Assessment of antagonistic activities of probiotic bacteria against pathogenic *Vibrio* spp. *in vitro* and *in vivo* in shrimp larvae

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Assessment of antagonistic activities of probiotic bacteria against pathogenic *Vibrio* spp. *in vitro* and *in vivo* in shrimp larvae

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DECLARATION

The results submitted in this thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor is it concurrently submitted for any degree.

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DEDICATED TO
MY
BELOVED PARENTS

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Abstract

Now-a-days probiotics attract the attention of scientists as because of their versatile antimicrobial activity and established health benefits on the host. In this study, three most farmer used commercial probiotics (Super Biotic, Zymetin & Aqua Clear-S) were selected to analysis the antagonistic activity against *Vibrio* spp. for evaluating effective antagonism of probiotic against pathogenic *Vibrio* spp. Shrimp hatchery owner and farmer often face *Vibrio* spp. attack in larval condition and want to use probiotics to prevent these pathogenic microbes. For *in vitro* and *in vivo* test disc diffusion tests and challenge tests in liquid media were conducted. The disc diffusion test revealed that the growth area of *Vibrio* spp. in the presence of super biotic was the lowest. The *Vibrio* spp. concentrations in this study considered as 10^6 CFU/mL in an *in vivo* challenge test with shrimp larvae. Probiotics co-culture was done in 37°c and incubates for 24 hour. In *in vivo* challenge test 50% mortality occurred in positive control group at 4^{th} day and among three probiotic treatment group the highest (31.65%) mortality occurred in the treatment group with aqua clear-s, and the lowest mortality (16.67%) in the treatment group with super biotic. So it has been found that both *in vitro* and *in vivo* condition commercial probiotic super biotic resist more the growth of *Vibrio* spp. than zymetin or aqua clear-s.

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Chapter 1

1. Introduction

Bangladesh has ranked third position in the world in terms of inland fish production in 2018, according to a report by the Food and Agriculture Organization (FAO) Fisheries sector contributes 3.57 percent to the national GDP. In the year 2018, Bangladesh earned Tk 4,500 corer by exporting around 69,000 metric tons of fish and fishery products. It has been predicted that by 2050, the total population of the planet will be 9 billion and aquaculture will have an important role in catering to the increased demand for food (Godfray et al. 2010). Day by day different types of new technologies are applied to increase the fish production. But various types of pathogenic microorganisms are trying to inhibit the production by creating various types of disease. Now-a-days Vibriosis is a common disease for fish culture and that is caused by different species of *Vibrio spp. Vibrio anguillarum* attacks and create disease to freshwater/marine fish, like eel *(A. anguilla)*, rui and seabass; *Vibrio harveyi* affects and create disease in culture of shrimp larvae, grouper, snapper; *Vibrio vulnificus* attacks and create disease to eel, and other marine fish; *Vibrio parahaemolyticus* creates disease in farmed *Penaeus vannamei*, *P. monodon*, *P. chinensis*, possibly other shrimp species (Haenen, 2017). Motile *Aeromonas* spp. *A. caviae, A .hydrophila, A. sobria, A. veronii, A. jandaei* create disease to various fresh-and brackish water fish species, like catfish, tilapia, Puntius, rohu, other cyprinids (Haenen, 2017). *Pseudomonas anguilliseptica* creates disease to Tilapia and *Pseudomonas fluorescens* creates disease to tilapia, tench, silver carp, bighead carp, rainbow trout (Haenen, 2017). To protect our cultured fish from pathogenic micro-organisms and to limit the fish mortality farmers frequently use different types of antibiotics. amoxyfish, amoxivet, tetravet, orgamysin, oxytetracycline, chlorotetracycline, amoxicillin, co-trimoxazole, sulphadiazine, azithromysin, eryvet are some antibiotics that are used by the fish farmer (Rasul, Majumdar & Akter, 2017). Most of the farmers use Oxy-Dox-F and Renamysin at the rate of 1-2g/kg feed for 7 days for its high activity against bacterial diseases (Rasul, Majumdar & Akter, 2017). Small concentration of Perefuran and Furazolion are used to control Vibrio infection in Bangladesh (Uddin & Kader, 2006). The use of antibiotics for aquatic animals may not only initiate environmental pollution problems but also can affect human health due to drug residues.

Considering the human health issue researcher find out a new way to control the effect of pathogenic micro-organisms. The use of beneficial bacteria known as probiotics instead of administering antibiotics in the animal industry is a better remedy and now gaining acceptance for the control of pathogens in aquaculture (Moriarty, 1999). Havenaar et al.

(1992) defined "a probiotic is a mono- or mixed culture of live microorganisms that, applied to animal or man, affect beneficially the host by improving the properties of the indigenous micro flora". Probiotics are living microbes that are beneficial and acts as antibiotics.

The present study was aimed to assess the antagonistic effect of probiotics against disease causing virulent *Vibrio spp.* At present this strain of bacteria creates various types of severe disease to our cultured fish species. To protect our cultured species from these virulent bacteria it is needed to find out a sustainable way and used of probiotics is that sustainable way.

1.1 Objectives

- To investigate antagonistic effect of commercial probiotics against pathogenic *Vibrio* spp.
- To investigate the protective activities of selected probiotics to shrimp larvae against *Vibrio* spp.

1.2 Literature review

1.2.1 Overview of aquaculture in Bangladesh

Bangladesh is considered as one of the most suitable regions for fisheries in the world, with the world's largest flooded wetland and the third largest aquatic biodiversity in Asia after China and India. Fish is one of the most popular complement to rice in the national diet, and it is saying that "a Bengali is made of fish and rice" (Ghose, 2014). The fisheries sector of Bangladesh is classified into 3 categories: Inland capture fisheries, Inland culture fisheries and Marine fisheries. The inland culture fisheries are contributing more than 55% of the total production (DoF, 2016). Total fish production of Bangladesh in 2014-2015 was reported to be 3,684,245 tons of which 1,023,991 tons (27.79%) where from inland open waters, 2,060,408 tons (55.93%) from inland closed waters and 599,846 tons (16.28%) from marine fisheries (DoF, 2016). The yearly increasing rate of overall fish production increase in Bangladesh went 7.20% to 3.84% during the years 2000-2001 to 2014-2015 (DoF, 2016).

In Bangladesh two kinds of aquaculture are practiced namely freshwater and coastal aquaculture. Freshwater and coastal aquaculture in South-western Bangladesh is different and it is mainly comprised integration with carps and prawn (e.g. *Paeneus monodon* and *Macrobrachium rosenbergii*) (Shamsuzzaman et al., 2017). In Bangladesh main aquaculture production systems are extensive, improved extensive, some are semi-intensive and few are intensive (Hossain, 2014). About 80% of total recorded aquaculture has been reported from pond aquaculture which is presently dominated by indigenous and exotic carps, Mekong Pangasius and Tilapia (Shamsuzzaman et al., 2017). It is worth mentioning that Bangladesh is one of the most suitable countries in the world for freshwater prawn farming for its biophysical resources (Ahmed & Diana, 2015). The total prawn culture area is 65,221 ha of which about 75% of the culture area is located in southwest region of Bangladesh. The total prawn production in Bangladesh was estimated at 43,713 tons in 2012-2013, an average annual productivity was 670 $kghec^{-1}$ (FRSS, 2014). Fisheries sector plays an important role in the national economy of the country and considering as the second largest export industry after readymade garments.

1.2.2 Problems in aquaculture

1.2.2.1 Disease problem

Fish diseases are caused by various types of causative agent. According to those causative agent fish diseases are classified into different type. Those are

i) Bacterial diseases

Different bacterial agents are responsible for bacterial disease. Furunculosis, Bacterial kidney Disease, Columnaris, etc are some example of bacterial disease.

ii) Viral Diseases

iii) Parasitic Diseases

iv) Nutritional Diseases

v) Diseases caused by environmental condition

vi) Neoplastic diseases

1.2.2.2 Climate Change impacts

According to Subasinghe et al. (2013), due to global warming day by day increased global temperature, sea level rise, more frequent occurrence of extreme weather events, changed weather patterns. There are bound to be many direct impacts on aquatic farming systems brought about by climate change. In the tropics, the main challenges will be to those farming activities that occur in deltaic regions, especially in the Mekong and Red River deltas in Viet Nam and the Ganges-Brahamaputra Delta in Bangladesh. In the temperate region, global warming could raise temperatures to the upper tolerance limits of some cultured species, thereby making such culture systems vulnerable to high temperatures. Climate change has both direct and indirect impacts in fish stock which are exploited commercially. It is evident that natural fish stock will be more resilient to climate impacts with significant food security consequences for certain populations. Climate changes directly effects on physiology and behavior of fish and alter growth, reproductive capacity, mortality and distribution (Issues and Challenges in Fisheries – Bangladesh, 2013).

1.2.2.3 Loss of Fish Biodiversity

The species composition in open-water has been out of balance because of disturbance to natural reproduction of the fish by overfishing and other natural and man-made causes. During migratory journey to and from floodplains and return to the safe habitat fish face many obstacle and hazards, which seriously disturb reproduction and survival in the system.

Physical loss, shrinkage and modification of habitats are major factors in depleting fish varieties. Thousands of physical structure constructed to control floods, cyclone and other natural calamities. Such structures resulted irreparable damages to fisheries diversities.

1.2.3 Disease caused by *Vibrio* sp. in shrimp

Chen et al. (2000) observed that Vibriosis is a bacterial disease responsible for mortality of cultured shrimp worldwide. Vibriosis is caused by gram-negative bacteria in the family Vibrionaceae.

Vibrio harveyi, a gram-negative, luminous bacterium, is one of the important etiologic agents of mass mortalities of *Penaeus monodon* larval rearing systems. A large number of shrimp hatcheries (over 280) along the coastline of our country involved in shrimp seed production often suffer setbacks due to luminescent bacterial disease and suffer enormous economic losses (Venkateswara, 2008). Luminescent *V. harveyi* appears to release exotoxins (Liuxy et al., 1996) and may cause 80-100% mortality in *P. monodon* hatcheries (Harris, 1995). *V. anguillarum*, *V. campbelli*, *V. nereis*, *V. cholerae* (non 01) and *V. splendidus* have also been reported in association with disease outbreaks in shrimps.

According to Jayasree et al. (2006) occurrence of five types of diseases: tail necrosis, shell disease, red disease, loose shell syndrome (LSS) and white gut disease (WGD) is by Vibrio spp. in *Penaeus monodon* from culture ponds of coastal Andhra Pradesh.

V. vulnificusis also an important Vibrio species which can cause wound infections and septicemia with a high mortality rate (Nascimento et al., 2001). *V. parahaemolyticusis* an organism of concern in shrimp culture not only because some strains are associated with diseases in shrimp (Lightner et al.,1992; Vaseeharan and Ramasamy, 2003) but also because some strains of this species are human pathogens, causing gastroenteritis (Powell, 1999). Most strains of *V. parahaemolyticus* associated with human disease produce a thermo stable direct haemolysin (TDH) or/and a TDH-related haemolysin (TRH) (DePaola et al., 2003).

1.2.4 Use of antibiotics in aquaculture

Antibiotics are drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of micro-organisms. Antibiotics that are sufficiently non-toxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases of human, animals and plants. Such chemical agents have been present in the environment for a long time, and have played a role in the battle between man and microbes (Serrano, 2005). Monsur (2012) found

Oxysentin 20%, Aquamycine, Captor and Acimox powder were used as antibiotics. Islam (2013) found Oxy-Dox-F, Renamox, Ciprovet and CT-Dox. Faruk et al., (2005) observed that Aquamycine, Oxy-Dox-F 100, Captor, Oxysentin 20%, Doxy-A Vet WSP, Tetravet WSP, Moxilin Vet WSP, Renamycin Soluble Powder and Oxy-D Vet were used as antibiotics to cure different bacterial diseases of cultured aquaculture species in Bangladesh.

1.2.4.1 Bad effects of antibiotics uses in aquaculture

Antibiotics are given to fish with their food, and sometimes in baths and injections. The unconsumed food, and fish faeces, containing antibiotics spread the sediment at the bottom of the raising pens; antibiotics are leached from the food and faeces and diffuse into the sediment and they can be washed by currents to distant sites (Sørum, 2006). In sediment remains residual of antibiotics that change the composition of the micro flora of the sediment and selecting for antibiotic-resistant bacteria (Sørum, 2006).

1.2.4.2 Effects on ecosystem

The usage of antibiotics in the fish and shrimp aquaculture has become a major problem which seepages through pond bottom and discharges as wastes and effluents mixing into run off water and covering coastal mangroves destroying important natural habitats for shrimps, fishes and to humans. The accumulation of these antibiotics in sediments will definitely alter the natural functions (Bhakta & Munekage, 2009).

1.2.4.3 Antibiotic resistance

The antibiotics that uses are often non-biodegradable and remain in the aquaculture environment for long periods of time. This encourages the growth of bacteria to survive in the presence of these antibiotics. So, bacteria of that environment acquired a resistance that is passed on to consequent generations. This encourages the growth of bacteria, which can survive in the presence of these antibiotics, acquiring a resistance that is passed on to consequent generations (Vignesh et al., 2011). Also the resistant strains of bacteria require even higher levels of dosing to treat diseases. That leads to more antibiotic reaching the natural environment (Bruun et al., 2003).

1.2.4.4 Effects on human health

As human have close contact with fish and need to consume fish so it is very much easy to transfer antibiotics from fish and this is very much harmful for human. It has been extensively shown that excessive and prophylactic use of antibiotics in animals has a negative influence on antibiotic therapy of animal and human bacterial infections because 1) zoonotic antibiotic resistant bacteria are able to infect human beings; and 2) animal and human pathogens can share genetic determinants for antibiotic resistance as the result of horizontal exchange of genetic information (Teuber 2001; Angulo et al., 2004; Cabello 2004; Molbak, 2004; Wassenaar, 2005).

1.2.5 Probiotics concept

The term probiotic, meaning "for life," is derived from the Greek language "pro" and "bios" (Gismondo et al., 1999). The term 'probiotic' was originally coined as an antonym of antibiotics; since probiotics are beneficial microorganisms selectively proliferate to exclude competitively the harmful microbes (Ninawe & Selvin, 2009). Therefore, Verschuere et al. (2000) have proposed a new definition, which allows a broader application of the term 'probiotic' in aquaculture. A probiotic is defined as "a live microbial adjunct that has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response toward disease, or by improving the quality of its ambient environment." According to Merrifield et al. (2010a), there are some essential criteria to select probiotics. Those are

- It must not be pathogenic, not only with regards to the host species but also with regards to aquatic animals in general and human consumers
- It should be registered for use as a feed additive
- It should display advantageous growth characteristics (e.g. short lag period, a short doubling time and/or growth at host rearing temperatures)
- It must be free of plasmid-encoded antibiotic resistance genes
- It must be resistant to bile salts and low pH favorable
- It should be able to adhere to and/or grow well within intestinal mucus
- It should be able to colonize the intestinal epithelial surface
- It should exhibit antagonistic properties towards one or more key pathogens
- It should produce relevant extracellular digestive enzymes (e.g. chitinase if chitin rich ingredients are to be incorporated into the diet or cellulose if the diet is rich in plant ingredients) and/or vitamins
- It should be indigenous to the host or the rearing environment
- It should remain viable under normal storage conditions and be robust enough to survive industrial processes

1.2.5.1 Successful and commercial probiotics in aquaculture

Bacillus sp. have been successfully used as probiotics in the aquaculture of black tiger shrimp (*Penaeus monodon*) in Thailand, where there was an improvement in the growth rate (47%) and survival rate when challenged with *Vibrio harveyi* (Rengpipat et al., 1998). *Pseudomonas fluorescens* (AH2) was shown to be strongly inhibitory against *Vibrio anguillarum* in model systems and it was found that this effect could be transferred to an in vivo situation where the mortality rate in rainbow trout infected with *V. anguillarum* was significantly reduced by the addition of the probiotic bacterium to the tank water (Gram et al., 1999).

Table 1: Prospective probiotics evaluated for shrimp aquaculture applications

Strain	Source	Evaluated for	Effective dose / mode of application	Reference
***Bacillus* S11**	Black tiger shrimp	Growth and survival of black tiger shrimp *Penaeus monodon*	1 kg wet wt (~100 g dry wt) of BS11 (~10^{10} CFU g^{-1}) in 3 kg of feed (2.5% BS11/3 kg)	Rengpipat et al., (2003)
***Bacillus subtilis* BT23**	Shrimp culture ponds	Against the growth of *Vibrio harveyi* isolated by agar antagonism assay from *Penaeus monodon*	10^6–10^8 CFU ml^{-1} for 6 d	Vaseeharan & Ramasamy (2003)
***Pseudomonas* sp. PM11 *Vibrio fluvialis* PM17**	Gut of farm reared sub-adult shrimp	Immunity indicators of *Penaeus monodon*	*Pseudomonas* sp. PM 11 @ 10^3 bacterial cells ml^{-1} for 3 days and *V. fluvialis* PM 17 @ 10^3 bacterial cells ml^{-1} for seven days	Alvandi et al., (2004)
***Arthrobacter* XE-7**	Isolated from *Penaeus chinensis*	Protection of *Penaeus chinensis* post-larvae from pathogenic vibrios such as *Vibrio parahaemolyticus*, *Vibrio anguillarum* and *Vibrio nereis*	10^6 CFU/ml	Li et al., (2006)
Bacillus subtilis* and *B. megaterium	Marine environment	Production of digestive enzymes proteases, carbohydrolases and lipases	Potential application in shrimp feeds	Leonel & Olmos-Soto (2006)
Paenibacillus* spp., *B. cereus* and *Pa.	Seawater, sediment and marine fish-	Against pathogenic Vibrios	10^4 and 10^5 CFU ml^{-1}	Ravi et al., (2007)

polymyxa	gut samples			
Synechocystis **MCCB 114 and 115**	Seawater	Antagonism against *V. harveyi*	Post–larvae fed on the cyanobacterial cultures	Preetha et al., (2007)
Bacillus licheniformis	Shrimp pond	Intestinal microbiota and immunity of the white shrimp *Litopenaeus vannamei*	*B. licheniformis* suspension of 10^5 CFU ml^{-1} for 40 days	Li et al., (2007)
Lactic-acid bacteria	Shrimp gut	Survival of marine shrimp, *Litopenaeus vannamei* challenged with *V. harveyi*	Liquid diet supplemented with B6 strain at 10(8) CFU/ mL	Vieira et al., (2007)
Pediococcus acidilactici	Strain MA 18/5M, CNCM	Survival of *Litopenaeus stylirostris* against Vibriosis caused by *Vibrio nigripulchritudo*	Probiotic-coated pellet feed	Castex et al., (2008)

Ten Harris dencia and de la pena (2001) found that antibiotic resistance was associated with historical and current use of antimicrobials which was found to be high in shrimp ponds using oxolinic acid in feed. According to Vignesh et al. (2011), in hatcheries, it is the common practice to use antibiotics as a prophylactic/therapeutic, measure especially when larval development is hindered. This is also meant to treat the microbial infections in stored water in hatchery tanks, while larval and post larval development continues. The practice can be replaced by using probiotics (helpful microbes).

So from this study we realized that it is high time to seek for alternative to antibiotic. From previous study we found that a single probiotics is used as an alternative to antibiotics but commercial probiotics are made in different composition. Now-a-days various commercial probiotics are found in market and it is easy to use that by farmer. So in this perspective our current experiment is conducted to show how much antagonism show the commercial probiotics against pathogenic *Vibrio* sp. By *in vivo* test we can realize is commercial probiotics able to prevent vibrio disease in shrimp larvae.

Chapter 2

2 Materials and methods

2.1 Collection of Fish Pathogens

A virulent strain of *Vibrio* spp. sample was collected from fish quality control laboratory of Fisheries and Marine Resource Technology Discipline, Khulna University. Pathogenic bacteria were isolated from the infected shrimp by pure culturing them in TCBS agar media, and subsequently in LB_{10} broth media. The isolated bacteria were stocked and stored in 80% glycerol at -80°C.

2.2 Assessment of Probiotic Bacterial composition

Three most popular commercial probiotics were used. Zymetin, Super Biotic and Aqua Clear-S were grown into LB solution. For preparing LB solution, 10g/L of Triptone water and 5g/L of yeast extract were added with required amount of distilled water then stir properly until dissolved completely and sterilized by autoclaving at 151bs pressure for 15 minutes.

Table 2: Composition of three probiotics

Name of probiotic	Composition
Super Biotic	*Bacillus* sp. 10^7 cfu/g
Zymetin	*Bacillus mesentericus, Streptococcus faecalis, Clostridium butyricum*
Aqua Clear-S	*Bacillus mesentericus*,. *Bacillus subtilis, Bacillus licheniformis, Lactobacillus acidophilus, Nitrosomonas* sp., *Nitrobacter* sp., *Aspergillus oryae, Saccharomyces cerevisiae.*

2.3 Bacteria Culture

2.3.1 Starch Agar media

For preparing agar media need starch agar powder in the concentration 30 gm/L water (H_2O). After taking required amount of starch agar powder heated to boiling to dissolve the medium. Sterilize by autoclaving at 151bs pressure 121°C for 15 minute.

2.3.2 Nutrient Agar media

For preparing agar media need starch agar powder in the concentration 28gm/L water (H_2O). After taking required amount of starch agar powder heated to boiling to dissolve the medium. Sterilize by autoclaving at 151bs pressure 121 °C for 15 minute.

2.3.3 Tryptone Soya Agar media

For preparing agar media need starch agar powder in the concentration 40gm/L water (H_2O). After taking required amount of starch agar powder in heated to boiling to dissolve the medium. Sterilize by autoclaving at 151bs pressure 121 °C for 15 minute.

2.4 Antagonistic effect of isolated probiotics against *Vibrio* spp.

Antagonism of isolated probiotics against *Vibrio* spp. by the bacterial isolates was assessed by disk and diffusion method. For this purpose, at first the media was dried to 60°C for 15 minutes and was poured into petri dishes and allowed to solidify.

To test the antagonistic effects of probiotics against *Vibrio* spp. on solid media, the disk diffusion test was performed. Agar plates containing 20 ml of agar. After that taking 1 ml of probiotic solution and spreading properly on the solid media. After few minute later soaked a blank disk into *Vibrio* spp. solution. Then, treated disk was aseptically placed using forceps onto the agar surface of the plates inoculated with the probiotic. The plates should incubate at 37°C for 24 hours.

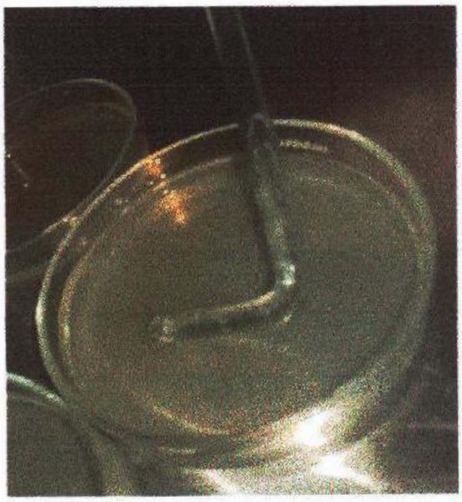


Figure 2: Spreading of probiotics

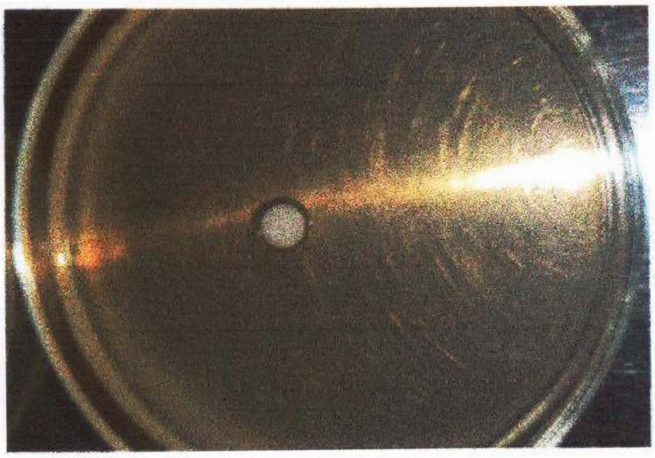


Figure 1: Putting the *Vibrio* soaked disk

2.5 *In Vivo* test

After completing *in vitro* test it is very essential to conduct *in vivo* test for determine that is it better suited for observing the overall effects of the experiment on a living subject. *In vivo* is crucial, because *in vitro* assays can sometimes yield misleading results.

2.5.1 Shrimp Post larvae collection and rearing

For conducting this study, wild shrimp post larvae (PL) was collected. Then the experiment was conducted in the Laboratories of Fisheries and Marine Resource Technology Discipline, Khulna University. This study was conducted to determine the antagonistic effect of probiotics by counting survival, of tiger shrimp against pathogenic *Vibrio* spp. through a challenge test.

2.5.2 Pathogen challenge test

The *Vibrio* spp. concentrations at this study considered as 10^6 CFU/mL in an *in vivo* challenge test with shrimp larvae. For the challenge test, we used 20 liters plastic tanks containing 15 L water, which was stocked with 20 PLs. The PLs were acclimatized for five days before inoculating the bacteria. At that time just feed and probiotics were given. The salinity of the tank water was adjusted to 10 ppt with 120 ppt brine water. Other environmental parameters like dissolve oxygen, temperature, pH etc. were maintained at a constant level. The tanks were distributed randomly at the laboratory to minimize other extraneous factors. After acclimatization the experimental tanks were inoculated with desired bacterial density through spectrophotometric observation. Only 5-10% water was adjusted daily to compensate the loss through evaporation and syphoning. Survival was checked daily.

During the 10 day challenge test period, the survival of prawn was recorded by the following

Formula:

Survival rate (%) = $(N_i - N_f) / N_i) \times 100$

N_i = Initial number of animals.

N_f = Final number of animals.

2.5.3 Experimental design

The experiment was conducted by challenging the shrimp post larvae against *Vibrio* spp. after giving probiotics treatment. The experimental design is given (Table 3)

Table 3: Experimental design

Control		**Treatment**		
Negative Control (No Probiotic+ No *Vibrio*)	**Positive Control (No Probiotic+ *Vibrio*)**	**PL+ *Vibrio*+ Zymetin**	**PL+ *Vibrio*+ Super Biotic**	**PL+ *Vibrio*+ Aqua Clear-S**
NC_1	PC_1	T_ZR_1	T_SR_1	T_AR_1
NC_2	PC_2	T_ZR_2	T_SR_2	T_AR_2
NC_3	PC_3	T_ZR_3	T_SR_3	T_AR_3

NC= Negative control, PC= Positive control, T_Z= Treatment with Zymetin, $T_{S=}$ Treatment with Super Biotic, T_{A-} Treatment with Aqua Clear-S.

2.6 Statistical analyses

All types of statistical analysis were carried out with both Microsoft office Excel (version 2007) and SPSS (version 23). One way ANOVA test was used to determine the comparison among three probiotics and comparison among the growth of *vibrio* spp. on three solid media. Before performing statistical test for *in vivo* result the normal distribution of the data were checked by K-S test, Shapiro-Wilk test and Q-Q plots.

Chapter 3

3 Result

3.1 *In vitro* test

In *in vitro* condition it was tested if our commercial probiotics hinder the growth of *Vibrio* spp. in laboratory condition. For that we use three solid media.

Figure 3 shows the comparison among three different agar media on the growth area diameter of *Vibrio* spp. in the present of commercial probiotic Aqua Clear-S. The ANOVA analysis result revealed that there was no significant difference among 3 media ($F_{(2,24)} = 0.438$, $p=0.651$).

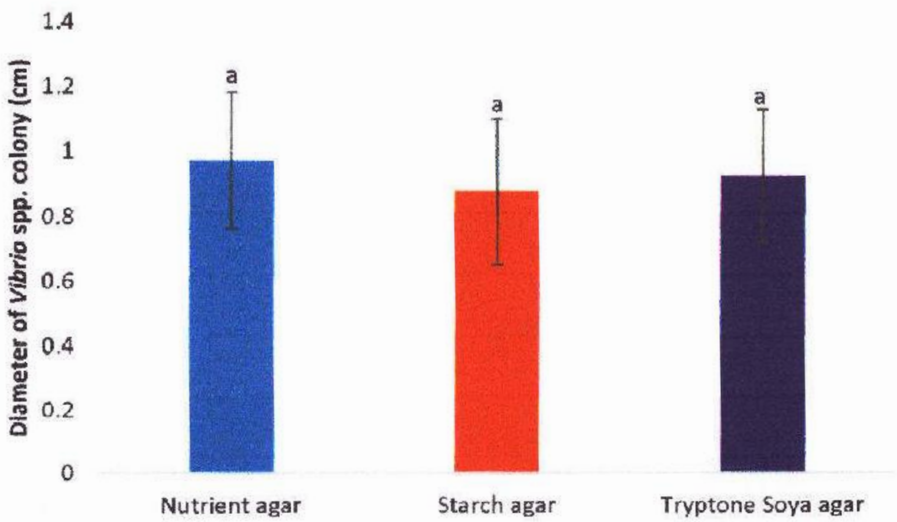


Figure 3: Antagonistic effect of Aqua Clear-S probiotics against *Vibrio* spp. in different agar media. Values are the mean ± SD. Same letter indicates there is no significant difference among the mean.

Figure 4 shows the comparison among three different agar media on the growth area diameter of *vibrio* spp. in the present of commercial probiotic Zymetin. The ANOVA analysis result revealed that there was no significant difference among 3 media ($F(2,24) = 1.141$, $p=0.336$).

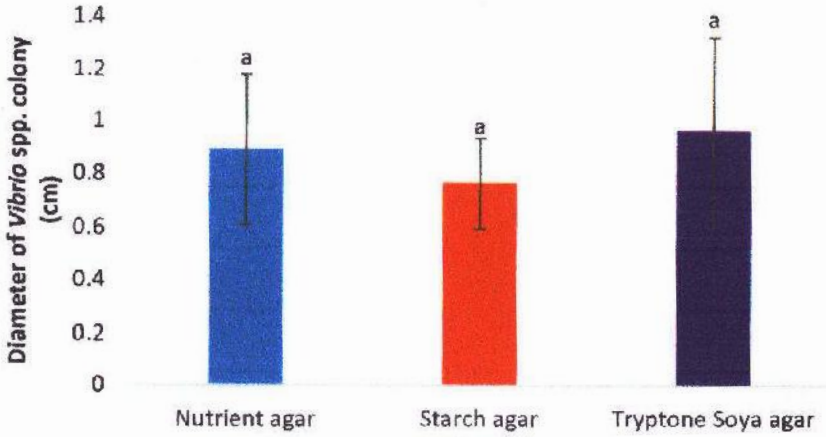


Figure 4: Antagonistic effect of Zymetin probiotics against *Vibrio* spp. in different agar media. Values are the mean ± SD. Same letter indicates there is no significant difference among the mean.

Figure 5 shows the comparison among three different agar media on the growth area diameter of *Vibrio* spp. in the present of commercial probiotic Super Biotic. The ANOVA analysis result revealed that there was no significant difference among 3 media ($F_{(2,24)}$=0.490, p=0.619)

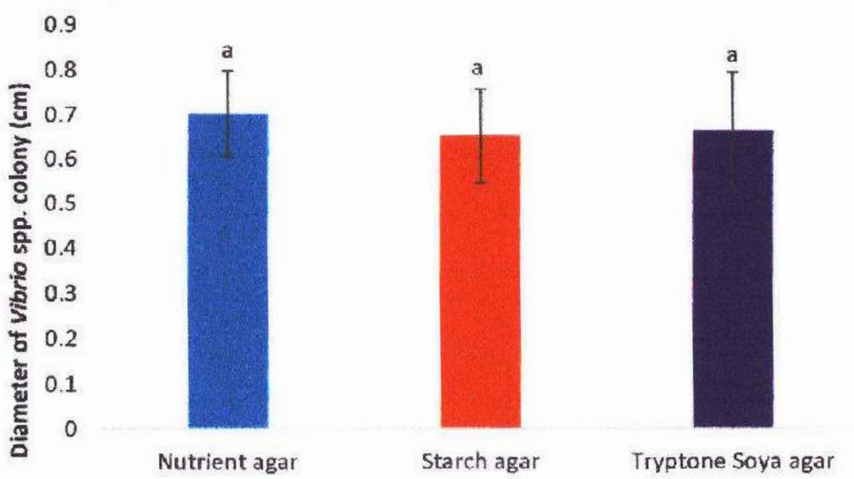


Figure 5: Antagonistic effect of Super Biotic probiotics against *Vibrio* spp. in different agar media. Values are the mean ± SD. Same letter indicates there is no significant difference among the mean.

To determine which probiotic show better defensive mechanism against *Vibrio* spp. comparison among three probiotics were done. In figure 6 showed, the growth area diameter of *Vibrio* spp. in the present of three different commercial probiotics in nutrient agar media.

The ANOVA analysis result revealed that there was significant difference among 3 probiotics antagonism in nutrient agar media ($F_{(2,28)} = 11.549$, p=0.000).

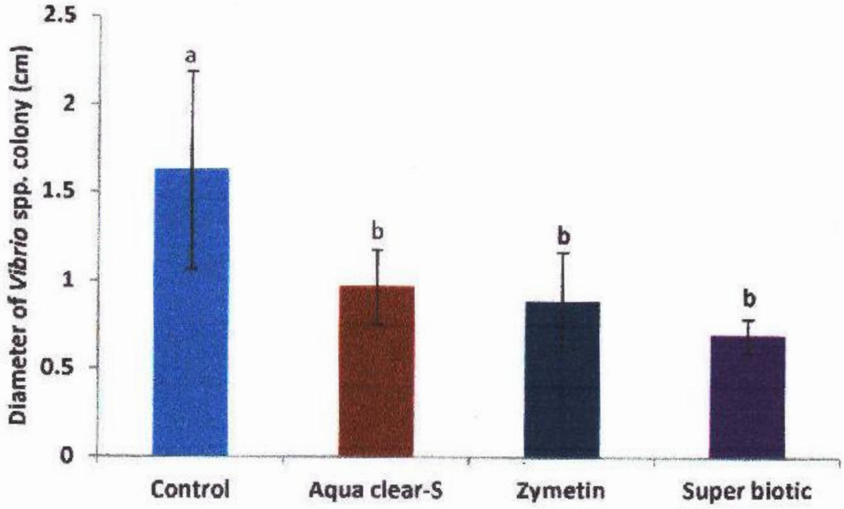


Figure 6: Antagonistic effect of 3 different commercial probiotics against *Vibrio* spp. in nutrient agar media. Values are the mean ± SD. Different letter indicates there was significant difference among the mean.

Figure 7 shows the growth area diameter of *Vibrio* spp. in the present of three different commercial probiotics in starch agar media. The ANOVA analysis result revealed that there was significant difference among 3 probiotics antagonism in starch agar media ($F_{(3,28)} = 88.274$, p=0.000).

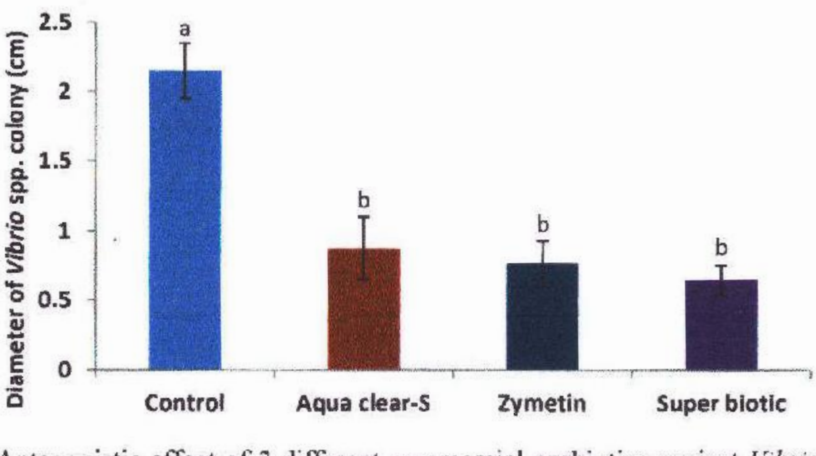


Figure 7: Antagonistic effect of 3 different commercial probiotics against *Vibrio* spp. in starch agar media. Values are the mean ± SD. Different letter indicates there was significant difference among the mean.

Figure 8 shows the growth area diameter of *Vibrio* spp. in the present of three different commercial probiotics in tryptone soya agar media. The ANOVA analysis result revealed that there was significant difference among 3 probiotics antagonism in tryptone soya agar media ($F_{(3,28)} = 35.955$, p=0.000).

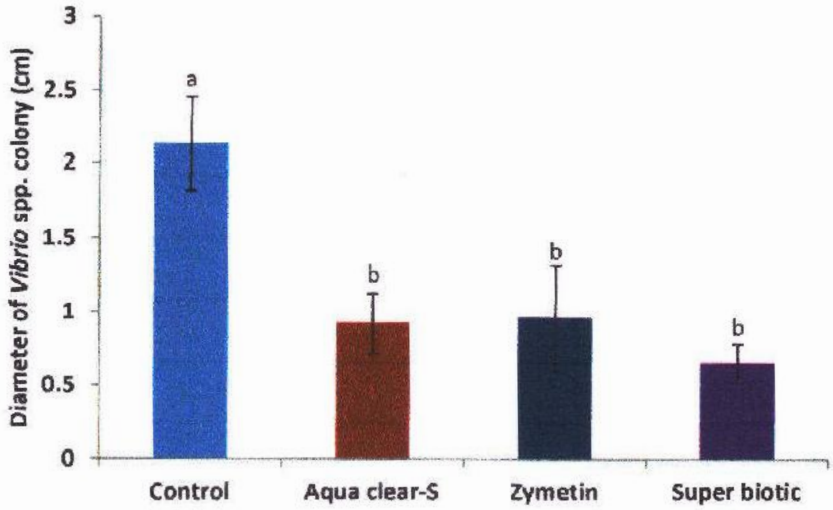


Figure 8: Antagonistic effect of 3 different commercial probiotics against *Vibrio* spp. in tryptone soya agar media. Values are the mean ± SD. Different letter indicates there is significant difference among the mean.

Figure 9 shows the total growth area diameter of *Vibrio* spp. in the present of three different commercial probiotics. The ANOVA analysis result revealed that there was significant difference among 3 probiotics antagonism ($F_{(2,78)}$ =11.414, p=0.000).

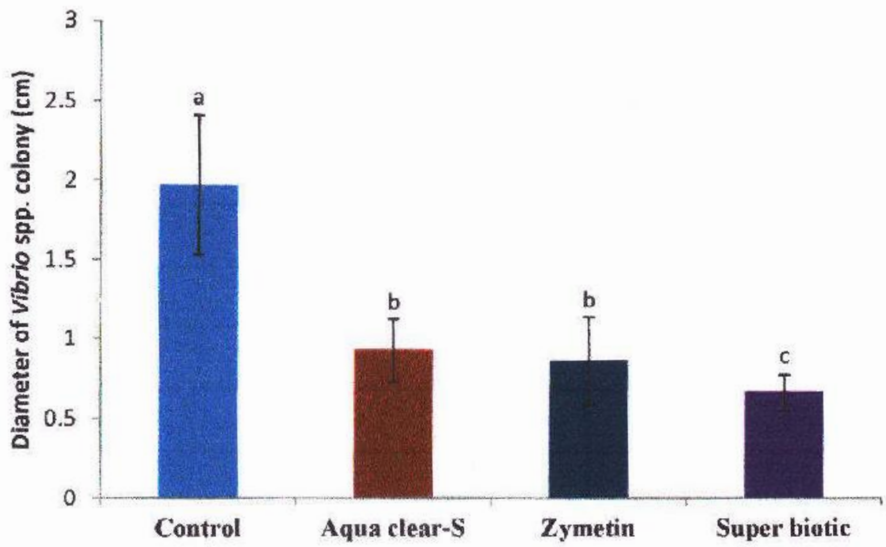


Figure 9: Antagonistic effect of 3 different commercial probiotics against *Vibrio* spp. Values are the mean ± SD. Different letter indicates there is significant difference among the mean.

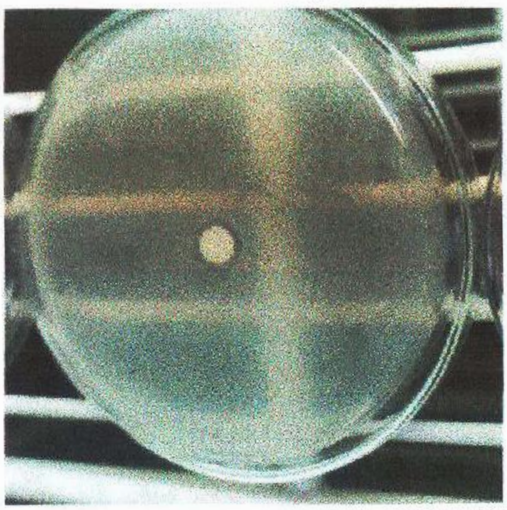


Figure 10: Growth area of *Vibrio* spp. in the presence of probiotic.

3.2 *In vivo* challenge test

In vivo challenge test was continuing in wet laboratories of Fisheries and Marine Resource Technology Discipline, Khulna University for 10 days.

3.2.1 Mortality of shrimp PL in the experiment

There was no mortality in the control group during the 10 days challenge test period. First mortality occurred on day 4 in the treatment groups. In positive treatment group 50.00%, in zymetin treatment group 23.33%, in super biotic treatment group 16.65% and in aqua clear-s treatment group 31.65% mortality occur. In 5^{th} day, mortality occurred in positive treatment group 23.35%, in zymetin treatment group 28.35%, in super biotic treatment group 23.35% and in aqua clear-s treatment group 31.65%. From the experiment it was observed that mortality just occurred in all treatment groups just 4^{th} and 5^{th} day.

Table 4: Mortality (%) of experimental shrimp post larvae

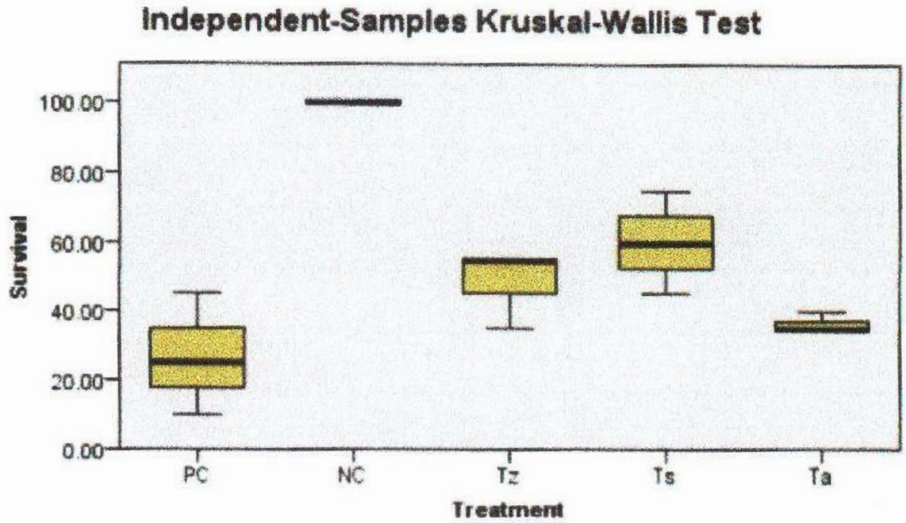
Day after challenges	Mortality %				
	PC	**NC**	**Tz**	**Ts**	**Ta**
Day 1	0	0	0	0	0
Day 2	0	0	0	0	0
Day 3	0	0	0	0	0
Day 4	50.00%	0	23.33%	16.67%	31.65%
Day 5	23.35%	0	28.35%	23.35%	31.65%
Day 6	0	0	0	0	0
Day 7	0	0	0	0	0
Day 8	0	0	0	0	0
Day 9	0	0	0	0	0
Day 10	0	0	0	0	0

NC= Negative control, PC= Positive control, T_Z= Treatment with Zymetin, T_{S-} Treatment with Super Biotic, $T_{A=}$ Treatment with Aqua Clear-S.

Figure 11: Shrimp post larvae that were challenged with *Vibrio* spp. no probiotics was used.

Figure 12: Shrimp post larvae that were challenged with *Vibrio* spp. in the presence of commercial probiotics.

From figure 10 and figure 11 we can understand the physical condition (e.g. black spot on tail, abdomen) of shrimp post larvae after challenge test.



NC= Negative control, PC= Positive control, T_Z= Treatment with Zymetin, T_S- Treatment with Super Biotic, T_A- Treatment with Aqua Clear-S.

Figure 13: Cumulative survive ability test of shrimp post larvae during 10 days challenge ($p<0.05$).

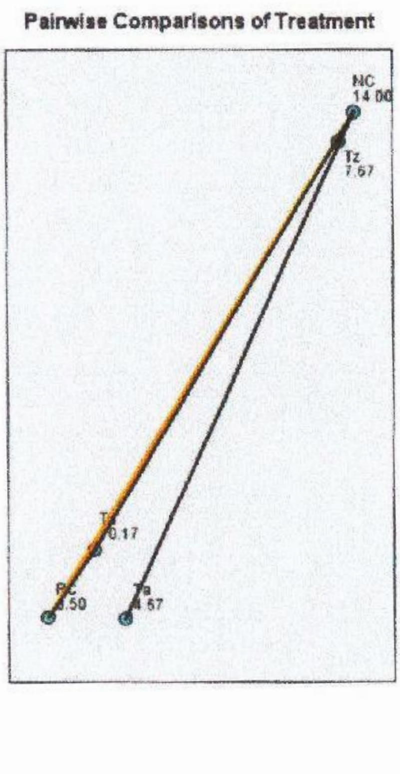


Figure 14: Pairwise comparison of treatment group (significant difference found between negative control and positive control; $p<0.05$)

Figure 14 shows the survival of shrimp larvae challenge with *Vibrio* spp. in the present of three different commercial probiotics. The ANOVA analysis result revealed that there was significant difference among treatment and control groups ($F_{(4,10)}$ =17.951, p=0.000).

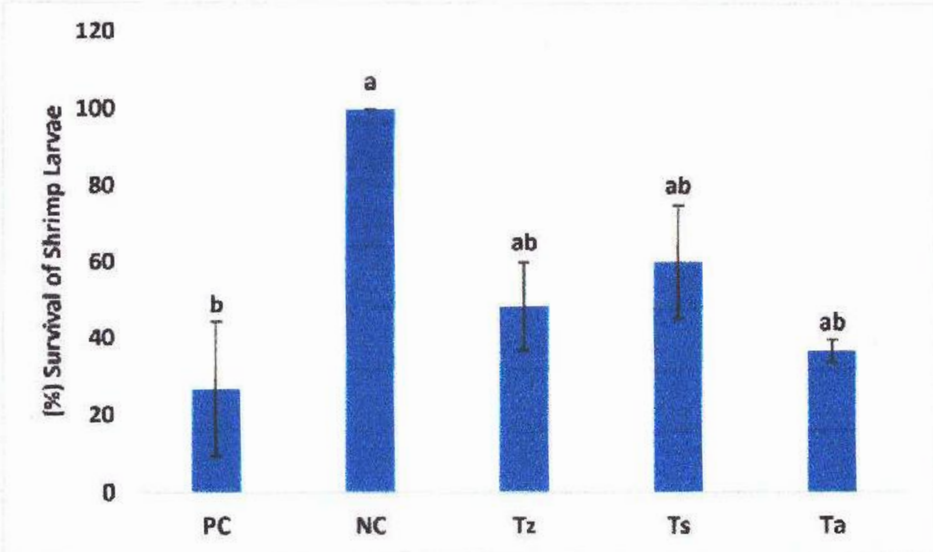


Figure 15: Antagonistic effect of three different probiotics against *Vibrio* spp. in *in vivo* condition. Values are the mean ± SD. Same letter indicates there is no significant difference among the mean.

Chapter 4

4 Discussion

The present study was attempted to evaluate the defensive power of three commercial probiotics against pathogenic *Vibrio* spp. both *in vitro* and *in vivo* condition in shrimp post larvae. Three types of solid media were used for *in vitro* experiment. After completing the experiment we found there was no significant difference found on the growth of different media. But from figure 6, 7, 8 and figure 9 it was found that there was significant difference among probiotics defensive power against pathogenic *Vibrio* spp.

From those figure it's shown that the growth area diameter is less in case of Super biotic than Aqua Clear-s or Zymetin. So Super Biotic shows more defensive power against pathogenic *Vibrio* and more actively inhibit the growth of pathogenic *Vibrio* spp.

From figure 10 we saw that due to the presence of probiotic *Vibrio* spp. cannot grow much area, probiotics hamper the growth. From *in vivo* test we have found that there was a significant difference between negative control group and positive control group. Significant difference also found among positive control group and three probiotic treatment group. No mortality occurred in negative control group because no *Vibrio* spp. was present in that group. Higher mortality found in positive control group because in positive control group shrimp larvae get direct challenge with *Vibrio* spp. No probiotics was used in positive control group. Among three probiotics treatments group higher mortality rate found in treatment with aqua clear-s. Lower mortality found in treatment with super biotic. So it can be concluded that super biotic resist the growth of *Vibrio* spp. more both in laboratory and in live condition.

From table 2 it was shown that super biotic contains *Bacillus* sp. 10^7 CFU/g that's why it showed more antagonism against *Vibrio* spp. According to Banat et al. (2010), Bacteria of the *Bacillus* genus (predominantly, *B. subtilis*) produces various bio-surfactants, which have a high potential for biotechnology and pharmacology. These compounds vary in structure and spectrum of activity and usually are responsible for antimicrobial effects of *Bacillus* bacteria (Cao et al., 2009; Fernandes et al., 2007).

Dalmin et al. (2001) reported that use of *Bacillus* spp. improved water quality, survival and growth rates and the health status of juvenile *Penaeus monodon* and reduced the pathogenic Vibrios. From table 2 it was shown that zymetin contains *Bacillus mesentericus*, *Streptococcus faecalis*, *Clostridium butyricum* bacteria but it cannot prevent the growth of *vibrio* sp. better than super biotic. Ling et al. (2015) found that supplementation with *C.*

butyricum combined with *B. infantis* probiotic mixture is a simple and effective method to treat antibiotic-associated diarrhea (AAD).

Venkateswara (2008) reported that *Lactobacillus* spp. acts as an effective probiotics against vibriosis and white spot diseases in *P. monodon*. Probiotics contain multiple strains of bacteria like *Bacillus acidophius*, *B. subtilus* , *B. lechniformis*, *Nitrobacter* spp., *Aerobacter* and *Sacharomyces carvevisiae*, application of probiotic through water of tanks and ponds may also have an effect on fish health by improving several qualities of water, since they modify the bacteria composition of the water and sediments.

Bacillus subtilis BT 23 strain show antagonism against the growth of *Vibrio harveyi* isolated from *Penaeus monodon* (Vaseeharan & Ramasamy, 2003). A study found that probiotic solutions containing *Streptomyces* in combination with bacteria of the genus *Bacillus* improved growth parameters and immune response in Pacific white shrimp (*Litopenaeus vannamei*) (Bernal et al., 2017). Similar findings were demonstrated in freshwater prawns (*Macrobrachium rosenbergii*) when their diet was supplemented with *Bacillus licheniformis* (Kumar et al., 2013).

In the present study three commercial probiotics Super biotic, Zymetin and Aqua clear-s were characterized for their activity against pathogenic *Vibrio* spp. From this study it was found that commercial probiotics Super biotic is more effective for preventing Vibrio disease.

Further study need to check the ability of other probiotics to resist vibrio disease more than super biotic. Commercial probiotic manufacturers should apply modern molecular techniques to ensure that the species of bacteria used in their products are correctly identified, for quality assurance as well as for safety.

Chapter 5

5 Conclusion

From previous study, it can be assume that attack of *Vibrio* spp. in shrimp is increasing day by day and the use of antibiotics and aquaculture drug to prevent that disease is also increasing day by day. Aquaculture in Bangladesh is heading towards commercialization and intensification. Different types of drugs and antibiotics have become an essential part of successful commercial aquaculture production but those drugs and antibiotics are showed adverse effect on environment and human health. To evaluate proper commercial probiotics that show more antagonism against *Vibrio* spp. in shrimp, the current study was conducted.

In the present study three commercial probiotics were used and challenge took place both in *in vitro* and *in vivo* condition. The findings resultant from this study revealed that there was significant difference of survive ability among three treatment groups. Also significant difference found among control groups and treatment groups. The *Vibrio* spp. concentrations for *in vivo* challenge test considered as 10^6 CFU/mL. First mortality occurs in both positive control group and three probiotics treatment groups in 4th day. Mortality occurred in positive treatment group 50.00%, in zymetin treatment group 23.33%, in super biotic treatment group 16.65% and in aqua clear-s treatment group 31.65%. In 5th day, mortality occurred in positive treatment group 23.35%, in zymetin treatment group 28.35%, in super biotic treatment group 23.35% and in aqua clear-s treatment group 31.65%. Survival rate is low in aqua clear-s. Super biotic also show more antagonism both in *in vitro* and *in vivo* condition.

Further study need to evaluate proper commercial probiotics that show more antagonism against *Vibrio* spp. than these three probiotics in shrimp.

Chapter 6

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Chapter 7

Annex

Oneway

Descriptives

Diameter of Aqua clea-s in different agar media

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Nutrient agar	9	.9722	.21082	.07027	.8102	1.1343	.60	1.35
Starch agar	9	.8778	.22515	.07505	.7047	1.0508	.60	1.25
Tryptone soya agar	9	.9278	.20632	.06877	.7692	1.0864	.65	1.30
Total	27	.9259	.20956	.04033	.8430	1.0088	.60	1.35

Test of Homogeneity of Variances

Diameter of Aqua clea-s in different agar media

Levene Statistic	df1	df2	Sig.
.113	2	24	.893

ANOVA

Diameter of Aqua clea-s in different agar media

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.040	2	.020	.438	.651
Within Groups	1.102	24	.046		
Total	1.142	26			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: **Diameter of Aqua clea-s in different agar media**

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nutrient agar	Starch agar	.09444	.10100	.624	-.1578	.3467
	Tryptone soya agar	.04444	.10100	.899	-.2078	.2967
Starch agar	Nutrient agar	-.09444	.10100	.624	-.3467	.1578
	Tryptone soya agar	-.05000	.10100	.874	-.3022	.2022
Tryptone soya agar	Nutrient agar	-.04444	.10100	.899	-.2967	.2078
	Starch agar	.05000	.10100	.874	-.2022	.3022

Homogeneous Subsets

Diameter of Aqua clea-s in different agar media

Tukey HSD[a]

VAR00002	N	Subset for alpha = 0.05
		1
Starch agar	9	.8778
Tryptone soya agar	9	.9278
Nutrient agar	9	.9722
Sig.		.624

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

Oneway

Descriptives

Diameter of Zymetin in different agar media

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Nutrient agar	9	.8944	.28553	.09518	.6750	1.1139	.60	1.40
Starch agar	9	.7667	.16956	.05652	.6363	.8970	.60	1.15
Tryptone soya agar	9	.9667	.36401	.12134	.6869	1.2465	.60	1.50
Total	27	.8759	.28601	.05504	.7628	.9891	.60	1.50

Test of Homogeneity of Variances

Diameter of Zymetin in different agar media

Levene Statistic	df1	df2	Sig.
4.342	2	24	.025

ANOVA

Diameter of Zymetin in different agar media

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.185	2	.092	1.141	.336
Within Groups	1.942	24	.081		
Total	2.127	26			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: **Diameter of Zymetin in different agar media**

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Nutrient agar	Starch agar	.12778	.13410	.613	-.2071	.4627
	Tryptone soya agar	-.07222	.13410	.853	-.4071	.2627
Starch agar	Nutrient agar	-.12778	.13410	.613	-.4627	.2071
	Tryptone soya agar	-.20000	.13410	.313	-.5349	.1349
Tryptone soya agar	Nutrient agar	.07222	.13410	.853	-.2627	.4071
	Starch agar	.20000	.13410	.313	-.1349	.5349

Homogeneous Subsets

Diameter of Zymetin in different agar media

Tukey HSD[a]

VAR00002	N	Subset for alpha = 0.05
		1
Starch agar	9	.7667
Nutrient agar	9	.8944
Tryptone soya agar	9	.9667
Sig.		.313

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

Oneway

Descriptives

Diameter of Super biotic in different agar media

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Nutrient agar	9	.7000	.09682	.03227	.6256	.7744	.60	.80
Starch agar	9	.6500	.10607	.03536	.5685	.7315	.60	.90
Tryptone soya agar	9	.6611	.13176	.04392	.5598	.7624	.60	1.00
Total	27	.6704	.11030	.02123	.6267	.7140	.60	1.00

Test of Homogeneity of Variances

Diameter of Super biotic in different agar media

Levene Statistic	df1	df2	Sig.
.058	2	24	.944

ANOVA

Diameter of Super biotic in different agar media

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.012	2	.006	.490	.619
Within Groups	.304	24	.013		
Total	.316	26			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: **Diameter of Super biotic in different agar media**

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Nutrient agar	Starch agar	.05000	.05305	.619	-.0825	.1825
	Tryptone soya agar	.03889	.05305	.746	-.0936	.1714
Starch agar	Nutrient agar	-.05000	.05305	.619	-.1825	.0825
	Tryptone soya agar	-.01111	.05305	.976	-.1436	.1214
Tryptone soya agar	Nutrient agar	-.03889	.05305	.746	-.1714	.0936
	Starch agar	.01111	.05305	.976	-.1214	.1436

Homogeneous Subsets

Diameter of Super biotic in different agar media

Tukey HSD[a]

VAR00002	N	Subset for alpha = 0.05: 1
Starch agar	9	.6500
Tryptone soya agar	9	.6611
Nutrient agar	9	.7000
Sig.		.619

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

Oneway

Descriptives

Diameter of *vibrio* spp. colony in nutrient agar media with different probiotics

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	1.6300	.55857	.24980	.9364	2.3236	1.25	2.60
Aqua clear-S	9	.9722	.21082	.07027	.8102	1.1343	.60	1.35
Zymetin	9	.8944	.28553	.09518	.6750	1.1139	.60	1.40
Super biotic	9	.7000	.09682	.03227	.6256	.7744	.60	.80
Total	32	.9766	.41015	.07251	.8287	1.1244	.60	2.60

Test of Homogeneity of Variances

Diameter of *vibrio* spp. colony in nutrient agar media with different probiotics

Levene Statistic	df1	df2	Sig.
3.807	3	28	.021

ANOVA

Diameter of *vibrio* spp. colony in nutrient agar media with different probiotics

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.884	3	.961	11.549	.000
Within Groups	2.331	28	.083		
Total	5.215	31			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: **Diameter of *vibrio* spp. colony in nutrient agar media with different probiotics**

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Aqua clear-S	.65778*	.16093	.002	.2184	1.0972
	Zymetin	.73556*	.16093	.000	.2962	1.1749
	Super biotic	.93000*	.16093	.000	.4906	1.3694

Aqua clear-S	Control	-.65778*	.16093	.002	-1.0972	-.2184
	Zymetin	.07778	.13601	.940	-.2936	.4491
	Super biotic	.27222	.13601	.212	-.0991	.6436
Zymetin	Control	-.73556*	.16093	.000	-1.1749	-.2962
	Aqua clear-S	-.07778	.13601	.940	-.4491	.2936
	Super biotic	.19444	.13601	.492	-.1769	.5658
Super biotic	Control	-.93000*	.16093	.000	-1.3694	-.4906
	Aqua clear-S	-.27222	.13601	.212	-.6436	.0991
	Zymetin	-.19444	.13601	.492	-.5658	.1769

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Diameter of *vibrio* spp. colony in nutrient agar media with different probiotics

Tukey HSD[a,b]

		Subset for alpha = 0.05	
VAR00002	N	1	2
Super biotic	9	.7000	
Zymetin	9	.8944	
Aqua clear-S	9	.9722	
Control	5		1.6300
Sig.		.282	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.500.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Oneway

Descriptives

Diameter of *vibrio* spp. colony in starch agar media with different probiotics

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
Control	5	2.1500	.19685	.08803	1.9056	2.3944	1.85	2.35
Aqua clear-S	9	.8778	.22515	.07505	.7047	1.0508	.60	1.25
Zymetin	9	.7667	.16956	.05652	.6363	.8970	.60	1.15
Super biotic	9	.6500	.10607	.03536	.5685	.7315	.60	.90
Total	32	.9813	.54503	.09635	.7847	1.1778	.60	2.35

Homogeneous Subsets

Diameter of *vibrio* spp. colony in starch agar media with different probiotics

Tukey HSD[a,b]

VAR00002	N	Subset for alpha = 0.05	
		1	2
Super biotic	9	.6500	
Zymetin	9	.7667	
Aqua clear-S	9	.8778	
Control	5		2.1500
Sig.		.084	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.500.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Oneway

Descriptives

Diameter of *vibrio* spp. colony in tryptone soya agar media with different probiotics

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	2.1400	.31898	.14265	1.7439	2.5361	1.65	2.45
Aqua clear-S	9	.9278	.20632	.06877	.7692	1.0864	.65	1.30
Zymetin	9	.9667	.36401	.12134	.6869	1.2465	.60	1.50
Super biotic	9	.6611	.13176	.04392	.5598	.7624	.60	1.00
Total	32	1.0531	.55197	.09757	.8541	1.2521	.60	2.45

Test of Homogeneity of Variances

Diameter of *vibrio* spp. colony in tryptone soya agar media with different probiotics

Levene Statistic	df1	df2	Sig.
4.929	3	28	.007

ANOVA

Diameter of *vibrio* spp. colony in tryptone soya agar media with different probiotics

	Sum of Squares	df	Mean Square	F	Sig.

Between Groups	7.498	3	2.499	35.955	.000
Within Groups	1.946	28	.070		
Total	9.445	31			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: **Diameter of *vibrio* spp. colony in tryptone soya agar media with different probiotics**

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Control	Aqua clear-S	1.21222*	.14706	.000	.8107	1.6137
	Zymetin	1.17333*	.14706	.000	.7718	1.5749
	Super biotic	1.47889*	.14706	.000	1.0774	1.8804
Aqua clear-S	Control	-1.21222*	.14706	.000	-1.6137	-.8107
	Zymetin	-.03889	.12429	.989	-.3782	.3005
	Super biotic	.26667	.12429	.164	-.0727	.6060
Zymetin	Control	-1.17333*	.14706	.000	-1.5749	-.7718
	Aqua clear-S	.03889	.12429	.989	-.3005	.3782
	Super biotic	.30556	.12429	.089	-.0338	.6449
Super biotic	Control	-1.47889*	.14706	.000	-1.8804	-1.0774
	Aqua clear-S	-.26667	.12429	.164	-.6060	.0727
	Zymetin	-.30556	.12429	.089	-.6449	.0338

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Diameter of *vibrio* spp. colony in tryptone soya agar media with different probiotics

Tukey HSD[a,b]

VAR00002	N	Subset for alpha = 0.05: 1	2
Super biotic	9	.6611	
Aqua clear-S	9	.9278	
Zymetin	9	.9667	
Control	5		2.1400
Sig.		.136	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.500.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Oneway

Descriptives

Diameter of *vibrio* spp. colony with different probiotics

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
Control	15	1.9733	.43869	.11327	1.7304	2.2163	1.25	2.60
Aqua clear-S	27	.9278	.19871	.03824	.8492	1.0064	.60	1.35
Zymetin	27	.8648	.27590	.05310	.7557	.9740	.60	1.50
Super biotic	27	.6704	.11030	.02123	.6267	.7140	.60	1.00
Total	96	1.0010	.50047	.05108	.8996	1.1024	.60	2.60

Test of Homogeneity of Variances

Diameter of *vibrio* spp. colony with different probiotics

Levene Statistic	df1	df2	Sig.
14.865	3	92	.000

ANOVA

Diameter of *vibrio* spp. colony with different probiotics

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.779	3	5.926	90.621	.000
Within Groups	6.016	92	.065		
Total	23.795	95			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: **Diameter of *vibrio* spp. colony with different probiotics**

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Control	Aqua clear-S	1.04556*	.08235	.000	.8301	1.2610
	Zymetin	1.10852*	.08235	.000	.8930	1.3240
	Super biotic	1.30296*	.08235	.000	1.0875	1.5184
Aqua clear-S	Control	-1.04556*	.08235	.000	-1.2610	-.8301
	Zymetin	.06296	.06960	.802	-.1192	.2451

	Super biotic	.25741*	.06960	.002	.0753	.4395
Zymetin	Control	-1.10852*	.08235	.000	-1.3240	-.8930
	Aqua clear-S	-.06296	.06960	.802	-.2451	.1192
	Super biotic	.19444*	.06960	.032	.0123	.3766
Super biotic	Control	-1.30296*	.08235	.000	-1.5184	-1.0875
	Aqua clear-S	-.25741*	.06960	.002	-.4395	-.0753
	Zymetin	-.19444*	.06960	.032	-.3766	-.0123

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Diameter of *vibrio* spp. colony with different probiotics

Tukey HSD[a,b]

		Subset for alpha = 0.05		
VAR00002	N	1	2	3
Super biotic	27	.6704		
Zymetin	27	.8648	.8648	
Aqua clear-S	27		.9278	
Control	15			1.9733
Sig.		.059	.842	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 22.500.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Oneway

Descriptives

Survival of shrimp larvae in challenge test

					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
PC	3	26.6667	17.55942	10.13794	-16.9534	70.2867	10.00	45.00
NC	3	100.0000	.00000	.00000	100.0000	100.0000	100.00	100.00
Tz	3	48.3333	11.54701	6.66667	19.6490	77.0177	35.00	55.00
Ts	3	60.0000	15.00000	8.66025	22.7379	97.2621	45.00	75.00
Ta	3	36.6667	2.88675	1.66667	29.4956	43.8378	35.00	40.00
Total	15	54.3333	28.08575	7.25171	38.7800	69.8867	10.00	100.00

Test of Homogeneity of Variances

Survival of shrimp larvae in challenge test

Levene Statistic	df1	df2	Sig.

2.381	4	10	.121

ANOVA

Survival of shrimp larvae in challenge test

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9693.333	4	2423.333	17.951	.000
Within Groups	1350.000	10	135.000		
Total	11043.333	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: **Survival of shrimp larvae in challenge test**

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
PC	NC	-73.33333*	9.48683	.000	-104.5553	-42.1114
	Tz	-21.66667	9.48683	.227	-52.8886	9.5553
	Ts	-33.33333*	9.48683	.035	-64.5553	-2.1114
	Ta	-10.00000	9.48683	.825	-41.2219	21.2219
NC	PC	73.33333*	9.48683	.000	42.1114	104.5553
	Tz	51.66667*	9.48683	.002	20.4447	82.8886
	Ts	40.00000*	9.48683	.012	8.7781	71.2219
	Ta	63.33333*	9.48683	.000	32.1114	94.5553
Tz	PC	21.66667	9.48683	.227	-9.5553	52.8886
	NC	-51.66667*	9.48683	.002	-82.8886	-20.4447
	Ts	-11.66667	9.48683	.736	-42.8886	19.5553
	Ta	11.66667	9.48683	.736	-19.5553	42.8886
Ts	PC	33.33333*	9.48683	.035	2.1114	64.5553
	NC	-40.00000*	9.48683	.012	-71.2219	-8.7781
	Tz	11.66667	9.48683	.736	-19.5553	42.8886
	Ta	23.33333	9.48683	.177	-7.8886	54.5553
Ta	PC	10.00000	9.48683	.825	-21.2219	41.2219
	NC	-63.33333*	9.48683	.000	-94.5553	-32.1114
	Tz	-11.66667	9.48683	.736	-42.8886	19.5553
	Ts	-23.33333	9.48683	.177	-54.5553	7.8886

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Survival of shrimp larvae in challenge test

Tukey HSD[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
PC	3	26.6667		
Ta	3	36.6667	36.6667	
Tz	3	48.3333	48.3333	
Ts	3		60.0000	
NC	3			100.0000
Sig.		.227	.177	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Treatment

Case Processing Summary

	Treatment	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Survival	PC	3	100.0%	0	0.0%	3	100.0%
	NC	3	100.0%	0	0.0%	3	100.0%
	Tz	3	100.0%	0	0.0%	3	100.0%
	Ts	3	100.0%	0	0.0%	3	100.0%
	Ta	3	100.0%	0	0.0%	3	100.0%

Descriptives

	Treatment			Statistic	Std. Error
Survival	PC	Mean		26.6667	10.13794
		95% Confidence Interval for Mean	Lower Bound	-16.9534	
			Upper Bound	70.2867	
		5% Trimmed Mean		.	
		Median		25.0000	
		Variance		308.333	

	Std. Deviation		17.55942	
	Minimum		10.00	
	Maximum		45.00	
	Range		35.00	
	Interquartile Range		.	
	Skewness		.423	1.225
	Kurtosis		.	.
NC	Mean		100.0000	.00000
	95% Confidence Interval for Mean	Lower Bound	100.0000	
		Upper Bound	100.0000	
	5% Trimmed Mean		100.0000	
	Median		100.0000	
	Variance		.000	
	Std. Deviation		.00000	
	Minimum		100.00	
	Maximum		100.00	
	Range		.00	
	Interquartile Range		.00	
	Skewness		.	.
	Kurtosis		.	.
Tz	Mean		48.3333	6.66667
	95% Confidence Interval for Mean	Lower Bound	19.6490	
		Upper Bound	77.0177	
	5% Trimmed Mean		.	
	Median		55.0000	
	Variance		133.333	
	Std. Deviation		11.54701	
	Minimum		35.00	
	Maximum		55.00	
	Range		20.00	
	Interquartile Range		.	
	Skewness		-1.732	1.225
	Kurtosis		.	.
Ts	Mean		60.0000	8.66025
	95% Confidence Interval for Mean	Lower Bound	22.7379	
		Upper Bound	97.2621	
	5% Trimmed Mean		.	
	Median		60.0000	
	Variance		225.000	
	Std. Deviation		15.00000	

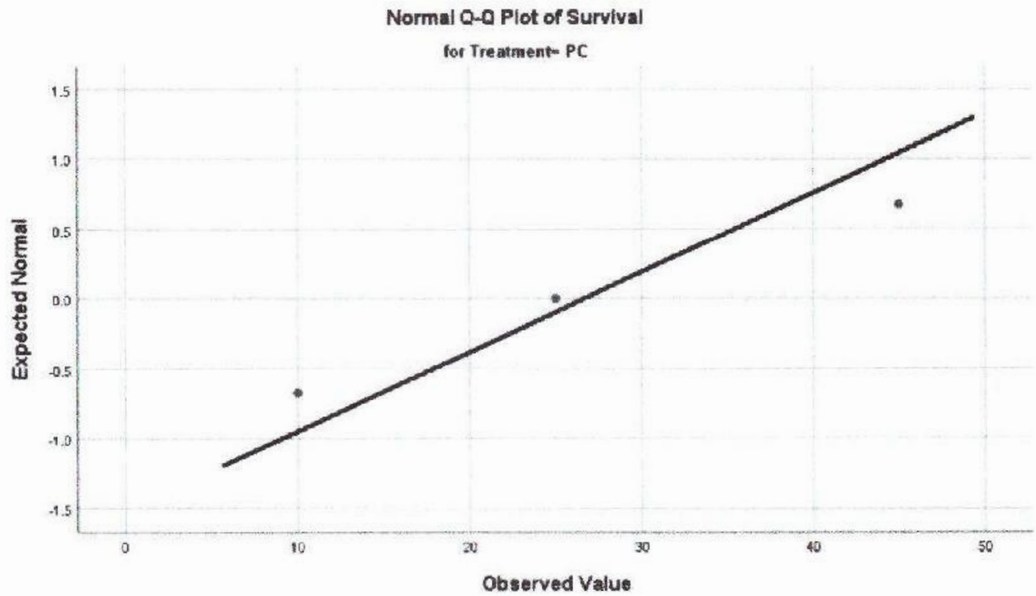
		Minimum		45.00	
		Maximum		75.00	
		Range		30.00	
		Interquartile Range		.	
		Skewness		.000	1.225
		Kurtosis		.	.
	Ta	Mean		36.6667	1.66667
		95% Confidence Interval for Mean	Lower Bound	29.4956	
			Upper Bound	43.8378	
		5% Trimmed Mean		.	
		Median		35.0000	
		Variance		8.333	
		Std. Deviation		2.88675	
		Minimum		35.00	
		Maximum		40.00	
		Range		5.00	
		Interquartile Range		.	
		Skewness		1.732	1.225
		Kurtosis		.	.

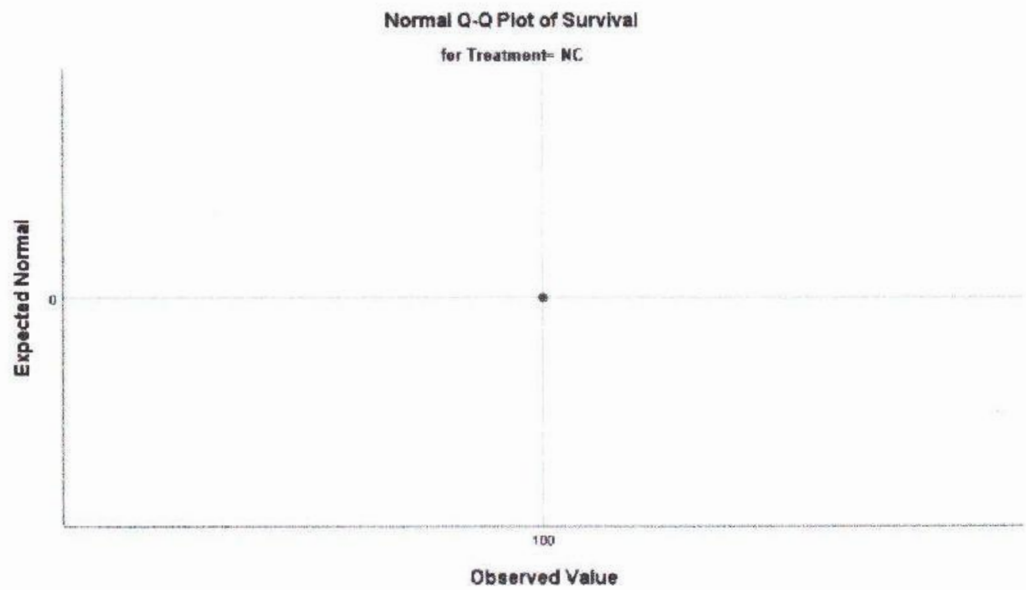
Tests of Normality

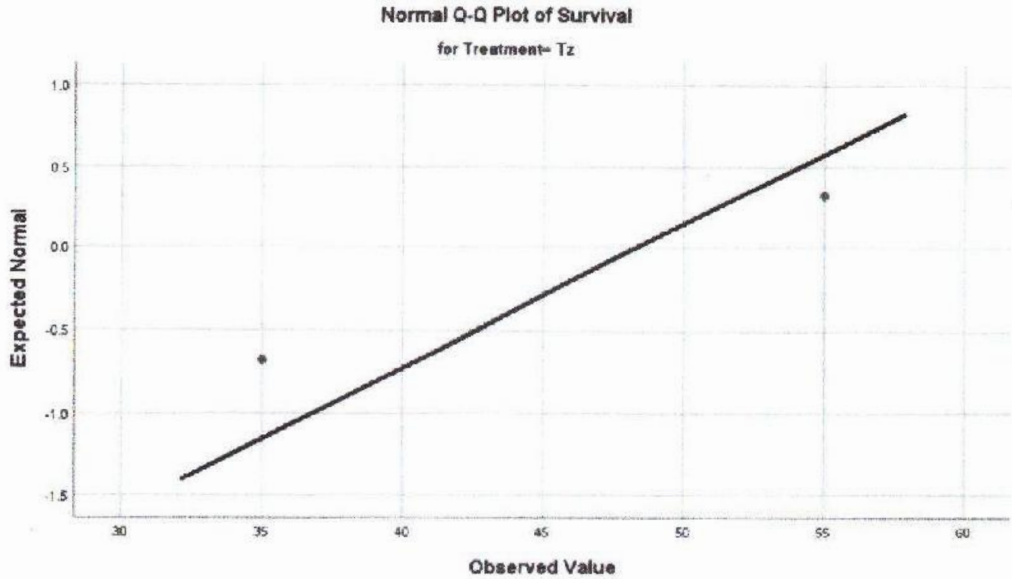
		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Treatment	Statistic	df	Sig.	Statistic	df	Sig.
Survival	PC	.204	3	.	.993	3	.843
	NC	.	3	.	.	3	.
	Tz	.385	3	.	.750	3	.000
	Ts	.175	3	.	1.000	3	1.000
	Ta	.385	3	.	.750	3	.000

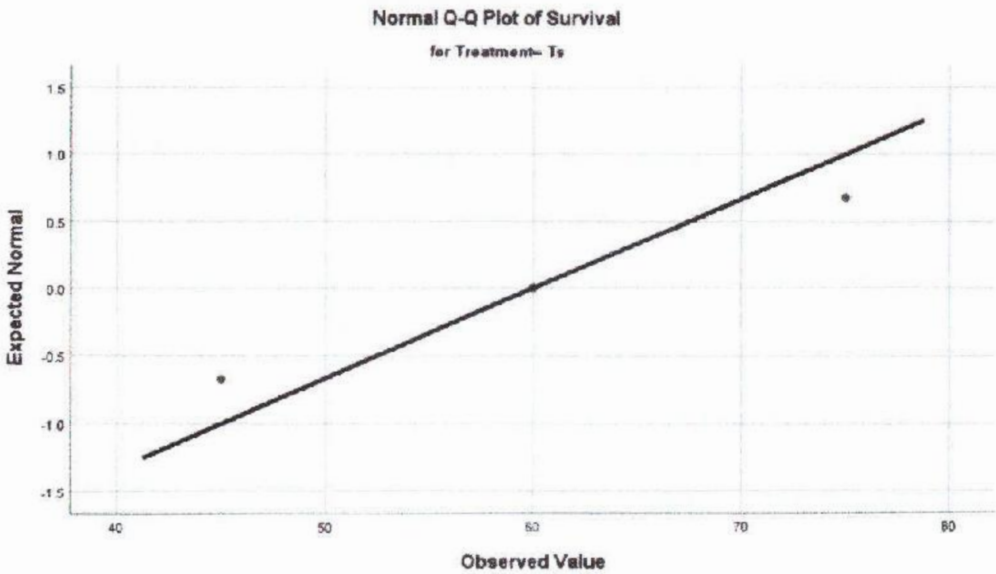
a. Lilliefors Significance Correction

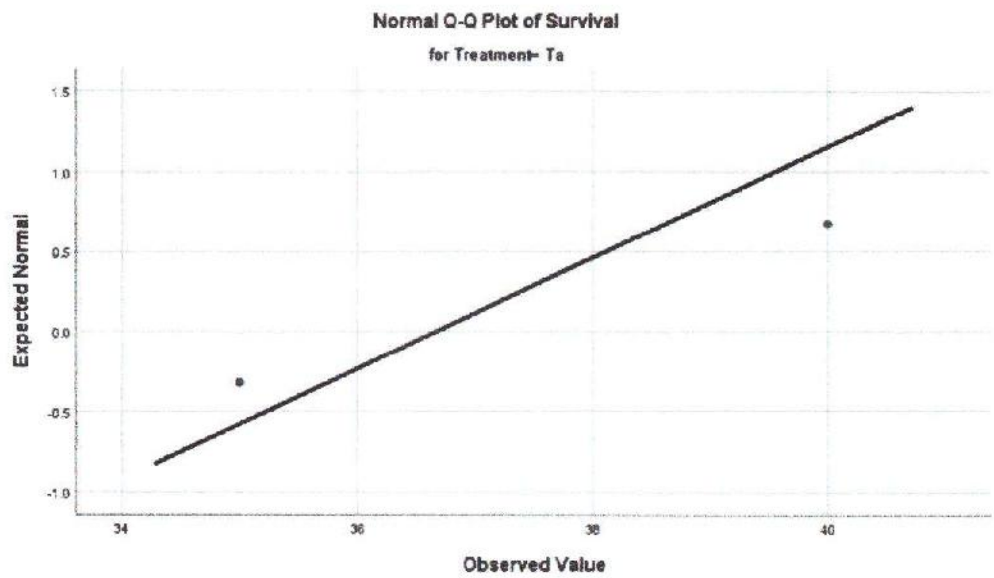
Survival
Normal Q-Q Plots


Normal Q-Q Plot of Survival
for Treatment= PC
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Expected Normal
0
10
20
30
40
50
Observed Value

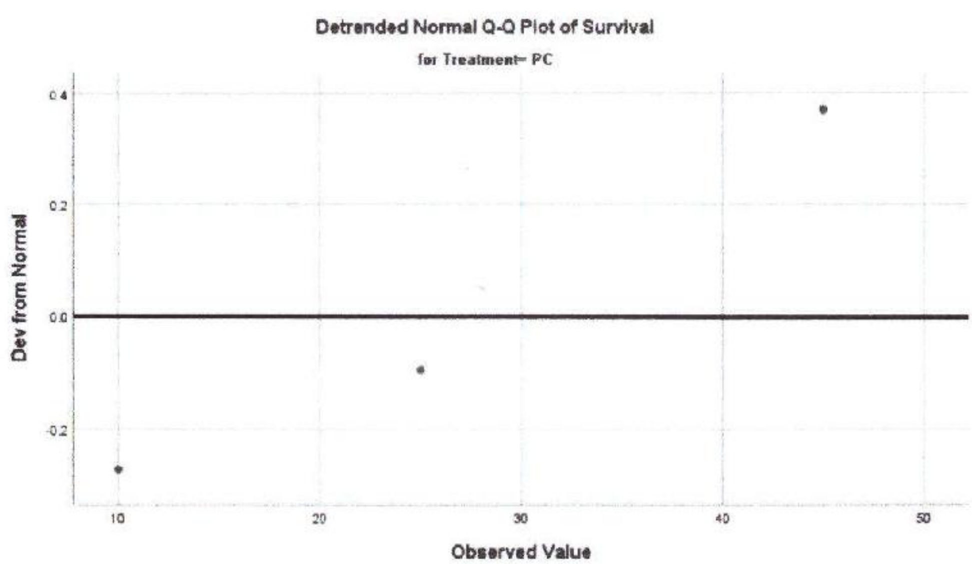

Normal Q-Q Plot of Survival
for Treatment= NC
Expected Normal
0
100
Observed Value

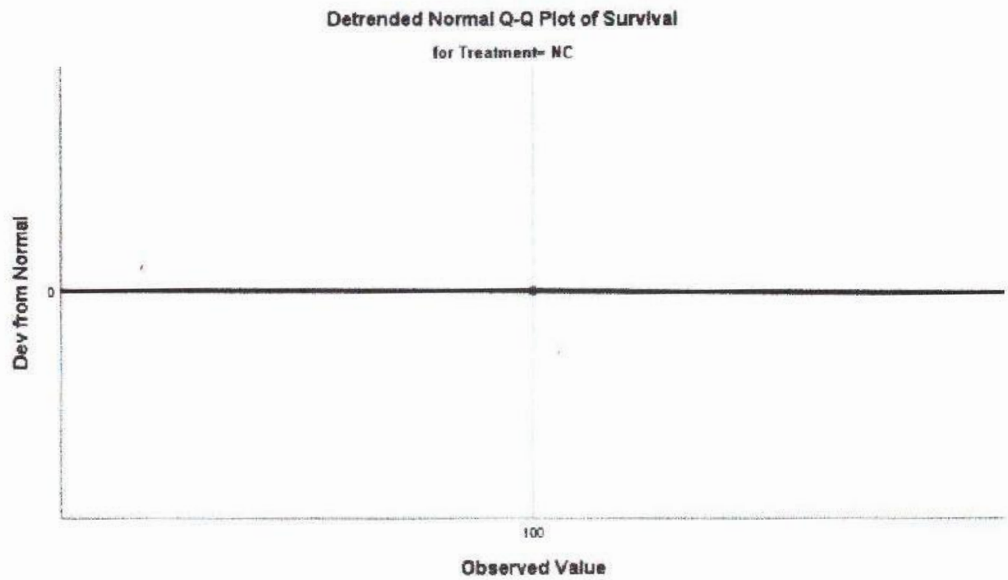

Normal Q-Q Plot of Survival
for Treatment= Tz
Expected Normal
Observed Value
1.0
0.5
0.0
-0.5
-1.0
-1.5
30
35
40
45
50
55
60

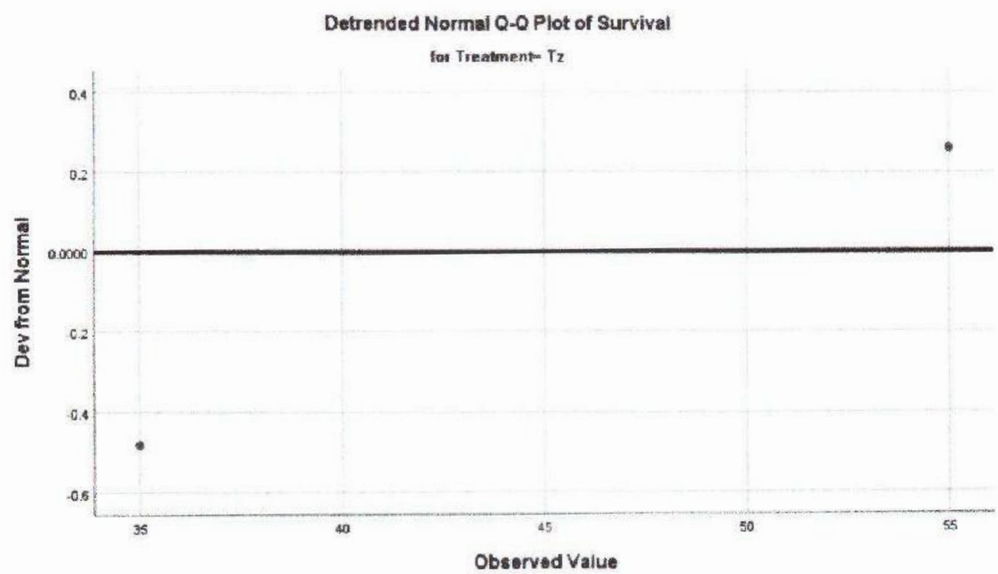

Normal Q-Q Plot of Survival
for Treatment= Ts
Expected Normal
Observed Value
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
40
50
60
70
80

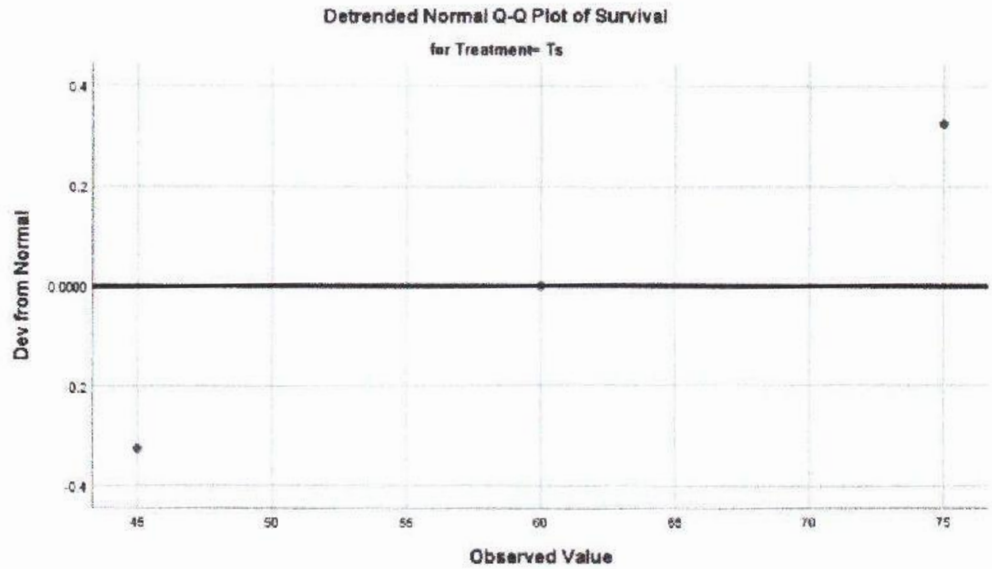


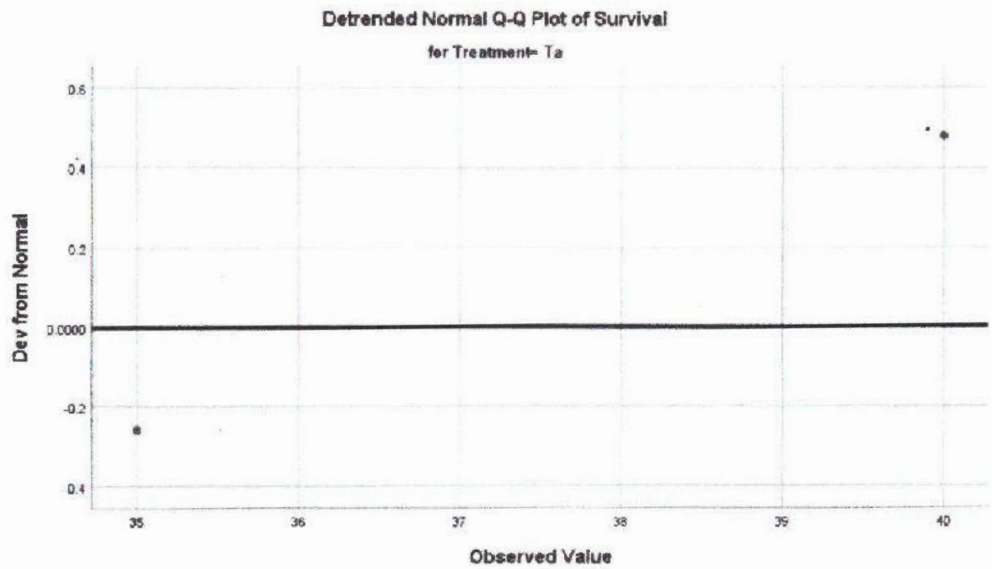
Detrended Normal Q-Q Plots




Detrended Normal Q-Q Plot of Survival
for Treatment= NC
Dev from Normal
0
100
Observed Value


Detrended Normal Q-Q Plot of Survival
for Treatment= Tz
Dev from Normal
0.4
0.2
0.0000
-0.2
-0.4
-0.6
35
40
45
50
55
Observed Value






```
*Nonparametric Tests: Independent Samples.
NPTESTS
  /INDEPENDENT TEST (Survival) GROUP (Treatment)
KRUSKAL_WALLIS(COMPARE=PAIRWISE) JONCKHEERE_TERPSTRA(ORDER=ASCENDING
COMPARE=PAIRWISE) HODGES_LEHMANN
  /MISSING SCOPE=ANALYSIS USERMISSING=EXCLUDE
  /CRITERIA ALPHA=0.05  CILEVEL=95.
```

Nonparametric Tests

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Survival is the same across categories of Treatment.	Independent-Samples Kruskal-Wallis Test	.026	Reject the null hypothesis.
2	The distribution of Survival is the same across categories of Treatment.	Independent-Samples Jonckheere-Terpstra Test for Ordered Alternatives	.645	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Each node shows the sample average rank of Treatment.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
PC-NC	-10.500	3.619	-2.902	.004	.037
Ta-NC	9.333	3.619	2.579	.010	.099
PC-Ts	-6.667	3.619	-1.842	.065	.654
Tz-NC	6.333	3.619	1.750	.080	.801
Ts-NC	3.833	3.619	1.059	.289	1.000
PC-Tz	-4.167	3.619	-1.151	.250	1.000
PC-Ta	-1.167	3.619	-.322	.747	1.000
Tz-Ts	-2.500	3.619	-.691	.490	1.000
Ta-Tz	3.000	3.619	.829	.407	1.000
Ta-Ts	5.500	3.619	1.520	.129	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.