Antimicrobial Activity of Estuarine Isolates against Shrimp Pathogenic *Aeromonas* Species.

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Abstract: Four isolates of *Pseudomonas* spp. from brackish water and four isolates of *Lactobacillus* spp. from healthy shrimp *(Penaeus monodon)* gut were screened for antimicrobial activity against shrimp pathogenic *Aeromonas* species using agar well diffusion assay. One isolate of *Pseudomonas* sp. (P2) and one isolate of *Lactobacillus* sp. (L2) were found active against all *Aeromonas* strains. The co-culture experiment showed that *Pseudomonas* P2 with $1.0x10^5$cfu/ml and *Lactobacillus* L2 with $1.0x10^5$cfu/ml were enough to suppress *Aeromonas* sp. (A2) within 12 hours. Therefore, the indigenously isolated antagonistic *Pseudomonas* sp. P2 and *Lactobacillus* sp. L2 could be used as effective biocontrol agents for management of aeromonasis in aquaculture.
[Caroline Nchedo Ariole and Edith Chikodiri Oha. **Antimicrobial Activity of Estuarine Isolates against Shrimp Pathogenic *Aeromonas* Species.** *Nat Sci* 2013;11(2):123-128]. (ISSN: 1545-0740). http://www.sciencepub.net/nature. 22

Keyword: Inhibitory activity; indigenous probiotics; shrimp pathogens

1. Introduction

Aquaculture has grown steadily over the past decade and has become an important economic activity in many countries (Ma *et al*., 2008; FAO, 2010). An important issue affecting production is the loss of stock through disease. Diseases caused by *Aeromonas* spp. are commonly implicated in episodes of mortality (Kesarcodin-Watson *et al*., 2008).

Good husbandry techniques and the usage of chemical additives, disinfectants, antimicrobials and vaccines are primarily practical to control bacterial infections in aquaculture (Wang *et al*., 2008). However, the increased use of antibiotics in aquaculture systems leads to complications such as increased stress among aquatic animals, development of drug resistance among fish and human pathogens subsequently via transfer of genes from drug resistant microbes (Wang *et al*., 2008; Heuer *et al*., 2009). Furthermore, the residues which are left behind adversely affect the environment and public health (Defoirdt *et al*., 2007). Consequently, the need for better and more effective alternatives is increasingly evident.

Probiotics ensure that the host maintains a beneficial microbial population in the gastrointestinal tract. They confer a healthy effect on the host as significant microbial food supplement in the field of prophylaxis (Geovanny *et al*., 2007). Probiotics have also found use in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds (Irianto and Austin, 2002; Sahu *et al*., 2008).

The use of probiotics in aquaculture for disease control is yet to gain popularity in Nigeria. Probiotics research is still at its infancy and data on aquatic indigenous probiotics are not available. Indigenous microorganisms are natural candidates for bio-control strategies because of their adaptations to local environmental constraints, hence are more likely to establish themselves in a particular habitat (Ortega-Morales *et al*., 2009).

Therefore, the objective of the present study was to investigate the inhibitory activity of indigenous *Pseudomonas* species isolated from brackish water and *Lactobacillus* species isolated from healthy shrimp (*Penaeus monodon*) gut against fish and shrimp pathogenic *Aeromonas*. This is a part of a long term screening and selecting indigenous probiotic strains from aquatic environment to suit the specific requirement in Nigeria.

2. Materials and Methods

Sample collection

Brackish water was collected in sterile plastic bottle while healthy and moribund shrimps (*Penaeus monodon*) were collected in sterile plastic bags from Sombriero River estuary in Buguma, Rivers State of Nigeria with the assistance of local fishermen.

2.1. Bacterial isolation

The Brackish water sample was diluted in a range 1:10 to 1:100. Sub samples of 0.1ml of both the diluted and the undiluted brackish water samples were cultured on Pseudomonas cetrimide agar (Oxoid). The healthy and moribund shrimps were cleaned externally with ethanol and their gastro-intestinal tracts dissected under sterile conditions. The gut contents were weighed and placed in a physiological solution and then diluted in a range 1:10 to 1:1000. Sub samples of 0.1ml of the dilutions from healthy shrimp were cultured on Man Rogosa (MRS) (Oxoid) (for *Lactobacillus* species) while sub samples of 0.1ml of the dilutions from moribund shrimp were cultured on Aeromonas medium with ampicillin supplement (Ryan) (Oxoid) (for *Aeromonas*

species). All the media were supplemented with 1.0% sodium chloride and incubated at 37^{O}C for 24 – 48hours.

Isolates with distinct colony morphology were picked and streaked repeatedly on Nutrient agar plates until pure. The purified isolates were identified to generic level based on their morphological and physiological characteristics (Holt *et al*., 1994).

2.2. Determination of antimicrobial activity

The antimicrobial activity was first determined by agar diffusion method (Baydar *et al*., 2004 and Dobner *et al*., 2003). Further study was made by broth assay where *Pseudomonas* P2 and *Aeromonas* A2 were mixed and *Lactobacillus* L2 and *Aeromonas* A2 were also mixed and survival determined by plate counting at various time intervals from 0 to 48hours (Chythanya *et al*., 2002).

2.3. Agar diffusion assay

Antimicrobial activity of four isolates of *Pseudomonas* spp. and four isolates of *Lactobacillus* spp. was carried out against the pathogenic *Aeromonas* strains. Wells were punched with a cork borer (6mm diameter) in plates of nutrient agar freshly seeded with 0.1ml of 24 hour old broth culture of *Aeromonas* strain. Exactly 0.1ml of a 24 hour old broth culture of each of the *Pseudomonas* and *Lactobacillus* strains and the control (nutrient broth containing 1.0% sodium chloride) were put into the wells. The plates were incubated for 24hours at 37^{O}C. The diameter of clear zones surrounding the wells were measured and recorded expressing the antibacterial activity.

2.4. Effect of *Pseudomonas* P2 and *Lactobacillus* L2 on growth of *Aeromonas* sp. A2 in sterile Nutrient broth.

Three 250ml flasks containing 100mL of nutrient broth containing 1.0% sodium chloride were sterilized at 121^{O}C for 15 minutes. Cell suspension of *Aeromonas* sp. A2 was then added to all flasks to get a cell density of approximately 1.0 x 10^5 cfu/ml. Cell suspension of *Pseudomonas* P2 adjusted to 1.0 x 10^5 cfu/ml final cell concentration was added to first flask and cell suspension of *Lactobacillus* L2 adjusted to 1.0 x 10^5 cfu/ml final cell concentration was added to the second flask while the third flask without probiotics added served as control. The cultures were incubated at 37^{O}C for 48 hours. *Pseudomonas* P2, *Lactobacillus* L2 and *Aeromonas* A2 were enumerated at 0, 12, 24 36 and 48 hour on Pseudomonas cetrimide agar (Oxoid), Man Rogosa Medium (Oxoid) and Aeromonas medium with Ampicillin supplement (Ryan) (Oxoid) respectively by standard spread plate method.

3. Results Analysis

The antibacterial activity of *Pseudomonas* spp. and *Lactobacillus* spp. against *Aeromonas* spp. are shown in Table 1 and Figure 1.

A total of four bacterial strains identified as *Pseudomonas* P1, *Pseudomonas* P2, *Pseudomonas* P3 and *Pseudomonas* P4 were isolated from brackish water. Two pathogenic isolates from moribund shrimp (*Penaeus monodon*) were identified as *Aeromonas* A1 and Aeromonas A2 and a total of four bacterial strains identified as *Lactobacillus* L1, *Lactobacillus* L2, *Lactobacillus* L3 and *Lactobacillus* L4 were isolated from healthy shrimp gut. *Pseudomonas* sp. P2 and *Lactobacillus* sp. L2 produced inhibition zones against all the pathogenic strains employed while *Pseudomonas* sp. P1, *Pseudomonas* sp. P3, *Pseudomonas* sp. P4, *Lactobacillus* L1 *Lactobacillus* L3 and *Lactobacillus* L4 had no antibacterial activity against the pathogens.

The inhibition of *Aeromonas* sp. A2 (1.0 x 10^5) cfu/ml) by *Pseudomonas* sp. P2 (adjusted to 1.0 x 10^5 cfu/ml final cell concentration) in nutrient broth containing 1.0% sodium chloride is shown in Figure 2. The inhibition of *Aeromonas* sp. A2 (1.0 x 10^5) cfu/ml) by *Lactobacillus* sp. L2 (adjusted to 1.0 x 10^5 cfu/ml final cell concentration) in nutrient broth containing 1.0% sodium chloride is shown in Figure 3. The *Pseudomonas* sp. P2 and *Lactobacillus* L2 could inhibit *Aeromonas* sp. A2 growth within 12 hours. It was found that the concentration of *Aeromonas* sp. A2 was constantly reducing but within 10^3 cfu/ml) until 48hours. For the control an increase of *Aeromonas* sp. A2 was observed from about 10^5 to 10^6 cfu/ml.

4. Discussion

The present study reports two estuarine probionts, *Pseudomonas* P2 and *Lactobacillus* L2, isolated respectively from brackish water and healthy shrimp gut, which showed antimicrobial activity against pathogenic *Aeromonas* A2 isolated from moribund shrimp (Table 1, Figures 1, 2 and 3).

Many bacterial isolates, which are common members of the non-pathogenic microflora of fish and shellfish culture systems, have been shown to inhibit fish and prawn pathogens *in vitro*. This has been demonstrated for Lactic acid bacteria (Joborn *et al*., 1997; Ajitha *et al*., 2004; Dhanasekaran *et al*., 2008) and *Pseudomonas* (Chythanya *et al*., 2002 ; Vijayan *et al*., 2006). Of the total active metabolites derived from microbes, more than 2.7% are obtained from *Pseudomonas* species (Berdy, 2005).

This explains why the *Pseudomonas* sp. P2 isolated from brackish water in this study was able to inhibit the growth of pathogenic *Aeromonas* sp. A2 both in agar diffusion assay and co-culture experiment.

The broad spectrum antagonistic activity of *Pseudomonas* spp. has been attributed to a number of factors, such as the production of phenazines, hydrogen cyanide or iron chelating siderophores and surface attachment inhibitors (Vijayan *et al*., 2006; Kennedy *et al*., 2009; Preetha *et al*., 2010; Pai *et al*., 2010).

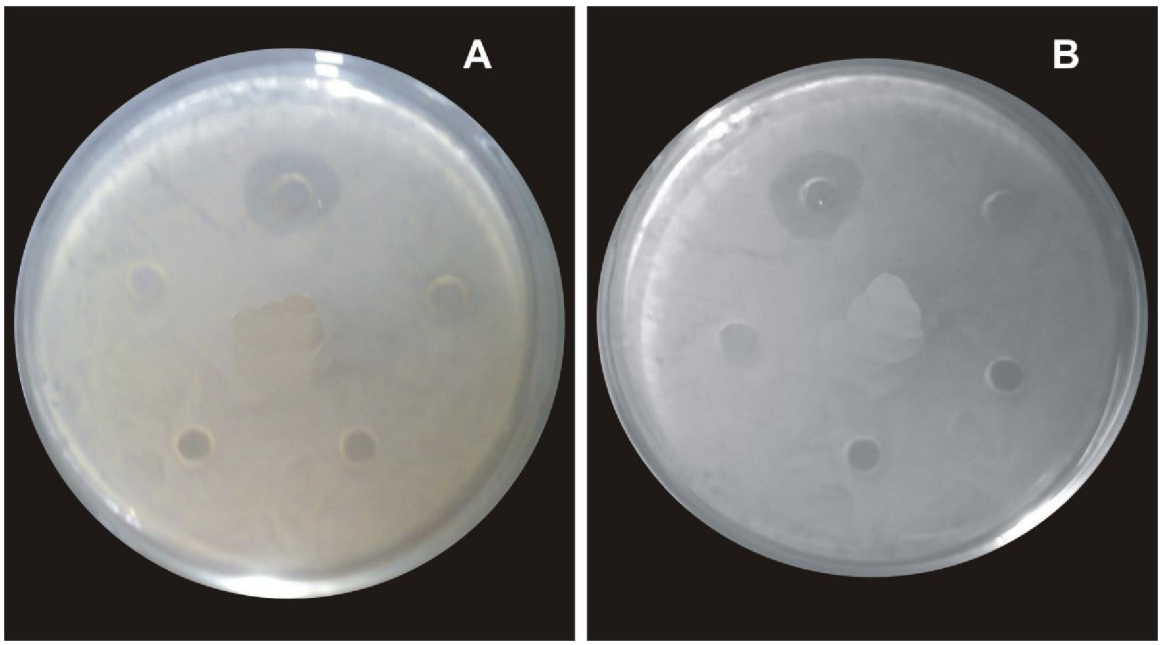

Figure 1: Inhibition zone of *Pseudomonas* sp. P2 (A) and *Lactobacillus* sp. L2 (B) against *Aeromonas* sp. A2

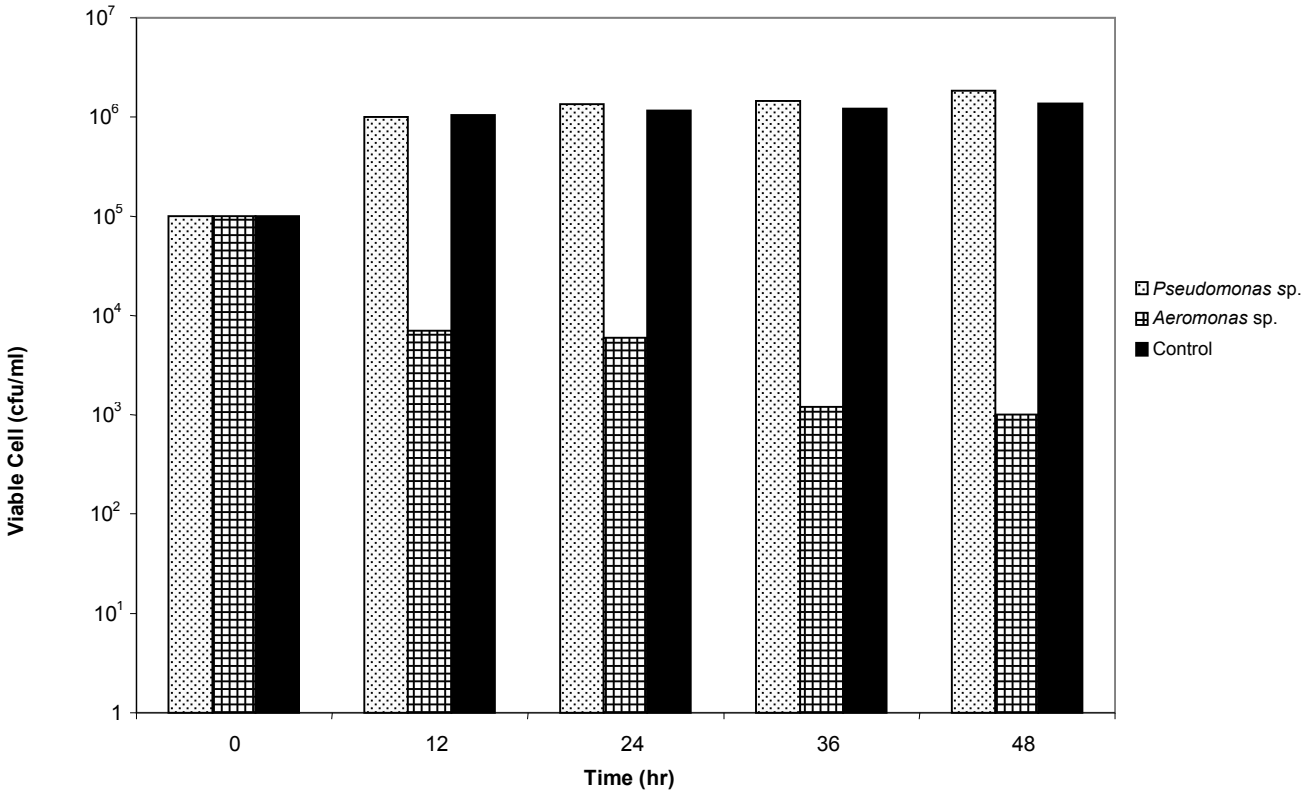

Figure 2: Effect of different concentrations of *Pseudomonas* sp. P2 on growth of *Aeromonas* sp. A2 in nutrient broth containing 1.0% sodium chloride

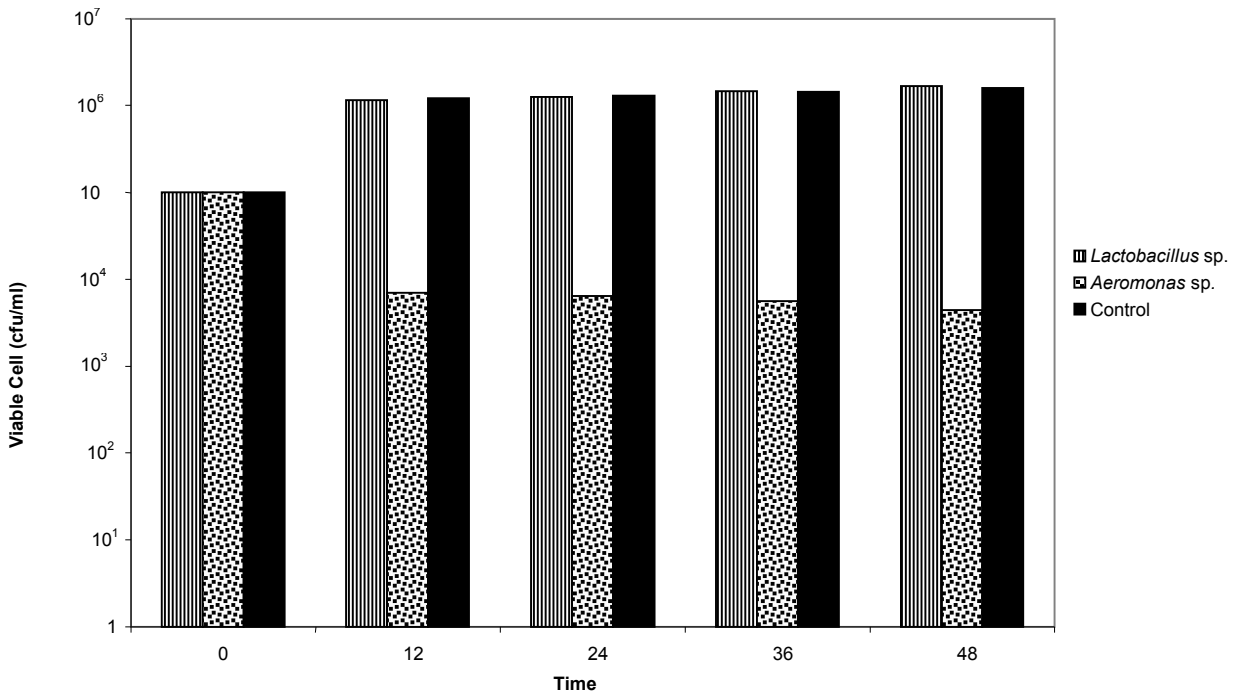

Figure 3: Effect of different concentrations of *Lactobacillus* sp. L2 on growth of *Aeromonas* sp. A2 in nutrient broth containing 1.0% sodium chloride

Table 1: Antimicrobial Activity of *Pseudomonas* species and *Lactobacillus* species against *Aeromonas* species

Bacterial Isolates	Inhibition Zone mm ± S.D.	
	Aeromonas sp. A1	*Aeromonas* sp. A2
Pseudomonas sp. P1	-	-
Pseudomonas sp. P2	20± 0.02	21± 0.02
Pseudomonas sp. P3	-	-
Pseudomonas sp. P4	-	-
Lactobacillus sp. L1	-	-
Lactobacillus sp. L2	20± 0.01	21± 0.01
Lactobacillus sp. L3	-	-
Lactobacillus sp. L4	-	-

The growth of *Aeromonas* sp. A2 was inhibited by *Lactobacillus* sp. because Lactobacilli have been found to produce metabolic products that play important role in controlling undesirable microflora in the gut (Itoh *et al*., 1995). Joborn *et al*., 1997 has reported the inhibitory activity of *Lactobacillus* against *Aeromonas salmonicida* in intestinal mucus of fish. This finding coincides with the findings of Dhanasekaran *et al*., 2008 who reported that three isolates of *Lactobacillus* showed anti-*Aeromonas* activity *in vitro*. In their in vivo studies, they reported that the antagonistic *Lactobacillus* was responsible for inhibition of *Aeromonas* populations in cat fish (*Clarias orientalis*).

Lactobacillus species are recognized for their fermentative ability as well as their health and nutritional benefits. *Lactobacillus* species also exert strong antimicrobial activity against many pathogenic microorganisms (Sanni *et al*., 1999; Rossland *et al*., 2003).

The Lactic acid bacteria (LAB) have been widely used and researched for human terrestrial animal purposes, and LAB are also known to be present in the intestine of healthy fish (Ringo and Gatesoupe, 1998; Hagi *et al*., 2004). LAB are natural residents of the human gastrointestinal tract (GIT) with the ability to tolerate the acidic and bile environment of the intestinal tract and function to

convert lactose into lactic acid, thereby reducing the pH in the GIT and naturally preventing the colonization by many bacteria (Mombelli and Gismondo, 2000; Klewicka and Klewicka, 2004).

A number of earlier studies have also shown that bacteria produce inhibitory substances that inhibit the bacterial pathogens in aquaculture systems (Austin *et al.,* 1995; Rengpipat *et al*., 1998; Gram *et al*., 1999). The use of such bacteria to inhibit pathogens by release of antimicrobial substances is now gaining importance in aquaculture as a better and more effective alternative than administering antibiotics to manage the health of fish and shrimp (Verschuere *et al*., 2000; Vine *et al*., 2004). Therefore, the isolated indigenous strains of *Pseudomonas* P2 and *Lactobacillus* L2 had the inhibitory property of biocontrol agents for use in shrimp farming and might be useful for replacing the commercial antibiotics. Further co-culture experiments to determine the minimum inhibitory concentration of the antagonists against the pathogenic strain, the species identification, optimization of *Pseudomonas* and *Lactobacillus* growth and the pathogenicity of the antagonists to shrimp larvae are going on in our Laboratory.

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1/23/2013