



Prophylactic Use of a Plant Extract and Probiotics against *Pseudomonas* Infection in African Catfish

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Abstract

Resistance of pathogens to antibiotics is not new in aquaculture industry, solution to this menace is discovering alternatives in managing disease outbreaks by pathogens. The objective of this work is to investigate the potential of fresh aqueous neem extract and probiotics in controlling *Pseudomonas aeruginosa* infection in *Clarias gariepinus*. A total of 120 post-juvenile *C. gariepinus* were divided into 6 groups of 20 fish each. The first 3 groups were uninfected (Control), group 1 was treated with normal saline, group 2 was treated with Neem leaf extract (3.75 g.L⁻¹) while group 3 was *B. subtilis* (1 g.L⁻¹) by immersion, in 15 litres of water, respectively. Groups 4 to 6 were infected orally with 1.6 x 10¹¹ CFU/100µl of *Pseudomonas aeruginosa* C₂BVB. Group 4 was untreated, while groups five and six were administered with Neem leaf extract and *B. subtilis* respectively, by immersion for fourteen days. Weight change, clinical signs and mortality were the parameters observed. Weight increase was observed in the control and uninfected bacillus treated groups, while weight decrease was observed in the uninfected neem treated and infected neem treated groups. No weight change was observed in infected and infected bacillus treated groups. Lesions like skin ulceration, skin haemorrhage were more in *P. aeruginosa* infected group but minimal to absent in infected neem treated and infected bacillus treated groups. Percentage mortality (PM) of *P. aeruginosa* infected group was 30%, while infected neem treated and infected bacillus treated groups were 10 and 5% respectively. Both treatments prevented *P. aeruginosa* infection in *C. gariepinus* based on the clinical signs observed, but PM was higher in infected neem treated group than in infected bacillus treated group. This study showed that *Bacillus subtilis* had better prophylactic effect on *P. aeruginosa* infected *C. gariepinus* than the leave extract of *Azadirachta indica*.

Keywords: Lesion, Mortality, Probiotics, Plant extract

Introduction

Gram-negative bacteria are the major cause of most diseases in tropical fish (Efuntoye *et al.*, 2012). In aquaculture, a wide range of antimicrobials are being used to control these diseases, (Rodgers and Furones, 2009). Unfortunately, the use of antibiotics for the control of bacterial diseases in aquaculture has proven unsustainable and ineffective due to development of multidrug antibiotic resistance in pathogens and retention of high levels of residues in animals (Cabello, 2006; Winkaler *et al.*, 2007). In recent studies, some of the bacteria flora in fish intestine have been isolated and cultured and used as probionts (Verschuere *et al.*, 2000; Vines *et al.*, 2006; Marzouk *et al.*, 2008). This innovative method has substituted for the use of chemotherapy in many fish diseases. However, another most promising natural compound is

azadirachtin (AZA), which is an active compound in neem tree (*Azadirachta indica*). Its antiviral, antibacterial and antifungal properties has been known for more than 2000 years (Isman *et al.*, 1990; Harikrishnan *et al.*, 2003). This study is aimed at investigating the use of plant extract of fresh aqueous neem leaves extract (*Azadirachta indica*) and probiotics (*Bacillus subtilis*) in preventing bacterial infection of *Pseudomonas aeruginosa* in *Clarias gariepinus*.

Materials and Methodology

Grouping of experimental fish and treatment administration

A total of 120 post-juvenile *Clarias gariepinus* procured from a local hatchery were used for this study and were allotted into six groups (groups 1 – 6) of 20 fish of two replicates each. First three groups (groups 1 – 3) were the uninfected



groups, and were treated with normal saline, fresh aqueous Neem leaf extract (3.75 g.L^{-1}) and *Bacillus subtilis* (1 g.L^{-1}), respectively by immersion for fourteen days. Group four to six were infected with $1.6 \times 10^{11} \text{ CFU.100}\mu\text{l}^{-1}$ of *P. aeruginosa* C₂BVB orally, however group five and six were administered fresh aqueous neem leaf extract and *B. subtilis* respectively for fourteen days in water medium.

Sources and extraction procedures

3.75 g.L^{-1} concentration of aqueous neem leaves extract were prepared by harvesting 60g of fresh neem leaves from Oyo State College of Agriculture and Technology, Igboora, Oyo State, Nigeria institutional environment. These were rinsed, blended in 1 litre of water, sieved and mixed with 15 litres of water before introducing the fish for 15 minutes, while the commercial probiotics; MaxCare® (Animal Care Consult, Nigeria) containing *Bacillus subtilis* was procured from a local Veterinary Pharmacy. $1.6 \times 10^{11} \text{ CFU.100}\mu\text{l}^{-1}$ of *P. aeruginosa* C₂BVB Accession number MT 785904 was sourced from Nigerian Institute for Oceanography and Marine Research, Lagos.

Observed parameters

Weight change, clinical signs and percentage mortality were observed and recorded during the course of the experimentation. Weight of different groups were measure before the commencement of the study (i.e. the initial weight) and at termination of the study (i.e. final weight). Weight change was determined by subtracting final weight from initial weight. The weight parameters were measured with sensitive weighing scale in gram. Mortality rate was measured by subtracting the number of dead fish from the number of experimented fish in each group, while percentage mortality (%) was measured by dividing the number of dead fish in a group with number of experimented fish then multiplied by 100.

Weight change (g) = initial weight (g) – final weight (g)

Mortality rate = number of experimented fish – number of dead fish

Percentage mortality (%) = number of dead fish ÷ number of experimented fish X 100

Statistical analysis

Weight change, mortality rate and frequency of mortality observed in this study were statistically

analyzed by general linear model using Statistical Package for the Social Science (SPSS, 27). To obtain the differences among the treatment means, data collected were subjected to Duncan Multiple Range Test.

Results

The result of weight change, clinical signs, mortality rate and frequency of mortality of *Clarias gariepinus* subjected to experimental treatments is represented in table 1 and 2. Report of weight change in this study goes as follows: control and uninfected bacillus group increased in weight by average of 15g and 5g respectively, uninfected and infected *Clarias gariepinus* treated with fresh aqueous neem leaves extract decreased in weight by average -10.00g and -15.00g respectively while infected and infected bacillus treated group neither increase nor decrease in weight. There was significant increase and decrease of the weights of fish before and after experimentation. Control group had a higher weight increase than uninfected bacillus group.

Mortality rate of different groups are as follows: control and uninfected neem treated group had 0.00 ± 0.00^b , uninfected bacillus treated grp and infected bacillus treated grp had 0.50 ± 0.50^{ab} , infected grp had 3.00 ± 0.00^a , infected neem treated grp had 1.00 ± 1.00^{ab} , $M \pm \text{SEM}$ (standard error of mean) is .345, while P -VALUE is .037. The percentage mortality (%) for this study is as follows: control and uninfected neem treated group had 0.00 ± 0.00^b , uninfected bacillus treated group and infected bacillus treated group had 5.00 ± 5.00^{ab} , infected group had 30.00 ± 0.00^a , infected neem treated group had 10.00 ± 10.00^{ab} , $M \pm \text{SEM}$ is 3.445, P -VALUE is .037. Clinical signs were not present in control, uninfected neem treated (replicate 1), uninfected bacillus treated (rep. 1) and infected neem treated (rep. 1) groups. Fin ulcer was observed at the dorsal fin with skin lightening in uninfected neem treated (rep. 2), there was change in skin colour of fish in uninfected bacillus treated (rep. 2) group (grp.), skin ulcerations and haemorrhage were observed on the skin of fish in infected grp. Injury and slow movement were observed in infected neem treated (rep. 2) grp, infected bacillus treated grp had change in skin colour, erosion on the dorsal fin, sign of wound, haemorrhage on the pectoral fin in some fish.



Table 1: Mortality rate, Percentage mortality and Weight change of different experimented groups

Groups	Mortality Rate	Percentage Mortality (%)	Weight change (g)
Uninfected Control	0.00±.00 ^b	0.00±.00 ^b	15.00±5.00 ^a
Uninfected Neem treated	0.00±.00 ^b	0.00±.00 ^b	-10.00±0.00 ^{cd}
Uninfected Bacillus treated	0.50±.50 ^{ab}	5.00±5.00 ^{ab}	5.00±5.00 ^{ba}
Infected	3.00±.00 ^a	30.00±.00 ^a	0.00±0.00 ^{bc}
Infected Neem treated	1.00±1.00 ^{ab}	10.00±10.00 ^{ab}	-15.00±5.00 ^e
Infected Bacillus treated	0.50±.50 ^{ab}	5.00±5.00 ^{ab}	0.00±0.00 ^{bc}
M±SEM	.345	3.445	3.128
P -VALUE	.037	.037	0.009

Values with different superscripts along the rows in table 1 indicate significance ($p < 0.05$) according to Duncan Multiple Range Test which was seen in Mortality Rate, Percentage Mortality (%) and Weight change (g).

Table 2: Clinical signs of exhibited by different experimented groups during experimentation

Groups	Replicate	Clinical signs
Control uninfected	1	Nil
Control uninfected	2	Nil
Uninfected Neem treated	1	Nil
Uninfected Neem treated	2	fin ulcer on the dorsal fin with skin lightening
Uninfected Bacillus treated	1	Nil
Uninfected Bacillus treated	2	Change in skin colour of fish in the group
Infected	1	Several fish had different skin ulcers
Infected	2	Patches of haemorrhage on the skin of fish
Infected Neem treated	1	Nil
Infected Neem treated	2	Injury and slow movement
Infected Bacillus treated	1	Change in skin colour in this group
Infected Bacillus treated	2	Change in skin colour, dorsal fin ulceration, wound, pectoral fin haemorrhage

Discussion

Probiotics, are live microorganisms that confer health benefits on the host, have been used in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds (Lawal *et al.*, 2019). Increase in weight of uninfected bacillus treated group contradicts Guimarães *et al.* (2019) who reported no significance difference in final weight of Nile tilapia experimentally given

probiotics (*Bacillus subtilis* and *Lactobacillus sp.*), it was observed in that study that there was increase in final weight of control group than the group where probiotics was added to the water medium just as was observed in this study. The increase in final weight seen in uninfected bacillus treated group was in line with report of Putra *et al.* (2017) where *Clarias gariepinus* were experimentally reared in a biofloc system which was enhanced with probiotic (*Bacillus* species). Fakhri *et al.* (2019) also reported similar result



where *Clarias gariepinus* were experimentally given probiotics (*Bacillus subtilis* and *Lactobacillus sp.*) as feed supplement and added to their rearing medium.

There was decline in average weight of uninfected neem treated group (Neem leave) and infected neem treated group (*P. aeruginosa* and neem leave) by average weight of -10g and -15g. Reduction observed in uninfected neem group and infected neem treated group contradicted Mona *et al.* (2015), where increase in weigh was reported in experimentation of carp exposed to *A. hydrophila* and *A. indica*, although, methods of experimentation differ, Mona *et al.* (2015) administration was dietary, while administration in this study was by dipping. Omoregie and Okpanachi, (1992) reported decline in weight gain of *Tilapia zilli* exposed to sublethal concentration of crude extracts of bark of *Azadirachta indica* in water medium. There was no difference between the initial weight and final weight of infected group (*Pseudomonas aeruginosa*) and infected bacillus treated group (*P. aeruginosa* and *Bacillus subtilis*). The result of weight changed obtained in infected group was also documented by El-Keredy and Naena, (2020), who challenged *Oreochromis niloticus* with *Pseudomonas aeruginosa* the report from this experimentation did not signify any weight difference between the control and the infected. Mortalities recorded in this group was responsible for weight stagnation due to disease infection. Therefore, it is inferred that mortality produced by *Pseudomonas aeruginosa* infection in *Clarias gariepinus* was the main cause of reduction in productivity, infectivity of the bacterium does not reduce weight gain. However, Kumar *et al.*, (2023) reported weight gain when similar experiment was conducted by introducing *B. subtilis*, *P. aeruginosa* and *S. cerevisiae* in water for rearing *Heteropneustes fossilis*. The increase in weight of uninfected bacillus group indicates that addition of this probiotic can improve productivity.

Significant difference p-value (< 0.05) was observed in the percentage mortality (PM) across the groups. Zero PM was recorded in Control group and uninfected neem treated group, while uninfected bacillus treated group and infected bacillus treated group had 5% PM, infected neem treated group had 10% and infected group had 30%. Result obtained in Control group and infected group correspond to Obisesan *et al.*

(2022); Luo *et al.*, 2015; Amrevuawho *et al.* (2014); Hossain *et al.* (2006) and Magdy *et al.* (2014) were pathogenicity of heteroclaris, Grass Carp (*Ctenopharyngodon idella*), *C. gariepinus* and *Oreochromis niloticus* were infected with *P. aeruginosa*. The control group had no mortality while the infected group had different ranges of mortality. Salah *et al.* (2022) confirm zero-mortality observed in uninfected neem treated group in Sea Bass (*Dicentrarchus labrax*) that was experimentally bath with different non-toxic dose neem leaves *Azadirachta indica*. Reduction in mortality observed in infected neem treated group corroborates Mona *et al.* (2015) findings where different concentration of neem leave extract were administered to Chinese carp and exposed to *A. hydrophila*, the groups with different concentration of neem leaf extract had reduction in mortality rate than the group without neem leaf extract. Cha *et al.* (2013) got similar result with what we had in infected bacillus treated group, he evaluated the effect of dietary and water additive of *Bacillus* spp. on olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae*, and recorded lesser mortality compared to group exposed to only *S. iniae*.

Clinical signs were observed in some groups and absence in some groups. Control group had no lesion at all, while the uninfected neem group and uninfected bacillus group lesions were absent in the first replicates but present in the second replicates. Both replicates in infected group and infected bacillus treated group had lesions. The lesion documented were fin and tail ulcer with skin lightening, skin dis-colouration, patches of haemorrhage on the skin, injury and slow movement. External lesions found in second replicates of uninfected neem treated group and uninfected bacillus treated group had not been documented in any article for neem and *Bacillus subtilis*. Lesions observed in infected group is in accordance with what was observed by Magdy *et al.* (2014) and Amrevuawho *et al.* (2014); the two authors experimentally infect *Pseudomonas aeruginosa* in heteroclaris and *Clarias gariepinus* and similar clinical signs were reported. Abd Allah *et al.* (2019) reported lesions in *Clarias gariepinus* that was infected with *Aeromonas hydrophila* and given *Bacillus subtilis* as was observed in infected bacillus treated group of this study. Lesion was not found in first replicate of infected neem treated group but was observed in the second



replicate. Absence of lesion was reported by Abd Allah *et al.* (2019) when *Aeromonas hydrophila* and oil extract of *Origanum vulgare* was included as feed additive in that study, therapeutic effect of aqueous *A. indica* leaf extract had been documented by Harishrishnan (2002) against *A. hydrophila*. Therefore, absence of lesion could be the effect of the neem extract.

Conclusion

It was observed that *Azadirachta indica* and *Bacillus subtilis* has the capacity to prevent infection of *Pseudomonas aeruginosa* in *Clarias gariepinus*, but the protective effect of *Bacillus subtilis* is higher than *Azadirachta indica*.

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