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In vitro antimicrobial activity of *Lactobacillus* isolates against shrimp (*Penaeus monodon*) pathogens

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Abstract

Four isolates of *Lactobacillus* spp. from the gut of a healthy shrimp (*Penaeus monodon*) were screened for antimicrobial activity against seven shrimp pathogens (*Vibrio* spp., *Aeromonas* spp, *Escherichia coli, Staphylococcus* sp. and *Salmonella* sp.) by agar well diffusion assay. Two isolates were found active against all the strains. The challenging experiment showed that *Lactobacillus* L2 at 1.0 x 10^6 cfu/ml was enough to suppress *Vibrio* sp. V2 within 12 hours. The isolated strains *Lactobacillus* L1 and L2 could have potential against *Vibrio* sp. V2 under *in vitro* condition and could be employed as potential probiotics in shrimp aquaculture system for control of bacterial infections.

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Introduction

Aquaculture is the fastest growing food producing sector globally with the greatest potential to meet the growing demand for aquatic food (FAO, 2006). Concomitant with the growth of aquaculture has been the recognition of the ever increasing importance of diseases, especially those caused by infectious agents.

The bacterial agents of penaeid shrimp has been reported to constitute the majority of bacteria present in the normal microflora of cultured and wild penaeid shrimp (Singh *et al*., 1998). These opportunistic pathogenic microbes apparently establish lethal infections as a result of other infectious diseases, nutritional diseases, extreme environmental stress and wounds. Infections by these bacteria display massive colonization of the appendages and foregut followed by infection of the midgut, hepatopancreas and a terminal septicemia (Ajitha *et al*., 2004).

Use of commercial antibiotic for disease treatment produces undesirable side effects, which may result in virulence of pathogens and causes for concern in promoting transfer of antibiotic resistance to human pathogens. Currently, a limited number of government approved antibiotics and chemotherapeutic agents are used for prevention and treatment of infectious aquatic diseases (Qi *et al*., 2009).

Therefore, there is an urgent need in aquaculture to develop microbial control strategies, since disease outbreaks are recognized as important constraints to aquaculture production. An alternative prophylactic treatment would be to support the natural non-specific host microbial and therapeutic defense mechanism by administration of live bacteria with demonstrable inhibitory effect upon pathogens as probiotics (Ajitha *et al*., 2004).

The research of probiotics in aquaculture is still at its early stage in Nigeria and data on aquatic indigenous probiotics are not available. Therefore, the aim of the present work was to isolate indigenous strains of *Lactobacillus* from the gut of healthy shrimp (*Penaeus monodon*) and evaluate their antagonistic effect on some isolated pathogenic bacteria from moribund shrimp. This is a part of a long term screening and selecting indigenous probiotic strains from aquatic environment to suit the specific requirement in Nigeria.

Materials and methods

Sample collection

Healthy and moribund shrimp (*Penaeus monodon*) were collected in sterile bags from Asaritoru River, in Amainagbo's creek, in Buguma, Rivers State of Nigeria with the assistance of local fishermen.

Bacterial isolation

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 plated on Man Rogosa (MRS) (Oxoid) (for *Lactobacillus* species) while sub samples of 0.1mL of the dilutions from moribund shrimp were plated on five different media. The media chosen were: Thiosulphate citrate bile salt sucrose (TCBS) agar (Oxoid) (for *Vibrio* species), Salmonella Shigella agar (Fluka) (for *Salmonella* species), Mannitol salt agar (Lab M) (for *Staphylococcus* species), MacConkey agar (BioTech) (for *Escherichia coli*) and Aeromonas medium with 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).

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 *Lactobacillus* L2 and *Vibrio* V2 were mixed and survival determined by plate counting at various time intervals from 0 to 48hours (Chythanya *et al.*, 2002).

Table 1. Antibacterial activity of isolated Lactobacillus spp. against isolated shrimp pathogens.

Lactobacillus spp.	Inhibition Zone (cm) ± S.D.						
	Vibrio sp. V1	*Vibrio* sp. V2	*Aeromonas* sp. A1	*Aeromonas* sp. A2	*Escherichia coli* E1	*Staphylococcus* sp. ST1	*Salmonella* sp. SM1
L1	1.2±0.00	1.6±0.01	1.4±0.02	2.0 ±0.00	2.1 ±0.03	1.0 ±0.01	1.2 ± 0.02
L2	1.8± 0.01	2.2±0.00	1.4±0.00	2.0 ±0.01	2.1± 0.01	1.2 ±0.01	0.9 ± 0.02
L3	-	-	-	-	-	-	-
L4	-	-	-	-	-	-	-

Agar diffusion assay

Antimicrobial activity of four isolates of *Lactobacillus* spp. was carried out against seven target 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 each tested bacterial stains. Exactly 0.1ml of a 24 hour old broth culture of each of the *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.

Effect of Lactobacillus L2 on growth of Vibrio V2 in sterile Nutrient broth

Two 250ml flasks containing 100mL of nutrient broth containing 1.0% sodium chloride was sterilized at 121^{O}C for 15 minutes. Cell suspension of *Vibrio* V2 was then added to all flasks to get a cell density of approximately 1.0 x 10^5 cfu/ml. Cell suspensions of *Lactobacillus* L2 adjusted to 1.0 x 10^6 cfu/ml final cell concentration were added to one flask while the other flask without *Lactobacillus* L2 added served as control. The cultures were incubated at 37^{O}C for 48 hours. *Lactobacillus* L2 and *Vibrio* V2 were enumerated at 0, 12, 24 36 and 48 hour on Man Rogosa Medium (Oxoid) and TCBS agar (Oxoid) respectively by standard spread plate method.

Results and discussion

The antibacterial activity of shrimp pathogens by *Lactobacillus* spp. are shown in Table 1 and Figure 1. A total of four bacterial strains identified as *Lactobacillus* L1, *Lactobacillus* L2, *Lactobacillus* L3 and *Lactobacillus* L4 were isolated from healthy shrimp gut. Seven pathogenic isolates from moribund shrimp (*Penaeus monodon*) gut were identified as *Vibrio* sp. V1, *Vibrio* sp. V2, *Aeromonas* sp. A1, *Aeromonas* sp. A2, *Escherichia coli* E1, *Staphylococcus* sp. ST1, and *Salmonella* sp. SM1. *Lactobacillus* sp. L1 and *Lactobacillus* sp. L2 produced inhibition zones higher than 8mm and against all the pathogenic strains employed while *Lactobacillus* L3 and *Lactobacillus* L4 had no antibacterial activity against the pathogens. 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).

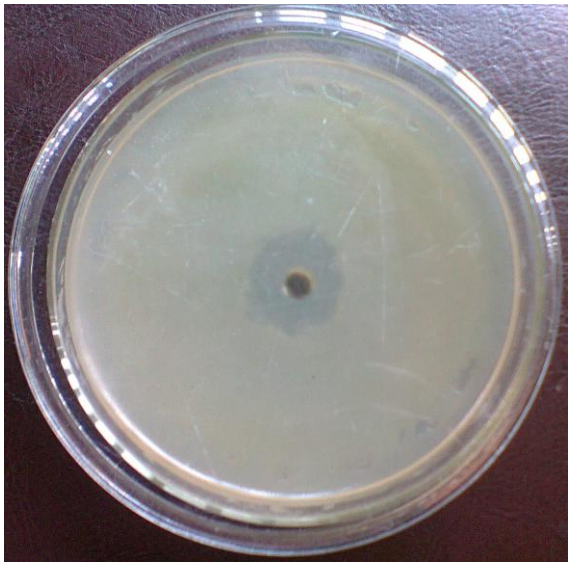

Fig. 1. Inhibition zone of *Lactobacillus* sp. L2 against *Vibrio* sp. V2.

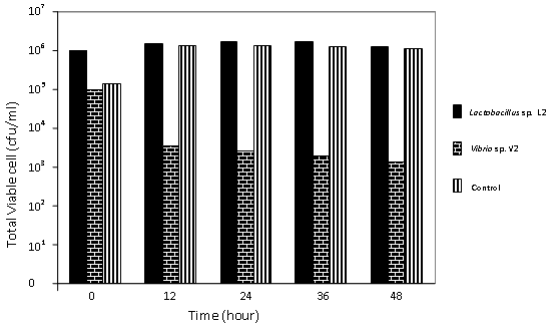

Fig. 2. Effect of *Lactobacillus* sp. L2 at 1.0 x 10^6 cfu/ml on growth of *Vibrio* sp. V2 in nutrient broth containing 1% sodium chloride.

The growth of *Vibrio* sp. V2 and other shrimp pathogens used in this study were inhibited by *Lactobacillus* spp. because Lactobacilli have been found to produce metabolic products that play an important role in controlling undesirable microflora in the gut (Itoh *et al*., 1995). Various strains of *Lactobacillus* species have been reported to be effective antagonists against *Vibrio* and *Aeromonas* spp. in both *in vitro* and *in vivo* studies (Dhanasekaran *et al*., 2008; Joborn *et al*., 1997; Ajitha *et al*., 2004). In this study also *Lactobacillus* spp. L1 and L2 isolated from healthy shrimp gut were found to have *in vitro* antimicrobial activity against *Vibrio* sp. V2 and other shrimp pathogens.

The inhibition of *Vibrio* sp. V2 (1.0 x 10^5) cfu/ml) by *Lactobacillus* sp. L2 (adjusted to 1.0 x 10^6 cfu/ml final cell concentration) in nutrient broth containing 1.0% sodium chloride is shown in Figure 2. The *Lactobacillus* L2 could inhibit *Vibrio* sp. V2 growth within 12 hours. It was found that the concentration of *Vibrio* sp. V2 was constantly reducing but within 10^3 cfu/ml) until 48hours. For the control an increase of *Vibrio* sp. V2 was observed from about 10^5 to 10^6 cfu/ml. Cell free extracts of four strains of lactic acid bacteria (LAB)- *Lactobacillus acidophilus*, *Streptococcus cremoris*, *Lactobacillus bulgaricus*-56 and *Lactobacillus bulgaricus* -57 inhibited growth of *Vibrio alginolyticus* in nutrient broth (Ajitha *et al*., 2004). In this study whole cell of two strains of *Lactobacillus* L1 and L2 also inhibited the growth of *Vibrio* sp. V2 in nutrient broth.

There are reports about the antibacterial activity of LAB against Gram positive bacteria (Piard and Desmazeaud, 1992). Lewus *et al*. (1991) showed the activity against *Aeromonas hydrophila* of 19 LAB strains including *Carnobacterium piscicola* and *Lactobacillus plantarum*.

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 in now gaining importance in fish and shrimp farming 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).

Lactobacillus L1 and L2, isolated in this study, had the inhibitory property of a biocontrol agent for use in control of shrimp pathogens 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 strains, the species identification and

optimization of *Lactobacillus* growth are going on in our laboratory.

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