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Effect of Organic Acids and Probiotics on the Pond Ecosystem in the Culture Ponds of *Litopenaeus vannamei*

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A B S T R A C T

The Organic acids and probiotics play a vital role in the culture of many terrestrial and aquatic organism, these have gained prominence as alternative biomedicine to antibiotics. produced by the probiotic bacteria and are produced by leading manufacturers these have a number of applications in many fields such as Aquaculture, food preparations, poultry, sewage water treatment, animal feed production, human consumption. The effect of dietary supplementations of citric acid, formic acid, lactic acid or their salts improved the growth of *Litopenaeus vannamei* was studied. All the organic acids and probiotics studied enhanced the activity of the gut and pond ecosystem when compared with the control. The percentage of decrease in total *vibrio* count (TVC) when the feed mixed with (3p/kg and 5g/kg organic acid) and (2kg/ha and 3kg/ha of Probiotics) applied in Experimental pond A and experimental pond B respectively. The results have been promising with the variance clearing measured the number of colonies formed in the cultures obtained from Control pond(with as low as 0.24×102 ±0.27 to 40.21 ×10² ± 0.13 in the pond water) (with as low as 0.15×102 ±0.13 to39.23 × 10² ± 0.85 in the gut), similarly the observations in both the Experimental pond A and experimental pond B have been tabulated.

Introduction

The study was conducted at Vaadachepurupalli, Visakhapatnam district in Andhra Pradesh. Organic acids act as growth promoters and enhance the antimicrobial activity of the organism; they also enhance the nutrient digestibility, survival and maintain a static endogenous micro floral quantity and composition. As organic acid is very essential and useful it's called "A gut environment modifier (GEM) designed to improve feed quality and an alternative to antibiotics".

It improves digestibility of the aquaculture feeds, increases the feed intake of aquatic species, has a strong antimicrobial effect and acts against gram negative bacteria leads to prevention of the diseases, it acts as a feed hygiene and feed quality regulator (anti-mould), it reduces buffering capacity of the feed, It helps in the decrease of pH in the feed which prevents the ammonia formation in the faecal matter, It also helps in the faster acidification of stomach content towards optimal pH for pepsin digestion, acidification

of the hepatopancreas, gut acidification, stimulation of enzyme secretion, improves protein digestion and also increases amino acid digestibility. In rearing ponds of *Litopenaeus vannamei*, the use of commercial probiotics has shown beneficial effects by improving survival, feed conversion, growth rate and keeping the parameters of water quality at optimum levels (Shariff *et al.,* 2001; Wang *et al.,* 2005). Studies of organic acids and probiotics to improve growth or survival in crustacean larvae are very scanty. The application of probiotics in the aquaculture ecosystems and in the feeds to the animal is one of the most promising areas where sustainable culture can be established and the practice of application of probiotics is reported by many aqua-culturists. Knowledge of probiotics has increased, currently it is know that these microorganisms have an antimicrobial effect through modifying the intestinal microbiota, secreting antibacterial substances like bacteriocins and organic acids (Myers., 2007). Organic acids and their salts are generally regarded as safe compounds and those with one or more carboxyl group (-COOH) in their structure are often used as antimicrobials in the livestock feed industry. In shrimp culture there are different bacterial strains used as probiotics and the popular probiotic bacteria belong to *Nitrosomonas* spp., *Cellulomonas* spp., *Bacillus* spp., *Pediococcus* spp., *Nitrobacter* spp., *Rhodococus* spp., *Rhodobacter* spp., *Enterobacter* spp., *Lactobacillus* spp., *Actinomycetes* spp., *Pseudomonas* spp., *Saccharomyces* spp., Denitrifying bacteria, *Bifidobacterium*, *Carnobacterium*, *Alteronomonas* spp., *Streptococcus* spp.,

Materials and Methods

The application of probiotics in the experimental ponds was followed uniformly in all the farms. The probiotic used for present study is SUPER BIOTIC (Plate 5 Fig B) which is a composition of water probiotics having the strength of 10 million colony forming units (CFU) i.e. 109 cfu/g of the probiotic was used. The probiotic strains in this SUPER BIOTIC were *Bacillus spp.* like *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megatirium* and *Bacillus polymixa*. The probiotic was applied at the rate of 2kgha-1 in the experimental pond A and 3kgha-1 in the experimental pond B at the frequency of every 15 days during the three years of the study starting from 45 DOC (Table 1(b)). The probiotics application followed the same procedure in all the experimental ponds during the study period. The application of probiotics were followed by soaking the probiotics material in 4 liters of water overnight for leaching in non-contaminated fresh water and later applied uniformly all over the ponds. For the studies on the role of organic acids, the commercial organic acid used in the present study is BAYERS' BAYMIX LATIBON (Plate 5, Fig. A) which is composed of formic, lactic, benzoic and propionic acids. The organic acid BAYMIX LATIBON is applied at the rate of 3 gkg-1 feed in the experimental pond A and 5 gkg-1 feed (Table 1(a)) in the experimental pond B in both summer and winter crops during the study period starting from 30 DOC based on the requirement. Application of organic acids was stopped two days prior to the application of SUPER BIOTIC and was started two days after the application of SUPER BIOTIC. The Organic acid is mixed in the feed with commercially available feed binder "Gell it" which was applied 10 ml/kg feed. In the process of top dressing in the feed the organic acid were weighed with simple balance of sensitivity 10 gm and mixed thoroughly with the binder "Gell It". The feed was broadcasted in the ponds after drying of feed pellets for 20 to 30 minutes in shade. The feed for the experimental study ponds for every feeding time was freshly mixed with organic acid. Later the feed was dried in the shade and

broadcasted in every feeding time. The application of the organic acid was followed every feeding time. The application of the organic acid was followed every day starting from 45 DOC during the culture in the entire crop period of both season's summer and winter in the three years of study period 2011 to 2013.

Estimation of Bacterial Load of Water

Water samples from the selected culture ponds were collected in sterile glass bottles and brought to the laboratory in cold condition and bacterial loads were estimated within one hour of collection, by employing standard pour plate method. The samples were prepared by serial dilution method. One ml of diluted water sample was taken asceptically into sterile, dry petridish with the help of a pipette. The nutrient agar medium (Himedia, Bombay) in lukeworm state was poured onto the sample contained in the petridish, and then petridish was rotated gently in both clock and anti-clockwise directions for uniform distribution of sample 41 solutions, triplicate sets were maintained for each direction. The petridishes were inverted after medium got solidified.

Estimation of Bacterial Load of Shrimp

Four shrimps collected from selected ponds at fortnightly interval for the estimation of bacteria loads, the standard methods were followed. The bacterial loads were estimated from gut, Hepatopancreas and Haemolymph. The weighed tissue sample approximately 60 to 80 mg was taken aseptically from freshly sacrificed specimen into a known volume of sterile and cooled peptone water (Himedia, Bombay). The tissues were homogenized with the help of sterile glass rod in ice cold conditions. One ml of homogenate was taken into dry, sterile petridish aseptically with a pipette. The bacterial load was estimated as reported earlier and expressed in CFU/g. In case of water, the volume of water sample was collected from various locations of the ponds during different times of the day. The bacterial loads estimated and expressed in CFU/ml Count was expressed in CFU/g.

Average number of colonies =

$$\frac{\text{No. of CFU/g of the gut X Dilution factor}}{\text{Weight of gut sample}}$$

Statistical Applications

The statistical package used for interpreting the available data was GRAPHPAD PRISM 6.0 Scientific Software for evaluation of the total *Vibrio* colony (TVC) counts. Histograms were used to interpret the growth data of the results obtained. The pie charts were used to represent the total percentage production of shrimp *Litopenaeus vannamei* in Andhra Pradesh during the year 2011 and 2013. The pie charts were applied for representing the data of the survival rate and growth during the study period in all the ponds of the eight work stations. The results of the immunological indices were tabulated and represented by using the statistical tool 2-D line charts.

Results and Discussion

At Vadacheepurupalli during the culture periods in the summer crop and winter crop from the year 2011 to 2013, the parameters of salinity, pH, and temperature in the study ponds were closely monitored and it was observed that the pH which was well maintained with the application of Organic acids and probiotics (). This control pond harvested at 19.0 g on 109th day with the effect of *Vibriosis spp.*.

The total *Vibrio* counts in the experimental pond A and pond B were recorded as $31.0\times10^2 \pm 0.25$ cfu ml^{-1} and $3.10\times10^2\pm0.23$ cfu ml^{-1} in the pond water and $20.0\times10^2\pm0.13$ cfu mg^{-1} and $4.15.12\times10^2 \pm 0.37$ cfu mg^{-1} in the shrimp

gut at 125 days of culture respectively. The experimental ponds A and B were harvested normally at 27.5 g and 30.0 g on 120th and 135th day respectively.

Table 1 Total vibrio count of culture ponds at Vadacheepurupalli during summer in the year 2011

Control Pond

S.No	Days of Culture	Salinity (ppt)	Ph	Temp (°C)	TVC water $cfuml^{-1}$	TVC gut $cfumg^{-1}$
1	Before stocking	25	8.1	29	0.24×10^2 ±0.27	-
2	25	30	8.5	30	0.28×10^2 ±0.60	-
3	50	31	7.9	31	1.55×10^2 ±0.41	1.23×10^2 ±0.34
4	75	35	8.3	32	30.0×10^2 ±0.47	24.8×10^2 ±0.41
5	100	39	8.5	32	32.7×10^2 ±0.81	25.7×10^2 ±0.63
6	125	-	-	-	-	-

Control Pond harvested due to *Vibriosis* at 19 g on 109th day

Experimental Pond A

S.No	Days of Culture	Salinity (ppt)	pH	Temp (°C)	TVC water $cfuml^{-1}$	TVC gut $cfumg^{-1}$
1	Before stocking	26	7.8	29	-	-
2	25	33	8.2	29	-	-
3	50	35	8.5	30	1.48×10^2 ±0.15	1.63×10^2 ±0.36
4	75	36	8.3	31	2.63×10^2 ±0.23	2.28×10^2 ±0.15
5	100	37	8.7	32	28.0×10^2 ±0.25	19.7×10^2 ±0.29
6	125	36	8.6	32	31.0×10^2 ±0.25	20.0×10^2 ±0.13

Pond A harvested normally at 27.5 g on 130th day

Experimental Pond B

S.No	Days of Culture	Salinity (ppt)	pH	Temp (°C)	TVC water $cfuml^{-1}$	TVC gut $cfumg^{-1}$
1	Before stocking	26	8.2	28	-	-
2	25	34	8.2	29	-	-
3	50	35	8.5	31	0.25×10^2 ±0.36	0.23×10^2 ±0.35
4	75	36	8.4	31	1.63×10^2 ±0.46	1.83×10^2 ±0.16
5	100	37	8.7	32	2.63×10^2 ±0.32	3.32×10^2 ±0.23
6	125	39	8.4	33	3.10×10^2 ±0.23	4.15×10^2 ±0.37

Pond B harvested normally at 30 g on 135th day

Table 2 Total vibrio count of culture ponds at Vadacheepurupalli during winter in the year 2011

Control Pond

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	23	8.2	28	-	-
2	25	25	8.6	29	0.28×10^{2} ±0.60	0.15×10^{2} ±0.13
3	50	-	-	-	-	-
4	75	-	-	-	-	-
5	100	-	-	-	-	-
6	125	-	-	-	-	-

Control Pond harvested due to *Vibriosis* at 3.2 g on 30th day

Experimental Pond A

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	22	7.8	28	-	-
2	25	24	8.1	27	0.11×10^{2} ±0.10	-
3	50	25	8.2	27	0.31×10^{2} ±0.52	0.27×10^{2} ±0.29
4	75	24	7.9	25	1.78×10^{2} ±0.38	2.45×10^{2} ±0.27
5	100	25	8.4	25	2.33×10^{2} ±0.30	2.77×10^{2} ±0.31
6	125	21	8.3	26	3.92×10^{2} ±0.55	3.50×10^{2} ±0.23

Pond A harvested normally at 28.0 g on 130th day

Experimental Pond B

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	16	7.5	28	-	-
2	25	17	7.9	27	-	-
3	50	19	8.4	26	0.17×10^{2} ±0.15	-
4	75	19	8.5	26	1.03×10^{2} ±0.45	1.00×10^{2} ±0.12
5	100	17	8.2	25	1.75×10^{2} ±0.16	1.30×10^{2} ±0.16
6	125	20	8.1	25	2.08×10^{2} ±0.13	2.04×10^{2} ±0.19

Pond B harvested normally at 29.0 g on 130th day

Table 3 Total vibrio count of culture ponds at Vadacheepurupalli during summer in the year 2012

Control Pond

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	25	8.2	30	0.22 × 10² ± 0.27	-
2	25	30	8.9	31	0.30 ×10² ± 0.60	-
3	50	35	7.8	33	1.57 ×10² ± 0.40	1.57 ×10² ± 0.40
4	75	39	8.5	33	30.0 ×10² ± 0.47	0.49 ×10² ± 0.24
5	100	25	8.2	30	37.25 × 10² ± 0.23	33.10 ×10² ± 0.47
6	125	30	8.9	31	40.21 ×10² ± 0.13	39.23 × 10² ± 0.85

Control Pond harvested normally at 26 g on 127th day

Experimental Pond A

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	26	7.8	28	-	-
2	25	27	8.5	30	-	-
3	50	32	8.5	32	-	-
4	75	35	7.9	33	0.22 × 10² ± 0.47	0.17 × 10² ± 0.22
5	100	36	8.6	32	2.25 ×10² ± 0.16	3.97 ×10² ± 0.42
6	125	32	8.2	32	3.25 ×10² ± 0.60	4.25 ×10² ± 0.13

Pond A harvested normally at 30 g on 132nd day

Experimental Pond B

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	25	7.9	28	-	-
2	25	28	8	30	-	-
3	50	32	8.6	32	-	-
4	75	35	8.6	33	0.26 × 10² ± 0.47	-
5	100	37	8.7	32	0.41 × 10² ± 0.49	0.26 × 10² ± 0.20
6	125	35	8.5	31	0.64 × 10² ± 0.61	0.33 × 10² ± 0.54

Pond B harvested normally at 33 g on 136th day

Table 4 Total vibrio count of culture ponds at Vadacheepurupalli during winter in the year 2012

Control Pond

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	14	8.5	28	-	-
2	25	17	7.9	29	8.97×10^{2} ±0.38	2.65×10^{2} ±0.19
3	50	18	8.8	27	5.62×10^{2} ±0.25	5.13×10^{2} ±0.33
4	75	17	8.7	26	9.79×10^{2} ±0.38	7.22×10^{2} ±0.19
5	100	17	8.5	26	26.5×10^{2} ±0.19	37.8×10^{2} ±0.46
6	125	18	8.8	27	29.0×10^{2} ±0.21	40.10×10^{2} ±0.17

Control Pond harvested normally at 22 g on 130th day

Experimental Pond A

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	15	8.1	28	-	-
2	25	15	7.9	29	0.14×10^{2} ±0.22	-
3	50	17	8.8	27	0.20×10^{2} ±0.38	0.16×10^{2} ±0.20
4	75	17	8.7	26	3.17×10^{2} ±0.62	2.62×10^{2} ±0.67
5	100	16	8.5	26	4.30×10^{2} ±0.21	3.50×10^{2} ±0.54
6	125	17	8.6	27	5.10×10^{2} ±0.23	4.09×10^{2} ±0.55

Pond A harvested normally at 31.5 g on 135th day

Experimental Pond B

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	15	8.2	26	-	-
2	25	16	8.1	27	-	-
3	50	16	8.8	26	-	-
4	75	17	8.7	26	0.12×10^{2} ±0.14	0.17×10^{2} ±0.14
5	100	16	8.9	25	2.17×10^{2} ±0.50	2.48×10^{2} ±0.35
6	125	17	8.7	25	3.30×10^{2} ±0.15	3.22×10^{2} ±0.23

Pond B harvested normally at 32.2 g on 135nd day

Table 5 Total vibrio count of culture ponds at Vadacheepurupalli during summer in the year 2013

Control Pond

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	18	7.8	28	-	-
2	25	20	8.7	30	-	-
3	50	20	8.8	32	$0.18 \times 10^{2} \pm 0.43$	-
4	75	19	7.8	31	$0.27 \times 10^{2} \pm 0.47$	$0.50 \times 10^{2} \pm 1.03$
5	100	19	8.5	30	$4.10 \times 10^{2} \pm 0.84$	$3.23 \times 10^{2} \pm 0.44$
6	125	20	8.8	32	$5.12 \times 10^{2} \pm 0.62$	$4.56 \times 10^{2} \pm 0.65$

Control Pond harvested normally at 22 g on 127th day

Experimental Pond A

No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	19	8.2	29	-	-
2	25	20	8.4	30	$0.16 \times 10^{2} \pm 0.52$	-
3	50	21	8.5	31	$0.20 \times 10^{2} \pm 0.54$	$0.17 \times 10^{2} \pm 0.21$
4	75	20	8.4	29	$0.22 \times 10^{2} \pm 0.23$	$1.42 \times 10^{2} \pm 0.61$
5	100	20	8.3	30	$1.39 \times 10^{2} \pm 0.45$	$2.86 \times 10^{2} \pm 0.85$
6	125	21	8.5	31	$28.7 \times 10^{2} \pm 0.63$	$9.27 \times 10^{2} \pm 0.56$

Pond A harvested normally at 31 g on 129th day

Experimental Pond B

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	18	8.1	28	-	-
2	25	19	7.9	30	$0.19 \times 10^{2} \pm 0.27$	-
3	50	20	8.5	30	$0.23 \times 10^{2} \pm 0.32$	-
4	75	18	8.1	29	$0.29 \times 10^{2} \pm 0.23$	-
5	100	20	8.2	30	$1.30 \times 10^{2} \pm 0.41$	-
6	125	20	8.5	32	$2.35 \times 10^{2} \pm 0.52$	$0.83 \times 10^{2} \pm 0.49$

Pond B harvested normally at 33.2 g on 129th day

Table 6 Total vibrio count of culture ponds at Vadacheepurupalli during winter in the year 2013

Control Pond

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	16	7.1	29	0.72×10^{2} ±0.56	-
2	25	19	7.3	28	2.28×10^{2} ±0.60	2.35×10^{2} ±0.70
3	50	-	-	-	-	-
4	75	-	-	-	-	-
5	100	-	-	-	-	-
6	125	-	-	-	-	-

Control Pond harvested due to White Spot Disease at 3 g on 25th day

Experimental Pond A

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	15	7.8	28	0.38×10^{2} ±0.46	-
2	25	17	8.1	28	1.31×10^{2} ±0.30	1.28×10^{2} ±0.63
3	50	-	-	-	-	-
4	75	-	-	-	-	-
5	100	-	-	-	-	-
6	125	-	-	-	-	

Pond A harvested due to White Spot Disease at 4 g on 25th day

Experimental Pond B

S.No	Days of Culture	Salinity (ppt)	pH	Temp (^{o}C)	TVC water cfuml^{-1}	TVC gut cfumg^{-1}
1	Before stocking	16	7.5	28	0.29×10^{2} ±0.60	-
2	25	17	8.3	27	1.52×10^{2} ±0.45	1.68×10^{2} ±0.71
3	50	-	-	-	-	-
4	75	-	-	-	-	-
5	100	-	-	-	-	-
6	125	-	-	-	-	-

Pond B harvested due to White Spot Disease at 4 g on 25th day

Fig.1 Total vibrio count of culture ponds at Vadacheepurupalli during summer in the year 2011

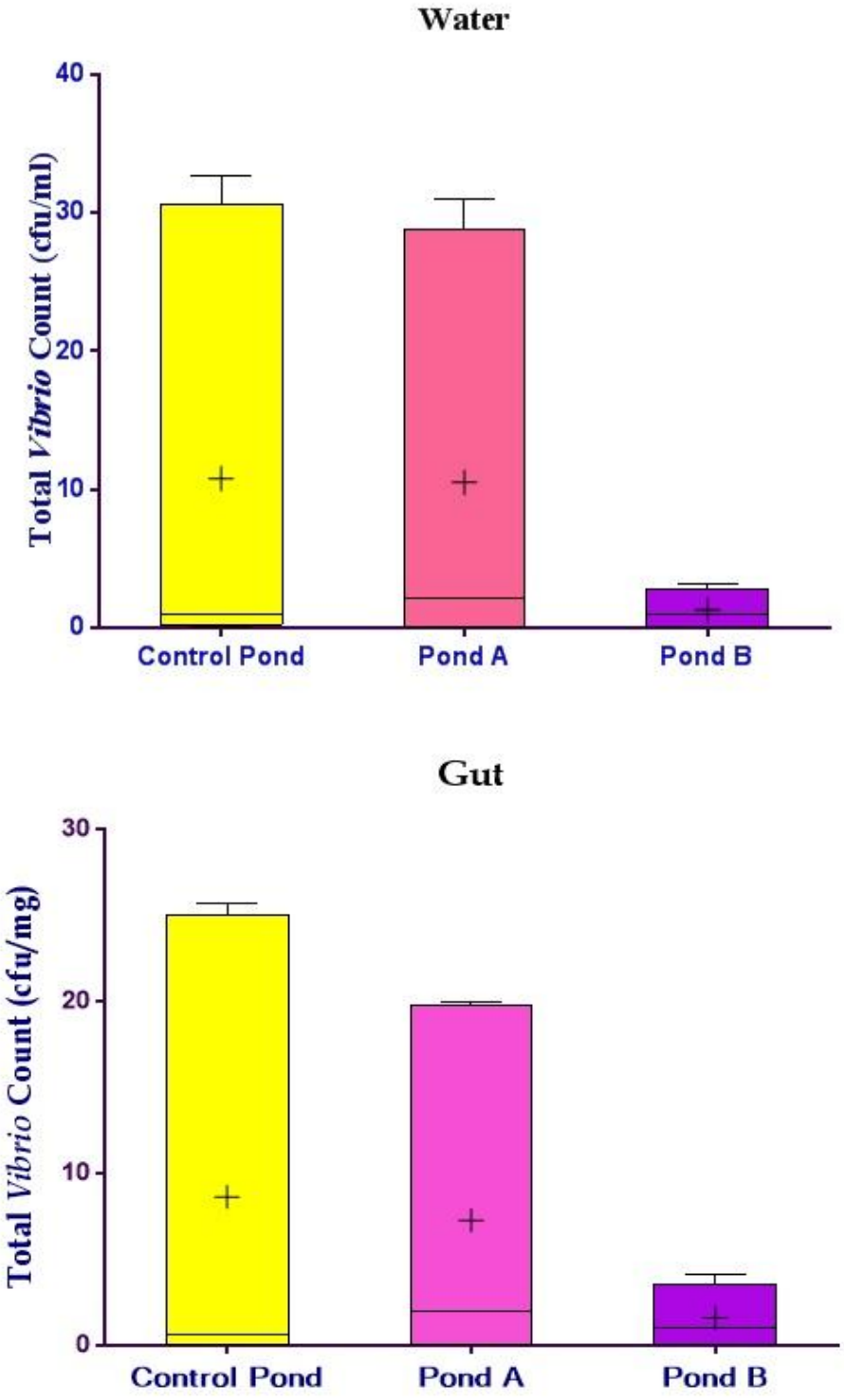


Fig.2 Total vibrio count of culture ponds at Vadacheepurupalli during winter in the year 2011

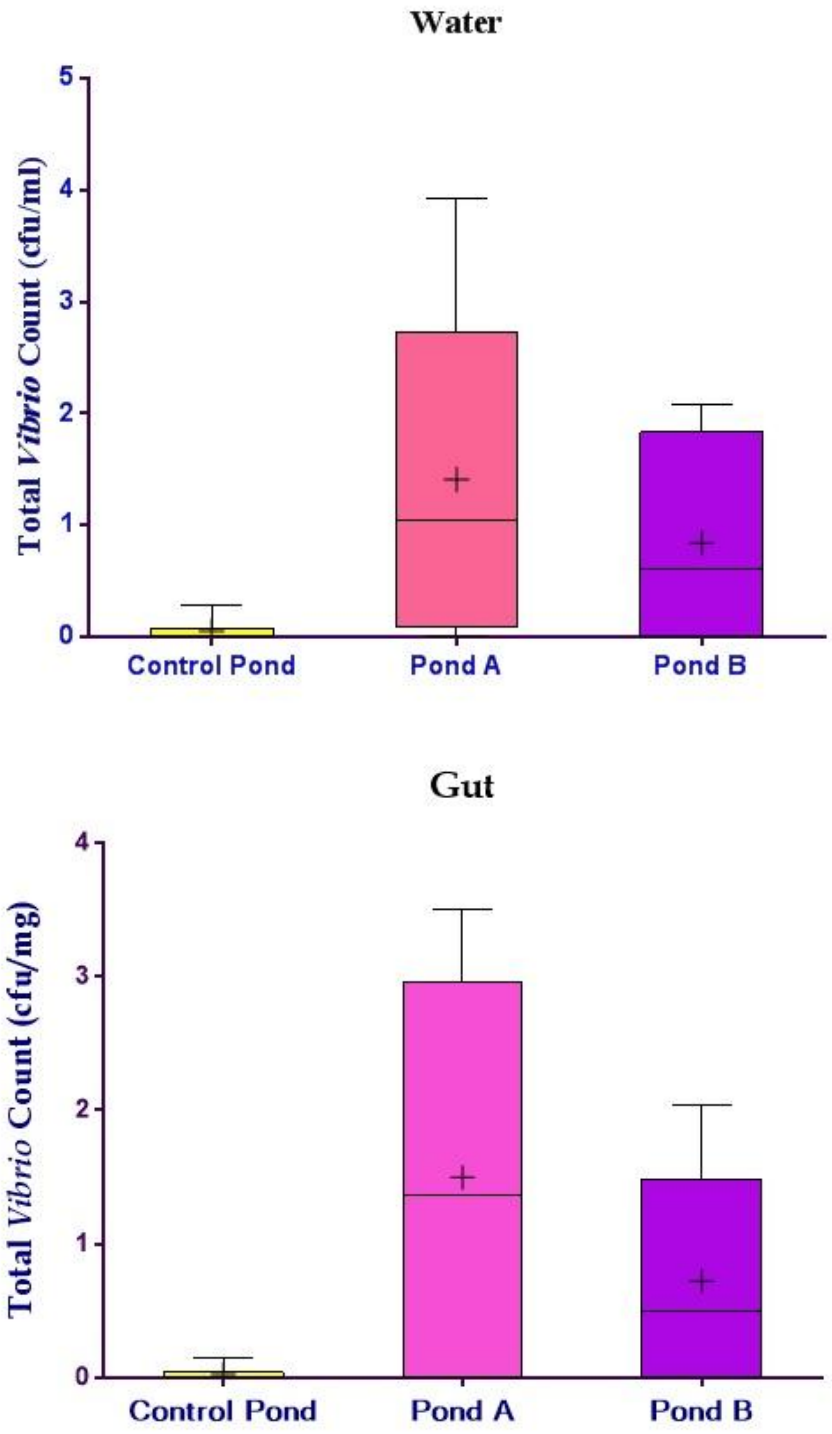


Fig.3 Total vibrio count of culture ponds at Vadacheepurupalli during summer in the year 2012

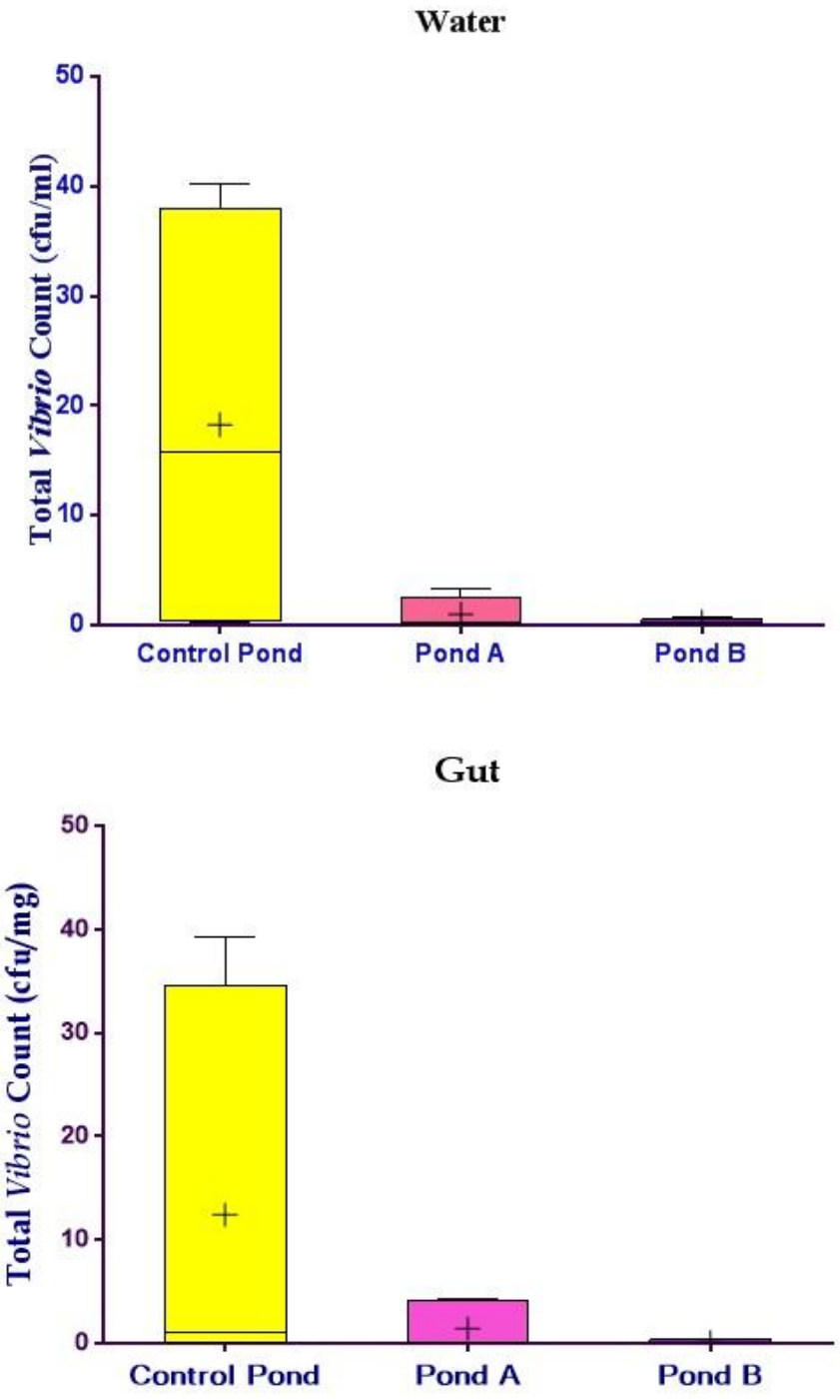


Fig.4 Total vibrio count of culture ponds at Vadacheepurupalli during winter in the year 2012

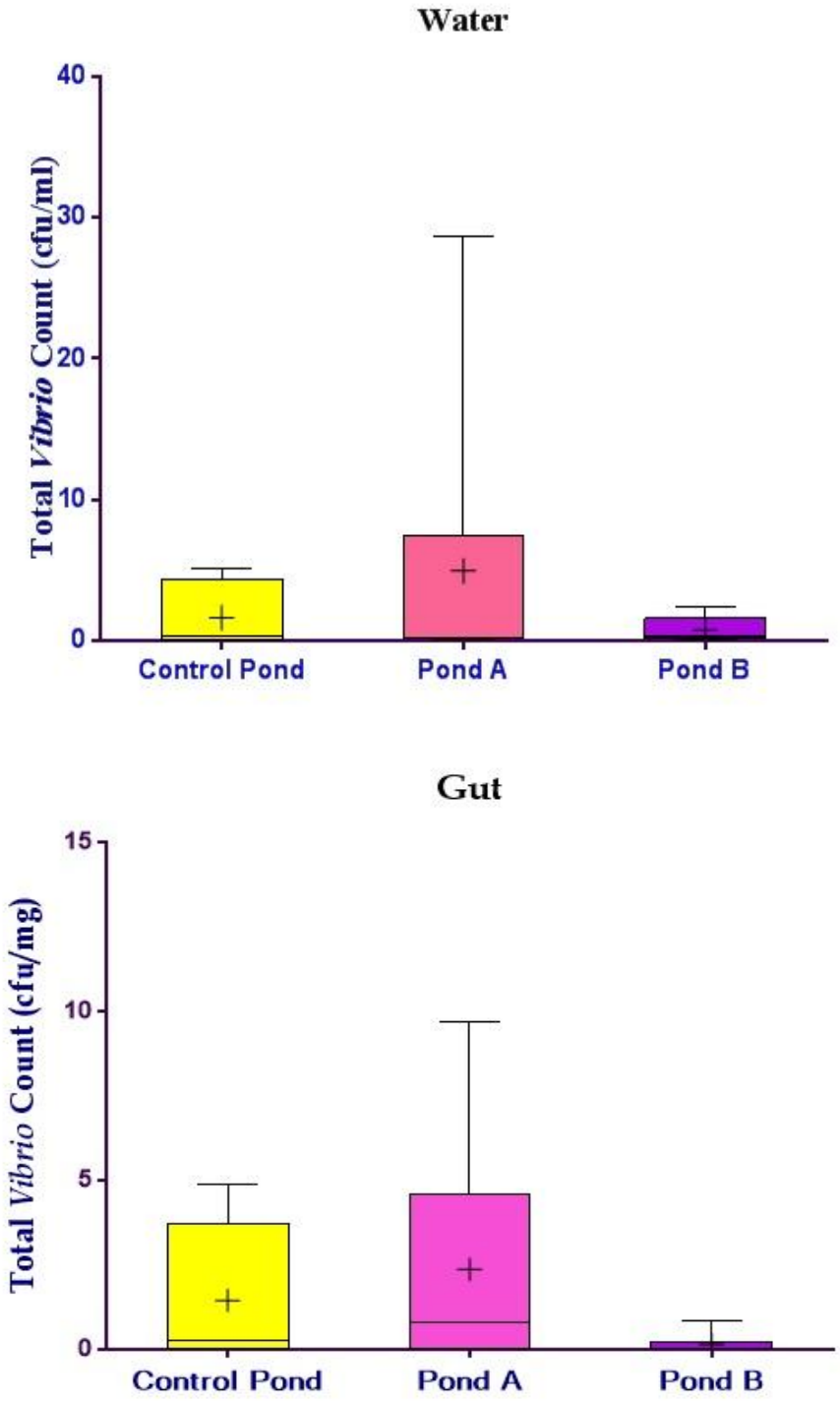


Fig.5 Total vibrio count of culture ponds at Vadacheepurupalli during summer in the year 2013

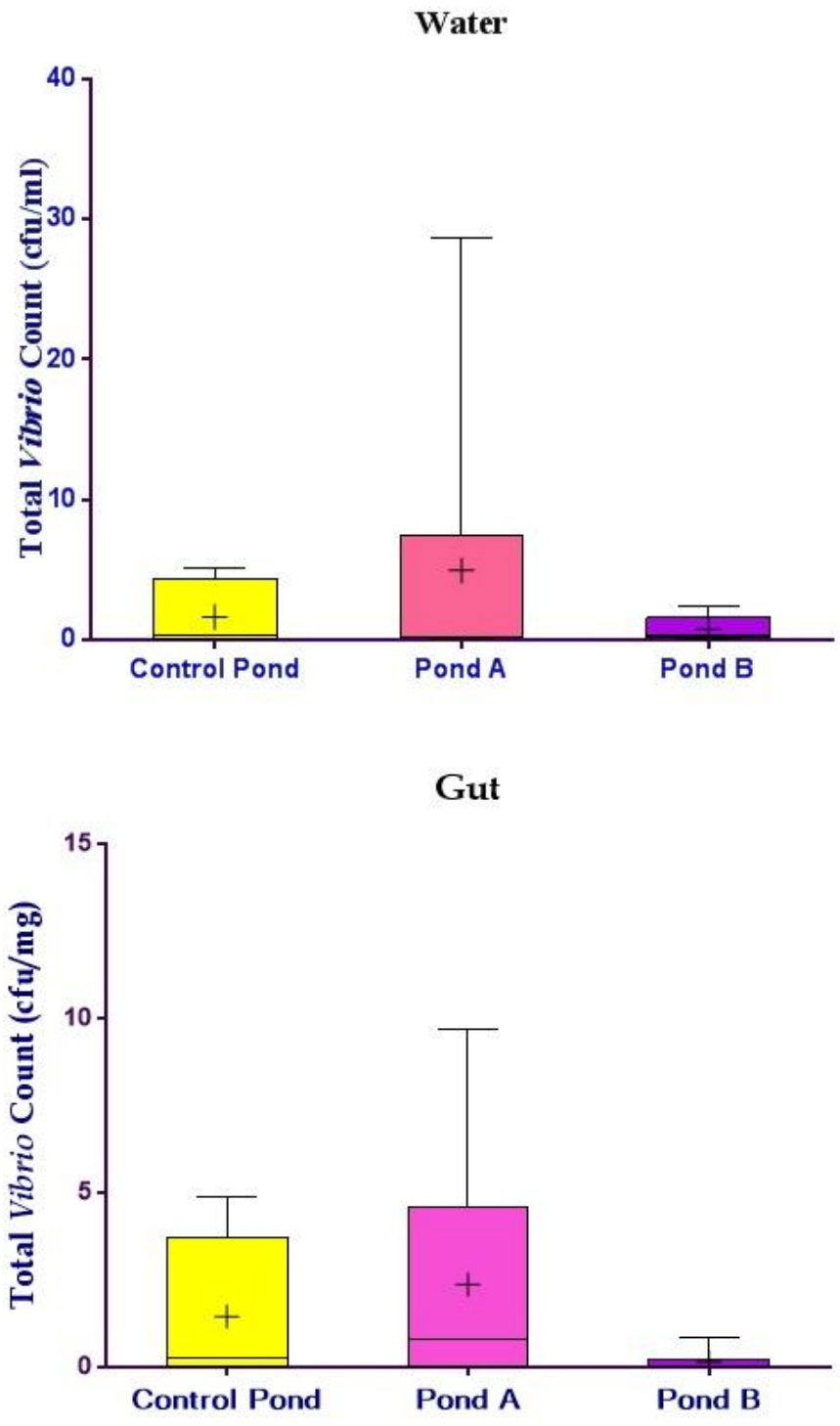
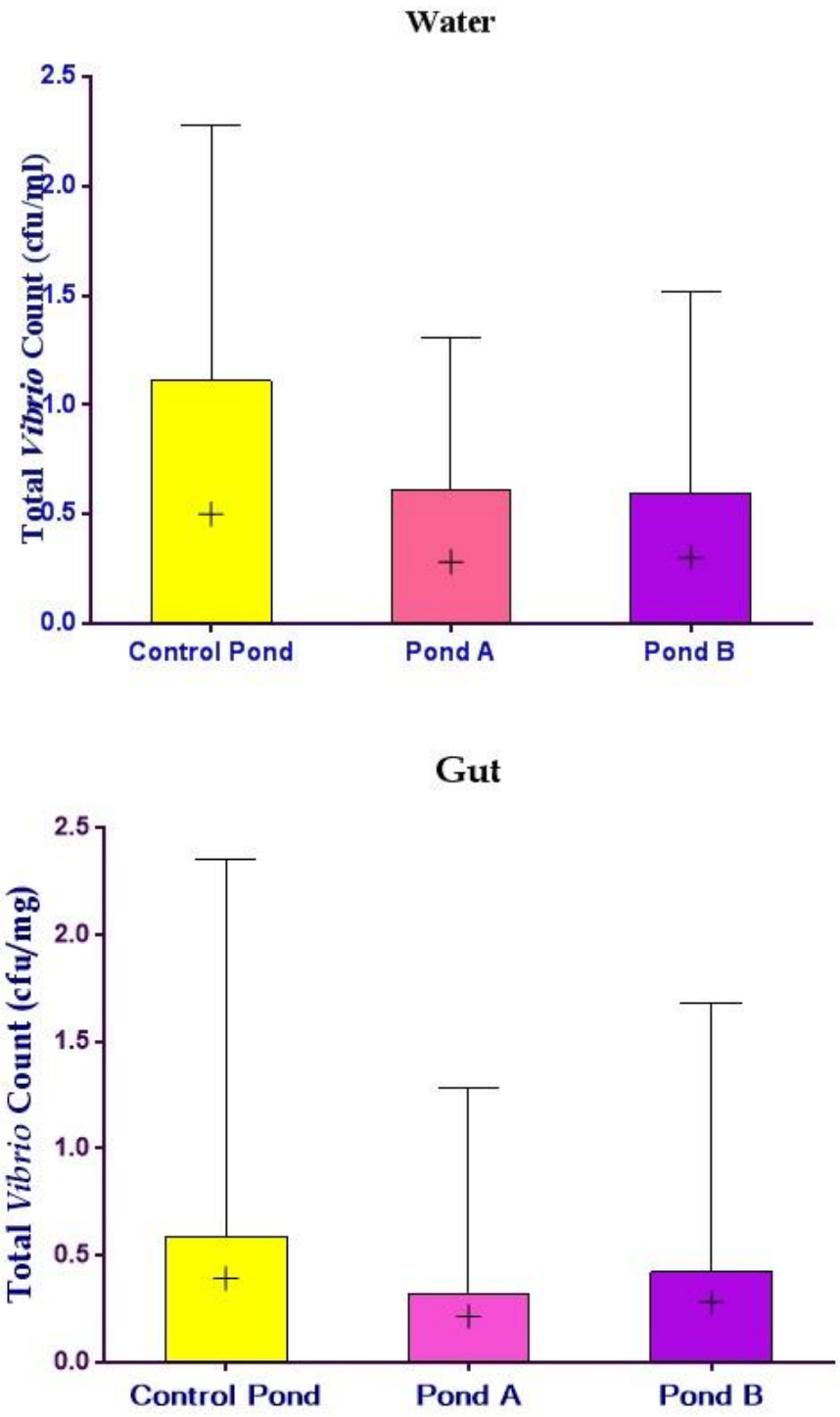


Fig.6 Total vibrio count of culture ponds at Vadacheepurupalli during winter in the year 2013



In conclusion, the present study was undertaken to study the effect of organic acids and probiotics in culture ponds of *Litopenaeus vannamei* over a period of three consecutive years from 2011 to 2013. The effect of Organic acids and probiotics created a healthy

and suitable environment for the shrimp culture. These have decreased the pathogens thereby decreasing the onset of various diseases. The growth and survival rate have been greatly influenced.

Application of these in the closed recirculating system in a semi intensive culture has been successful and the output has been marginally well over the conventional method. Based on the results of the present study, it was revealed that the application of probiotics have controlled the pathogenic *Vibrio* spp.in the shrimp culture pond and in the gut by the effect of organic acids. It is evident from the experimental ponds A & B, which showed considerable reduction in the Total vibrio count (TVC) during the culture period

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