Study on the effect of dietary probiotic bacteria Arthrobacter species, *β*-1,3 glucan and *Moringa oleifera* leaf on protection of *Penaeus indicus* Juveniles from pathogenic *Vibrio harveyi*

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Abstract: A strain of Arthrobacter sp., isolated from *Penaeus indicus* culture water was evaluated for potential use as a probiotic for shrimp Juveniles comparing with β-1,3 glucan and Moringa *oleifera* leaf protecting *Penaeus indicus* from pathogenic vibrios. In vitro studies demonstrated antagonism against *V. harveyi*. *Penaeus indicus* Juvenile divided into four groups 150 each with three replicates. First three groups supplied with Arthrobacter sp. ($1x10^8$ CFU/ml), *β*-1,3 glucan (10gm/kgm diet) and *Moringa oleifera* leaf (10mg/kg diet) were added to diet of shrimp for 4 weeks while 4th group given a basal diet without any additives. During the 5 day after the experiment challenge experiment was started the shrimp Juvenile were reared in water with addition of *V. harveyi*. The cumulative mortality was recorded for the whole challenge experiment. Both Arthrobacter sp and *β*-1,3 glucan are able to protect the shrimp Juvenile from the pathogenic vibrios, *Moringa oleifera* leaves also can protect shrimps but Arthrobacter sp and *β*-1,3 glucan improve the non – specific immune response, weight gain, immunological and physiological state. Arthrobacter sp. can be regarded as a probiotic bacterium for the culture of shrimp while *β*-1,3 glucan and, *Moringa oleifera* leaf were considered as immunostimulants for cultured of shrimp *Penaeus indicus* Juvenile against pathogenic vibrios Juvenile.

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1. Introduction

Vibriosis is a systemic disease of marine, estuarine and some freshwater fishes, caused by bacteria of the genus *Vibrio.* The disease has been known for centuries;Vibriosis is one of the major disease problems in shellfish and finfish aquaculture. It is a bacterial disease responsible for mortality of cultured shrimp worldwide (Adams, 1991;Lightner *et al*., 1996). Vibrio species are widely distributed in culture facilitates throughout the world. Numerous Vibrio species have been reported as pathogenic bacteria to various penaeid shrimps (Chanratchakool *et al*., 1995; Alapide-Tendencia and Dur- eza, 1997**)**.

Usage of probiotic bacteria as a better remedy than administering antibiotics is accepted for the control of pathogens in aquaculture. Probiotic bacteria were used in shrimp larviculture and fish hatchery, including Lactobacillus spp. (Gatesoupe, 1991; Garcia-de-la-Banda *et al*., 1992; Li *et al.,*2006; Pope *et al.*,2011), Bacillus spp. (Rengpipat *et al*., 2000), The antagonism mechanism, based on the removal of the pathogen by the beneficial population, (Fuller, 1989; Gatesoupe, 1999). probiotic strains were able to improve the water quality through degradation of waste organic matter and heavy metals removal **(**El-Rayes 2012). Some studies primarily have attributed the enhancement of animal growth to the nutritional benefits of probiotic bacteria, such as vitamin production, availability of minerals and trace elements, and production of important digestive enzymes (Holzapfel *et al.,* 1998).

There were reports of Arthrobacters producing antimicrobial compounds (Hentschel *et al.,* 2001; Li et al.,2006). Some studies have found that Arthrobacter sp. can use a wide and diverse range of organic substances as carbon and energy sources including nicotine, nucleic acids and various herbicides and pesticides (Hagedorn and Holt, 1975). As reviewed by Abrashev *et al.* (1998)**,** some Arthrobacter species have the ability to produce a number of valuable substances like amino acids, vitamins, enzymes, specific growth factors, and polysaccharides. So arthrobacter sp. possess many advantageous properties and nutritional benefits to be probiotics in aquaculture (Li *et al*., 2006).

β-glucan has been shown to increase host resistance to a diverse range of microbial pathogens, stimulate anti-tumour mechanisms and is also immunostimulatory (Di-Luzio, 1985; Seljelid *et al.,* 1987**).** The stimulation of non-specific and specific immunity, and increased protection against microbial infections in healthy and stressed fish after glucan exposure have been demonstrated in many

aquaculture species (Anderson, 1992; Sahoo & Mukherjee (2001).

Plant oil from seeds and leaves such as *Moringa oleifera* are in high demand for their medicinal value. Apart from the medicinal uses, *Moringa oleifera* was reported to be a good source of vitamins and amino acids **(**Olugbemi *et al*; 2010). *Moringa oleifera* was claimed to boost immune systems (Fuglier, 1999; Olugbemi *et al.,* 2010). *Moringa oleifera* leaf powder was reported to have antibacterial properties and conclusion was made to investigate it as a phytotherapeutic agent to combat infectious agents (Patel, 2011; Ogbe and Affiku,2011).

The aim of the present study is to investigate the antibacterial ability of Arthrobacter sp. against two pathogenic vibrios strains, including *V. harveyi*., in of *Penaeus indicus* Juvenile comparing with *β*-1,3 glucan and *Moringa oleifera* leaf powder as medicinal plant.

2. Materials and methods:

Bacterial strains:

The Arthrobacter sp. used in this study was isolated on Marine Agar (MA) from *P. indicus* culture water sample collected from Red Sea, Jeddah, Sudia Arabia. This organism was identified as *Arthrobacter globiformis*. (Irregular rods with V-shape and clubbed ends) based on the characteristics described in the Bergey's Manual of Systematic Bacteriology. The Arthrobacter was used as inducing anti-bacterial substances for *V. harveyi*. in inhibition experiments in vitro. Among challenge experiment, protection of *P. indicus* Juvenile against *V. harveyi* by Arthrobacter was evaluated. The pathogenic *V. harveyi* was isolated from diseased *P. indicus* died with (red leg) disease, The pathogenic vibrios was used as target bacteria for inhibition experiments in vitro and for inducing infection during the challenge experiment in vivo.

Culture of bacteria:

Arthrobacter sp. and pathogenic *V. harveyi*. were cultured in marine broth (MB) and incubated at 27 ^{o}C for 24 h, cell cultures were suspended in sterile, saline solution (0.8%), washed and centrifuged three times at 4 ^{o}C 800 rpm for 15 min (Li *et al*.,2006)..

Inhibition experiment in vitro:

The inhibition experiments in vitro were carried out using method reported by Li *et al*.(,2006) The culture of *V. harveyi*. strain was prepared by pouring 0.1 ml of a young culture (16–18 h in marine broth MB, about 1x10^{7} CFU/ml) on the marine agar then air drying the plate in the incubator (27 ^{o}C) for15 min. Then, the test strain (Arthrobacter sp.) was scratched and dotted on the medium using the general inoculator. After the plates were incubated at 27 ^{o}C for 24 h, clear inhibitory zones were observed around the Arthrobacter sp. zones of inhibition was measured.

Prepratation of *Moringa oleifera* leaf powder:

Moringa oleifera leaves were harvested from an orchard at early flowering stage. Stem and branches were cut from Moringa trees and spread out under the shade to dry in the sun at 35^{O}C for 5 days. The leaves were then removed by hand and grounded into powder by milling using a Miller machine (Ogbe and Affiku, 2011).

Experimental design:

Healthy shrimp *P. indicus* Juvenile 18-22 gm weight were obtained from a commercial hatchery along the Red Sea coast in Jeddah, Sudia Arabia. The shrimp were held in one tank before the experiment. Acclimated for 1 week, then divided into 12 experimental groups, each containing 150 *P.indicus* Juveniles, they were placed into 12 tanks each. Four treatments with three replicates for each. 1st group (G1) supplied with diet with Arthrobacter IFO 12137 (1x10^{8} CFU/ml) 2nd group (G2) supplied a diet with β-1.3.Glucan (10 gm/kgm diet) (Sahoo and Mukhrjee 2001). 3rd group (G3) supplied a diet with *Moringa oleifera* leaves powder (50 gm/kg diet) (Patel, 2011**)**. While 4th group (G4) supplied a basal diet without any additions. (Li *et al*.,2006).

Pathogen challenge test:

After feeding for three weeks, shrimp were weighted challenged with *V. harveyi* which had been cultured and maintained using thiosulphate citrate bile salt TCBS broth and agar (Difco). Shrimp in the control and treated groups (150 shrimp per treatment) were immersed in a suspension of *V. harveyi* at 1x10^{7} CFU ml^{-1} according to Austin et al. (1995). Shrimp survival was recorded after 5 days of challenge. At the same time, three shrimp from each treatment were randomly sampled. The whole shrimp was cut into small pieces using sterile surgical sciccors and transferred to a sterile tube. Bacterial determinations were then made using serial dilutions, followed by plating on TSA and TCBS agar. After 24-48 h of incubation at 37^{O}C, colonies were counted and recorded. Shrimp survival and weight gain were determined for each treatment after 5 days of the challenge. Shrimp were reared in a closed,

continuously aerated water system, and were fed three times daily with a standard commercial diet at about 4% body weight. The challenge experiment was conducted as a completely randomized design. (Li *et al*.,2006).

Lysozyme determination:

Lysozyme determination carried out according to method described by Tahir and Secombes (1996) briefly Hemolymph shrimp is collected with syringe 2 ml volume, (at first abdomen), by using 26 Gauge hypodermic needle, settled for 1 hour or more at room temperature. Hemolymph was centrifuged
5000 rpm, for 5 minute, later on supernatant serum was collected. Vibrio is dissolved in Buffer citrate (Phosphate Buffer Saline (PBS), 0,1 M, pH:7,8,Buffer: Buffer citrate + 0,09% Na Cl). (1-3%). HEWL (Sigma) Hen Egg White Lysozyme dissolved in Buffer citrate saline with dose: 60,50,40,30,20,0 µg/ml. Shrimp "serum" is taken 25 µl (in"duplicate") and was put into "Wells" plate 96-well and so it is with standard solution in duplicate. "serum" and standard solution then enhanced 175 µl substrats (*.Vibrio harveyi)* Gaug above "micprolate reader" at wavelength 450 nm with pattern kinetic. Standard Curve is made and dose calculation is calculated according to standard equation, with linear regression calculation Rantetondok and Rukminasari (2010).

Phagocytosis activity:

Haemolymph (0.5 ml) was drawn from the ventral sinus of the shrimp into a 5 ml. syringe containing K-199 medium supplemented with 2.5% L-cysteine as an anticoagulant. The haemocytes were collected and washed twice with K-199, by centrifugation at 1.000 rpm, 4°C for 5 minutes. Haemcytes ($1x10^5$ cells/ml) were mixed with fluorescent labeled latex beads (polysciences, Inc. Warrington) $1x10^7$ beads/ml) in K-199 on glass disc placed in a well plate. After incubation at 25°C for 1 h. fixed in haemocytes with 20% glutaraldehyde. Non-phagocytised latex beads were removed by washing with 0.15 MNaCl and distilled water. Phagocyte monolayer was stained with Giemsa stain, rinsed with distilled water and air- dried. The number of cells which had ingested beads and the total number of beads ingested were counted from 200 cells observed using fluorescent microscope (Raa et al.,1992). The following parameters were calculated:

phagocytosis percentage = No of cells ingesting beads/ No of cells observed x 100

Phagocytic index = No of cells ingesting beads / No of cells observed x Number of beads ingested/ number of cells observed x 100

Bactericidal activity:

Vibrio harveyi was cultured in tryptic soy broth with 1.5% NaCl overnight at 25°C. bacteria was collected by centrifugation and washed once in 2% sterile saline then diluted with saline to obtain the bacterial suspension at optical density of 0.1 (540 nm). The haemolymph was prepared by centrifugation at 9.700 rpm for 20 min. with 2.5% L-cystein (anticoagulant) then 100 µl of bacterial suspension was incubated with 100 µl of cell free haemolymph. The samples were incubated in sterile microtube for 3 h. at 25oC. Aliquots of 100 µl were taken from each microtube and spread onto thiosulphate citrate bile salts agar (TCBS) plates in order to count the colony forming untis (cfu) (Adams,1991). the positive control were bacteria suspensded in saline incubated in K-199 with 2.5% L-cysteine (Song and Huang, 1999).

Percentage inhibition = +ve control cfu – sample cfu / +ve control cfu / 100

Statistical analysis:

Results were analyzed using the analysis of variance (ANOVA) and LSD's multiple range test ($p \leq 0.05$) using the SPSS Program.

3.Results

Inhibition experiment in vitro

Arthrobacter globiformis IFO 12137 grown on the MA and there were zones around the spots of *Arthrobacter globiformis* IFO 12137. The zone diameters of the inhibition for *V. harveyi*. was 3.67 cm, respectively. The results indicate that *Arthrobacter globiformis* IFO 12137 inhibits these pathogenic vibrios in vitro.

Survival of shrimp Juvenile:

As shown in table 1, there were significant (ANOVA, $p \leq 0.05$) differences in the survival of shrimp Juvenile among the four treatments during the 5-day experimental period. Treatment (G1) (added *Arthrobacter globiformis* IFO 12137) had the hightest survival (94.7%), followed by (G2) (dietary β-1.3 glucan (89.3%,) followed by (G3) dietary *Moringa oleifera* leaves (86.4%). While the control group (G4) had the lowest survival (46.9 %). weight gain, lysozyme concentration, percentage of inhibition, phagocytic activity and phagocytic index were showed in the table 1 and Fig 1&2&3.

Table 1: Showing survival %, weight gain, lysozyme concentration, anti-bacterial, phagocytic activity and phagocytic index of shrimp

group	Survival %	Weight gain gm	Lysozyme Concentration µg/ml	Percentage inhibition	Phagoctic activity %	Phagocytic index
G1(Arthrobacter)	94.7%*	3.2±0.16	13±0.65*	33.17*	37*	9.92*
G2 B-1.3.Glucan	89.3%,*	4.1±0.20*	23±1.15*	28.90*	43*	7.15
G3 *M.oleifera* leaf	86.4%)*.	6.6±0.33*	6±0.30	21.11	28	7.02
G4 (control)	46.9 %	2.3±0.11	4±0.20	18.98	25	5.23

*Significant mean ± standard error n=3

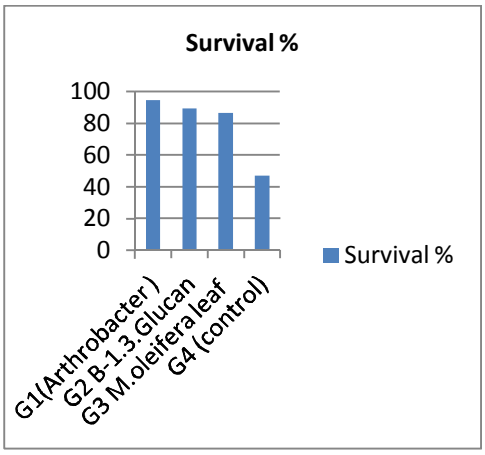

Fig. 1. Effects of dietary *Arthrobacter globiformis* IFO 12137, β-1.3 glucan and *Moringa oleifera* leaves on the survival of shrimp *P. indicus* juveniles challenged with pathogenic vibrios.

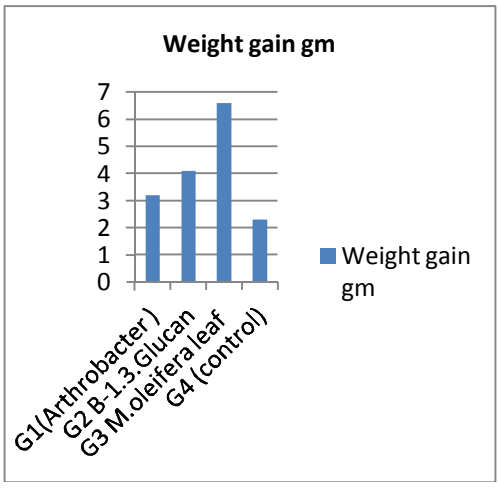

Fig. 2. Effects of dietary *A. globiformis*, β-1.3 glucan and *Moringa oleifera* leaves on the weight gain (gm) of *P. indicus* juveniles

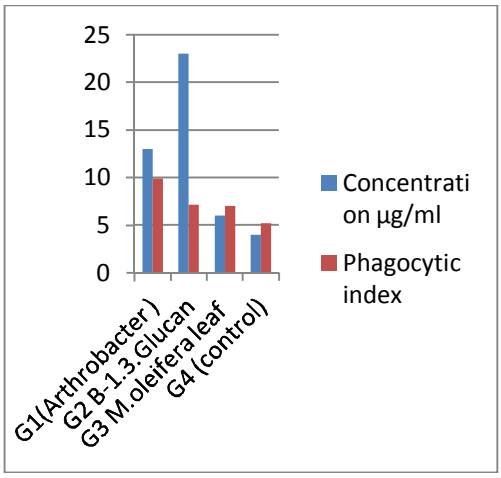

Fig. 3. Effects of dietary *A. globiformis*, β-1.3 glucan and *M. oleifera* leaves on lysozyme conc. and phagocytic index in haemolymph of *P. indicus* juveniles

Total bacterial count in shrimp specimens after challenge:

Total bacterial count (cfu) in shrimp specimens after challenge for determination the amounts of bacteria V. harveyi carried by shrimp. The highest burden bacteria was in control (supplied the basal diet) followed by group supplied β-1.3.Glucan followed by group supplied *M. oleifera* leaf while the lowest bacterial count was recorded in the group supplied probiotic *Arthrobacter globiformis* IFO 12137.

4. Discussion:

The immune stimulatory effects of immunostimulants like bacteria, glucans, peptidoglycans, lipopolysaccharides (LPS) and other polysaccharides have been widely studied in fish and crustaceans (Smith et al.,2003). Present study was carried out investigating comparative study between *Arthrobacter globiformis* IFO 12137, β-1.3.Glucan as important immunostimulant for shrimps and *M. oleifera* leaf as medicinal and anti-bacterial plant in protection of *P. indicus* Juvenile from pathogic vibrio harveyi.

This study demonstrated promising results for survival enhancement in *P. indicus* Juvenile caused by probiotics and immunostimulants used, with different degrees. Treatment G1 (added only with *Arthrobacter globiformis* IFO 12137) produced significant higher survival than the control (added nothing). There were reports of Arthrobacters producing antimicrobial compounds (Hentschel et al., 2001; Jayanth et al., 2001). The results nearly agree with that obtained by Li et al.,(2006) who reported that the beneficial bacterium Arthrobacter XE-7 isolated from the natural microflora is probiotic. They added that results of the inhibition experiment showed that Arthrobacter XE-7 has the ability to inhibit pathogenic vibrios (V. parahaemolyticus, V. anguillarum and V. nereis). Parallel to the inhibition experiment, the shrimp challenge experiment confirmed that the Arthrobacter XE-7 is not only able to antagonize the pathogenic vibrios but also able to reduce the concentration of NH3–N. Therefore, the effect of probiotic nature of Arthrobacter XE-7 is based on both the competitive exclusion of the pathogen and the water quality improvement.

Results of shrimp survival clearly show that *Arthrobacter globiformis* IFO 12137 is non-pathogenic to shrimp *P. indicus* Juvenile especially it was supplied with the diet at very high level ($1x10^8$ CFU/ml), *Arthrobacter globiformis* have the comparable protection of shrimp post larvae from the pathogenic vibrio (*V.harvei*) Li *et al*.,(2006).

Numerous studies concern the protection from pathogenic bacteria by probiotics with penaeid shrimp and other crustaceans (Nogami and Maeda, 1992; Garri- ques and Arevalo, 1995), and the advantage of probiotics over antibiotics has been discussed by Moriarty (1998), but only a few other published reports deal with the comparative study between the probiotics and antibiotics Li *et al.*,(2006).

Competitive exclusion might be an important factor for the *Arthrobacter globiformis* IFO 12137 enhancing survival of shrimp. In vitro. It produced wide zones of inhibition against pathogenic vibrio harveyi several. the results cofirm the results obtained by Carnio et al., (1999) who reported that there is antagonistic activity of Arthrobacter sp. against pathogen such as Listeriosis.

Dietary β-glucans when introduced to the basal diet of shrimp *Penaeus indicus* significantly enhance survival (89.3%,) in challenged shrimp improve the weight gain (4.1gm), lysozyme

concentration (23±1.15), percentage inhibition (28.90), phagocytic activity (43) and phagocytic index (7.15) comparatively with control group.

β-glucans are glucose polymers that are major structural components of the cell wall of yeast, fungi, and bacteria, but also of cereals like oat and barley. There is much structural variation in the β-glucans from these different sources, which may influence their physiological functions. β-glucans derived from fungi and yeast and alginate have immune modulating properties. Most frequently evaluated are effects on macrophage activation and on lysozyme, respiratory burst and leukocyte activity, which have been suggested to contribute to the increased resistance against infections, the results of the present study confirmed by the results obtained by Thanardkit *et al.,* (2002), Lopez *et al*.,(2003) and Felix *et al*.,(2008).

It is for the first time in aquaculture *Moringa oleifera* leaf used as anti- bacterial, immunostimulant and growth promoter in shrimp *Penaeus indicus* Juvenile. Present study revealed that dietary *Moringa oleifera* leaves affect significantly on growth stimulatining weight gain, Survival percentage but non - significally affect on percentage inhibition, lysozyme activity, pagocytic activity and index, the results indicated that *Moringa oleifera* leaves in aquaculture successfully used as nutritious as a essential additive in diet of shrimp because it contain high amounts of vitamins, minerals and amino acids important for metabolism and growth of shrimp *P..indicus.*

Plant oil from seeds and leaves such as *Moringa oleifera* are in high demand for their medicinal value. Apart from the medicinal uses, *Moringa oleifera* was reported to be a good source of vitamins and amino acids (Olugbemi *et al*; 2010). *Moringa oleifera* was claimed to boost immune systems (Fuglier, 1999; Olugbemi *et al.,* 2010). The leaves and green fresh pods are used as vegetables by man and are rich in carotene and ascorbic acid (vitamin C) with a good profile of amino acids (Makkar and Becker, 1996). They are also used in livestock feed and the twigs are reported to be very palatable to ruminants (Kimoro, 2002; Kakengi *et al.,* 2007). The edible leaves are very nutritious and are consumed in Nigeria. The Moringa seed oil is high in (80.4%) polyunsaturated fatty acid (Anwar and Rashid, 2007; Ogbunugafor *et al.,* 2011). *Moringa oleifera* extract was reported to have antibacterial properties and conclusion was made to investigate it as a phytotherapeutic agent to combat infectious agents (Patel, 2011). Most parts of the plant been used in folk medicine in Africa and South Asia (Fahey, 2005). The medicinal effects of the plant was ascribed to their possession of anti-oxidants, which are known to suppress formation of reactive oxygen species (ROS) and free radicals (Sofidiya *et al.,* 2006; Olugbemi *et al.,* 2010; Ogbunugafor *et al.,* 2011; Ogbe and Affiku, 2011).

In this study, it was concluded that the beneficial bacterium *Arthrobacter globiformis* IFO 12137 isolated from the natural habitatof shrimp (water culture) is a good aquaculture probiotic for shrimp *P. indicus* Juvenile. Results of the inhibition experiment in vitro showed that *Arthrobacter globiformis* IFO 12137 has antagonistic effect against vibrio bacteria. It has the ability to inhibit pathogenic vibrios (V. harveyi). Parallel to the inhibition experiment, the shrimp challenge experiment confirmed that the *Arthrobacter globiformis* is not only able to antagonize the pathogenic vibrios but also able to act as growth promoter, therefore, the study propose that the probiotic nature of *Arthrobacter globiformis* IFO 12137 is based on both the competitive pathogenic exclusion and enhancing growth. In addition, a reduction of in vivo mortality.

The study indicated also that dietary β-1.3.glucan consider as immunostimulant of choice for shrimp *P. indicus* affecting survival weight gain (growth) lysozme concentration bactericidal phagocytic activity and index acting as anti-bacterial and growth promoter. Present study investigate the effect of dietary *Moringa oleifera* leaf for the first time in aquaculture as medicinal plant for protection of *P. indicus* from pathogenic *V.harveyi* the study indicated that *Moringa oleifera* leaves contained appreciable amounts of carbohydrate, protein and minerals, which are nutritional requirements of poultry. Possibly, the leaves from this plant could be useful as feed supplement and as medicine in poultry to improve health and growth performance. Thus, further studies are needed to investigate the effects of *Arthrobacter globiformis* on other species of shrimp and fish investigating other physiological and immunological parameters also that *Moringa oleifera* must be further studied introducing it in feeding of aquatic animals.

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