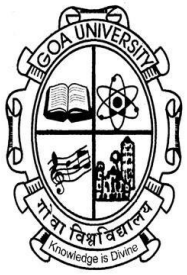
Halotolerant bacteria as probiotics in shrimp aquaculture

A Thesis submitted to Goa University



for the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

By

Samantha Fernandes

Research Guide

Prof. Savita Kerkar

Goa University

Taleigao, Goa

June, 2019

Halotolerant bacteria as probiotics in shrimp aquaculture

A Thesis submitted to Goa University for the

Award of the Degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

By

Samantha Fernandes

Research Guide

Prof. Savita Kerkar

Goa University

Taleigao, Goa

June, 2019

Dedicated to

My Family

DECLARATION

I hereby declare that the thesis entitled "**Halotolerant bacteria as probiotics in shrimp aquaculture"**, submitted for the degree of **Doctor of Philosophy (Ph.D.)** in **Biotechnology** to Goa University, is based on studies carried out by me at the Department of Biotechnology, Goa University, under the supervision of **Prof. Savita Kerkar** (Research Guide).

The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University / Institute. Materials obtained from other sources have been duly acknowledged in the thesis.

Place:

Date:

Samantha Fernandes

(Research Scholar)

ACKNOWLEDGEMENT

At the outset I thank God for bestowing me with the physical and mental capacity to carry out my research work. It is because of His grace that I ‘ve been able to accomplish everything that I have today.

I thank and appreciate the valuable time set aside for me by my guide **Prof. Savita Kerkar**, Professor, Department of Biotechnology. The door to her office was always open whenever I required her guidance. Her constant support, encouragement and trust helped shape me into a better person professionally and personally. No words can describe how grateful I am having gained immensely from her profound knowledge in the subject; her positive attitude, commitment, generosity, patience, enthusiasm, constant encouragement and guidance at every juncture during the course of my research.

I would also like to thank **Prof. Varun Sahni,** the Vice Chancellor of Goa University and **Dr. Satish Shetye** (former Vice Chancellor) of Goa University for the access to the University Laboratory and all the required apparatus, facilities and infrastructure that allowed me to effectively and efficiently conduct my research.

I express my gratitude to the present and former Deans of Faculty of Life Sciences, **Prof. P. K. Sharma**, **Prof. M.K. Janarthanam**, **Prof. Saroj Bhosle** and **Prof. G. N. Nayak** for their valuable advice and helpful encouragement.

I am extremely thankful to **Dr. Ramaiah** (Vice Chancellor’s Nominee) for his constant guidance and critical evaluation of my research.

I am grateful to the University Grants Commission, India for the financial assistance through the UGC-MANF fellowship program.

My profound gratitude to the Head, Department of Biotechnology, for providing me with laboratories well equipped with the necessary staff, instruments and chemicals needed for my research.

I take this opportunity to thank **Dr. Urmila Barros**, **Prof. Usha D. Muraleedharan**, **Prof. Sanjeev C. Ghadi** for their valuable advice and feedback all throughout my

tenure at the university. I also express my gratitude to **Prof. UMX Sangodkar**, **Dr. Dharmendra Tiwari**, **Dr. Sanika Samant**, **Dr. Trupti Asolkar** and **Dr. Abhishek Mishra** for their support. I have learnt so much from all of you.

I appreciate the ever willing and cheerful assistance of the non-teaching staff of our Department. I express my heartfelt gratitude to **Mr. Serrao, Mrs. Sanjana, Mrs. Sandhya, Mr. Ulhas, Mr. Parijat, Mr. Tulsidas, Mrs. Ruby, Mr. Sameer, Mr. Rahul, Ms. Jaya, Mr. Aashish, Mr. Amonkar and Mrs. Sumati**. I also fondly remember the very helpful **Late. Mr. Martin** who would go out of his way to help us out with anything, he will always be missed. I would also like to thank **Mrs. Gulabi** for her assistance in the animal house and **Mrs. Kunda** and **Mrs. Manda** for ensuring a very clean atmosphere for researchers to work.

To my dear lab mates and friends with whom I have had the pleasure of spending all these years with – **Kirti, Shuvankar, Amruta, Michelle, Ruchira, Manasi, Priti, Srijay, Lillian, Poonam, Preethi, Alisha, Perantho, Imran, Surya, Krupali, Rupesh, Judith, Pingal, Nicola, Delicia, Priyanka, Sreekala, Varsha, Anchit, Deepti** and **Nuha** thank you so much for all the help, support and for creating a cheerful work environment. You all have left me with so many wonderful memories that I will cherish for life. A special thank you to my seniors **Tonima, Kuldeep** and **Flory**, their excellent research work served as a benchmark and a constant reference throughout my research.

I also thank **Avelyno D'Costa** and **Praveen Kumar** for assisting with parts of my research, especially with the toxicology studies.

I am extremely thankful to the Head, Department of Zoology for providing me with the necessary aqua culture lab facilities for my shrimp trials.

I am extremely thankful to the National Institute of Oceanography, Goa for providing me with aquatic facilities and the National Chemical Laboratory, Pune for providing the necessary facilities for the proteomic analysis that was a part of my research.

I am greatly thankful to **Dr. Rivonkar**, **Mr. Praveen Madkaikar** and all the staff at Dr Rivonkars aquaculture farm for providing me with industry grade facilities and the required manpower assistance. It was because of their generosity that I was able to

carry out my field trials. A sincere gratitude to **Mr. Rajesh** for providing me with the shrimp larvae, feed and expert advice on shrimp aquaculture.

A special thanks to **Prof. Julio B. Fernandes** for his invaluable guidance and advice at different occasions during my Ph.D. tenure. I am previledged to have an uncle like you.

I also remember all my teachers, staff and colleagues from St. Aloysius college - Mangalore, DCT's Dhempe college of Arts and Science- Miramar, St. Xavier's Higher secondary school- Mapusa, Our Lady of the Rosary high school- Dona Paula and Primrose primary school - Porvorim. You all have played an important role in moulding me into the person I am today.

To my parents **Mrs. Antonette** and **Dr. John Fernandes**, my inlaws **Dr. Alba** and **Mr. Baylon DMello**, my husband **Algernon**, my sister **Liesl**, my brothers in law **Elroy** and **Alston**, and my darling nephew **Zayne**; words cannot express how grateful I am to all of you. Your love, support and sacrifice all throughout my Ph. D. journey has been the greatest blessing of all and I am forever indebted to all of you. I am also grateful to my extended family for their care and advice all along the way.

Lastly, I place on record, my gratitude to all those who have directly or indirectly lent a helping hand in my research and in the completion of my thesis in some way or the other, whether directly or indirectly and my sincere apologies to all those whom I could not mention individually.

---- Samantha Fernandes

CONTENTS

ABBREVIATIONS

%	Percentage
°C	Degrees Centigrade
µ	Micron(s)
µg	Micrograms
µg/µL	Micrograms/ microlitre
µL	Micro litre
µm	Micro meter
µmol	Micro mole
µmol/L	Micro moles per litre
ABW	Average body weight
ACN	Acetonitrile
APHA	American Public Health Association
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Beads/mL	Beads per milliliter
BLAST	Basic Local Alignment Search Tool
BOD	Biological oxygen demand
bp	Base pairs
BSA	Bovine serum albumin
Cd	Cadmium
Cells/mL	Cells per millilitre
CFU	Colony forming units
CFU/mL	Colony forming units per millilitre
CLSI	The Clinical and Laboratory Standards Institute
cm	Centimetre
Cu	Copper
CuSO4	Copper sulphate
DMPD	N,N-dimethyl-p-phenylene-diamine sulphate
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
DO	Dissolved oxygen
DTT	Dithiothreitol
EHT	Electron high tension
EMS	Ethyl methanesulfonate
FA	Formic acid
FAO	Food and Agricultural Organization
FCR	Feed conversion ratio
FE	Feed efficiency
g	Grams
g/L	Gram per litre
g/m3	Gram per cubic meter

GIT	Gastrointestinal tract
h	Hour(s)
HLS	Hemocyte lysate supernatant
IAA	Indole acetic acid
IDA	Information dependent acquisition
Kb	Kilo base pairs
Kg/ha	Kilogram/hectare
KX	1000 times
L	Litre
Larvae/ m2	Larvae/square meter
LC-MS	Liquid chromatography mass spectrometry
L-DOPA	L-3, 4-dihydroxy phenylalanine
m	Metre
M	Molar
m2	Square meter
mA	Milliampere
mg	Milligram(s)
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
min	Minute(s)
mL	Millilitre
mm	Millimetre
mM	Millimolar
MS	Mass spectrometry
MTCC	Microbial Type Culture Collection and Gene Bank
N	Normality
NaCl	Sodium chloride
NBT	Nitro- blue tetrazolium
NCBI	National Center for Biotechnology Information
NCL	National Chemical Laboratory
NH4Cl	Ammonium chloride
nm	Nanometre
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
pH	Hydrogen ion concentration
PL	Post-larvae
pNPP	Paranitrophenyl palmitate
PO	Phenoloxidase
ppm	Parts per million
ppt	Parts per thousand
PRPs	Pattern recognition proteins
ProPO	Prophenoloxidase
psu	Percentile salinity units

PVC	Polyvinyl chloride
RB	Respiratory burst
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
s	Second(s)
SD	Standard deviation
SEM	Scanning electron microscopy
SGR	Specific growth rate
sp.	Species
spp.	Species (plural)
subsp.	Subspecies
SPF	Specific pathogen free
SWATH	Sequential window acquisition of all theoretical fragment-ion spectra
TCB	Total culturable bacteria
TCBS	Thiosulphate citrate bile salts sucrose
TDS	Total dissolved solids
THC	Total hemocyte count
TPP	Total plasma protein
TVC	Total Vibrio count
U/mg	Units per milligram
UK	United Kingdom
USA	United States of America
UV	Ultra violet
W	Weight
WD	Working distance
ZMA	Zobell marine agar

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER 1:

INTRODUCTION

1. Introduction

Marine ecosystems provide a rich source of essential products and services such as seafood, biological metabolites with pharmaceutical potential, fuel, nutrient and waste management, etc that are necessary for human population (Lloret, 2010; Chivian and Bernstein, 2008). Globally, seafood is among the highest traded food commodities that include both, fish and shellfish obtained from aquaculture production and capture fisheries. It contributes to more than 15% of the average animal protein consumption providing nutrition, livelihood and income to people in many developing countries (Smith et al., 2010). The increasing demand for seafood is attributed to the growing human population, escalating affluence, urbanisation, and corresponding consumer preferences (Longo et al., 2019, Béné et al. 2015, York & Gossard 2004). Over the last 6 decades, the use of mechanised fishing crafts, efficient means of catching and preserving fish have successfully strengthened seafood supplies thereby outstripping the growth of population (Little et al., 2016). However, the Food and Agriculture Organization (FAO, United States) reported that nearly 90 % of the fisheries worldwide have either been overfished or exploited to maximum capacity (FAO, 2016). The lack of information on the maximum sustainable yield for fishery has imposed heavy pressure on the global capture fisheries (Pauly et al. 2003; Hilborn 2011). One notable strategy to manage and limit overfishing is by adapting to modern aquaculture. Although aquaculture has been in the food industry for only 4 - 5 decades, opportunity due to necessity has been its major driving force (Little et al., 2016).

Aquaculture deals with the farming of aquatic organisms, including fish, crustaceans, molluscs, and aquatic plants; giving ownership to an individual or corporate of the stock being cultivated. It involves the intervention in the rearing processes such as feeding, stocking, monitoring the water conditions, protection from predators etc to enhance production (John et al., 2018, FAO, 2016). The exponential rise in the human population, along with the per capita consumption of fish and increasing wealth (mainly in the developed western countries), has promoted aquaculture as an enterprise at small scale and corporate level (Little et al., 2016). It is a flourishing industry providing one of the most sustainable sources of edible protein globally. According to Food and Agriculture organization (FAO, 2014), a 50 % increase in the aquaculture production has been reported over the past decade, with more than 220 species of shellfish and finfish being cultivated

worldwide. Among the crustaceans, shrimp are the highest farmed species throughout the world, grown mainly in coastal ponds (Naylor et al., 2000).

Shrimp aquaculture is a promising economic activity of the food industry, dealing with the controlled cultivation of shrimp to a final commercial size in small water bodies (Fernandes et al., 2019). The global shrimp production has shown an increasing trend from 1 to approximately 4 million tonnes over the last two decades (Food and Agriculture Organization, FAO 2016) with Asia contributing to 90 % of this production (Hoseinifar et al., 2010, Sahu et al., 2008). The Government of India has listed shrimp farming as an important sector for increasing exports, thereby contributing to the foreign exchange of the country [Swathi al., 2013]. Two species that have dominated more than 90 % of the farmed shrimp market are Whiteleg shrimp *Litopenaeus vannamei and Penaeus monodon.* However, a significant change in the world penaeid production has been noticed over a period of time with respect to species cultivated. A remarkable decrease in the share of *P. monodon* has been observed from 51.0 % in 1998 to 17.8 % in 2007; while the share of *Litopenaeus vannamei* has shown an increasing trend from 19.63 % in 1998 to 70.25 % in 2007. This trend has continued to increase due to a switch in the preference over *monodon* in many Asian countries such as China, Thailand etc.

Litopenaeus vannamei, also known as Pacific white shrimp is a tropical marine species farmed globally in intensive, semi-intensive and extensive culture systems (Li et al., 2017). This species has been cultivated in inland waters with low salinity in many Asian countries as it can tolerate a wide range of salinity and temperature (Li et al, 2018, Cheng et al., 2016). In Asia, the cultivation of Whiteleg shrimp (*Litopenaeus vannamei*), is the species of choice owing to the availability of specific pathogen-free (SPF) brood stock, lesser protein requirement, lower production cost, tolerance to wider range of salinity, its unique taste and high nutritive value, making it a promising candidate for aquaculture over *P. monodon* (Limhang et al. 2005, Lin and Chen 2003, 2001). Further, *L. vannamei* is a favourable choice of the processors, as it has a higher meat yield of 66 – 68 % as compared to *P. monodon* and is also preferred by the consumers of the United States (world's biggest shrimp market) (Liao and Chien, 2011, Rosenberry 2002). In India, *L. vannamei* is the largest cultured shrimp species in terms of productivity with its production being more than 3 million tonnes per year (FAO, 2016, Zhang et al., 2016).

However, the intensification and increased commercialization of aquaculture production, has inevitably resulted in disease related problems. In large-scale production systems, where the farmed shrimp are exposed to stressful situations, problems related to deterioration of environmental conditions and diseases have resulted in serious production crashes. Since the last decade, disease outbreaks have become a predominant constraint to the aquaculture industry, impeding both social and economic development in many countries (Qi et al., 2009, Bondad-Reantaso et al., 2005). Under stressful conditions, *Litopenaeus vannamei* is susceptible to various diseases caused by viruses such as yellow head virus, white spot syndrome virus and prevalent pathogenic bacteria such as *Vibrio* species (*V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* etc) causing vibriosis (Li et al., 2018, Ramos-Carreno et al., 2014, Lin et al., 2012). The diseased shrimp show slow growth, low survival, low stress tolerance, decreased disease resistance and symptoms such as lethargy, tail cramping, disoriented spinning behavior etc., especially at low salinities (Li et al., 2018). The diseases caused by *Vibrio* spp. frequently implicate in episodes of mortality, leading to production crashes and serious economic loss in many Asian countries. The application of antimicrobials and antibiotics are the most widely used methods for prevention and treatment of aquaculture diseases (Liu et al., 2017). These agents are derived from natural or synthetic sources and are capable of killing or inhibiting the growth of microorganisms. Although the discovery of antibiotics has been a boon to mankind since the 20th century, its successful use has been compromised by the development of tolerance to that compound since it was first administered (Davies and Davies, 2010). Hence, the application of antibiotics and chemotheraphy to combat bacterial diseases has promoted the development of antibiotic-resistant bacteria, environmental hazards and accumulation of antibiotic residues in tissues [Cabello, 2006]. The unchecked usage of these antibiotics and overcrowding of shrimp in an aquaculture pond deteriorates the water quality, leading to stress induced immune suppression and mass mortality of shrimp in many Asian countries [Tseng et al., 2008]. Further, the genes responsible for drug resistance in some of these multiple antibiotic-resistant bacteria could be transferred to bacteria in humans posing a critical threat to human health (Kathleen et al. 2016). These alarming drawbacks have provoked the aquaculture industry to identify and develop new strategies which are equally effective as antibiotics, sustainable and eco-friendly [Standen et al., 2014, Lazado et al., 2015].

One such remedy that is gaining importance within the aquaculture industry to control pathogens is the use of "probiotic" bacteria [Maftuch et al., 2013, Navinchandran et al., 2013]. Probiotics are defined as "live microorganisms that beneficially affect the host when ingested in required amounts" [Navinchandran et al., 2013]. Their mode of action includes: production of inhibitory compounds, competition for adhesion sites, competition for available energy, improvement of water quality, enzymatic contribution to digestion and enhancement of immune response [Verschuere et al., 2000]. Thus probiotics can be used to replace antibiotics as they produce antagonistic compounds against pathogens, compete with the pathogens for space and nutrients, and boost the immunity of the host to combat diseases. Further, probiotics produce enzymes that break down a large number of sugars and complex nutrients in the intestine, thus providing fatty acids and essential amino acids to the host. This results in better feed absorption and improved growth rate of the cultured species [El-Haroun et al., 2006, Balcazar et al., 2006]. An essential characteristic to be considered while designing a probiotic is that the selected bacteria should be non-pathogenic to the host, humans or other aquatic organisms (Romero et al., 2012). Probiotics include several types of bacteria, yeast, bacteriophages or microalgae which are mono or multi-strains delivered either through dietary supplementation or addition to water directly or via injection (Van Doan et al., 2017, Llewellyn et al., 2014, LaPatra et al., 2014, Moriarty 1998). Some bacteria that have been widely used as probiotics in aquaculture include genera *Aeromonas,* strains of *Bacillus, Enterococcus, Microbacterium, Staphylococcus* spp., *Lactobacillus, Pseudoalteromonas, Nitrobacter, Arthrobacter* etc. (Valipour et al., 2019, Louis et al., 2018, Zorriehzahra et al., 2016, Zheng and Wang, 2016, Wang and He, 2011, Pai et al., 2010, Swain et al., 2009, Li et al. 2008, Balcazar et al., 2006). These probiotics have been obtained from various sources and are specific to their hosts.

For decades, farmers have been cultivating shrimp around salt pans in many parts of the world such as India, Thailand, Portugal, Vietnam, Philippines and Bangladesh (FAO 2018, Ansari et al., 2012, Rodrigues et al., 2011). These salt pans are extreme hypersaline environments or interconnected multi-pond systems with a constant influx of sea water that is evaporated for the manufacture of natural salt. Being coastal ecosystems, they harbour microorganisms that can tolerate a wide range of salinity, known as halophilic (salt loving) and halotolerant (tolerate salt but do not essentially require salt for growth) (Ballav et al., 2014). These microorganisms are prokaryotic and eukaryotic having the

ability to resist the denaturing effects of salts and balance the osmotic pressure of the environment. Salt pans are rich bio-reserves of unique microbes that produce novel chemical entities of biotechnological significance. Salt pan bacteria have been used in the production of enzymes, compatible solutes, biosurfactants, bioremediation of metals and production of pharmaceutically important compounds [Sinanoglou et al., 2015, Zhang et al., 2013, Kamat and Kerkar, 2011, Madern et al., 2000]. These diverse microorganisms are repositories of stable compounds that function at a wide range of salinity, temperature, pH and extreme conditions [Kerkar 2004]. The application of these indigenous hypersaline bacteria in shrimp aquaculture has remained unexplored.

Thus, the aim of our research work was to explore the potential of halotolerant bacteria isolated from the salt pans of Goa as probiotics in shrimp aquaculture.

Our work was carried out with the following objectives:

- Screening of salt pan bacteria for their potential probiotic properties.
- Evaluating the inhibitory effect of the bacterial metabolites against various shrimp pathogens.
- Checking the ability of the halotolerant isolates in enhancing the immunity of shrimp.
- Assessing the water quality of the aquaculture ponds after the application of halotolerant probionts.
- Evaluating the effect of the probiotic bacteria on the growth of *Litopenaeus vannamei* and identification of the potential probionts.

Significance of the thesis

Shrimp aquaculture is an essential economic activity owing to the growing demand of shrimp in the global market, high nutritive value of shrimp and limited supply from capture fisheries. The production of shrimp by aquaculture has improved remarkably over the last two decades, with India being the second largest producer after China. However, the shrimp industry has experienced serious losses in many Asian countries due to diseases caused by opportunistic bacteria, poor water quality, increased cost of feed, and stress induced immune suppression. The persistent use of antibiotics to combat aquaculture diseases has led to the development of antibiotic resistant bacteria and accumulation of antibiotic residues in tissues. Therefore, recent studies have focused on finding alternate methods of disease control such as vaccines, immunostimulants and environment friendly microorganisms known as "probiotics". Probiotics are live microorganisms that beneficially affect the host when ingested in required amounts. They produce inhibitory compounds against pathogens, compete for adhesion sites and available energy, improve the water quality, enhance the host immunity and enzymatically contribute to digestion. They thereby boost the overall growth and survival of the host.

The microorganisms in extreme environments such as marine salt pans have a unique adaptation strategy making them useful candidates for biotechnological applications. These organisms have the ability to produce an array of natural products which can be unlocked only by constant research efforts. Salt pans along the coastal ecosystem harbour both halophilic (salt loving) and halotolerant microbial communities. The halotolerant bacteria can tolerate a wide range of salinities but do not essentially require salt for growth. These salt pan bacteria have been reported to produce a wide range of compounds with biomedical potential, industrial and agricultural application and metal bioremediation. The use of these microorganisms has not yet been explored in the aquaculture industry. As farmers have been cultivating shrimp around salt pans in many parts of the world, the use of these indigenous salt pan bacteria as probiotics would be a novel aspect in the field of aquaculture. Our study exemplifies the probiotic potential of saltpan bacteria in enhancing the feed utilization efficiency and boosting the immunity of *Litopenaeus vannamei* against vibriosis. The data procured could be used to formulate new probiotics that would successfully improve the growth and survival of shrimp in aquaculture.

CHAPTER 2:

REVIEW OF LITERATURE

2. Review of Literature

Aquaculture is broadly defined as the development of man-made enclosures to rear aquatic life forms, such as fish, shellfish and seaweed for the purpose of human consumption. Aquaculture has emerged as a progressively significant food source for the advanced population expansion. Over the last two decades, Asia has accounted for about 89 % of the world's aquaculture production, with China contributing to around 61.5 % followed by India (7.1 %) (Food and Agricultural Organization, 2018). With an increase in the demands of the growing population, this trend is predicted to continue or accelerate in the near future. As India has a vast coastline of 7,517 km and a large river and canal system of approximately 195.210 km, aquaculture has become a prominent activity along the coastal regions (Kumar, 2016). It plays an important role in providing employment to thousands of skilled and unskilled workers, promoting economic income to farmers and catering to the increasing demand of seafood worldwide (Kumar et al., 2016). The United Nations FAO predicts that by 2020, 50 % of the world's seafood requirement will be met by aquaculture, due to the over exploitation of capture fisheries (Moriarty, 1998). Based on the salinity of water, the aquaculture production can be classified as: marine aquaculture, brackish water aquaculture and fresh water aquaculture. Aquaculture can be carried out directly in the sea (mariculture) or in inland structures adjacent to the sea, such as gated lagoons or coastal ponds. Inland aquaculture includes marine shrimp species, such as Whiteleg shrimp, *Litopenaeus vannamei* that can grow in inland saline-alkaline water or freshwater after acclimatisation (FAO, 2018).

2.1 Shrimp Aquaculture

Shrimp aquaculture has grown tremendously from a traditional small-scale occupation in Southeast Asia to a global industry, with an annual growth rate of 5.8 % in the last decade. It is an important source of foreign exchange for a number of developing countries in Asia and Latin America. Based on the stage of growth, shrimp aquaculture industry is divided into two types: hatcheries (young shrimp are bred until they attain nauplii or post-larvae stage) and farms (adolescents are reared until they reach marketable size). Shrimp farms are further divided into three categories viz. extensive shrimp farms, semi-intensive shrimp farms and intensive shrimp farms based on their stocking densities (Dieu et al., 2011). Extensive shrimp farms practice traditional low-density methods with 2 – 3 post larvae

stocked per square meter. In such farms, feeding is carried out by naturally occurring organisms as water is exchanged via tides. The Annual yield is approximately 50 to 500 kg/ha of shrimp with very low production cost. It represents 55 – 60 % of all shrimp farms globally. Semi-intensive shrimp aquaculture represents 25 – 30 % of the total shrimp farms worldwide possessing a stocking density of 10 to 30 post larvae per square meter. Oxygen depletion is prevented by the use of aerators and the shrimp are fed with formulated commercial pellets or by introducing live feed supplements in the ponds (Gunalan, 2015). On the other hand, Intensive shrimp farms are smaller ponds (1000 – 1500 m^2) with high stocking densities ranging from 30 - 300 post larvae /m^2. The shrimp are stocked from hatcheries and the water quality is maintained by frequent water exchange and mechanised aerators. Shrimp are fed 4 - 5 times a day with specially designed industrial feed and often supplemented with vitamins and immune-stimulators. These farms require advanced technical infrastructure with adequately trained professionals (Dieu et al., 2011). In Asian intensive cultures, the crop is partially harvested after the first 3 months. Besides these culture methods, recent research has focused on growing specific pathogen free (SPF) broodstock larvae in super intensive raceway systems that are enclosed in greenhouses. These systems do not exchange or discharge water but only replace the water lost due to evaporation (Li et al., 2009). It is hence projected as an eco-friendly, biosecure, and cost-efficient method to grow high quality shrimp.

In the international market, shrimp is the most traded aquaculture product. Some of the mainly cultivated shrimp species include *Litopenaeus vannamei*, *Penaeus monodon*, *P. merguiensis*, *P. chinensis*, *P. japonicus*, *P. indicus*, *P. stylostris* and *Metapenaeus* spp. (FAO, 2016). Among these, Whiteleg shrimp *Litopenaeus vannamei* and *Penaeus monodon* are the two species that dominate the shrimp market. However, a remarkable decrease in the share of *P. monodon* has been observed in the last two decades with an exponential increase in the production of *Litopenaeus vannamei* globally (Liao and Chien, 2011, Rosenberry 2002).

2.2 Whiteleg shrimp *Litopenaeus vannamei*

Litopenaeus vannamei, is a tropical marine species, native to the Eastern Pacific coastal region with water temperature above 20 °C throughout the year. The optimum temperature for its growth is 28 – 32 ºC (Krummenauer, 2011). Although *L. vannamei* can tolerate 0 to

50 ppt salinity, maximum growth is observed between 10 - 40 ppt (Jennyfers et al., 2014). The optimum pH for its growth is 8.0, however it can tolerate a pH range of 7 to 9. This species is very susceptible to hypoxic conditions, and hence the dissolved oxygen concentration must be maintained above 4.5 ppm. Depending on the stocking density, *L. vannamei* attains marketable size of approximately 20 g, within 100 - 120 days.

The main producers of *L. vannamei* include China, India, Indonesia, Thailand, Ecuador, Brazil, Venezuela, Mexico, Malaysia, Vietnam, Taiwan, Pacific Islands, Colombia, the United States of America, Cambodia and Philippines (FAO, 2016). *L. vannamei* was experimentally introduced into Asia in 1978 and by 2008, 82% of the entire world production of *Litopenaeus vannamei* was attributed to Asia (Liao and Chien, 2011). The availability of specific pathogen free (SPF) broodstock, its ability to grow at a faster rate, tolerance to higher stocking density and wider range of salinity (Limhang et al. 2005, Lin and Chen 2003) has made it the species of choice of many farmers across the globe. *L. vannamei* has been farmed globally in intensive, semi-intensive and extensive systems (Li et al., 2017). However, in certain cases farming of shrimp at very high stocking densities could lead to environmental problems such as nutrient loading. In aquaculture practices, the accumulation of metabolic waste products i.e. animal excreta and decomposing organic solids such as remnants of feed have resulted in the generation of high ammonia and nitrite concentration which is toxic to the cultured shrimp. Besides, the ammonia nitrogen discharged into nearby water bodies will deplete the water quality causing pollution, eutrophication and a threat to the ecological balance. Removal of nitrite and ammonia is very critical and can be accomplished by the application of autotrophic bacteria (David et al., 2010).

2.3 Shrimp diseases

With the development of shrimp farming from traditional small-scale practices to modern intensive aquaculture, the complexity of shrimp diseases has equally magnified in India. The interaction among the pathogen, its host and the surrounding pond environment play a key role in the development of diseases (Kautsky et al., 2000). The frequent outbreak of viral and bacterial diseases has resulted in serious economic losses to farmers and the aquaculture industry. The major bacterial disease affecting shrimp aquaculture is vibriosis caused by gram negative bacteria from the family *Vibrionaceae*. Some *Vibrio* sp. which

cause vibriosis include *Vibrio harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, *V. anguillarum*, *V. splendidus* [Jayasree et al., 2006]. In hatcheries, vibriosis manifests as luminescence in pond water or the shrimp body, causing reduced feeding, disruption of the gut, necrosis and high mortality. High levels of *Vibrio* sp. in grow-out ponds are associated with internal and external necrosis, low feeding, red discoloration of shrimp (particularly tail), chronic mortality and often a secondary infection that decreases shrimp immunity, making it susceptible to viral infections (FAO, 2016). In recent decades, the control and prevention of these pathogens have led to the extensive use of antimicrobials and veterinary medicines (Balcazar et al., 2006). Antibiotics have been the immediate treatment for bacterial infections, playing a critical role in modern medicine. Nevertheless, the efficacy of antibiotics as a precautionary measure has been questioned, as their exploitation can result in the emergence of resistant bacterial strains. This is seen mainly in shrimp farming where massive rise in production, overcrowding of animals and uncontrolled antibiotic usage has led to the development of several antibiotic resistant bacteria and production crashes in many countries (Karunasagar et al., 1994, Moriarty 1999). The aquaculture industry is thus focussing on finding alternate methods to control or eliminate the use of antimicrobials, thus maintaining a healthy pond environment. One method that is gaining interest within the aquaculture industry is the use of probiotic microorganisms (Sahu et al., 2008).

2.4 Probiotics in Aquaculture

The word “probiotic” originates from Greek words pro and bios meaning “prolife” [Cruz et al., 2012]. Parker in 1974 had defined probiotics as “organisms and substances that contribute to intestinal microbial balance” (Parker, 1974). Later, in 1989 Fuller modified the definition as “live microbial feed supplement that benefits the host (human or animal) by improving the microbial balance of the body” (Fuller, 1989). The work carried out by Elie Metchnikoff is regarded as the first study conducted on probiotics, describing them as “microbes ingested with the aim of promoting good health” (Fuller 1992). To summarise, probiotics multiply a few beneficial microbes to compete with and suppress the growth of the harmful ones (Sahu et al., 2008). These microbial supplements used in aquaculture benefit their host by inhibiting pathogenic microorganisms, improving feed value, contributing enzymatically to digestion, secreting growth promoting factors, stimulating

the host immune response and improving the pond water quality (Zhou et al., 2009, Liu et al., 2012). Probiotics include certain yeast, actinomycetes and bacteria that are not harmful when continuously used for a long period of time (Sahu et al., 2008).

If a probiotic eliminates a pathogen by merely producing antagonistic extracellular metabolites, the pathogen may eventually develop resistance to the metabolite. Therefore, it is important that a probiotic has different mechanisms to outcompete pathogens in order to grow, which include the production of antagonistic compounds, ability to attach to the intestinal mucus, considerable growth characteristics (short lag-period and short doubling time) and production of compounds beneficial to the host. Understanding the underlying mechanism used by probiotics to compete with pathogens is an essential criterion while designing a protocol for probiotic selection (Vine et al., 2006). The following briefly describes the mode of action of probiotics:

2.4.1 Production of antagonistic compounds

Microorganisms produce primary and secondary metabolites that may have bactericidal or bacteriostatic effect on other organisms. These metabolites include siderophores, hydrogen peroxide, lysozymes, bacteriocins, proteases, organic acids, antibiotics etc. (Vine et al., 2006). Some bacteria such as *Bacillus* and Lactic acid bacteria produce proteins or protein complexes known as bacteriocins that could restrain the growth of other organisms (Farzanfar, 2006). A study conducted by Campos et al. (2006) reported three lactic acid bacteria, *Lactococcus lactis*, *Enterococcus faecium* and *Enterococcus mundtii* that inhibited the growth of *Listeria monocytogenes* and *Staphylococcus aureus* by production of heat-resistant bacteriocins. Zokaeifar et al.(2012), reported *Bacillus subtilis* strain with anti-*Vibrio* activity against *Vibrio harveyi* and *Vibrio parahaemolyticus* with probiotic potential in *Litopenaeus vannamei*.

Probiotics also protect the host from pathogens by modulating the immune response of the host.

2.4.2 Immunomodulation

Shrimp have an underdeveloped immune system, depending mainly on their nonspecific immune response (Vadstein, 2000). The innate immune system is categorized as humoral or cellular, mediated by factors present in the plasma hemolymph or executed directly by intact hemocytes. There are three types of hemocytes viz. hyaline cells, semi-granular cells

and granular cells (Chen et al, 2014). These circulating hemocytes play a significant role in innate immune responses; including pattern-recognition system, release of antimicrobial peptides, prophenoloxidase (proPO) activating system, phagocytosis, encapsulation and nodule formation (Sirirustananun et al, 2011). Phagocytosis is the primary defence mechanism in shrimp which is mainly carried out by the semi-granular cells. Hyaline cells also assist in phagocytosis and production of superoxide anion. The granular hemocytes in addition to their ability to form superoxides and peroxides, contain high concentration of phenol oxidase and acid phosphatase activities that play a major role in antibacterial activity (Xue et al., 2000). The Pattern recognition proteins (PRPs) in shrimp trigger the innate immune system by recognizing and binding to foreign polysaccharide particles, known as pathogen-associated molecular patterns (PAMPs), and in turn activate phagocytosis and the prophenoloxidase (proPO) activating system. ProPO is synthesized and localized in granules of granular and semi-granular cells and is released into the plasma by exocytosis after the binding of foreign polysaccharides and PRPs. ProPO is proteolytically converted to PO, which further catalyses the oxidation of phenol to quinines, subsequently polymerizing into melanin (Lee and Soderhall, 2002; Subramanian 2014). During phagocytosis, there is a release of superoxide anions and other reactive oxygen species (ROS) having microbicidal activity. This aerobic process is known as respiratory burst (Kitikiew 2012). The assessment of shrimp immune response to immune stimulators will lead to a deeper understanding of their role in defence reactions against specific pathogens (Subramanian et al, 2014). Studies have reported that supplementation of probiotics including *Bacillus subtilis* (Tseng et al, 2009), *Bacillus cere*us (Navinchandran et al, 2014), *Bacillus S*11 (Rengpipat et al 2000), *Arthrobacter* sp. (Xia et al, 2013), *Lactobacillus* sp. (Phianphak et al, 1999), *Bacillus coagulans* (Zhou et al, 2009), *Lactobacillus plantarum* (Chiu et al, 2007) have profoundly enhanced the nonspecific immunity and survival of shrimp in aquaculture.

Besides their effect on the immune system and production of antagonistic compounds to combat pathogens, probiotics are also known to compete with pathogens for nutrients, chemicals and available space.

2.4.3 Competitive exclusion

Microbial interactions play an important role in regulating the equilibrium between competing beneficial and pathogenic microorganisms (Balcazar et al., 2006). One credible

mechanism to prevent the colonization by pathogens in the gut or other tissue surfaces is competition for adhesion sites (Verschuere et al., 2000). Most pathogenic bacteria need to attach to the mucosal lining of the host's gastrointestinal tract during the initial stages of infection (Verschuere et al., 2000, Adams, 2010). The ability of microorganisms to colonize the host's gastrointestinal tract, thereby inhibit the attachment of pathogens, is an essential criterion for selection of probiotics (Balcazar et al., 2006, Lazado et al., 2015). Microbial manipulation by addition of beneficial bacteria in aquaculture is more effective if the probiotic is added at an early stage in larviculture indicating a prophylactic application of probionts (Verschuere et al., 2000). Rengpipat et al.(2000), reported that *Bacillus* S11 added to *Penaeus monodon* pL-10 improved the survival of the larvae against *Vibrio harveyi*.

The existence of any microbial population is dependent on its capacity to compete for chemicals, nutrients and available energy with the other microorganisms in the same environment (Zorriehzahra et al., 2016, Verschuere et al., 2000). Verschuere et al.(2000) selected various strains with a positive effect on the growth and survival of *Artemia* juveniles. The filtrate experiments and *in vitro* antagonism tests demonstrated that no extracellular antagonistic compounds were involved in the protective role of these strains against *Vibrio proteolytics* CW8T2 pathogen. The study inferred that the selected bacteria protected *Artemia* by competing with the pathogenic *Vibrio* sp. for chemicals and available energy.

Apart from their action against pathogenic diseases, probiotics have also been reported to play an essential role in the production of enzymes and other beneficial compounds that aid in digestion and improvement of water quality.

2.4.4 Production of other beneficiary compounds

Probiotic organisms have been reported to improve the nutrition of the host by detoxifying harmful compounds in the feed, producing vitamins such as vitamin B12 and biotin and denaturing indigestible ingredients in the diet by producing extracellular enzymes that aid in digestion (Irianto and Austin, 2002). Therefore, the intake of probiotics improves the intestinal microbial equilibrium, resulting in enhanced absorption of food, better feed value and increased growth rate of the cultured species (Balcazar et al., 2006, Vine et al., 2006). Gupta and Dhawan, reported an increase in the digestive enzyme activity (protease,

amylase and lipase) of *Macrobrachium rosenbergii* when exposed to *Bacillus coagulans*, resulting in better feed absorption and growth of the host (Gupta and Dhawan, 2013).

2.4.5 Influence on water quality

As aquaculture activities generate a large amount of nitrogenous compounds such as toxic ammonia which needs to be treated, probiotics have been explored for their effect on the water quality of the host environment. Probiotics such as *Bacillus licheniformis* and *B. subtilis* have been reported to improve the water quality of aquaculture by maintaining the ammonia concentration within acceptable range (Taoka et al., 2006). Two groups of bacteria, viz. ammonia-oxidizing bacteria (oxidizing ammonia to nitrite) and nitrite oxidizing bacteria (oxidizing nitrite to nitrate), transform ammonia to nitrate by a process called nitrification. This process plays a key role in preventing the accumulation of toxic ammonia in aquaculture ponds (Qi et al, 2009). Wang et al.(2005) reported that a commercial probiotic comprising of *Saccharomyces cerevisiae*, *Bacillus* sp, *Nitrobacter* sp and *Nitrosomonas* sp. increased the beneficial bacterial microbiota of *Litopenaeus vannamei* and reduced the inorganic nitrogen (from 3.74 mg/L to 1.79 mg/L) and phosphate concentration (from 0.11 mg/L to 0.03 mg/L). Studies have also suggested that the use of *Bacillus* sp. as probiotics have improved the water quality of aquaculture ponds by converting organic matter back to carbon dioxide (Balcazar et al., 2006). During growing seasons, high levels of probiotics in aquaculture ponds have been found to reduce the accumulation of particulate and dissolved organic carbon.

With an increase in demand for environment friendly aquaculture, many scientists are exploring the use of new probiotics for aquatic animals. The most frequently used bacterial probiotic species include genera *Lactobacillus*, strains of *Bacillus, Aeromonas, Bifidobacterium, Plesiomonas, Pseudomonas, Fusubacterium, Bacteroides, Agrobacterium, Eubacterium, Carnobacterium, Enterococcus, Bacteroides, Clostridium, Brevibacterium, Microbacterium* and *Staphylococcus* spp. (Balcazar et al., 2006, Zorriehzahra et al., 2016). Besides bacteria, other probiotics such as yeast, algae and actinomycetes have also been used in many countries. The cell-wall of *Saccharomyces cerevisiae* contains glucan, mannoproteins and chitin making it a suitable immunostimulating probiotic in mariculture (Scholz et al., 1999).

2.5 Screening and development of probiotics

The development of probiotics involves rigorous *in vitro* and *in vivo* screening of microorganisms from various sources that would benefit the host by conferring disease resistance, growth enhancement or improving the pond environment. Several *in vitro* assays such as pathogen inhibition (Guo et al., 2009), production of antimicrobial substances (Vázquez et al., 2005), production of digestive enzymes (Ochoa and Olmos, 2006), ability to adhere to the intestinal epithelium (Vine et al., 2006) and growth rate (Vine et al., 2006) are performed to shortlist potential strains for *in vivo* assays. Fig 1 illustrates the basic steps involved in the development of a new probiotic.

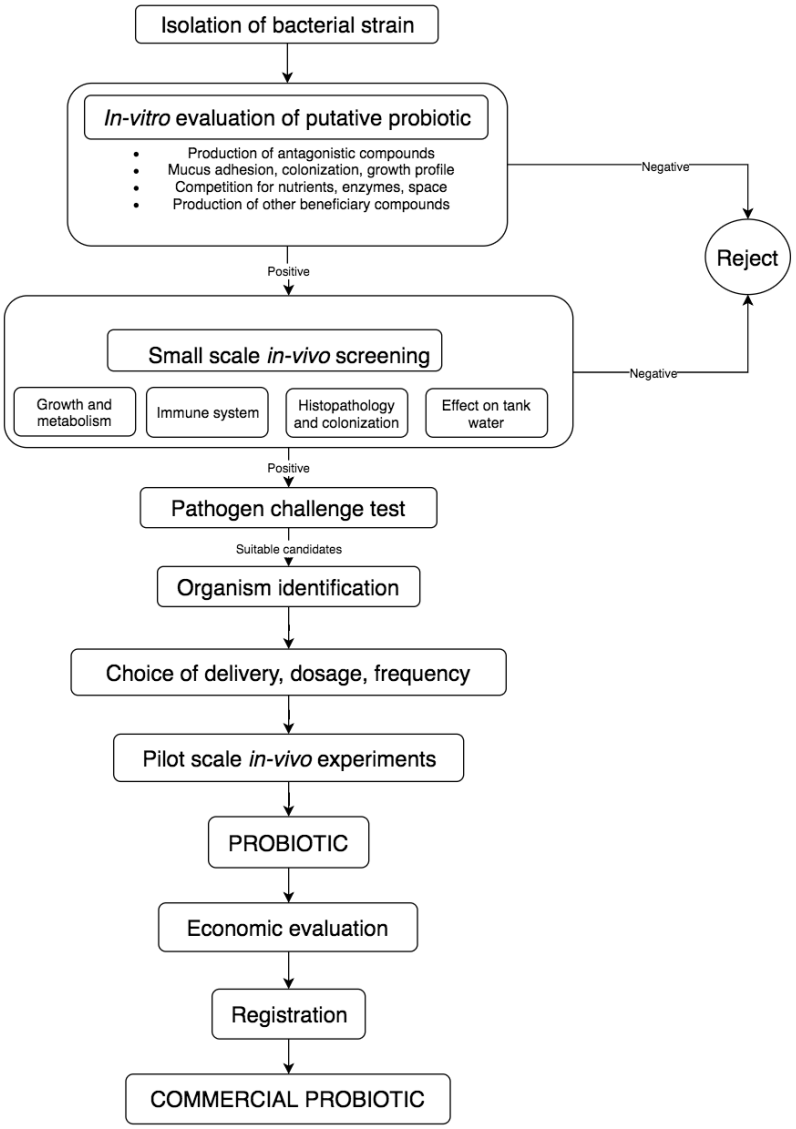


Fig. 1: Protocol for the development of a commercial probiotic for aquaculture.

2.5.1 *In vitro* screening for antimicrobial activity

The selection of appropriate microorganisms is one of the most essential criteria for designing a probiotic. It involves methodically screening a vast number of potential microorganisms by *in vitro* tests to exclude the less-promising candidates, thus reducing the quantity of *in vivo* trials required to authenticate the effectiveness of the probiont (Vine et al., 2006). One such test involves *in vitro* antagonism, in which pathogens are exposed directly to the candidate probionts or their extracellular metabolites in a solid medium such as well diffusion assay, or in liquid medium by tests such as co-culture method (Balcazar et al., 2006; Vine et al., 2006; Sotomayor and Balcazar 2003). However, the results of in *vitro* antagonism may not be a confirmed method to predict a promising *in vivo* effect. A study conducted by Olsson et al.(1992), inferred that the same organism, when grown in two different media, produced varying quantities of inhibitory metabolites. Therefore, besides screening for inhibitory compounds, further tests involving screening for production of primary metabolites, competition for space and nutrients need to be performed.

2.5.2 *In vitro* tests for evaluation of probiotics

2.5.2.1 Mucus adhesion

The ability of microorganisms to adhere to and colonize the gut or the external surface of the host, thereby preventing the colonization of pathogens, is an important criterion for selection of potential probiotics (Vine et al., 2006). This involves the sustainability of the candidate probiotic within the host and within its culture environment for a significant time period. Some bacteria only secrete metabolites during the stationary phase of growth (Monaghan et al., 1999), which may not occur *in vivo* due to constant flushing in the gut. A common practice involves selecting bacteria that produce antimicrobial metabolites; however, determining the stage of growth for the production of metabolites and their ability to compete for attachment sites is also essential (Vanbelle et al., 1990). Mucus adhesion prolongs the longevity of the bacteria in the gastrointestinal tract of the host, hence is considered as a prerequisite for colonization (Apostolou et al., 2001,Vine et al. 2004;Kesarcodi et al., 2008). The incompetence to attach to the mucus of the gut wall indicates that these bacteria may not multiply adequately to compensate for being flushed

during gut evacuation (Vine et al., 2006). Therefore, potential probionts must be tested for mucus adhesion property *in vitro* prior to large-scale trials, as the putative probiotic may be transient *in vivo* having no effect on the health of the host organism (Vine et al, 2006).

The growth profiles of the putative probiotics can be assessed in a standard microbiological media and compared to eliminate bacteria having similar growth and antagonistic abilities, thereby reducing the pool of probiotics to be tested further. For a probiotic to multiply and effectively colonize the gut of aquatic organisms, it is important to survive in the bile salts present in the gut of the host (Viera et al., 2013). Bile salts can emulsify the fatty acids and phospholipids present in microorganisms, thereby having a microbicidal effect. Some bacteria are capable of hydrolyzing these bile salts using specific enzymes (Erkkila¨ et al.,2000). Therefore, the putative probiotic must be screened for its resistance to bile salts (Vieira et al., 2013). Another important aspect to be considered while designing a probiotic is its survival at varying pH concentrations. The pH values of aquaculture ponds are not constant, ranging from approximately 6 in super-intensive shrimp farms under bioflake to 9 in eutrophied ponds (Vinatea et al., 2010; Momoyama, 2004). Thus, the bacteria selected as a putative probiotic must be capable of resisting a wide range of pH.

The putative probiotic bacteria must also be screened for the production of enzymes and other beneficial compounds, and their ability to outcompete the pathogens for suitable nutrients.

2.5.2.2 Pathogenicity test

Before a microorganism is used as a probiotic, it is mandatory to confirm that it is non-toxic and non-pathogenic to the host. Some *Bacillus* spp. produce hemolysin toxin that may be harmful to the host (Liu et al., 2009). The haemolytic ability of the bacteria can be tested by streaking on blood agar (Chang et al, 2000). The safety of the probiotic can also be assessed by challenging the host with the putative probiotic under both ordinary and stress conditions (Verschuere et al., 2000). This can be validated by small-scale challenge experiments of the host species using temporary baths in the bacterial suspension, injection challenges, or direct addition of the putative probiotic to the tank water (Balcazar et al., 2006). Pathogenicity test can be combined with small-scale *in vivo* screening under monoxenic conditions. The probiotic under study should preferably not contain any virulence or antibiotic resistance genes (Watson et al., 2008). However, all ‘pathogenic’

bacteria are not necessarily harmful (Balcazar et al., 2006). Some opportunistic bacteria such as *V. alginolyticus* have been used as a probiotic in fish aquaculture (Austin et al., 1992), and algal production (Gomez-Gil et al., 2002). Therefore, a pathogen for one species in aquaculture may turn out to be a probiotic for another species and vice versa.

2.5.2.3 Organism identification

Once the putative probiotic has been proven to be beneficial to the host by *in vitro* and small-scale *in vivo* tests, it should preferably be identified to species level using 16S rRNA gene sequencing or fatty acid profile technique. The information about the identity of the organism would be useful in tracing the history of pathogenicity, duplicate organisms, culture requirements, and its suitability as a candidate probiont.

2.5.2.4 Route of delivery, dosage, and frequency

The delivery of probiotics should preferably begin in the early stages of larval development preceding exogenous feeding. Probiotics can be administered to the host or incorporated in its aquatic environment via live food, adding it to the culture water, injecting intramuscularly or intraperitoneally, addition to artificial diet or bathing [Balcazar et al., 2006, Moriarty 2009, Gram et al., 1999, Rengpipat et al., 2000]. Probiotics are more efficient if supplied on a regular basis or if they are able to colonize and persist in the aquatic host or in its ambient environment (Watson et al., 2008). Vine et al.(2006) suggested that the probability of probiotics being ingested either directly or via live food is increased if the larvae are exposed to a probiotic concentration higher than that naturally occurring in the water. This would result in better establishment of the probiotic in the mucus and gut epithelium, thereby suppressing the attachment of pathogenic bacteria. Daily inoculation of *L. vannamei* larval tanks with 10^5 CFU/mL probiotic bacteria prevented the colonization of bacterial pathogens during larval culture (Balcazar et al., 2006).

A bacterium that exhibits antagonistic properties in the laboratory may not necessarily be inhibitory when associated with the host *in vivo* (Watson et al., 2008). Therefore validating the efficacy of the putative probiotic *in vivo* is an essential step while designing a probiotic.

2.5.2.5 *In vivo* validation

In vivo screening of candidate probiotics involves exposing the bacteria to the host under culture, followed by monitoring the growth, survival, immune and physicochemical parameters for a defined time period. Vaseeharan and Ramasamy reported a decrease in the cumulative mortality of *Penaeus monodon* challenged with *V. harveyi* when treated with *B. subtilis* probiotic (Vaseeharan and Ramasamy, 2003). A researcher must also be able to re-isolate the organism from the gastrointestinal tract or the surface of the host in order to confirm resistance due to the putative probiotic. Zokaeifar et al. showed the colonization of *B. subtilis* strain L10 and G1 in the gut of *L. vannamei* after receiving a diet containing the two strains for 8 weeks. The study also reported a significant decrease in *Vibrio* spp. in the shrimp gastrointestinal tract of the host (Zokaeifar et al., 2012).

If the microorganism has successively passed the screening stages discussed above, it can be termed as a "probiotic" (Vine et al., 2006). However, for an industry to commercialize the probiotic, it is necessary to study the shelf life and viability of the organisms, storage conditions and production cost of the probiotic.

2.6 Recent probiotics used in aquaculture

With an increase in demand for environmentally friendly aquaculture, many scientists are exploring the use of new probiotics for aquatic animals. Table 1 represents the recent probiotics used to control pathogens in crustacean aquaculture and their beneficial effects.

Table 1: Recent probiotics used in crustacean aquaculture

Probiotic organism	Host organism	Mode of action	Mode of application	Reference
Lactobacillus plantarum	*Astacus leptodactylus*	Enhanced immune response, digestive enzyme activity	Incorporated in feed	Valipour et al., 2019
Pseudoalteromonas sp. NC201	*Litopenaeus stylirostris*	Increased survival against *Vibrio nigripulchritudo*, enhanced immunity of the host	Added to culture water	Louis et al., 2018

Combination of *Bacillus mesentericus, Clostridium butyricum, Enterococcus faecalis*	*Macrobrachium rosenbergii*	Enhanced immunity and increased survival against *Vibrio* spp. and *Aeromonas* spp.	Incorporated in feed	Azad et al., 2018
Pseudoalteromonas sp. NC201	*Litopenaeus stylirostris*	Immunostimulatory effect, increased survival against *Vibrio parahaemolyticus nigripulchritudo*	Added to culture water	Louis et al, 2018
Clostridium butyricum	*Macrobrachium rosenbergii*	Antagonistic effect against *V. harveyi*	Incorporated in feed	Sumon et al, 2018
Streptomyces sp. *RL8, Streptomyces* sp. N7 individual or in combination with *Lactobacillus* and *Bacillus*	*Litopenaeus vannamei*	Enhanced growth and immune response, modulated the host and water microbiota, increased resistance to disease	Incorporated in feed	Bernal et al., 2016
Lactobacillus pentosus	*Litopenaeus vannamei*	improved the growth performance, digestive enzyme activities and feed utilization, decreased mortality against pathogenic *Vibrio* sp.	Incorporated in feed	Zheng and Wang, 2016
Saccharomyces cerevisiae	*Macrobrachium rosenbergii*	Increased growth, feed utilization and enzyme activity	Incorporated in feed	Seenivasan et al., 2014
(Bacillus subtilis DCU, Bacillus cereus HL7, Bacillus pumilus BP,	*mud crab (Scylla paramamosain)*	Immunostimulatory effect, increased survival against *Vibrio parahaemolyticus*	Incorporated in feed	Wu et al, 2014
Rhodosporidium paludigenum	*Litopenaeus vannamei*	Improved growth performance and immune response	Incorporated in feed	Yang et al., 2011
Lb. rhamnosus and two commercial probiotics (including *Ped. acidilactici)*	*Penaeus monodon*	Increased disease resistance against *V. anguillarum*	Added to culture water	Panigrahi et al., 2011
Bacillus MCCB101, *Micrococcus* MCCB104 in	*Penaeus monodon*	Improved immune response and increased disease resistance	Incorporated in feed	Antony et al., 2011

combination or independently				
Combination of Nitrobacter sp., *Bacillus* sp., *Nitrosomonas* sp., and *Lactobacillus*	*Litopenaeus vannamei*	Decreased nitrogen and phosphate pollution in pond sediments	Added to culture water	Wang and He, 2011
Combination of *B. thuringiensis, B. polymyxa, B. subtilis, B. megaterium and B. licheniformis*	*Penaeus monodon*	Improved growth performance and feed utilization	Incorporated in feed	Boonthai et al., 2011
B. subtilis E20	*Litopenaeus vannamei*	Improved immunity and growth performance	Added to culture water	Liu et al., 2011
Pseudomonas sp., *Bacillus* sp., *Arthrobacter* sp. Independently	*Penaeus monodon*	Improved diseased resistance against *V. harveyi*, immunostimulatory effect and improved digestive enzyme activity	Added to culture water	Pai et al., 2010
Ped. acidilactici	*Litopenaeus stylirostris*	Improved disease resistance against *V. nigripulchritudo*	Incorporated in feed	Castex et al., 2010
Combination of Bacillus spp.	*Homarus gammarus*	Enhanced growth and survival	Incorporated in feed	Daniels et al., 2010
Vibrio gazogenes	*Litopenaeus vannamei*	Immunostimulatory effect and anti-*Vibrio* activity	Injection and incorporated in the diet	Thomson et al, 2010
Enterococcus faecium MC13, *Streptococcus phocae* PI80	*Penaeus monodon*	Increased survival against *V. harveyi* and *V. parahaemolyticus*	Incorporated in feed	Swain et al., 2009
Ps. synxantha, Ps. aeruginosa independently and combined	*Penaeus latisulcatus*	Improved growth and disease resistance	Incorporated in feed	Hai et al., 2010
Lb. plantarum	*Litopenaeus vannamei*	Immune resistance	Incorporated in feed	Vieira et al., 2008
Arthrobacter XE-7	*Litopenaeus vannamei*	Disease resistance against *V. parahaemolyticus*	Incorporated in feed	Li et al., 2008

Microbial communities in extreme environments are untapped resources with enormous potential for the discovery of novel products of biotechnological significance. These microorganisms are a prolific source of numerous essential biomolecules that have resulted from their evolution and adaptation mechanisms (Ballav et al, 2015).

Saltpans are extreme hypersaline environments or interconnected multi-pond systems permitting a discontinuous salinity gradient [Kamat and Kerkar, 2011]. Majority of these ponds are thalassic in nature with a constant influx of sea water that is evaporated for the manufacture of natural salt. The evaporation of water in different ponds leads to the progressive development of diverse microbial species that get adapted to varying concentrations of salinity (DasSarma and DasSarma, 2017). In Goa, since 2002, only around 16 salt pans have been utilized for commercially producing natural salt (Kamat and Kerkar, 2011). The salinity in these salt pans reduces to as low as 5-10 ppt during the monsoon seasons and exceeds up to 350 ppt during the salt manufacturing or non-monsoon season. During high tide, sea water gains entry into the salt crystallizer ponds and gets concentrated by evaporation up to saturation levels. These crystallizer ponds symbolize a distinctive marine hypersaline environment, with salinity ranging from 10 to 350 psu; temperature 10 ºC to 42 °C and pH 6 to 9 (Ballav et al., 2015). Marine salterns being coastal ecosystems harbour halotolerant bacteria that possibly enter from terrestrial ecosystems but over time have adapted to hypersaline conditions (Ballav et al., 2015). These rich genera of microorganisms that include bacteria, archae, fungi, cyanobacteria and algae have adapted to major changes in salinity, oxygen, pH and temperature in salt pans. They have the ability to produce an array of natural products of biotechnological interest which can be unlocked only by constant research efforts [Manikandan and Senthilkumar, 2017].

Presently, hypersaline microorganisms are used for production of industrially important enzymes, bioactive compounds and secondary metabolites such as proteins, amino acids, enzymes, etc (Enache and Kamekura, 2010). Amylase enzyme produced by hypersaline bacteria have been effectively used in the treatment of digestive disorders and other pharmaceuticals applications [Margesin and Schinner, 2001]. The secondary metabolites produced by hypersaline microbes contain several bioactive compounds like polypeptides, lipopeptides, macrolactins and isocoumarins [Manikandan and Senthilkumar, 2017]. Hypersaline bacteria are also known to produce organic compounds such as pigments, antimicrobials, lipids and compatible solutes that are active and stable under extreme

conditions. The products of these organisms have been used in bioremediation of metals, in food colouring, as a feed additive, in the detergent industry, etc [Sinanoglou et al., 2015, Zhang et al., 2013].

The use of salt pan microorganisms has not yet been explored in the aquaculture industry. As aquaculture has been traditionally practiced around salt pan areas in many parts of the world, exploring the potential of halotolerant bacteria as probiotics could promote the use of these native organisms in shrimp aquaculture.

CHAPTER 3:

MATERIALS AND METHODS

3. Materials and Methods

3.1 Growth and maintenance of salt pan bacteria

The bacterial isolates used in the present study were previously isolated from water, sediments, and biofilms of the Ribandar, Batim and Agarwado salt pans of Goa, India. The salinity of these salt pans ranged from 5 ppt (during monsoons) to 400 ppt (during the salt making season). These isolates were designated with codes SK, ABSK, MFSK, SBSK, TSK and FSK (Kamat, 2012; Pereira, 2013; Ballav, 2016; Bartakke, 2018) and preserved in Professor Savita Kerkar's departmental culture collection. The bacteria were maintained on their respective medium of isolation viz. Zobell marine agar, Nutrient agar, 25 % Nutrient agar, Media D agar and Actinomycetes isolation agar (appendix). All the media components and chemicals were of analytical grade.

3.2 Growth and maintenance of shrimp pathogens

Shrimp pathogens *Vibrio alginolyticus* and *Vibrio vulnificus* were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. *Vibrio parahaemolyticus, Vibrio harveyi, and Vibrio cholerae* were procured from National Institute of Oceanography, (CSIR-NIO, Goa). All the pathogens were maintained on Nutrient agar supplemented with 2.5 % NaCl and incubated at 30 °C for 24 h.

3.3 Screening of halotolerant bacteria for production of bioactive compounds against shrimp pathogens.

A total of 300 halotolerant bacteria were screened for their inhibitory activity against shrimp pathogens. The following methods were used for antimicrobial studies:

3.3.1 Agar well diffusion assay

The halotolerant bacteria (300) were individually inoculated in 50 mL Nutrient broth (appendix I) supplemented with sea water at 28 ± 2 °C for 48 h on an orbital shaker at 120 rpm. The supernatant was separated from the cells by centrifugation at 10,000 rpm for 5 minutes at 4 ˚C.

Shrimp pathogens: *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* were separately grown in 50 mL flask with 25 mL Nutrient broth supplemented with 2.5 % NaCl. The flasks were placed on an orbital shaker at 120 rpm, 28 ± 2 °C for an incubation period of 24 h. Subsequently, the shrimp pathogens were spread plated with sterile cotton swabs on Mueller Hinton agar plates (Appendix I). A total of 100 µL of each of the halotolerant bacterial cell free supernatant was added to 8 mm agar wells and incubated at 28 °C for 24 – 48 h. Anti-*Vibrio* activity was recorded from the diameter of the zone of clearance (mm) around the well (Nakayama et al, 2009).

3.3.2 Co-culture method

Halotolerant bacteria (eighteen) (showing prominent anti-*Vibrio* activity in the well diffusion method) and the pathogenic *Vibrio* species were pre-cultured separately in 100 mL of 50 % Nutrient broth supplemented with seawater and incubated at 120 rpm at 28 °C overnight. The colony forming units (CFU) of the individual cultures was determined by serial dilution and plating on Nutrient agar after 24 h. The halotolerant bacteria were inoculated in 25 mL Nutrient broth at an initial concentration of 10^7 CFU/ mL, followed by inoculation of individual *Vibrio* species at a cell concentration of 10^3 CFU/ mL. The co-cultured broths were incubated at 28 °C for 48 h at 120 rpm on an orbital shaker. Flasks containing individual *Vibrio* cultures without the halotolerant bacteria served as control. The effect of the halotolerant bacteria on *Vibrio* pathogens was determined by ten-fold serial dilution and plating 100 µL on Thiosulphate citrate bile salts sucrose (TCBS) agar, and recorded as colony forming units (CFU) after 24 h incubation (Vaseeharan and Ramaswamy, 2002).

$$Colony\ forming\ units\ (CFU/\ mL) = (Number\ of\ colonies\ x\ dilution\ factor)\ /\ volume\ of\ culture\ plated.$$

3.4 Screening of halotolerant bacteria for ammonia degradation

The ability of the halotolerant organisms to degrade ammonia was studied by Nesslerization method with slight modification (Golterman., 1991). The halotolerant organisms were inoculated in enrichment medium (appendix I) containing 1 g/L

ammonium sulphate. The media were incubated for 7 days at 28 °C at 120 rpm on an orbital shaker. The concentration of ammonia was estimated on day 0, 3, 5 and 7 by Nesslerization method as given below.

The Enriched broth (5 mL) containing the halotolerant bacteria was centrifuged at 10,000 rpm, 4 °C for 5 min (Remi cooling centrifuge, India) to separate the bacterial cells from the broth. Sodium Potassium tartarate (appendix I) (100 µL) was added to the broth, followed by 100 µL Nesslers reagent (appendix I). The mixture was incubated for 5 minutes at room temperature. The absorbance was recorded at 425 nm using a spectrophotometer (UV mini1240, Shimadzu, USA). The concentration of ammonia was calculated based on a standard graph.

3.5 *In-vitro* screening for probiotic potential

To assess the probiotic potential of the halotolerant bacteria, the mucus adhesion property, tolerance to bile salts, salinity and pH tolerance, ability to produce hydrolytic enzymes (qualitatively as well as quantitatively), bacterial growth profile and biocompatibility were evaluated.

3.5.1 Mucus adhesion

The ability of the cultures to adhere to shrimp mucus *in vitro* was studied by Crystal violet method (Balakrishna, 2013). The salt pan bacteria (100 µL) were added into a 96 well microtitre plate pre-coated with 150 µL of fish intestinal mucus. A higher volume of mucus was maintained compared to the volume of bacteria, to avoid contact of the stain with the plate. Bacterial adhesion was carried out by incubating the plate at 37 ^{o}C for 1 h, followed by washing the plate three times with 250 µL of 0.1 M phosphate buffered saline (PBS) to remove the non-adherent cells. The adhered bacteria were fixed for 20 min at 60 ^{o}C and stained with 100 µL crystal violet (0.1 %) for 45 min. The wells were washed 5 times with PBS to remove excess stain. The stain bound to the bacteria was released by adding 100 µL citrate buffer (pH 4.3) and incubated for 45 min at room temperature. The absorbance was read at 640 nm using a 96 well microtitre plate reader (Bio-Rad, India). Stained mucus without bacteria was used as negative control and the absorbance of this negative control was subtracted from the absorbance value of the test samples.

3.5.2 Tolerance to Bile salts

Halotolerant cultures that showed anti-microbial activity against shrimp pathogens were screened for bile salt tolerance. The halotolerant bacteria were inoculated in Nutrient broth containing 0 %, 2 % and 4 % bile salts and incubated at 30 °C at 120 rpm on an orbital shaker. The growth of the bacteria was monitored after 24 h and 48 h by noting the absorbance at 600 nm (Shimadzu UV-1800, Japan). The tolerance of the halotolerant bacteria to bile salts was determined as the percentage reduction in absorbance in relation to the growth medium without added bile salts (Vieira, 2013).

3.5.3 Salinity tolerance test

The halotolerant bacteria were grown in Nutrient broth containing 0, 3 and 5 % NaCl at 30 °C on an orbital shaker at 120 rpm. The tolerance of the bacteria to varied salt concentrations was assessed by recording the absorbance at 600 nm after 24 h.

3.5.4 pH tolerance test

The halotolerant bacteria were inoculated in Nutrient broth containing pH ranging from 5 to 9 and placed on an orbital shaker at 30 °C. After 24 h, the absorbance of each broth was recorded at 600 nm. The tolerance of the halotolerant bacteria to varied pH concentration was determined as the percentage reduction in absorbance in relation to the growth medium of pH 7 (Vieira 2013).

3.5.5 Production of extracellular enzymes

The ability of the halotolerant bacteria to produce hydrolytic enzymes that would aid in shrimp digestion was studied qualitatively and quantitatively.

3.5.5.1 Qualitative estimation

The qualitative estimation of enzyme production was carried out by plate assays on minimal media containing specific substrates. The amylolytic activity was determined by spot inoculation on 1 % starch agar plates (appendix I) , followed by incubation at 28 ± 2 °C for 24 – 48 h. The plates were then flooded with 1 % Lugol's iodine solution (Sigma

L6146) and observed for a zone of clearance around the colony (Alariya et al, 2013). Similarly, protease production was studied by inoculating the cultures on 1 % casein agar (appendix I) at 28 ± 2 ℃ for 24 – 48 h followed by the addition of Coomassie brilliant blue and observed for a zone of clearance around the colony (ShivaKumar, 2012). Lipase production was indicated by a clearance zone around the colony on 1% tributyrin agar plates after 48 h at 28 ± 2 °C (Kumar et al, 2012). To evaluate for cellulase production, the bacterial strains were spot inoculated on 1 % Carboxy-methyl cellulose agar (appendix I) and incubated at 28 ± 2 °C for 24 – 48 h. The plates were flooded with Congo red dye and observed for a zone of clearance around the colony (Liang et al, 2014). Chitinase production was estimated by spot inoculating the halotolerant bacteria on minimal agar supplemented with 1 % colloidal chitin (appendix I) and incubating the plates at 28 ± 2 °C for 24 – 48 h. The cultures showing a zone of clearance against a cream background were noted as chitinase producing bacteria (Saima et al., 2013).

3.5.5.2 Quantitative estimation

Bacterial colonies that produced a zone of clearance exceeding 15 mm in the plate assay were selected for enzyme activity in broth. The isolates were individually inoculated into minimal broth (appendix I) supplemented with 1 % starch/ casein/ carboxy-methylcellulose/ p-nitrophenyl palmitate as substrate for amylase / protease / cellulase / lipase respectively. Subsequently, the broths were incubated at 150 rpm, 30 °C for 24 h. The cells were harvested by centrifugation at 8,000 RPM for 10 min and the cell free supernatant was used for respective enzyme activities as described below.

Amylase

Amylase activity was assayed by 3, 5- dinitrosalicylic acid (DNS) method (Worthington, 1993). The cell free supernatant (0.5 mL) was incubated at 25 °C for 3 min. 1 % starch solution (0.5 mL) was added to this and incubated for 5 min at room temperature. DNS reagent (1 mL) was added and the tubes were heated in a boiling water bath for 5 min, cooled and the volume was made up to 10 mL with distilled water. The absorbance was read at 540 nm using a spectrophotometer (UV mini 1240, Shimadzu, USA). One unit amylase activity was defined as the number of micromoles of maltose released per min under assay conditions.

Protease

Protease activity was measured according to Lowry et al. (1951). The cell free supernatant solution (1 mL) was incubated with 1 mL of casein at 50 °C for 30 min. Trichloroacetic acid (2 mL) was added to the tubes and incubated on ice for 15 min. The tubes were centrifuged and the supernatant was collected. 1 mL of this solution was mixed with 4.5 mL of alkaline reagent and incubated at 28 ± 2 °C for 10 min, followed by addition of 0.5 mL of Folin's phenol reagent. The contents were mixed and the absorbance was recorded at 640 nm after 15 min. A calibration curve was prepared using BSA (200 µg mL^{-1}) as the standard. One unit of protease activity was defined as the number of micromoles of tyrosine released per min per mg of protein.

Cellulase

Cellulase activity was measured following the method of Ghose (1987). The cell free supernatant (0.5 mL) was added to 0.5 mL 1 % Carboxymethyl cellulose prepared in 0.5 M Sodium citrate buffer (pH 4.8), mixed, and incubated at 50 °C for 30 min. The reaction was terminated by adding 3 mL DNS and placing the tubes in a 100 °C boiling water bath (Bio – Technics, India) for 5 min. After boiling, the tubes were cooled and diluted with 20 mL of distilled water. The absorbance was read at 540 nm. One unit of cellulase activity was defined as the amount of enzyme that released 1 µmole of glucose equivalent per min under assay conditions

Lipase

A reaction mixture containing 75 µL paranitrophenyl palmitate (pNPP), 5 – 50 µL test sample and tris buffer (0.05 M, pH 8.5) to make the final volume to 3 mL, was incubated at 45 °C water bath for 20 min. The reaction was arrested by adding 1 mL of acetone: ethanol mixture. A control containing heat inactivated enzyme was maintained. The absorbance (A_{410}) of control was subtracted from the absorbance of the corresponding test sample. One unit of lipase activity was defined as micromole(s) of paranitrophenol released by hydrolysis of pNPP by one mL of enzyme under assay conditions (Kanwar et al, 2005).

3.5.6 Viability of halotolerant bacteria in aquaculture pond water agar

The ability of the halotolerant bacteria to survive in aquaculture pond water was assessed by growing the isolates in pond water agar (appendix I). The halotolerant bacteria were streaked on plates exclusively containing aquaculture pond water with 2 % agar. The isolates were incubated at 28 °C ± 2 for 24 – 48 h and the growth of the bacteria was recorded.

3.5.7 Hemolysis test

Hemolysis test was performed to study the hemolytic activity of the potential cultures on human erythrocytes and shrimp hemocytes.

3.5.7.1 Human blood agar

The halotolerant cultures were streaked on plates containing blood-based agar supplemented with 6 % human sterile blood (appendix I). The plates were incubated at 37 °C for 24 h. Slight destruction of erythrocytes producing a green zone around the bacterial colony indicated α- hemolysis. A clear hemolysis zone around the bacterial colony indicated β – hemolysis, while no change in agar around the colony was recorded as γ – hemolysis (Villasenor et al, 2012).

3.5.7.2 Shrimp hemolymph agar

Shrimp hemolymph was drawn from the ventral sinus cavity of an adult shrimp (approximately 30 g) using a sterile 1 mL syringe containing 0.5 mL marine anticoagulant solution (appendix II). The hemolymph was immediately transferred to a sterile tube and stained with 3 % Rose Bengal dye, dissolved in anti-coagulant solution and gently tapped to achieve complete mixing. Under sterile conditions, 1 mL of the stained hemolymph solution and 15 mL sterile agar base (appendix I), were gently mixed to attain homogenous distribution of hemocytes, and poured into petri plates. The halotolerant bacteria were streaked on the agar and incubated at 30 °C for 24 – 48 h. Hemolytic activity was recorded based on the formation of a clearance zone around the colony (Chang *et al*, 2000).

3.6 Growth profile and production of antimicrobial metabolite

The growth profile of six halotolerant bacteria showing probiotic potential *in vitro* was studied. The bacteria were inoculated in Zobell Marine Broth (Himedia, M385) and incubated at 28 °C for 24 h. When the absorbance of the starter culture was 0.9 ± 0.02 at 600 nm, the culture was inoculated in fresh Zobell Marine broth (prepared in sea water) in triplicates and placed on an orbital shaker at 120 rpm. The initial absorbance was noted at 600 nm. The broth (1 mL) was withdrawn every 4 h for a period of 56 h and the absorbance was measured. Simultaneously, the stage of production of anti-*Vibrio* metabolite was studied every 4 h by well diffusion assay (previously described in 3.3.1).

3.7 Biocompatibility of the halotolerant isolates

The biocompatibility of the six halotolerant bacteria was assessed by cross streak method (Kamat and Kerkar, 2011). Each culture was streaked on separate Nutrient agar plates (prepared in sea water) as a band and incubated at 28 ± 2 °C for 24 h. The remaining halotolerant bacteria were streaked perpendicular to the band, and incubated at 28 ± 2 °C for 24 – 48 h. The present test was done to check the compatibility of the six halotolerant bacteria to grow together without inhibiting each other. These six halotolerant bacteria exhibiting maximum compatibility were termed as “candidate probionts”.

3.8 Preparation of single bacterial suspension for *in vivo* studies

Suspensions of the candidate probionts were prepared using individual colonies inoculated in Nutrient broth with sea water and incubating them at 28 °C on an orbital shaker for 24 – 48 h depending on their respective growth profile. The culture broth was centrifuged at 5000 rpm for 10 min to obtain the bacterial pellet. The pellet was resuspended and washed twice in sterile 0.1 M Phosphate buffered saline (PBS), pH 7.5 (appendix I). The viable count was determined by ten-fold serial dilutions and plating 100 µL on Nutrient agar. A standard graph of colony forming units/ mL vs A_{630} of the bacterial suspension was prepared. Bacterial suspensions of 10^8, 10^9 and 10^{10} CFU/mL in PBS were prepared to standardise the dosage for the *in vivo* trials.

3.9 *In vivo* trial of individual halotolerant bacteria on *Litopenaeus vannamei*

3.9.1 Larval collection and maintenance

Specific pathogen free (SPF) brood stock of *Litopenaeus vannamei* post-larvae (PL-12) were procured from a hatchery in Tamil Nadu, India. The post-larvae were acclimatized for two weeks in stock tanks before distributing them in experimental tanks for *in vivo* trials.

3.9.2 Experimental design

Litopenaeus vannamei post-larvae (PL) were equally distributed in isolated glass tanks and acclimatized for one week before the experiment (Plate 1). The tank water was maintained using filtered seawater and was continuously aerated using mechanized aerators. All shrimp had an approximate initial weight of 0.62 ± 0.04 g. One set served as a control and the other six sets were inoculated with candidate probiont SK07, SK27, ABSK55, FSK444, TSK17 and TSK71 at a dosage of 10^8, 10^9 and 10^{10} CFU/mL. Each candidate probiont was exposed to *L. vannamei* in the above three different cell concentrations (in triplicates). The tanks were arranged in completely randomized design. The candidate probiotic suspension was inoculated weekly in the tank as a water additive. Shrimp were fed twice a day (4 – 6 % body weight) with a commercial feed "Bay White enriched *vannamei* feed" by Waterbase Ltd, India (comprising minimum 40 - 41% crude protein, 5-6 % crude fat, 3 % crude fibre and maximum 12 % moisture) for a period of 120 days.

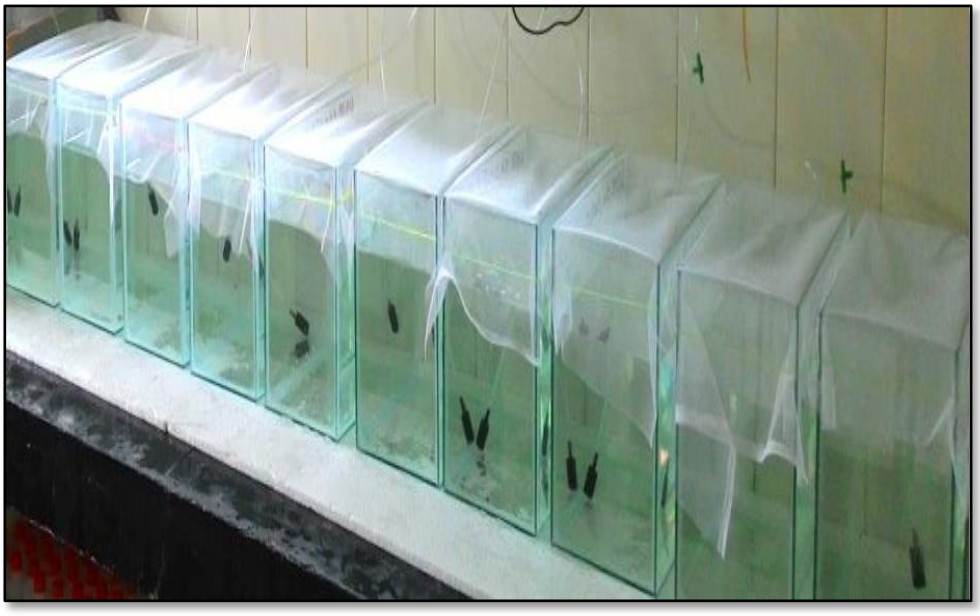


Plate 1: Experimental setup of tanks for trials with individual halotolerant bacteria

3.9.3 Analysis of water quality

The physico-chemical and microbial parameters of the water of each experimental tank were assessed weekly to study the impact of the halotolerant bacteria on the water quality as described in the following sections.

3.9.3.1 Physico-chemical parameters

Temperature, dissolved oxygen (DO), total dissolved solids and salinity of the water of each tank was measured using a hand held multi-parameter (CyberScan PC 650, Eutech Instruments, Thermo Fisher Scientific, India). The pH of water was measured with a digital pH meter (pH 700, Eutech instruments, Thermo Fisher Scientific, India) previously calibrated with standard buffer solutions of pH 9.2, 7 and 4.

3.9.3.2 Estimation of dissolved ammonia

Dissolved ammonia was estimated spectrophotometrically by Nesslerization method as described in section 3.4. Water was collected from each tank (5 mL) in screw capped test tubes to avoid evaporation of ammonia. To this, 0.1 mL of potassium sodium tartrate (appendix II) and 0.1 mL Nessler's reagent (SDFCL, India) was added. The solution was mixed well using a vortex mixer and incubated for 5 min. The absorbance was read at 425 nm using a UV mini 1240 spectrophotometer (Shimadzu, USA). The amount of dissolved NH_3-N ions present in the sample was calculated from the standard graph of NH_4Cl.

3.9.3.3 Estimation of Nitrite

Nitrite estimation was carried out spectrophotometrically according to APHA (2012). Water sample (25 mL) was collected from each tank in glass test tubes and 0.5 mL sulphanilamide reagent (appendix II) was added to each tube. The contents were mixed well and incubated for 5 minutes at room temperature. This was followed by addition of 0.5 mL N-(1-napthyl) ethelene diamine hydrochloride solution (appendix II). The contents of the tubes were mixed well and incubated for 15 – 20 min at room temperature. The absorbance was measured at 543 nm.

3.9.3.4 Estimation of Nitrate

Preparation of Nitrate reducing cadmium column:

Cadmium (Cd) granules were soaked in 1 % HCl overnight. The Cd granules were then transferred to a glass stoppered bottle and washed with distilled water 3 – 4 times until the water was clear. After discarding the distilled water, 2 % $CuSO_4$ solution (appendix II) was added to the glass bottle and shaken for 30 min to coat $CuSO_4$ onto the Cd granules. The granules were washed with distilled water till the water appears clear. The column was filled with dilute ammonium chloride buffer (appendix II), pH 8. The glass wool was placed at the bottom of the column using a wire or glass rod. The Cu coated Cd granules were then transferred to the column, followed by addition of glass wool above the granules. The column was activated by flushing it with ammonium chloride buffer twice, followed by passing distilled water twice to adjust the flow rate (8 min for 100 mL).

Estimation of Nitrate of the tank water

Water samples (100 mL) were collected from each tank and introduced into the Cd column. The first 40 mL was discarded and next 50 mL was collected in glass test tubes and labelled. Sulphanilamide reagent (appendix II) (1 mL) was added to each tube. The contents were mixed well and incubated for 5 min at room temperature. This was followed by addition of 1 mL N-(1-napthyl) ethelene diamine hydrochloride solution (appendix II). The contents of the tubes were mixed and incubated for 15 – 20 min at room temperature. The absorbance was measured at 543 nm. The column was washed with distilled water between each sample. The Nitrate concentration was calculated with reference to a standard graph ranging from 0 – 9 μmoles/ L Potassium nitrate (APHA, 2012).

3.9.3.5 Estimation of Sulphide

Sulfide estimation was carried out according to APHA, 2012. The water sample (1 mL) was fixed in 9 mL of 2 % zinc acetate solution (appendix II) in screw capped test tubes and brought to the laboratory for analysis. The contents were transferred to a 50 mL volumetric flask. To this, 5 mL N- N, dimethyl – p- phenylene – diamine sulphate (DMPD) solution (appendix II) and 0.25 mL Ferrous (III) ammonium sulphate solution (appendix II) was

added and allowed to stand for 10 min at room temperature. The volume was made up to 50 mL with distilled water and absorbance was measured at 670 nm against blank. The blank solution was prepared using distilled water. The sulfide concentration was estimated using a standard graph of sodium sulfide (appendix II) ranging from 50 mg/L to 250 mg/L.

3.9.3.6 Estimation of Alkalinity

Total alkalinity was estimated according to Haraldsson et al., 1997. The pH meter was standardised against different buffer solution (pH 7, pH 4 and pH 10). The steps were repeated until a constant pH reading of buffer solution was obtained. The tank water sample (100 mL) was transferred in to a 250 mL beaker and 25 mL of standard 0.01 N HCl was added to it. The solution was mixed thoroughly using a glass rod and the pH was recorded after 2 to 3 min. The obtained pH was converted to hydrogen ion activity to measure alkalinity.

3.9.3.7 Biological oxygen demand

Biological oxygen demand (BOD) was calculated by Winklers method (Golterman, 1983). The water sample was collected by immersing a glass BOD bottle in the tank, ensuring no bubbles are formed during sample collection. One bottle was labelled as ‘Day 0’ and fixed immediately using 1 mL Winklers A and 1 mL Winklers B solution (appendix II). The bottle was closed and mixed well and the precipitate was allowed to settle for 30 min. The second bottle was labelled as ‘Day 5’ and kept in a dark chamber for 5 days. To the ‘Day 1’ bottle, 3 mL of 50 % sulphuric acid was added to dissolve the precipitate and 50 mL was withdrawn into a conical flask. The solution was titrated against 0.01 N Sodium thiosulphate (appendix II) until pale colour developed. To this, 1 mL starch indicator was added and the solution was again titrated until the blue colour disappeared.

$$\text{BOD (mg/L)} = \frac{\text{(Initial DO – Final DO) x Average volume of bottle}}{\text{Volume of sample taken}}$$

$$\text{DO (mg/L)} = \frac{\text{Normality of Sodium thiosulphate x volume of Sodium thiosulphate x 1000 x 8}}{\text{Volume of sample taken}}$$

3.9.3.8. Microbiological analysis

The water samples from the tanks were collected in sterile PVC bottles and transported to the laboratory. The samples were serially diluted 10-fold up to 10^6 using 0.85 % saline. For total culturable bacterial count, the samples were spread plated on Nutrient agar (appendix I) and for total *Vibrio* count on TCBS agar (appendix I) and incubated at 30 ± 2 °C (Classic scientific, India). The CFU/ mL were calculated as mentioned in section 3.3.2.

3.9.4. Analysis of shrimp growth and survival

The growth of the shrimp was monitored every 15 days by measuring the weight and length of five shrimp per tank. The weight was measured by placing a live shrimp on a weighing balance (Denver instrument, USA) and immediately releasing it back into the water. Similarly the length was measured from the tip of the cephalothorax (rostrum) to the end of the uropod using a ruler.

The effect of the halotolerant bacteria on the shrimp growth and survival was calculated according to Zokaeifar et al. (2012) and Hasan et al. (2012) with slight modification.

Weight gain (g) = Final weight (g) – Initial weight (g)

Specific growth rate (SGR %) = [(ln Wt (g) – ln W0 (g)) / t] x 100

Where t is the culture period in days, ln W_0 is the natural logarithm of initial shrimp weight, ln Wt is the natural logarithm of shrimp weight at day t.

Feed efficiency (FE) = (Final weight (g) – initial weight (g)) / total feed intake (g)

Survival rate (%) = (Final numbers/initial numbers) x 100

Yield (weight in g / tank volume) = Total number of shrimp x average body weight (g)

3.9.5 Analysis of the immune system of shrimp after exposure to the candidate probionts

3.9.5.1 Haemolymph collection

The haemolymph (100 µL) of 90 day old *Litopenaeus vannamei* was collected from the ventral-sinus cavity of randomly picked shrimp per treatment group using a 26-gauge needle coupled with a 1 mL syringe containing 0.4 mL of marine anticoagulant solution (appendix II) and mixed gently (Rengipat et al, 2000).

3.9.5.1.1 Total Hemocyte count

The cell count of the collected hemolymph-anticoagulant mixture (100 µL) was enumerated using a Neubauer hemocytometer slide and expressed as number of cells /mL. They were observed under light microscope at 400 X magnification (BX53 Olympus, Japan).

3.9.5.1.2 Phagocytic activity assay

Hemolymph (100 µL) was mixed with 400 µL of anticoagulant solution. The hemocytes were separated from the hemolymph by centrifuging at 2500 rpm for 10 min at 4 °C. Approximately 10^7 cells/mL hemocytes were mixed with 100 µL of latex beads (~10^8 beads/mL, polystyrene 1.1 µm particle size, Sigma) in 1 X phosphate buffered saline (appendix II), pH 7.4 on a clean glass slide. The mixture was incubated at room temperature for 30 min in a moist chamber, followed by fixation with 2.5 % glutaraldehyde. The slides were washed with 1 X phosphate buffered saline to remove non adherent hemocytes, dried and stained with modified giemsa. The number of phagocytising cells were counted from 100 observed cells using a light microscope (BX53 Olympus, Japan) at 1,000 X magnification (Rengipat et al, 2000).
Phagocytic activity was calculated according to Rengipat et al, 2000.

Percentage phagocytosis = (no. of cells ingesting beads / no. of cells observed) x 100

3.9.5.1.3 Preparation of Hemocyte lysate supernatant (HLS)

Haemolymph (100 µL) was collected and mixed with 400 µL of marine anticoagulant solution, centrifuged at 2500 rpm for 10 min at 4 °C. The pellet containing hemocytes were washed and collected in ice cold cacodylate (CAC) buffer (appendix II). The mixture was homogenized in a sonicator (output 5 with 50 % duty cycle for 3 s) followed by centrifugation at 14,000 rpm for 20 min at 4 °C. The resultant hemocyte lysate supernatant was used as an enzyme source (Smith and Soderhall, 1991).

3.9.5.1.4 Phenoloxidase concentration

Phenoloxidase (PO) activity was measured spectrophotometrically using L-3, 4-dihydroxy phenylalanine (L-DOPA, GRM360 Hi Media, India) as substrate and trypsin (Sigma-Aldrich, India) as the elicitor. Hemocyte lysate supernatant (0.2 mL) was mixed with 0.2 mL of 0.1 % trypsin in cacodylate buffer (appendix II) and incubated for 30 min at room temperature. This was followed by addition of 0.2 mL of L-DOPA (appendix II). The reaction mixture was diluted with 0.6 mL cacodylate buffer, mixed and the absorbance was measured at 490 nm. The absorbance was measured against a blank containing cacodylate buffer, trypsin and L-DOPA. One unit of enzyme activity was calculated by an increase in absorbance of 0.001 per min per mg of protein (Wang and Gu, 2010). Bradford assay was used to measure the protein content in HLS using bovine serum albumin as standard (Bradford, 1976).

3.10 Formulation of the bacterial consortium

Subsequently four potential candidate probionts SK07, SK27, ABSK55 and TSK71 were selected for future experiments as the bacterial consortia to be tested *in vivo* on *Litopenaeus vannamei*.

3.10.1 Large scale production of bacterial suspension

The four candidate probionts were mass cultured separately in Nutrient broth supplemented with sea water as described in section 3.8. The broth was centrifuged at 4500 g for 15 min at 4 °C. The bacterial cell pellet was collected and washed in sterile phosphate buffered saline (appendix II), mixed with 10 % sucrose solution and stored at –80 °C. The cells were freeze dried in a lyophilizer (ScanVac, CoolSafe) and the resultant powder was used for further studies.

3.10.2 Bacterial viability and preparation of bacterial consortium

The viability and purity of the lyophilized powder was studied by dissolving 100 mg of the powder in sterile 0.85 % saline, serially diluting it and plating on Nutrient agar supplemented with sea water (appendix I). The cell viability was calculated as described in

section 3.3.2. Based on the *in vivo* trials carried out, cell concentration of 10^9 CFU/ mL of each bacterial culture were used to prepare the final mixed consortium of 10^9 CFU/mL.

3.10.3 Shelf life of the bacterial consortium

The shelf life of the formulated bacterial consortium was studied at two temperatures: 4 °C and room temperature (28 °C ± 2). Aliquots of 1 g of bacterial consortium was introduced in sterile microvials and kept at the respective temperatures. A total of 48 vials were prepared to carry out the study at 15 day interval for a period of 365 days. Every 15 days, one vial was aseptically resuspended in 9 mL of 0.85 % sterile saline and used for analysis. The viability of the bacteria in the consortium was calculated in terms of CFU/mL after 10 fold serial dilutions of the bacterial suspension.

3.11 *In vitro* synergistic effect of the formulated bacterial consortium

The bacterial consortium (1 x 10^9 CFU/mL) was inoculated in respective media for 24 h, to study its synergistic effect on the production of anti-*Vibrio* metabolites; production of amylase, protease, cellulase and lipase enzymes and ammonia degradation activity as described in sections 3.3.1, 3.5.5.1 and 3.4.

3.12 *In-vivo* synergistic effect of the formulated bacterial consortium on *Litopenaeus vannamei*

Subsequent to the *in vitro* synergistic effect of the consortium, lab-scale trials were conducted to determine the *in vivo* synergistic effect of the formulated consortium on *Litopenaeus vannamei.*

3.12.1 Larval collection and experimental set up

Specific pathogen free (SPF) brood stock of *Litopenaeus vannamei* post-larvae (PL-12) were procured from a hatchery in Tamil Nadu, India. The post-larvae were acclimatized for two weeks in 40 L stock tanks before distributing them in experimental tanks for *in vivo* trials. The post-larvae (PL) were equally distributed in 9 isolated glass tanks and acclimatized for one week before experimentation (represented in Plate 2). The tanks were arranged in a completely randomized design. Three treatment groups were maintained: (i)

control (uninoculated tanks), (ii) SFSK4 (tanks inoculated with 1 x 10^9 CFU/ mL halotolerant bacterial consortium) and (iii) CP1 (tanks treated with commercial probiotic at a cell concentration of 10^9 CFU/mL). The tanks were continuously aerated to maintain the dissolved oxygen concentration. All shrimp had approximate initial weight of 0.60 ± 0.04 g. The lyophilized bacterial consortium was dissolved separately in minimal amount of tank water before inoculating it into the tank. Shrimp were fed with a commercial feed (Bay White enriched *vannamei* feed, Waterbase Ltd, India) twice a day (4 – 6 % body weight) according to the manufacturer's instructions for 120 days.

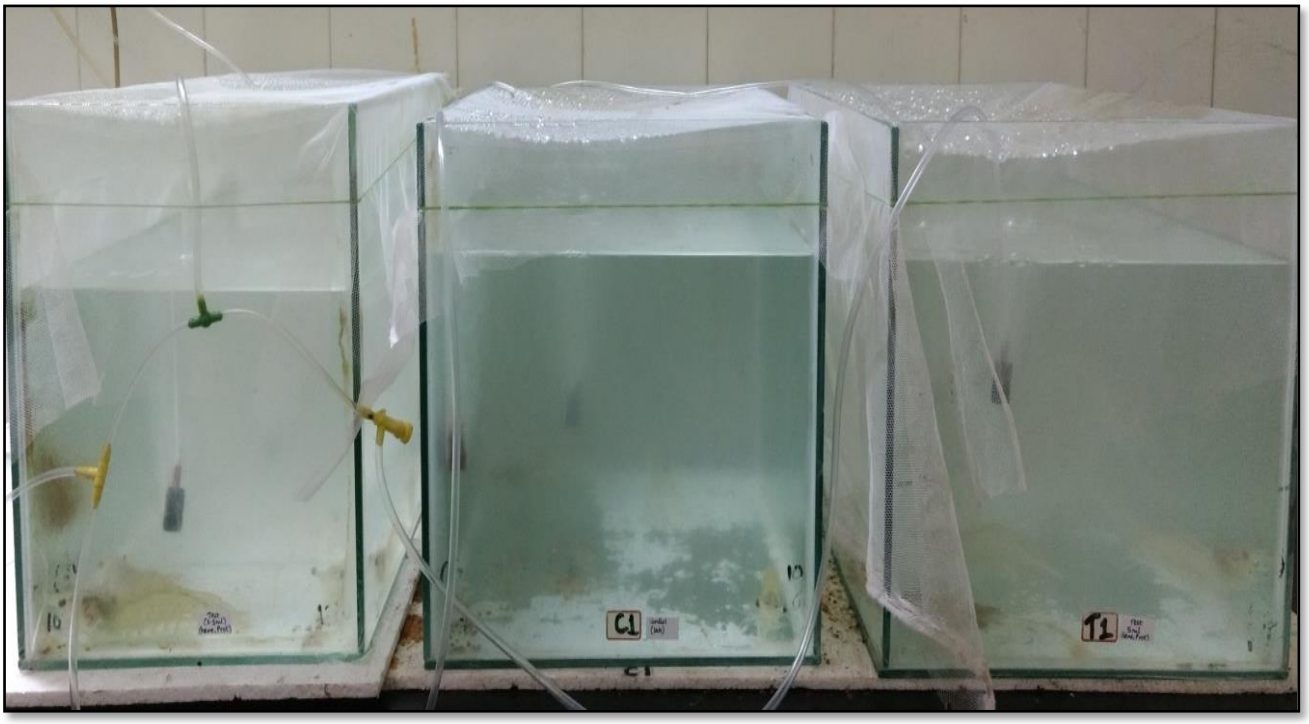


Plate 2: Representation of experimental tanks used to study *in vivo* synergistic effect of SFSK4.

3.12.2 Analysis of the physico-chemical and biological parameters of tank water

The physico-chemical parameters such as temperature, pH, dissolved oxygen, total dissolved solids, salinity, ammonia, nitrite, nitrate, sulphide and alkalinity along with

microbial parameters (total cultivable bacteria and total *Vibrio* count) was analysed weekly for a period of 120 days as described in section 3.9.3.

3.12.3 Analysis of growth parameters

The length and weight of five randomly picked shrimp from each tank were measured every 15 days for a period of 120 days. The weight gain, feed efficiency, specific growth rate and percent survival were calculated to study the effect of the consortium on the growth and feed utilization efficiency, as described in section 3.9.4.

3.12.4 Analysis of shrimp immune system

The hemolymph of the control, test and commercial probiotic treated shrimp was extracted after 60, 90 and 120 days of trial and analyzed to study the effect of the respective treatments on the shrimp immune system. The total hemocyte count, phagocytic activity and phenoloxidase activity was estimated as described in section 3.9.5. Besides these parameters, the total plasma protein, respiratory burst and bacterial clearance efficiency was analyzed as follows:

3.12.4.1 Total Plasma Protein

The collected hemolymph (100 µL) was centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant was separated from the hemocytes and the plasma protein concentration was estimated by Bradford method (Bradford, 1976).

3.12.4.2 Respiratory burst

Respiratory burst was estimated following the protocol of Subramanian et al (2014). The assay measured the reduction of nitro-blue tetrazolium (NBT) by hemocytes which determined the amount of superoxide anion (respiratory burst activity). Hemolymph (100 µL) was inoculated in a microtitre plate and incubated at room temperature. After 30 min, the supernatant was discarded and 0.3 % NBT (50 µL) was added and incubated at room temperature for 2 h. This was followed by removal of the supernatant and addition of 200 µL absolute ethanol to fix the hemocytes. The hemocytes were washed with 200 µL of 70

% methanol twice and allowed to dry. The formazan deposits that were formed were dissolved in 120 µL potassium hydroxide (2 mol/ L) and 140 µL dimethyl sulfoxide. The absorbance was read at 630 nm using a microtitre plate reader (iMark Bio-Rad, USA).

3.12.4.3 Bacterial clearance efficiency

Bacterial clearance efficiency was estimated according to Navinchandran et al (2014) with slight modification. The shrimp pathogens *Vibrio harveyi* and *Vibrio alginolyticus* were grown separately in Nutrient broth (HiMedia M002) overnight at 28 °C on an orbital shaker (REMI, India). The broth were centrifuged and washed in sterile 1 X PBS (appendix), and further diluted with PBS in order to obtain an O.D of 0.1 at 600 nm. The hemolymph (100 µL) was centrifuged for 20 min at 9700 rpm using 400 µL sterile marine anticoagulant solution (appendix I). The bacterial suspension (100 µL) was incubated with cell free hemolymph (100 µL) in a sterile microtube for 3 h at 28 °C. From each microtube, 100 µL samples were withdrawn and spread plated on Thiosulphate citrate bile salts sucrose agar (appendix I) and incubated at 28 ± 2 °C for 24 h. The colony forming units were calculated as described in section 3.3.2. A positive control consisting of 100 µL bacterial suspension incubated with 100 µL PBS in anticoagulant solution was also maintained. Bacterial clearance efficiency was calculated as follows:

Percent inhibition (%) = 100 – [(mean CFU/mL of test sample) / (mean CFU/mL of positive control)] x 100

3.12.5 Analysis of anti-*Vibrio* activity *in vivo*

The effect of the halotolerant bacterial consortium on the shrimp pathogens (*Vibrio harveyi* and *Vibrio alginolyticus*) was studied by modified Patil et al. (2013) method. *Vibrio harveyi* and *Vibrio alginolyticus* were individually grown in Nutrient broth for 24 h at room temperature on an orbital shaker at 120 rpm. The broth was centrifuged at 4500 g for 10 min to obtain the cell pellet. The pellet was washed with 1 X PBS and the viable count was calculated as described in section 3.3.2.

3.12.5.1 Pathogen challenge test

The juvenile shrimp (60 days) from the control and test probiotic SFSK4 treated sets were challenged separately with fresh bacterial suspension of *V. harveyi* and *V. alginolyticus*

(10^6 CFU/mL) in 2 L glass beakers. However, in the test probiotic beakers, the inoculation of *V. harveyi* and *V. alginolyticus* was followed by the addition of the test probiotic SFSK4 consortium. Likewise, a positive control containing shrimp exposed to the respective pathogens without test probiotic SFSK4 was also maintained. Uninoculated shrimp tanks served as negative control. The experiment was conducted in triplicates for 168 h and the mortality (%) of the juvenile shrimp was noted.

3.12.5.2 Histopathological analysis of shrimp exposed to *Vibrio* pathogens

The control tank shrimp and the shrimp treated with SFSK4 for 90 days were transferred to 10 L glass tanks, followed by exposure to *V. harveyi* pathogen (10^6 CFU/mL). The test set tanks were further exposed to SFSK4 (10^9 CFU/mL). Similarly, a positive control containing shrimp exposed only to the pathogen and an uninoculated tank (negative control) was maintained. The hepatopancreas of the positive control, negative control and test shrimp were dissected for histological examination and immersed in 10 % formalin for 24 h and transferred to 70 % ethanol until processing. About 5 – 7 µm sections were made using a rotary microtome, followed by staining with hematoxylin - eosin and examined under 100 x and 400 x magnification using a light microscrope (Ng et al, 2015).

3.13 Identification of the probiotic bacterial isolates

The four halotolerant bacteria used in the consortium were characterized and identified based on biochemical, morphological and molecular characteristics as illustrated in Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986).

3.13.1 Cell morphology and gram characteristic

The selected cultures were Gram stained according to Hans Christian Gram (1884). The bacteria were also characterized morphologically as cocci (spherical cells) or rods (cylindrical cells).

3.13.2 Spore staining

The Gram positive cultures were stained according to Schaeffer-Fulton's method (1933). The position of the spore was studied and recorded as central, sub-terminal or terminal.

3.13.3 Cell size

The size of the bacterial cell was measured by scanning electron microscopy (Bratbak, 1993). To prepare the samples, a clean coverslip (1 cm^2) was fixed with cellotape on a glass slide. Freshly grown bacterial culture (10 µL) was spread onto the coverslip, air dried and fixed with 2.5 % glutaraldehyde overnight. The next day, the coverslip was washed with sterile Phosphate buffered saline (pH 7.4) followed by successive dehydration with 30 %, 50 %, 70 %, 80 % and 90 % acetone for 10 min each and 100 % acetone for 30 min. The slides were subsequently air dried. The coverslips were cut into small pieces and attached to a stub with cellotape, and sputter coated with gold (SC7620 Mini sputter coater, Quorum technologies, UK). The cells were viewed under a scanning electron microscope (Evo 18, Carl Ziess, Germany) with magnification from 2 KX to 40 KX. The size of the cells was measured and the detailed morphology was photographed and noted.

3.13.4 Motility test

The motility of the cultures was determined by hanging drop method (Collins and Lyne, 1984).

3.13.5 Catalase test

The bacterial culture was grown on Nutrient agar plates for 24 h. A loopful of the culture was taken on a glass slide and 3 % hydrogen peroxide (2 drops) was added. The bacterial culture was observed for production of effervescence (Vasanthakumari, 2009).

3.13.6 Oxidase test

Oxidase test was carried out by oxidase discs (HiMedia DD018). The bacterial culture (10 µL) was inoculated on a disc and the colour change was observed. Microorganisms were documented as oxidase positive when the disc colour changed to purple within 5 to 10 s. Change beyond 10 seconds or no colour change was considered oxidase negative.

3.13.7 Biochemical tests

The biochemical tests were carried out using Himedia kits as per manufacturer's instructions. KB013 HiBacillus™ identification kit was used for *Bacillus* spp., KB002 HiAssorted™ biochemical test kit for Gram negative rods, KBM001 HiMotility™ Biochemical kit for *E.coli* and KB007 HiVibrio™ identification test kit for *Vibrio*.

3.13.8 Carbohydrate utilization test

Studies on carbohydrate utilization of the bacteria was carried out using KB009 HiCarboTM kits (HiMedia KB009A/ KB009B1 / KB009C). The kit contains a total of 35 tests for utilization of carbohydrates. The utilization profile was determined as per the manufacturer's instructions (HiMedia Laboratories Pvt. Ltd, India). A pure colony of the bacteria under study was inoculated in 50 mL Nutrient broth and incubated on an orbital shaker for 18 h at room temperature. Each well of the strip was inoculated with 50 µL of bacterial suspension aseptically. The strips were incubated for 24 - 48 h at room temperature. The colour change was noted and results were interpreted according to the chart provided with the strips.

3.13.9 Metabolic fingerprinting

Metabolic fingerprinting was carried out using the BIOLOG assay kit according to the manufacturer's instructions (Hayward, CA94545, USA). Biolog GN2 MicroPlate™ and Biolog GP2 MicroPlate™ comprising of 95 biochemical tests were used for Gram negative and Gram positive cultures respectively. The metabolic activity was also studied using Biolog GEN III MicroPlate™ containing 71 carbon source utilization tests and 23 chemical sensitivity assays. Freshly grown bacterial culture (150 µL) was added to each well and incubated for 48 h at room temperature. The colourless wells which resembled the blank were recorded as negative, whereas the wells that turned purple were recorded as positive.

3.13.10 Molecular identification

The genomic DNA of the four bacteria was isolated using HiPurA™ Bacterial Genomic DNA purification kit (HiMedia, India) according to manufacturer's instructions. The isolated genomic DNA was used as template to amplify the 16S rRNA gene by Polymerase chain reaction. The universal bacterial primers for 16S rRNA gene: forward primer 27F: AGAGTTTGATCCTGGCTCCAG and reverse primer 1492R: TACGGTTACCTTGTTACGACTT were utilized for PCR reaction. The PCR reaction mixture contained 20 µL MilliQ water, 3 µL template, 1 µL forward primer, 1 µL reverse primer and 25 µL 2X Taq polymerase mix. PCR was performed with an initial

denaturation at 95 °C of 5 min followed by 35 cycles of 95 °C for 1 min; 54 °C for 1 min and 72 °C for 1 min. A final extension step was carried out at 72 °C for 10 min. The PCR amplified products were run on 0.8 % low melting agarose gel along with StepUp™ 500 bp DNA ladder (Genei, India). The expected 1.5 kb length band was excised using a sterile scalpel and eluted by AxyPrep^{TM} DNA Gel Extraction Kit following the manufacturer's instructions (Axygen Biosciences, USA). The DNA sequence of the amplified excised gene segment was obtained from AgriGenome Labs, Pvt. Ltd, India. The obtained raw sequences were used to assemble the contigs using BioEdit software, version 7.2.5 (Hall, 1999) and aligned. The contigs (approximately 1.5 kb) were matched with GenBank database using NCBI-BLAST (Altschul et al., 1990). MEGA 6 software was used to construct the 16S rRNA gene sequence based phylogenetic tree (Tamura et al., 2013) by Neighbour-joining method. The evolutionary distances were computed by Tamura-Nei method with 1000 bootstrap replicates. The analysis included 21 nucleotide sequences. The positions consisting of less than 95 % site coverage were eliminated. Therefore, fewer than 5 % alignment gaps, ambiguous bases and missing data, were allowed at any position. The sequences of the 4 bacteria have been deposited in GenBank.

3.14 Toxicity evaluation of the test probiotic consortium SFSK4

The prepared halotolerant probiotic consortium was assessed for its safety before farm trials. The toxicity evaluation included LC_{50} (lethal concentration 50), cytotoxicity and genotoxicity study. The individual cultures were also screened for their susceptibility to various antibiotics.

3.14.1 Antibiotic susceptibility test

The susceptibility of the halotolerant bacteria SK07, SK27, ABSK55 and TSK71 to antibiotics was assayed according to Kirby-Bauer disk diffusion method. The antibiotic containing disks (Himedia) were placed on Muller Hinton agar plates previously swabbed with the individual bacterial strains. The antibiotics tested were Amoxyclav (30 µg), Amikacin (30 µg), Ampicillin (10 µg), Chloramphenicol (30 µg), Cephalothin (30 µg), Cephalexin (30 µg), Ciprofloxacin (5 µg), Co-trimoxazole (25 µg), Cefotaxime (30 µg), Clindamycin (2 µg), Doxycycline hydrochloride (30 µg), Erythromycin (15 µg), Gentamycin (10 µg), Levofloxacin (5 µg), Nitrofurantoin (300 µg), Neomycin (30 µg),

Nalidixic acid (30 µg), Ofloxacin (5 µg), Streptomycin (10 µg), Vancomycin (30 µg), Penicillin-G (10 µg), Tobramycin (10 µg), Tetracyclin (30 µg) and Kanamycin (30 µg). The diameters of zone of inhibition were measured after incubating the plates at 30 °C for 24 h. The strains were categorized as susceptible, intermediate (moderately susceptible) or resistant as recommended by the Clinical and Laboratory Standards institute (CLSI) (2013).

3.14.2 Lethal concentration 50 (LC_{50})

This experiment was conducted to determine if the maximum dosage of test probiotic consortium SFSK4 is fatal to the postlarvae of *L. vannamei*. The post larvae (PL – 20) were exposed to test probiotic at a cell concentration of 10^8, 10^9, 10^{10}, 10^{11}, 10^{12} CFU/mL (10^{12} is the maximum concentration causing froth formation in the tank). The temperature was maintained at 28 ± 2 °C, salinity 30 ± 2 ppt, pH at 7.6 ± 0.2 with continuous aeration. Similarly, a positive control tank (challenged with 10^6 CFU/mL *V. alginolyticus*) and a negative control tank (untreated) was maintained. The survival rate of the post-larvae was noted daily in the control and test tanks for a period of one week. The experiment was conducted in triplicates to note 50 % mortality (Roque et al, 2005).

3.14.3 Experimental conditions for cytotoxicity and genotoxicity assay

L. vannamei post larvae (PL-20) and juveniles were exposed to 1 x 10^{10} and 1 x 10^{12} CFU/mL concentration of the test probiotic SFSK4 for 7 days in 2 L glass beakers. Two positive controls containing shrimp challenged with 10^6 CFU/mL *V. alginolyticus* and shrimp exposed to Ethyl methane sulfonate were maintained. Untreated shrimp tanks served as negative control. After 7 days of challenge, the juveniles were carefully dissected using a sterile scalpel and the organs were detached and placed on ice. The hemolymph was extracted as described in section 3.9.5.1. The post larvae and the tissues obtained from the juvenile shrimp were separately suspended in 500 µL sterile PBS (1 X, pH 7.4), homogenized and filtered through a sterile muslin cloth. The filtrate was centrifuged at 4 ºC for 5 min at 3000 rpm. The obtained pellet was resuspended in 200 µL sterile PBS and the cell suspension was used for toxicity evaluation.

3.14.3.1 Cytotoxicity evaluation

The cytotoxicity study was performed by Trypan blue dye exclusion test (Strober 2001). The cell suspension (100 µL) was mixed with 100 µL of 0.4 % trypan blue in a sterile vial and incubated for 2 – 3 min at room temperature. The dead cells took up the stain due to disintegration of the intact plasma membrane. 100 cells were scored and the numbers of stained and unstained cells were counted separately.

% Cellular Viability = (Number of viable cells/ Total number of cells counted) x 100

3.14.3.2 Genotoxicity evaluation by Single cell gel electrophoresis

The single cell gel electrophoresis or comet assay is a technique for quantifying DNA damage and repair *in vivo* and *in vitro* in any eukaryotic cell and some prokaryotic cells. The comet assay was carried out according to the procedure outlined by D'Costa et al. (2017) and PraveenKumar et al. (2017). The suspension of shrimp hepatopancreatic cells (20 µL) was mixed with 80 µL low melting agarose (0.5 % in PBS) and spread on a frosted microscopic slide (Fisherfinest premium) previously coated with 1 % normal melting point agarose and placed in a cold lysing solution at 4 °C, overnight. The procedure was performed in dim light to avoid photo-oxidation of DNA. DNA was then allowed to unwind by placing the slides in electrophoresis buffer (pH 10) for 15 – 20 min. Electrophoresis was carried out under alkaline conditions (pH 10) at 300 mA, 25 V for 30 min. The slides were placed in 400 mM Tris base neutralization buffer (pH 7.5) for 5 min. The DNA gel was stained with ethidium bromide and analyzed under a fluorescence microscope (BX53 Olympus, Japan) containing a green filter at 200x magnification. Randomly choosen non-overlapping cells were captured by ProgRes® Capture Pro 2.7. The comet images were analysed using computer software, CASP and the percent tail DNA was documented. The percent tail DNA is the amount of DNA (%) present in the tail of the "comet" and is used as an indicator of DNA damage.

3.15 Farm trial of the test probiotic consortium SFSK4 on *Litopenaeus vannamei*

After evaluating SFSK4 for its safety to eukaryotes, field trials were conducted in a shrimp aquaculture farm for 120 days, to ascertain its efficiency in an outdoor culture system.

3.15.1 Design of the experiment

The experiment was conducted at Dr. Rivonkar's shrimp aquaculture farm at Batim, Goa, India (15°27'35.3"N 073°53'47.8"E) adjacent to the Batim salt pans (Fig. 2). The farm received water from the adjoining Zuari estuary.

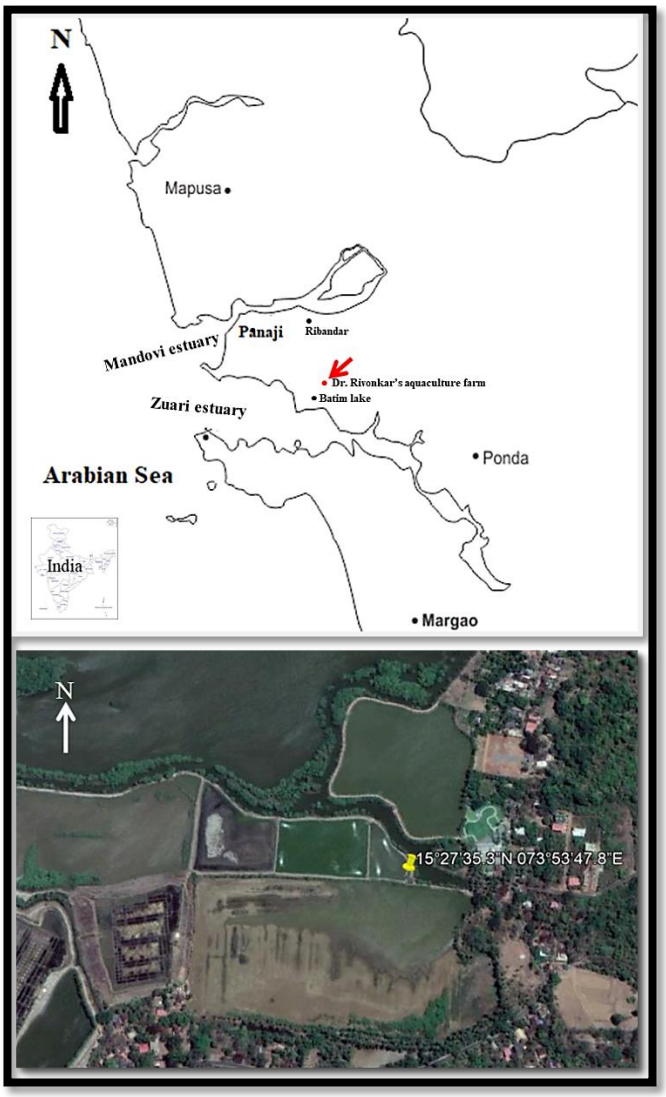


Fig. 2: Location of the shrimp aquaculture farm selected for trials.

Litopenaeus vannamei post-larvae (PL-20) were stocked at a stocking density of 25 shrimp /m^2 in 1000 L capacity tanks (represented in Plate 3). The following treatment sets were maintained: (i) SFSK4 (tanks treated with halotolerant bacterial consortium at a dosage of 1 x 10^9 CFU/mL tank water), (ii) Control (untreated tanks) and (iii) CP1 (tanks treated with a commercial shrimp probiotic at a dosage of 1 x 10^9 CFU/mL tank water). The tanks were continuously aerated using aerators (SB-648A, SEBO) except during feeding. Shrimp were

fed with commercial feed pellets (Bay White enriched *vannamei* feed, Waterbase Ltd., Chennai, India) four times a day (4 – 6 % body weight) according to the manufacturer's instructions and farmers practice. The tanks were cleaned every week with 50 % water exchange in a flow through system.

Plate 3: Representation of experimental tanks used to study the effect of SFSK4 in an outdoor culture system

3.15.2 Analysis of physico-chemical and microbial parameters of tank water

The physico – chemical parameters such as temperature, pH, dissolved oxygen, total dissolved solids, salinity, ammonia, nitrite, nitrate, sulphide and alkanity along with microbial parameters (total culturable bacteria and total *Vibrio* count) was analysed weekly for a period of 120 days as described in section 3.9.3.

3.15.3 Analysis of growth parameters

The length and weight of five randomly picked shrimp from each tank were measured every 15 days for a period of 120 days. The weight gain, feed efficiency, specific growth rate and percent survival were calculated to study the effect of the consortium on the growth and feed utilization efficiency, as described in section 3.9.4.

3.15.4 Analysis of shrimp gastrointestinal tract (GIT)

The effect of the test probiotic consortium (SFSK4) on the gut of *Litopenaeus vannamei* was studied by analysing the bacteria and the enzyme activity in the gastrointestinal tract.

3.15.4.1 Bacteriological analysis

The spread plate technique was used to estimate the presence of the halotolerant bacteria in the shrimp GIT according to Zokaeifar et al (2012). Three shrimp from each tank were aseptically dissected with sterile surgical scissors and the isolated GIT was homogenized in a sterile homogenizer with 0.01 M phosphate buffered saline (pH 7.4). The samples were serially diluted and plated on Nutrient agar (HiMedia M001) in sea water and Thiosulfate citrate bile salts sucrose agar (HiMedia, M189) in triplicates to record the total cultivable bacterial count and total *Vibrio* count in the shrimp gut. The DNA of 8 bacterial colonies that were morphologically and biochemically similar to the target halotolerant bacteria was isolated, amplified and sequenced by 16S rRNA gene sequencing as described in section 3.13.10.

3.15.4.2 Total enzyme activity in shrimp gastrointestinal tract

The crude extract of the gastrointestinal tract was used to study the effect of the test probiotic consortium on the digestive enzyme activity of the shrimp. The shrimp from the control, SFSK4 and commercial probiotic CP1 treated tanks were dissected aseptically to isolate the gastrointestinal tract. The GIT was weighed and homogenized with cold deionised water (1:10). The homogenate was centrifuged at 5000 g for 20 min at 4 °C. The supernatant was carefully separated and passed through 0.45 mm pore size filters

(Sartorius, Germany). The total enzyme activity of the supernatant was estimated in triplicates as described in section 3.5.5.2.

3.15.5 Analysis of shrimp immune system

The hemolymph of the control, SFSK4 and commercial probiotic CP1 treated shrimp was extracted after 60, 90 and 120 days of trial and analyzed to study the effect of the respective treatments on the shrimp immune system. The total hemocyte count, phenoloxidase activity, respiratory burst, total plasma protein, phagocytic activity and bacterial clearance efficiency was estimated as described in section 3.9.5.

3.15.6 Proteomic analysis

The proteomic analysis of the control and SFSK4 treated shrimp hemolymph was carried out to identify the differentially expressed proteins in response to SFSK4. The analysis was carried out at National Chemical Laboratory (NCL), Pune.

3.15.6.1 Sample preparation (In Solution digestion)

The protein concentration in the shrimp hemolymph sample was estimated using Bradford method (1976) with bovine serum albumi as standard (Huang et al, 2018). Ammonium bicarbonate (50mM) was prepared in LCMS grade water in a clean washed test tube. After making up 1µg/ul protein, all samples were incubated at 80°C for 15 min. 3 µl of 100 mM dithiothretol solution was added in each sample tube and incubated at 60°C for 15 min. The tubes were spun and allowed to cool to room temperature. Iodoacetamide (3 ul) was added and incubated in dark at room temperature for 30 min. 10ul proteomics grade trypsin solution (pH 7.5) was added to each protein sample at a ratio of 1:25, mixed and incubated at 37 °C for 16 h with 800 rpm mixing. After 12 h, 2 ul of trypsin was added and further incubated at 37 °C at 800 rpm. After 18 h, 2 ul of formic acid was added to stop the trypsin reaction. The sample was centrifuged for 30 min at 4 °C at 13000 rpm and the supernatant was collected. The obtained supernatant was desalted and eluted with ZipTip column. In brief, the ZipTips were conditioned with 15 µL of 50 % acetonitrile and equilibrated with 15 µL of 0.1 % formic acid.

3.15.6.2 LC-MS/MS data aquisition

The digested solution (containing peptides) was then loaded onto the ZipTip column and washed with 15 µL 0.1 % formic acid (four times repeated washing). Finally, the peptides were eluted two times with 15 µL 50% acetonitrile containing 0.1% formic acid. For IDA, maximum sample loaded was 3.5ug, Bgal standard digest spiking 500 fm and analysed on an Agilent 1260 HPLC system coupled to a TripleTOF 5600+ mass spectrometer with NanoSource III (AB SCIEX). Briefly,1 µg of peptides for each sample was loaded onto a C18 trap column and separated on a 75µm×15 cm long analytical column with a linear gradient of 2–40% of Buffer B (98% ACN, 0.1% FA) in 90 min and a flow rate of 200 nl/min. Full MS scans were acquired in the mass range of m/z 340–1500 in positive ion mode and most intense ions with charge state from 2+ to 5+ were selected for fragmentation and the second MS scans were acquired in mass range of m/z 100–1500.

3.15.6.3 SWATH MS data acquisition

For SWATH analysis, maximum sample loaded was 2ug, Bgal standard digest spiking 200fm. The samples were mixed well and transferred to fresh vials. SWATH MS data for both the samples were acquired on the same MS instrument using the identical LC conditions. Acquired datasets were searched by ProteinPilot 5.0.1 software (AB SCIEX) against a Uniprot *Litopenaeus vannamei* database added with crustacean database. The generated spectral libraries from the resulting ProteinPilot group files using following settings: maximum 2 missed cleavages, carbamidomethylation of cysteines set as fixed modification, asparagine and glutamine deamidation, oxidation of methionine and hydroxylation of lysine and proline were set as variable modifications, precursor charge state from 2+ to 5+, 99% confidence of the correct peptide identification. Other parameters were used in default settings.

3.15.6.4 SWATH data processing and analysis

The qualitative processing and analysis of acquired SWATH data was done by using the Peak view 2.2 software with the default analysis settings and the following minor changes: FDR of protein identification was set to 5% (with PSM-FDR threshold maintained at 1%) and maximum number of 6 precursors was used for quantification. Peak area of fragment ions was used for peptide quantification. To quantify proteins, the mean value of the

peptides quantities was calculated, reviewed and compared. The results of protein quantification were exported as a tsv file and further processed and statistically analysed in Marker view 1.2.1.

3.16. Statistical analysis of data

Statistical analysis was performed by using IBM© SPSS version 23.0 statistical software (IBM Corporation, USA, 2015). The results were expressed as mean with their corresponding standard deviation (S.D.). The data were log transformed to improve linearity prior to analysis. The results of *in vitro* screening of bacteria were subjected to one-way analysis of variance (ANOVA). For data pertaining to *in vivo* trials, the individual samples were considered to be nested within tanks and the results were analyzed using nested ANOVA. Levene's test was performed to check homogeneity of variance. Subsequently, *post-hoc* multiple comparisons with Tukey HSD test was used to assess the significant difference between the treatment groups. The data were considered to be statistically significant at $p < 0.05$. Pearson's correlation analysis was used to calculate between the different physicochemical parameters, the shrimp growth, enzyme activity and bacteriological analysis during the field trials.

CHAPTER 4:

RESULTS

4. Results

4.1 Screening of halotolerant bacteria for production of bioactive compounds against shrimp pathogens

4.1.1 Agar well diffusion assay

Out of the 300 salt pan bacteria and actinomycetes screened for bioactivity against *Vibrio* pathogens, as seen in Fig. 3, 12 biofilm associated bacteria, 12 from salt pan water, 3 from salt pan sediments and 2 actinomycetes show bioactivity against shrimp pathogens. Amongst the 4 niches selected, the biofilm associated bacteria and the salt pan water isolates, were found to be exhibiting maximum bioactivity against *Vibrio* spp. Out of the 51 actinomycetes from salt pans, only 2 gave promising results.

Table 2 represents the salt pan cultures showing more than 15 mm zone of clearance against 2 or more shrimp pathogens. Twelve cultures showed activity against *Vibrio cholerae*, 11 against *V. parahaemolyticus*, 13 against *V. harveyi*, 15 against *V. alginolyticus* and 8 against *V. vulnificus.* Plate 4 represents the halotolerant bacteria showing anti-*Vibrio* activity by agar well diffusion assay.

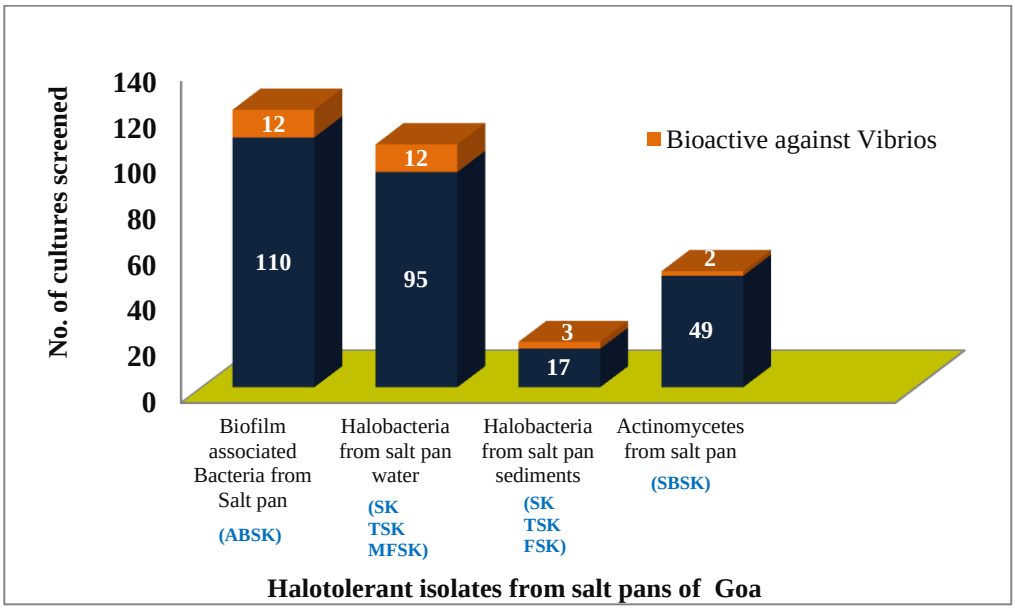


Fig. 3: Halotolerant isolates showing anti-*Vibrio* activity by well diffusion assay.

Table 2: Halotolerant cultures showing more than 15 mm zone of clearance against 2 or more shrimp pathogens.

Sr. no	Culture code	Zone of inhibition (mm)				
		Vibrio cholerae	*Vibrio parahaemolyticus*	*Vibrio harveyi*	*Vibrio alginolyticus*	*Vibrio vulnificus*
1	**SK07**	-	-	17.6 ± 1.2	19.3 ± 0.6	-
2	**SK14**	17.0 ± 1.0	-	16.0 ± 1.0	15.3 ± 1.5	-
3	**SK27**	-	**20.0 ± 1.0**	13.3 ± 0.6	18.0 ± 0.0	-
4	**ABSK 34**	19.3 ± 1.2	-	15.3 ± 0.6	15.0 ± 1.0	14.0 ± 1.0
5	**ABSK 55**	20.0 ± 1.0	-	**22.0 ± 1.0**	**20.3 ± 0.6**	15.0 ± 0.0
6	**ABSK 140**	18.3 ± 0.6	14.0 ± 1.0	-	16.0 ± 1.0	-
7	**ABSK 740**	14.0 ± 1.0	16 ± 1.0	16 ± 1.0	15.6 ± 1.2	-
8	**FSK140**	15.0 ± 1.0	-	14.0 ± 0.6	-	15.0 ± 1.0
9	**FSK327**	20.6 ± 0.6	15.3 ± 0.6	14.0 ± 1.0	17.6 ± 0.6	-
10	**FSK444**	**22.0 ± 1.2**	19.3 ± 0.6	10.0 ± 1.0	15.0 ± 1.0	-
11	**MFSK 15**	-	16.0 ± 1.0	-	16.6 ± 1.5	14.6 ± 0.6
12	**MFSK 23**	17.6 ± 0.6	14.6 ± 1.5	14.3 ± 0.6	-	-
13	**MFSK 77**	14.0 ± 1.0	-	-	15.3 ± 0.6	19.6 ± 0.6
14	**SBSK 201**	-	15.0 ± 2.0	18.0 ± 1.0	-	15.3 ± 0.6
15	**SBSK 203**	14.3 ± 0.6	-	18.0 ± 1.0	19.0 ± 0.6	-
16	**TSK 06**	-	14.6 ± 2.1	16.6 ± 0.6	15.3 ± 1.0	20.0 ± 1.0
17	**TSK 17**	**22.0 ± 1.0**	14.6 ± 0.6	-	15.3 ± 0.6	-
18	**TSK71**	-	16.0 ± 1.0	-	18.0 ± 0.6	**21.0 ± 1.0**

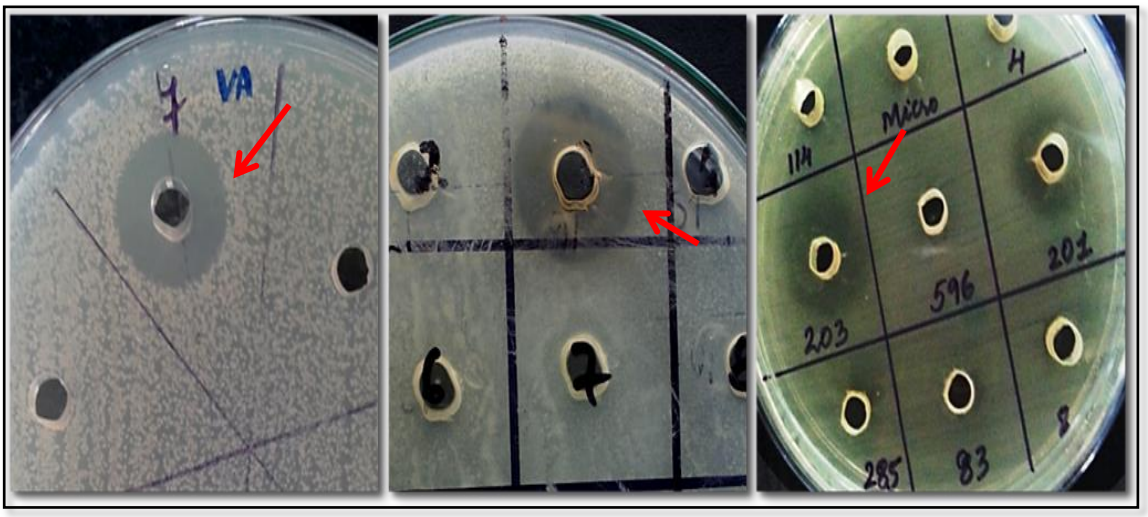


Plate 4: Halotolerant bacteria showing anti-*Vibrio* activity by Agar well diffusion method

4.1.2 Co-culture method

The co-culture method was a successful assay to visualise the inhibitory effect of the halotolerant bacteria against the pathogens used. Each culture showed varying inhibitory patterns towards the different *Vibrio* sp. used. Culture FSK444, when co-cultured with shrimp pathogens showed a two-fold decrease in *Vibrio cholerae* colony count as compared to the control. Culture SK27 decreased the colony count of *V. parahaemolyticus* by more than 2 fold, followed by FSK444 and TSK71. Similarly, a two-fold decrease in the colony count of *V. harveyi* and *V. alginolyticus* was observed when co-cultured individually with culture SK07. A four – fold decrease in *V. alginolyticus* was observed when co-cultured with ABSK55. Culture TSK71 remarkably decreased the counts of *V. alginolyticus* by three-fold and *V. vulnificus* by two-fold as compared to the control (Table 3). Plate 5 depicts the *Vibrio* count on TCBS agar after co-culturing with halotolerant bacteria in Nutrient broth.

Plate 5: Plates representing the *Vibrio* count on TCBS agar after co-culture of *Vibrio* with halotolerant bacteria

Table 3: Anti-*Vibrio* activity of halotolerant bacteria by co-culture method

Culture	*Vibrio* count on TCBS (x 10^8 CFU/mL)				
	V. cholerae	*V. parahaemolyticus*	*V. harveyi*	*V. alginolyticus*	*V. vulnificus*
SK07	-	-	**37.0 ± 3.7**	**12.7 ± 1.5**	-
SK27	36.3 ± 7.5	**11.0 ± 3.6**	40.6 ± 5.5	24.0 ± 3.6	-
SK14	52.3 ± 3.1	-	53.0 ±2.6	20.0 ± 2.7	-
ABSK 34	36.3 ± 3.8	-	58.0 ±3.6	27.0 ± 5.3	35.7 ± 6.5
ABSK 55	**30.0 ± 3.61**	-	**30.7 ±7.0**	**8.3 ± 1.5**	34.0 ± 2.7
ABSK 140	48.0 ± 6.9	23.0 ± 5.0	-	22.6 ± 6.0	-
ABSK 740	43.6 ± 4.5	24.3 ± 2.5	50.6 ±3.2	22.7 ± 4.7	-
FSK140	46.0 ± 7.0	-	76.3 ±7.4	-	47.0 ± 4.4
FSK327	36.3 ± 2.5	33.0 ± 2.7	91.3 ±8.3	18.7 ± 3.1	-
FSK 444	**27.0 ± 2.0**	**17.0 ± 1.7**	46.0 ± 10.4	18.0 ± 3.0	-
MFSK 15	-	25.3 ± 3.5	-	26.0 ± 4.6	40.0 ±7.8
MFSK 23	46.0 ± 4.4	22.7 ± 4.7	44.7 ±6.1	-	-
MFSK 77	48.3 ± 4.0	-	-	29.3 ± 3.2	33.0 ± 3.5
SBSK 201	-	21.7 ± 3.0	47.3 ±3.2	-	34.7 ± 1.5
SBSK 203	36.6 ± 4.5	-	42.3 ±3.5	15.0 ± 3.0	-
TSK 06	-	25.0 ± 1.73	41.6 ± 3.79	24.3 ± 2.5	32.7 ± 3.5
TSK 17	35.3 ± 3.5	22.3 ± 4.2	-	20.7 ± 2.5	-
TSK71	-	**17.6 ± 3.1**	-	**9.7 ± 2.1**	**26.3 ± 2.1**
V_c Control	**53 ± 3.2**				
V_p Control		**25.0 ± 2.65**			
V_h Control			**75.3 ± 6.1**		
V_a Control				**33.6 ± 3.5**	
V_v Control					**57.7 ± 4.5**

Legend: V_c - *Vibrio cholerae*, V_p - *Vibrio parahaemolyticus*, V_h - *Vibrio harveyi*, V_a - *Vibrio alginolyticus*, V_v - *Vibrio vulnificus*.

4.2. Screening of halotolerant bacteria for ammonia degradation

In vitro studies revealed that twelve halotolerant bacteria degraded 40 % ammonia in 7 days, while 56 cultures degraded 30 – 39 % ammonia, 98 degraded 20 – 29 % ammonia and 84 degraded only 10 – 19.9 % ammonia from the broth. It was observed that 14 cultures increased the concentration of ammonia as compared to day 0 (Fig. 4).

Table 4 represents the 12 cultures that showed more than 40 % decrease in ammonia concentration as compared to day 0. Culture ABSK55 showed highest ammonia degrading ability of 12.3 % per day, followed by TSK17 with 8.32 % degradation per day. The total degradation of ammonia by ABSK55 at the end of the experiment was found to be 86.10 % and by TSK71 was 58.26 %.

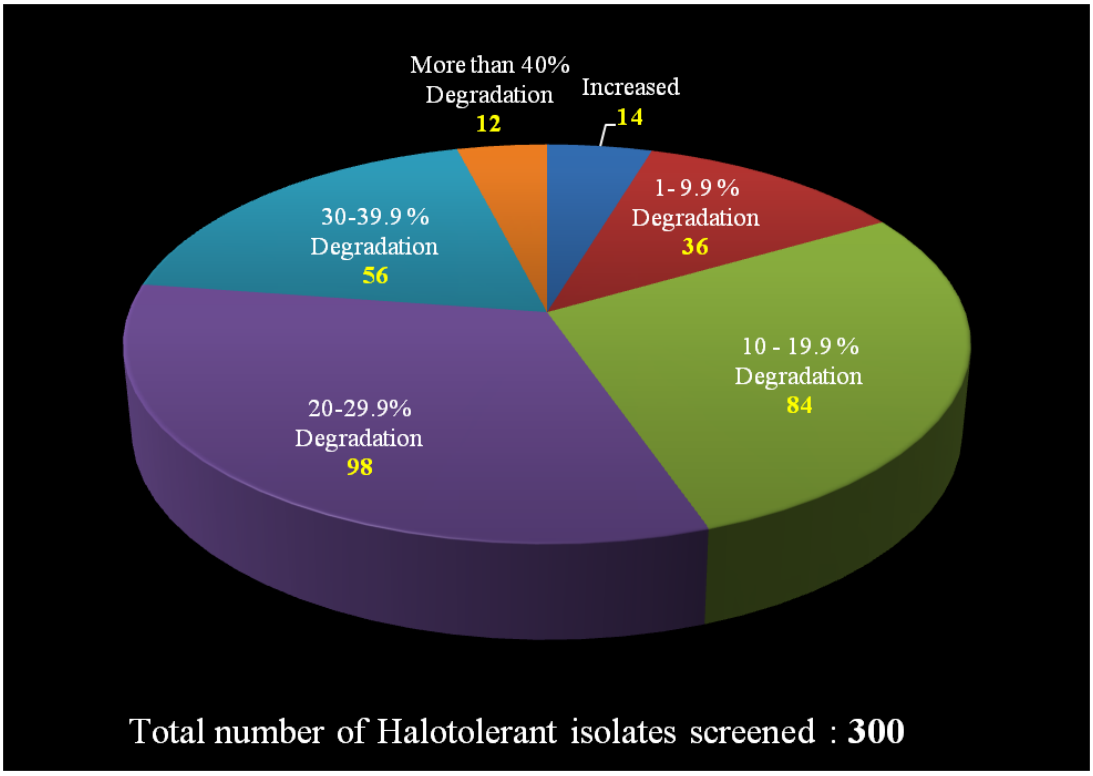


Fig. 4: Halotolerant bacteria showing ammonia degradation over a period of 7 days.

Table 4: Halotolerant bacteria showing above 40 % ammonia degradation in 7 days.

Culture code	Ammonia concentration (mg /L)				Decrease in NH_3 concentration (%)	Per day Decrease in NH_3 concentration (%)
	0 day	3 day	5 day	7 day		
SK256	20.1 ± 0.08	15.3 ± 0.20	14.1 ± 0.03	11.8 ± 0.04	40.89	5.84
ABSK32	19.9 ± 0.40	15.8 ± 0.10	12.1 ± 0.05	11.1 ± 0.03	44.32	6.33
ABSK55	**19.9 ± 0.41**	**16.0 ± 0.04**	**13.4 ± 0.02**	**2.8 ± 0.07**	**86.10**	**12.30**
ABSK91	19.9 ± 0.19	16.4 ± 0.60	11.7 ± 0.03	11.9 ± 0.02	40.02	5.71
ABSK108	19.7 ± 0.36	16.6 ± 0.03	13.4 ± 0.02	11.8 ± 0.04	40.41	5.77
ABSK2	19.9 ± 0.26	12.5 ± 0.01	10.9 ± 0.02	10.5 ± 0.03	47.48	6.78
ABSK171	19.5 ± 0.23	16.6 ± 0.03	13.6 ± 0.01	11.6 ± 0.06	40.83	5.83
ABSK53	19.7 ± 0.36	17.6 ± 0.02	15.6 ± 0.02	10.8 ± 0.03	45.16	6.45
FSK444	20.1 ± 0.09	16.9 ± 0.02	13.9 ± 0.02	12.0 ± 0.03	40.32	5.76
MFSK15	19.9 ± 0.29	19.3 ± 0.02	13.2 ± 0.03	11.8 ± 0.04	41.05	5.86
FSK140	19.9 ± 0.40	13.4 ± 0.03	12.9 ± 0.02	11.0 ± 0.07	44.62	6.37
TSK17	**19.8 ± 0.29**	**13.6 ± 0.02**	**12.3 ± 0.02**	**8.3 ± 0.02**	**58.26**	**8.32**

4.3 *In- vitro* screening for probiotic potential

The halotolerant bacteria (twenty five) that showed prominent anti-*Vibrio* activity and ammonia degradation were further screened for probiotic potential *in-vitro*.

4.3.1 Mucus adhesion

Fig. 5 illustrates the twenty five halotolerant cultures screened for mucus adhesion. As seen in the graph, 9 cultures showed more than 70 % mucus adhesion. Halotolerant culture TSK71 showed highest mucus adhesion (86 ± 5.9 %) followed by SK27 (85.4 ± 5.5 %) and SK07 (85.4 ± 6.3 %). The results highlighted the ability of certain halotolerant bacteria to adhere to shrimp mucus, thereby having the potential to compete with pathogens for adhesive sites in the gastrointestinal tract and the surface of the host. This is an important characteristic to be considered for the selection of probiotics for aquaculture.

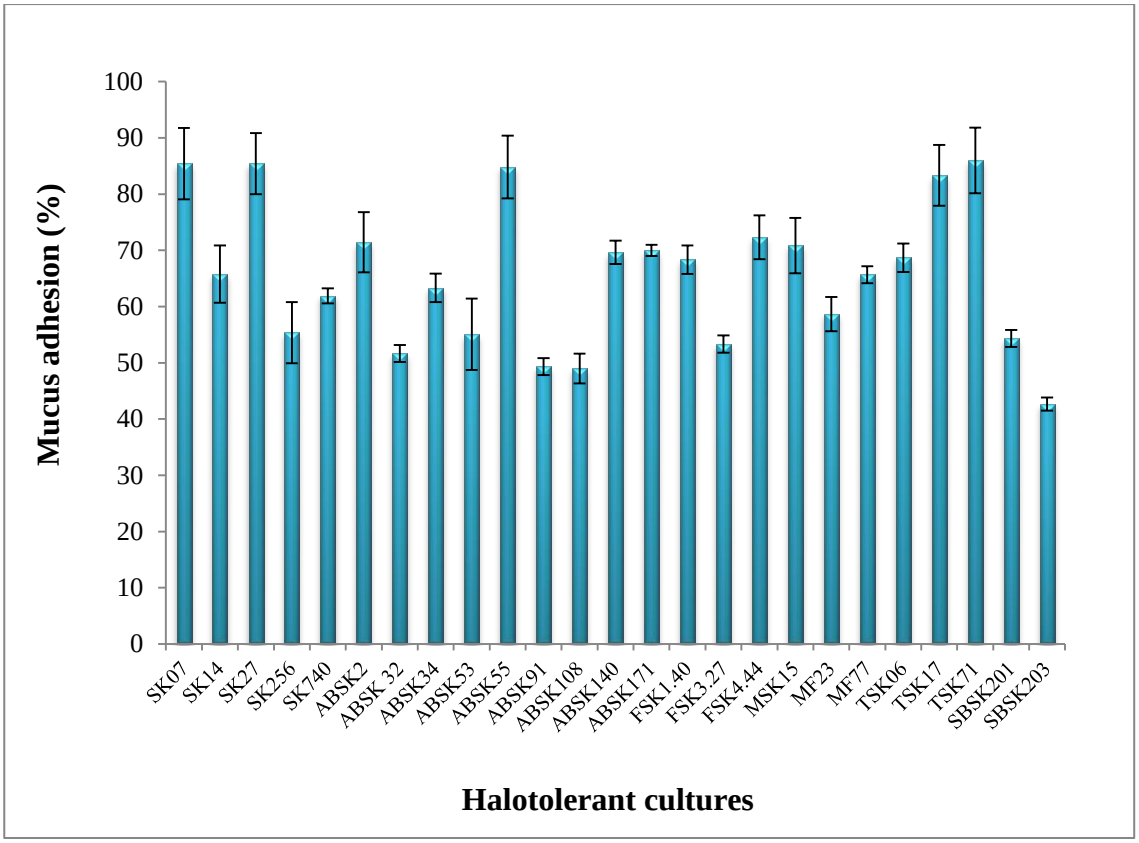


Fig. 5: Halotolerant cultures showing mucus adhesion.

4.3.2 Production of extracellular enzymes

The results of spot inoculation assay revealed the potential of the halotolerant isolates to produce a wide range of extracellular enzymes. Among the screened cultures, 23 bacteria produced extracellular enzymes (Table 5). A significant (p <0.001) difference in the enzyme production of different halotolerant bacteria was observed, indicating that each organism had a unique profile in producing specific enzymes. Extracellular amylase production was observed in 18 cultures (Plate 6A). Halotolerant bacteria SK27 showed highest amylase production in 24 h (21.0 ± 1.0 mm), followed by SK07 (19.33 ± 2.0 mm) and TSK71 (19.0 ± 2.0 mm). A total of 14 cultures produced protease (Plate 6B), with the highest protease production in culture SK27 (23.33 ±1.5 mm) followed by SK07 (20.67 ±1.5 mm). Cellulase production was observed in 17 cultures (Plate 6C), while 12 cultures produced lipase (Plate 6D). Culture TSK71 showed maximum cellulase (20.67 ± 0.5 mm)

and lipase production (22.33 ±1.3 mm), followed by ABSK55 with 21.0 ± 1.3 mm clearance zone on tributyrin agar. No chitinase production was detected.

When screened for enzyme activity in broth after 24 h of incubation, SK27 showed maximum amylase activity (3.85 ± 0.6 U/mg) followed by TSK71 (3.82 ± 0.6 U/mg). Maximum protease activity was exhibited by SK07 (3.44 ± 0.3 U/mg) and TSK71 (3.09 ± 0.6 U/mg). ABSK55 exhibited highest cellulase (3.83 ± 0.4 U/mg) and lipase (3.15 ± 0.1 U/mg) activity as compared to the other cultures (Fig. 6).

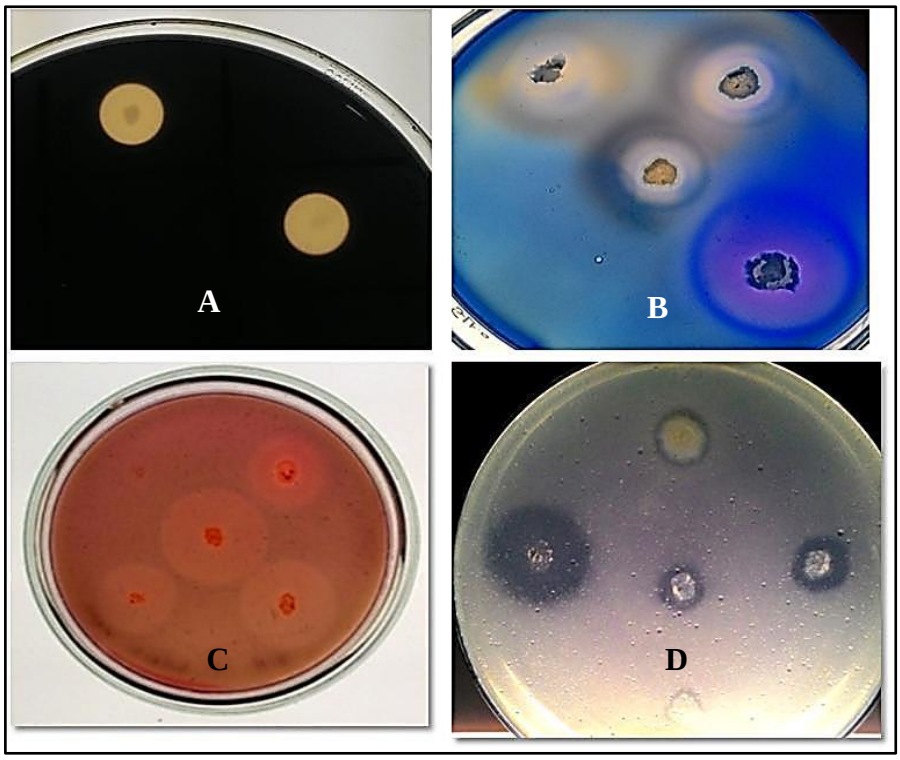


Plate 6: Production of extracellular enzymes by spot inoculation: (A) Amylase (B) Protease (C) Cellulase (D) Lipase.

Table 5: Production of extracellular enzymes (zone in mm) by halotolerant bacteria by spot inoculation method.

Sr. No	Culture code	Amylase	Protease	Cellulase	Lipase	Chitinase
1	SK07	**19.33 ± 2.0**	**20.67 ±1.5**	**19.33 ±1.5**	N.D.	N.D
2	SK14	12.33 ± 0.5	N.D.	13.0 ± 1.0	N.D.	N.D
3	SK27	**21.0 ± 1.0**	**23.33 ±1.5**	13.67 ± 0.5	15 ± 1.1	N.D
4	SK256	13.67±1.5	N.D.	14.67 ±1.5	N.D.	N.D
5	ABSK2	11.0 ± 1.0	N.D.	15.0 ± 1.2	N.D.	N.D
6	ABSK 32	10.33 ±0.58	11 ± 1	N.D.	9.67 ± 0.58	N.D
7	ABSK34	N.D.	11.67 ± 0.58	N.D.	N.D.	N.D
8	ABSK53	17.67 ± 1.5	N.D.	N.D.	9.67 ±1.15	N.D
9	ABSK55	N.D.	N.D.	**19.0 ± 2.0**	**21.0 ± 1.3**	N.D
10	ABSK91	10.33 ± 0.58	9.67 ± 0.58	10 ± 0 05	11 ± 1	N.D
11	ABSK108	11.33 ± 0.58	12 ± 1	9.33 ± 0.58	N.D.	N.D
12	ABSK140	N.D.	12.67 ± 0.58	9.67 ± 0.58	11 ± 1	N.D
13	ABSK171	N.D.	N.D.	10.33 ± 0.58	N.D.	N.D
14	ABSK740	N.D.	10.0 ± 1.1	14.0 ±1.5	12.33 ±1.5	N.D
15	FSK140	11.67 ± 0.58	11 ± 1	11 ± 1	10.67 ± 0.58	N.D
16	FSK327	10 ± 1.0	N.D.	N.D.	11 ± 1	N.D
17	FSK444	15.0 ±1.0	**19.13 ±1.5**	N.D.	N.D.	N.D
18	MSK15	11.0 ±1.1	N.D.	14.6 ±1.5	N.D.	N.D
19	MF23	11.33 ± 0.58	ND.	10.33± 0.58	N.D.	N.D
20	MF77	10.67 ± 1.15	11.67 ± 2.08	10 ± 1	11.67 ± 0.58	N.D
21	TSK06	12.67 ± 0.58	13 ± 1	N.D.	N.D.	N.D
22	TSK17	14.33 ±1.5	18.0 ± 1.0	18.0 ± 1.3	**18.0 ±1**	N.D
23	TSK71	**19.0 ±2.0**	18.0 ±1.0	**20.67 ± 0.5**	**22.33 ±1.3**	N.D
24	SBSK201	N.D.	N.D.	N.D.	N.D.	N.D
25	SBSK203	N.D.	N.D.	N.D.	N.D.	N.D

N.D represents ‘not detected’

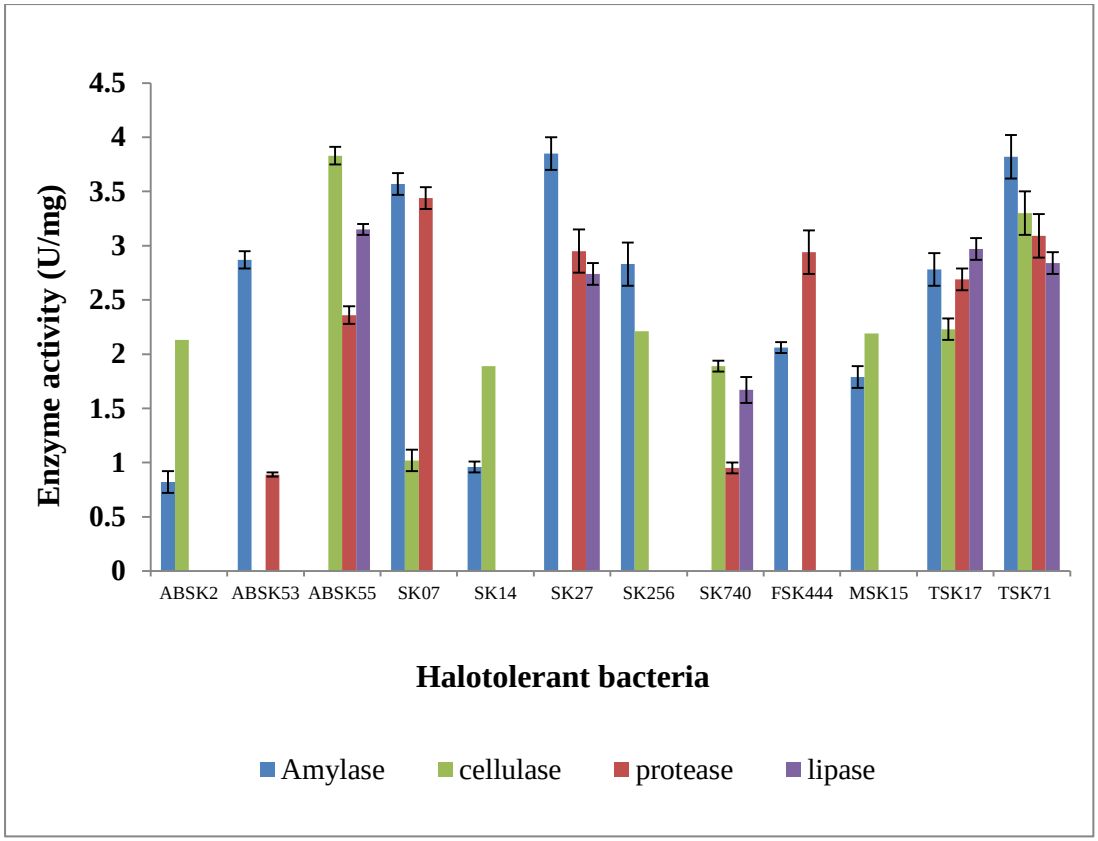


Fig. 6: Halotolerant bacteria showing enzyme activity in broth.

4.3.3 Tolerance to bile salts, pH, salinity and growth on pond water agar

An essential criterion for the selection of bacteria to be a candidate probiotic is its ability to survive at a wide range of bile salts (in the gastrointestinal tract), pH and salinity; and grow in aquaculture pond water.

Table 6 represents the tolerance of salt pan bacteria to varying concentrations of bile salts, pH and salinity. Out of the 25 cultures screened, 13 cultures confirmed the ability to grow at 0 – 3 % bile salts, 16 cultures survived at a pH range from 5 to 9, and 24 cultures exhibited optimum growth from 0 – 5 % salinity. When streaked on pond water agar plates, 14 salt pan cultures showed favourable growth. The results indicate the conducive nature of the salt pan bacteria to survive in fluctuating environmental conditions.

Table 6: Bile salt, pH and salinity tolerance of salt pan bacteria.

Culture code	Bile salt concentration (%)				pH concentration					NaCl concentration (%)			Growth on Pond water agar
	0	1	2	3	5	6	7	8	9	0	3	5	
SK07	++	++	++	++	++	++	++	++	++	++	++	++	+
SK14	++	+	-	-	++	++	++	++	++	+	++	-	+
SK27	++	++	++	++	++	++	++	++	++	++	++	+	+
SK256	++	+	-	-	++	++	++	++	+	++	+	+	-
SK740	++	++	+	-	-	++	++	++	+	+	++	+	+
ABSK2	++	+	+	-	-	+	++	++	++	++	++	++	-
ABSK 32	++	++	+	-	+	+	++	++	++	++	++	+	+
ABSK34	++	++	++	+	+	++	++	++	+	+	++	+	-
ABSK53	++	++	+	+	-	++	++	++	++	++	++	+	-
ABSK55	++	++	++	+	++	++	++	++	++	++	++	++	+
ABSK91	++	++	+	-	+	++	++	++	+	++	++	+	+
ABSK108	++	++	+	+	-	+	++	++	+	+	++	++	-
ABSK140	++	+	+	-	-	+	++	++	+	+	++	+	-
ABSK171	++	++	+	+	+	++	++	++	+	++	++	++	-
FSK140	++	+	+	+	+	+	++	++	++	++	++	+	+
FSK327	++	+	+	-	+	++	++	++	++	++	++	+	-
FSK444	++	++	++	+	+	++	++	++	++	++	++	++	+
MSK15	++	++	+	+	+	++	++	++	++	++	++	+	+
MF23	++	+	+	-	-	+	++	++	++	+	++	++	+
MF77	++	++	+	+	+	+	++	++	+	++	++	++	-
TSK06	++	++	+	-	-	+	++	++	++	+	++	++	+
TSK17	++	++	++	+	++	++	++	++	++	++	++	++	+
TSK71	++	++	++	++	++	++	++	++	++	++	++	++	+
SBSK201	+	+	+	-	-	+	++	++	++	+	++	++	-
SBSK203	+	+	-	-	-	-	++	++	+	-	++	++	-

Absorbance at 600 nm :

<0.05: '-', absorbance between 0.051 to 0.50: '+', absorbance > 0.51 : '++'

For growth on pond water agar: '+' indicates growth in 24 h.

4.3.4 Hemolytic activity

Hemolytic activity test is a preliminary safety assessment performed to eliminate those cultures that produced hemolysin toxin against human blood agar or shrimp hemolymph agar. When streaked on human blood agar, a total of 13 halotolerant cultures showed no hemolysis (γ hemolysis), while 12 exhibited complete hemolysis (β hemolysis) (Table 7). All the 25 cultures showed γ hemolysis on shrimp hemolymph agar. The tests were compared with shrimp pathogens, where *V. parahemolyticus* showed 26 mm β hemolysis on human blood agar and 16 mm β hemolysis on shrimp hemolymph agar (Plate 7). The cultures that produced hemolysin against human erythrocytes and shrimp hemocytes were rejected, while the ones showing no hemolysis were considered for further studies based on their respective bioactivities.

Table 7: Hemolytic activity on human blood agar and shrimp hemolymph agar.

Culture code	Growth on human blood agar	Growth on shrimp hemolymph agar
SK07	γ hemolysis	γ hemolysis
SK14	β hemolysis – 16 mm	γ hemolysis
SK27	γ hemolysis	γ hemolysis
SK256	β hemolysis – 8 mm	γ hemolysis
SK740	γ hemolysis	γ hemolysis
ABSK2	β hemolysis – 13 mm	γ hemolysis
ABSK32	β hemolysis – 11 mm	γ hemolysis
ABSK34	γ hemolysis	γ hemolysis
ABSK53	β hemolysis – 8 mm	γ hemolysis
ABSK55	γ hemolysis	γ hemolysis
ABSK91	β hemolysis – 18 mm	γ hemolysis
ABSK108	β hemolysis – 10 mm	γ hemolysis
ABSK140	γ hemolysis	γ hemolysis
ABSK171	β hemolysis – 10 mm	γ hemolysis
FSK140	γ hemolysis	γ hemolysis
FSK327	β hemolysis – 27 mm	γ hemolysis
FSK444	γ hemolysis	γ hemolysis
MSK15	β hemolysis – 19 mm	γ hemolysis
MFSK23	β hemolysis – 17 mm	γ hemolysis
MFSK77	γ hemolysis	γ hemolysis
TSK06	β hemolysis - 14 mm	γ hemolysis
TSK17	γ hemolysis	γ hemolysis
TSK71	γ hemolysis	γ hemolysis
SBSK201	γ hemolysis	γ hemolysis
SBSK203	No growth	γ hemolysis
V. alginolyticus	γ hemolysis	γ hemolysis
V. parahaemolyticus	β hemolysis – 26 mm	β hemolysis – 16 mm

β hemolysis : Complete hemolysis, α hemolysis : Partial hemolysis, γ hemolysis : No hemolysis

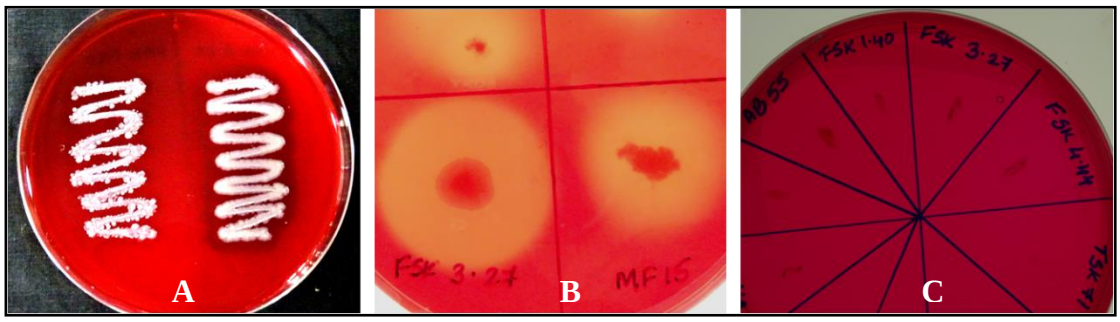
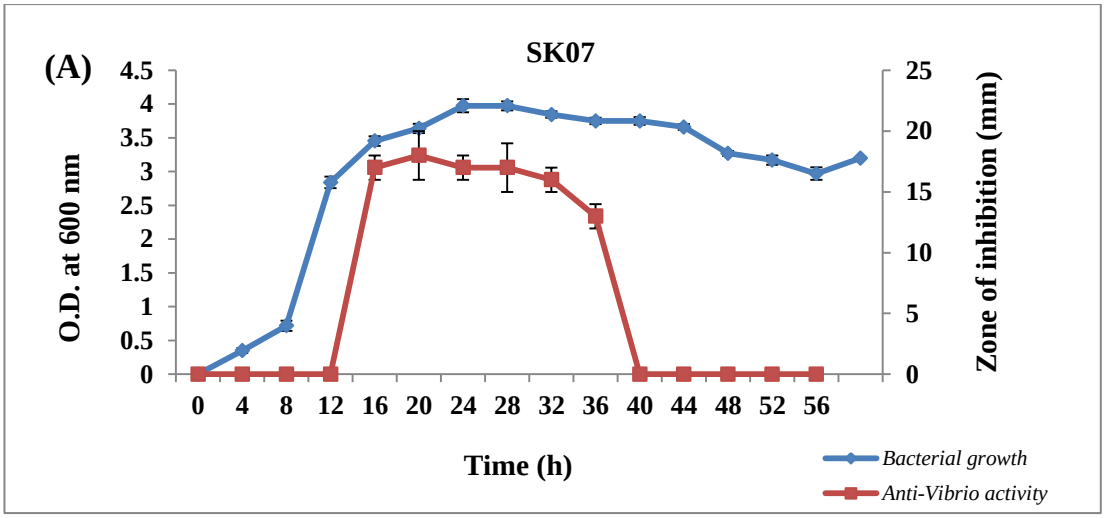
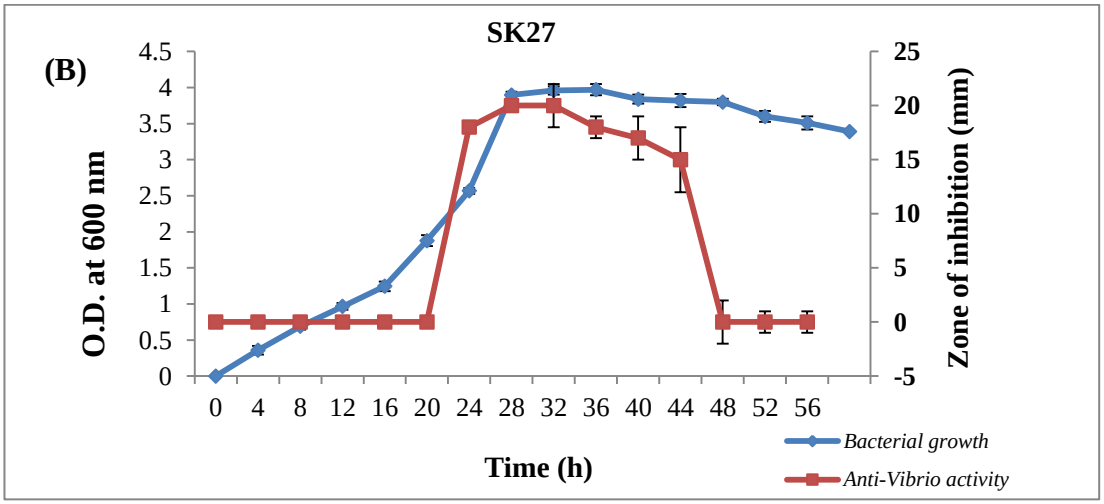


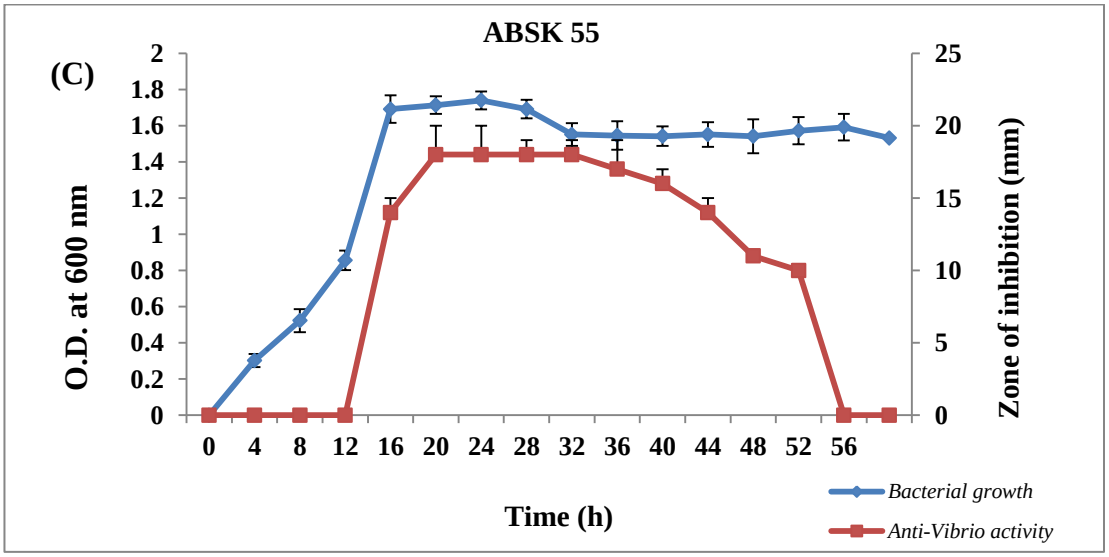
Plate 7: Plates indicating hemolysis by salt pan bacteria. (A) γ hemolysis (left) and β hemolysis (right) on human blood agar. (B) β hemolysis on human blood agar with zone of clearance measured in mm (diameter). (C) γ hemolysis on shrimp hemolymph agar

4.3.5 Growth profile and stage of production of antimicrobial metabolite

Based on the *in vitro* tests carried out, six potential halotolerant bacteria (SK07, SK27, ABSK55, FSK444, TSK17 and TSK71) were selected to study their growth profile and the stage at which the antimicrobial metabolites were produced. All the six cultures exhibited a sigmoidal curve, with each culture having a distinct lag and log phase. Culture SK07, SK27, FSK444 and TSK71 showed bioactivity against *V. alginolyticus, V. parahemolyticus, V. cholerae* and *V. alginolyticus* respectively during their late log phase to early stationary phase (Fig. 7 A,B,D,F). SK07, SK27 and ABSK55 exhibited a short lag phase with the production of anti-*Vibrio* metabolite beginning between 16 to 24 h of growth (Fig. 7 A, B, C). Anti-*Vibrio* activity by ABSK55, FSK444 and TSK17 was observed up to 52 - 56 h (Fig. 7 C, D, E). The growth profile of shrimp pathogens *Vibrio harveyi* and *V. alginolyticus* exhibited a short lag phage with an exponential phase ending at 20 – 24 h (Fig. 8).


(A)
SK07
O.D. at 600 nm
Zone of inhibition (mm)
Time (h)
Bacterial growth
Anti-Vibrio activity


(B)
SK27
O.D. at 600 nm
Zone of inhibition (mm)
Time (h)
Bacterial growth
Anti-Vibrio activity


(C)
ABSK 55
O.D. at 600 nm
Zone of inhibition (mm)
Time (h)
Bacterial growth
Anti-Vibrio activity

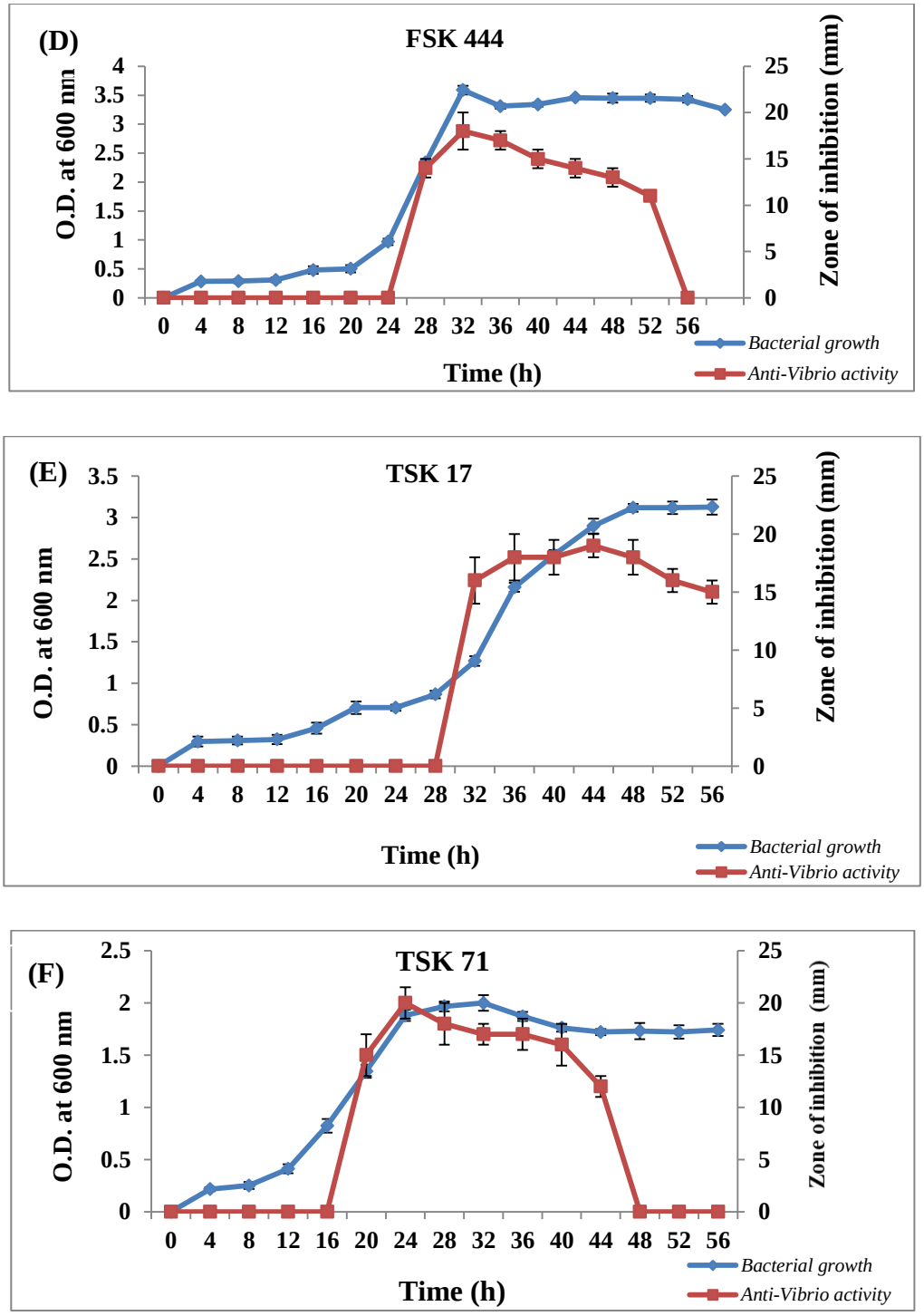


Fig. 7: Growth profile and anti-*Vibrio* activity of (A) SK07 against *V. alginolyticus,* (B) SK27 against *V. parahaemolyticus,* (C) ABSK55 against *V. harveyi,* (D) FSK444 against *V. cholerae,* (E) TSK17 against *V. cholerae,* (F) TSK71 against *V. alginolyticus.*

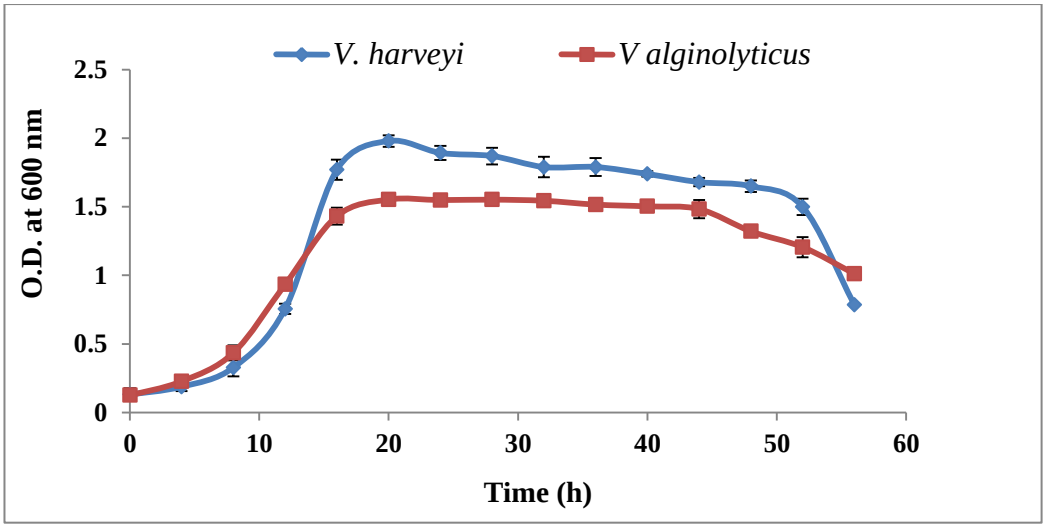


Fig. 8: Growth profile of shrimp pathogens *Vibrio harveyi* and *V. alginolyticus*.

4.3.6 Biocompatibility between the selected salt pan bacteria

The six salt pan isolates showed no inhibitory effect by cross streak method when grown simultaneously in one plate (Plate 8). The results confirmed the compatibility of the isolates with each other with reference to their growth. The consolidated data of the selected salt pan cultures showing probiotic potential *in vitro* is tabulated in Table 8. These bacteria were selected to further study their effect *in vivo* on Whiteleg shrimp, *Litopenaeus vannamei*.

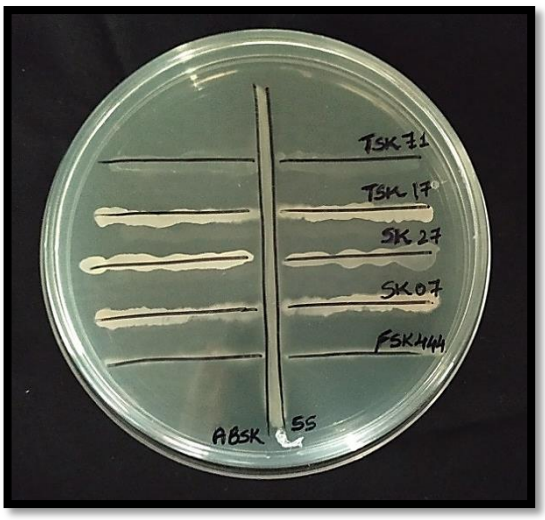


Plate 8: Biocompatibility between the selected salt pan bacteria.

Table 8: Selected candidate probionts based on *in vitro* experiments for *in vivo* trials.

***In vitro* tests**	**SK07**	**SK27**	**ABSK55**	**FSK444**	**TSK17**	**TSK71**
Anti-*Vibrio* activity	*V. parahaemolyticus* *V. harveyi* *V. alginolyticus*	*V. cholerae* *V. harveyi* *V. alginolyticus*	*V. cholerae* *V. harveyi* *V. alginolyticus* *V. vulnificus*	*V. cholerae* *V. harveyi* *V.parahaemolyticus* *V. vulnificus*	*V. cholerae* *V.parahaemolyticus* *V. alginolyticus*	*V.parahaemolyticus* *V. alginolyticus* *V. vulnificus*
Rate of ammonia degradation per day (%)	-	-	12.30	5.76	8.32	-
Mucus adhesion (%)	85.4 ± 6.3	85.4 ± 5.5	85.4 ± 5.5	72.3 ± 3.9	83.3 ± 5.4	86 ± 5.9
Bile salt tolerance (%)	0 – 3	0 – 3	0 – 3	0 – 3	0 – 3	0 – 3
pH tolerance	5 – 9	5 – 9	5 – 9	5 – 9	5 – 9	5 – 9
Salinity tolerance (%)	0 – 5	0 – 5	0 – 5	0 – 5	0 – 5	0 – 5
Enzyme production	Amylase, protease, cellulase	Amylase, protease, cellulase Lipase	protease, cellulase lipase	Amylase, protease,	Amylase, protease, cellulase lipase	Amylase, protease, cellulase Lipase
Growth on Pond water agar	+	+	+	+	+	+
Hemolysis test	γ hemolysis	γ hemolysis	γ hemolysis	γ hemolysis	γ hemolysis	γ hemolysis
Stage of production of anti-*Vibrio* compounds	16 – 36 h	24 – 44 h	16 – 52 h	28 – 52 h	32 – 56 h	20 - 44 h
Biocompatibility	compatible	compatible	compatible	compatible	compatible	compatible

4.4 Preparation of bacterial suspension for *in vivo* assays

Bacterial suspensions of individual cultures containing a fixed viable count (10^8, 10^9 and 10^{10} CFU/mL) were used for the preliminary *in vivo* assays. Plate 9 is a representation of a bacterial suspension used for individual *in vivo* studies on *Litopenaeus vannamei*.

Plate 9: Bacterial suspension used for preliminary *in vivo* studies on *Litopenaeus vannamei*.

4.5 Lab-scale trial of individual halotolerant bacteria on *Litopenaeus vannamei*

Subsequent to the *in vitro* experiments conducted, the six halotolerant bacteria termed as "candidate probionts" or "candidate probiotic bacteria" were tested for their individual effects *in vivo* at different cell concentrations.

4.5.1 Analysis of water quality of the experimental tanks

Data representing the effect of the candidate probionts on the water quality of shrimp culture tanks is presented in Table 9A and 9B. As seen in the table, pH of all the tanks ranged from 8.01 ± 0.41 to 8.36 ± 0.23 and temperature from 27.99 ± 1.33 to 28.51 ± 1.17 ºC. The total dissolved solids (TDS) was detected within the range of 17.43 ± 3.3 to 18.76 ± 3.8 ppt and salinity was between 29.26 ± 1.02 to 29.74 ± 1.21 ppt. No significant difference ($p<0.05$) was observed in the physical parameters across all the treatment groups. The dissolved oxygen (DO) concentration was maintained between the range of 4.71 ± 1.08 to 5.62 ± 0.60 mg/L with continuous aeration. The average biological oxygen demand (BOD) was detected within the range of 18.67 ± 4.51 to 30.67 ± 4.04 mg/L. No significant difference in the BOD and DO was observed between the control and treatment groups (Table 9A).

In contrast, the data of Nitrogen analysis showed remarkable results in the treatment groups as compared to the control. A significant difference ($p< 0.01$) was observed between the control (0.055 ± 0.005 mg/L) and the tanks treated with 10^8, 10^9, 10^{10} CFU/mL (0.023 ± 0.002, 0.013 ± 0.004, 0.011 ± 0.003 mg/L respectively). Similarly, the nitrate and ammonia concentration was significantly lower ($p<0.05$) in tanks treated with ABSK55 at all three concentrations as compared to control. No sulfide was detected across the different treatment groups. No significant difference in alkalinity and total culturable bacterial (TCB) count was observed between the control and the treatment groups. Interestingly, a ten-fold decrease in the total *Vibrio* count was observed in the tanks treated with 10^9 and 10^{10} CFU/mL of SK07, ABSK55 and TSK71 (Table 9B).

Table 9: Effect of individual candidate probionts on the water quality of shrimp culture tanks. (A) Physical and chemical, (B) Chemical and microbial parameters.

	(CFU/ mL)	pH	Temp (°C)	TDS (ppt)	Salinity (ppt)	DO (mg/ L)	BOD (mg/ L)
Control	-	8.08±0.11	28.27±1.11	18.45±4.0	29.26±1.02	5.28±0.62	25.33±4.51
SK07	10^8	8.11±0.24	28.26±0.87	17.94±3.5	29.62±1.22	5.12±0.91	21.67±4.04
	10^9	8.29±0.15	28.21±1.12	18.14±3.6	29.41±0.92	5.62±0.60	19.33±4.51
	10^{10}	8.33±0.05	28.30±1.21	17.55±3.9	29.57±0.66	5.60±0.74	23.67±3.06
SK27	10^8	8.05±0.24	28.10±0.97	18.13±3.7	29.38±1.02	4.90±0.84	19.33±3.21
	10^9	8.02±0.20	28.42±0.92	17.49±3.9	29.43±0.86	5.58±0.81	24.00±6.74
	10^{10}	8.23±0.23	28.23±1.14	17.43±3.3	29.35±1.03	4.71±1.08	23.00±5.29
ABSK 55	10^8	8.02±0.22	28.22±0.84	17.55±3.2	29.37±1.07	5.38±0.77	22.33±3.21
	10^9	8.21±0.25	28.33±1.08	17.79±4.4	29.39±1.15	5.57±0.69	23.67±4.51
	10^{10}	8.06±0.32	28.04±1.09	17.95±3.5	29.62±0.90	5.28±1.08	20.00±7.55
FSK444	10^8	8.23±0.20	28.31±1.04	17.58±3.5	29.70±0.67	5.16±0.42	19.67±4.04
	10^9	8.30±0.31	28.25±0.87	17.94±3.7	29.32±0.98	5.40±0.59	25.00±2.65
	10^{10}	8.36±0.23	28.22±1.05	18.73±3.3	29.31±0.94	5.07±0.32	29.00±2.00
TSK 17	10^8	8.12±0.29	27.99±1.33	17.89±3.5	29.50±1.13	5.07±1.08	30.67±4.04
	10^9	8.14±0.35	28.21±0.97	17.67±3.6	29.74±1.21	5.27±0.95	22.00±2.65
	10^{10}	8.08±0.49	28.51±1.17	18.76±3.8	29.39±0.85	5.34±0.73	24.67±7.77
TSK 71	10^8	8.06±0.52	28.34±0.82	17.50±3.2	29.36±0.74	4.91±0.80	18.67±4.51
	10^9	8.11±0.03	28.33±0.87	17.80±4.1	29.53±1.04	4.88±0.74	19.00±2.00
	10^{10}	8.01±0.41	28.20±1.09	18.64±3.7	29.50±1.02	5.13±0.89	23.00±4.58

9 (A) Physical and chemical parameters

Each value is the mean ± SD of three replicates (n = 3). Values have been recorded weekly for a period of 120 days and calculated as average which is tabulated above.

Table 9 (B) Chemical and microbial parameters

	(CFU /mL)	Nitrite (mg/L)	Nitrate (mg/ L)	Ammonia (mg/ L)	Alkalinity (mg/ L)	TVC (Log CFU/ mL)	TCB (Log CFU/ mL)
Control		0.055±0.005	0.38±0.06	0.25±0.09	97.5±8.7	6.44±0.08	9.31 ± 0.17
SK07	10^8	0.045±0.003	0.35±0.01	0.18±0.08	115.0±4.1	6.40±0.03	9.25 ± 0.27
	10^9	0.047±0.006	0.33±0.01	0.20±0.06	107.5±8.7	5.87±0.09*	10.14±0.06*
	10^{10}	0.051±0.002	0.32±0.01	0.18±0.09	113.8±4.8	5.75±0.14*	10.55±0.07*
SK27	10^8	0.045±0.001	0.34±0.01	0.15±0.06	113.8±9.5	6.36±0.06	9.67±0.12
	10^9	0.047±0.002	0.38±0.04	0.15±0.07	115.0±4.1	6.13±0.03*	10.05±0.03*
	10^{10}	0.046±0.004	0.36±0.01	0.14±0.05	102.5±6.5	6.12±0.07*	10.72±0.07*
ABSK55	10^8	0.023±0.00*	0.30±0.0**	0.16±0.07	97.5±2.9	6.07±0.04*	9.13 ± 0.15
	10^9	0.013±0.00**	0.30±0.0**	0.06±0.02*	102.5±8.7	5.12±0.13*	10.04±0.06*
	10^{10}	0.011±0.00**	0.28±0.0**	0.08±0.03*	101.3±6.3	5.08±0.16*	10.99±0.06*
FSK444	10^8	0.057±0.003	0.39±0.05	0.22±0.09	116.3±4.8	6.40±0.08	9.43 ± 0.17
	10^9	0.047±0.005	0.39±0.06	0.17±0.04	112.5±8.7	6.36±0.02	10.24±0.04*
	10^{10}	0.052±0.002	0.40±0.01	0.15±0.04	106.3±4.8	6.42±0.02	10.83±0.04
TSK 17	10^8	0.042±0.00	0.36±0.01	0.17±0.07	103.8±7.5	6.38±0.08	9.80 ± 0.16
	10^9	0.044±0.006	0.34±0.01	0.19±0.05	95.0±4.1	6.16±0.04	10.45±0.05*
	10^{10}	0.041±0.00	0.34±0.01	0.21±0.10	115.0±4.1	6.15±0.06	10.78±0.04*
TSK 71	10^8	0.038±0.00	0.33±0.02	0.18±0.09	107.5±2.9	6.32±0.09	9.44 ± 0.16
	10^9	0.041±0.00	0.32±0.0*	0.15±0.08	100.0±4.1	5.43±0.12*	10.12±0.04*
	10^{10}	0.042±0.00	0.32±0.0*	0.13±0.70	100.0±4.8	5.27±0.08*	10.91±0.04*

Each value is the mean ± SD of three replicates (n = 3). * and ** indicate statistically significant from control (ANOVA; $p < 0.05$, $p < 0.01$ and subsequent post hoc multiple comparison with Dunnett's test).

Values have been recorded weekly for a period of 120 days and calculated as average which is tabulated above.

4.5.2 Analysis of shrimp growth and survival

Table 10 illustrates the growth performance and feed utilization efficiency of *Litopenaeus vannamei* when treated with individual candidate probionts at different cell concentrations. As seen in the Table, the final body weight of shrimp treated with halotolerant isolates SK07 (10^8, 10^9 and 10^{10} CFU/mL), SK27 (10^9, 10^{10} CFU/mL) and TSK71 (10^9 and 10^{10} CFU/mL) was significantly higher as compared to the control tank (13.93 ± 0.75 g). The highest final body weight was observed in shrimp treated with SK07: 10^9 CFU/mL (19.84 ± 0.20 g) followed by SK27: 10^{10} CFU/mL (19.55 ± 1.20 g). Similar results were observed with respect to weight gain and feed efficiency (FE) with maximum feed efficiency seen in shrimp treated with SK27: 10^9 CFU/mL (0.66 ± 0.05) followed by SK07 : 10^9 and 10^{10} CFU/mL (0.64 ± 0.01 respectively) as compared to control (0.45±0.03). Halotolerant culture TSK71 also showed a significant increase ($p<0.05$) in weight gain and feed efficiency of *Litopenaeus vannamei* when treated with a concentration of 10^9 and 10^{10} CFU/mL. No significant difference was observed in the specific growth rate (SGR) ($p=0.393$) and percentage of survival ($p=0.114$) across the different treatment sets. The results of the effect of the candidate probiotic bacteria on the growth and feed utilization efficiency suggested that SK07, SK27 and TSK71 increased the growth of *Litopenaeus vanna*mei compared to the control. There was no significant difference observed among the different concentrations of SK07 (10^8, 10^9, 10^{10} CFU/mL), SK27 (10^9, 10^{10} CFU/mL) and the different concentrations of TSK71 (10^9, 10^{10} CFU/mL), thus indicating that irrespective of the concentrations used (10^9 or 10^{10} CFU/mL), these cultures play a role in increasing the growth of *Litopenaeus vannamei*.

Table 10: Growth performance and feed utilization efficiency of *Litopenaeus vannamei* supplemented with different concentrations of individual candidate probionts.

	(CFU/mL)	Initial length (cm)	Final length (cm)	Initial weight (g)	Final weight (g)	Weight gain (g)	Specific growth rate (SGR)	Feed efficiency (FE)	Survival (%)
CONTROL	-	2.78 ± 0.06	11.25 ± 0.25^{a}	0.62 ± 0.11	13.93 ± 0.75^{a}	13.31 ± 0.83^{a}	2.65 ± 0.18	0.45 ± 0.03^{a}	71.67 ± 5.77
SK07	10^{8}	2.57 ± 0.10	13.5 ± 0.7^{bcd}	0.63 ± 0.12	19.34 ± 0.49^{e}	18.71 ± 0.60^{d}	2.69 ± 0.17	0.61 ± 0.02^{e}	78.33 ± 2.88
	10^{9}	2.75 ± 0.10	13.50 ± 0.9^{bcd}	0.65 ± 0.13	19.84 ± 0.20^{e}	19.19 ± 0.22^{d}	2.86 ± 0.16	0.64 ± 0.01^{e}	76.67 ± 2.88
	10^{10}	2.63 ± 0.12	13.3 ± 0.6^{bcd}	0.60 ± 0.06	19.61 ± 0.20^{e}	19.00 ± 0.25^{d}	2.90 ± 0.09	0.64 ± 0.01^{e}	78.33 ± 5.77
SK27	10^{8}	2.63 ± 0.15	13.1 ± 0.4^{abcd}	0.65 ± 0.07	18.33 ± 1.01^{de}	17.69 ± 0.97^{cd}	2.83 ± 0.06	0.56 ± 0.03^{cde}	80 ± 5
	10^{9}	2.63 ± 0.15	13.9 ± 0.3^{d}	0.62 ± 0.06	19.47 ± 1.40^{e}	18.85 ± 1.36^{d}	2.94 ± 0.06	0.66 ± 0.05^{e}	81.67 ± 2.88
	10^{10}	2.75 ± 0.48	13.25 ± 0.2^{bcd}	0.59 ± 0.07	19.55 ± 1.20^{e}	18.60 ± 1.14^{d}	2.94 ± 0.14	0.63 ± 0.04^{e}	81.67 ± 5.77
ABSK 55	10^{8}	2.70 ± 0.08	12.77 ±0.4^{abcd}	0.65 ± 0.07	17.03±0.21^{abcde}	16.38 ±0.15^{abcd}	2.72 ± 0.08	0.54 ±0.00^{abcd}	78.33 ± 7.63
	10^{9}	2.80 ± 0.33	13.17 ±0.9^{abcd}	0.60 ± 0.13	17.13 ±1.10^{abcde}	16.53 ±1.09^{abcd}	2.81 ± 0.05	0.55 ±0.04^{abcd}	81.67 ± 5.77
	10^{10}	2.63 ± 0.15	13.1 ± 0.4^{abcd}	0.62 ± 0.07	17.37 ±1.40^{abcde}	16.74 ±1.37^{abcd}	2.77 ± 0.09	0.58 ± 0.05^{de}	80 ± 5
FSK444	10^{8}	2.70 ± 0.10	11.9 ± 0.7^{abc}	0.62 ± 0.05	14.77 ± 1.07^{abc}	14.15 ± 1.07^{ab}	2.75 ± 0.10	0.48 ± 0.02^{ab}	71.66 ± 2.88
	10^{9}	2.70 ± 0.09	12.26 ± 0.8^{ab}	0.61 ± 0.09	14.53 ± 1.26^{ab}	13.92 ± 1.25^{ab}	2.67 ± 0.04	0.47 ± 0.04^{ab}	73.33 ± 7.63
	10^{10}	2.70 ± 0.10	11.8 ± 0.8^{ab}	0.65 ± 0.08	14.29 ± 1.13^{a}	13.63 ± 1.15^{a}	2.68 ± 0.13	0.46 ± 0.04^{ab}	70 ± 5
TSK 17	10^{8}	2.63 ± 0.21	13.5 ± 0.9^{bcd}	0.62 ± 0.09	15.55 ± 0.83^{abcd}	14.93 ±0.87^{abc}	2.84 ± 0.15	0.50 ± 0.03^{abcd}	75 ± 5
	10^{9}	2.72 ± 0.22	13.02±0.75^{bcd}	0.66 ± 0.08	15.77 ± 0.10^{abcd}	15.11 ± 0.30^{abc}	2.66 ± 0.23	0.51 ± 0.01^{abcd}	71.67 ± 7.63
	10^{10}	2.63 ± 0.15	13.2 ± 0.9^{bcd}	0.67 ± 0.05	15.11 ± 0.66^{abcd}	14.44 ±0.64^{abc}	2.55 ± 0.05	0.46 ± 0.02^{ab}	80 ± 5
TSK 71	10^{8}	2.60 ± 0.10	13.53 ± 0.3^{bcd}	0.63 ± 0.09	17.93 ± 1.18^{bcde}	17.30 ±1.27^{bcd}	2.80 ± 0.18	0.53 ± 0.04^{abcd}	81.67 ± 5.77
	10^{9}	2.70 ± 0.10	14.2 ± 0.5^{d}	0.64 ± 0.06	18.27 ± 1.70^{de}	17.64 ± 1.65^{cd}	2.80 ± 0.04	0.58 ± 0.05^{de}	80.00 ± 5
	10^{10}	2.73 ± 0.14	13.77 ± 0.4^{cd}	0.62 ± 0.16	18.44 ± 2.61^{de}	17.82 ± 2.52^{cd}	2.76 ± 0.08	0.59 ± 0.08^{de}	78.33 ± 5.77

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

4.5.3 Analysis of the immune system of shrimp after exposure to individual candidate probionts

Fig. 9 represents the effect of the individual halotolerant bacteria on the total hemocyte count (THC) of *Litopenaeus vannamei*. As seen in the graph, an increasing trend in the THC was observed across all the treatment groups except TSK17 (10^9 and 10^{10} CFU/mL) as the number of days increased. The THC of shrimp treated with all the three concentrations of ABSK55, SK07 and SK27 was significantly higher ($p<0.05$) as compared to control at 60, 90 and 120 day analysis. More than 40 % increase in THC was observed in shrimp treated with halotolerant culture ABSK55 (10^8, 10^9 and 10^{10} CFU/mL) and SK07 (10^9 and 10^{10} CFU/mL) as compared to the control.

At 60, 90 and 120 day analysis, a significant ($p<0.05$) increase in phagocytosis was observed in shrimp treated with a cell concentration of 10^8, 10^9 and 10^{10} CFU/mL of ABSK55 and SK27; and a cell concentration of 10^9 and 10^{10} CFU/mL of SK07. Halotolerant bacteria ABSK55 at 10^9 CFU/mL showed highest phagocytic activity of 42.67 ± 2.31 %, followed by SK27: 10^{10} CFU/mL (41.50 ± 6.66 %). No significant difference in phagocytosis was observed in shrimp tanks treated with FSK444 and TSK17 at 60, 90 and 120 days analysis (Fig 10).

Shrimp treated with ABSK55, SK07 and SK27 at all three cell concentrations, showed a significant increase in phenoloxidase activity as compared to the control after 120 days. Highest phenoloxidase activity was recorded on the 120^{th} day in shrimp treated with 10^8, 10^9, 10^{10} CFU/mL of ABSK55 (0.28 ± 0.03, 0.28 ± 0.00 and 0.26 ± 0.01 units/min/mg protein respectively) as compared to the control (0.15 ± 0.01 units/min/mg protein) (Fig. 11).

The results of immune system analysis revealed the ability of the halotolerant bacteria ABSK55, SK07 and SK27 to stimulate the immune response of *Litopenaeus vannamei* at varying cell concentrations.

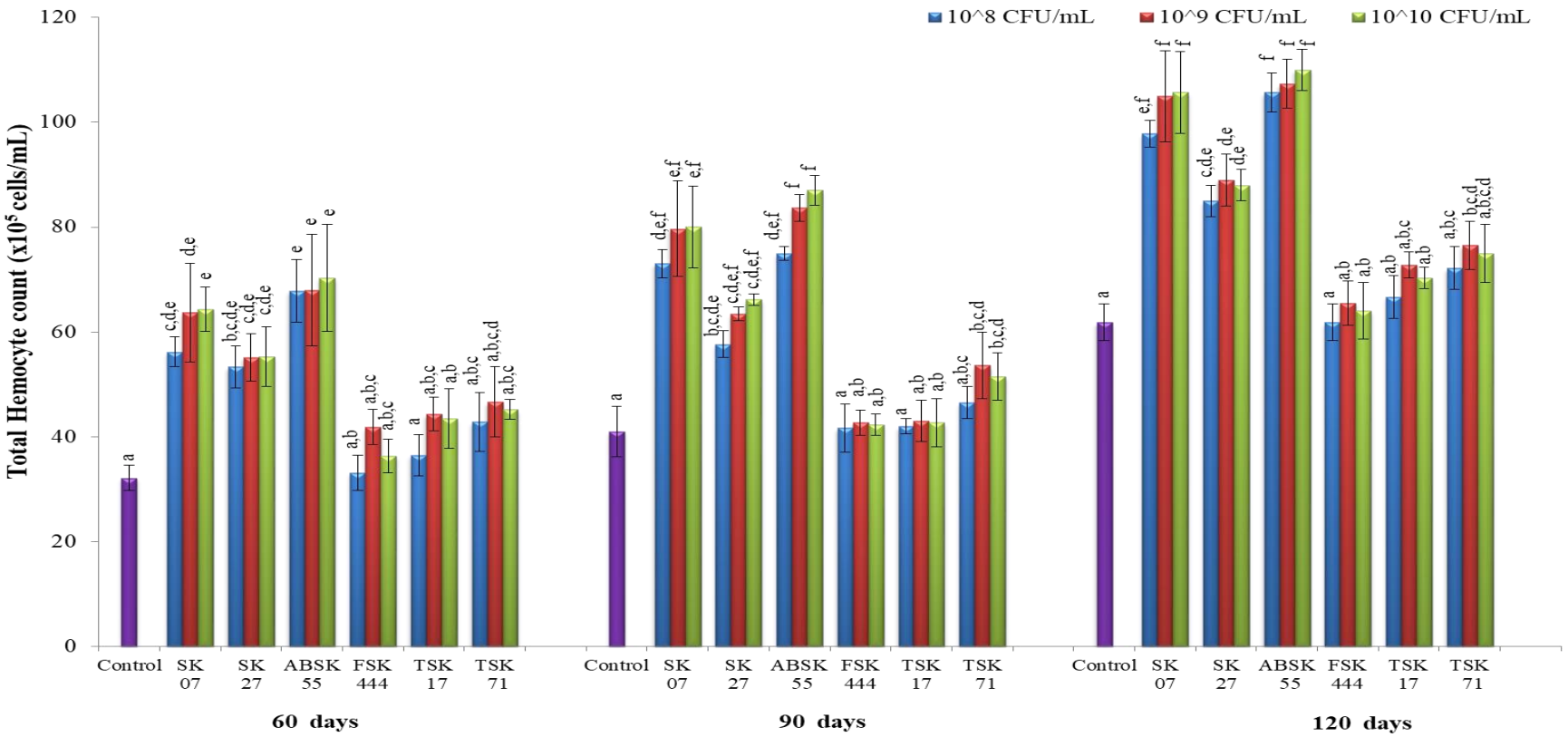


Fig. 9: Effect of individual candidate probionts on the total hemocyte count of *Litopenaeus vannamei*.

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

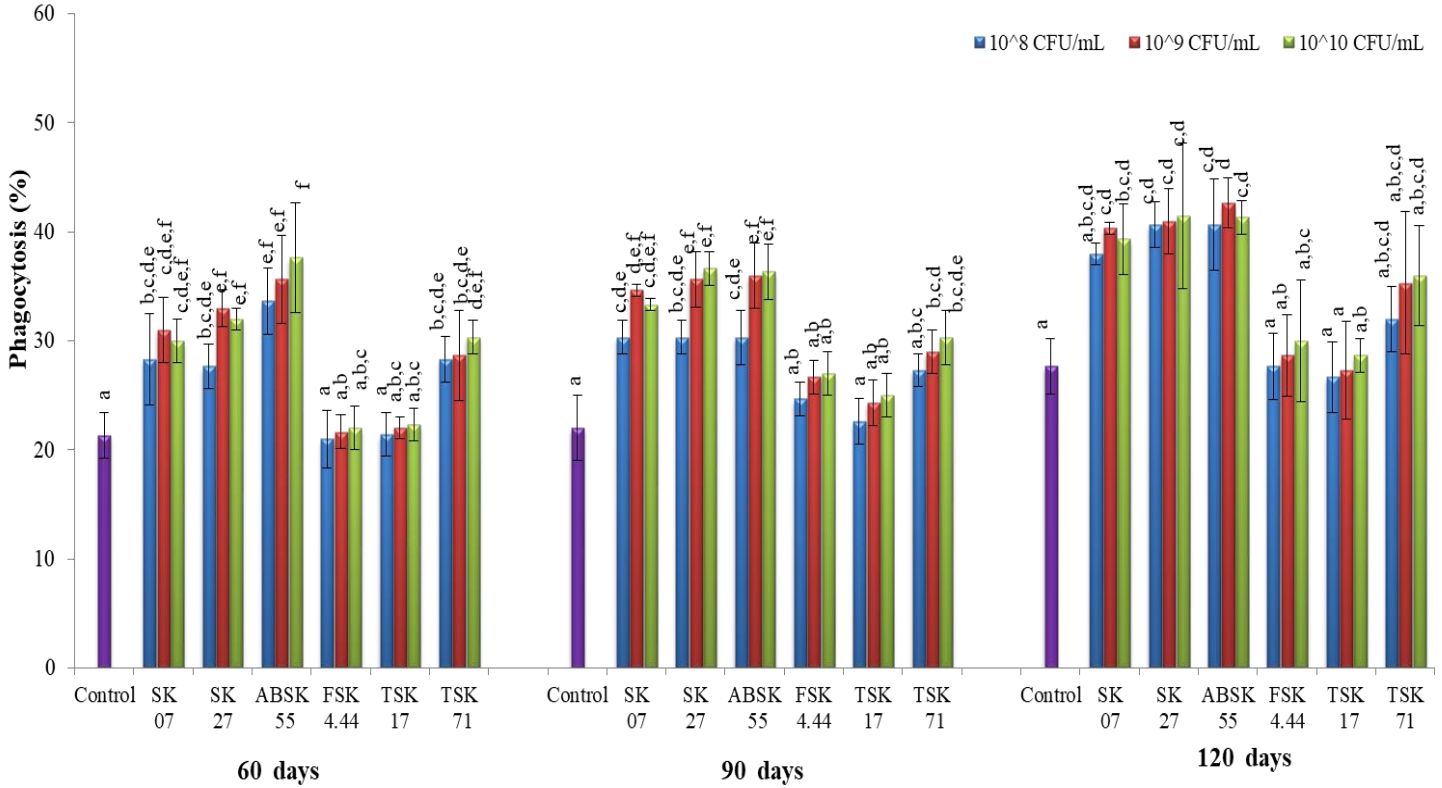


Fig. 10: Effect of individual halotolerant bacteria on the Phagocytic activity of *Litopenaeus vannamei*.

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

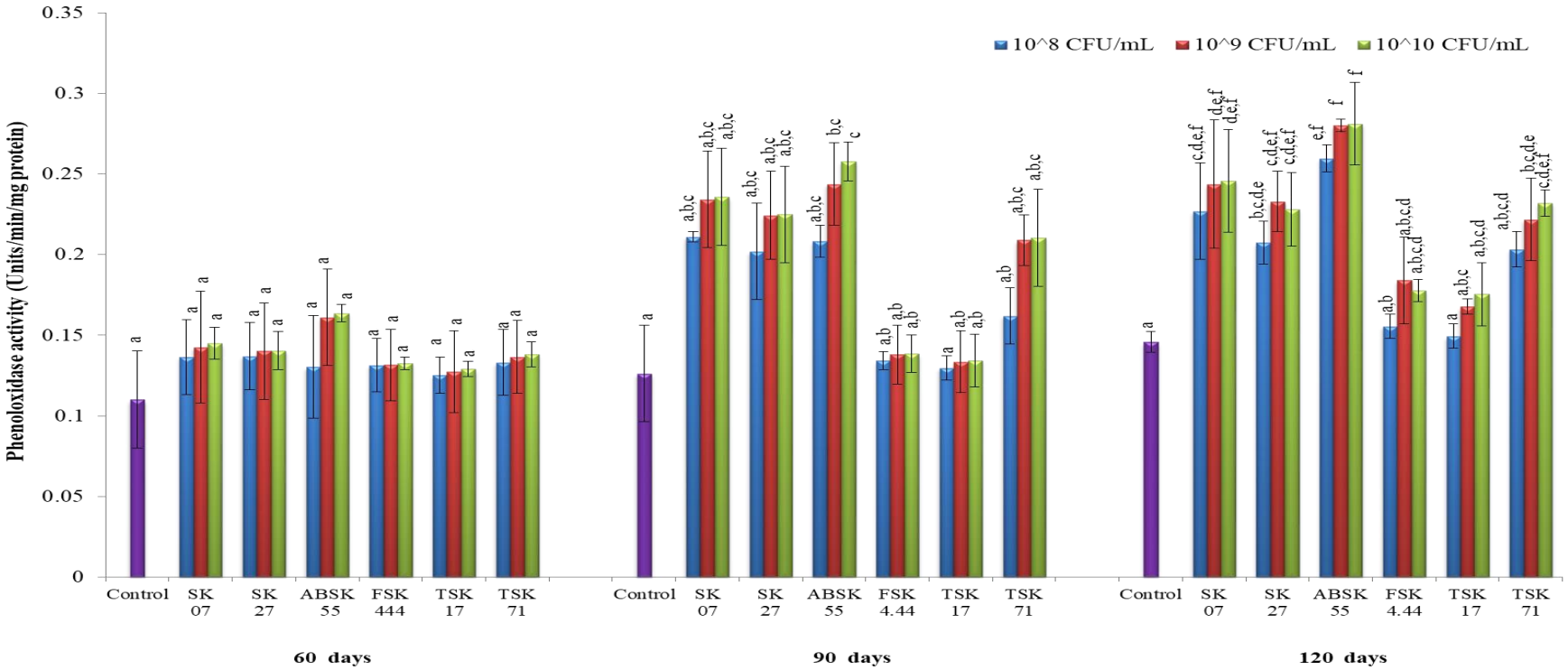


Fig. 11: Effect of individual halotolerant bacteria on the Phenoloxidase activity of *Litopenaeus vannamei*.

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

4.6 Formulation of the bacterial consortium

Based on the *in vivo* trials carried out in the laboratory, four salt pan bacteria viz. SK07, SK27, ABSK55 and TSK71 were selected for the preparation of the test probiotic consortium. Table 11 represents the parameters based on which the halotolerant bacteria were selected for the formulation of the test consortium. As seen in section 4.5.2, there was no significant difference (p>0.05) observed between the different concentrations of SK07 (10^8, 10^9, 10^{10} CFU/mL), SK27 (10^9, 10^{10} CFU/mL) and the different concentrations of TSK71 (10^9, 10^{10} CFU/mL) on the growth of *Litopenaeus vannamei*. Similarly, no significant difference was observed between the effect of the varying concentrations of 10^9 and 10^{10} CFU/mL of ABSK55, SK07 and SK27 on the immune response of *Litopenaeus vannamei* (section 4.5.3). Hence, the minimum optimal dosage of 10^9 CFU/mL was considered for the preparation of the test probiotic consortium for further studies. Plate 10 represents the lyophilized halotolerant bacterial consortium prepared at a dosage of 10^9 CFU/mL.

Table 11: Bacteria selected for formulation of the test probiotic consortium.

Parameters	SK07	SK 27	ABSK 55	TSK 71
Bioactivity (Bactericidal)	*V. harveyi*, *V. parahemolyticus*	*V. cholerae*, *V. harveyi*	*V. harveyi*, *V. alginolyticus*, *V. cholerae*	*V. alginolyticus*, *V. vulnificus*, *V. parahemolytic*us
Water quality	-	NH_3 degradation	NH_3 degradation	-
Enzyme production	Amylase, Protease	Amylase, Protease	Lipase	Cellulase, Lipase
Growth	Increased ABW, FE	Increased ABW, FE	-	Increased ABW, FE
Immune	THC, Phenoloxidase	Phagocytosis	Highest Phagocytosis, THC, Phenoloxidase	-

- ABW- Average body weight, FE – Feed efficiency, THC – Total hemocyte count

Plate 10: Test probiotic consortium SFSK4.

4.7 Shelf life of the prepared bacterial consortium

Fig. 12 represents the shelf life and bacterial viability (Log CFU/mL) of the individual halotolerant bacteria and the test probiotic studied at room temperature (28 ± 2 ºC) and at refrigerated conditions (6 ± 2 ºC) for a period of 180 days. As seen in Fig. 12 A, the bacterial viability was stable up to 120 days at room temperature, following which a gradual decrease in viable count was observed up to 180 days. However, at 6 ± 2 ºC, stable viable count was seen up to the end of the experiment i.e. 180 days (Fig. 12B). The viable count of the test consortium was found to be higher than individual cultures at both, room temperature and refrigerated condition. The consortium which comprised of all the four halotolerant isolates had the highest stability and shelf life as compared to the stability of the individual cultures (Fig. 12).

4. 8 *In vitro* synergistic effect of the bacterial consortium

Prior to evaluating the effect of the test consortium on *Litopenaeus vannamei*, the synergistic effect of the bacterial consortium was tested *in vitro*. It was observed that the bacteria synergistically exhibited anti-*Vibrio* activity against *V. harveyi, V. alginolyticus, V. cholerae, V. parahaemolyticus*, ammonia degradation and enzyme production when stored at both 28 ± 2 ºC (Table 12) and 6 ± 2 ºC (Table 13). A gradual decrease in the Anti-*Vibrio* activity, ammonia degradation and enzyme production was observed as the

number of days increased (180 days), however the difference was not significant ($p>0.05$) as compared to day 0.

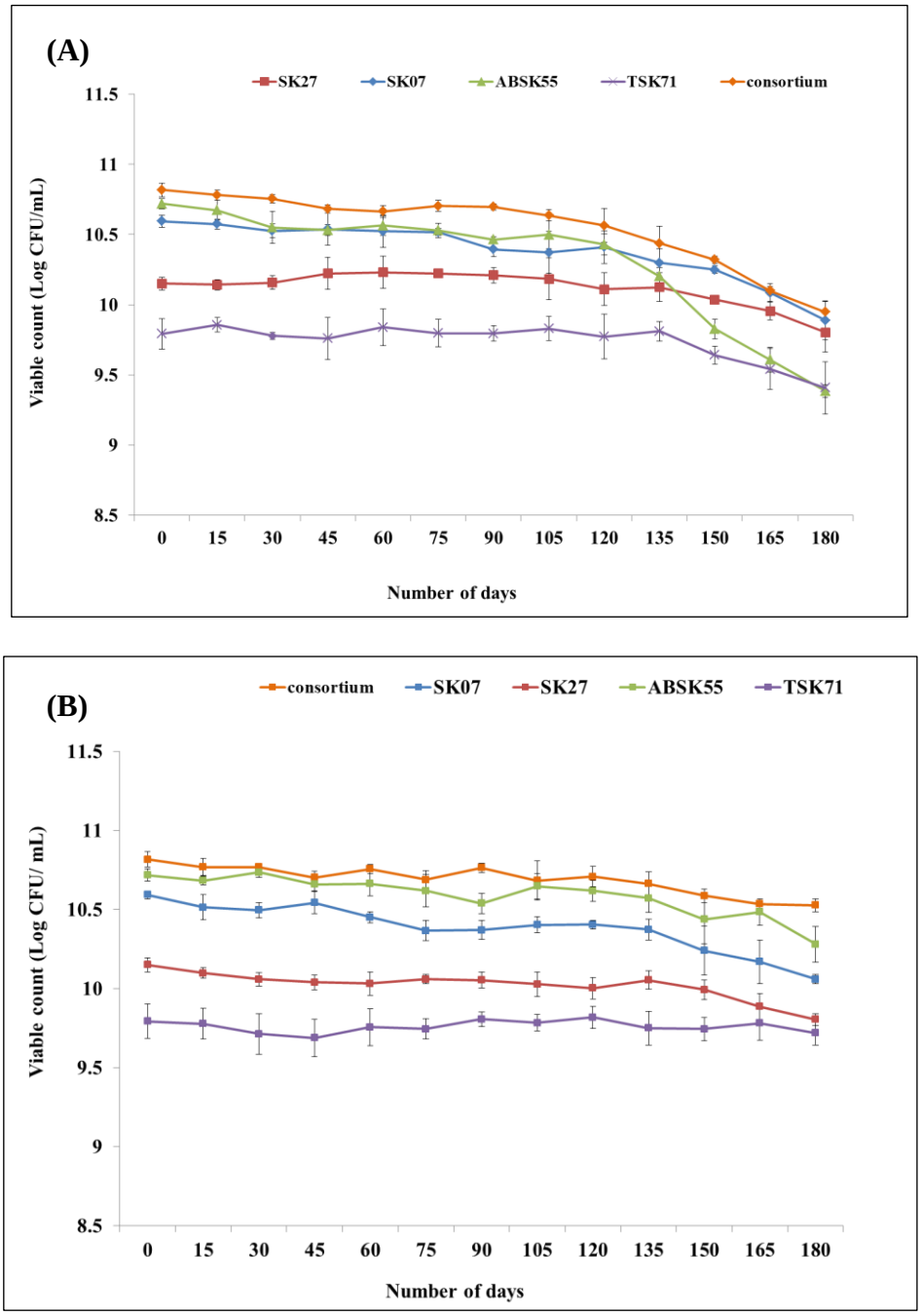


Fig. 12: Bacterial viability of the selected isolates stored at (A) Room temperature (28 ± 2 ºC) and (B) 6 ± 2 ºC).

Table 12: *In vitro* synergistic effect of the bacterial consortium stored at room temperature (28 ± 2 ºC).

Period →	0 day	60 day	120 day	180 day
Anti-*Vibrio* activity (Zone in mm) *V. harveyi* *V. alginolyticus* *V. cholerae* *V. parahaemolyticus*	 24.00 ±1.00 24.33 ±1.15 21.33 ±1.15 21.67 ±0.58	 22.33 ±2.08 22.67 ±1.15 22.67 ±1.15 22.00 ±2.65	 21.00 ±2.65 19.67 ±1.53 20.67 ±1.53 21.00 ±1.00	 21.33 ±2.08 20.33 ±1.53 20.67 ±0.58 19.67 ±0.58
Ammonia degradation (%)	10.29 ± 0.74	9.79±0.84	7.75 ± 1.30	7.54 ±0.38
Enzyme production (Zone in mm) Amylase Protease Cellulase Lipase	 23.00 ± 1.09 26.00 ± 0.58 20.33 ± 1.53 22.00 ± 2.00	 23.33 ± 0.58 25.67 ± 1.00 20.00 ±1.15 22.33 ± 1.15	 22.67 ±1.53 25.00 ±0.58 20.33 ±1.00 21.00 ±1.09	 19.33 ±1.53 23.66 ±1.53 19.5 ±1.00 18.33 ±1.53

Table 13: *In vitro* synergistic effect of the bacterial consortium stored at 6 ± 2 ºC.

Period →	0 day	60 day	120 day	180 day
Anti-*Vibrio* activity (Zone in mm) *V. harveyi* *V. alginolyticus* *V. cholerae* *V. parahaemolyticus*	 23.33±0.58 25.00±1.00 23.33±1.15 22.00±1.00	 22.00±1.00 22.00±1.00 21.67±1.53 21.67±2.31	 20.67±1.53 20.33±2.08 20.00±0.58 19.33±0.58	 20.33±2.52 20.33±0.71 20.00±1.00 18.33±1.53
Ammonia degradation (%)	10.15 ± 0.68	9.38 ± 0.68	8.05 ± 0.51	7.91 ± 0.68
Enzyme production (Zone in mm) Amylase Protease Cellulase Lipase	 24.00 ± 1.00 26.00 ± 0.58 20.33 ± 1.53 22.00 ± 2.00	 23.33 ± 0.58 25.67 ± 1.00 19.33 ± 1.15 22.33 ± 1.15	 22.67 ± 1.53 25.00 ± 0.58 20.00 ± 1.15 21.00 ± 1.00	 19.33 ± 1.53 23.66 ± 1.53 20.33 ± 1.00 20.00 ± 2.08

4. 9 *In vivo* synergistic effect of the bacterial consortium on *Litopenaeus vannamei*

Subsequent to the success of the *in vitro* studies carried out, the effect of the same bacterial consortium was further evaluated *in vivo*.

4.9.1 Effect of the halotolerant bacterial consortium on the physico-chemical and microbial parameters of tank water

Table 14 represents the effect of the halotolerant bacterial consortium (SFSK4) on the physico-chemical and microbial parameters of the shrimp culture tanks. As seen in the Table, the pH of the tanks ranged from 8.06 ± 0.35 to 8.09 ± 0.31, temperature from 27.37 ± 1.16 to 27.63 ± 1.36 ºC, Total dissolved solids (TDS) from 19.15 ± 3.11 to 19.41 ± 2.87 ppt and salinity from 29.34 ± 1.01 to 29.46 ± 0.86 ppt. No significant ($p>0.05$) difference was observed in the physical parameters of water quality across the different treatment groups. The Dissolved oxygen and Biological oxygen demand ranged from 5.34 ± 0.29 to 5.58 ± 0.66 mg/L and 20.01 ± 5.12 to 21.39 ± 5.91 mg/L respectively. A significant decrease ($p=0.02, F=7.907$) in the ammonia concentration in the tanks treated with SFSK4 (0.08 ± 0.02 mg/L) was observed as compared to the control tanks (0.22 ± 0.02 mg/L). The nitrate concentration was higher in the tanks treated with SFSK4 as compared to the control, however the difference was insignificant ($p>0.05$). Similarly, no significant difference in the nitrite concentration was observed across the different treatment groups. The alkalinity and total culturable bacterial (TCB) count ranged from 87.61 ± 2.48 to 91.77 ± 5.53 mg/L and from 10.0 3± 0.06 to 10.16 ± 0.04 Log (CFU/mL) respectively. No significant difference was observed in the alkalinity and total culturable bacterial count (TCB) across the different treatment groups. A ten-fold decrease in the total *Vibrio* count (5.63 ± 0.28 Log CFU/mL) was observed in the shrimp tanks treated with SFSK4 as compared to the control tanks (6.27 ± 0.11 Log CFU/mL) ($p= 0.013, F = 9.657$).

No significant difference was observed between the TVC of the control and commercial probiotic treated shrimp tanks ($p<0.05$).

Table 14: Effect of the halotolerant bacterial consortium (SFSK4) on the physico-chemical and microbial parameters of tank water.

Parameter	Control	Test Probiotic SFSK4	Commercial Probiotic CP1	*p* value	*F* value
pH	8.09±0.31	8.06±0.35	8.08±0.37	0.994 (NS)	0.006
Temp (°C)	27.63±1.36	27.37±1.16	27.47±1.12	0.964 (NS)	0.037
TDS (ppt)	19.24±2.80	19.15±3.11	19.41±2.87	0.987 (NS)	0.013
Salinity (psu)	29.34±1.01	29.46±0.86	29.38±0.89	0.988(NS)	0.012
DO (mg/L)	5.34±0.29	5.54±0.55	5.58±0.66	0.837 (NS)	0.184
BOD (mg/L)	21.39±5.91	20.01±5.12	20.93±5.70	0.910 (NS)	0.095
Ammonia (mg/L)	0.22±0.02^{a}	**0.08±0.02^{b}**	0.14±0.05^{ab}	0.021	7.907
Nitrite (mg/L)	0.10±0.03	0.05±0.02	0.07±0.02	0.187 (NS)	2.225
Nitrate (mg/L)	0.51±0.13	0.68±0.15	0.57±0.20	0.461(NS)	0.884
Alkalinity (mg/L)	91.77±5.53	90.16±5.50	87.61±2.48	0.584 (NS)	0.589
TCB (Log CFU/mL)	10.16±0.04	10.03±0.06	10.05±0.08	0.115 (NS)	3.170
TVC (Log CFU/mL)	6.27±0.11^{a}	**5.63±0.28^{b}**	6.03±0.08^{ab}	0.013	9.657

Each value is the mean ± SD of three replicates (n = 3).Within each row, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

4.9.2 Effect of the Halotolerant bacterial consortium (SFSK4) on the growth and feed utilization efficiency of *Litopenaeus vannamei*

Fig. 13 represents the effect of the test probiotic consortium (SFSK4) on the growth and feed utilization efficiency of *Litopenaeus vannamei.* A significant ($p<0.05$) increase in the average body weight of shrimp treated with the test probiotic (17.93 ± 1.78 g) was observed as compared to the control tank shrimp (14.01 ± 1.04 g). No significant difference ($p>0.05$) in shrimp body weight was observed between the shrimp treated with the commercial probiotic (15.85± 0.92 g) and the control and the shrimp treated with the commercial probiotic and the test probiotic (Fig. 13 A).

A steady increase in the weight gain of shrimp in all the three treatment groups was observed, with a significantly higher ($p<0.05$) weight gain in shrimp treated with test probiotic as compared to control (Fig. 13 B).

The specific growth rate (SGR) showed a gradual decrease over a period of 120 days across all the treatment groups. However, the SGR was significantly higher ($p<0.05$) in the test probiotic treated shrimp as compared to control at 30, 60 and 90 analysis, while at 120 day analysis no significant difference was observed between the different treatment groups (Fig. 14 A).

The feed efficiency (FE) was found to be significantly ($p<0.05$) higher in the test probiotic treated shrimp at 60, 90 and 120 day analysis as compared to the control tank shrimp. At 60 day and 90 days, it was observed that the feed efficiency of the test probiotic treated shrimp was also significantly higher ($p<0.05$) as compared to the commercial probiotic (Fig. 14 B).

The percentage of shrimp survival showed a gradual decrease in all the treatment groups as the number of days increased. No significant difference ($p>0.05$) was observed between the different treatment groups over a period of 120 days (Fig. 15).

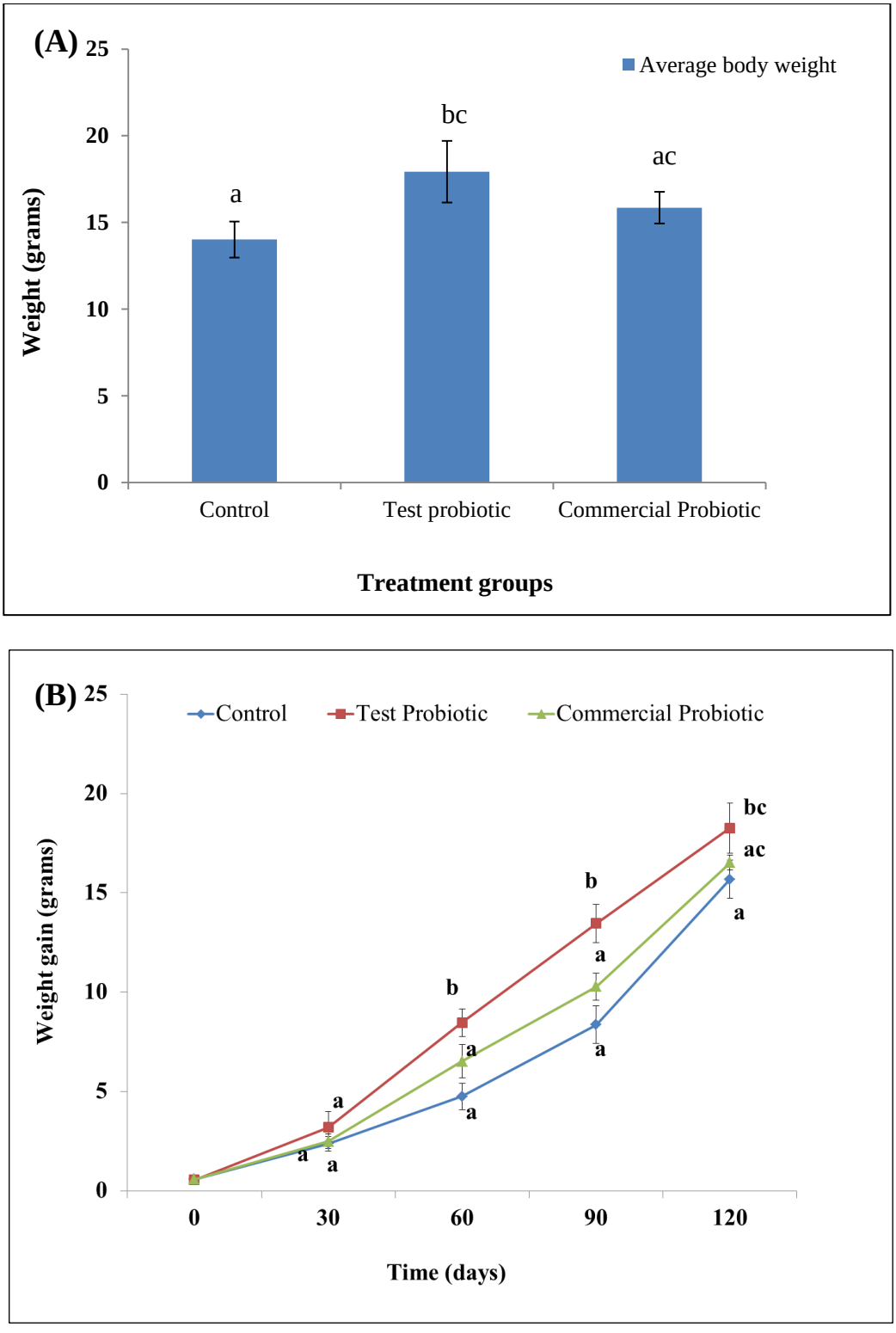


Fig. 13: Effect of the bacterial consortium on (A) Average body weight and (B) weight gain of *Litopenaeus vannamei.*

Each value is the mean ± SD of the three replicates (n = 3). Different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test)

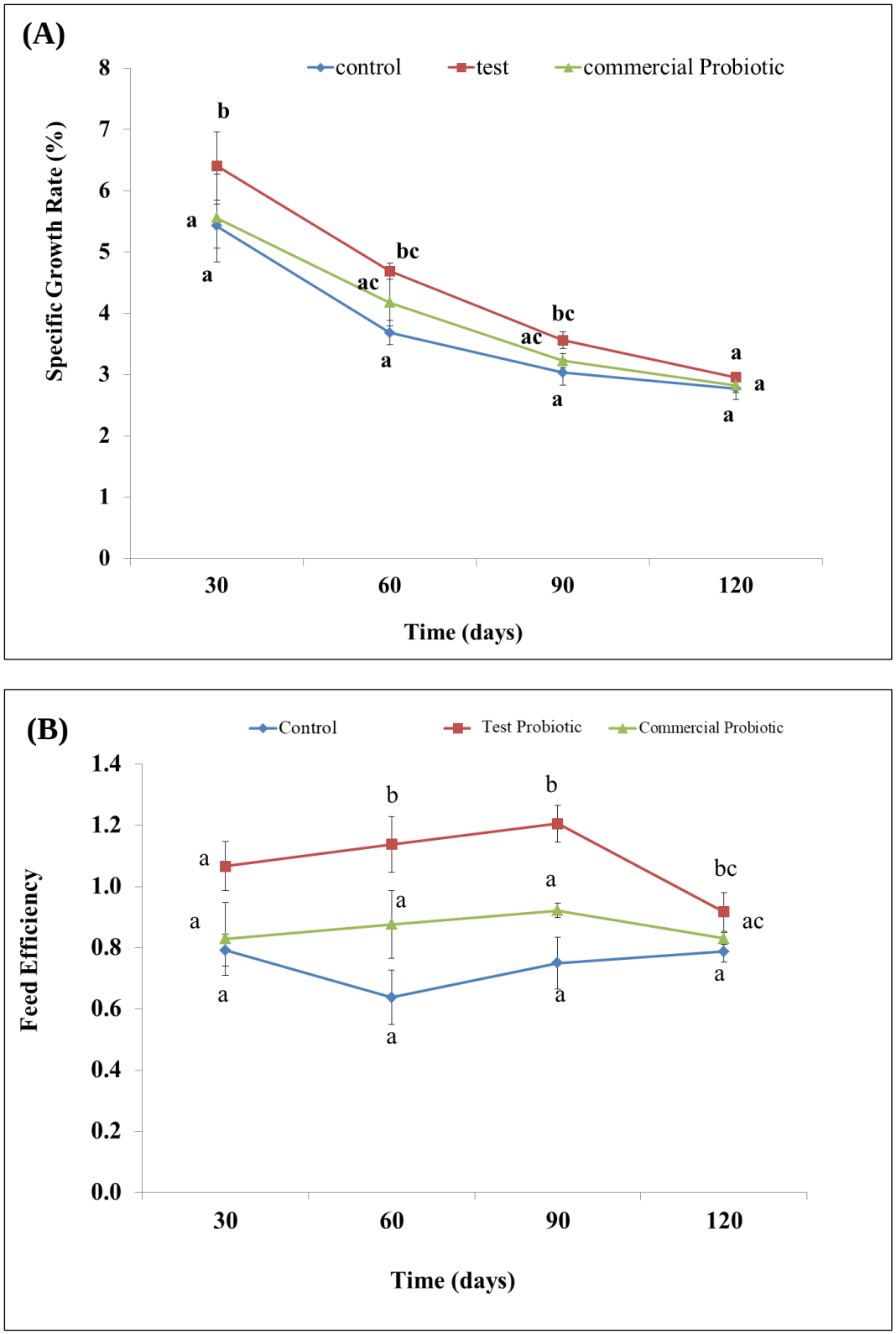


Fig. 14: Effect of the bacterial consortium on (A) Specific growth rate and (B) Feed Efficiency of *Litopenaeus vannamei.*

Each value is the mean ± SD of the three replicates (n = 3). Different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test)

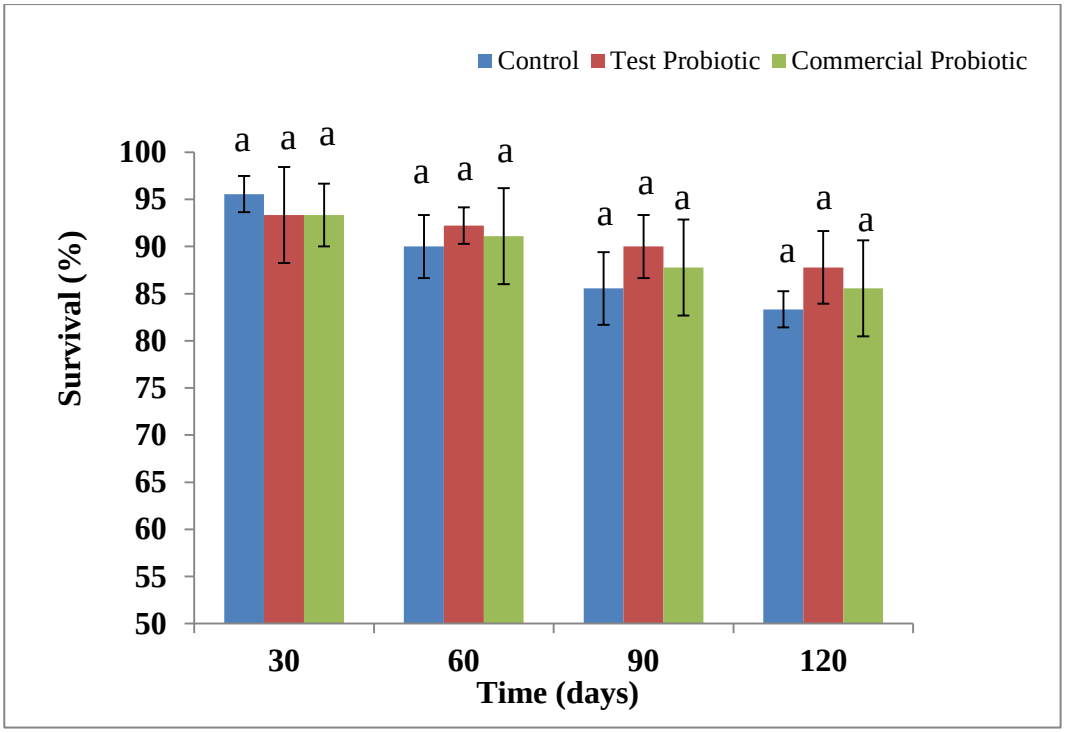


Fig. 15: Effect of the bacterial consortium on survival of *Litopenaeus vannamei.*

Each value is the mean ± SD of the three replicates (n = 3). Different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test)

4.9.3 Effect of the Halotolerant bacterial consortium on the immune response of *Litopenaeus vannamei*

4.9.3.1 Total Hemocyte count

Fig. 16 represents the effect of the test probiotic consortium on the total hemocyte count of *Litopenaeus vannamei* in comparison with the control and commercial probiotic treated shrimp. An increase in the total hemocyte count was observed across all the treatment groups as the number of days increased. The THC of the test probiotic and the commercial probiotic was significantly higher ($p<0.05$) as compared to the control tank shrimp on 60, 90 and 120 day analysis. No significant difference in the THC of the test probiotic and commercial probiotic treated shrimp was observed during the entire trial period.

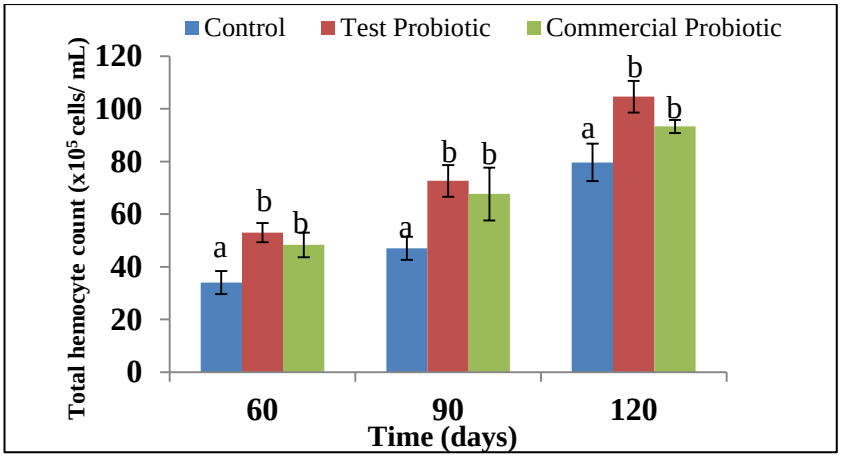


Fig. 16: Effect of the test probiotic consortium on the total hemocyte count of *Litopenaeus vannamei.*

Each value is the mean ± SD of the three replicates (n = 3). Different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test)

4.9.3.2 Phagocytic activity

An increase in the phagocytic activity of *Litopenaeus vannamei* was observed in all the treatment groups over a period of 120 days. The percent phagocytosis of the test probiotic treated shrimp was significantly higher ($p<0.05$) than the control on 60, 90 and 120 day analysis and significantly higher than the commercial probiotic on 120 day analysis (Fig. 17).

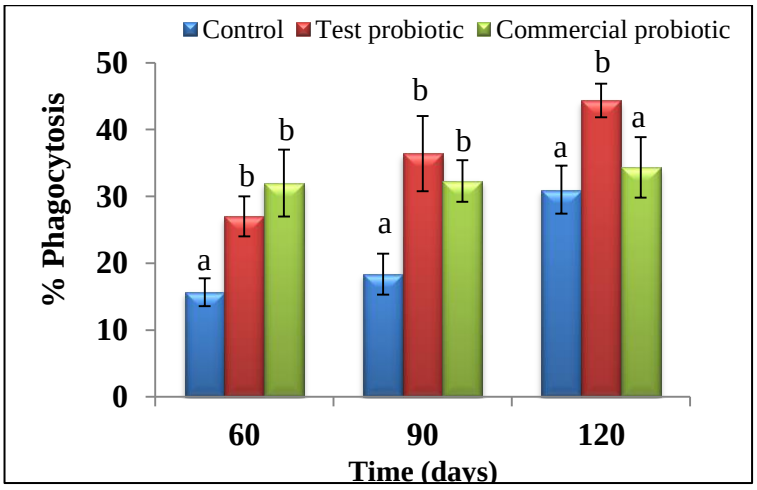


Fig. 17: Effect of the test probiotic consortium on the phagocytic activity of *Litopenaeus vannamei.*

4.9.3.3 Phenoloxidase activity

The phenoloxidase (PO) activity in *Litopenaeus vannamei* showed an increasing trend as the number of days increased (Fig. 18). The PO in shrimp treated with the test probiotic was significantly higher ($p<0.05$) than the control at 60, 90 and 120 day analysis, and significantly higher than the commercial probiotic at 90 and 120 day analysis. No significant difference ($p>0.05$) was observed between the shrimp treated with the commercial probiotic and the control.

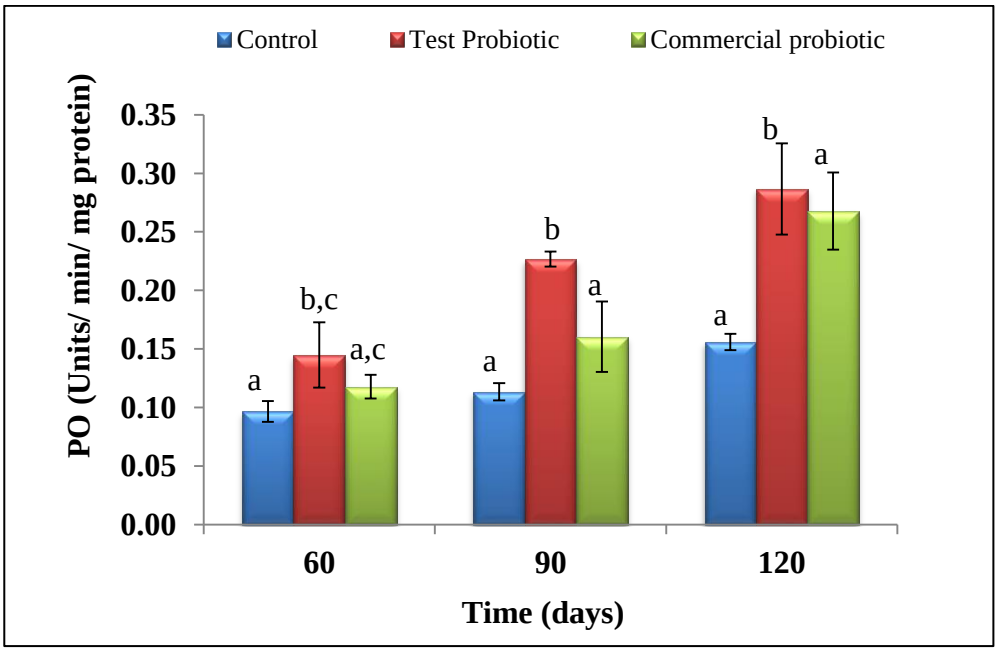


Fig. 18: Effect of the test probiotic consortium on the phenoloxidase activity of *Litopenaeus vannamei.*

Each value is the mean ± SD of the three replicates (n = 3). Different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test)

4.9.3.4 Total Plasma protein

Fig. 19 represents the effect of the test probiotic consortium in comparison with the control and commercial probiotic treated shrimp on the total plasma protein of *Litopenaeus vannamei.* A significant (p<0.05) increase in the total plasma protein of shrimp treated with the test probiotic and the commercial probiotic was observed at 90 and 120 day analysis. No significant difference (p>0.05) was observed in the test and commercial probiotic treated shrimp.

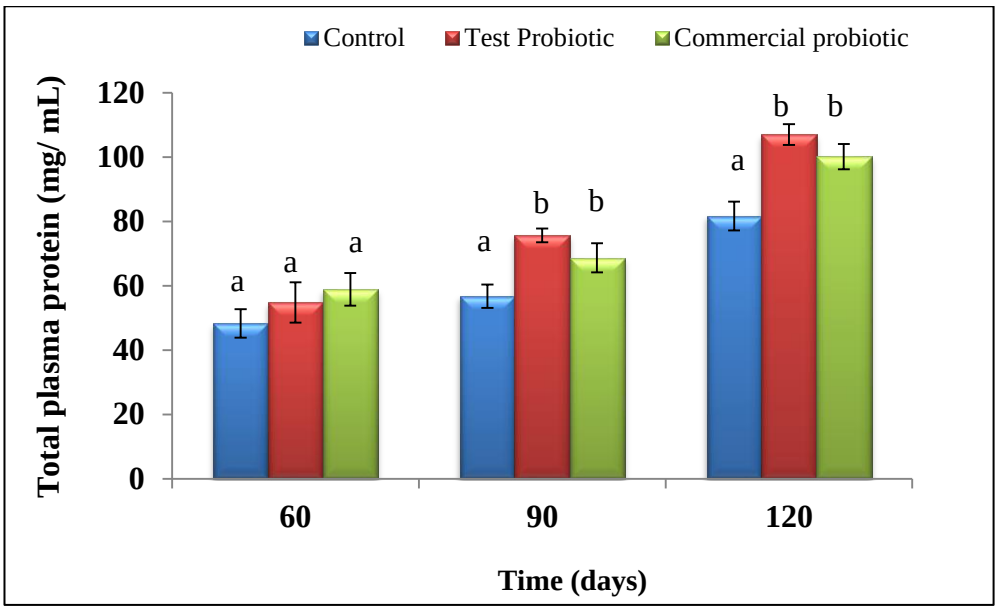


Fig. 19: Effect of the test probiotic consortium on the total plasma protein of *Litopenaeus vannamei*.

Each value is the mean ± SD of the three replicates (n = 3). Different superscript letters are statistically significant (ANOVA; p < 0.05 and subsequent post hoc multiple comparison with Tukey HSD test)

4.9.3.5 Respiratory burst

An increase in the respiratory burst (RB) was observed across all the treatment groups as the number of days increased. The RB in the test probiotic was significantly higher (p<0.05) than the control at 60 day analysis, however, no significant difference was observed at 90 and 120 day analysis across the different treatment groups (Fig 20).

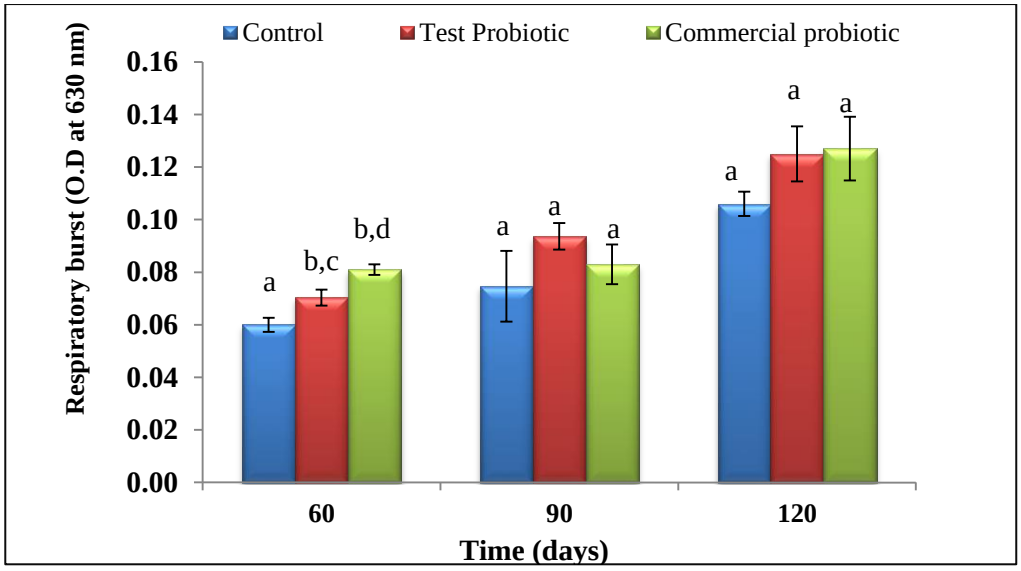


Fig. 20: Effect of the test probiotic consortium on respiratory burst of *Litopenaeus vannamei*.

Each value is the mean ± SD of the three replicates (n = 3). Different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test)

4.9.3.6 Bacterial clearance efficiency

The bacterial clearance efficiency against *V. alginolyticus* and *V. harveyi* was significantly higher ($p<0.05$) in the shrimp treated with the test probiotic as compared to control (Fig. 21).

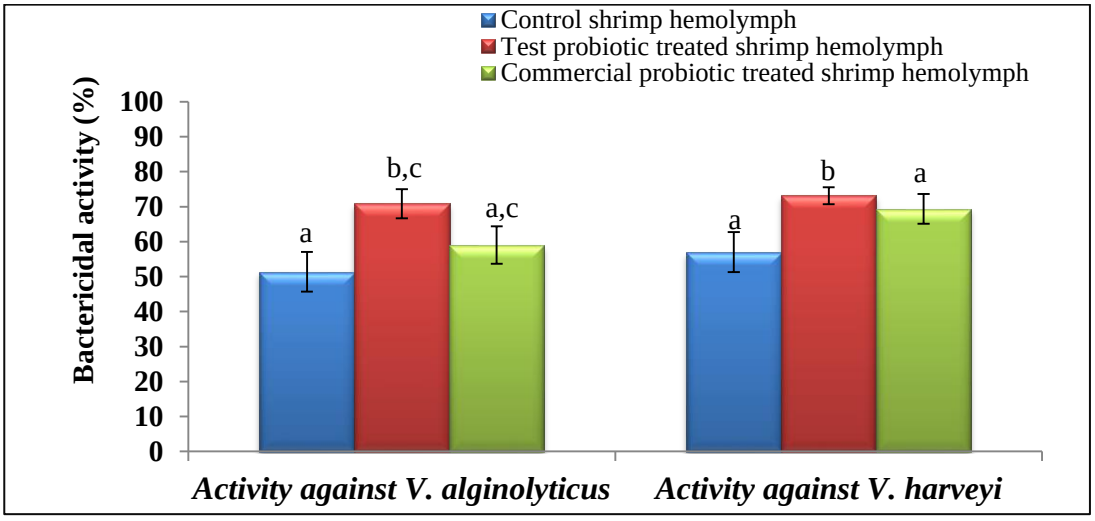


Fig. 21: Effect of the test probiotic consortium on the bacterial clearance efficiency of *Litopenaeus vannamei*

4.9.4 Analysis of anti-*Vibrio* activity *in vivo*

4.9.4.1 Pathogen challenge test

Fig. 22 represents the anti-*Vibrio* activity of the test probiotic consortium against *V. alginolyticus and V. harveyi* for 168 h. When challenged with *V. alginolyticus*, the test probiotic treated shrimp tanks showed a significant ($p<0.01$) decrease in shrimp mortality by 50 % as compared to the tank challenged only with *V. alginolyticus* (Fig. 22 A). Similar results were observed in shrimp tanks challenged with *V. harveyi*, where more than 53 % decrease in shrimp mortality was seen when treated with test probiotic as compared to the tanks challenged only with *V.harveyi* (Fig 22 B). The control (unchallenged) shrimp tanks and the tanks treated only with the test probiotic showed less than 3 % shrimp mortality in 168 h. The results confirmed the anti-*Vibrio* activity of the halotolerant bacteria in *Litopenaeus vannamei.*

4.9.4.2 Histopathological analysis of shrimp exposed to *Vibrio* pathogens

Plate 11 represents the histology of the hepatopancreas of *Litopenaeus vannamei* exposed to (A) Control, (B) Test probiotic consortium, (C) *V. harveyi* (D) *V. harveyi* with test probiotic consortium, (E) *V. alginolyticus*, (F) *V. alginolyticus* with test probiotic consortium. As seen in the image, the hepatopancreatic cells of the control tank shrimp and the shrimp treated with the test probiotic consortium showed intact cell membrane and nucleus (Plate 11 A and B). However, in the hepatopancreatic cells of shrimp treated with *V. harveyi* (Plate 11 C) and *V. alginolyticus* (Plate 11 E) a loss of membrane integrity and necrosis were observed. Interestingly, a comparatively intact membrane and lesser tissue disruption was observed in the shrimp cells challenged with the pathogens along with the test probiotic. The results indicated a probable repair mechanism in the probiotic treated shrimp cells (Plate 11 D and F).

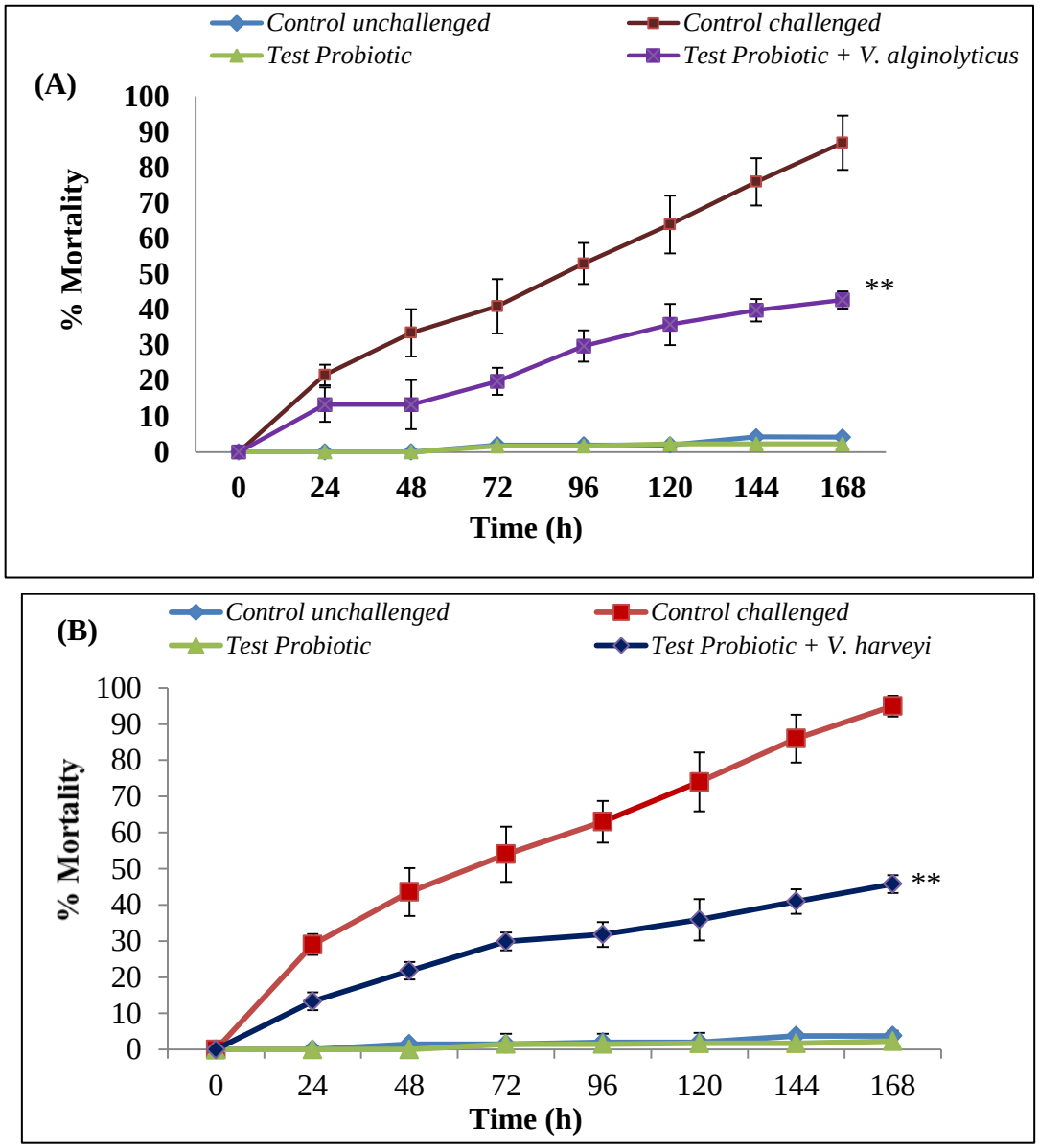


Fig. 22: Pathogen challenge test against (A) *V. alginolyticus* (B) *V. harveyi.*

Each value is the mean ± SD of the three replicates (n = 3). ** indicates the *Test probiotic + Vibrio* tank exhibited significant decrease in mortality compared to control challenged tank. (ANOVA; $p < 0.01$ and subsequent post hoc multiple comparison with Dunnett's test).

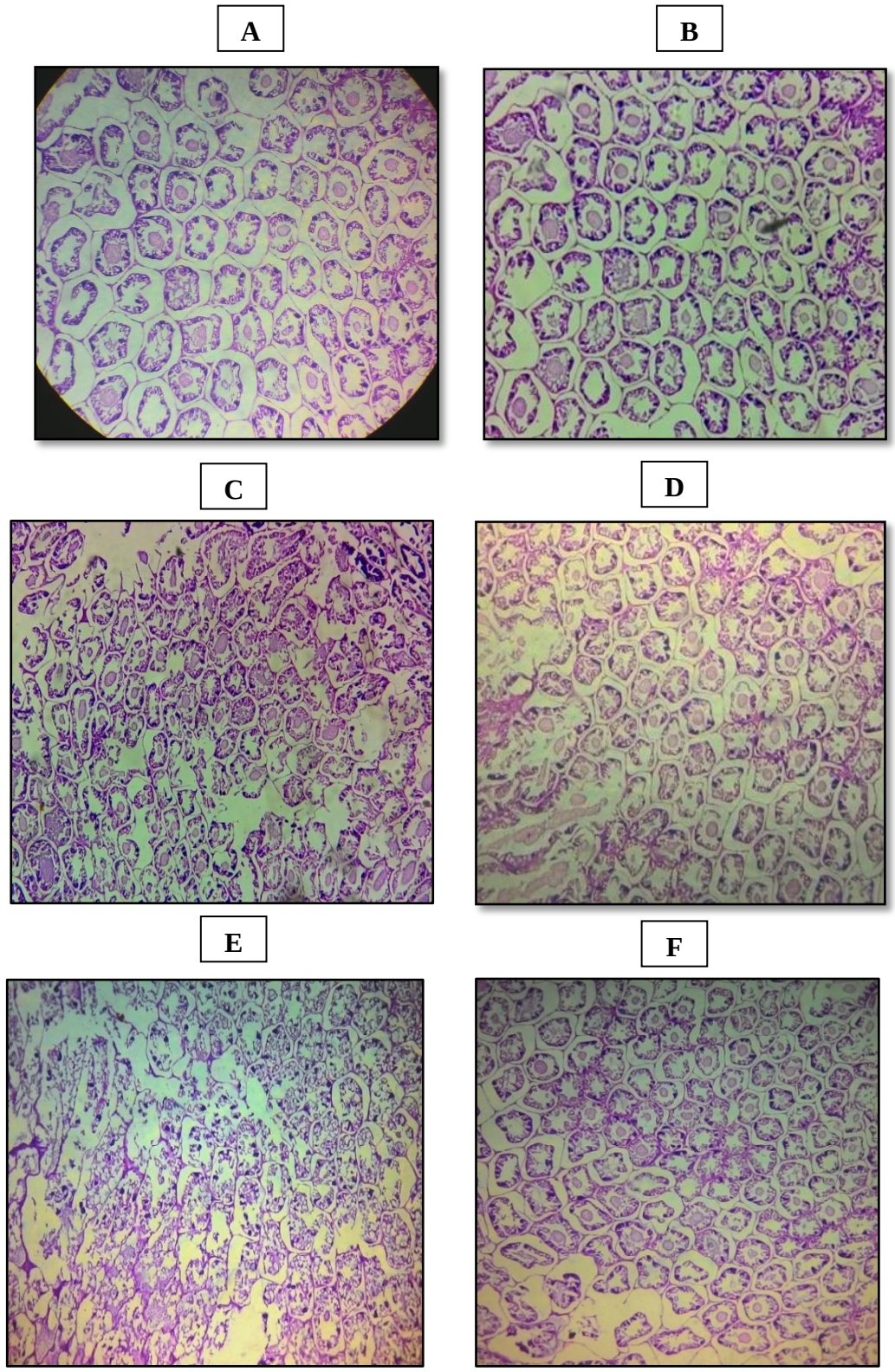


Plate 11: Histology of the hepatopancreas of *Litopenaeus vannamei* exposed to (A) Control, (B) Test probiotic consortium, (C) *V. harveyi* (D) *V. harveyi* + Test probiotic consortium, (E) *V. alginolyticus*, (F) *V. alginolyticus* + Test probiotic consortium.

4.10 Identification of the probiotic bacterial isolates

The potential halotolerant bacteria SK07, SK27, ABSK55 and TSK71 were identified using classical and molecular methods. Plate 12, 13, 14 and 15 illustrate the morphology of the respective bacterial colony on agar plate along with the bacterial morphology by SEM analysis. Table 15 further describes the morphological and biochemical characteristics of each individual culture. Subsequently the biochemical characterisation was confirmed by BIOLOG plates which briefly outlined the substrates utilized, bacterial tolerance to NaCl and pH concentrations and the reaction to antibiotics (Table 16 to 23).

Table 24 describes the molecular identification details of the respective bacteria. Salt pan bacteria SK27 (1428 bp) showed 99 % nBLAST similarity with *Bacillus amyloliquefaciens* subsp. plantarum strain FZB42. SK07 (1417 bp) showed 99 % similarity with *Bacillus subtilis* subsp. spizzenii strain NBRC. TSK71 (1421 bp) showed 99 % similarity to *Bacillus licheniformis* strain DSM 13 in NCBI database. ABSK55 (1403 bp) showed 99 % similarity to *Pseudomonas chengduensis* strain MBR and *Pseudomonas alcaliphila* strain NBRC 102411. These sequences have been submitted to GenBank database under accession numbers MF599413 (SK27), MF599414 (SK07), MF599415 (ABSK55) and MF593892 (TSK71). The taxonomic position of the four salt pan bacteria in the phylogenetic tree along with their closest relatives is illustrated in Fig. 23. The figure validates the results of identification as the bootstrap values were found to be close to 100. Neighbor-Joining method was used to infer the evolutionary history of the respective cultures. The percentage of replicates in which the related taxa clustered together in the bootstrap test (1000 replicates) are depicted next to the nodes. The tree has been drawn to scale, using the same units of branch lengths as that of the evolutionary distances used to deduce the phylogenetic tree.

Plate 12: Colony morphology and scanning electron micrograph of SK07.

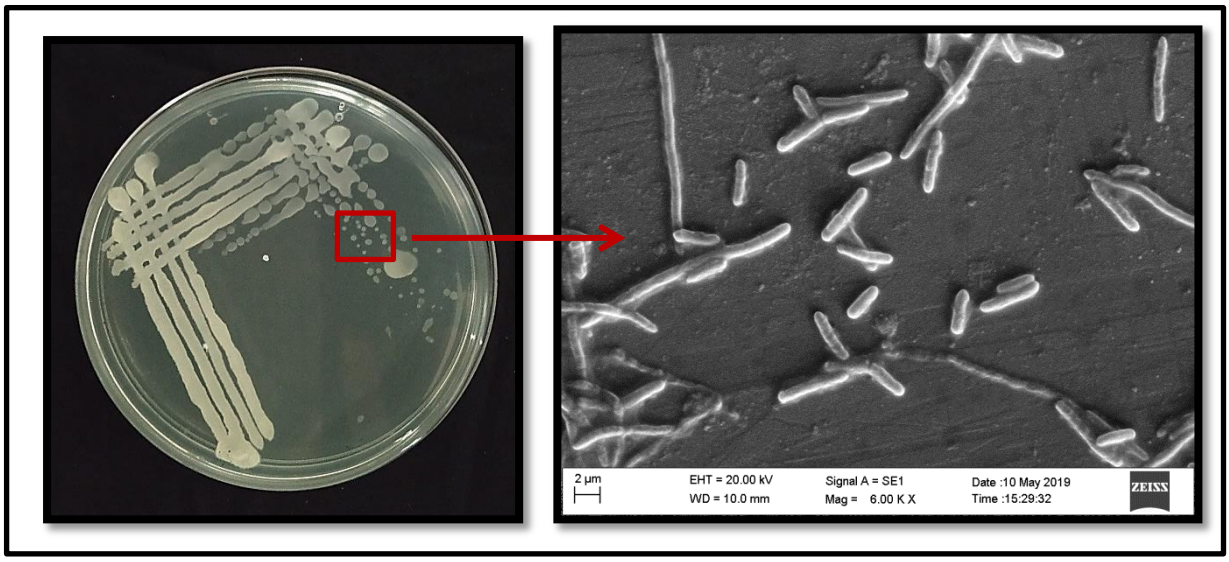


Plate 13: Colony morphology and scanning electron micrograph of SK27.

Plate 14: Colony morphology and scanning electron micrograph of ABSK55.

Plate 15: Colony morphology and scanning electron micrograph of TSK71.

Table 15: Morphological and biochemical characteristics of the selected isolates.

	SK07	SK27	ABSK55	TSK71
Gram character	Gram positive	Gram positive	Gram negative	Gram positive
Shape	Rods	Rods	Rods	Rods
Average size	1.906 µm x 0.692 µm	3.539 µm x 0.906 µm	1.656 µm x 0.492 µm	1.898 µm x 0.557 µm
Catalase	+	+	+	+
Oxidase	+	+	+	±
Endospore	sub-terminal	central	absent	central
Motility	Motile	Motile	Motile	Motile
Carbohydrates utilized	Cellobiose, Fructose, Glucose, Maltose, Mannitol, Mannose, Salicin, Sucrose, Trehalose, ONPG, Esculin	Dextrose, Sucrose, Mannose, Glycerol, Esculin, Mannitol, Cellobiose, Trehalose, Raffinose, Sorbitol, Fructose	Fructose, Glycerol, Sorbitol, Adonitol, Sorbose, Citrate,	Lactose, Xylose, Maltose, Fructose, Dextrose, Raffinose, Trehalose,Sucrose, L-Arabinose, Mannose, Inulin, Na gluconate, Glycerol, Salicin, Sorbitol, Mannitol, Adonitol, Erythritol, Cellobiose, ONPG, Esculin, D-Arabinose
H2S production	+	-	-	-
Nitrate reduction	+	+	-	+
Voges proskauer	-	+	-	+
Arginine utilization	-	-	+	+
Ornithine utilization	-	-	-	-
Lysine utilization	-	-	-	-
Urease	+	-	+	-
Phenylalanine deaminase	-	-	-	-

Legend: '-' activity absent, '+' activity present

Table 16: Metabolic profile of SK07 on BIOLOG GEN III MicroPlateTM.

1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 -D-Lactose	B3 D-Melibiose	B4 -Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl- -D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 -D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 -Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 -Amino-Butryric Acid	H3 -Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 -Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Positive reactions are highlighted in yellow.

Table 17: Metabolic profile of SK07 on BIOLOG GP2 MicroPlateTM.

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-β-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-Inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl-D-Galactoside	C10 β-Methyl-D-Galactoside	C11 3-Methyl Glucose	C12 α-Methyl-D-Glucoside
D1 β-Methyl-D-Glucoside	D2 α-Methyl-D -Mannoside	D3 Palatinose	D4 D-Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxybutyric Acid	E8 β-Hydroxybutyric Acid	E9 γ-Hydroxybutyric Acid	E10 p-Hydroxy-Phenylacetic Acid	E11 α-Ketoglutaric Acid	E12 α-Ketovaleric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Pyruvatic Acid Methyl Ester	F7 Succinic Acid Mono- methyl Ester	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl - L-Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 D-Fructose-6-Phosphate	H10 α-D-Glucose-1-Phosphate	H11 D-Glucose-6-Phosphate	H12 D-L-α-Glycerol Phosphate

Positive reactions are highlighted in yellow.

Table 18: Metabolic profile of SK27 on BIOLOG GEN III MicroPlateTM.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 -D-Lactose	B3 D-Melibiose	B4 -Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl- -D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 -D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 -Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 -Amino-Butryric Acid	H3 -Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 -Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Positive reactions are highlighted in yellow.

Table 19: Metabolic profile of SK27 on BIOLOG GP2 MicroPlateTM.

A1 Water	**A2** α-Cyclodextrin	**A3** β-Cyclodextrin	**A4** Dextrin	**A5** Glycogen	**A6** Inulin	**A7** Mannan	**A8** Tween 40	**A9** Tween 80	**A10** N-Acetyl-D-Glucosamine	**A11** N-Acetyl-β-D-Mannosamine	**A12** Amygdalin
B1 L-Arabinose	**B2** D-Arabitol	**B3** Arbutin	**B4** D-Cellobiose	**B5** D-Fructose	**B6** L-Fucose	**B7** D-Galactose	**B8** D-Galacturonic Acid	**B9** Gentiobiose	**B10** D-Gluconic Acid	**B11** α-D-Glucose	**B12** m-Inositol
C1 α-D-Lactose	**C2** Lactulose	**C3** Maltose	**C4** Maltotriose	**C5** D-Mannitol	**C6** D-Mannose	**C7** D-Melezitose	**C8** D-Melibiose	**C9** α-Methyl-D-Galactoside	**C10** β-Methyl-D-Galactoside	**C11** 3-Methyl Glucose	**C12** α-Methyl-D-Glucoside
D1 β-Methyl-D-Glucoside	**D2** α-Methyl-D - Mannoside	**D3** Palatinose	**D4** D-Psicose	**D5** D-Raffinose	**D6** L-Rhamnose	**D7** D-Ribose	**D8** Salicin	**D9** Sedoheptulosan	**D10** D-Sorbitol	**D11** Stachyose	**D12** Sucrose
E1 D-Tagatose	**E2** D-Trehalose	**E3** Turanose	**E4** Xylitol	**E5** D-Xylose	**E6** Acetic Acid	**E7** α-Hydroxybutyric Acid	**E8** β-Hydroxybutyric Acid	**E9** γ-Hydroxybutyric Acid	**E10** p-Hydroxy-Phenylacetic Acid	**E11** α-Ketoglutaric Acid	**E12** α-Ketovaleric Acid
F1 Lactamide	**F2** D-Lactic Acid Methyl Ester	**F3** L-Lactic Acid	**F4** D-Malic Acid	**F5** L-Malic Acid	**F6** Pyruvatic Acid Methyl Ester	**F7** Succinic Acid Mono- methyl Ester	**F8** Propionic Acid	**F9** Pyruvic Acid	**F10** Succinamic Acid	**F11** Succinic Acid	**F12** N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	**G2** D-Alanine	**G3** L-Alanine	**G4** L-Alanyl-Glycine	**G5** L-Asparagine	**G6** L-Glutamic Acid	**G7** Glycyl - L-Glutamic Acid	**G8** L-Pyroglutamic Acid	**G9** L-Serine	**G10** Putrescine	**G11** 2,3-Butanediol	**G12** Glycerol
H1 Adenosine	**H2** 2'-Deoxy Adenosine	**H3** Inosine	**H4** Thymidine	**H5** Uridine	**H6** Adenosine-5'-Monophosphate	**H7** Thymidine-5'-Monophosphate	**H8** Uridine-5'-Monophosphate	**H9** D-Fructose-6-Phosphate	**H10** α-D-Glucose-1-Phosphate	**H11** D-Glucose-6-Phosphate	**H12** D-L-α-Glycerol Phosphate

Positive reactions are highlighted in yellow.

Table 20: Metabolic profile of ABSK55 on BIOLOG GEN III MicroPlateTM.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 -D-Lactose	B3 D-Melibiose	B4 -Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl- -D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 -D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 -Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 -Amino-Butyric Acid	H3 -Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 -Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Positive reactions are highlighted in yellow.

Table 21: Metabolic profile of ABSK55 on BIOLOG GN2 MicroPlateTM.

A1 Water	A2 α -Cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-galactosamine	A8 N-Acetyl-D-glucosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α -D-Glucose	B7 m-Inositol	B8 α -D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β -Methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Methyl Pyruvate	C12 Mono-Methyl-Succinate
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α -Hydroxy Butyric Acid	D11 β -Hydroxy Butyric Acid	D12 γ -Hydroxy Butyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α -Keto Butyric Acid	E4 α -Keto Glutaric Acid	E5 α -Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromo Succinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 Glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Ornithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 γ -Amino Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyethylamine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α -Glycerol Phosphate	H11 Glucose-1-Phosphate	H12 Glucose-6-Phosphate

Positive reactions are highlighted in yellow.

Table 22: Metabolic profile of TSK71 on BIOLOG GEN III MicroPlateTM.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 -D-Lactose	B3 D-Melibiose	B4 -Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl- -D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 -D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 -Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 -Amino-Butryric Acid	H3 -Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 -Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Positive reactions are highlighted in yellow.

Table 23: Metabolic profile of TSK71 on BIOLOG GP2 MicroPlateTM.

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-β-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-Inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl-D-Galactoside	C10 β-Methyl-D-Galactoside	C11 3-Methyl Glucose	C12 α-Methyl-D-Glucoside
D1 β-Methyl-D-Glucoside	D2 α-Methyl-D - Mannoside	D3 Palatinose	D4 D-Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxybutyric Acid	E8 β-Hydroxybutyric Acid	E9 γ-Hydroxybutyric Acid	E10 p-Hydroxy-Phenylacetic Acid	E11 α-Ketoglutaric Acid	E12 α-Ketovaleric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Pyruvatic Acid Methyl Ester	F7 Succinic Acid Mono- methyl Ester	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl - L-Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 D-Fructose-6-Phosphate	H10 α-D-Glucose-1-Phosphate	H11 D-Glucose-6-Phosphate	H12 D-L-α-Glycerol Phosphate

Positive reactions are highlighted in yellow.

Table 24: Molecular identification of the selected bacterial isolates.

Sr. no	Culture code	GenBank accession number	Length of the sequence	Closest match	Percent similarity	Culture identification
1.	SK07	MF599414	1417 bp	*Bacillus subtilis* subsp. spizzenii strain NBRC	99 %	*Bacillus subtilis* strain SK07
2.	SK27	MF599413	1428 bp	*Bacillus amyloliquefaciens* subsp. plantarum strain FZB42	99 %	*Bacillus amyloliquefaciens* strain SK27
3.	ABSK55	MF599415	1403 bp	*Pseudomonas chengduensis* strain MBR and *Pseudomonas alcaliphila* strain NBRC 102411.	99 %	*Pseudomonas sp*. strain ABSK55
4.	TSK71	MF593892	1421 bp)	*Bacillus licheniformis* strain DSM 13	99 %	*Bacillus licheniformis* strain TSK71

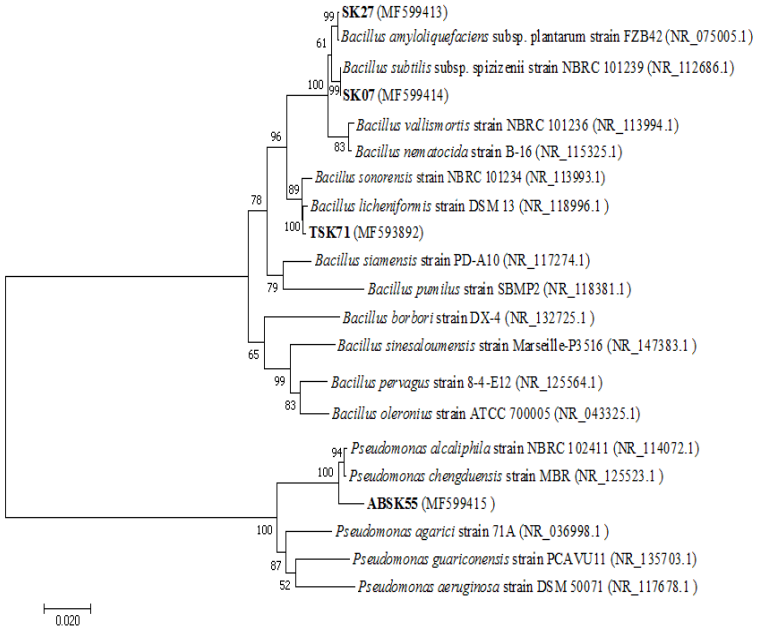


Fig. 23: Phylogenetic tree depicting the relationship of the four halotolerant bacteria with other isolates based on 16S rDNA sequence similarity.

The number at the nodes indicates bootstrap values based on 1000 replicates.

4.11 Safety evaluation of the test probiotic consortium

To ensure the safety of the probiotic under study, certain *in vitro* and *in vivo* toxicity tests were carried out.

4.11.1 Antibiotic susceptibility test

Table 25 represents the sensitivity of the four salt pan bacteria to 24 antibiotics belonging to different groups: cell wall inhibitors (Amoxyclav, Ampicillin, Cephalothin, Cephalexin, Cefotaxime, Penicillin, Vancomycin), protein synthesis inhibitors (Amikacin, Chloramphenicol, Clindamycin, Doxycyclinehydrochloride, Erythromycin, Gentamycin, Kanamycin, Neomycin, Streptomycin, Tetracyclin, Tobramycin), nucleic acid synthesis inhibitors (Ciprofloxacin, Levofloxacin, Co-trimoxazole, Nalidixic acid, Ofloxacin) and Nitrofurantoin, along with the interpretive criteria according to Clinical and laboratory standards institute. The four bacteria were susceptible to antibiotics belonging to all the tested groups. Bacteria SK07 was susceptible to 21 antibiotics, SK27 to 23, ABSK55 to 19 and TSK71 to 21 antibiotics. Plate 16 represents an image of the antibiotic susceptibility result of salt pan bacteria SK27.

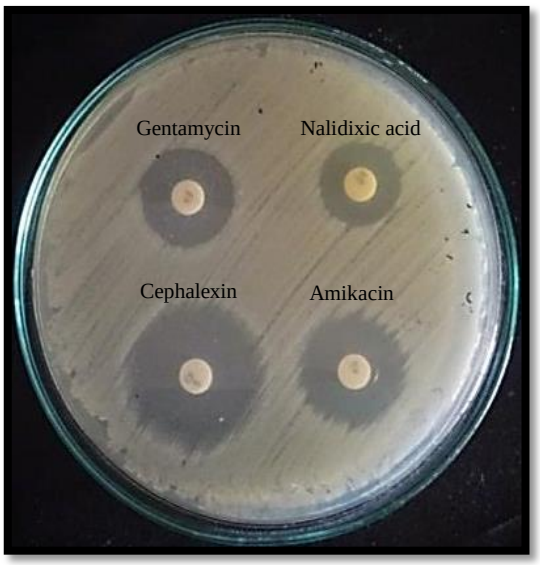


Plate 16: Antibiotic susceptibility test of SK27 by Kirby-Bauer disk diffusion method.

Table 25: Antibiotic susceptibility of the salt pan bacterial strains.

Antibiotic	Zone of inhibition in mm				Interpretive criteria (mm)
	Test cultures				
	SK07	SK27	ABSK55	TSK71	
Inhibitors of cell wall synthesis					
Amoxyclav (30 µg)	18^{S}	42^{S}	18^{S}	21^{S}	S (≥18), I (14 - 17), R (≤13)
Ampicillin (10 µg)	24^{S}	27^{S}	14^{I}	18^{S}	S (≥17), I (14 - 16), R (≤13)
Cephalothin (30 µg)	24^{S}	42^{S}	15^{I}	18^{s}	S (≥18), I (15 - 17), R (≤14)
Cephalexin (30 µg)	26^{S}	40^{S}	21^{S}	15^{I}	S (≥18), I (15 - 17), R (≤14)
Cefotaxime (30 µg)	18^{R}	30^{S}	23^{I}	26^{S}	S (≥26), I (23 - 25), R (≤22)
Penicillin (10 µg)	19^{I}	23^{S}	19^{I}	18^{I}	S (≥22), I (12 - 21), R (≤12)
Vancomycin (30 µg)	19^{S}	24^{S}	17^{S}	21^{S}	S (≥17), I (15 - 16), R (≤14)
Inhibitors of protein synthesis					
Amikacin (30 µg)	24^{S}	30^{S}	23^{S}	32^{S}	S (≥17), I (15 - 16), R (≤14)
Chloramphenicol (30 µg)	25^{S}	31^{S}	22^{S}	26^{S}	S (≥18), I (13 - 17), R (≤12)
Clindamycin (2 µg)	21^{S}	18^{I}	24^{S}	21^{S}	S (≥21), I (15 - 20), R (≤14)
Doxycyclinehydrochloride (30 µg)	32^{S}	32^{S}	20^{S}	29^{S}	S (≥14), I (11 - 13), R (≤10)
Erythromycin (15 µg)	18^{I}	31^{S}	23^{s}	24^{S}	S (≥23), I (14 - 22), R (≤13)
Gentamycin (10 µg)	28^{S}	25^{S}	25^{S}	30^{S}	S (≥15), I (13 - 14), R (≤12)
Kanamycin (30 µg)	23^{S}	31^{S}	20^{S}	25^{S}	S (≥18), I (14 - 17), R (≤13)
Neomycin (30 µg)	20^{S}	18^{S}	19^{S}	20^{S}	S (≥17), I (13 - 16), R (≤12)
Streptomycin (10 µg)	20^{S}	16^{S}	18^{S}	15^{S}	S (≥15), I (12 - 14), R (≤11)
Tetracyclin (30 µg)	27^{S}	30^{S}	21^{S}	29^{S}	S (≥15), I (12 - 14), R (≤11)
Tobramycin (10 µg)	24^{S}	27^{S}	18^{S}	26^{S}	S (≥15), I (13 - 14), R (≤12)
Inhibitors of nucleic acid synthesis					
Ciprofloxacin (5 µg)	34^{S}	36^{S}	27^{S}	37^{S}	S (≥21), I (16 - 20), R (≤15)
Co-trimoxazole (25 µg)	35^{S}	36^{S}	11^{I}	20^{S}	S (≥16), I (11 - 15), R (≤10)
Levofloxacin (5 µg)	28^{S}	31^{S}	30^{S}	34^{S}	S (≥17), I (14 - 17), R (≤13)
Nalidixic acid (30 µg)	20^{S}	22^{S}	19^{S}	21^{S}	S (≥19), I (14 - 18), R (≤13)
Ofloxacin (5 µg)	24^{S}	34^{S}	24^{S}	33^{S}	S (≥16), I (13 - 15), R (≤12)
Other					
Nitrofurantoin (300 µg)	19^{S}	18^{S}	17^{S}	20^{S}	S (≥17), I (15 - 16), R (≤14)

Legend:'S' represents susceptible, 'I' represents intermediate and 'R' represents resistant (according to the Clinical and Laboratory Standards institute, 2013)

4.11.2 Lethal concentration 50

After challenging the shrimp with increasing dosages of the probiotic for 12 days, the shrimp were monitored for behavioural and pigmentation changes. The larvae were observed to be dead when seen to be opaque and immobile. No significant difference (p>0.05) in mortality was observed between the control (untreated) and the probiotic treated tanks up to 12 days of culture (Table 26). A significantly higher (p<0.05) mortality rate was observed in tanks challenged with shrimp pathogen *V. alginolyticus* with more than 50 % mortality after 3 days when challenged with a dose of 1 x 10^6 CFU/mL, and 6 days when challenged with a dose of 1x 10^5 CFU/mL. Moreover, administration of the highest dose of test probiotic (1x 10^{12} CFU/mL) did not show any adverse effect on shrimp survival. These results suggest that the formulated probiotic is avirulent to *Litopenaeus vannamei* post larvae.

Table 26: Percentage of mortality of *Litopenaeus vannamei* when exposed to different concentrations of probiotic consortium and shrimp pathogens.

Experimental tanks	Challenge dose	No. of shrimp	% Mortality			
			3 days	6 days	9 days	12 days
Negative control	-	45	0	0	8.89 ± 3.85^a	13.33 ± 6.67^a
Probiotic Dose 1	1x 10^8	45	0	0	6.67 ± 0.00^a	8.89 ± 3.85^a
Probiotic Dose 2	1x 10^9	45	0	0	4.44 ± 3.85^a	6.67 ± 0.00^a
Probiotic Dose 3	1x 10^{10}	45	0	0	2.22 ± 3.85^a	6.67 ± 3.85^a
Probiotic Dose 4	1x 10^{11}	45	0	0	4.44 ± 3.85^a	13.33 ± 3.85^a
Probiotic Dose 5	1x 10^{12}	45	0	0	6.66 ± 0.00^a	8.89 ± 3.85^a
Positive Control 1 (*V. alginolyticus*)	1x 10^5	45	33.33 ± 6.67	57.77 ± 3.85	73.33 ± 6.67^b	80 ± 6.67^b
Positive Control 2 (*V. alginolyticus*)	1x 10^6	45	51.11 ± 3.85	93.33 ± 6.67	95.56 ± 3.85^c	100.00 ± 0^c

Each value is the mean ± SD of three replicates. Different superscript letters are statistically significant (ANOVA, p<0.05 and subsequent post hoc multiple comparison with Tukeys HSD test).

4.11.3 Cytotoxicity evaluation

The cellular viability of the post larvae (PL-20) and juvenile shrimp gut, hepatopancreas and haemolymph are given in Table 27. No significant difference (p>0.05) in the cellular viability was observed in post larvae exposed to test probiotic at a dosage of 10^{12} CFU/mL as compared to the negative control. A significant increase (p<0.05) in the viability of hepatopancreatic and stomach cells was observed in shrimp treated with probiotic at a dose of 10^{10} CFU/mL as compared to the negative control. In contrast, the shrimp treated with positive controls viz. *Vibrio alginolyticus* and ethyl methanesulfonate (EMS) exhibited a significant decrease in cell viability as compared to the control and probiotic treated shrimp.

Table 27: Cellular percentage viability of postlarvae (PL-20) and juvenile shrimp exposed to probiotic SFSK4 and shrimp pathogen *Vibrio alginolyticus.*

TREATMENTS	Percentage Viability (%)			
	Post larvae	**Juvenile hepatopancreas**	**Juvenile stomach**	**Juvenile haemolymph**
Negative Control	72.33 ± 1.52^{ab}	55.0 ± 3.60^{a}	63.0 ± 4.0^{a}	62.33 ± 3.51^{a}
Probiotic dose 1: 10^{10}CFU/mL	75.33 ± 1.52^{b}	66.0 ± 3.0^{b}	76.0 ± 4.0^{b}	65.33 ± 7.50^{a}
Probiotic dose 2: 10^{12}CFU/mL	70 ± 2^{a}	55.66 ± 2.51^{a}	69.0 ± 3.0^{ab}	60.33 ± 3.51^{a}
*Vibrio alginolyticus (*1x 10^{6})	36.33 ± 0.57^{c}	32.0 ± 4.0^{c}	42.33 ± 5.5^{c}	36.33 ± 6.5^{b}
Ehyl methane sulfonate	-	30.33 ± 2.08^{c}	35.33 ± 1.5^{c}	23.0 ± 2^{c}

Each value is the mean ± SD of three replicates. Different superscript letters are statistically significant (ANOVA, p<0.05 and subsequent post hoc multiple comparison with Tukeys HSD test).

4.11.4 Evaluation of Genotoxicity

Plate 17 (A and B) represents the % tail DNA in the hepatopancreatic cells of *Litopenaeus vannamei* juveniles after exposure to the test probiotic SFSK4 and ethyl methane sulphonate (EMS) reagent. As seen in the figure, the hepatopancreatic cells exposed to EMS showed the presence of comets, indicating DNA damage in the nuclei (Plate 17 A). However, no comets were observed in shrimp cells treated with probiotic SFSK4 at a concentration of $1x10^{10}$ and $1x10^{12}$ CFU/mL, signifying no DNA damage (Plate 17 B).

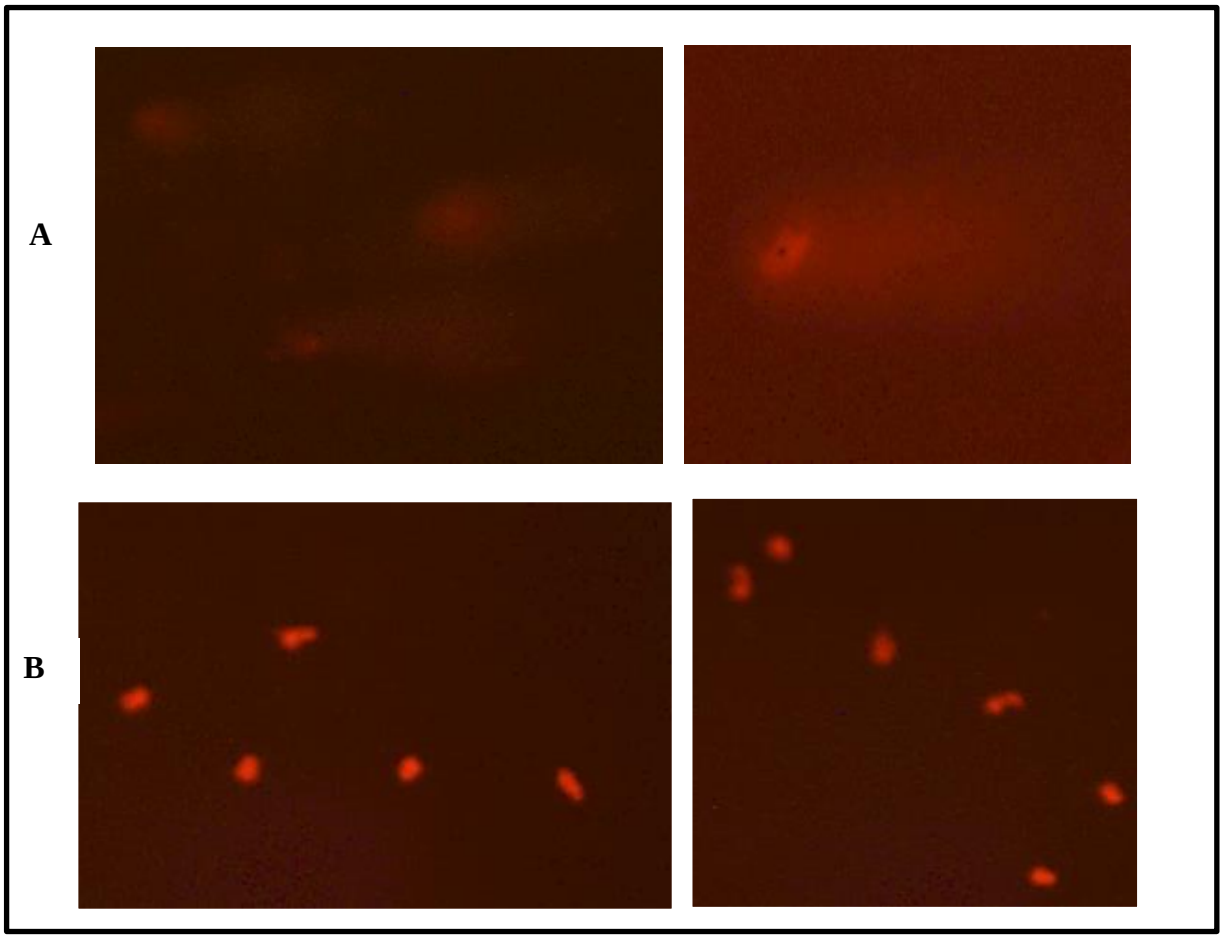


Plate 17: (A) Appearance of comets in the hepatopancreatic cells of shrimp exposed to EMS. (B) Hepatopancreatic cells exposed to high dose of test probiotic at 10^{10} and 10^{12} CFU/mL showing absence of comets.

4.12 Farm trial of the test probiotic consortium SFSK4 on *Litopenaeus vannamei*

Once the beneficial effect of the test probiotic SFSK4 was confirmed on *Litopenaeus vannamei* and its safety to the host was ensured in the laboratory, the formulated probiotic was mass cultured and tested for its efficacy in a shrimp aquaculture farm environment.

4.12.1 Effect of the test probiotic consortium SFSK4 on the water quality of *Litopenaeus vannamei* tanks

Table 28 represents the average physico-chemical and microbial parameters of *Litopenaeus vannamei* tanks after treatment with SFSK4. As seen in the table, the pH of the tanks ranged from 7.61 ± 0.25 to 7.82 ± 0.33, temperature from 27.16 ± 1.76 to 27.8 ± 1.87 ºC, Total dissolved solids from 47.65 ± 1.00 to 48.45 ± 0.57 ppt and salinities from 33.86 ± 3.09 to 33.95 ± 3.16 psu. The dissolved oxygen in the tanks was between 4.82 ± 0.59 mg/L to 5.04 ± 0.69 mg/L and the biological oxygen demand was in the range of 9.75 ± 0.99 to 13.07 ± 0.36. The ammonia concentration between the different treatment sets was within the range of 0.09 ± 0.02 to 0.22 ± 0.02 mg/L. The Nitrite concentration was found to be between 0.16 ± 0.04 to 0.20 ± 0.03 mg/L and nitrate between 0.62 ± 0.013 to 0.73 ± 0.15 mg/L. The total Alkalinity was in the range of 85.28 ± 5.07 to 89.16 ± 3.49 mg/L. No significant (p>0.05) difference in the physico-chemical parameters was observed among the different treatment sets. The Total culturable bacterial count (TCB) in the shrimp tanks ranged from 10.03 ± 0.08 to 10.16 ± 0.04 Log CFU/mL.A significant *(p=0.013, F=9.657)* ten-fold decrease in the total *Vibrio* count was observed in shrimp treated with SFSK4 (5.63 ± 0.28 Log CFU/mL) as compared to the control (6.27±0.11 Log CFU/mL) and commercial probiotic treated shrimp tanks (6.03 ± 0.08 Log CFU/mL). The decrease in the total *Vibrio* count indicates the anti-*Vibrio* activity of the consortium in *Litopenaeus vannamei in vivo.*

Table 28: Physico-chemical and microbial parameters of *Litopenaeus vannamei* aquaculture tanks.

Parameter	Control	Test Probiotic SFSK4	Commercial Probiotic	*p* value	*F* value
pH	7.82±0.33	7.61±0.25	7.73±0.27	0.662	0.442
Temp (°C)	27.8±1.87	27.53±1.85	27.16±1.76	0.915	0.091
TDS (ppt)	47.65±1.00	48.35±0.46	48.45±0.57	0.387	1.118
Salinity (psu)	33.86±3.29	33.95±3.16	33.86±3.09	0.999	0.001
DO (mg/L)	5.04±0.69	4.98±0.73	4.82±0.59	0.635	0.491
BOD (mg/L)	12.13±1.82	9.75±0.99	13.07±0.36	0.635	0.491
Ammonia (mg/L)	0.22±0.02	0.09±0.02	0.13±0.07	0.141	2.638
Nitrite (mg/L)	0.20±0.03	0.16±0.04	0.19±0.12	0.178	2.231
Nitrate (mg/L)	0.62±0.013	0.73±0.15	0.62±0.20	0.459	0.873
Alkalinity (mg/L)	88.07±9.97	89.16±3.49	85.28±5.07	0.778	0.262
TCB (Log CFU/mL)	10.16±0.04	10.03±0.08	10.05±0.08	0.115	0.392
TVC (Log CFU/mL)	6.27±0.11	**5.63±0.28***	6.03±0.08	0.013	9.657

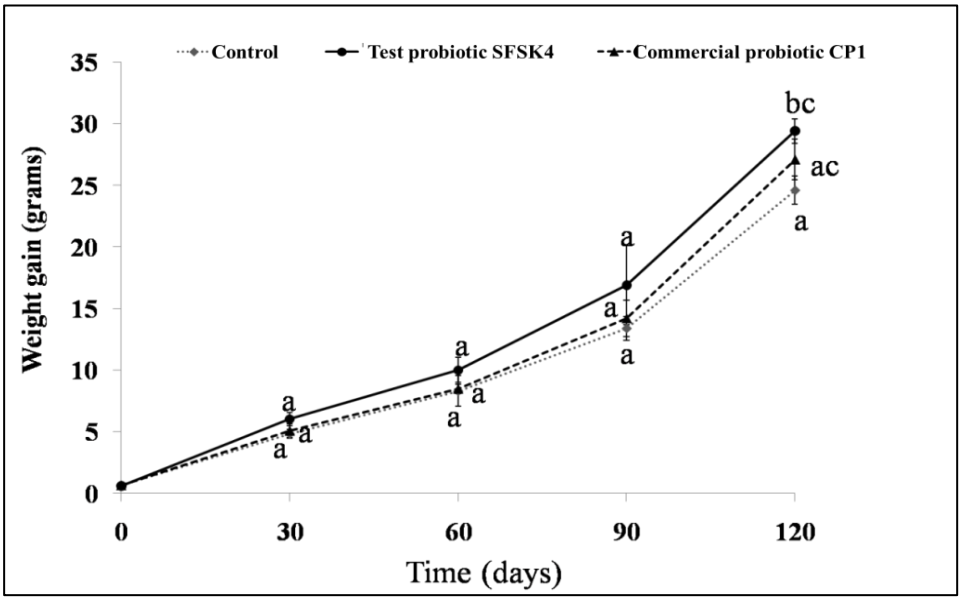
* and ** indicate statistically significant from control (ANOVA; $p < 0.05$, $p < 0.01$ and subsequent post hoc multiple comparison with Dunnett's test).

4.12.2 Effect of the test probiotic consortium SFSK4 on the growth and feed utilization efficiency of *Litopenaeus vannamei*

The final weight gain in shrimp treated with the test probiotic SFSK4 (29.39 ± 0.98 g) was significantly higher (p = 0.012, F = 10.278) than that of the control shrimp (24.59 ± 1.15 g). Shrimp treated with commercial probiotic showed no significant difference in body weight (27.09 ± 1.67 g) compared to the test probiotic SFSK4 (p = 0.154) and control (p = 0.122) at the end of the experiment (Fig. 24 A). The FE of test probiotic was significantly higher (p = 0.005, F = 20.120) as compared to control. No significant difference in FE was observed between commercial probiotic and control (p = 0.072) and between commercial probiotic and test probiotic treated shrimp (p = 0.053) (Fig. 24 B).

The specific growth rate was found to be significantly higher by 1.53 % in the test probiotic treated shrimp (p = 0.01) and by 1 % in the commercial probiotic treated shrimp (p = 0.030) at 30 days compared to control. No significant difference (p > 0.05) was observed in the SGR between the treatments at 60, 90, and 120 days (Fig. 25 A). A decrease in the survival rate was observed in all three treatment groups over a period of 120 days, with no significant difference (p = 0.422) across the treatments (Fig. 25 B). No significant difference in yield was observed between control and commercial probiotic (1056.57 ± 84.84 g/m3) (p = 0.293) and between test probiotic and commercial probiotic treated shrimp (p = 0.268). However, the total yield at the end of the experimental trial was significantly higher (p = 0.040, F = 5.784) in the test probiotic SFSK4 treated shrimp (1160.33 ± 59.43 g/m3) as compared to control (957.17 ± 73.01 g/m3) (Fig. 26). The results further confirmed that the test probiotic consortium SFSK4 demonstrated a better synergistic performance with 21.2 % increase in yield as compared to control (untreated) shrimp. Plate 18 represents the size and growth of *Litopenaeus vannamei* from pL12 to 120 days of farm trial.

A



B

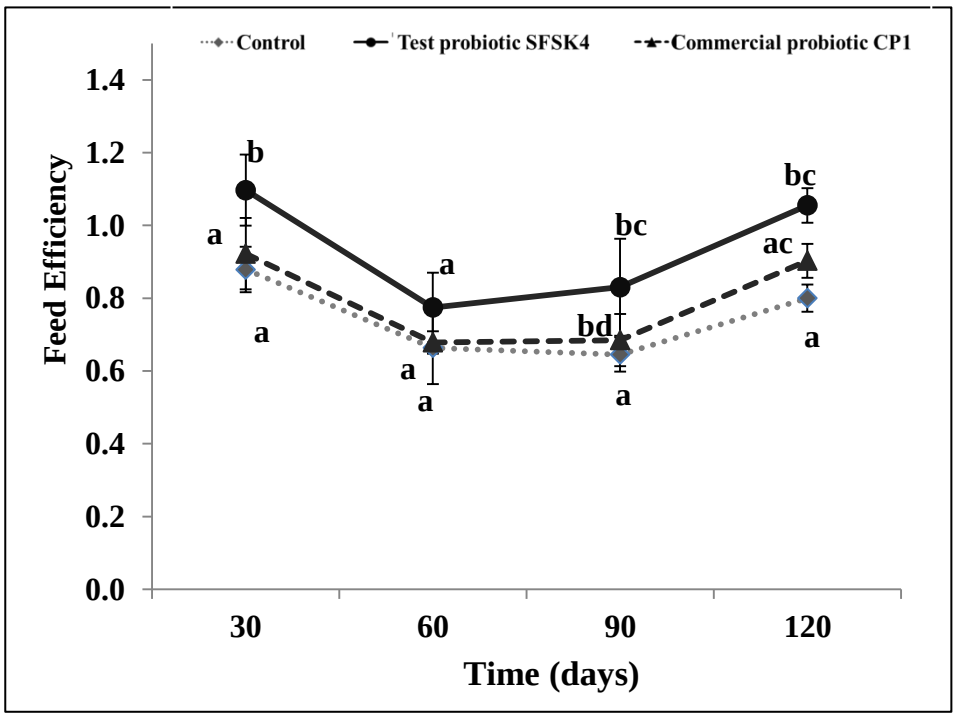
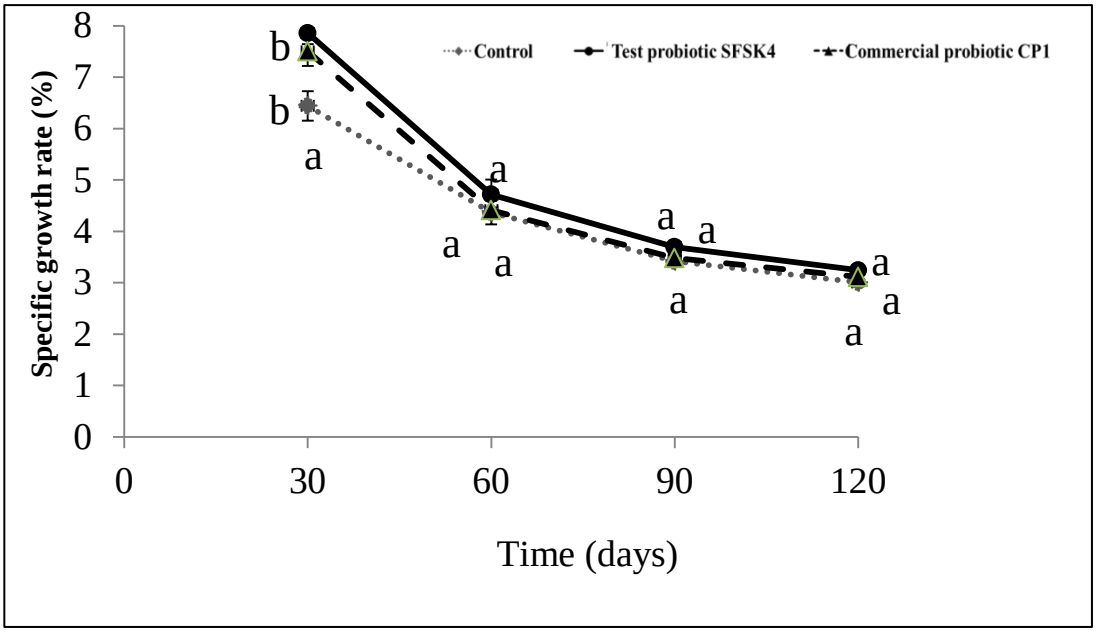


Fig. 24: (A) Weight gain, (B) Feed efficiency of *Litopenaeus vannamei* fed without bacteria (control), with test probiotic (SFSK4) and commercial probiotic (CP1) for 120 days.

Each value is the mean ± SD of three replicates. Different superscript letters are statistically significant (ANOVA, $p<0.05$ and subsequent post hoc multiple comparison with Tukeys HSD test).

A



B

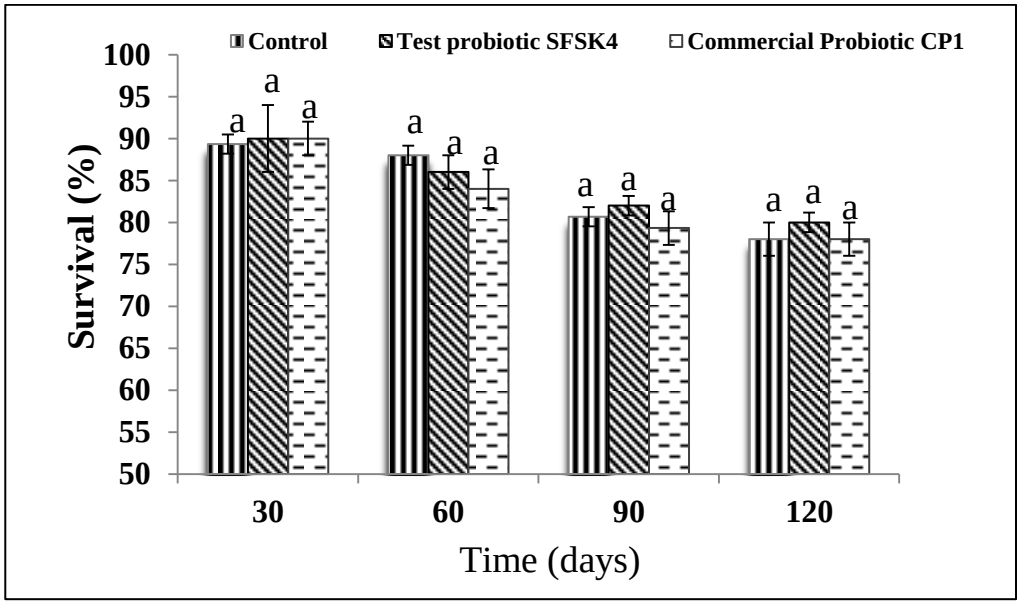


Fig. 25: (A) Specific growth rate, (B) Survival of *Litopenaeus vannamei* fed without bacteria (control), with test probiotic (SFSK4) and commercial probiotic (CP1) for 120 days.

Each value is the mean ± SD of three replicates. Different superscript letters are statistically significant (ANOVA, $p<0.05$ and subsequent post hoc multiple comparison with Tukeys HSD test).

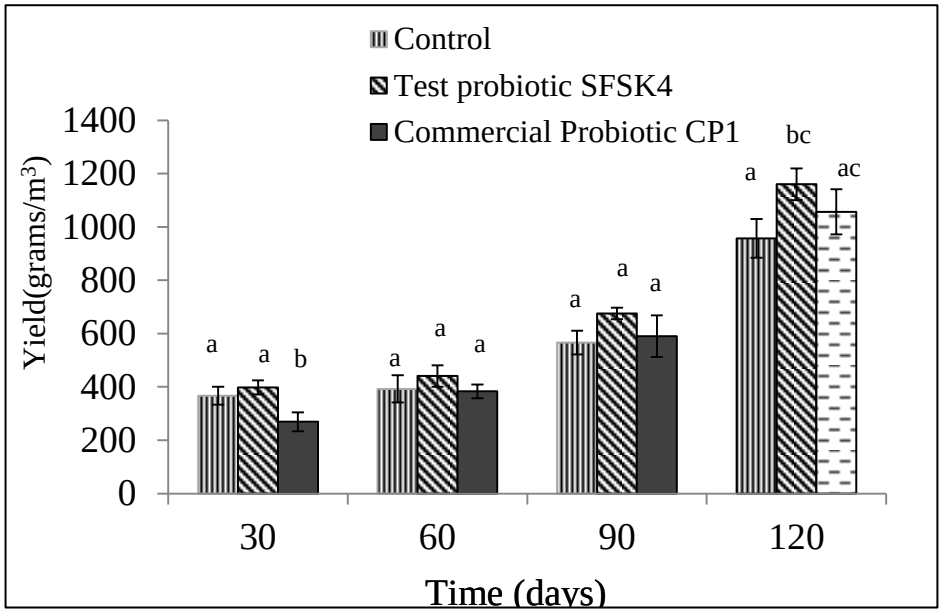


Fig. 26: Final Yield of *Litopenaeus vannamei* fed without bacteria (control), fed with test probiotic (SFSK4) and commercial probiotic

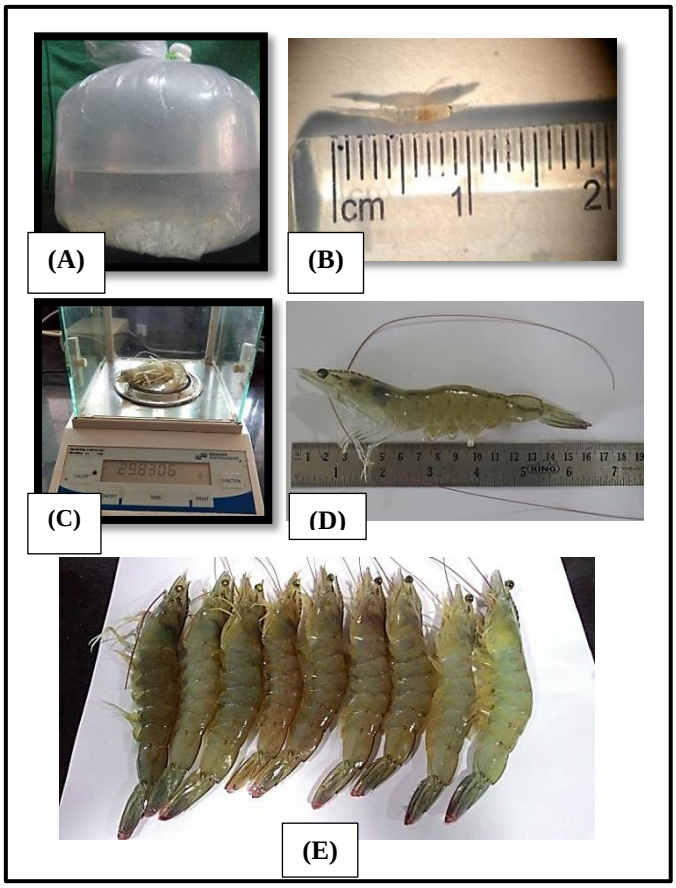


Plate 18: Image representing the growth of *Litopenaeus vannamei* (A) size at pL12, (B) size at pL30, (C) weight at day 90, (D) length at day 90, (E) size at day 120.

4.12.3 Effect of the test probiotic consortium SFSK4 on the gastrointestinal tract of *Litopenaeus vannamei*

The effect of the test probiotic consortium on the total enzyme activity was studied as explained in 4.12.3.1. Further, the presence of these bacteria in the gastrointestinal tract of shrimp and its effect on the *Vibrio* count in the gut was studied as described in section 4.12.3.2.

4.12.3.1 Total Enzyme Activity in Shrimp Gastrointestinal Tract

The digestive enzyme activity in the gastrointestinal tract of shrimp after 120 days of growth is represented in Table 29 (A). As seen in the table, the amylase activity was significantly higher (p = 0.001) in the test probiotic SFSK4 treated shrimp by 0.37 U/mg activity as compared to control and by 0.31 U/mg activity as compared to commercial probiotic CP1 treated shrimp. Similarly, a significant increase in the protease (p = 0.004) and lipase (p = 0.006) activity was seen in shrimp treated with SFSK4 than untreated control shrimp. The protease activity in shrimp treated with SFSK4 was also significantly higher (p = 0.003) compared to the shrimp treated with CP1. Although the lipase activity in SFSK4 was higher as compared to CP1 treated shrimp, the difference was not significant (p = 0.860). The cellulase activity in the gut of shrimp treated with CP1 was significantly higher than control (p = 0.004) and test (p = 0.002) shrimp. Further, positive correlations were observed between yield and amylase, yield and protease, yield and lipase, amylase and protease, amylase and lipase and protease and lipase as per Pearson's correlation analysis. Negative correlation was observed between total *Vibrio* count and yield, amylase, protease and lipase ($p < 0.01$, $p < 0.05$) as shown in Table 30.

4.12.3.2 Bacteriological Analysis of Shrimp Gastrointestinal Tract

The bacteriological analysis of the shrimp gut showed no significant difference in the total culturable bacterial count of control (5.842 ± 0.420 log CFU/mL) as compared to test probiotic SFSK4 (6.017 ± 0.603 log CFU/mL) and commercial probiotic CP1 (6.230 ± 0.161 log CFU/mL) treated shrimp. However, a significant decrease in the total *Vibrio* count of SFSK4 treated shrimp (3.71 ± 0.24 log CFU/mL) was detected in comparison

with the control (4.32 ±0.13 log CFU/mL). The CP1 treated shrimp showed no significant difference in the total *Vibrio* count (3.98 ± 0.34 log CFU/mL) as compared to SFSK4 and control (Table 29 B).

The presence of the salt pan bacterial isolates in the gut of shrimps treated with the test probiotic SFSK4 was confirmed by 16S rDNA sequencing. The results of the bacteria isolated from the gut of *L. vannamei* revealed the presence of the halotolerant bacteria in the gut of SFSK4 treated shrimp and absence in control. Among the isolates obtained from the gut of the test shrimp, one isolate showed 99 % nBLAST similarity with *B. amyloliquefaciens* (1408 bp sequence), one exhibited 99 % nBLAST similarity with *B. subtilis* (1329 bp sequence) and one showed 99 % nBLAST similarity with *B. licheniformis* (1387 bp sequence).

Table 29: (A) Total enzyme activity, (B) Bacterial count in the gastrointestinal tract of *Litopenaeus vannamei* treated without bacteria (control), treated with test probiotic (SFSK4) and treated with Commercial probiotic (CP1) after 120 days of culture.

	Control	**Test Probiotic SFSK4**	**Commercial Probiotic CP1**
(A) Enzyme activity (U/mg protein)			
Amylase	1.09 ± 0.02^{a}	1.46 ± 0.05^{b}	1.15 ± 0.04^{a}
Protease	1.04 ± 0.07^{a}	$1.55 \pm 0.07^{b,c}$	$1.29 \pm 0.03^{b,d}$
Cellulase	0.89 ± 0.03^{a}	0.98 ± 0.04^{a}	1.16 ± 0.04^{b}
Lipase	0.66 ± 0.08^{a}	1.04 ± 0.08^{b}	1.00 ± 0.10^{b}
(B) Bacterial count (Log (CFU/mL))			
Total culturable bacteria	5.842 ± 0.42	6.017 ± 0.603	6.230 ±0.161
Total *Vibrio* count	4.32 ± 0.13^{a}	3.71 ± 0.24^{bc}	3.98 ± 0.34^{ac}

Each value is the mean ± SD of three replicates. Different superscript letters are statistically significant (ANOVA, $p<0.05$ and subsequent post hoc multiple comparison with Tukey's HSD test).

Table 30: Pearson's correlation analysis between yield, enzyme activity and bacterial count in the shrimp gastrointestinal tract.

	Yield	Amylase	Protease	Cellulase	Lipase	TCB	TVC
Yield	1						
Amylase	0.797**	1					
Protease	0.723**	0.881**	1				
Cellulase	0.185	-0.080	0.359	1			
Lipase	0.689*	0.694*	0.883**	0.535	1		
TCB	0.080	0.194	0.337	0.208	0.424	1	
TVC	-0.798**	-0.789**	-0.784**	-0.145	-	-0.191	1

** $p<0.01$, *$p<0.05$

4.12.4 Effect of the test probiotic consortium SFSK4 on the immune response of *Litopenaeus vannamei*

The total haemocyte count (THC) was found to be significantly ($p<0.05$) lower in untreated control shrimps, compared to shrimps treated with SFSK4 and CP1 at 60, 90 and 120 days, with a maximum THC count of 143.67 ± 6.03 x 10^5 cells/mL in SFSK4 treated shrimps. No significant difference in THC was recorded between shrimps treated with SFSK4 and CP1 (Fig. 27). A significant increase ($p<0.05$) in the phagocytic activity was seen in shrimps treated with SFSK4 at 60, 90 and 120 days with highest activity at 120 days (46.15 ± 5.33 %) as compared to control (36.74 ± 6.21 %). Shrimps treated with CP1 showed significantly higher phagocytic activity than control at 60 and 90 day analysis, however no significance was observed at 120 days. An increasing trend in the percent phagocytosis was observed across all the three treatment groups at 60, 90 and 120 day analysis (Fig. 28). The phenoloxidase activity was significantly higher ($p<0.05$) in shrimps treated with SFSK4 and CP1 at 90 and 120 day analysis. A two-fold increase in PO was observed in shrimps treated with SFSK4 (0.417 ± 0.006 U/min/mg protein) compared to control (0.215 ± 0.028 U/min/mg protein) at 120 day analysis (Fig. 29). No significant difference was observed between SFSK4 and CP1 treated shrimps. The respiratory burst (RB) and total plasma protein (TPP) showed a steady rise across all three treatment groups over an increasing time period. No significant difference in RB and TPP was observed at 60 and 90 day analysis. However, the RB and TPP were significantly higher in SFSK4 treated shrimps (0.230 ± 0.012 and 138.88 ± 2.07 mg/mL) as compared to control shrimps (0.194 ± 0.008 and 107.67 ± 14.19 mg/mL) at 120 day analysis (Fig. 30 and Fig. 31). The bactericidal activity or bacterial clearance efficiency against *V. alginolyticus* was found to

be significantly higher in shrimps treated with SFSK4 as compared to control shrimps and shrimps treated with CP1. A significant increase in bactericidal activity was recorded against *V. harveyi* when treated with SFSK4 and CP1 treated shrimp (Fig. 32). Further, positive correlations were observed between the different cellular and humoral immunity parameters (Table 31). The results confirmed an immunostimulatory effect of the formulated probiotic against shrimp pathogens *V. harveyi* and *V. alginilyticus*.

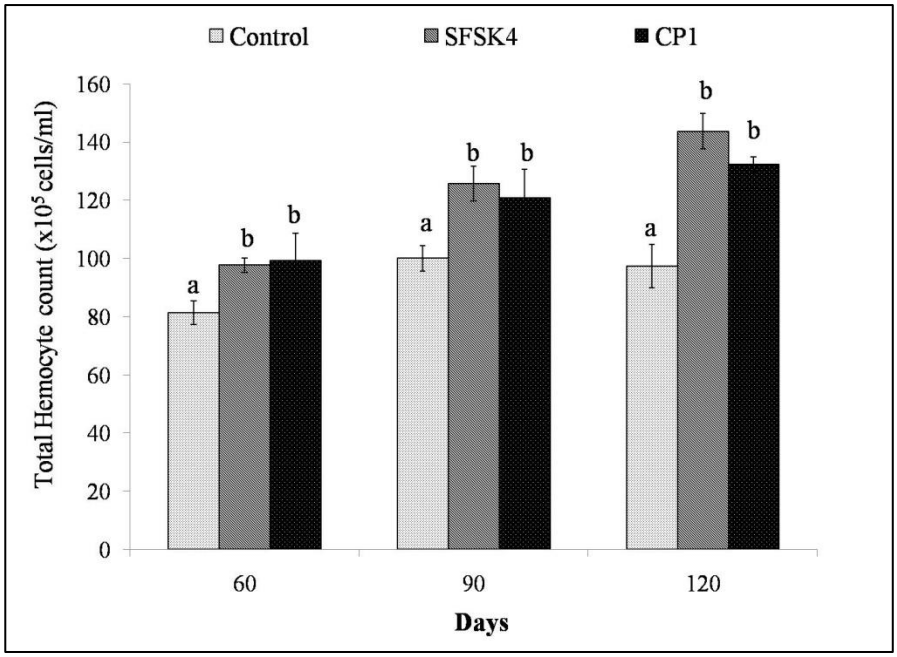


Fig. 27: Effect of the control, bacterial consortium SFSK4 and commercial probiotic CP1 on the Total Hemocyte count of *Litopenaeus vannamei.*

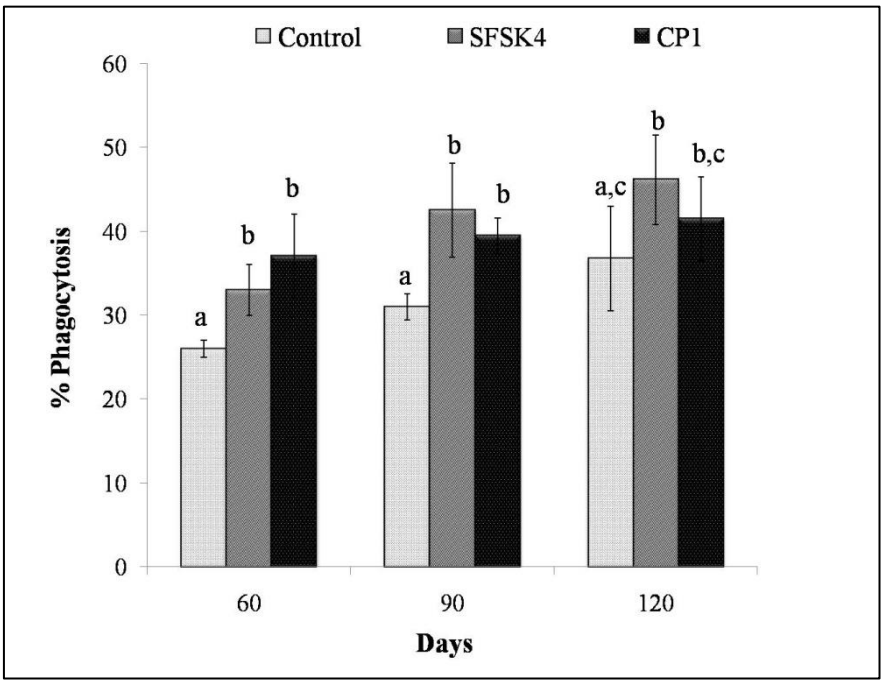


Fig. 28: Effect of the control, bacterial consortium SFSK4 and commercial probiotic CP1 on the phagocytic activity of *Litopenaeus vannamei.*

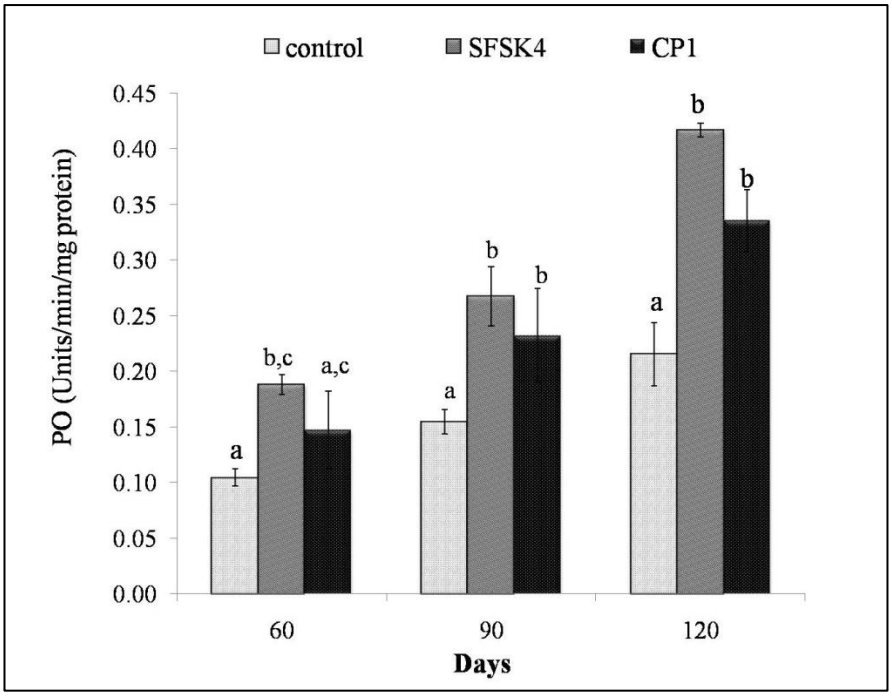


Fig. 29: Effect of the control, bacterial consortium SFSK4 and commercial probiotic CP1 on phenoloxidase activity of *Litopenaeus vannamei*.

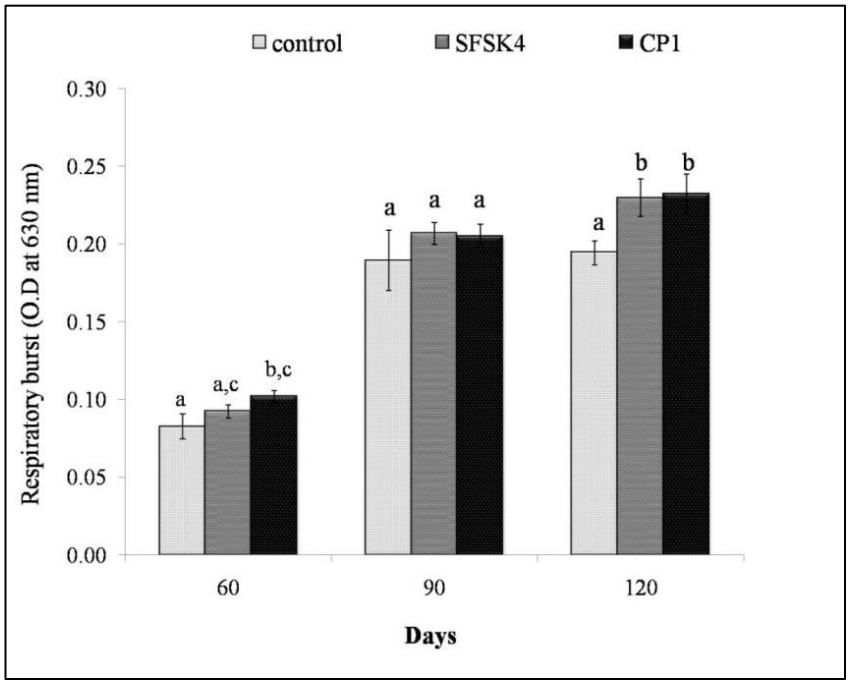


Fig. 30: Effect of the control, bacterial consortium SFSK4 and commercial probiotic CP1 on Respiratory burst of *Litopenaeus vannamei*.

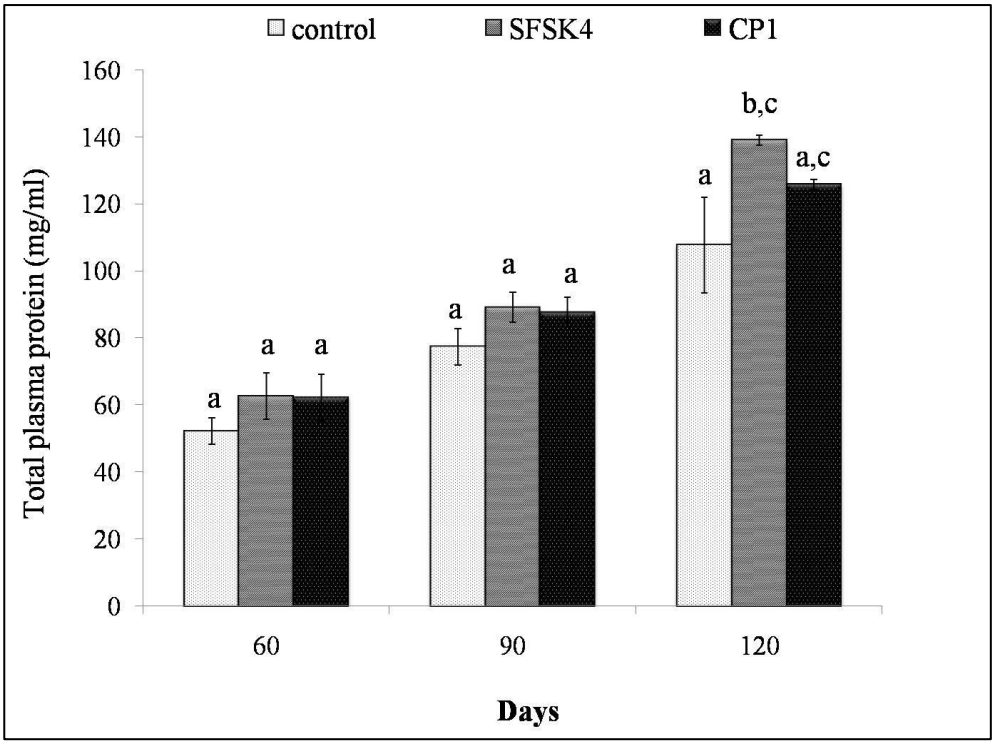


Fig. 31: Effect of the control, bacterial consortium SFSK4 and commercial probiotic CP1 on total plasma protein of *Litopenaeus vannamei*.

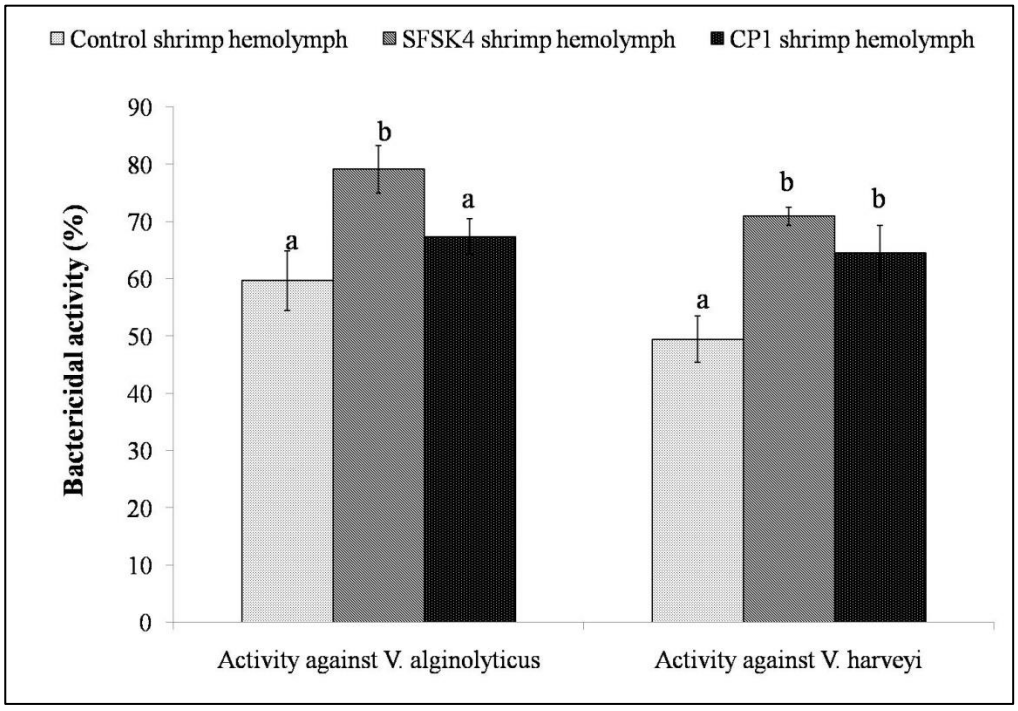


Fig. 32: Effect of the control, bacterial consortium SFSK4 and commercial probiotic CP1 on the bactericidal activity of *Litopenaeus vannamei*.

Table 31: Pearson's correlation analysis between total hemocyte count (THC), phagocytosis, phenoloxidase (PO), respiratory burst (RB), bactericidal activity against *V. alginolyticus* (Va) and *Vibrio harveyi* (Vh), total plasma protein (TPP) and survival of *Litopenaeus vannamei* after treatment with probiotic.

	THC	Phagocytosis	PO	RB	Bactericidal activity (Va)	Bactericidal activity (Vh)	TPP	Survival
THC	1							
phagocytosis	0.797*	1						
Phenoloxidase	0.874**	0.891**	1					
RB	0.801**	0.674*	0.830**	1				
Bactericidal activity (Va)	0.746*	0.728*	0.821**	0.552	1			
Bactericidal activity (Vh)	0.896**	0.822**	0.829**	0.680*	0.867**	1		
TPP	0.914**	0.865**	0.884**	0.788*	0.670*	0.752*	1	
Survival	0.347	0.411	0.411	0.155	0.534	0.386	0.511	1

*. Represents $p<0.05$, ** represents $p<0.01$. N = 9

4.13 Proteomic analysis of the Probiotic treated shrimp hemolymph

Table 32 represents the comparative analysis of the control and probiotic treated (SFSK4) hemolymph proteome. A total of 1622 proteins were identified using multiplex LC-MS/MS and SWATH MS analysis in control and probiotic treated shrimps. Fifty proteins showed a significant change in expression between the two groups. Out of these 50 proteins, 39 were up-regulated and 11 proteins were down regulated in SFSK4 treated shrimp as compared to the control. Four major immune modulation proteins were identified in the shrimp hemolymph treated with SFSK4. These proteins are involved in pattern recognition and cell mediated immune response (phagocytosis, apoptosis and invading pathogen encapsulation) in crustaceans.

Table 32: Proteomic profile of SFSK4 treated shrimp hemolymph.

Protein [Species]	Accession No.	Fold change	Function
UPREGULATED			
Caspase 2 [*Litopenaeus vannamei*]	R4PWN6	**19.91**	activation of cell mediated immune response in the host (Role in apoptosis several stimuli, including oxidative stress).
GTPase activating protein and VPS9 domain containing protein 1like (*Megachile rotundata*)	A0A0K2SXG9	**13.51**	involved in signal transduction pathways that regulate cell growth, cell migration, and apoptosis.
Hemocyanin V4 [*Litopenaeus vannamei*]	A0A0G2YAK1	**12.29**	respiratory protein, converted to enzymes : antimicrobial
Hemocyanin [*Palaemon carinicauda*]	G9DE16	**11.07**	metal ion binding, oxidoreductase activity
Heat shock cognate 70 [*Litopenaeus vannamei*]	Q6GUA8	**9.75**	nucleotide binding, ATP binding
Hemocyanin Fragment [*Litopenaeus vannamei*]	G9BYP1	**9.32**	metal ion binding, oxidoreductase activity

Hemocyte transglutaminase [*Litopenaeus vannamei*]	A8QL63	**7.89**	metal ion binding, protein-glutamine gamma-glutamyltransferase activity, peptide cross-linking
Glucan pattern-recognition lipoprotein [*Fenneropenaeus chinensis*]	I6YF21	**6.75**	signaling pattern recognition receptor activity, Activates ProPhenolOxidase system
Alpha-enolase-like protein (Fragment) [*Daphnia magna*]	A0A0P6CIM0	**6.32**	magnesium ion binding, phosphopyruvate hydratase activity, glycolytic process
Hc subunit 3 (Fragment) [*Skogsbergia lerneri*]	A0A060RCQ5	**5.86**	metal ion binding, oxidoreductase activity
Pacifastin light chain-like serine proteinase inhibitor [*Litopenaeus vannamei*]	A0A0K2CT08	**5.24**	Role in Prophenoloxidase system, peptidase inhibitor activity, endopeptidase inhibitor activity,serine-type endopeptidase inhibitor activity,
Uncharacterized protein [*Scylla olivacea*]	A0A0P4WJH8	**4.75**	ATP binding, nucleotide binding
Hemocyanin HC3 [*Limnoria quadripunctata*]	D5J6V8	**4.52**	metal ion binding, oxidoreductase activity
Clottable protein [*Marsupenaeus japonicus*]	A7YIH6	**3.92**	lipid transporter activity
REVERSED Glucosamine-6-phosphate isomerase (Fragment) [*Limnadia lenticularis*]	D0UPV5	**3.83**	glucosamine-6-phosphate deaminase activity, hydrolase activity, isomerase activity, carbohydrate metabolic process etc
Fibrinogen-like protein [*Fenneropenaeus merguiensis*]	A0A0K0QRA8	**3.78**	
Alpha2 macroglobulin isoform 2 [*Fenneropenaeus chinensis*]	D6BMV2	**3.62**	endopeptidase inhibitor activity,extracellular region, extracellular space
Hemocyanin (Fragment) [*Litopenaeus vannamei*]	Q9NFY6	**3.49**	metal ion binding, oxidoreductase activity
Hemocyanin subunit L2 (Fragment) [*Litopenaeus vannamei*]	A0A059TFW7	**3.20**	metal ion binding, oxidoreductase activity
Hemocyanin subunit L2 (Fragment) [*Litopenaeus vannamei*]	A0A059TEX0	**3.15**	metal ion binding, oxidoreductase activity
Cytoplasmic actin [*Portunus trituberculatus*]	U3M6R0	**2.91**	ATP binding, nucleotide binding
Hemocyanin [*Macrobrachium*	A0A0A0PM26	**2.86**	metal ion binding,

nipponense]			oxidoreductase activity
Hemocyanin (Fragment) [*Marsupenaeus japonicus*]	Q5GJ05	**2.74**	
70 kDa heat shock protein (Fragment) [*Aratus pisonii*]	X2G9I3	**2.43**	Nucleotide binding, ATP binding, stress response
Hemocyanin alpha subunit 1 [*Atyopsis moluccensis*]	I4EC45	**2.41**	metal ion binding, oxidoreductase activity
Hemocyanin 2 [*Pacifastacus leniusculus*]	Q6Y0Z1	**2.17**	metal ion binding, oxidoreductase activity
Beta-1,3-glucan-binding protein [*Litopenaeus vannamei*]	P81182	**2.09**	signaling pattern recognition receptor activity, immune system process, cell surface pattern recognition receptor signaling pathway, innate immune response, regulation of innate immune response, extracellular region, lipid transport,
Hemocyanin-like protein (Fragment) [*Metapenaeus ensis*]	A9Q7C4	**2.03**	metal ion binding, oxidoreductase activity
Hemocyanin subunit L5 (Fragment) [*Litopenaeus vannamei]*	A0A059TIS0	**1.83**	
Hemocyanin A chain [*Panulirus interruptus]*	P04254	**1.62**	oxygen carrier activity, metal ion binding, oxidoreductase activity, oxygen transport (specific in hemolymph)
Hemocyanin (Fragment) [*Penaeus monodon*]	Q95V28	**1.36**	metal ion binding, oxidoreductase activity
Hemocyanin gamma subunit 1 [*Atyopsis moluccensis*]	I4EC49	**0.36**	metal ion binding, oxidoreductase activity
Hemocyanin [*Litopenaeus vannamei*]	Q26180	**1.30**	metal ion binding, oxidoreductase activity
Hemocyanin subunit [*Metacarcinus magister*]	Q5G2A5	**1.09**	metal ion binding, oxidoreductase activity
Hemocyanin subunit L3 (Fragment) [*Litopenaeus vannamei*]	A0A059TIR8	**1.34**	metal ion binding, oxidoreductase activity
Clottable protein [*Litopenaeus vannamei*]	A8DR94	**1.61**	lipid transporter activity
Sarcoplasmic calcium-binding protein (Fragment) [*Chionoecetes opilio]*	P86909	**1.88**	
Uncharacterized protein [*Lepeophtheirus salmonis*]	A0A0K2U4H6	**1.74**	
Hemocyanin [*Litopenaeus vannamei*]	A0A088MK65	**1.05**	metal ion binding, oxidoreductase activity

DOWNREGULATED			
Hemocyanin [*Fenneropenaeus merguiensis*]	S5ZHH2	**2.87**	metal ion binding, oxidoreductase activity
Hemocyanin [*Pacifastacus leniusculus*]	Q8MUH8	**2.83**	metal ion binding, oxidoreductase activity
Hemocyanin [*Litopenaeus vannamei*]	X2KWE4	**2.63**	metal ion binding, oxidoreductase activity
Adiponectin receptor protein-like [*Hirondellea gigas*]	A0A2P2HW26	**2.29**	Receptor (integral part of membrane)
Hemocyanin subunit L1 (Fragment) [*Litopenaeus vannamei*]	A0A059TGC6	**1.98**	metal ion binding, oxidoreductase activity
Hemocyanin subunit L [*Marsupenaeus japonicus*]	B0L611	**1.89**	metal ion binding, oxidoreductase activity
Hemocyanin [*Fenneropenaeus chinensis*]	B9VR33	**1.72**	metal ion binding, oxidoreductase activity
Hemocyanin subunit 1 [*Macrobrachium nipponense*]	M4IQR3	**3.82**	metal ion binding, oxidoreductase activity
Hemocyanin [*Nebalia kensleyi*]	C8BP49	**1.51**	metal ion binding, oxidoreductase activity
Hemocyanin subunit Y [*Marsupenaeus japonicas*]	B0L612	**1.53**	metal ion binding, oxidoreductase activity
REVERSED Uncharacterized protein [*Daphnia pulex*]	E9GKS8	**1.35**	cell adhesion, integrin-mediated signaling pathway, integrin complex

CHAPTER 5:

DISCUSSION

5. DISCUSSION

5.1 Halotolerant bacteria and their *in vitro* probiotic potential

The development of probiotics requires rigorous *in vitro* and *in vivo* characterization and safety analysis to understand their exact mode of action. Studies have suggested that probiotics can have a beneficial effect on the host by competitive exclusion of pathogenic bacteria, improvement of water quality, stimulation of immune response, enzymatic contribution to digestion and enhancing the host nutrition (Hai NV 2015, Merrifield et al. 2010, Gomez and Balcazar, 2008, Irianto & Austin 2002). The selection of appropriate microorganisms is very critical as incompatible micro-organisms can cause adverse effects to the host and its environment (Sánchez et al., 2013). Therefore, several *in vitro* screening tests including experiments to assess the production of antagonistic compounds, production of other beneficial compounds such as enzymes, ability to attach to mucus, tolerance to pH and bile salts, growth profile of the bacteria, primary safety assessment etc. need to be carried out prior to *in vivo* trials (Vine et al. 2006, Sánchez 2013). The results of these *in vitro* assays are used to contemplate whether the candidate strains are suitable to be analysed *in vivo* (Sorroza et al. 2012).

In our present study, 300 halotolerant bacteria and actinomycetes were screened for production of antagonistic compounds and ammonia degradation *in vitro*. The cultures (25) showing highest anti-*Vibrio* activity and ability to degrade more than 40 % ammonia in 7 days were further screened for enzyme activity, mucus adhesion, tolerance to varying concentrations of pH, bile salts, salinity and haemolytic activity. Based on the above test, 6 best cultures viz. SK07, SK27, ABSK55, FSK444, TSK17 and TSK71 were selected for *in vivo* studies.

Probiotics exhibit antagonism by production of antimicrobial metabolites such as antibiotics, bacteriocins, lytic enzymes, organic acids or by competing for intestinal space, essential nutrients and other resources with the pathogenic microorganisms (Balcazar et al., 2006, Verschuere et al., 2000). Competitive exclusion is a mechanism in which an established microbiota reduces or inhibits the colonization of competing microorganisms for the same nutrients and attachment sites (LaraFlores & Aguirre-Guzman 2009). In our study, 9.66 % of the tested salt pan isolates produced antagonistic compounds against different *Vibrio* species *viz. V. cholerae, V. parahaemolyticus, V. alginolyticus, V. harveyi,*

and V. vulnificus tested by well diffusion assay. Gao et al. (2008) reported the *in vitro* antimicrobial activity of *Bacillus pumilus* H2 isolated from marine sediments against 29 *Vibrio* sp. and attributed this activity to the production of an antimicrobial compound, amicoumacin A. Similarly, *Bacillus pumilus* B16 and *Bacillus mojavensis* isolated from seafood inhibited pathogenic *Vibrio* species including *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus* (Liu et al., 2015) when tested *in vitro*. Zhang et al. (2017) reported *Pseudomonas aeruginosa* strain PA31x isolated from marine sediments to produce an antagonistic compound (Phenazine-1-Carboxylic Acid) against *Vibrio anguillarum in vitro*. Subsequently, in our study halotolerant bacteria SK07, SK27 and ABSK55, reduced the *Vibrio* count by two to four folds as compared to the control when co-cultured with *Vibrio* pathogens in broth. The results indicate that the halotolerant bacteria produced antagonistic compounds against the *Vibrio* pathogens and competed with them for available nutrients or energy which was evident from the co-culture method. Our results were also in agreement with Vaseeharan and Ramasamy (2003), where co-culture of *V. harveyi* with *Bacillus subtilis* BT23 competitively inhibited the growth of *V. harveyi in vitro*. Their study reported that for an effective inhibitory activity the candidate probiotic must be present at significantly higher concentration than the test pathogen. In a similar study, *B. cereus* competitively excluded the growth of *Aer. hydrophila* by more than 70 % due to its higher growth rate and ability to favourably uptake essential nutrients like glucose and iron when co-cultured *in vitro* (Lalloo et al., 2010). Evaluating the growth profile and stage of production of anti-*Vibrio* metabolites is essential as most metabolites are produced during the stationary phase of growth, and this phase might not occur *in vivo* due to the constant flushing of the gut (Monaghan et al., 1999). To prevent the proliferation of pathogens, an ideal probiotic must possess a fast growth rate with a short lag phase and a small doubling time (Balakrishna and Keerthi, 2012). In our work, all the growth parameters of the halotolerant bacteria were kept constant to allow direct comparison of the individual growth profiles. The results showed a short lag phase in culture SK07, SK27, FSK444 and TSK71 showing anti-*Vibrio* activity beginning at 16 to 24 h of growth (late log phase to early stationary phase). However, the actual growth profile of bacteria *in vivo* may be influenced by factors such as the availability of nutrients, the composition of the host gut and the physico-chemical parameters of the aquaculture pond (Vine et al., 2004). Therefore, besides having a short lag phase, it is essential that the candidate probiotic has the ability to attach to the host mucus membrane and colonize the intestinal or skin mucus, thereby competitively excluding the pathogens for adhesion sites

(Vine et al. 2006, Balcazar et al. 2006). Balcazar et al. demonstrated that three strains of *Lactobacillus* decreased the adhesion of fish pathogens *Carnobacterium piscicola, A. salmonicida and Yersinia ruckeri* to the mucus of rainbow trout. The study recommended the use of adhering bacteria when designing probiotic supplements, as the inability to compete for attachment sites may expel the bacteria from the mucus lining during gut evacuation (Balcazar et al., 2007). In our study the selected halotolerant bacteria TSK71, SK27 and SK07 exhibited above 85 % mucus adhesion. The ability of these cultures to adhere to an abiotic surface *in vitro* demonstrates their capability to survive in the gastrointestinal tract for several days and participate in the elimination of pathogens, digestive processes and the establishment of a healthy gut environment (Mahdhi et al. 2010). Many studies have suggested that stronger the adhesion of the bacteria to the mucus membrane, longer will be its resident time in the intestinal tract or the skin surface, implying a higher probiotic potential (Sanchez et al., 2013, Luis-Villasenor et al. 2011). Thus, the high mucus adhesion, anti-*Vibrio* activity and faster growth of the halotolerant strains SK07, SK27, ABSK55 and TSK71 were important characteristics considered while selecting these bacteria as candidate probiotics against shrimp pathogens *in vivo*.

Besides antimicrobial activity, probiotics are also known to play an important role in improving the water quality of aquaculture ponds. One of the most critical issues in intensive farms is the accumulation of toxic nitrogenous waste such as ammonia, accounting for 60 – 70 % nitrogen excretion (Chen et al., 1990). The present study shows halotolerant culture ABSK55 decreased more than 80 % of ammonia concentrations over a period of 7 days. In penaeid shrimp, high levels of ammonia reduces the oxygen transport in hemolymph, damages the gills and has a negative impact on the growth and survival of the shrimp (Racotta & Herna´ndez-Herrera 2000, Zin & Chu 1991; Chen & Lin 1992). Studies have reported that microorganisms reduce the nitrite and ammonia as well as enhance the decomposition of organic matter in aquaculture ponds (Cha et al. 2013, Wang et al. 2005, Verschuere et al. 2000). Similar results were reported by Xu et al. (2017) in which *Pseudomonas putida* Y-9 performed heterotrophic nitrification with ammonium. Huang et al. (2017) reported *in vitro* ammonia degradation by simultaneous nitrification and denitrification by *Bacillus litoralis* N31 isolated from shrimp mariculture water. The survival of the halotolerant strains at a high concentration of ammonia and its ability to utilize ammonium sulphate as the sole nitrogen source indicates their suitability for bioremediation of aquaculture water with high nitrogen concentrations

Probiotics are also known to produce a variety of metabolites such as extracellular enzymes that help in breaking down complex feed components (e.g. carbohydrates, proteins, and lipids), resulting in feed absorption and enhanced growth of the host (Gómez and Shen, 2008). The enzymes produced also aid in degrading the organic matter, which helps in improving the water quality of aquaculture ponds. Our study reports 23 halotolerant bacteria with a distinctive ability to produce different digestive enzymes, namely amylase, cellulase, protease and lipase. Among these, halotolerant bacteria SK27 showed highest amylase (21.0 ± 1.0 mm) and protease (23.33 ±1.5 mm) production followed by TSK71 with highest cellulase (20.67 ± 0.5 mm) and lipase (22.33 ±1.3 mm) production. Hypersaline bacteria are excellent sources of exogenous enzymes that carry out reactions efficiently under extreme conditions. Mellado et al. (2004) reported *Halomonas, Bacillus-Salibacillus* and *Salinovibrio* species isolated from hypersaline environments in Spain to produce hydrolytic amylase, DNAase, protease and pullulanase when screened *in vitro.* Vijayabaskar et al., (2012) reported *Bacillus cereus* isolated from the salt pans of Tamil Nadu, India to produce hydrolytic amylase of Industrial significance. Gesheva and Vasileva-Tonkova (2012) reported a halophilic *Antarctic Nocardioides* sp. that produced multiple hydrolytic enzymes on different growth media. Our studies reveal a wide range of bacteria with the capability to hydrolyse structurally different non-related polymers. This is the first study that has used salt pan bacterial enzymes to boost the digestion of shrimps in aquaculture. An added feature of the salt pan bacteria SK07, SK27, ABSK55 and TSK71 is that they tolerate a wide range of pH (5 - 9), salinity (0 - 5 %) and bile salts (0 - 3 %). Salinity tolerance is an essential factor as aquaculture pond salinities vary from zero to 38 ppt depending on the season, time of the year and region of farming (Araneda et al., 2008). Like salinity, pH tolerance of probiotics is a critical factor as the pH of an aquaculture pond is not constant, varying from 6 (in superintensive cultures) to 9 (in eutrophied ponds) (Vinatea et al., 2010; Momoyama, 2004). The probiotic must also be able to survive the acidic pH of the host gastrointestinal tract. Vieira et al. (2008) suggested that bile salt tolerance is also important as the probiotic must be able to resist the microbicidal effect of the bile salts present in the gut and effectively colonize the digestive tract. In a study conducted by Hyronimus et al. (2000), *B. racemicus* IAM 12395 and *B. laevolacticus* DSM 6475 showed resistance to pH 3, however were weakly tolerant or sensitive to bile salts above 0.3 %.

Certain bacteria produce hemolysin toxin, which could cause a threat to the host (Liu et al., 2009). However, the four bacteria SK07, SK27, TSK71 and ABSK55 exhibited no hemolysis (γ-hemolysis) on both, human blood agar and shrimp hemolymph agar indicating the non-toxic nature of these bacteria. Similar results were reported by Luis-Villasenor et al. (2011) where four *Bacillus* strains including *B. amyloliquefaciens* isolated from the gut of *L. vannamei* exhibiting γ-hemolysis on blood and hemolymph agar were good candidates for probiotic application, which is comparable to our findings.

5.2 Bacterial identification and synergistic effect of the mixed probiotic consortium

The four isolates were identified as *Bacillus subtilis* strain SK07, *Bacillus amyloliquefaciens* strain SK27, *Bacillus licheniformis* strain TSK71 and *Pseudomonas* sp. strain ABSK55. All the cultures were motile, producing enzymes, tolerant to varying concentrations of pH (5 to 9), salinity (0 to 5 %) and bile salts (0 to 3 %) as well as exhibited strong mucus adhesion and anti-*Vibrio* activity *in vitro.* Although, three of the cultures were identified to be *Bacillus* sp., each of them possessed unique characteristics when screened *in vitro. Bacillus subtilis* strain SK07 was selected for its bactericidal activity against *V. harveyi* and *V. parahaemolyticus.* This *Bacillus* strain also produced amylase and protease with the ability to increase the average body weight and feed efficiency of *Litopenaeus vannamei.* The strain also stimulated the shrimp immune response by increasing the total hemocyte and phenoloxidase activity. *Bacillus amyloliquefaciens* strain SK27 was selected for its inhibition to *V. cholerae* and *V. harveyi,* ability to degrade ammonia, production of amylase and protease and its ability to enhance shrimp growth and phagocytosis. *Bacillus licheniformis* strain TSK71 was chosen for its bioactivity against *V. alginolyticus*, *V. vulnificus* and *V. parahemolyticus*, cellulase and lipase production and ability to increase shrimp body weight. *Bacillus* species are Gram-positive, spore forming bacteria that remain viable for a long period of time (Amin et al., 2015). They are aerobes or facultative anaerobes comprising approximately 200 species (Yang et al., 2013). They represent the most heterogeneous group with reference to genotypic and phenotypic characters. *Bacillus* sp. have been reported in soil (Yang et al., 2013); in extreme environments with high salt concentration (Annamalai et al., 2013), high pH (Sturr et al., 1994), high temperature (Yang et al., 2013), in marine environments (Ichimatsu et al., 2000, Motta et al., 2004) as well as the gastrointestinal tract of aquatic

animals (Ray et al., 2012). Further, *Bacillus* spores can persist in the gastrointestinal tract by tolerating the bile salts and the extreme acidic conditions of the stomach. Studies have claimed that *Bacillus* species produce spores that protect against gut stress, help in adhering to the intestine and increase their stability during processing and storage (Tam et al. (2006), Elshaghabee et al., 2017). This makes them a favourable choice for the scientists and manufactures (Elshaghabee et al., 2017). Moreover, Seenivasan et al. (2012) reported that 3 % of *Bacillus subtilis* supplemented in the diet of *Macrobrachium rosenbergii* enhanced the nutritional indices, growth, energy utilization performance and survival of the host. Similarly, an increase in the digestive enzyme activity viz. amylase, protease and lipase was observed in *Fenneropenaeus indicus* when exposed to a *Bacillus* probiotic comprising of (*Bacillus polymyxa, Bacillus subtilis, Bacillus circulans* and *Bacillus laterosporus*) (Ziaei-Nejad et al., 2006). Balcázar et al. (2007) demonstrated the anti-*Vibrio* effect of four bacterial strains (*Vibrio alginolyticus* UTM 102, *Pseudomonas aestumarina* SLV22, *Bacillus subtilis* UTM 126 and *Roseobacter gallaeciensis* SLV03) isolated from the gut of *Litopenaeus vannamei* against *Vibrio parahaemolyticus* PS017. Furthermore, a study conducted by Li et al. (2007) revealed that *Bacillus licheniformis* applied as a water additive to *Litopenaeus vannamei* tanks competitively excluded *Vibrio* species and increased the total haemocyte count, phenoloxidase activity and superoxide dismutase activity, thereby enhancing the shrimp immunity. Gullian et al. (2004) demonstrated the immunostimulatory effect and competitive exclusion mechanism of probiotic *Bacillus* P64 on *Litopenaeus vannamei* to treat wild shrimp diseases caused by *V. harveyi.*

Halotolerant bacteria *Pseudomonas* sp. ABSK55 was selected for its inhibitory activity to *V. harveyi, V. alginolyticus, V. cholerae*., ammonia degradation, lipase production and ability to increase phagocytosis, total hemocyte count and phenoloxidase in *L. vannamei.* Pseudomonads commonly inhabit aquatic environments including shrimp aquaculture ponds (Otta et al.,1999) and are found to be associated with the skin, gills and the intestinal tract of live aquatic organisms (Cahill, 1990). Chythanya et al. (2002) reported that *Pseudomonas* I-2 showed prominent inhibition of pathogenic *V. harveyi* with 17 mm inhibition zone when screened *in vitro* by well diffusion method. In our study, *Pseudomonas* sp. ABSK55 showed 22 mm zone against *V. harveyi* in well diffusion assay and exhibited a two-fold decrease in the *Vibrio* count in co-culture method. Preetha et al. (2015) reported a *Pseudomonas* sp. isolated from brackish water showing antagonism to a

wide range of *Vibrio* spp. These results are on par with our results where our marine *Pseudomonas* sp. ABSK55 being a salt pan bacteria showed bioactivity against 4 of the 5 tested *Vibrio* species.

Further, our study evaluated the *in vitro* synergistic effect of the halotolerant bacteria in consortium by co-culturing the potential isolates, followed by assessing them for anti-*Vibrio* activity, enzyme production and ammonia degradation. The synergistic effect of bacteria is generally observed in nature, e.g. microorganisms co-exist in communities and mutually interact with each other to release diverse metabolites in water. Strain compatibility is a very essential preliminary step before designing a multifunctional probiotic consortium. In our study, the biocompatibility between the candidate probionts was confirmed by cross-streak assay. The results suggest the compatibility of four bacteria *B. subtilis* strain SK07, *Bacillus amyloliquefaciens* strain SK27, *Bacillus licheniformis* strain TSK71 and *Pseudomonas* sp. strain ABSK55 to grow together. The consortium showed no significant change ($p>0.05$) in the ammonia degrading ability and production of cellulase and lipase as compared to when used individually. However, it was observed that the *in vitro* anti-*Vibrio* effect of the consortium against *V. harveyi, V. alginolyticus, V. cholerae* and *V. parahaemolyticus* was significantly higher ($p<0.05$) as compared to the inhibition exhibited by the individual isolates. Besides, the amylase and protease production was found to be boosted in the consortium (23.00 ± 1.09 mm and 26.00 ± 0.58 mm zone respectively) as compared to the individual cultures (21.0 ± 1.0 and 23.33 ±1.5 mm zones respectively). Anti-*Vibrio* activity, ammonia degradation and enzyme production of the mixed consortium lasted for 180 days (maybe more), as the experimental period was confined to 180 days. No significant difference ($p>0.05$) in activities was observed between the consortium kept at room temperature and 4 ºC. This indicated that storage period up to 180 days and temperature had no significant effect on the activity of the consortium when tested in respective broth *in vitro*. The formulation of a bacterial consortium having synergistic effects, such as enzyme production, anti-*Vibrio* activity, ammonia degrading ability makes it a multifunctional single product with a great potential in aquaculture. Further, our results revealed that the lyophilized bacterial consortium was stable up to 120 days at room temperature (28 ± 2 ºC) and up to 180 days (which was the assessed period) at 6 ± 2 ºC. The viable count of the consortium was found to be higher than individual cultures at both, room temperature and refrigerated conditions which revealed a positive effect when the halotolerant strains were grown together.

5.3 Trials of the formulated probiotic consortium on shrimp (*Litopenaeus vannamei*)

The *in vivo* trials on *Litopenaeus vannamei* were carried out after extrapolating their positive effects in the *in vitro* experiments. These six cultures were designated as candidate probiotic bacteria or candidate probionts. Simultaneously, the six bacteria were assessed for their activity in different concentrations ranging from 10^8 to 10^{10} CFU/mL. Both 10^9 and 10^{10} CFU/mL concentrations gave promising results, however considering the economics of the production, the concentration of 10^9 was selected as the appropriate concentration for the formulation. It was observed that 2 bacterial isolates viz. FSK444 and TSK17 did not show the same activity witnessed in the *in vitro* experiments. The decrease in activity of the above isolates could be because of the non-conducive aerated environment of the tanks which was alien to FSK444 and TSK17. Hence, it was decided to eliminate these two cultures from the formulation. Subsequently the four isolates which were compatible in their growth as well as activity were collectively used in the probiotic formulation denoted as SFSK4.

5.3.1 Effect of SFSK4 on the growth of *Litopenaeus vannamei*

The probiotic consortium SFSK4 was used in a lyophilized powdered form which was dissolved in minimal amount of tank culture water prior to inoculation. The lab-scale trials conducted with SFSK4 indicated a significant ($p<0.05$) increase in the body weight of shrimp at the end of the experiment as compared to the control (shrimp tanks not exposed to SFSK4). Similar results were observed in the farm trial where the shrimp weight of the tanks treated with the SFSK4 was significantly ($p<0.05$) higher as compared to the control. Similar results were reported by Fernandes et al. (2011) where an increase in the body weight, feed utilisation and survival of *Penaeus indicus* was observed when *L. acidophilus*, *Streptococcus cremoris* NCIM 2285, *Lactobacillus bulgaricus* NCIM 2285 and *L. bulgaricus* NCIM 2285 (2057) were orally administered to juvenile shrimps. Das et al. (2010) reported an increase in the body weight of *Penaeus monodon* supplemented with *Streptomyces* sp. The study suggested that *Streptomyces* sp. secrete hydrolytic enzymes which improve the amylolytic and proteolytic activity in the shrimp digestive tract, leading to efficient uptake of feed nutrients. With reference to Kongnum and Hongpattarakere

(2011), the average body weight of shrimp fed with *L. plantarum* MR03.12 supplemented diet was higher than control group with significant difference in feed conversion ratio, relative growth rate and percent survival. *Lactobacillus acidophilus* and *Lactobacillus sporogenes* significantly improved the growth of *Macrobrachium rosenbergii* post larvae when administered for 60 days (Venkat et al., 2004). Our SFSK4 formulation comprised of three strains of Bacillus viz. *Bacillus licheniformis* TSK71, *Bacillus amyloliquefaciens* SK27, *Bacillus subtilis* SK07 and one *Pseudomonas* sp. ABSK 55. The formulation was quite stable in its shelf life for more than 180 days. As mentioned above, *Bacillus* is capable of spore formation, resistant to physical stress and chemical effects that determine their longevity in the environment (Henriques and Moran, 2000; Nicholson et al., 2000). They adapt to the gastrointestinal tract by forming a symbiotic association that improves the physiological response of the host. After colonisation, they release relevant digestive enzymes and other necessary growth factors that facilitate nutrient assimilation, resulting in prevention of intestinal disorders and enhanced growth in shrimp and fish (Sahu et al., 2008; Nimrat et al., 2012, Wang 2007). A significant difference in the growth of *L. vannamei* over control was in accordance with other investigators that used *Bacillus* as probiotics. Solano and Soto demonstrated *B. subtilis* and *B. megaterium* from marine sediments as producers of proteases, amylases and lipases in shrimp feed (Solano and Soto, 2006). Navinchandran et al. reported that *B. cereus* isolated from the gut of wild shrimp *P. monodon* significantly stimulated the growth of *P. monodon* at a concentration of 0.4 % per 100 g feed, after 90 days of culture (NavinChandran et al., 2014). A similar trend was seen when *Bacillus sp*. was administered to *Fenneropenaeus indicus* in hatchery and farming stages up to 120 days of culture (Ziaei-Nejad et al., 2006). Several studies have demonstrated the beneficial effect of probiotics on growth performance of the host. However, the exact mode of action is not well understood. One explanation for improvement of shrimp growth is due to the induction of digestive enzymes (Zokaeifar et al., 2012). In the present study, a 33 % increase in the amylase, 48.5 % increase in protease and 56.7 % lipase activity was observed in shrimp tank treated with SFSK4. The increase in shrimp yield by 22 % strongly correlates (**$p < 0.01$; *$p < 0.05$) to the digestive enzyme activity of the experimental shrimps. The amylase, protease and lipase activity in the gut of the test shrimps was significantly higher than that of the control shrimps. Yanbo and Zirong (2006) noted a remarkable increase in amylase, protease and lipase activity in *Cyprinus carpio* (common carp) when treated with a mixture of photosynthetic bacteria and *Bacillus* sp. Similar results were also reported by Zhou et al. (2009) where B.

coagulans SC816 supplemented as water additive significantly increased the survival and digestive enzyme activity of shrimp larvae. Ziaei-Nejad et al. (2006) reported significantly higher specific activity of amylase and lipase in Indian white shrimp (*F. indicus*) when fed with a commercial *Bacillus* probiotic for 6 days compared to control. An enhanced feed digestion in the panaeids may be attributed to an increase in the digestive enzyme activity and a subsequent increase in the appetite of the treated shrimps, resulting in enhanced feed utilisation efficiency and body weight. Another possible explanation for growth enhancement is due to competitive exclusion, wherein probiotics produce bactericidal compounds or compete with pathogens for nutrients and adhesion sites, thereby creating an antagonistic environment for pathogen colonisation (Zokaeifar et al., 2012). In the present study, this mechanism was demonstrated by molecular detection of the halotolerant isolates in the gastrointestinal tract, along with a decrease in the *Vibrio* count in the GIT of the treated shrimps as compared to the control. These results are in agreement with Li et al., (2007) where a significant decrease in total *Vibrio* count was observed in the gut of *L. vannamei* exposed to *B. licheniformis* probiotic. The study suggests that *B. licheniformis* plays a role as a competitive exclusion agent which modifies the microflora of the gut and reduces the population of *Vibrio* spp. (Li et al.,2007). Similar results have also been documented in *P. monodon* administered with *Bacillus* spp. (Gullian et al., 2004), (Vaseeharan et al., 2003). Likewise, in *Penaeus japonicus* fed with *B. licheniformis* and *B. subtilis* (Zhang et al., 2011) and in *Marsupenaeus japonicus* fed with *Bacillus* spp. (Dong et al., 2013), a decrease in the *Vibrio* count of the gut was reported on exposure to probiotic bacteria as compared to control. In our study, as the halotolerant bacteria were added to the shrimp tanks since the post-larval stage of growth, we attribute the decrease in *Vibrio* count to competitive exclusion mechanism. These results are consistent with the *in vitro* experiments displaying antagonistic activity of these strains against *Vibrio* spp. Correlation analysis intriguingly revealed a significant association between yield; digestive enzymatic activity (amylase, protease, cellulase) and total *Vibrio* count ($p < 0.01$). This was similar to the results reported by Gamboa-Delgado et al.,(2003) where a positive correlation was observed between the digestive enzyme activity and body weight in juvenile shrimp *L. vannamei*.

5.3.2 Effect of SFSK4 on the water quality and Anti-*Vibrio* activity

The anti-*Vibrio* activity of SFSK4 against *V. harveyi* and *V. alginolyticus* was continuously assessed for 168 h with a gap of 24 h. SFSK4 treated shrimp tanks showed a significant ($p<0.01$) decrease in shrimp mortality by 50 % as compared to the tank challenged only with *V. alginolyticus* without SFSK4. When challenged with *V. harveyi*, SFSK4 shrimp tanks showed a significant ($p<0.01$) decrease in shrimp mortality by 53 % as compared to the tank challenged only with *V. harveyi.* The results confirmed the anti-*Vibrio* activity of SFSK4. Further, the histopathological analysis of the shrimp hepatopancreatic cells showed loss of membrane integrity and necrosis in shrimp challenged with the pathogens. Comparatively, an intact membrane and mild tissue disruption was seen in shrimp challenged with the pathogen along with SFSK4. Similar results were reported by Zokaeifar et al. (2014) where disease resistance and an improvement in immune response was observed in *Litopenaeus vannamei* treated with 10^8 CFU/mL of *Bacillus subtilis* for 8 weeks. In addition, Similar results were reported by Nimrat et al. (2012) where an improved growth and survival of *Litopenaeus vannamei* as well as improved tank water quality (decrease in ammonia and nitrite) was observed when *Litopenaeus vannamei* was administered with a consortium of *Bacillus* spp. isolated from the intestine of *Penaeus monodon.*

In our study, the assessment of water quality of laboratory trials showed a significant ($p<0.05$) decrease in the ammonia concentration of tanks treated with SFSK4 as compared to the control tanks. However, no significant difference was observed in the ammonia concentration when SFSK4 was applied to the farm tanks. Both, the lab-scale and the farm trial experiments showed a ten-fold decrease in *Vibrio* count in the shrimp tanks treated with SFSK4 as compared to the control. The results thus confirmed the anti-*Vibrio* activity of SFSK4 in an outdoor culture system. Siminar results were reported by Liu et al., (2010), where administration of 10^9 CFU/mL of *Bacillus subtilis* E20 to the larval rearing tank improved the immunity, disease resitance and survival of *Litopenaeus vannamei* but had no effect on the ammonia or nitrite concentration.

5.3.3 Immune system analysis

As shrimps possess a non-specific immune system, immunostimulation or vaccination may provide only temporary protection against specific pathogens. On the other hand, effective probiotic treatments would deliver broad-spectrum disease protection due to enhancement of serological immunity and competitive exclusion in the shrimp gut (Rengipat, 2000). Moreover, the need for environment-friendly aquaculture practices has triggered the demand for new probiotics in aquaculture (Liu et al., 2015). The cell wall of bacteria possess highly conserved signature molecules such as lipopolysaccharides (LPSs) or peptidoglycans (PGNs) which are absent in multicellular organisms (Vazquez et al., 2009). These molecules are recognized by the pattern recognition receptors of the host which in turn activate distinct signalling pathways that play an important role in stimulating the shrimp immune system (Vazquez et al., 2009). Shrimps have a relatively simple immune system; hence probiotics are expected to play a significant role in stimulating their immune response (Liu et al., 2014). Many researchers have reported the use of bacteria as effective probiotics to stimulate the immune system in fish and crustaceans. Studies have also demonstrated that using mixed cultures of probiotics improves feed conversion, total survival and final yield of farmed *Litopenaeus vannamei* (Wang et al., 2005).

In the present study, *Litopenaeus vannamei* was treated with a probiotic consortium SFSK4 comprising of four halotolerant bacteria. The probiotic consortium exhibited an enhanced immune stimulation resulting in improved resistance against shrimp pathogens as compared to the untreated control tank shrimps. Research has suggested that there is a direct link between haemolymph and the bacterial concentration in the water (Yanbo and Zirong 2006). An increase in the total haemocyte count in the hemolymph facilitates heightened immune competence during stress conditions, resulting in disease resistance in shrimps (Nicholson et al., 2000; Ziaei-Nejad et al., 2006 ; Chiu et al., 2007). Besides this, the components of the phenoloxidase system stimulate numerous cellular defence reactions such as phagocytosis, encapsulation, nodule formation and hemocyte migration. Activation of the phenoloxidase system has been explored by many scientists to measure immunostimulation in shrimps (Sharma et al., 2010). The results obtained in our study were comparable to other researchers that used probiotics to stimulate the immunity of cultured crustaceans. A significant increase in the total hemocyte count (by 47.6 %),

phenoloxidase (by 93.4 %) and phagocytic activity (by 28.7 %) as compared to the control was observed in shrimp exposed to SFSK4. The results were similar to Navinchandran et al. where *Penaeus monodon* fed with a diet supplemented with 0.4 % *Bacillus cereus* exhibited significantly higher ($p<0.05$) total hemocyte count, phenoloxidase and bactericidal activity as compared to the control (Navinchandran et al., 2013). Xia et al. (2013) reported an increase in the phenoloxidase concentration, phagocytic activity and bacterial clearance efficiency of *L. vannamei* when inoculated with *Arthrobacter* sp. CW9. In our study, an increasing trend in all the above mentioned immune parameters was observed at 60, 90 and 120 day analysis across all the treatment groups. A 28.9 % increase in the total plasma protein and 18.4 % increase in respiratory burst was observed as compared to the control at 120 day analysis. Respiratory burst has been widely used to estimate the defence mechanism against invading pathogens in shrimps (Chiu et al, 2007). The phagocytes in the hemolymph produce superoxide anions to attack intrusive pathogens during phagocytosis. A study conducted by Nikoskelainen et al. highlighted that addition of 10^5 CFU/mL of *Lactobacillus rhamnosus* stimulated the respiratory burst activity in *Oncorhynchusmykiss* (Nikoskelainen et al., 2003). Similarly a significant increase in the respiratory burst was observed in *Litopenaeus vannamei* fed with 10^7 CFU/ kg of basal diet containing 2007). A mixed consortium of *S. xiamenensis* A-2, *S. xiamenensis* A-1 and *A. veronii* A-7 when supplemented in the diet at 10^8 CFU/g improved the innate immune response and disease resistance in *Ctenopharyngodon idellus* (grass carp) as compared to those fed with a single probiotic (Wu et al., 2015). Wang and Gu (2010) reported increased growth and immune response in *Litopenaeus vannamei* treated with *Rhodopseduomonas palustris* GH642, *Lactobacillus acidophilus* RS058, and *Bacillus coagulans* NJ105 as water additives. The cumulative effect of the elevated THC, phenoloxidase, phagocytic activity, and respiratory burst coupled with bactericidal activity in our study, suggests that the halotolerant probiotic consortium SFSK4 stimulates the immune system of *Litopenaeus vannamei.* The study thus confirms that SFSK4 enhances both cellular and humoral response, thus rendereing *L. vannamei* more resistant to *V. harveyi* and *V. alginolyticus.*

Further, a proteomic analysis revealed about 1642 proteins which were identified using multiplex LC-MS/MS and SWATH MS analysis in control and SFSK4 *treated* shrimps. A total of 50 proteins showed significant expression changes between the two groups, which include 39 up-regulated and 11 down-regulated proteins The differentially expressed

proteins can be categorized as immune related proteins, stimulus response proteins, signal transduction proteins, hemocynins, glucose and lipid metabolism proteins, cytoskeleton proteins, DNA or protein binding proteins, oxidoreductases and transmembrane transporters. Four major immune modulation proteins were identified in the proteomic study which are involved in pattern recognition and cell mediated immune response. The shrimp cell mediated immune responses involves phagocytosis, apoptosis and invading pathogen encapsulation. The strong 20 fold upregulation of Caspase 2 in SFSK4 treated shrimp suggest the activation of cell mediated immune response in the host. Caspase 2 is evolutionarily most conserved caspase which plays an important role in apoptosis induced by several stimuli, including oxidative stress (Lopez-Cruzan et al., 2016). Interestingly, this was coupled with GTPase activating protein are involved in a variety of signal transduction pathways that regulate cell growth, intracellular trafficking, cell migration, and apoptosis (Scheele et al., 2007). Glucan pattern-recognition lipoprotein and β-1,3-glucan-binding protein were upregulated in SFSK4 treated shrimp which are involved in signaling pattern recognition receptor activity and innate immune response. β-glucan-binding proteins react with β-glucans and the formed glucan-BGBP complex induces degranulation of hemocytes and the activation of proPO system [Vargas-Albores et al, 2000]. Previous reports suggests that the dietary administration of **β**-1,3-glucan has also been documented to improve immunological responses and resistance against infection in *Penaeus monodon* pathogens (Chang *et al,* 2000).

Hemocyanin has been reported as a novel and important defense molecule in mollusks and arthropods. Besides its primary function as a respiratory protein, it has been suggested that hemocyanin could be functionally converted into phenoloxidase like enzyme, antiviral agent, antimicrobial protein, agglutinin and hemolysin (Zhang et al., 2009; Lee and Soderhall, 2002). In this study, hemocyanin expression was altered, 21 were up-regulated whereas 9 hemocyanin were down-regulated. Previous reports suggest that crustacean hemocyanin could generate some fragments with obvious antibacterial and antifungal activities (Destoumieux-Garzón, 2001; Lee and Soderhall, 2002). It is reported that three-quarters of C-terminal hemocyanin fragments showed up-regulation, but two-thirds of N-terminal fragments displayed down-regulation during TSV virus infection in hemocytes of *Penaeus vannamei* (Chongsatja, 2007). Overall, our study suggests that hemocyanin might play an important role in improvement of the immune activity of shrimp by generating multiple fragments with different functions and these fragments could be developed as a

proteomic marker of immune stimulation in shrimp. Similarly, hemolymph clottable protein functions mainly as a substrate for transglutaminase to form stable clots. It has been shown that polymerization of clottable proteins by the hemocyte transglutaminase can trigger hemolymph clotting in crustaceans (Komatsu, 1998). Moreover, the concentration of clottable protein is regulated to suit the growth status of shrimp. Here, we found that hemolymph clottable protein could also response to the probiont stimulation and might mediate the improvement of shrimp immunity.

We speculate that the lipopolysaccharides induction in the haemocytes lead to the oxidative stress which increased the production of reactive oxygen species (ROS) which gradually damages the macromolecules (DNA, proteins and lipids) present in the haemocytes and induced cell apoptosis.

5.4 Safety assessment of the SFSK4

As probiotics play a vital role in stimulating the immunity of the cultured organisms, it is very essential to ensure that they are not harmful to their respective hosts. The toxicity of live bacterial cultures or their bioactive metabolites on the target organisms must be analysed to rule out any adverse effect before commercial application (Neu, 2014). Snydman (2008) recorded three concerns pertaining to the safety of probiotics: occurrence of disease, toxic effect on the host gastrointestinal tract and the transfer of antibiotic resistant genes (Abe, 2010). Very few researchers have focussed on the toxicity evaluation of aquaculture probiotics. The purpose of our study was to determine if the formulated probiotic consortium SFSK4 produced any toxins that could be harmful to eukaryotic systems. The *in vitro* antibiotic susceptibility tests along with hemolysis test were used as the first level of assessment of potential toxicity of the live bacteria. The results indicated that all the halotolerant bacteria showed more that 95 % susceptibility to the 28 tested antibiotics. Studies have suggested that some bacteria possess resistance of “intrinsic type” (i.e., chromosomally encoded), generally because of membrane impermeability or lack of affinity of the antibiotic for the target bacteria or production of innate enzymes that inactivate the antibiotic (Kaktcham et al, 2018; Delcour 2009; Abebe et al, 2016). This type of resistance presents no risk of horizontal proliferation and does not possess safety concern (Salminen 1998).

A study conducted by Villasenor et al., 2012 reported that four *Bacillus* strains including *B. amyloliquefaciens* isolated from the gut of *Litopenaeus vannamei* exhibited no

hemolysis (γ hemolysis) whereas *B. licheniformis* strains showed α haemolysis with slight destruction of hemocytes. In our study, all the four bacteria *Bacillus licheniformis* TSK71, *Bacillus amyloliquefaciens* SK27, *Bacillus subtilis* SK07 and *Pseudomonas sp.* ABSK 55 exhibited no hemolysis (γ hemolysis) on human blood agar. The results indicate the absence of the production of hemolysin.

Further the LC_{50} analysis revealed that the formulated probiotic SFSK4 was avirulent to *Litopeneaus vannamei* when challenged at a maximum dosage of $1x10^{12}$ CFU/mL. This was evident from the healthy appearance, feeding habit and % survival of the cultured shrimps. The results were in agreement to Chu et al. (2010) where oral administration of probiotic *Bacillus* spp. at a dosage of 10^{11} CFU to *Carassius auratus gibelio* fingerlings exhibited no adverse effect on fish growth and activity. The results of LC_{50} analysis was also supported by the cytotoxicity studies, which confirmed the non-cytotoxic effect of the formulated probiotic on post-larvae and juvenile shrimps.

Some bacteria produce certain toxins known as “bacterial genotoxins” which are effectors that cause single and double strand DNA breaks in eukaryotic cells (Frisan 2015; Grasso 2015). Genotoxicity studies were carried out to ensure that the formulated probiotic does not produce genotoxins against its host. The comet assay that was performed to validate the safety and non toxic property of SFSK4, indicated that the formulated probiotic was non-toxic to the shrimp DNA at a cell concentration of $1x10^{10}$ and $1x10^{12}$ CFU/mL.

The consortia SFSK4 comprising of four salt pan bacterial isolates, three being Gram positive *Bacilli* and one Gram negative *Pseudomonas* species makes it a suitable combination as far as its distribution in the aquaculture pond is concerned. *Bacillus* cultures generally inhabit the sediments and the surface of sediments whereas Pseudomonads are ubiquitous in soil and water, assuring their continuous distribution and vigilance against opportunistic pathogens gaining entry into these aquaculture ponds. Further, these salt pan *Bacilli* and *Pseudomonas* cultures which were found to be tolerant to a wide range of salinities (0 to 5 %) shows a possibility of using this consortia in fresh water as well as sea water ecosystems for aquaculture.

SUMMARY

The thesis on "**Halotolerant bacteria as probiotics in shrimp aquaculture**" explores the potential of salt pan bacteria as probiotics in shrimp aquaculture. The salient features of our research are presented below:

Screening of halotolerant bacteria for probiotic potential *in vitro*

- A total of 300 halotolerant bacteria were screened for production of bioactive metabolites against shrimp pathogens. Anti-*Vibrio* activity was observed in 29 bacteria against different *Vibrio* sp. viz. *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* by well diffusion assay. Eighteen cultures exhibited anti-*Vibrio* activity against more than two shrimp pathogens with more than 15 mm zone of inhibition. These cultures were further confirmed for Anti-*Vibrio* activity by co-culture method.
- Co-culture method revealed that bacteria SK07 and SK27 decreased the colony count of *V. parahaemolyticus* by more than 2 fold, followed by FSK444 and TSK71. Similarly, more than 50 % decrease in the colony count of *V. harveyi* and *V. alginolyticus* was observed when co-cultured individually with culture SK07 and ABSK55. A four – fold decrease in *V. alginolyticus* was observed when co-cultured with ABSK55. Culture TSK71 remarkably decreased the counts of *V. alginolyticus* by three-fold and *V. vulnificus* by two-fold as compared to the control.
- The ability of 300 halotolerant bacteria to degrade ammonia was also evaluated. Twelve bacteria showed more than 40% decrease in ammonia concentration in 7 days when tested *in vitro*.
- The best cultures (twenty five) showing anti-*Vibrio* activity and ammonia degrading ability were further assessed for their probiotic potential.
- Out of the 25 screened cultures, 18 produced amylase, 14 protease, 7 cellulase and 12 produced lipase. Mucus adhesion of more than 70 % was observed in 9 cultures which were also found to be tolerant to pH (5 to 9), salinity (0 to 5 %), bile salts (0 to 3%) with no hemolytic activity.

- Based on the anti-*Vibrio* activity, ammonia degrading ability, mucus adhesion, enzyme production, tolerance to pH, bile salts, salinity and growth on pond water water agar, six cultures were selected as candidate probionts and experimented at different cell concentrations (10^8 to 10^{10} CFU/mL) to study their effect on *Litopenaeus vannamei*. These cultures were found to be biocompatible when grown synergistically, each exhibiting a unique growth profile.

***In vivo* studies with individual Halotolerant bacteria**

- *In vivo* studies with the 6 cultures revealed that 4 cultures viz. SK07, SK27, ABSK55 and TSK71 showed promising growth promoting activity and immune stimulation.
- Culture ABSK 55 at a cell concentration of 10^9 and 10^{10} CFU/mL significantly decreased the ammonia concentration in the tanks.
- The final body weight of shrimp treated with halotolerant isolates SK07: (10^8, 10^9 and 10^{10} CFU/mL), SK27: (10^9, 10^{10} CFU/mL) and TSK71 (10^9 and 10^{10} CFU/mL) was remarkably higher than control.
- The results of immune system analysis revealed the ability of halotolerant bacteria ABSK55, SK07 and SK27 to stimulate the immune response of *Litopenaeus vannamei* at varying cell concentrations. Shrimp treated with ABSK55 (10^8, 10^9 and 10^{10} CFU/mL) showed highest total hemocyte count, phagocytic activity and phenoloxidase activity.

Formulation of the bacterial consortium

- The four halotolerant cultures showing promising activity individually on *Litopenaeus vannamei* were selected to formulate the bacterial consortium (denoted as SFSK4) at a cell concentration of 10^9 CFU/mL.
- The consortium comprising of the above lyophilized bacteria was stable for 120 days at room temperature (28 ± 2°C) and 180 days at 6 ± 2°C. The consortium synergistically showed anti-*Vibrio* activity, ammonia degrading ability and enzyme production *in vitro*. The shelf life and Anti-*Vibrio* activity of the consortium was found to be higher than the individual cultures.
- Based on the morphological, biochemical and molecular characterization, the four Halotolerant bacteria were identified as *Bacillus subtilis* strain SK07, *Bacillus*

amyloliquefaciens strain SK27, *Pseudomonas sp*. strain ABSK55 and *Bacillus licheniformis* strain TSK71.

Lab-scale trial of the formulated consortium on *Litopenaeus vannamei*

- The pH, temperature, TDS, salinity, DO, BOD, alkalinity and Total bacteria count were comparable with each other across the different treatment sets. The total *Vibrio* count and ammonia concentration was significantly lower in the shrimp tanks treated with SFSK4 as compared to the control.
- The test probiotic SFSK4 significantly increased the average body weight and feed efficiency of the shrimp in 120 days.
- A significant increase in the total hemocyte count, phagocytic activity, phenoloxidase activity, total plasma protein and bacterial clearance efficiency was observed in shrimp treated with SFSK4.
- The pathogen challenge test revealed more than 50 % decrease in shrimp mortality when exposed to SFSK4.

Safety assessment of SFSK4

- The four cultures used in SFSK4 were susceptible to most of the tested antibiotics. Bacteria SK07 was susceptible to 21 antibiotics, SK27 to 23, ABSK55 to 19 and TSK71 to 21 antibiotics, suggesting the absence of antibiotic resistant genes in the selected strains.
- The results of LC_{50} analysis confirmed the non-virulence of SFSK4 to *Litopenaeus vannamei* post larvae.
- Cytoxicity and genotoxicity tests confirmed the nontoxic nature of SFSK4 to *Litopenaeus vannamei*.

Farm trial of SFSK4 on *Litopenaeus vannamei*

- On confirming the safety of SFSK4 farm trial were carried out in an outdoor culture system.
- Farm trials confirmed the ability of SFSK4 to enhance the growth and feed efficiency of *Litopenaeus vannamei.* A 22 % increase in yield was observed.

- SFSK4 significanty increased the amylase, protease and lipase activity in the shrimp gut by 33 %, 49 % and 57 % as compared to the control.
- Bacteriological analysis of the shrimp gut revealed the presence of *Bacillus subtilis, Bacillus licheniformis* and *Bacillus amyloliquefaciens* on treatment with SFSK4. A ten-fold decrease in the total *Vibrio* count in the shrimp gut was also observed as compared to the control.
- The total hemocyte count, phagocytic activity, phenoloxidase activity, total plasma protein and bacterial clearance efficiency was found to be significantly higher in shrimp treated with SFSK4.
- Proteomic analysis of the shrimp hemolymph revealed the differential expression of 50 immune proteins, in which 39 were upregulated and 11 were downregulated. Four major immune modulation proteins involved in pattern recognition and cell mediated immune response (phagocytosis, apoptosis and invading pathogen encapsulation) in crustaceans were identified in SFSK4 treated shrimp hemolymph.

The consortium "SFSK4", comprising of *Bacillus subtilis* strain SK07, *Bacillus amyloliquefaciens* strain SK27, *Pseudomonas sp*. strain ABSK55 and *Bacillus licheniformis* strain TSK71 showed remarkable results in both lab-scale and field trial experiments which validates its function as a probiont in aquaculture for *Litopenaeus vannamei*.

CONCLUSION

This study reveals that halotolerant bacteria have the ability to stimulate the growth and immunity of *Litopenaeus vannamei*, thus increasing the yield and survival of shrimp against pathogenic *Vibrio* spp., which are a great menace in aquaculture farms. The formulated consortium SFSK4 proved to have a broad spectrum effect in promoting the digestive enzyme activity, augmenting the immune response and competitively excluding shrimp pathogens. Therefore, we conclude that SFSK4 is a multifunctional single probiotic with immense potential to combat vibriosis in shrimp aquaculture. To the best of our knowledge, this is the first study to report the probiotic potential of salt pan bacteria in shrimp aquaculture.

BIBLIOGRAPHY

- Abe F, Muto M, Yaeshima T, Iwatsuki K, Aihara H, Ohashi Y, Fujisawa T (2010) Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability. Anaerobe 16(2):131-136.
- Abebe E, Tegegne B, Tibebu S (2016) A Review on Molecular Mechanisms of Bacterial Resistance to Antibiotics. EJAS 8 (5): 301-310.
- Adams CA (2010) The probiotic paradox: live and dead cells are biological response modifiers. Nutr Res Rev 23:37-46.
- Alariya SS, Sethi S, Gupta S, Lal B, Lal GB (2013) Amylase activity of a starch degrading bacteria isolated from soil. Arch Appl Sci Res 5:15–24.
- Amin M, Rakhisi Z, AZ Ahmady (2015) Isolation and Identification of Bacillus Species From 393 Soil and Evaluation of Their Antibacterial Properties. Avicenna J Clin Microb Infec 2:10–13.
- Annamalai N, Rajeswari MV, Elayaraja S, Balasubramanian T (2013) Thermostable, haloalkaline cellulase from *Bacillus halodurans CAS 1* by conversion of lignocellulosic wastes. Carbohydr Polym 94:409–415.
- Ansari ZA, Pandiyarajan RS, Ayajuddin M (2012) Khazan land of Goa and its fishery potential. Fishcoop 3–6.
- Antony SP, Singh BIS, Jose RM, Kumar APR , Philip R (2011) Antimicrobial peptide gene expression in tiger shrimp, *Penaeus monodon* in response to Gram-positive bacterial probionts and white spot virus challenge. Aquaculture 316: 6–12.
- APHA (2012) Standard Methods for the Examination of Water and Waste Water. 22nd Edition, American Public Health Association, American Water Works Association, Water Environment Federation.
- Apostolou E, Kirjavainen PV, Saxelin M, Rautelin H, Valtonen V, Salminen S, Ouwehand AC, (2001) Good adhesión properties of probiotics: a potential risk for bacteremia? FEMS Immunol Med Microbiol 31:35-39.
- Araneda M, Pérez EP, Gasca-leyva E (2008) White shrimp *Penaeus vannamei* culture in freshwater at three densities: condition state based on length and weight. Aquaculture 283(1-4): 13-18.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403-410.

- Apud, F. D. (1985). Extensive and semi-intensive culture of prawn and shrimp in the Philippines. In Taki, Y., Primavera, J. H., Llobrera, J. A. (Eds.), Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps, 4-7 December 1984 (pp. 105-113). Iloilo City, Philippines: Southeast Asian Fisheries Development Center, Aquaculture Department.
- Austin B, Baudet E, Stobie M (1992) Inhibition of bacterial fish pathogens by Tetraselmis suecica. J Fish Dis 15:55-61.
- Azad MAK, Islam SS, Sithi IN, Ghosh AK, Banu GR, Khandaker JB, Huq A (2018) Effect of probiotics on immune competence of giant freshwater prawn Macrobrachium rosenbergii. Aquac Res 50(2): 644-657.
- Balakrishna A (2013) *In vitro* evaluation of adhesion and aggregation abilities of four potential probiotic strains isolated from guppy (Poecilia reticulata). Braz Arch Biol Technol 56: 793–800.
- Balakrishna A, Keerthi TR (2012) Screening of potential aquatic probiotics from the major microflora of guppies (Poecilia reticulata). Front Chem Sci Eng 6(2): 163–173.
- Balcazar JL, Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Muzquiz JL (2006) The role of probiotics in aquaculture. Vet Microbiol 114(3-4):173-186.
- Balcazar JL, Vendrell D, de Blas I, Ruiz-Zarzuela I, Girones O, Muzquiz JL (2007) *In vitro* competitive adhesion and production of antagonistic compounds by lactic acid bacteria against fish pathogens. Vet Microbiol 122: 373–380.
- Ballav S (2016) Exploring the Biodiversity of culturable actinomycetes in the salt pans of Goa and their anti-microbial potential. Thesis, Goa University.
- Ballav S, Kerkar S, Thomas S, Augustine N (2015) Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites. J Biosci Bioeng 119(3):323-330.
- Balouiri M, Sadiki M, Ibnsouda SK (2016) Methods for *in vitro* evaluating antimicrobial activity: A review. Int J Pharmaceut Anal 6(2): 71–79.
- Bartakke A (2018) Formulation of a biofertilizer for salt tolerant rice grown in khazan soils, using salt pan bacteria. Thesis, Goa University.
- Bauer AW, Kirby MM, Sherris JC, Tuurck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45:493–496.

- Béné C, Barange M, Subasinghe R, Pinstrup-Andersen P, Merino G, Hemre GI, Williams M (2015) Feeding 9 billion by 2050—putting fish back on the menu. Food Security 7(2):261-274.
- Bergey DH, Krieg NR, Holt JG (1984) Bergey's Manual of Systematic Bacteriology Volume 1. 1st Edition John G. Holt, Editor-in-Chief, Baltimore, MD. Gram negative Bacteria of general, medical, or industrial importance.
- Bratbak G (1993) Microscope methods for measuring bacterial biovolume: Epifluoresence microscopy, Scanning electron microscopy and transmission electron microscopy. In: Kemp PF, Cole JJ, Sherr BF, Sherr EB (ed) Handbook of methods in aquatic microbial ecology. CRC Press, , pp 312-314.
- Bernal MG, Marrero RM, Campa-Córdova ÁI, Mazón-Suástegui, JM (2016) Probiotic effect of Streptomyces strains alone or in combination with *Bacillus* and *Lactobacillus* in juveniles of the white shrimp *Litopenaeus vannamei.* Aquacult Int 25 (2):927–939.
- Bondad-Reantaso MG, Subasinghe RP, Arthur JR, Ogawa K, Chinabut S, Adlard R, Tan Z, Shariff M (2005). Disease and health management in Asian aquaculture. Vet Parasitol 132: 249–272.
- Boonthai T, Vuthiphandchai V , Nimrat S (2011) Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaeus monodon*). Aquacult Nutr 17: 633–644.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
- Bratbak G (1993) Microscope method for measuring bacterial biovolume: Epifluoresence microscopy, Scanning electron microscopy and transmission electron microscopy. In: Paul F. Kemp, Jonathan J. Cole, Barry F. Sherr, Evelyn B. Sherr (eds) Handbook of methods in aquatic microbial ecology. CRC press, pp 312-314.
- Bray WA, Lawrence AL, Leung-Trujillo JR (1994) The effect of salinity on growth and survival of *Penaeus vannamei*, with observations on the interaction of IHHN virus and salinity. Aquaculture 122:133-146.
- Bray WA, Moya M, Lawrence AL, Collins CA (1999) Brood stock culture of *Litopenaeus vannamei* in low salinity desert groundwater of 2.3 ppt: Summary of

growth and sperm development. In: Book of Abstracts World Aquacult Soc Ann Conf. Sydney,pp:101.

- Cabello F (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol 8(7):1137–1144.
- Campos CA, Rodriguez O, Calo-Mata P, Prado M, Barros Velázquez J (2006) Preliminary characterization of bacteriocins from *Lactococcus lactis*, *Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (*Psetta maxima*). Food Res Int 39:356-364.
- Castex M, Lemaire P,Wabete N and Chim L (2010) Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge. Fish Shellfish Immunol 28: 622–631.
- Cha JH, Rahimnejad S, Yang SY, Kim KW, Lee KJ (2013) Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. Aquaculture 402–403: 50–57.
- Chang C, Liu W, Shyu C, (2000) Use of prawn blood agar hemolysis to screen for bacteria pathogenic to cultured tiger prawns *penaeus monodon.* Dis Aquat Organ 43: 153–157.
- Chen JC, Liu PC, Lei SC (1990) Toxicity of ammonia and nitrite to *Penaeus monodon* adolescents. Aquaculture 89: 127–137.
- Chiu CH, Guu YK, Liu CH, Pan TM, Cheng W (2007) Immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*. Fish Shellfish Immunol 23:364-377.
- Chong-Robles J, Charmantier G, Boulo V, Lizárraga-Valdéz J, Enríquez-Paredes LM, Giffard-Mena I. (2014) Osmoregulation pattern and salinity tolerance of the white shrimp *Litopenaeus vannamei* (Boone, 1931) during post-embryonic development. Aquaculture 422–423:261-267.
- Chongsatja PO, Bourchookarn A, Lo CF, Thongboonkerd V, Krittanai C (2007) Proteomic analysis of differentially expressed proteins in Penaeus vannamei hemocytes upon Taura syndrome virus infection. J Proteomics 7(19):3592-3601.

- Chu W, Lu F, Zhu W, Kang C (2011) Isolation and characterization of new potential probiotic bacteria based on quorum-sensing system. J Appl Microbiol 110(1):202-208.
- Chythanya R, Karunasagar I, Karunasagar I (2002) Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas I-2* strain. Aquaculture 208(1-2):1–10.
- Claus D, Berkeley RCW (1986) Genus Bacillus In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's Manual of Systematic Bacteriology vol 2. The Williams & Wilkins Co, Baltimore, pp 1105-1139.
- Collins CH, Lyne PM (1984) Microbiological Methods 5th edn. Butterworths Co Ltd, London.
- Cruz PM, Ibanez AL, Hermosillo OAM, Saad HCR (2012) Use of Probiotics in Aquaculture. ISRN Microbiol 1–13.
- Daniels CL, Merrifield DL, Boothroyd DP, Davies, SJ, Factor JR , Arnold KE (2010) Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (Homarus gammarus L.) larvae growth performance, gut morphology and gut microbiota. Aquaculture 304:49–57.
- Das S,Ward LR, Burke C (2010) Screening of marine *Streptomyces* spp. for potential use as probiotics in aquaculture. Aquaculture 305:32–41.
- DasSarma S, DasSarma P (2017) Halophiles. In: eLS. Wiley, UK, pp 1–13.
- Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74 (3): 417–433.
- Dcosta A, Shyama SK, Kumar P and Furtado S (2017) The Backwater Clam (*Meretrix casta*) as a bioindicator species for monitoring the pollution of an estuarine environment by genotoxic agents. Mutat Res Genet Toxicol Environ Mutagen 825:8-14.
- Delcour AH (2009) Outer Membrane Permeability and Antibiotic Resistance. Biochim Biophys Acta 1794(5): 808–816.
- Destoumieux-Garzón D, Saulnier D, Garnier J, Jouffrey C, Bulet P, Bachere E (2001) Crustacean Immunity: Antifungal Peptides Are Generated From The C-terminus Of Shrimp Hemocyanin In Response To Microbial Challenge. 276(50): 47070-47077.
- Dieu BTM, Vlak JM, Zwart MP (2011) Effects of extensive and intensive shrimp farming on the genetic composition of white spot syndrome virus populations. In:

Bondad-Reantaso MG, Jones JB, Corsin F Aoki T (eds) Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Malaysia, pp 145-156.

- El-Haroun ER, Goda AMAS, Chowdhury MAK (2006) Effect of dietary probiotic biogens supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia Oreochromis niloticus (L). Aquac Res 37:1473–1480.
- Elshaghabee FMF, Rokana N, Gulhane RD, Sharma C, Panwar H (2017) Bacillus As Potential Probiotics: Status, Concerns, and Future Perspectives. Front Microbiol 8: 1490.
- Enache M, Kamekura M (2010) Hydrolytic enzymes of halophilic microorganisms and their economic values. Rom J Biochem 47: 47-59.
- Farzanfar A (2006) The use of probiotics in shrimp aquaculture. FEMS Immunol Med Microbiol 48:149–158.
- FAO (2010) Regional review on status and trends in aquaculture development in Asia Pacific. pp 89.
- FAO (2016) The State of World Fisheries and Aquaculture. pp 200.
- Fernandes R, Sridhar M, Sridhar N (2011) Effect of lactic acid bacteria administered orally on growth performance of *Penaeus indicus* (H.Milne Edwards) juveniles. Res J Microbiol 6(5):466–479.
- Fernandes S, Kerkar S, Leitao J, Mishra A (2019) Probiotic Role of Salt Pan Bacteria in Enhancing the Growth of Whiteleg Shrimp, *Litopenaeus vannamei*. Probiotic and Antimicro. prot. https://doi.org/10.1007/s12602-018-9503-y
- Fuller R (1989) Probiotics in man and animals. J Appl Bacteriol 66(5):365–378.
- Fuller R (1992) History and development of probiotics. In: Fuller R (ed) Probiotics: the Scientific Basis. Chapman & Hall, New York, pp 1–8.
- Gao XY, Liu Y, Miao, LL, Li EW, Hou TT, Liu ZP (2017) Mechanism of anti-*Vibrio* activity of marine probiotic strain *Bacillus pumilus H2*, and characterization of the active substance. AMB Express 7(1): 23
- Gesheva V, Vasileva-Tonkova E (2012) Production of enzymes and antimicrobial compounds by halophilic Antarctic Nocardioides sp.grown on different carbon sources. World J Microbiol Biotechnol 28:2069–2076.
- Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268.

- Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens AH2*, a possible probiotic treatment of fish. Appl Environ Microbiol 65(3):969-973.
- Golterman HL (1991) Direct nessierization of ammonia and nitrate in fresh-water. Annls Limnol. 27 (1): 99-101.
- Golterman HL (1983) The Winkler Determination. In: Gnaiger E, Forstner H (eds) Polarographic Oxygen Sensors. Springer, Berlin, pp 346-351.
- Gomez GD, Balcazar JL (2008) A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunol Med Microbiol 52: 145–154.
- Gómez RG, ShenMA (2008) Influence of probiotics on the growth and digestive enzyme activity of white Pacific shrimp (*Litopenaeus vannamei*). J Ocean Univ China 7:215–218.
- Gomez-Gil B, Roque A, Velasco-Blanco G (2002) Culture of *Vibrio alginolyticus C7b*, a potential probiotic bacterium, with the microalga Chaetoceros muelleri. Aquaculture 211 (1):43-48.
- Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens AH2*, a Possible Probiotic Treatment of Fish. J Appl Environ Microbiol 65 (3): 969-973.
- Gullian M, Thompson F, Rodriguez J (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture 233:1–14.
- Gunalan B (2015) Semi-intensive Culture Techniques for Shrimp Farming. In: Perumal S, AR T, Pachiappan P (eds) Advances in Marine and Brackishwater Aquaculture. Springer, New Delhi, 151-162.
- Guo J, Liu K, Cheng S, Chang C, Lay Y, Yang J, Chen T, (2009) Selection of probiotic bacteria for use in shrimp larviculture. Aquac Res 40: 609-618.
- Gupta A, Dhawan A (2013) Probiotic based diets for freshwater prawn Macrobrachium rosenbergii (de Man). Indian J Fish 60:103–109.
- Hai N, Fotedar R (2009) Comparison of the effects of the prebiotics (Bio-Mos® and β-1,3-D-glucan) and the customised probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). Aquaculture 289: 310–316.

- Hai NV (2015) The use of probiotics in aquaculture. J Appl Microbiol 119 (4):917-935.
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98.
- Haraldsson C, Anderson LG, Hassellöv M, Hulth S, Olsson K (1997) Rapid, high-precision potentiometric titration of alkalinity in ocean and sediment pore waters. Deep Sea Res Part 1 Oceanogr Res 44(12): 2031–2044.
- Hasan B, Guha B, Datta S (2012) Optimization of feeding efficiency for cost effective production of *Penaeus monodon Fabricius* in Semi-intensive pond culture system. J Aquac Res Dev 3: 1 – 7.
- Henriques AO, Moran CP (2000) Structure and assembly of the bacterial endospore coat. Methods 20:95–110.
- Hoseinifar SH, Zare P, Merrifield DL (2010) The effects of inulin on growth factors and survival of the Indian white shrimp larvae and postlarvae (*Fenneropenaeus indicus*). Aquac Res:1365–2109.
- Huang F, Pan L, Lv N, Tang X (2017) Characterization of novel Bacillus strain N31 from mariculture water capable of halophilic heterotrophic nitrification–aerobic denitrification. J Biosci Bioeng 124(5): 564–571.
- Hyronimus B, Le Marrec C, Sassi AH, Deschamps A (2000) Acid and bile tolerance of spore-forming lactic acid bacteria. Int. J. Food Microbiol. 61: 193–197.
- Ichimatsu T, Mizuki E, Nishimura K, Akao T, Saitoh H, Higuchi K, Ohba M (2000) Occurrence of *Bacillus thuringiensis* in fresh waters of Japan. Curr Microbiol 40: 217–220.
- Irianto A, Austin B (2002) Probiotics in aquaculture. J Fish Dis 25: 633–642.
- Irianto A, Austin B (2002) Use of probiotics to control furunculosis in rainbow trout, Oncorhynchus mykiss (Walbaum). J Fish Dis 25:1-10.
- Kaktcham PM, Temgoua JB, Zambou FN, Diaz-Ruiz G, Wacher C, Pérez-Chabela ML (2018) In Vitro Evaluation of the Probiotic and Safety Properties of Bacteriocinogenic and Non-Bacteriocinogenic Lactic Acid Bacteria from the Intestines of Nile Tilapia and Common Carp for Their Use as Probiotics in Aquaculture. Probiotics & Antimicro Prot 10:98–109.

- Kamat T (2012) A study of an antibacterial compound from salt pans of Goa. Thesis, Goa University.
- Kamat T and Kerkar S (2011) Bacteria from Salt Pans: A Potential Resource of Antibacterial Metabolites. Recent Res Sci Technol 3(9):46-52.
- Kanwar SS, Verma HK, Kaushal RK, Gupta R, Chimni SS, Kumar Y, Chauhan GS (2005) Effect of solvents and kinetic parameters on synthesis of ethyl propionate catalysed by poly (AAc-co-HPMAcl- MBAm)-matrix-immobilized lipase of Pseudomonas aeruginosa BTS-2. World J Microbiol Biotechnol 21:1037–1044.
- Karunasagar I, Pai R, Malathi GR (1994) Mass mortality of Penaeus monodon larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture 128:203–209.
- Kathleen MM, Samuel L, Felecia C, Reagan EL, Kasing A, Lesley M, Toh SC (2016) Antibiotic resistance of diverse bacteria from aquaculture in Borneo. Int J Microbiol 2016 (4):1-9.
- Kautsky N, Ronnback P, Tedengren M, Troell M (2000) Ecosystem perspectives on management of disease in shrimp pond farming. Aquaculture 191 (1-3):145-161.
- Kerkar S (2004) Ecology of hypersaline microorganisms. In: Ramaiah N (ed) Marine microbiology facets and opportunities. NIO Goa, India, pp 53–67.
- Kesarcodi WA, Kaspar H, Lategan MJ, Gibson L, (2008) Probiotics in aquaculture: the need principles and mechanisms of action and screening processes. Aquaculture 274 (1): 1-14.
- Keysami MA, Saad CR, Sijam K, Daud HM and Alimon AR (2007) Effect of Bacillus subtilis on growth development and survival of larvae Macrobrachium rosenbergii (de Man). Aquac Nutr 13: 131–136.
- Kitikiew S, Chen J, Putra DF, Lin Y, Yeh S, Liou C (2013) Fucoidan effectively provokes the innate immunity of white shrimp *Litopenaeus vannamei* and its resistance against experimental *Vibrio alginolyticus* infection. Fish Shellfish Immunol 34: 280–290.
- Komatsu, M, Ando, S, (1998) A very high-density lipoprotein with clotting ability from hemolymph of sand crayfish Ibacus ciliatus. Biosci Biotechnol Biochem 62: 459–463.

- Kongnum K, Hongpattarakere T (2011) Effect of *Lactobacillus plantarum* isolated from digestive tract of wild shrimp on growth and survival of white shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi*. Fish Shellfish Immunol 32:170–177.
- Krieg NR, Ludwig WWW, Hedlund BP, Paster BJ, Staley JT, Ward N, Brown D, Parte A (1989) Bergey's Manual of Systematic Bacteriology Volume 4, Springer-Verlag, New York.
- KM Cheng, Hu CQ, Liu YN, Zheng SX, and Qi XJ (2006) Effects of dietary calcium, phosphorus and calcium/phosphorus ratio on the growth and tissue mineralization of *Litopenaeus vannamei* reared in low-salinity water. Aquaculture, 251 (2-4): 472–483.
- Krummenauer D, Peixoto S, Cavalli RO, Poersch LH, Wasielesky W (2011) Superintensive Culture of White Shrimp, *Litopenaeus vannamei*, in a Biofloc Technology System in Southern Brazil at Different Stocking Densities. J World Aquacult Soc 42 (5):726–733.
- Kuddus SM, Ahmad RIZ (2013) Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. Genet Eng Biotechnol J 11 (1): 39–46.
- Kuhn DD, Smith SA, Boardman GD, Angier MW, Marsh L, Flick GJ (2010) Chronic toxicity of nitrate to Pacific white shrimp, *Litopenaeus vannamei*: Impacts on survival, growth, antennae length, and pathology. Aquaculture 309:109–114.
- Kumar P, Shyama SK, Dcosta A, Samit K et al (2017) Evaluation of DNA damage induced by gamma radiation in gill and muscle tissues of Cyprinus carpio and their relative sensitivity. Ecotoxicol Environ Saf 144: 166-170.
- Kumar S, Karan R, Kapoor S, Singh SP, Khare SK (2012) Screening and isolation of halophilic bacteria producing industrially important enzymes. Braz J Microbiol 43:1595–1603
- Kumar V, Roy S, Meena DK, Sarkar UK (2016) Application of Probiotics in Shrimp Aquaculture: Importance, Mechanisms of Action, and Methods of Administration. Rev Fish Sci Aquac 24(4): 342-368.
- Lalloo R, Moonsamy G, Ramchuran S, Görgens J, Gardiner N (2010) Competitive exclusion as a mode of action of a novel Bacillus cereus aquaculture biological agent. Lett Appl Microbiol 50(6): 563–570.

- Lalloo R, Ramchuran S, Ramduth D, Görgens J, Gardiner N (2007). Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish. J Appl Microbiol, 103(5): 1471–1479.
- LaPatra SE, Fehringer TR, Cain KD (2014) A probiotic Enterobacter sp. provides significant protection against *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) after injection by two different routes. Aquaculture 433:361–366.
- Lara-Flores M, Aguirre-Guzman G (2009) The use of probiotic in fish and shrimp aquaculture. A review. In: Perez-Guerra N, Pastrana-Castro L (eds) Probiotics: Production, Evaluation and Uses in Animal Feed. Research Signpost, India, pp 75–90.
- Lazado CC, Caipang CM, Estante EG (2015) Prospects of host associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. Fish Shellfish Immunol 45(1):2-12.
- Lee SY and Soderhall K (2002) Early events in crustacean innate immunity. Fish Shellfish Immunol 12:421–437.
- Li E, Wang X, Chen K, Xu C, Qin JG, and Chen L (2017) Physiological change and nutritional requirement of Pacific white shrimp *Litopenaeus vannamei* at low salinity. Rev Aquacult 9: 57–75.
- Li E, Xu C, Wang X, Wang S, Zhao Q, Zhang M, Qin JG, Chen L (2018). Gut Microbiota and its Modulation for Healthy Farming of Pacific White Shrimp *Litopenaeus vannamei*. Rev Fish Sci Aquac 26(3): 381–399.
- Li J, Tan B, Mai K, Ai Q, Zhang W, Liufu Z, Xu W (2008) Immune responses and resistance against *Vibrio parahaemolyticus* induced by probiotic bacterium *Arthrobacter XE-7* in Pacific white shrimp, *Litopenaeus vannamei*. J World Aquacult Soc 39: 477–489.
- Li J, Xu Y, Jin L, Li X (2015) Effects of a probiotic mixture (Bacillus subtilis YB-1 and Bacillus cereus YB-2) on disease resistance and non-specific immunity of sea cucumber, Apostichopus japonicus (Selenka). Aquac Res 46(12): 3008-3019.
- Li S, Willits DH, Browdy CL, Timmons MB, Losordo TM (2009) Thermal modeling of greenhouse aquaculture raceway systems. Aquacult Eng 41:1–13.
- Liang YL, Zhang Z, Wu M, Wu Y, Feng JX (2014) Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region

of China and optimization of cellulase production by *Paenibacillus terrae ME27-1*. Biomed Res Int 2014:1–13

- Liao IC, Chien YH (2011) The Pacific White Shrimp, *Litopenaeus vannamei*, in Asia: The World's Most Widely Cultured Alien Crustacean. In: Galil B, Clark P, Carlton J (eds) In the Wrong Place - Alien Marine Crustaceans: Distribution, Biology and Impacts. Invading Nature - Springer Series in Invasion Ecology vol 6, Dordrecht.
- Limhang K, Limsuwan C, Chuchird N (2005) A comparison of growth rate and production between rearing black tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) in low salinity water. Fisheries : 420-427.
- Lin YC, Chen JC (2001) Acute toxicity of ammonia on *Litopenaeus vannamei* Boone juveniles at different salinity levels. J Exp Mar Biol Ecol 259:109–119.
- Lin YC, Chen JC (2003) Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. Aquaculture 224:193–201.
- Lin YC, Chen JC, Li CC, Wan ZWM, Suhaili ASNA, Kuo YH, Chang YH, Chen LL, Tsui WC, Chen YY (2012) Modulation of the innate immune system in white shrimp *Litopenaeus vannamei* following long-term low salinity exposure. Fish Shellfish Immunol 33: 324–331.
- Little D, Newton R, Beveridge M (2016) Aquaculture: A rapidly growing and significant source of sustainable food? Status, transitions and potential. Proc Nutr Soc 75(3) : 274-286.
- Liu CH, Chiu CS, Ho PL , Wang SW, (2009) Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease-producing probiotic, *Bacillus subtilis E20*, from natto. J Appl Microbiol 107: 1031- 1041.
- Liu KF, Chiu CH, Shiu YL, Cheng W, Liu CH (2010) Effects of the probiotic, *Bacillus subtilis E20*, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish Shellfish Immunol 28: 837–844.
- Liu W, Bao Q, Jirimutu, Qing M, Siriguleng, Chen X, Sun T, Li M, Zhang J, Yu J, Bilige M, Sun T, Zhang H (2012) Isolation and identification of lactic acid bacteria from Tarag in Eastern Inner Mongolia of China by 16S rRNA sequences and DGGE analysis. Microbiol Res 167:110–115.

- Liu X, Steele JC, Meng XZ (2017). Usage, residue, and human health risk of antibiotics in Chinese aquaculture: A review. Environ Pollut 223:161–169.
- Liu XF, Li Y, Li JR, Cai LY, Li XX, Chen JR, Lyu SX (2015) Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahaemolyticus* for use as probiotics in aquaculture. World J Microbiol Biotechnol 31(5) : 795–803.
- Llewellyn MS, Boutin S, Hoseinifar SH, Derome N (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Front. Microbiol.* 5:207.
- Lloret J (2010) Human health benefits supplied by Mediterranean marine biodiversity. Mar Pollut Bull 60(10): 1640-1646
- Longo SB, Clark B, York R, Jorgenson AK (2019) Aquaculture and the displacement of fisheries captures. Conservation Biology. doi:10.1111/cobi.13295.
- Lopez-Cruzan M, Sharma R, Tiwari M, Karbach S, Holstein D, Martin CR, Lechleiter JD, Herman B (2016) Caspase-2 resides in the mitochondria and mediates apoptosis directly from the mitochondrial compartment. Cell Death Discov pii: 16005.
- Louis S, Nelly W, Dominique A, Jean-René M, Marie P, Cong Z, Lindivata M, Bouloa V, Dominique P (2018) Survival improvement conferred by the *Pseudoalteromonas* sp. NC201 probiotic in *Litopenaeus stylirostris* exposed to *Vibrio nigripulchritudo* infection and salinity stress. Aquaculture 495:888-889.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1): 265–275.
- Lucas JS, Southgate PC, Tucker CS (2018) Technology & Engineering. Aquaculture: Farming Aquatic Animals and Plants. A clear illustration of the important role of aquaculture in supporting food security, livelihoods, and economic development around the world. Wiley-Blackwell.
- Luis-Villasenor E, Macias-Rodriguez ME, Gomez-Gil B, Ascencio-Valle F, Campa-Cordova A (2011) Beneficial effects of four Bacillus strains on the larval cultivation of *Litopenaeus vannamei*. Aquaculture 321:136–144.
- Madern D, Ebel C, Zaccai G (2000) Halophilic adaptation of enzymes. Extremophiles 4: 91-98.
- Maftuch E, Prasetio A, Rozik M, Nurdiyani R, Sanusi E, Nursyam H, Fariedah F, Marsoedi, Murachmam (2013) Improvement of innate immune responses and

defense activity in tiger shrimp (*Penaeus monodon Fab*.) by intramuscular administration of the outer membrane protein *Vibrio alginolyticus*. Springer plus 2:432.

- Mahdhi A, Chaieb K, Kamoun F, Bakhrouf A (2010) Use of Pseudomonas stutzeri and Candida utilis in the Improvement of the Conditions of Artemia Culture and Protection against Pathogens. Braz J Microbiol 41: 107–115.
- Manikandan P and Senthilkumar PK (2017) An Overview of Saltpan Halophilic Bacterium. Int J Bio Res 2:28-33.
- Margesin R, Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 5: 73-83.
- Mayer-Harting A, Hedges AJ, Berkeley RCW (1972) Methods for studying bacteriocins. Methods Microbiol 7:315-422.
- Mellado E, Sánchez-Porro C, Martín S, Ventosa A (2004) Extracellular Hydrolytic Enzymes Produced by Moderately Halophilic Bacteria. In: Ventosa A (eds) Halophilic Microorganisms. Springer, Berlin, 285–295.
- Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RTM, Bogwald J, Castex M, Ringoc E (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302: 1–18.
- Mishra, A, Mandoli A, Jha, B (2008) Physiological characterization and stress-induced metabolic responses of Dunaliella salina isolated from salt pan. J Ind Microbiol Biotechnol 35(10): 1093–1101.
- Momoyama K (2004) Mortalities of farm-raised Kuruma prawn *Penaeus japonicus* caused by high pH ambient water due to blooms of a blue-green alga, *Chroococcus turgidus*. Fish Pathol 39: 129-135.
- Monaghan RL, Gagliardi MM, Streicher SL (1999) Culture preservation and inoculation development. In: Demain AL, Davies JE (eds) Manual of Industrial Microbiology and Biotechnology American Society for Microbiology 2nd ed, ASM Press, Washington DC, pp. 28-48.
- Moriarty DJW (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164:351–358.
- Moriarty DJW (1999) Disease control in shrimp aquaculture with probiotic bacteria. In: Bell CR, Brylinsky M, Johnson-Green P (eds). Microbial Biosystems:

New Frontiers: Proceedings of the 8th International Symposium on Microbial Ecology. Atlantic Canada Society for Microbial Ecology, Canada.

- Motta AS, Cladera-Olivera F, Brandelli A (2004) Screening for antimicrobial activity among bacteria isolated from the Amazon basin. Braz J Microbiol 35: 307–310.
- Nakayama T, Lu H and Nomura N (2009) Inhibitory effects of Bacillus probionts on growth and toxin production of *Vibrio harveyi* pathogens of shrimp. Lett Applied Microbiol 49:679-684.
- NavinChandran M, Iyapparaj P, Moovendhan S, Ramasubburayan R, Prakash S, Immanuel G, Palavesam A (2014) Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in P. monodon. Fish Shellfish Immunol 36:38–45.
- Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MCM, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature 405: 1017–1024.
- Neu AK, Månsson M, Gram L, Prol-García MJ (2014) Toxicity of bioactive and probiotic marine bacteria and their secondary metabolites in Artemia sp. and Caenorhabditis elegans as eukaryotic model organisms. Appl Environ Microbiol 80(1):146-153.
- Ng W, Koh CB, Teoh CY and Romano N (2015) Farm-raised tiger shrimp, *Penaeus monodon*, fed commercial feeds with added organic acids showed enhanced nutrient utilization, immune response and resistance to *Vibrio harveyi* challenge. Aquaculture 449:69–77.
- Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64: 548–572.
- Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius EM (2003) Immune enhancement in rainbow trout (Oncorhynchus mykiss) by potential probiotic bacteria (Lactobacillus rhamnosus). Fish Shellfish Immunol 15(5):443-452.
- Nimrat S, Suksawat S, Boonthai T, Vuthiphandchai V (2012) Potential Bacillus probiotics enhance bacterial numbers, water quality and growth during early

development of white shrimp (*Litopenaeus vannamei*). Vet Microbiol 159: 443–450.

- Olsson JC, Westerdahl A, Conway PL, Kjelleberg S (1992) Intestinal colonization potential of turbot (Scophthalmus maximus) and dab (Limanda limanda) associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl Environ Microbiol 58 (2):551-556.
- Pai SS, Anas A, Jayaprakash NS, Priyaja P, Sreelakshmi B, Preetha R, Philip R, Mohandas A, Sarogeni I, Singh B (2010) *Penaeus monodon* larvae can be protected from *Vibrio harveyi* infection by pre-emptive treatment of a rearing system with antagonistic or non-antagonistic bacterial probiotics. Aquac Res 41: 847–860.
- Panigrahi A, Otta SK, Gopal C, Patil PK, Sivagnanam S, Ravichandran P, Ponniah AG (2011) Evaluation of probiotic vis-à-vis antibiotics based seed production in penaeid shrimp larviculture. In: 8th Symposium on Diseases in Asian Aquaculture, Book of Abstracts, pp 114.
- Parker RB (1974) Probiotics, the other half of the antibiotics story. Anim Nutr Health 29: 4–8.
- Pauly D, Alder J, Bennett E, Christensen V, Tyedmers P, Watson R (2003) The future for fisheries. Science 302:1359-1361.
- Pérez-Sánchez T, Ruiz-Zarzuela I, de Blas I, Balcázar JL (2013) Probiotics in aquaculture: a current assessment. Rev Aquacult 6(3): 133–146.
- Pereira F (2013) Metal tolerance in heterotrophic bacteria from marine salterns of ribandar Goa. Thesis, Goa University.
- Phianphak W, Rengpipat S, Piyatiratitivorakul S and Menasveta P (1999) Probiotic Use of *Lactobacillus* spp. for Black Tiger Shrimp, *Penaeus monodon*. J Sci Res 24:1-47.
- Preetha R, Vijayan KK, Jayapraksh NS, Alavandi SV, Santiago TC, Singh IS B (2015) Optimization of culture conditions for mass production of the probiotics *Pseudomonas MCCB 102* and *103* antagonistic to pathogenic *Vibrio*s in aquaculture. Probiotics Antimicrob. 7: 137–145.
- Ray AK, Ghosh K, Ringø E (2012) Enzyme-producing bacteria isolated from fish gut: a review. Aquac Nutr 18: 465–492.

- Qi Z, Zhang XH, Boon N, Bossier P (2009) Probiotics in aquaculture of China — Current state, problems and prospect. Aquaculture 290 (1-2): 15–21.
- Ramos-Carreno S, Valencia-Yanez R, Correa-Sandoval F, Ruız-Garcia N, Dıaz-Herrera F, Giffard-Mena I (2014) White spot syndrome virus (WSSV) infection in shrimp (*Litopenaeus vannamei*) exposed to low and high salinity. Arch Virol 159: 2213–2222.
- Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S & Menasaveta P (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus S11*). Aquaculture 191:271–288.
- Rice EW, Baird RB, Eaton AD, Clesceri LS (Eds.) (2012) In: Standard Methods for the Examination of Water and Wastewater 22nd Edition, American Public Health Association, Washington DC.
- Rodrigues CM, Bio A, Amat F, Vieira (2011) Artisanal salt production in Aveiro/Portugal—an ecofriendly process. Saline Syst 7(3):1–14.
- Romero J, Feijoó CG and Navarrete (2012) Antibiotics in Aquaculture – Use, Abuse and Alternatives. In: Carvalho ED, David GS, Silva RJ (eds) Health and Environment in Aquaculture.InTechOpen, pp 159-198.
- Roque A, Abad-Rosales S, Betancourt-Lozano M, García-de la PL, Baird D et al (2005) Evaluation of susceptibility of the cultured shrimp *Litopenaeus vannamei* to vibriosis when orally exposed to the insecticide methyl parathion. Chemosphere. 60:126-134.
- Rosenberry B (2002) World shrimp farming 2002. Shrimp News International, San Diego, pp 276.
- Sahu MK, Swarnakumar NS, Sivakumar K, Thangaradjou T, Kannan L (2008) Probiotics in aquaculture: importance and future perspectives. Indian J Microbiol 48:299–308.
- Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, de Vos WM, Fondén R, Saxelin M, Collins K, Mogensen G, Birkeland SE, Mattila-Sandholm T (1998) Demonstration of safety of probiotics -- a review. Int J Food Microbiol 44(1-2):93-106.
- Schaeffer A, Fulton D (1933) A simplified method for staining spores. Science 77: 194-195.

- Scheele JS, Marks RE, Boss GR (2007) Signaling by small GTPases in the immune system. Immunol Rev 218:92-101.
- Scholz U, Diaz GG, Ricque D, Suarez LEC, Albores FV, Latchford J (1999) Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products. Aquaculture 176:271–283.
- Seenivasan C, Radhakrishnan S, Muralisankar T, Bhavan PS (2012) *Bacillus subtilis* on survival, growth, biochemical constituents and energy utilization of the freshwater prawn *Macrobrachium rosenbergii* post larvae. Egypt J Aquat Res 38: 195–203.
- Seenivasan C, Radhakrishnan S, Muralisankar T, Bhavan SP (2014) Effects of Probiotics on Survival, Growth and Digestive Enzymes Activities in Freshwater Prawn Macrobrachium rosenbergii (De Man 1879). Proc Zool Soc 69 (1): 52–60.
- Shivakumar S (2012) Production and characterisation of an acid protease from a local Aspergillus sp. by solid substrate fermentation. Rch Appl Sci Res 4:188–199.
- Sinanoglou VJ, Zoumpoulakis P, Heropoulos G, Proestos C, Ciri CA et al (2014) Lipid and fatty acid profile of the edible fungus *Laetiporussulphurous*. Antifungal and antibacterial properties. J Food SciTechnol 52: 3264-3272.
- Sinanoglou VJ, Zoumpoulakis P, Heropoulos G, Proestos C et al (2015) Lipid and fatty acid profile of the edible fungus Laetiporus sulphurous antifungal and antibacterial properties. J Food Sci Technol 52:3264-3272.
- Sirirustananun N, Chen J, Lin Y, Yeh S, Liou C, Chen L, Sim S, Chiew S (2011) Dietary administration of a Gracilaria tenuistipitata extract enhances the immune response and resistance against *Vibrio alginolyticus* and white spot syndrome virus in the white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 31:848-855.
- Smith MD, Roheim CA, Crowder LB, Halpern BS, Turnipseed, M, Anderson, J L, Asche F, Bourillón L, Guttormsen AG, Khan A, Liguori LA, McNevin A, O'Connor MI, Squires D, Tyedmers P, Brownstein C, Carden K, Klinger DH, Sagarin R, Selkoe KA (2010) Sustainability and Global Seafood. Science 327(5967): 784–786.
- Snydman DR (2008) The safety of probiotics. Clin Infect Dis 46(2):104-111.
- Solano JLO, Soto JO (2006) The functional property of Bacillus for shrimp feeds. Food Microbiol 23:519–525.

- Sorroza L, Padilla D, Acosta F, Roman L, Grasso V, Vega J, Real F (2012) Characterization of the probiotic strain Vagococcus fluvialis in the protection of European sea bass (Dicentrarchus labrax) against vibriosis by *Vibrio anguillarum*. Vet Microbiol 155: 369–373.
- Standen BT, Rawling MD, Davies SJ, Castex M, Foey A, Gioacchini G (2013) Probiotic Pediococcus acidilactici modulates both localised intestinal and peripheral immunity in tilapia (Oreochromis niloticus). Fish Shellfish Immunol 35:1097-1104.
- Stearns SC (2009) Sustaining Life: How Human Health Depends on Biodiversity. Environmental Health Perspectives 117(6): A266.
- Strober W (2001) Trypan blue exclusion test of cell viability. Curr Protoc Immunol 111(1): A3.B.1-A3.B.3.
- Sturr MG, Guffanti AA, Krulwich TA (1994) Growth and bioenergetics of alkaliphilic *Bacillus firmus OF4* in continuous culture at high pH. J Bacteriol 176: 3111–3116.
- Subramanian K, Balaraman D, Balachandran DN, Thirunavukarasu R, Renuka PS, Kumarappan A (2014) Immune response of shrimp (*Penaeus monodon*) against *Vibrio furnissii* pathogen. J Coast Life Med 2(4):281-286
- Subramanian K, Balaraman D, Dhayanithi NB, Thirunavukarasu R, Gopal S *et al* (2014) Immune response of shrimp (*Penaeus monodon*) against V. furnissiipathogen. J Coast Life Med 2:281-286.
- SwathiLekshmi PS, Chandrakandan K, Balasubramani N (2013) Discriminating Factors of High and Low Adopters of Shrimp Farmers in Nagapattinam of Tamil Nadu. Indian Res J Ext Edu 13(1): 120 – 122.
- Tamura K, Stecher G, Peterson D, et al (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30: 2725–2729
- Tan LTH, Chan KG, Lee LH, Goh BH (2016) Streptomyces Bacteria as Potential Probiotics in Aquaculture. Front Microbiol 7:79.
- Taoka Y, Maeda H, Jo JY et al (2006) Use of live and dead probiotic cells in tilapia Oreochromis niloticus. Fish Sci 72(4):755–766.
- Taoka Y, Maeda H, Jo JY, Jeon MJ, Bai SC, Lee WJ, Yuge K, Koshio S (2006) Growth, stress tolerance and nonspecific immune response of Japanese flounder

Paralichthys olivaceus to probiotics in a closed recirculating system. Fisheries Sci 72(2):310-321.

- Tseng D, Ho P, Huang S, Cheng S, Shiu Y et al (2009) Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei* by the probiotic *Bacillus subtilis E20*. Fish Shellfish Immunol 26: 339–344
- Vadstein O (2000) The use of immunostimulation in marine larviculture: possibilities and challenges. Aquaculture 155:401–417.
- Valipour A, Nedaei S, Noori A, Khanipour AA, Hoseinifar SH (2019) Dietary *Lactobacillus plantarum* affected on some immune parameters, air-exposure stress response, intestinal microbiota, digestive enzyme activity and performance of narrow clawed crayfish (Astacus leptodactylus, Eschscholtz). Aquaculture 504 : 121–130.
- Van DH, Hoseinifar SH, Dawood MAO, Chitmanat C, Tayyamath K (2017) Effects of Cordyceps militaris spent mushroom substrate and *Lactobacillus plantarum* on mucosal, serum immunology and growth performance of Nile tilapia (*Oreochromis niloticus*). Fish Shellfish Immunol 70:87–94.
- Vasanthakumari R (2009) Practical microbiology. BI publications pvt Ltd, New Delhi.
- Vaseeharan B and Ramasamy P (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis BT23*, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett Appl Microbiol 36:83-7.
- Vázquez J, González M, Murado M, (2005) Effects of lactic acid bacteria cultures on pathogenic microbiota from fish. Aquaculture 245 (1-4): 149-161.
- Vazquez L, Alpuche J, Maldonado G, Agundis C, Pereyra-Morales A, Zenteno E (2009) Immunity mechanisms in crustaceans. Innate Immun 15(3):179-88.
- Venkat HK, Sahu NP, Jain KK (2004) Effect of feeding *Lactobacillus*-based probiotics on the gut microflora, growth and survival of postlarvae of *Macrobrachium rosenbergii (de man)*. Aquac Res 35:501–507.
- Verschuere L, Heang H, Criel G, Dafnis S, Sorgeloos P, Verstraete W (2000) Protection of Artemia against the pathogenic effects of *Vibrio proteolyticus CW8T2* by selected bacterial strains. Appl Environ Microbiol 66:1139–1146.
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671.

- Vieira FDN, Neto CCB, Mouriño JLP, Jatobá A., Ramirez C, Martins ML, Barracco MAAA, Vinatea LA (2008) Time-related action of *Lactobacillus plantarum* in the bacterial microbiota of shrimp digestive tract and its action as immunostimulant. Pesq Agropec Bras 43: 763–769.
- Vieira FN, Jatobá A, Mouriño JLP, Vieira EA, Soares M, Silva BCD, Seiffert WQ, Martins LM Vinatea LA (2013) *In vitro* selection of bacteria with potential for use as probiotics in marine shrimp culture. Pesq Agropec Bras 48(8): 998-1004.
- Vinatea L, Gálvez AO, Browdy CL, Stokes A, Venero J, Haveman J, Lewis BL, Lawson A, Shuler A, Leffler JW (2010) Photosynthesis, Water Respiration And Growth Performance Of *Litopenaeus vannamei* in a super-intensive raceway culture with zero water exchange:interaction of water quality variables. Aquacult Eng 42 (1):17-24.
- Vine NG, Leukes WD, Kaiser H (2004) *In vitro* growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. FEMS Microbiol Lett 231: 145–152.
- Vine NG, Leukes WD and Kaiser H (2006) Probiotics in marine larviculture. FEMS Microbiol Rev 30:404–427.
- Wang H, Ren L, Yu X, Hu J, Chen Y, He G, Jiang Q (2017) Antibiotic residues in meat, milk and aquatic products in Shanghai and human exposure assessment. Food Control 80:217–225.
- Wang Y, Gu Q (2010) Effect of probiotics on white shrimp (*Penaeus vannamei*) growth performance and immune response. Mar Biol Res 6: 327-332.
- Wang YB (2007) Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269:259–264
- Wang YB, Xu ZR, Xia MS (2005) The effectiveness of commercial probiotics in northern white shrimp *Penaeus vannamei* ponds. Fisheries Sci 71(5):1036–1041.
- Watson AK, Kaspar H, Lategan MJ, Gibson L (2008) Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture 274:1-14.
- Worthington V (1993) Worthington enzyme manual. Enzymes and related biochemicals. Worthington Chemical, Columbus.

- Wu ZQ, Jiang C, Ling F, Wang GX (2015) Effects of dietary supplementation of intestinal autochthonous bacteria on the innate immunity and 739 disease resistance of grass carp (Ctenopharyngodon idellus). Aquaculture 438: 105-114.
- Xia Z, Zhu M and Zhang Y (2014) Effects of the probiotic Arthrobacter sp. CW9 on the survival and immune status of white shrimp (*Penaeus vannamei*). Lett Appl Microbiol 58(1):60-4.
- Xu Y, He T, Li Z, Ye Q, Chen Y, Xie E, Zhang X (2017). Nitrogen Removal Characteristics of Pseudomonas putida Y-9 Capable of Heterotrophic Nitrification and Aerobic Denitrification at Low Temperature. Biomed Res Int 2017: 1–7.
- Yanbo W, Zirong X (2006) Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. Anim Feed Sci Technol 127:283–292.
- Yang G, Chen M, Yu Z, Lu Q, Zhou S (2013) *Bacillus composti* sp. nov. and *Bacillus thermophilus* sp. nov., two thermophilic, Fe(III)-reducing bacteria isolated from compost. Int J Syst Evol Microbiol 63: 3030–3036.
- York R, Gossard MH (2004) Cross-national meat and fish consumption. Ecol Econom 48:293-302.
- Zhang D, Wang F, Dong S, Lu Y (2016) De novo assembly and transcriptome analysis of osmoregulation in *Litopenaeus vannamei* under three cultivated conditions with different salinities. Gene 578(2):185–193.
- Zhang L, Tian X, Kuang S, Liu G, Zhang C, Sun C (2017) Antagonistic Activity and Mode of Action of Phenazine-1-Carboxylic Acid, Produced by Marine Bacterium Pseudomonas aeruginosa PA31x, Against *Vibrio anguillarum In vitro* and in a Zebrafish *In vivo* Model. Front Microbiol 8: 289.
- Zhang WY, Huo YY, Zhang XQ, Zhu XF, Wu M (2013) Halolaminasalifodinae sp. nov. and sp. nov., two extremely halophilicarchaea isolated from a salt mine. Int J Syst Evol Microbial 63: 4380-4385.
- Zheng CN, Wang W (2016) Effects of *Lactobacillus pentosus* on the growth performance, digestive enzyme and disease resistance of white shrimp, *Litopenaeus vannamei* (Boone, 1931). Aquac Res 48 (6): 2767-2777.
- Zhou X, Wang Y, Li W (2009) Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. Aquaculture 287:349–353.

- Ziaei-Nejad S, Rezaei MR, Takami GA, Lovett DL, Mirvaghefi AR, Shakouri M (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture 252: 516– 524.
- Zokaeifar H, Babaei N, Saad CR, Kamarudin MS, Sijam K, Balcazar JL (2014) Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 36: 68–74.
- Zokaeifar H, Balcazar JL, Kamarudin MS, Sijam K, Arshad A, Saad CR (2012) Selection and identification of non-pathogenic bacteria isolated from fermented pickles with antagonistic properties against two shrimp pathogens. J Antibiot 65(6):289-94.
- Zokaeifar H, Balcazar JL, Saad CR, Kamarudin MS, Sijam K, Arshad A et al (2012) Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 33:683–689.
- Zorriehzahra MJ, Delshad ST, Adel M, Tiwari R, Karthik K, Dhama K, Lazado CC (2016) Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. Vet Q 36(4):228-241.

APPENDIX

Media composition

Media	Name of media	Composition	
		Ingredients	g/L
M1	**Zobell Marine Agar**	Peptone	5.000
		Yeast extract	1.000
		Ferric citrate	0.100
		Sodium chloride	19.45
		Magnesium chloride	8.800
		Sodium sulphate	3.240
		Calcium chloride	1.800
		Potassium chloride	0.550
		Sodium bicarbonate	0.160
		Potassium bromide	0.080
		Strontium chloride	0.034
		Boric acid	0.022
		Sodium silicate	0.004
		Sodium fluorate	0.0024
		Ammonium nitrate	0.0016
		Disodium phosphate	0.008
		Agar	15.000
		Distilled water	1000 mL
		Final pH (at 25°C)	7.6 ± 0.2

Media	Name of media	Composition	
		Ingredients	g/L
M2	**Nutrient Agar**	Peptone Sodium chloride Beef extract Yeast extract Agar Seawater Final pH (at 25°C)	5.000 5.000 1.500 1.500 15.000 1000 mL 7.4 ± 0.2
M3	**Media D Agar**	Soy Peptone Sodium chloride Tryptone Distilled water Final pH (at 25°C)	0.500 0.500 1.500 1000 mL 7.4 ± 0.2
M4	**Actinomycetes isolation agar**	Sodium caseinate L-Asparagine Sodium propionate Dipotassium phosphate Magnesium sulphate Ferrous sulphate Agar Distilled water Final pH (at 25°C)	2.000 0.100 4.000 0.500 0.100 0.001 15.00 1000 mL 8.1 ± 0.2

Media	Name of media	Composition	
		Ingredients	**g/L**
M5	**Nutrient Broth**	Peptone Beef extract Sodium chloride Distilled water Final pH (at 25°C)	10.00 10.00 5.000 1000 mL 7.2 ± 0.2
M6	**Muller Hinton agar**	HM infusion B Acicase Starch Agar Distilled water Final pH (at 25°C)	300.0 17.50 1.500 17.00 1000 mL 7.4 ± 0.1
M7	**Thiosulphate citrate bile salts (TCBS) agar**	Proteose peptone Yeast extract Sodium thiosulphate Sodium citrate Bile Sucrose Sodium chloride Ferric citrate Bromo thymol blue Thymol blue Agar Distilled water Final pH (at 25°C)	10.000 5.000 10.000 10.000 8.000 20.000 10.000 1.000 0.040 0.040 15.000 1000 mL 8.6 ± 0.2

Media	Name of media	Composition	
		Ingredients	**g/L**
M8	**Enriched medium containing 1g/L ammonia**	Glucose Ammonium sulphate Dipotassium hydrogen phosphate Sodium chloride Magnesium sulphate Ferrous sulphate pH	5.00 1.00 1.00 2.00 0.5 0.4 7.2 ± 0.2
M9	**Starch Agar medium**	Disodium hydrogen phosphate Potassium dihydrogen phosphate Sodium chloride Ammonium chloride Agar Distilled water Soluble starch Final pH (at 25°C)	6.000 3.000 0.500 1.000 20.000 1000 mL 10.000 7.6 ± 0.2

Media	Name of media	Composition	
		Ingredients	**g/L**
M10	**Tributyrin agar medium**	Disodium hydrogen phosphate	6.000
		Potassium dihydrogen phosphate	3.000
		Sodium chloride	0.500
		Ammonium chloride	1.000
		Agar	20.000
		Distilled water	1000 mL
		Tributyrin	1% (w/v)
		Final pH (at 25°C)	7.6 ± 0.2
M11	**Carboxy-methyl cellulose agar medium**	Disodium hydrogen phosphate	6.000
		Potassium dihydrogen phosphate	3.000
		Sodium chloride	0.500
		Ammonium chloride	1.000
		Agar	20.000
		Distilled water	1000 mL
		Carboxy-methyl cellulose	10.000
		Final pH (at 25°C)	7.6 ± 0.2

Media	Name of media	Composition	
		Ingredients	**g/L**
M12	**Minimal agar**	Disodium hydrogen phosphate	6.000
		Potassium dihydrogen phosphate	3.000
		Sodium chloride	0.500
		Ammonium chloride	1.000
		Agar	20.000
		Distilled water	1000 mL
		Final pH (at 25°C)	7.6 ± 0.2
M13	**Minimal broth**	Disodium hydrogen phosphate	6.000
		Potassium dihydrogen phosphate	3.000
		Sodium chloride	0.500
		Ammonium chloride	1.000
		Distilled water	1000 mL
		Final pH (at 25°C)	7.6 ± 0.2
M14	**Pond water agar**	Agar	20.000
		Aquaculture Pond water	1000 Ml
		Final pH (at 25°C)	7.6 ± 0.2

Media	Name of media	Composition	
		Ingredients	**g/L**
M15	**Blood based agar supplemented with 6% human sterile blood**	**P**eptone	10.000
		Tryptose	10.000
		Sodium chloride	5.000
		Agar	15.000
		Sterile human blood	5% (v/v)
		Final pH (at 25°C)	7.3 ± 0.2
M16	**Zobell Marine Broth**	Peptone	5.000
		Yeast extract	1.000
		Ferric citrate	0.100
		Sodium chloride	19.45
		Magnesium chloride	8.800
		Sodium sulphate	3.240
		Calcium chloride	1.800
		Potassium chloride	0.550
		Sodium bicarbonate	0.160
		Potassium bromide	0.080
		Strontium chloride	0.034
		Boric acid	0.022
		Sodium silicate	0.004
		Sodium fluorate	0.0024
		Ammonium nitrate	0.0016
		Disodium phosphate	0.008
		Distilled water	1000mL
		Final pH (at 25°C)	7.6 ± 0.2

Media	Name of media	Composition	
		Ingredients	**g/L**
M17	**Caesin Agar**	Disodium hydrogen phosphate	6.000
		Potassium dihydrogen phosphate	3.000
		Sodium chloride	0.500
		Ammonium chloride	1.000
		Agar	20.000
		Distilled water	1000mL
		Caesin	10.000
		Final pH (at 25°C)	7.6 ± 0.2

Reagents

- **Ammonia estimation**

Nessler's reagent constituents	*(g/100 mL)*
Mercuric chloride	10.00
Potassium iodide	7.000
Sodium hydroxide	16.00
Final pH (at 25°C)	13.2±0.05

Sodium Potassium tartrate
50 g of Sodium Potassium tartrate was dissolved in 100mL of distilled water (free of ammonia – boil the solution to expel ammonia)

Ammonia free water: Boiled distilled water

Standard NH_3-N solution (1000ppm)
3.819g of anhydrous ammonium chloride (oven dried at 100^0C for 1hr) was weighed and diluted with 1000ml of ammonia free water.

- **Mucus Adhesion/ Formulation of the bacterial consortium**

1 X Phosphate-Buffered Saline

Constituents	**(g/1000 mL)**
Sodium chloride	8.000
Potassium chloride	0.200
Disodium hydrogen phosphate	1.440
Potassium dihydrogen phosphate	0.240
Final pH (at 25°C)	7.4

Crystal Violet (0.1%): 2 g crystal violet was dissolved in 20 ml of 95% ethyl alcohol. 0.8 g ammonium oxalate monohydrate was dissolved in 80 ml deionized water. The crystal violet and ammonium oxalate monohydrate solutions were mixed to make the crystal violet stain and filtered.

Citrate buffer (pH 4.3)

Constituents	**(g/1000 mL)**
Sodium Citrate dehydrate	11.975 g
Citric Acid	11.385 g

11.975 g Sodium Citrate dehydrate was accurately weighed and added to 800ml of distilled water. 11.385 g of Citric Acid accurately weighed was added to the solution and solution. The final desired pH of the solution was adjusted using HCl and/or NaOH. The volume was then made up to 1 L with distilled water.

- **Production of extracellular enzymes (Qualitative)**

  - **Protease**

Coomassie brilliant blue: 0.2g of dye was dissolved in 100 ml distilled water, warmed and cooled. 100 ml 2N H_2S0_4 was added to the solution and incubated at room temperature for 3 hours to overnight and then filtered and used.

  - **Cellulase**

Congo red dye (0.1%) : 0.1 g of congo red was dissolved in 100mL of distilled water.

- **Production of extracellular enzymes (Quantitative)**

  - **Amylase**

3,5-dinitrosalicylic acid reagent **(DNS) :** 1.0 gm of 3,5-dinitrosalicylic acid was weighed and dissolved in 50 ml of distilled water. 30.0 gms sodium potassium tartrate tetrahydrate added to this solution slowly. 20 ml of 2 N NaOH was further added and diluted to a final volume of 100 ml with reagent distilled water.

  - **Protease**

Trichloroacetic acid **(20%):** 100 g of TCA accurately weighed was added to 45.4 mL of distilled water. 10mL of this solution was further diluted to 50mL with distilled water.

  - ***Bovine Serum Albumin (BSA)***

For a 10% (100 mg/mL) stock solution of BSA, 1 g powdered molecular biology grade BSA was dissolved in 10 mL of distilled water with gentle mixing to avoid clumping.

  - **Cellulase**

1% ***Carboxymethyl cellulose*****:**

1g of powdered carboxymethylcellulose sodium was added to 100 ml of water, while stirring to produce a uniform dispersion. The solution was stirred continuously until a clear solution obtained. Few drops of 1-naphthol was added to the solution.

0.5 M *Sodium citrate buffer* (pH 4.8)

Constituents	**(g/1000 mL)**
Citric Acid	48.330g
Sodium Citrate	79.410g

48.33 g of Citric Acid was accurately weighed and added to 800mL of distilled water. 79.41 g of Sodium Citrate accurately weighed was added to the solution. The final desired pH of the solution wasadjusted using HCl and/or NaOH. The volume was then made up to 1 L with distilled water.

- **Lipase**

***p-nitrophenyl palmitate* pNPP (20 mM):** 0.151 g of pNPP was dissolved in 20 ml of isopropanol.

***Tris-HCl* buffer (0.05 M, pH 8.5):** 0.606 g of Tris-base was solubilized in 80 ml of distilled water, pH was adjusted to 8.0 using 1 M HCl and final volume was adjusted to 100 ml using distilled water.

- **Hemolysis test -Shrimp blood agar preparation**

***Marine anticoagulant* solution (on ice)**

Constituents	**(g/1000 mL)**
Sodium chloride	26.300
Glucose	18.000
Tri sodium citrate	08.800
Citric acid	05.000
EDTA	03.700
pH	05.400

- **Estimation of Nitrite:**

***Sulphanilamide reagent*:** 5g of sulphanilamide accurately weighed was dissolved in a mixture of 50ml of concentrated hydrochloric acid and about 300ml of distilled water. The volume was made up to 500ml with distilled water.

***N-(1-naphthyl) ethylene diamine hydrochloride* solution:**

100mg of 0.5mL N-(1-naphthyl) ethylene diamine hydrochloride was accurately weighed and mixed with 70ml of acetone and 30 ml of water.

- **Estimation of Nitrate:**
  - **Preparation of Nitrate reducing cadmium column:-**

2%CuSO4 solution: 2 g cupric sulphate pentahydrate, $CuSO_4*5H_2O$ was dissolved in 1 litre distilled water.

- ***Dilute ammonium chloride buffer:***

Concentrated ammonium chloride solution: 125 g of reagent grade ammonium chloride (NH_4Cl) was accurately weighed and dissolved in 500 ml of distilled water.

Dilute ammonium chloride solution: 50 ml of the concentrated ammonium chloride (NH_4Cl) solution was accurately weighed and dissolved to 2000 ml with distilled water.

- **Dissolved oxygen by Winkler's Method**

(All reagents prepared in distilled water)

• ***Winkler's A solution:***
Manganous chloride ($MnCl_2.4H_2O$) 600 g/L

• ***Winkler's B solution:***

Alkaline iodide:	KOH	700 g/L
	KI	150 g/L

Sodium thiosulphate (0.01N) 2.482 g/L

Starch indicator 1 g %

Potassium dichromate (0.01N) 0.49 g/L

- **Phagocytic activity assay**

Phosphate buffered saline , pH 7.4

Constituents	**(g/1000 mL)**
NaCl	8 g
KCl	200 mg
Na_2HPO_4	1.44 g
KH_2PO_4	240 mg

8 g of NaCl was accurately weighed and added to.800 ml of distilled water. 200 mg of KCl was added to the solution. Accurately weighed 1.44 g of Na_2HPO_4 and 240 mg of KH_2PO_4 were added to the solution. The final desired pH of the solution was adjusted using HCl and/or NaOH. The volume was then made up to 1 L with distilled water

- **Preparation of Hemocyte lysate supernatant (HLS)**

Ice cold cacodylate (CAC) buffer

Cacodylate formulation: (0.05M)
Solution A: 42.8 g of Sodium cacodylate trihydrate was added to 1 L of distiiled water.
Solution B: 0.2 M HCl Concentrated HCl (36-38%) 10 ml of distiiled water.
The desired pH was obtained by adding soln B to 50 ml of soln A and diluting to a total volume of 200 ml with distiiled water.

- **Respiratory burst**

- ***Nitro-blue tetrazolium (NBT):*** (N 6876, Sigma) stock solution was prepared at 10 mg/ml in distilled water and kept at 48°C in the dark. From this stock solution, a NBT working solution was prepared in culture medium at 0.3% just before utilization.
- ***Potassium hydroxide (2mol/L)****:* 112.2 g of KOH accurately weighed was diluted to 1 L with distilled water.

PUBLICATIONS

Manuscript published:

- **Fernandes S**, Kerkar S, Leitao J, Mishra A (2019) **Probiotic role of Salt Pan Bacteria in Enhancing the Growth of Whiteleg Shrimp, *Litopenaeus vannamei*.** Probiotics and Antimicrobial proteins. doi: 10.1007/s12602-018-9503-y. (**Impact Factor: 2.345**).

Book chapter published:

- **Fernandes S** and Kerkar S (2019) **Bacterial probiotics over antibiotics: a boon to aquaculture.** In: Surya Nandan Meena and Milind Mohan Naik, editors. Advances in Biological Science Research - A Practical Approach. Academic press, Elsevier, USA. p. 215 – 231.

Abstracts published in International and National conferences:

- **"Enhancement of immune response in *Litopenaeus vannamei* by a formulated probiotic consortium"** by **Samantha Fernandes** and Savita Kerkar at the **Third International Conference on Innovative Research in Science and Technology** organized by Gopal Krishna Gokhale college, Kolhapur on 7 - 8th November 2017. **[**Won **Best Poster Presentation - First Place].**

- **"Inducible Immuno-stimulatory effect of Hypersaline Bacteria on *Litopenaeus vannamei*"** by **Samantha Fernandes** and Savita Kerkar at the **International conference on Recent advances in Aquaculture (RAA-2016)** organized by the Department of Marine Living Resources, Andhra Pradesh at Vishakhapatnam, India on 16th to 17th December 2016 [Won **Best Oral Presentation – Third prize]**

- "**Hypersaline bacteria as a feed supplement in shrimp aquaculture"** by **Samantha Fernandes** and Savita Kerkar for the 3 minutes thesis competition held

at BITS Pilani, KK Birla campus on 29th April, 2017 **[**Won **Best Oral presentation - First place].**

- **"Evaluation of cytotoxic and genotoxic effects of potential hypersaline bacteria as a live feed supplement for *Litopenaeus vannamei*"** by Moreska Costa, **Samantha Fernandes**, Avelyno D'Costa, SK Shyama, Savta Kerkar at the **National conference of young researchers (NCYR 2017) on New frontiers in Life Sciences and Environment** organized by the Department of Life Sciences and Environment, Goa University on 16th and 17th March, 2017.

- **"Salt pan bacteria as potential inhibitors of shrimp pathogens"** by **Samantha Fernandes** and Savita Kerkar at the National seminar on **"Life and Life Processes Sustainable Development"** held at Goa University from 19th to 21st February, 2015.

- **"Assessment and evaluation of the anti-*Vbrio* activity of bacteria from marine salterns as candidate probionts in shrimp aquaculture"** by **Samantha Fernandes** and Savita Kerkar at the **56th Annual conference of Association of Microbiologists of India (AMI – 2015) and International symposium on "Emerging Discoveries in Microbiology"** held at Jawaharlal Nehru university, New Delhi from December 7 – 10, 2015.

Manuscripts in pipeline:

- **Fernandes S**, D'Costa A, Costa M, Mishra A, Shyama SK, Das KR, Kerkar S. (2019) **Immuno-stimulatory effect and safety evaluation of a probiotic consortium SFSK4 for shrimp aquaculture**. (Fish and Shellfish immunology – Under review).

- **Fernandes S**, Bartakke AB, Kerkar S (2019) ***Pseudomonas* sp. strain ABSK55 and *Bacillus* species from salt pans of Goa as biocontrol agents in shrimp aquaculture**.