PATHOGENICITY OF *Aeromonas hydrophila* ASSOCIATED WITH SEED PRODUCTION IN *Clarias gariepinus*

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Abstract

Aeromonas hydrophila causes haemorrhagic septicaemia in fish. Its role in hatchery propagation of Clarias gariepinus is not well elucidated, thus necessitating the investigation. Pathogenicity test was conducted using β-haemolytic Aeromonas hydrophila on Clarias gariepinus seeds in Petri-dishes. Aeromonas hydrophila (10^9, 10^{10} and 10^{11} cfu/ml) effect on fertilized eggs (200), day-old larvae (720) and fry (240) exposed by bath for 20 minutes (eggs), and 15, 30 and 60 minutes (larvae and fry) were determined. The larvae and fry were fed artemia ad-libitum, and observed for seven and four days respectively. Eighty fingerlings were orally challenged with similar Aeromonas hydrophila doses, monitored for clinical signs (30 days) and bacterial isolation determined. Data were analysed using standard tests at $\alpha_{0.05}$. Hatchability significantly improved with increasing bacterial dose. Larval mortality significantly increased from 0.33±0.33 (pre-fed) to 4.33±0.67 (post-fed) on first-exogenous feeding. Aeromonas hydrophila significantly reduced post-fed larval mortality to 1.33±0.44 (10^{11}cfu/ml) and 2.00±0.41 (60-minutes exposure), and insignificantly reduced fry mortality from 1.83±0.31 to 0.83±0.48 (10^{10} cfu/ml) and 0.83±0.31 (60-minutes exposure). Challenged fingerlings showed no clinical sign throughout the period. Aeromonas hydrophila was isolated from fingerlings' gut and liver. Studied Aeromonas hydrophila was non-pathogenic, enhanced the seeds' health and is a prospective gut stabilizer.

Key words: Clarias gariepinus seeds, Aeromonas hydrophila, pathogenicity.

Introduction

Clarias gariepinus is a fast growing, disease resistant freshwater fish of growing economic value in the African aquaculture industry (FDF, 2007, Goda *et al*., 2007; Osman *et al.*, 2007). However, one of the factors limiting the supply of its seeds to farmers is mass mortality arising from bacterial infection (Macharia *et al.*, 2005; Ariole and Okpokwasili, 2012). One of such pathogens is *Aeromonas hydrophila.*

Aeromonas hydrophila is regarded as a major pre-dominating freshwater and fish ectodermal bacterium (Ringo and Birkbeck, 1999; Al-Harbi and Uddin, 2006; Tomas, 2012).The bacterium is reported as being pathogenic in some fish species and humans (Albert, 2000; Cipriano, 2001; Seatha *et al*, 2004; Yardimci and Aydin, 2011). Clinical manifestations of *A. hydrophila* infection on affected fish include septicaemia, exophthalmia, reddening of the skin, oedema in the scale pockets – "washboard" appearance, dermal ulcers, haemorrhagic lesions on the gills and acute diarrhea. Others may be associated with corneal ulcers, peritonitis and meningitis (Albert, 2000; Seatha *et al*, 2004; Citarasu *et al*, 2011; Tomas, 2012).

Biochemical properties and virulence factors produced by the microbe include β-haemolytic activity (haemolysins), proteases, toxins (enterotoxin, endotoxin, exotoxin and cytotoxins), exopolysaccharide, S-layers iron-binding systems, extracellular enzymes, secretion systems, fimbriae and other non-filamentous adhesins, motility and flagella. These factors have been reported as potential indicators of pathogenicity (Dibua and Okpokwasili, 2006; Yousr *et al.*, 2007; Pandey *et al.*, 2010). The antibacterial activity of crude cell extract of the bacterium against indicator pathogenic bacteria – *Staphylococcus arlettae* strain An1, *Acinetobacter* sp. strain An2, *Vibrio parahaemolyticus* strain An3 and *Alteromonas aurentia* SE3, was demonstrated by Pandey *et al.* (2010).

Some strains of the bacterium, according to Zhang *et al.*, (2000), are considered non-pathogenic. Most of the described diseases associated with *A. hydrophila* infection has been found difficult to reproduce in animal models (Tomas, 2012), except those conducted through systemic route (Rey *et al*., 2009). According to Tomas (2012), the virulence of the bacterium is hypothetically believed to depend on the bacterial strain, the infection route, and the animal used as model organism. *A. hydrophila* was documented to exhibit a probiotic effect on rainbow trout (Irianto and Austin, 2002) and germ-free Artemia, *franciscana nauplii* (Gunasekara *et al.*, 2010) when administered via non-parenteral routes.

However, not much was said about its pathogenicity on hatchery propagation of *Clarias gariepinus*

Thus, this study was designed to determine the non-parenteral pathogenicity of a β-haemolytic *Aeromonas hydrophila* on hatchery propagation of *Clarias gariepinus*

Methodology

Bacterial Isolate

A fish bacterium isolate that was typed as β-haemolytic *Aeromonas hydrophilia*was obtained from AnimalCare® laboratory in Ogere, Ogun state, Nigeria. The isolate was cultured on nutrient agar at 35ºc and identified using the morphological and biochemical characterisation as summaried in table 1. A serial dilution of 1.35 x 10^{12} cfu/ml *A. hydrophila* suspension (10^9, 10^{10} and 10^{11} cfu/ml) was used for the study. This is in accordance with the specification of the Public Health Agency of Canada (2012) that gave the oral infective dose in human and animals as greater than 10^{10}cfu.

Experimental Procedure

Experimental infection of fertilized eggs

The first challenge was carried out on a total of about 200 fertilized eggs. From a bowl of fertilized eggs, 0.2ml (~50) eggs were each drawn into four Petri dishes arranged into three treatments and a control. The three treatments were challenged with graded doses of 10^9, 10^{10} and 10^{11} cfu/ml of *A. hydrophila.* Each dose-group was exposed for 20 minutes, rinse and incubated at 26ºC in 30 ml of distilled water (pH and total hardness adjusted to 7.0 and 60ppm respectively) in Petri-dishes. Percentage hatchability was thereafter determined.

Experimental infection of *Clarias gariepinus* seeds

Clarias gariepinus seeds (larvae, fry and fingerlings) were separately challenged with the *Aeromonas hydrophila* to evaluate the clinico-pathological effects.

A total of 720 apparently healthy day-old larvae and 240 apparently healthy seven days old frys were selected from a monitored production tank. The fish seeds were cultured using the adjusted distilled water. They were distributed into three bacterial dose groups (10^9, 10^{10} and 10^{11}cells/ml of *A. hydrophila*) and a control group. Each group had three exposure-time (15, 30 & 60 minutes) units in triplicates of 20 larvae per Petri-dish and duplicates of 10 frys per Petri-dish (figure 1). The treatments were topically applied via the culture medium. The adjusted culture water was daily renewed and the mortality pattern / clinical manifestation monitored for seven (larvae) and four (frys) days post-infection. The larvae and frys were fed on dried decapsulated artemia cysts. The larvae were fed from day 5to7

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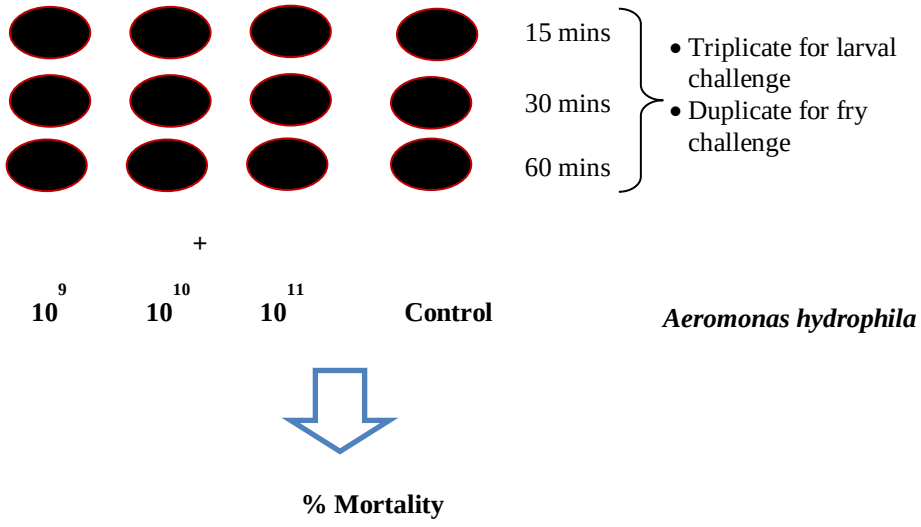

Figure I: Design of experimental infection trial

Eighty fingerlings of 7.5g average weight were purchased from a commercial farm, separated into four intensively stocked groups (equivalent to 100kg m^{-3}) of twenty fingerlings per group in clean

plastic aquaria, and acclamatized for twenty-four hours before the study. Three of the four groups were orally challenged, with each group fish receiving 2 drops (0.1ml) of the graded doses of *Aeromonas hydrophila* suspension, and monitored for clinico-pathological effects for a period of thirty days.

Statistical analysis

The data obtained from the experimental infections were subjected to statistical analysis using descriptive statistics, ANOVA and Tukey-Kramer Multiple Comparisons Test ($p<0.05$).

Results

Bacteria Isolate

Organism identification probability (using Gideon online software)

Aeromonas hydrophila, 99%
Aeromonas jandaei, 1%
Aeromonas veronii biovar veronii, <1%
Aeromonas veronii biovar sobria, <1%

Experimental infection of fertilized eggs

The percentage hatchability of fertilized eggs challenged with *Aeromonas hydrophila* for 20 minutes exposure-time improved from 12.8% hatch rate (control) to 77.1% hatch rate (10^{11}cfu/ml), as represented in figure 2. The increment was statistically significant ($P < 0.05$) at 10^{11}cfu.

Experimental infection of *Clarias gariepinus* seeds

The results obtained from the challenge of *Clarias gariepinus* seeds (larvae and fry) with a β-haemolytic strain of *A. hydrophila* are presented in figures 3-8.

All the larval groups were observed to have 0 - 1 (that is 0 - 5%) larva mortality within the first four pre-feeding days, but 0 - 5 (that is 0 - 25%) larval mortality for the three post-feeding days (5^{th} to 7^{th} day).No meaningful difference was observed in the pre-feeding mortality pattern of exposed larvae and the control.

The mean post-fed mortality of *A. hydrophila* infected larvae is shown in Figures 4 and 5. In Figure 4, the mean post-fed larval mortality reduced from 3.0 to 2.2 (26.8% reduction) at 10^{9}cfu, 2.0 to 0.9 (55% reduction) at 10^{10}cfu, and 1.8 to 0.9 (50% reduction) at 10^{11}cfu, while a reduction in larval mortality of 3.5 to 1.9at 15 minutes' exposure time (45.7% reduction), 1.55 to 0.95 at 30 minutes' exposure time (38.7% reduction), and 2.3 to 0.9 at 60 minutes' exposure time (60.9% reduction) was obtained, as indicated in Figure 5.

The percentage reduction in post-fed larval mean mortality and overall mean mortality of infected larvae ranged from 14.0 to 76.7% and 14.9 to 78.7% respectively, when compared to the control. The highest post-fed larval mean mortality reduction recorded was 76.7% at 10^{10} c.f.u. / 30 minutes; 10^{10} c.f.u. / 60 minutes; 10^{11} c.f.u. / 30 minutes and 10^{11} c.f.u. / 60 minutes when compared with the controlled experiment, while the highest overall reduction in group mean mortality of 78.7% occurred at 10^{11} c.f.u. / 30 minutes and 10^{11} c.f.u. / 60 minutes (bacterial dose and time of exposure to the bacterium respectively). When comparing the effects of *A. hydrophila* at 10^{11} c.f.u. / 30 minutes and 10^{11} c.f.u. / 60 minutes on post-fed larval mean mortality and the overall group-mean mortality, a 97.5% of the overall reduction in mean mortality occurred after first exogenous feeding.

On the exponential curve in figure 2, a reduction in the mean larval mortality from 3.3 to 2.7 at 10^{9}cfu (18.2% reduction); 2.6 to 1.2 at 10^{10}cfu (53.8% reduction), and 2.0 to 0.85 at 10^{11}cfu (57.5% reduction) was recorded. Also, a reduction in the mean mortality from 3.9 to 2.2 at 15 minutes' exposure time (43.6% reduction), 2.1 to 1.1 at 30 minutes' exposure time (47.6% reduction), and 2.9 to 0.9 at 60 minutes' exposure time (69% reduction), was observed by considering the effect of each bacterial dose per exposure time on infected larvae (Figure 3).

Statistically, larval first-exogenous artemia feeding resulted in pre-fed mortality range of 0.22 ± 0.15 to 0.44 ± 0.18 (all groups), while post-fed mortality decreased from 4.33 ± 0.67 (control) to 1.33 ± 0.44 (10^{11} cfu/ml) and 1.78 ± 0.36 (30 minutes exposure) with *Aeromonas hydrophila* introduction, irrespective of the treatment applied. The difference between pre-fed mortality (0.37 ± 0.089) and the post-fed mortality (2.07 ± 0.271), using unpaired t test with Welch correction, is significant ($P < 0.0001$) irrespective of the treatment applied. The level of significant differences between the pre-fed and post-fed larval mortalities decreased from 0.33 ± 0.333 (pre-fed, control) / 4.33 ± 0.667 (post-fed, control) to 0.22 ± 0.147 (pre-fed, 10^{11} cfu/ml) / 1.33 ± 0.441 (post-fed, 10^{11}cfu/ml).

Table 1: *Aeromonas hydrophila* identification test

Gram	**Negative**
Bacterial shape	Bacillus
Growth on ordinary blood agar	growth + β-haemolysis
Motility	Positive
Catalase test	Positive
Oxidase test	Positive
Citrate test	Positive
Indole test	Positive
VogesProskauer	Negative
Urea hydrolysis	Positive
Methyl red	Positive
Lactose fermenter	Positive
Glucose fermenter	Positive
Gas from glucose	Positive
Starch hydrolysis	Negative
D-Mannitol	Positive
Maltose	Negative
Sucrose	Positive

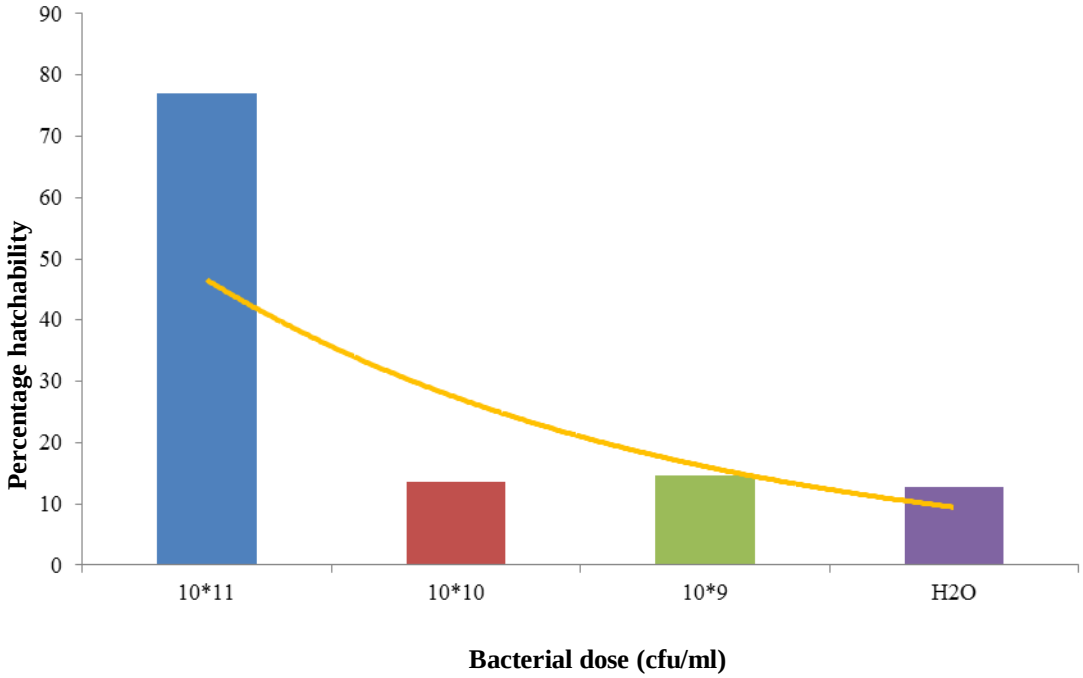

Fig 2: Percentage hatchability of *Aeromonas hydrophila*-challenged *Clarias gariepinus* eggs

Key
10*11 = 10^{11}; 10*10 = 10^{10}; 10*9 = 10^{9}; H2O = water (H_2O)

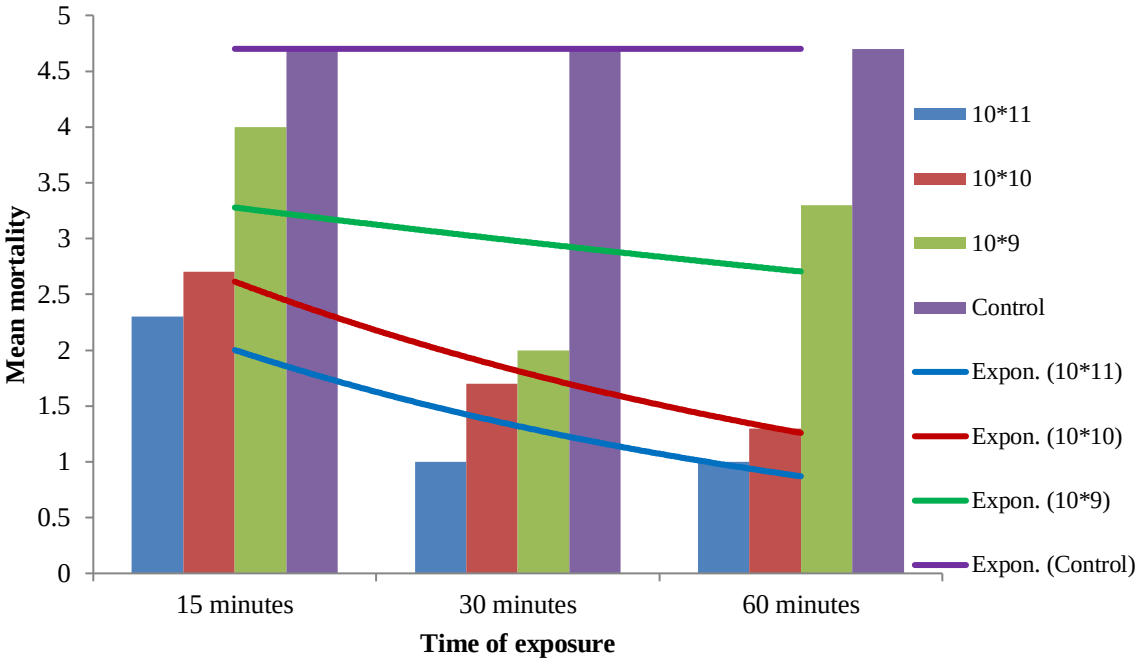

Figure 3: Mean mortality pattern of varied larval exposure time to *A. hydrophila* doses

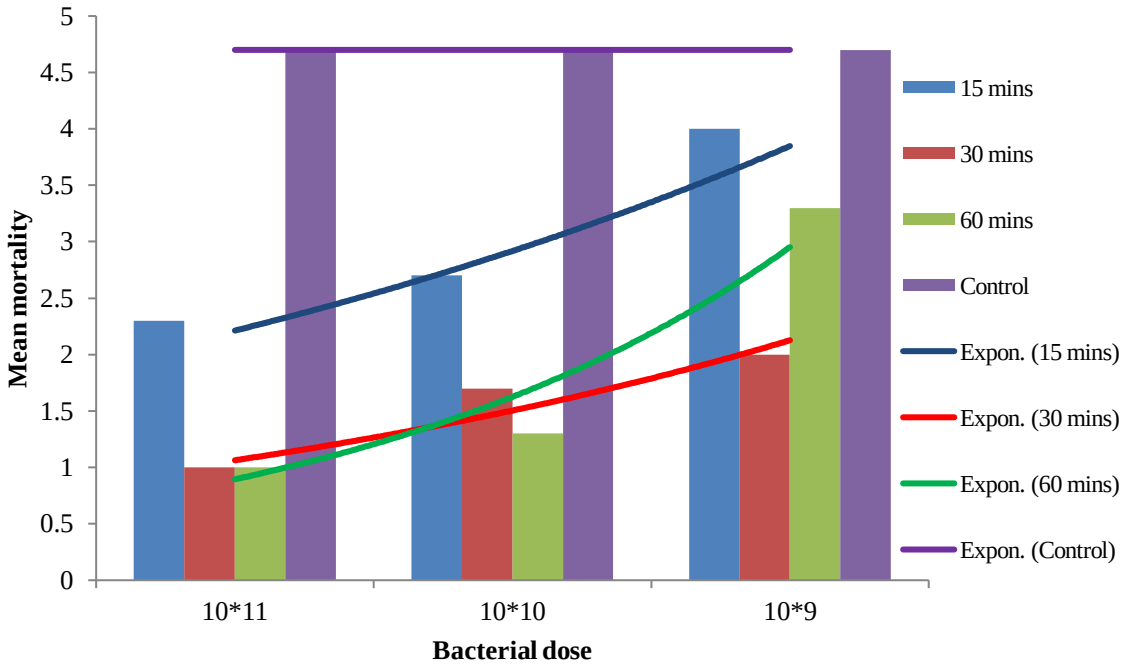

Figure 4: Mean mortality pattern of varied *A. hydrophila* doses per exposure time on infected larvae

Key

10*11 = 10^{11}; 10*10 = 10^{10}; 10*9 = 10^{9}; H2O = water (H_2O)

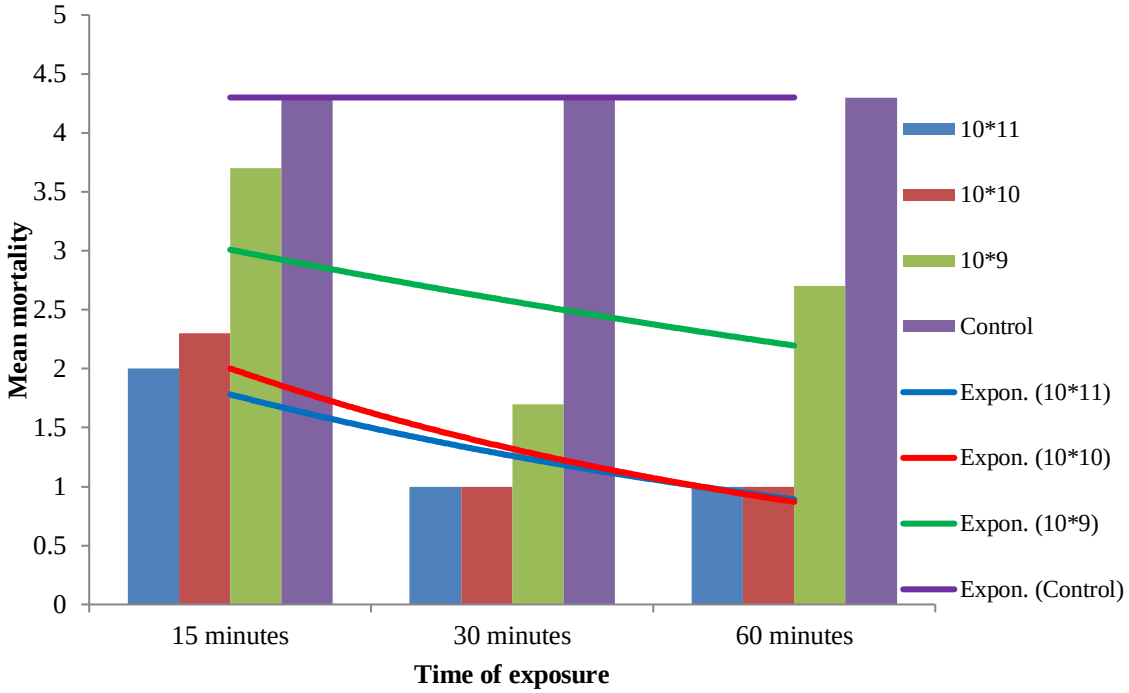

Figure 5: Mean post-fed mortality of varied larval exposure time to *A. hydrophila* doses

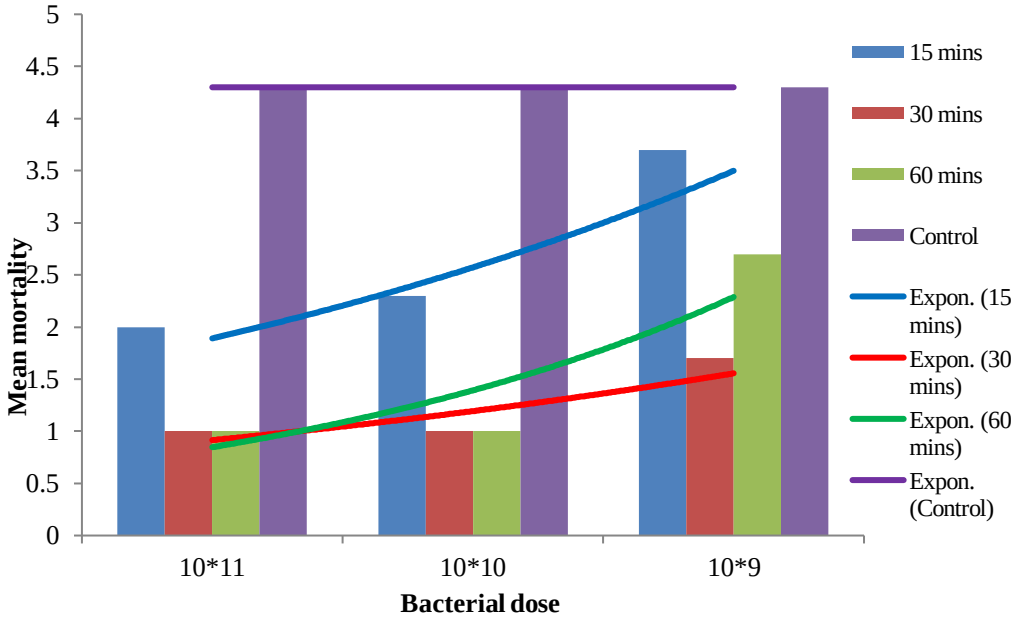

Figure 6: Mean post-fed mortality of varied *A. hydrophila* doses per exposure time on infected larvae

Key

$10*11 = 10^{11}$; $10*10 = 10^{10}$; $10*9 = 10^{9}$; H2O = water (H_2O)

Figure 7 reveals the effects of varied time of fry exposure to bacterial doses. The average mortality per group ranged from 0.5 to 3.0 fry within the four-day period. A persistent reduction from 1.5 to 0.5 average fry mortality was recorded with increasing bacterial dose when exposed for more than 30 minutes to *A. hydrophila*. The least mortality figure of 0.5 fry was obtained at 10^{10} and 10^{11}cfu/ml bacterial doses, and 60 minutes' exposure-time. On the exponential trendline curve, a reduction in fry mortality from 2.65 to 1.3 was recorded when the time of exposure to 10^{9} bacterial dose was increased from 15 to 60 minutes, that is less 50.9% mortality, and a reduction of about 83.9% from 2.8 to 0.45 fry mortality when the time of exposure to 10^{11} bacterial dose was increased from 15 to 60 minutes.

The exponential curves in Figure 8 showed the effects of increasing the bacterial dose on the infected fry that were exposed for specified time frame. A reduction in fry mortality occurred from 1.65 (10^{9}cfu) to 1.1 (10^{11}cfu) at 30 minutes of exposure time to the bacterium, that is 33.3% reduction in mortality, and a pronounced reduction from 1.25 (10^{9}cfu) to 0.4 (10^{11}cfu) fry mortality within the four days of the experimental study, that is 68% reduction in mortality. There was no noticeable change at 15 minutes of exposure.

Statistically, an irregular reduction in fry mortality occurred from 2.0 ± 0.365 at 10^{9}cfu to 0.833± 0.477 at 10^{10}cfu (58.4% reduction in mortality), and to 1.5 ± 0.5 at 10^{11}cfu (33.3% reduction), irrespective of the time of exposure to the bacterium. However, a consistent reduction in fry mortality from 2.167 ± 0.543 (15 minutes' exposure-time) to 1.333 ± 0.422 at 30 minutes' exposure-time (38.5% reduction), and to 0.833 ± 0.307 at 60 minutes' exposure-time (61.6% reduction) was recorded, irrespective of the bacterial dose.

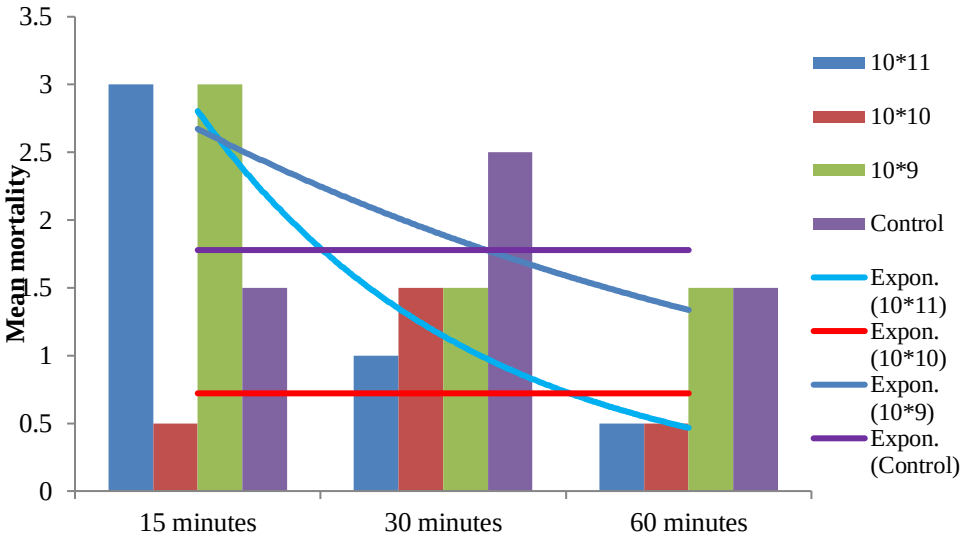

Figure 7: Mean mortality patternof varied exposure time to *A. hydrophila* doses on infected fry

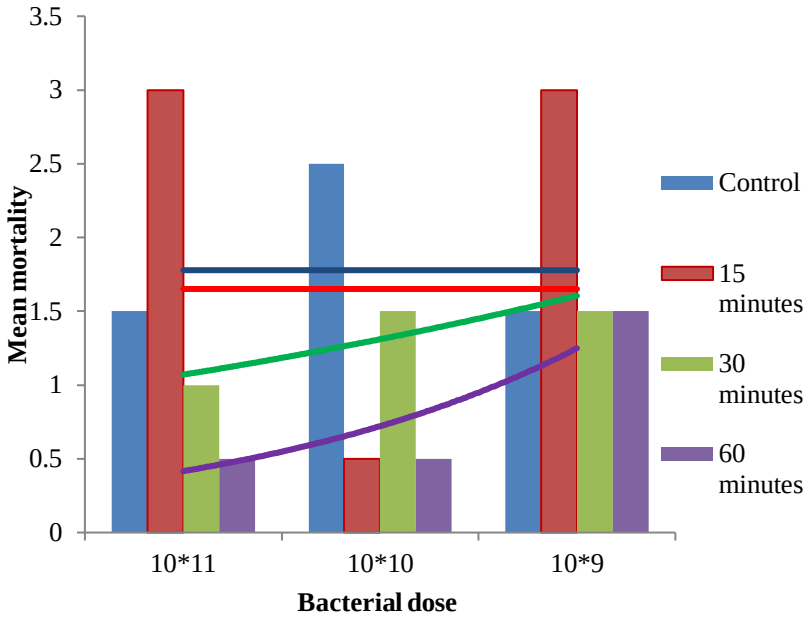

Figure 8: Mean mortality pattern of varied *A. hydrophila* doses per exposure time on infected fry

The oral challenge with 0.1ml of a serially diluted (10^0, 10^{-1}, 10^{-2} and 10^{-3}) concentration of 1.35 x 10^{12} cfu ml^{-1} produced no obvious clinical manifestation of disease, gross pathological changes or fish mortality in any of the four groups infected within the thirty days period. On carrying out laboratory (microbiology) analysis of various organs / tissues, the organism was re-isolated from the intestine and liver of infected fingerlings. No trace of the bacterium was found in the kidney, muscle and brain of the fish.

Discussion

The *Aeromonas hydrophila* used for the challenge was observed to be non-pathogenic, as it increased fish seed hatchability and survivability. The increase in hatchability of the challenged eggs with increasing bacterial dose was probably due to the increasing dose of the bacteriocin released by *Aeromonas hydrophila* (Chen and Hoover, 2006; Dibua and Okpokwasili, 2006; Pandey *et al.*, 2010). Bacteriocin is expected to reduce the colonization of egg surface by other bacterial agents (Pandey *et al.*, 2010), thus improving hatchability. According to Hansen and Olafsen (1989), and Sauter *et al.* (2006), reduction in surface colonization of eggs could improve hatchability. Egg washing is expected to minimize surface bacterial load and *A. hydrophila* toxins. The 20-minute exposure-time (before the eggs were washed) was probably not enough for the organism to establish itself and overgrow the external surface of such eggs, since the bacterial incubation period is given as 1-2 days post-infection (FDA, 2016).

A reduction in fish larval and fry mortality was observed with increasing dose and period of exposure to *Aeromonas hydrophila* via bath. This inverse association between bacterial dose /time of exposure and fish mortality implies that the bacterium has a positive influence on the fish health and survivability. This suggests that the challenging bacterium may probably be a non-pathogenic variant (Zhang *et al.*, 2000).The released anti-bacterial factors (Pandey *et al.*, 2010) are expected to limit other bacterial activities, thus promoting the fish health status. This observation is in agreement with the findings of Irianto and Austin (2002) and Gunasekara *et al.* (2010), who documented that *A. hydrophila* enhanced rainbow trout survival rate and improved the development of the digestive tract of germ-free *Artemia*, *Franciscana* nauplii.

The fish seeds (eggs, larvae and fry) seem to be insensitive to *A. hydrophila* toxins, contrary to the hypothesis of Dibua and Okpokwasili (2006) and Yousr *et al*. (2007). This might be due to factors such as: the presence of transferred maternal antibody in the fish (fish seed immunity); inability of the bacterium to attain its predilection site due to the route of exposure; and or the bacterium being non-pathogenic (Tomas, 2012). Since *Aeromonas hydrophila* is present in most freshwater bodies (whether treated or not) and are pre-dominant co-

inhabitant of fish gut (Al-Harbi and Uddin, 2006; Tomas, 2012), the probable development and transfer of maternal antibody from the broodstock to the eggs and consequently the resulting larvae and fry, cannot be ruled out. The isolation of the bacterium from the liver of apparently healthy (control and infected) fingerlings further suggests the probable development of immunity against *Aeromonas hydrophila.*

Introduction of fish larval to first exogenous feed (dry, decapsulated artemia cysts) was observed to increase larval mortality, similar to the report of Gisbert *et al.* (2004) on California halibut larvae exposure to first feed. The sudden increase in post-fed larval mortality might have resulted from either a rapid increase in gut pathogen due to an upsurge in nitrogenous substrate, and or due to the release of 'toxic' metabolites of the ingested artemia cysts, which is foreign to the system of the larvae at the time. The subsequent stability of the post-fed larval gut was possibly due to the microbial regulatory role of *Aeromonas hydrophila* released bacteriocin (Dibua and Okpokwasili, 2006; Pandey *et al.*, 2010) on gut pathogens. As the concentration of the bacteriocin increases with increase in bacterial dose, the effectiveness of the inhibitory function on probable gut pathogens increases (Pandey *et al.*, 2010). The probable involvement of *Aeromonas hydrophila* in nutrient metabolism or in the reduction of toxic metabolites cannot be overruled, as was similarly reported in artemia nauplii by Gunasekara *et al.* (2010). This is further buttressed by the fact that there was a decrease in fry mortality with increasing exposure time and dose, though not significant. Thus, the organism may be assumed to act as a gut probiont associated with microbial regulatory bacteriocin production and metabolic waste degradation.

Oral infection of *C. gariepinus* fingerlings with *A. hydrophila* likewise produced no disease condition in any of the three challenged groups, despite the stress introduced through handling (restraint and manual dose administration).

The specified infective dose of not less than 10^{10} cfu suggested by the Public Health Agency of Canada (2012) was observed to be non-pathogenic to the infected *Clarias gariepinus* seeds (larvae, fry andfingerlings) in this study. According to Yardimci and Aydin (2011), microbial infectivity and disease establishment are determined by factors such as the virulence of the challenging organism, period of exposure to the microbe, fish species' tolerance, prior exposure and or level of immunity. Since the bacterium was a fish isolate, and the cultured larvae and fry were not previously exposed to *A. hydrophila,* it might be inferred that there is an immunological species-specific tolerance (Lieschke1 and Trede, 2009) to the bacterium, or that *A. hydrophila* is a commensal (Eurell *et al.*, 1978; FSA, 2000), or that other factors are required to prompt a disease condition (FDA, 2016).

Based on the findings of Morgan *et al.* (1985) on *A. hydrophila* infection in humans, there was no significant clinical disease manifestation when 57 human volunteers were orally challenged using five pathogenic strains of the bacterium with doses ranging from 10^4 to10^{10} cfu. Similarly, a reported pathogenic strain of *A. hydrophila* isolated and tested by Kawula *et al*. (1996) was reported as being unable to invade both fish and human cultured cells. The Food Standards Agency (2000) is also of the opinion that the bacterium is a non-pathogenic microbe. Eurell *et al.* (1978) and Michel (1981) stated that *Aeromonas hydrophila* is not a fish pathogen but a secondary invader of already compromised hosts. Its fast propagation on wounds and necrotic tissues (Rahim *et al.*, 1985; Citarasu *et al.*, 2011), coupled with the release of anti-bacterial factors (Pandey *et al.*, 2010), probably explains why it is sometimes incriminated in some disease cases (Austin and Austin, 2007).

The bacteriological analysis conducted on the infected *Clarias gariepinus* fingerlings' tissues and organs at the end of the thirty-day period of study showed that the introduced bacterium (*Aeromonas hydrophila*) was able to establish itself or has been established in the gastro-intestinal tract prior to the challenge, as commonly experienced in most freshwater fishes (Trust and Sparrow, 1974; Ringo and Birkbeck, 1999). The ability of the bacterium to utilize fructose, galactose, maltose, mannitol, trehalose, dextrin, glycogen and glycerol (Hsu *et al.*, 1985), elastin, casein and fibrinogen (Shotts *et al.*, 1985) might have enhanced its establishment and successful co-inhabitation within the gut. Moreover, the production of virulence factors such as β-haemolytic activity and toxins, as revealed by Dibua and Okpokwasili (2006) and Pandey *et al*. (2010), might have been responsible for its ability to out-compete other microbes in order to establish itself in the gut.

The organism isolation from the fish liver is a demonstration of its invasive nature, although its inability to hydrolyzed collagen (Shotts *et al* 1985) was expected to create a limitation. However, being isolated in the liver, with no sign of disease, might depict the development of immunity by the host fish against the organism. Its inability to invade the kidney, muscle and brain showed that the organism's predilection sites exclude the kidney, muscle and brain, but include the gastro-intestinal tract of *Clarias gariepinus*, while the liver probably helps in immune development.

Conclusion

The studied *Aeromonas hydrophila* is considered a commensal with protective ability when applied externally or orally at a dose of 10^{10} and 10^{11}cfu/ml. It is a good probiotic candidate for *Clarias gariepinus* seed production, being able to improve hatchability, and larval and fry survivability at high dose (10^{11}cfu). The bacterium may also be regarded as a gut stabilizer (or gut probiont) since it was able to considerably reduced mortality associated with larval first exogenous feeding.

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