ISSN 2349-7203

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals

HUMAN

Human Journals
Research Article
March 2015 Vol.:2, Issue:4

Investigation on Virulence Dose and Antagonistic Activity of Selected Probiotics against *Aphanomyces invadans* and *Aeromonas hydrophila*

ISSN 2349-7203

IJPPR
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Submission: 28 February 2015
Accepted: 7 March 2015
Published: 25 March 2015

Keywords: *Aphanomyces invadans, Aeromonas hydrophila* , EUS, Probiotics, LD50

ABSTRACT

The use of antibiotics to prevent and control bacterial diseases in aquaculture, has led to an increase in antibiotic-resistant bacteria. In the present study the lethal dose (LD_{50}) of *Aeromonas hydrophila* and *Aphanomyces invadans* in *Heteropneustes fossilis* was determined and *in vitro* antimicrobial susceptibility of probiotics against *A. invadans* and *A. hydrophila* isolates was also assessed. Isolated *A. invadans* and *A. hydrophila* were injected into test fishes which showed slight to severe dermomuscular lesions. *H. fossilis* infected with *A. invadans* at 10^8 cfu/ml and 10^7cfu/ml showed cent percent mortality. They produced severe necrotic lesions in infected tissues and at the end of the trial they lost the layer of skin and all the individuals succumbed. Similarly, *A. hydrophila* (10^6cfu/ml) injected fishes showed 89.47 % mortality and severe lesions and wound were noticed in the infected portions. The injured tails appeared reddish in colour and loss of skin layer was observed. The determined LD_{50} for *A. invadans* was 7.9 x 10^5 cfu/ml and for *A. hydrophila* was 2.4 x 10^6cfu/ml. The highest zone of inhibition was recorded by *B. subtilis (*12 ± 0.2 mm) followed by *B. coagulans* (10 ± 0.7 mm), *L. acidophilus* (9 ± 0.3 mm), *S. cerevisiae* (4 ± 0.7mm), *P. fluorescens* (2 ± 0.5 mm) and *B. licheniformis* (2 ± 0.2 mm) against *A. hydrophila*. In case of *A. invadans*, the highest zone of inhibition was recorded against *B. subtilis* (7 ± 0.6 mm) followed by *B. coagulans* (6 ± 0.5 mm) and *L. acidophilus* (5 ± 0.8 mm).

INTRODUCTION

Epizootic Ulcerative Syndrome (EUS) is one of the most important problems in aquaculture. Dhanaraj *et al.* [1] reported *Aphanomyces invadans* as the primary causative agent of EUS and the pathogenicity of *Aeromonas hydrophila* consistently associating with EUS affected fish [2]. Dykstra *et al.*[3] isolated *Aphanomyces* from ulcerative mycosis affected fish in the Eastern USA. Hatai [4] noticed fish mortality and reported the susceptibility and resistance of 11 species of fish against *Aphanomyces* infection, while Khan *et al*. [5] showed progressive histopathological changes in tilapia (*Oreochromis niloticus*), rosy barb (*Puntius schwanenfeldi*), rainbow trout (*Oncorhynchus mykiss*), roach (*Rutilus rutilus*) and stickleback (*Gasterosteus aculeatus*) to demonstrate differential host susceptibility to the fungus.

Byers *et al*. [6] have shown that *A. hydrophila* can produce siderophores that confer resistance against the ability of serum transferrin to inhibit bacterial growth. Many studies have been attempted further to describe the virulence mechanisms of motile aeromonads. Kou [7] found that many of the virulent, avirulent, and attenuated aeromonads possessed hemorrhagic factors and lethal toxins. The virulent bacteria had quantitatively more toxic potential than the avirulent or attenuated counterparts.

The use of antibiotics to prevent and control bacterial diseases in aquaculture, have led to an increase in antibiotic-resistant bacteria [8-9]. The discovery and development of antimicrobial agents to treat systemic bacterial infections is one of the most fascinating stories in the history of microbiology [10]. There have been many studies aimed at developing effective prophylactic methods for use in aquaculture as alternatives to chemotherapy [11-12]. Hence, alternative strategies such as probiotics have been proposed as biological control agents.

Probiotics administered in the form of feed additives have shown improvement in the intestinal microbial balance and the health status by colonizing the gut and acting as antagonists to pathogens by increasing resistance to pathogens [13-15]. Plumb [16] reported vaccines cannot completely eliminate pathogens or prevent the target organisms from being present in vaccinated populations. Thus, in order to treat the pathogen, several antimicrobial agents such as amoxacillin, ampicillin, chloramphenicol, erythromycin, flumequine, oxolinic acid, oxytetracycline, nitrofurazone, sulphadiazine-trimethoprim and tetracycline [17-19] have been

used. However, over the last decade, drug-resistant strains carrying a transferable R-plasmid have developed [20-21] making treatment with antimicrobial chemotherapeutics less successful. For a treatment to be effective, antimicrobial susceptibility experiments should be carried out to evaluate the susceptibility and resistance development to antimicrobial agents. The present study was designed to determine the lethal dose (LD_{50}) of *A. hydrophila* and *A. invadans* on healthy catfish, *Heteropnuestes fossilis* and to evaluate and compare the *in vitro* antimicrobial susceptibility using selected probiotics against *A. invadans* and *A. hydrophila* isolates.

MATERIALS AND METHODS

Isolation of *Aphanomyces invadans*

Infected *H. fossilis* with moderate, pale, raised, dermal lesions were selected for the study. The scales around the peripheral portion of the lesion were removed and the underlying skin was seared using a sterile spatula for surface sterilization. Using a sterile scalpel blade and sterile fine-pointed forceps, a piece of muscle (2 mm) was cut underlying the seared area and placed on a petridish containing CzapekDox agar with penicillin G (100 units/ml) and oxolinic acid (100 ug/ml) [22]. The plates were sealed, incubated at room temperature and examined daily. The emerging hyphal tips were repeatedly transferred to fresh plates of CzapekDox agar until cultures are free of contamination. The mother culture was examined daily with microscope for at least 5 days and subculture was maintained. Recovered fungi were identified by sporulation features, hyphal diameter, growth rate at 22°C and failure to grow at 37°C.

Aeromonas hydrophila growth studies

A. hydrophila was easily cultured using *Aeromonas* isolation agar. Growth of the bacterial cells was measured by direct count using Haemocytometer and total plate count method [23]. The number of cells was calculated after measuring the sample intensity or cell count at intervals of 0, 3, 6, 9, 12, 15, 18, 24, 48 and 72 h after inoculation of the cells in the fresh medium and the cells were harvested by centrifugation at 5000 rpm for 15 min. The pellet was serially diluted and total count was taken using Neubaur counting chamber. For viable count, 0.1 ml from the dilution was spread plated on agar plates, incubated at 37^0C for 24 h and the colonies were counted.

Determination of LD_{50}value of *A. invadans* and *A. hydrophila* on *H. fossilis*

H. fossilis of average length 20 ± 3 cm and average weight 65 ± 2.5g were randomly selected and distributed into 3m x 1.5m x 1m cement tank filled with well water at the stocking rate of 10 fingerlings per tank separately for *A. invadans* and *A. hydrophila* treatments. Triplicates were maintained for each treatment for a period of 10 days and mortalities were recorded. To find out the LD_{50} value of *A. invadans* (viable spores) and *A. hydrophila*, 18 h old broth culture (logarithmic phase) containing different loads of bacteria in physiological saline (0.85 % NaCl; pH 7.2) were inoculated intraperitoneally. Ten fishes were administered with the dose of *A. invadans* (10^2 to 10^8) and *A. hydrophila* (10^3 to 10^9) cells per 0.2 ml. The LD_{50} value was calculated following Reed and Muench [24]. The fishes were observed carefully for visible external symptoms and behavioral changes. Time taken to lose the balance and the individual death were noted. The fishes were considered to be dead when there was no opercular movement. The mortality of the challenged fish was recorded and death due to *A. invadans* and *A. hydrophila* was confirmed by re-isolation of organism from the liver, spleen, body fluids and intestine.

Antagonistic activity of probiotic bacteria against *A. invadans* and *A. hydrophila*

Antagonistic activity of *Bacillus subtilis, B. coagulans, B. licheniformis, Saccharomyces cerevisiae, Pseudoomonas fluorescens* and *Lactobacillus acidophilus* against target fungi *A. invadans* and bacteria *A. hydrophila* were assessed by well diffusion assay. For *A. hydrophila*, antagonistic activity was performed using the plates containing solidified Muller Hinton agar (20 ml) and inoculated with 0.5 ml of overnight culture of *A. hydrophila* (10^6cfu/ml). A well having six mm diameter was made in the agar using cork borer and 50 µl of culture supernatant of *B. subtilis, B. coagulans, B. licheniformis, S. cerevisiae, P. fluorescens*and *L. acidophilus* were transferred into each well. The bacterial plates were incubated for 18 h at 37^0C in aerobic environment and width of the zone of incubation (mm) was measured [25]. Similarly for *A. invadans*, CzapekDox Agar was used and the inoculated plates were incubated at 25^0C for 72h. The MIC were observed and recorded.

Statistics

Values are given as mean and ± indicates standard deviation which was calculated using Microsoft excel software.

RESULTS AND DISCUSSION

A. invadans and *A. hydrophila* injected test fishes showed slight to severe dermomuscular lesions. *A. invadans* concentrations of 10^8 cfu/ml and 10^7 cfu/ml injected fish showed cent percent mortality. They produced severe necrotic lesions in infected tissues and at the end of the trial they lost the layer of skin and all the individuals died. *A. hydrophila* (10^6 cfu/ml) injected fishes showed 89.47 % mortality, severe lesions and wound were noticed in the infected portions. The injured tails were reddish in colour and loss of skin layer was observed. 10^5 cfu/ml dose injected fish showed 56.25 % cumulative mortality. They showed slight lesions and swelling on the infected portion. No mortality was found in 10^2 cfu/ml and 10^3 cfu/ml concentration injected fishes. The determined LD_{50} for *A. invadans* was 7.9 x 10^5 cfu/ml (Table 1).

A. hydrophila injected fishes showed reddening and swelling at the site of infection and changes were noticed at 7 h and 12 h with 10^9 cfu/ml, 10^8 cfu/ml and 10^7 cfu/ml concentrations with mortalities of 100 %, 96.66 % and 83.33 % respectively. Initially, slight lesions were produced which turned like a blanched area along with slight swelling followed by deep lesions. In 10^6 cfu/ml dose injected fishes, 59.09 % mortality was observed. The determined LD_{50} for *A. hydrophila* was 2.4 x 10^6cfu/ml (Table 2). No mortality was found in 10^3 cfu/ml injected fishes but swelling and mild lesion were observed.

The present study was attempted to find out the antagonistic activity of selected probiotics against EUS causative pathogens *A. invadans* and *A. hydrophila*. *B. subtilis, B. coagulans, S. cerevisiae, B. licheniformis, P. fluorescens* and *L. acidophilus* exhibited zones of inhibition against *A. hydrophila* (10^6 cfu/ml) and *B. subtilis, B. coagulans* and *L. acidophilus* exhibited zones of inhibition against *A. invadans* (10^5cfu/ml). The highest zone of inhibition was recorded for *B. subtilis (*12 ± 0.2 mm) followed by *B. coagulans* (10 ± 0.7 mm), *L. acidophilus* (9 ± 0.3 mm), *S. cerevisiae* (4 ± 0.7mm), *P. fluorescens* (2 ± 0.5 mm) and *B. licheniformis* (2 ± 0.2 mm) against *A. hydrophila*. In case of *A. invadans*, the highest zone of inhibition was recorded by *B. subtilis* (7 ± 0.6 mm) followed by *B. coagulans* (6 ± 0.5 mm) and *L. acidophilus* (5 ± 0.8 mm) (Table 3). B. *licheniformis, S. cerevisiae* and *P. fluorescens* didn't produce zones of inhibition against *A. invadans*. In the case of *B. subtilis, B. coagulans* and *L. acidophilus* zones of inhibition were observed against both pathogens *A. invadans* and *A. hydrophila*.

The present study showed differences in susceptibility of *H. fossilis* to *A. invadans* and *A. hydrophila*. *A. invadans* and *A. hydrophila* have been consistently associated with EUS and the pathogenicity of EUS susceptible fish has already been reported [2, 26]. In the present study, *A. invadans* injected test fish at concentrations of 10^8 cfu/ml and 10^7 cfu/ml showed 100 % mortality. The LD_{50} for *A. invadans* was 7.9 x 10^5 cfu/ml. Hatai [4] injected goldfish with 5000 spores/fish while Wada *et al*. [27] injected common carp, *C. carpio* with 3000 spores. However, initial natural challenges are unlikely to be of such magnitude under field conditions and therefore, they developed a method of reproducing EUS which uses more realistic numbers of infective zoospores of *A. invadans* given by intramuscular injection at approximately the same and they found mortality at higher level. *A. invadans* (10^5 cfu/ml) administered fishes showed moderate, pale, raised, dermal lesions. Similarly, Kiryu *et al*. [28] reported the cyst stages are infectious and they attach to the intact skin and produce germination tubes that penetrate the skin and produce lesions.

In the present study, severe lesions were observed following *A. hydrophila* administration in 10^9cfu/ml during the LD_{50} assay. Similarly, Lio-po*et al*.[29]stated *A. hydrophila* injected intramuscularly at a concentration of 10^9 cfu/ml induced severe dermomuscular necrotic lesions in both catfish and snakeheads. Khali and Mansour [30] found that *A. hydrophila* was found to produce haemolytic and proteolytic exotoxin, that are lethal to tilapia and the LD_{50} value was 2.1 x 10^4 cells/fish. The lethal effect was also attributed to the unknown virulent factors that were responsible for 20% mortality. Lipton [31] observed that *P. aeruginosa* had a lethal dose of 1.5 x 10^5 cfu/ml for *C. carpio* and 4.2 x 10^5 cfu/ml for *O. mossambicus* and *A. hydrophila* had 2.1 x 10^6 cfu/ml and 3.2 x 10^6 cfu/ml for *C. carpio* and *O. mossambicus* respectively.

In the antagonistic study, *B. subtilis* (7 ± 0.6 mm) and *B. coagulans* (6 ± 0.5 mm) showed maximum zones of inhibition against *A. invadans*. Similarly, Matsumo *et al.* [32] and Shigeru *et al.* [33] reported that many species of *Bacillus* are capable of producing biologically active substances which are able to disintegrate fungal cell walls. Podile and Parkash [34] examined *B. subtilis* strain AF1 which produced extracellular protein that disintegrates fungal cell walls by lysis of chitin. Similar results were obtained by Gulewicz and Trojanowska [35], who observed the susceptibility of *Aspergillus niger* when *B. subtilis* AF1 was added within 12 h of the growth of fungi. Itami *et al*.[36] reported that *Bacillus*sp surface antigens or their metabolites act as

immunogens for shrimp by stimulating phagocytic activity of granulocytes. Gulewicz and Trojanowska [35] isolated active lupine from strains of the *Bacillus* sp, the majority of which showed antifungal properties.

In the present investigation, *L. acidophilus* (5 ± 0.8 mm) showed minimum inhibition against *A. invadans* when compared with *B. subtilis* and *B. coagulans.* Among the probiotics, *B. licheniformis, S. cerevisiae*and *P. fluorescens* did not produce zone of inhibition against *A. invadans* whereas, *B. subtilis, B. coagulans, L. acidophilus, S. cerevisiae, B. licheniformis* and *P. fluorescens* produced zones of inhibition against *A. hydrophila.* Among the probiotics, *B. subtilis* (12 ± 0.2 mm) and *B. coagulans* (10 ± 0.7 mm) produced maximum zone of inhibition against *A. hydrophila*. *Bacillus sp has* produced secondary metabolite of extracellular compounds such as bacteriocin, hydrogen peroxidase and other organic acids [37-38] and also inhibited pathogenic bacteria in fish and shellfish by successful colonization in the gut of the host [13,39]. Skjermo and Vadstein [40] and Rengipipat *et al*.[41] also reported that *Bacillus* spores have been used as biocontrol agents to reduce *Vibrio* sp in shrimp culture practices. Vaseeharan and Ramasamy [42] investigated the inhibitory activity of *B. subtilis* BT23, isolated from shrimp culture ponds, against pathogenic *Vibrio harveyi* under *in vitro* and *in vivo* conditions.

In the present study, *L. acidophilus* showed maximum zone of inhibition (9 ±0.3mm) against *A. hydrophila.* Similarly, Manohar [43] has found the maximum antagonistic activity of *L. bulgaricus* (5.5 ± 0.6 mm) and *L. acidophilus* (5.3 ± 0.5 mm) against *A. hydrophila*. Griffith [44] reported the effect of Lactic Acid Bacteria in increasing disease resistance to *Vibrio* pathogens in shrimps. Mishra and Lambert [45] also reported the maximum antagonistic activity of *Lactobacillus* sp against different pathogenic organisms like *Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis.*

Several bacterial strains which are common members of the non-pathogenic microflora of fish are capable of inhibiting fish pathogenic bacteria and fungi in *in vitro* assay and this has been demonstrated for lactic acid bacteria by Gatesoupe [46] and Joborn *et al*. [47]. Smith and Davey [48], Austin *et al.* [49], Moriarty [50] and Gram *et al*. [51] have found that the addition of antagonistic bacteria to the water results in reduction of number of fish pathogenic bacteria in water. Gram *et al*. [51] reported that *P. fluorescens* AH2 was strongly inhibitory against *Vibrio anguillarum* in model systems and this effect could be transferred to an *in vivo* situation. Further,

they observed significantly reduced mortality in the experimental fish infected with *V. anguillarum* following the addition of probiotics to the tank water. Indeed there has already been intensive research on probiotics for use in aquaculture [46,50]. Probiotics for human and terrestial animals are mainly lactic acid bacteria (LAB) of different species and *Bacillus* sp [39, 52-54].

In our previous studies carried out at our CARE centre, probiotics incorporated feed improved the health status of the fresh water air breathing fish *Channa striatus* [55]. The growth as well as the survival of *C. striatus* was enhanced against the fish pathogen *A. hydrophila*. Similarly, Dhanaraj *et al.* [56] reported the enhanced growth performance of Koi Carp (*Cyprinuscarpio*) fed with probiotics. Juvenile common carp (*Cyprinus carpio)* fed with the diet containing probiotics and/or spirulina also showed increased survival with high growth rate when compared to the control group [57].

CONCLUSION

All fishes injected with high concentration of *A. invadans* showed mortality whereas for *A. hydrophila* severe lesions were noticed at the site of injection. Lower doses did not significantly kill the fish. Among the probiotics, *B. subtilis, B. coagulans* and *L. acidophilus* only produced zone of inhibition against *A. invadans* and *A. hydrophila*. Hence, these three probiotics can be chosen for further studies on growth performance and disease challenge of *H. fossilis*. Further studies to explore the mechanism of action of the pathogens as well as the probiotics are essential for the extensive use of these probiotics in large scale aquaculture industry.

ACKNOWLEDGEMENT

We acknowledge the financial assistance received from UGC (F6-6/2012-2013/Emeritus.2012-2013-OBC-675/(SA-II) dt.25.04.2013) to carry out this study. We are grateful to the Principal, St. Xavier's College (Autonomous), Palayamkottai, for providing necessary facilities.

Table 1 Determination of LD_{50} for virulent *Aphanomyces invadans* in *H. fossilis* by intraperitonial route (Reed and Muench, 1938)

No. of fungal spore (cfu/ml)	Initial number	Died	Survived	Death ratio	Survival ratio	Mortality	Cumulative mortality (%)
10^8	10	10	0	37	0	37/37	100.00
10^7	10	10	0	27	0	27/27	100.00
10^6	10	8	2	17	2	17/19	89.47
10^5	10	5	5	9	7	9/16	56.25
10^4	10	4	6	4	13	4/17	23.52
10^3	10	0	10	0	23	0/23	0.00
10^2	10	0	10	0	33	0/33	0.00

$$\text{Proportionate distance} = \frac{\text{Mortality above 50\% - 50}}{\text{Mortality above 50\% - Mortality below 50\%}}$$

$$56.25 - 50.00 \,/\, 56.25 - 23.52$$

$$6.25 / 32.73 = 0.19$$

LD_{50} = Dilution above 50% - Proportionate distance
= 5 + 0.19 = 5.19

Antilog 5.19 = 7.9×10^5

LD_{50} = 7.9×10^5

Table 2 Determination of LD_{50} for virulent *Aeromonas hydrophila* in *H. fossilis* by intraperitonial route (Reed and Muench, 1938)

No. of bacterial cells (cfu/ml)	Initial number	Died	Survived	Death ratio	Survival ratio	Mortality	Cumulative mortality (%)
10^9	10	10	0	39	0	39/39	100.00
10^8	10	9	1	29	1	29/30	96.66
10^7	10	7	3	20	4	20/24	83.33
10^6	10	5	5	13	9	13/22	59.09
10^5	10	5	5	8	14	8/22	36.36
10^4	10	3	7	3	21	3/24	14.28
10^3	10	0	10	0	31	0/31	0.00

$$\text{Proportionate distance} = \frac{\text{Mortality above 50\% - 50}}{\text{Mortality above 50\% - Mortality below 50\%}}$$

$$59.09 - 50 \,/\, 59.09 - 36.36$$

$$9.09 / 22.73 = 0.39$$

LD_{50} = Dilution above 50% - Proportionate distance

= 6 + 0.39 = 6.39

Antilog 6.39 = 2.4×10^6

LD_{50} = 2.4×10^6

Table 3 Antimicrobial activities of probiotics against *A. hydrophila* and *A. invadans* by agar well diffusion method.

Probiotics	Zone of inhibition	
	A. hydrophila	***A. invadans***
B. subtilis	12 ± 0.2mm	7 ± 0.6mm
B. lichniformis	2 ± 0.2mm	-
B. coagulans	10 ± 0.7mm	6 ± 0.5mm
L. acidophilus	9 ± 0.3mm	5 ± 0.8mm
S. cerevisiae	4 ± 0.7mm	-
P. fluorescens	2 ± 0.5 mm	-

Values are given as mean and ± indicates standard deviation

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