this study *S. aureus* and *S. pyogenes* were also reported. The probiotic bacteria isolated in this studied commercial diet was *Pediococcus pentosaceus*. *Staphylococcus aureus* is an opportunist commensal bacterium that asymptomatically colonise the oral mucosa of about 30% of the human population which can gain virulence and cause dangerous infection (Gorwitzet *et al.* 2008). This bacterium was reported to be present in different fish species sampled in local markets (Bujjamma and Padmavathi 2015), as affirmed by the presences of the bacterium in diet D4 containing fish meal; thus its present in the commercial diet may be attributed to the diets protein source. Similarly, *S. pyogenes* is an animal related bacterium thus may be sourced from the animal protein used for the diet. *Pediococcus pentosaceusis* the only probiotic bacteria isolated in the commercial diets. This bacterium exhibit excellent antibacterial activity against several important fish pathogens (Gong *et al.* 2019); if fed to the fish, it may improve the fish immunity against *S. aureus* and *S. pyogenes*.

TABLE 3 Characterisation of fungi isolates identified in formulated fish diets with supplemented insect protein.

Diet	Macroscopic Appearance	Microscopic Appearance	Probable fungi
A	Light yellow greenish colony. Ovoid in shape colonies	Conidial head are radicate. Condiosphores was thick walled, hyaline and slightly roughened, erect, long aseptate with a vesicle at the top with phialides and short conidial chains.	*Aspergillus flavus*
B	No growth	No growth	-
C	Greenish to black, white mycelia at the margin, white droplet, yellow golden in the media.	It has subglobose conidia shape that is smooth finely roughed, septate hyhae	*Penicillium* sp.
D	No growth	No growth	-
E	Greenish to black, white mycelia at the margin, white droplet, yellow golden in the media.	It has subglobose conidia shape that is smooth finely roughed, septate hyhae	*Penicillium* sp.
F	No growth	No growth	-

A, 100% grasshopper meal; B, 100% fishmeal; C, 1:1 (grasshopper: fishmeal); D, 1:1 (cockroach: fishmeal); E, 100% cockroach meal; F, commercial diet. -, absent

The isolation of two probiotic bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *B. subtilis*) in Diet A containing 100% grasshopper is an indication that the diet is healthy with the bacteria isolates. *Bacillus subtilis* is a probiotic bacteria that has excellent fermentation properties with high product yield. This bacterium has the highest survival rate amongst other bacteria in fish diet (Zmyslowska and Lewandowska 1999). The bacterium is reported to have potential biotechnology application (Sharma *et al.* 2019). *Lactobacillus delbrueckii* subsp. *bulgaricus*, a thermophilic lactic acid bacteria, has been reported to be present in grasshopper (Ng'ang'a *et al.* 2018; Muratore *et al.* 2020;). However, the presence of *S. pyogenes* revealed that grasshopper also harbour pathogenic bacteria (Muratore *et al.* 2020) Thus, grasshopper are good sources of probiotic bacteria.

Diet B recorded the isolation of three bacteria. The presences of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* may be attributed to the presences of grasshopper in the meal as Muratore *et al.* (2020) reported the isolation of *Lactococcus* sp. in grasshopper. Whilst the presences of *S. aureus* may be attributed to the fishmeal in the diet as earlier postulated. *Staphylococcus aureus* isolated in diet with cockroach meal may be attributed to presence of cockroach in the diet (Guzman and Vilcinskas (2020). Six species of cockroaches were isolated the bacterium by Guzman and Vilcinskas (2020). Similarly other researchers have isolated the bacterium from cockroaches (Cotton *et al.* 2000; Menasria *et al.* 2014; Guzman and Vilcinskas 2020). *Bifidobacterium* spp. are now prevalent in cockroach, as they are important in biotechnology as they produce ranges of specialised metabolites (Lampert *et al.* 2019).

The two isolated fungi in this study were one of the most important genera of toxigenic fungi in the tropics, which are always related to the use of plant ingredients in feed formulation (Bankole *et al.* 2006). *Aspergillus flavus* is a toxigenic fungus. It is the most detected fungus in fish and fish feeds (Hassan *et al.* 2011; Fallah *et al.* 2014; Njagi 2016; Marijani *et al.* 2017; Namulawa *et al.* 2020) where they are mostly attributed to the plant ingredient sources; may have be contained in the plant sources used in diet preparation. Another fungus associated with fish and fish diets is *Penicillium* spp. (Hassan *et al.* 2011; Fallah *et al.* 2014; Greco *et al.* 2015; Njagi 2016; Namulawa *et al.* 2020).

The presence of these fungi may be attributed to some physical properties of certain feed types that could favour moisture retention, which favours the colonization of the substrate by the fungi (Namulawa *et al.* 2020). However, the low colonisation of the diets may be attributed to the production process (Namulawa *et al.* 2020).

5| CONCLUSIONS

This study, thereof, revealed that the diets with insect proteins were rich in probiotic bacteria than the fishmeal and commercial diets; an indication that grasshopper and cockroach meal are potentially good candidates to replace fishmeal in fish diet.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

This work was carried out in collaboration among all authors. KMA and MA designed and supervised the study; YMM, TTA, HM and KII wrote the protocols and collected sample from field and performed laboratory analysis; KMA, YMM and MH managed the data analyses and literature search of the study. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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