Isolation and characterisation of coral mucus bacilli with quorum quenching potential as a biocontrol agent for shrimp gut protection against Early Mortality Syndrome (EMS)

by

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ABSTRACT

A shrimp disease, known commonly as Early Mortality Syndrome (EMS), has been a major problem for shrimp aquaculture in Southeast Asia due to its epizootic prevalence within the region since the first regional case was reported in 2009. Although the mechanism of pathogenicity is not well understood, the pathogenic strain associated with the cause of EMS is *V. parahaemolyticus*, known to cause near 100% mortality among juvenile shrimps in aquaculture ponds. The Coral Probiotic Hypothesis affirms that a coral species may acquire long term adaptive immunity simply by allowing natural selective forces to act upon the microbial community associated with it. As the coral holobiont survives environmental stresses together, the microbial community associated with an individual coral experiences dynamic shifts in abundance and diversity in response to external changes. In this study, bacilli strains isolated from coral mucus were tested for antagonistic effects against quorum sensing and *Vibrio parahaemolyticus*. Positive results indicated that some bacilli isolated from South China Sea coral mucus had the potential to be further developed as a biocontrol agent against EMS. From the initial pool of bacilli strains, phenotypic characterisation was carried out. While attention was given to bacilli biofilms, other parameters such as temperature-growth responses and biosurfactant production were also assessed quantitatively. Three phenotypically distinct strains showing notable potential were chosen to undergo co-cultivation as a method for strain improvement via long term exposure to the pathogenic *V. parahaemolyticus*. This experiment resulted in two bacilli strains showing improved competitive fitness against the target organism over the 6 weeks experiment duration. An oxygen stress response test and plate-based assays yielded the desired outcome of significant improvement in growth or antagonistic activity of coevolved bacilli strains as compared to respective wild types. For ease of *in vitro* visualization, *V. parahaemolyticus* was successfully tagged with green fluorescent protein via triparental conjugation technique. Co-culture with coevolved bacilli strains show significant growth inhibition of pathogen. Potentials of both coevolved bacilli strains and green fluorescent protein-tagged *V. parahaemolyticus* in battling against EMS were discussed.

ACKNOWLEDGEMENTS

For every stumble and hurdle along the way, I dare say "*Be strong and courageous, do not be afraid or tremble at them, for the Lord your God is the one who goes with you. He will not fail you or forsake you*" (Deuteronomy 20:1). I thank Him for all that He has done for me.

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DECLARATION

I hereby declare that my thesis titled "*Isolation and characterisation of coral mucus* bacilli *with quorum quenching potential as a biocontrol agent for shrimp gut protection against Early Mortality Syndrome (EMS)*" is original and contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of candidate's knowledge contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, discloses the relative contributions of the respective workers or authors.

(ANGELICA FIONA TAN) DATE: 5TH MAY 2017

In my capacity as the Principal Coordinating Supervisor of the candidate's thesis, I verify that the given information is true to best of my knowledge.

(Dr. Moritz Müller) DATE: 5TH MAY 2017

PUBLICATIONS ARISING FROM THIS THESIS

Early work has been presented in the following conferences and contributed to the content presented in this thesis:

I. Angelica Fiona Tan, Yao Long Lew, Hui Luh Ang, Janice Sing May Ong, Shien Ping Ong, Ing Jye Sim, Aazani Mujahid and Moritz Müller 'Construction of a stable GFP-tagged *Vibrio parahaemolyticus* strain as an assessment of antibiosis' Monash Science Symposium. 2016 (Poster presentation)

II. Angelica Fiona Tan, Andrew J. Spiers, Yao Long Lew, Aazani Mujahid and Moritz Müller 'Co-cultivation of coral mucus-associated bacilli as a method for strain improvement to combat Early Mortality Syndrome (EMS) in shrimp aquaculture' IOC Western Pacific (WESTPAC) 10th International Scientific Conference. 2017 (Oral presentation)

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Abbreviation	Definition
AHL	*N*-acyl homoserine lactone
AiiA	Gene responsible for autoinducer inactivation enzyme
AHPNS	Acute hepatopancreatic necrotic syndrome
A-L	Air-liquid
ANOVA	Analysis of variance
BHI	Brain Heart Infusion media
C6-HSL	*N*-hexanoyl homoserine lactone
CAA	Casamino acid
CFS	Cell free supernatant
CV	Crystal violet
DF	Degrees of freedom
dH_2O	Distilled water
EMS	Early Mortality Syndrome
EPS	Extracellular polymeric substances
EtOH	Ethanol
F	f-statistic
FAO	Food and Agriculture Organization of the United Nations
FM	Floccular mass
GFP	Green fluorescent protein
GLM	General linear model
HDPE	High-density polyethylene
HP	Hepatopancreas
KB	Kings B
KB-Fe	Iron-supplemented King's B
Kn-TCBS	Kanamycin-supplemented Thiosulphate citrate bile salt media
LB	Luria Bertani medium
LB-NaCl	Salt-supplemented Luria Bertani
LSMeans	Least Square Means
LST	Liquid surface tension
LSTRA	Liquid surface tension reducing activity
M9	Minimal salt media

MATH	Microbial adhesion to hydrocarbons
MDM	Maximum deformation mass assay
n	Number of samples
NB	Nutrient broth
NP	Number of parameters
OD	Optical density
P	p-value
PC	Physically cohesive
PCR	Polymerase chain reaction
QQ	Quorum quenching
QS	Quorum sensing
R^2	Coefficient of determination
rDNA	Ribosomal deoxyribonucleic acid
SEM	Standard error mean
TK-HSD	Tukey-Kramer honestly significant difference
TSA/TSB	Tryptic soy agar/ Tryptic soy broth
UV	Ultraviolet
VM	Viscous mass
VP	*Vibrio parahaemolyticus*
VP_{ATCC}	Standard V. parahaemolyticus strain obtained from German Collection of Microorganisms and Cell Cultures
VP_{EMS}	Pathogenic strain of V. parahaemolyticus, responsible for EMS
VP-GFP / GFP-tagged VP	V. parahaemolyticus strain tagged with green fluorescent protein
WA	Waxy aggregation
X^2	Chi-square test-statistic

1. Introduction

1.1 Shrimps

Shrimps are crustaceans belonging to the order decapoda, meaning “ten-footed”. Other members of decapoda includes crabs and lobsters, but shrimps are visually distinct from these two groups by having a long slender abdomen, unlike crabs which have short and wide ones; as well as having thin slender legs for perching, unlike lobsters and crabs which have strong rigid legs for walking or crawling. Shrimps can be found in a wide range of habitats, including both fresh and saltwater environments. Most shrimps are omnivorous scavengers, living off nutrients obtained from environmental detritus (Ruello 1973; Moss 1993) and sometimes directly from other organisms (Becker and Grutter 2004). The word shrimp can cover a very large taxonomical group, but organisms typically classified as shrimps possess key physiological features as highlighted in Figure 1.

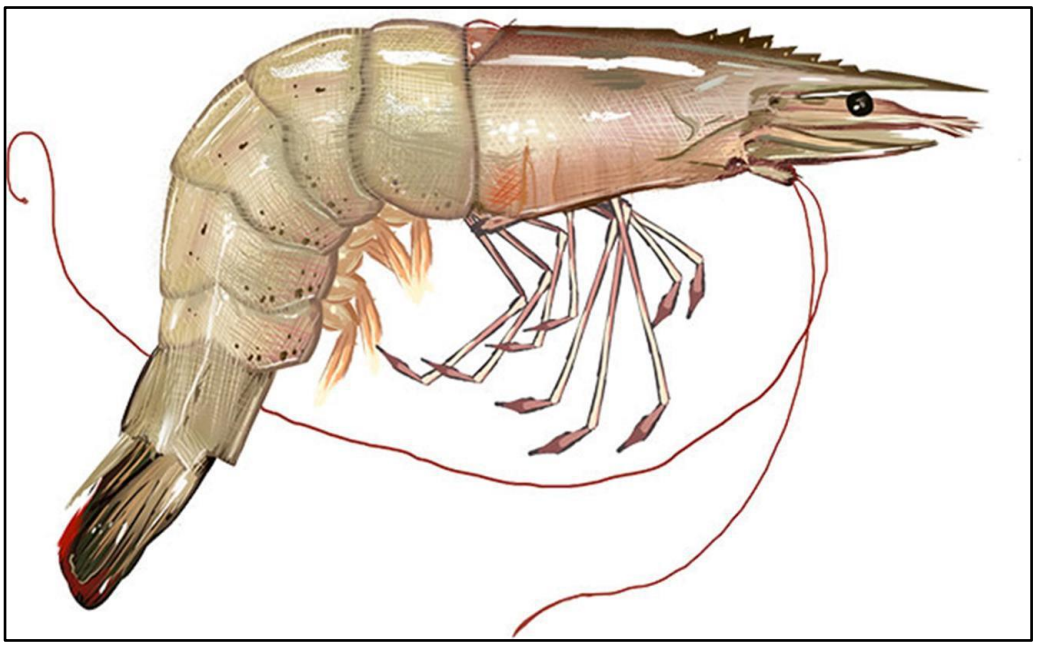


Figure 1. Side view of shrimp (clearly indicate fused head and thorax to give the cephalothorax, five pairs of legs, long segmented abdomen, pleopods, tail) (JSOnline 2016).

Colloquially, the terms “shrimps” and “prawns” are often interchangeably used to great confusion, thus for the purpose of this thesis, the word “shrimp” will be used to describe the group of organisms represented in the figure above as well as the samples obtained used in this study, belonging to the species *Penaeus vannamei*.

1.2 Shrimp aquaculture

Since the early 1980s', the production of seafood went through a dramatic transition, mainly due to the increasing trend of fish farming, or aquaculture. One of the most lucrative and widely traded aquaculture products are shrimps.

Shrimp farming has come a long way since the 1980s', intensifying rapidly following technological advances, fuelled primarily by strong demands in industrialized countries. Shrimp farming can be done virtually anywhere in which a steady supply of clean water can be obtained, preferably near rivers (Kungvankij *et al*. 1986). The easiest way to start shrimp aquaculture is by way of simple earthen ponds, this process is as simple as digging up the soil to form a pond, wait for the rain to fill it up, stock it with juvenile shrimps, feed them, and wait for them to reach maturity before harvest (Tacon *et al*. 2002, see Figure 2). Another mode of rearing shrimps involve a lined pond in which the above mentioned earthen ponds are further lined with a layer of HDPE, which prevents leaching of unwanted materials from soil to pond water as well as drainage of desired compounds into the soil (Burford *et al*. 2003). Shrimp cultivation can also be done using cages, but this method does not yield commercial quantities required for export. A point could be made for cage aquaculture of shrimps as it appears to be a more sustainable model for shrimp farming, especially if carried out in polyculture with other commercial species (Lombardi *et al*. 2006).

Figure 2. Photograph of workers harvesting shrimps from an earthen pond in a local Malaysian shrimp farm (Kurtz 2013).

The commercial sale of shrimp has become a substantial source of revenue for many developing countries (FAO 1998; Weidner & Rosenberry 1992). Shrimp aquaculture in Asia has contributed to 78% of the 712,000 metric tonnes of global farmed shrimp production recorded in 1995 (Shang *et al*. 1998). Common species of shrimps reared in cultivation ponds primarily consists of tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Penaeus vannamei*) (Naylor *et al*. 1998; Rosenberry 1998; see Figure 3).

Figure 3. Photographs of: Left: a tiger shrimp, *Penaeus monodon* (21food.com 2008); Right: two Pacific white shrimps, *Penaeus vannamei* (TradeKey 2007).

While shrimp farming is easily started as described above, several hindrances leading to intensive shrimp mortalities exist due to poor soil and water quality as well as diseases. In many of such cases, the ponds often fail in productivity recovery.

The nature of shrimp farming which are supported by increased pumping and aeration of water as well as commercial feed and chemicals makes shrimps highly susceptible to diseases such as Early Mortality Syndrome.

1.3 Early Mortality Syndrome (EMS)

Early Mortality Syndrome (EMS) refers to the unusual, acute mortality in shrimp approximately within the first 35 days after stocking new cultivation ponds (Joshi *et al*. 2014). EMS manifests itself as a form of acute hepatopancreatic necrotic syndrome (AHPNS), which is a severe disruption to the digestive system of juveniles due to cellular death. This “organ failure” of the shrimp digestive system results in nearly 100% mortality to all infected shrimps, what few individuals which survive typically will not reach full-sized maturity.

1.4 Global impact of EMS

In the FAO report (2013), it was highlighted that the shrimp farming industry of Southeast Asia makes up the largest and most productive shrimp farming region in the world (see Figure 4).

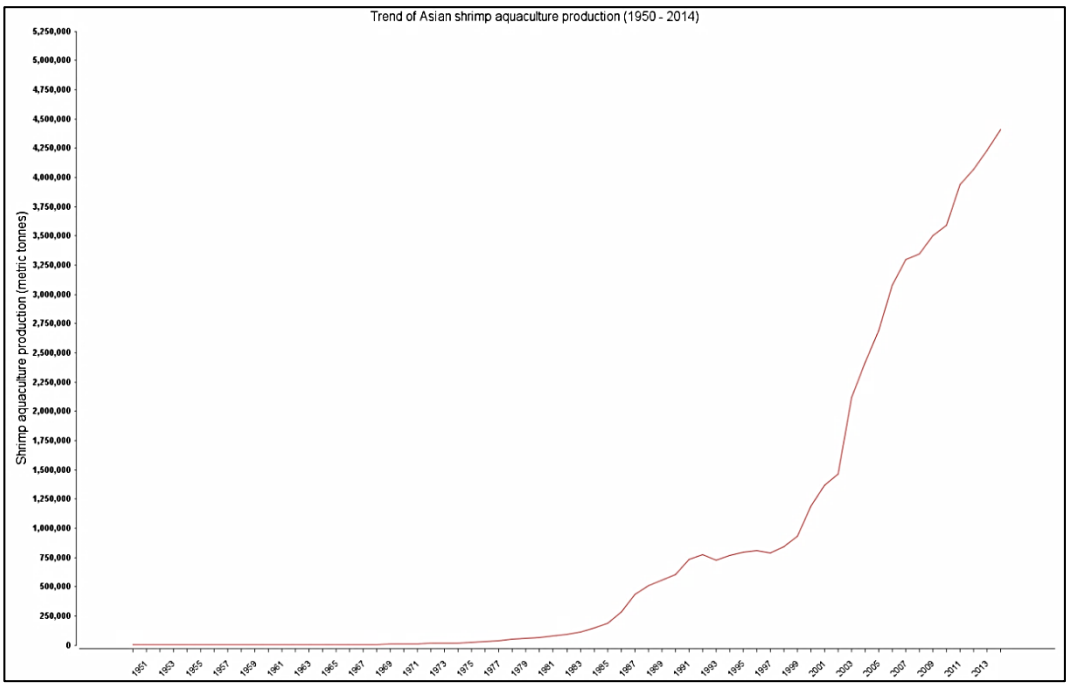


Figure 4. Trend of Asian shrimp aquaculture production until 2014 (FAO 2016).

In the year 2009, China began reporting an unknown emerging disease causing significant mortalities known as “covert mortality disease”. By 2010, the first occurrence of EMS was reported to have affected China shrimp aquaculture sector when the range of affected farms had expanded (Lightner *et al*. 2012). Confirmed occurrences of the disease following the China outbreak were Vietnam and Malaysia in 2011, then south-eastern farms of Thailand in 2012.

Secondary impacts of the disease include employment, social welfare and international market presence (FAO 2013).

1.5 Infection mechanism/Histopathology

EMS is known to infect common aquaculture shrimp species, such as *Penaeus monodon*, *P. vannamei* and *P. chinensis* (FAO 2013). A summary of histopathology studies shows that tissues obtained from affected hepatopancreas of shrimps are atrophied, pale and have black spots or streaks (see Figure 5). Shells of affected shrimps are often found to be soft and have almost empty guts.

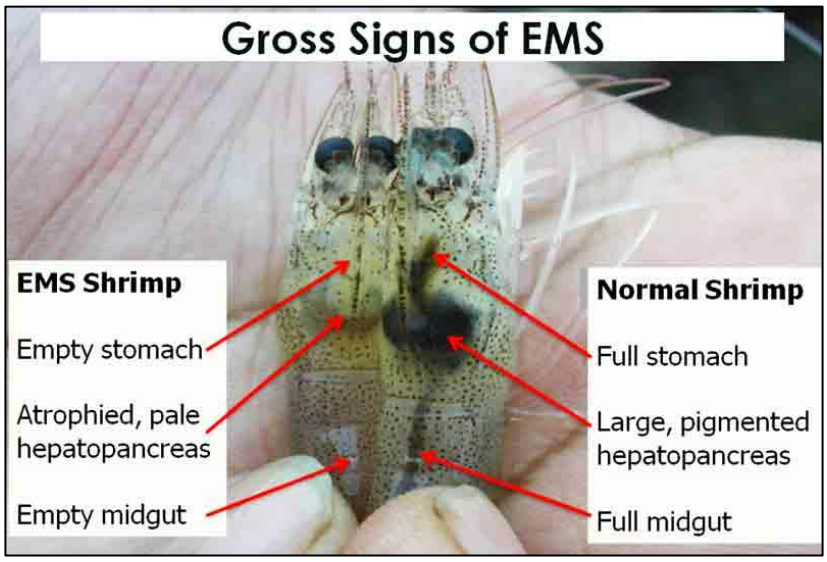


Figure 5. Comparison image between healthy and EMS-infected shrimps (Bodnar 2015).

The causative agent of EMS was found to be a bacterium known as *Vibrio parahaemolyticus*.

1.6 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus (VP) is a gram negative, curved, rod-shaped bacterium (Figure 6), ubiquitous in brackish water or briny water, resulting from mixing of salty seawater with fresh water, commonly present in the transition zones between freshwater rivers with the open ocean (Cavallo *et al*. 2009). This most common interaction which this halophile has with humans is to cause gastroenteritis and inflammatory diarrhoea; most often due to poorly prepared seafood as well as accidental ingestion of tainted water (Su and Liu 2007). Worldwide outbreaks occur often during summer and early fall as VP thrives at elevated seawater temperatures (McLaughlin *et al*. 2005; Nair *et al*. 2007).

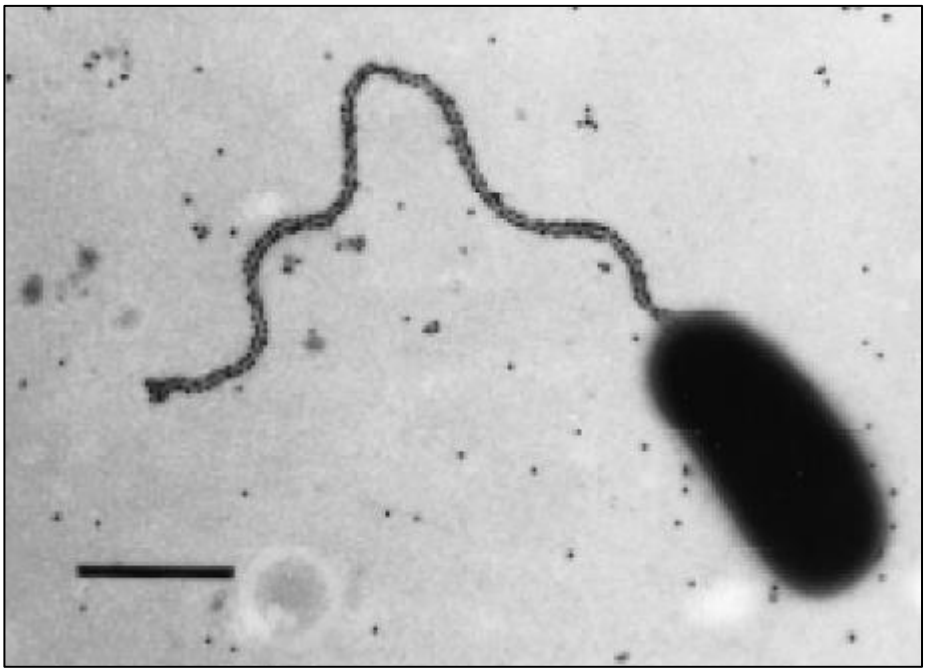


Figure 6. Photograph of a scanning electron microscope image of *Vibrio parahaemolyticus* [Bar = 1 µm] (Pathogen Profile Dictionary 2010).

Recently, aquaculture livestock issues have also been raised due to the problems caused by *V. parahaemolyticus*. The role of this bacterium was initially thought to be a secondary infection due to prominence of bacterial colonization only in the near-terminal stages of EMS (FAO 2013). One of the earliest publications on associating a virulent strain of *V. parahaemolyticus* with EMS-infected shrimp was by Zhang *et al.* 2012 in Guangxi Province, China. This led to a discovery that identifies certain strains of *V. parahaemolyticus* as the causative agent of EMS by Lightner and his team (Lightner *et al*. 2012).

Since then, numerous reports have been made regarding the involvement of this pathogen in shrimp aquaculture (Balcázar *et al*. 2007; Schryver *et al*. 2014; Sani *et al*. 2013; Joshi *et al*. 2014), but little is known regarding its exact mechanism of pathogenicity in shrimps other than the inflammation of shrimp hepatopancreases. However, it is known that bacterial intercellular communication regulate the expression of virulence factors, such is the case of a pathogenic strain of *Vibrio harveyi* which was found to cause significant mortality in *Macrobrachium rosenbergii* larviculture (Pande *et al*. 2012). It is believed that inhibition of signalling between *V. parahaemolyticus* might be the best non-chemotherapeutic solution against EMS.

1.7 Quorum sensing

The mechanism whereby bacteria coordinate expression of certain genes in response to population density by producing, releasing and detecting autoinducers is known as quorum sensing (Bai *et al*. 2008). This intercellular communication of bacteria plays vital roles, common reasons being to coordinate behavioural widespread response to any environmental changes and population density, therefore regulating the expression of specific genes in population-density dependent manners (García-Aljaro *et al*. 2008).

There are many classes of signalling molecules, among them are a widely studied group of autoinducers, known as N-acylhomoserine lactones (AHLs) (see Figure 7). AHLs have been found to be widely used signalling molecules associated with Gram-negative bacteria. The highly conserved nature of their quorum sensing systems allow for interspecies communication as well (Dong *et al*. 2000).

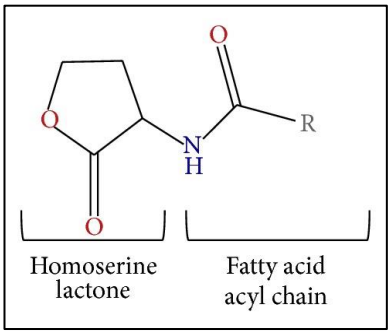


Figure 7. Chemical structure of quorum sensing molecule, N-acyl homoserine lactone (AHL) (Chan *et al*. 2014).

Expression of AHLs is regulated by cell density; activation of related regulatory genes are only possible at high cell densities, as illustrated in Figure 8 (Fuqua *et al*. 1996).

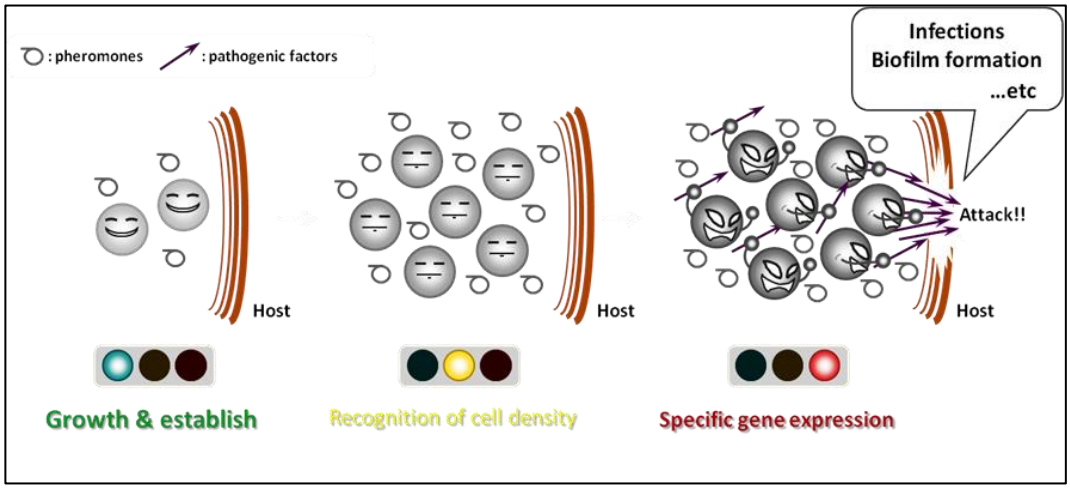


Figure 8. Summary diagram of cell density dependent gene (virulence) expression in quorum sensing (Laboratory of Microbial Technology 2013).

Pathogenic bacteria are known for their activation of virulence genes via the quorum sensing mechanism (Passador *et al*. 1993; Miller and Bassler 2001). The pathogenic strain of *Vibrio parahaemolyticus* (VP) responsible for EMS (called VP_{EMS} in the following) has been isolated from a significant number of shrimp farms, but it is not for sure that a site with the correct VP strain will have its shrimp stock decimated. It was therefore postulated that the VP utilises quorum sensing to determine whether or not "critical mass" was achieved for expression of their *tox* gene responsible for causing AHPNS in postlarvae shrimps (Zorriehzahra and Banaederakshan 2015).

As the concentration of AHLs is a factor in virulence gene expression in pathogenic bacteria, it is possible to modulate the availability of AHLs as signalling molecules in order for prevention of pathogenicity.

1.8 Quorum quenching

Quorum quenching is a term that describes a system that opposes or inactivates autoinducers. Quorum quenching can be broadly generalised to a chemical and biological approach. The biological approach is widely studied, with research being carried out on the receptor protein structures and functional groups of the communication molecules.

Many studies have looked into AHL-degrading enzymes, broadly categorised as autoinducer inactivation proteins. One such enzyme is AHL-lactonase, or AiiA (the gene responsible for this autoinducer inactivation enzyme), which was initially isolated from an unidentified *Bacillus* sp (Dong *et al*. 2000), and subsequently from *B. subtilis* and *B. thuringiensis* (Pan *et al*. 2008; Park *et al*. 2008) as a tool for overcoming interspecies competition (Huma *et al*. 2011).

Studies have used the idea of this censorship of inter-cellular communication as novel therapeutic strategies to counteract pathogenic diseases (Bai *et al*. 2007; Chen *et al*. 2010).

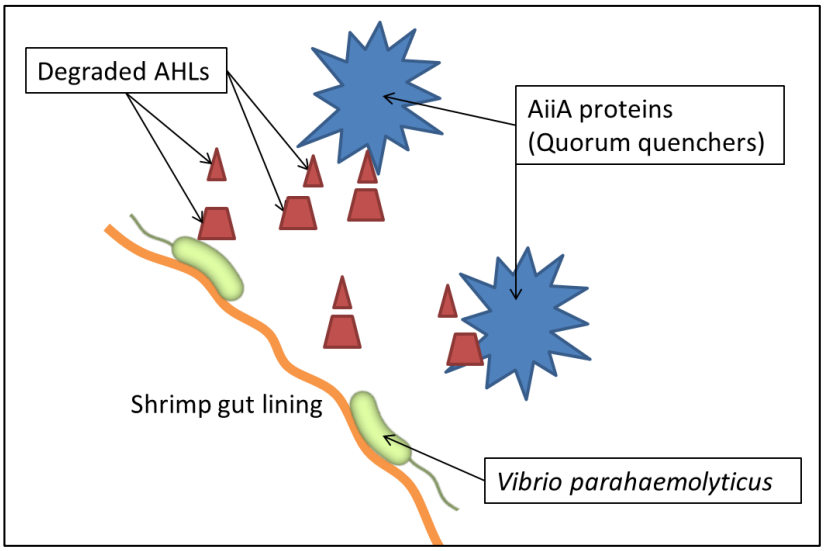


Figure 9. Schematic diagram of *in vivo* quorum quenching action on quorum sensing molecules.

Rather than a cure for EMS, the approach undertaken would be a prophylactic one. The application of quorum quenching enzymes as an anti-infective strategy to counteract vibriosis has been explored previously (Bai *et al*. 2007) (see Figure 9). It is theorised that VP loads in shrimps and surrounding waters during EMS outbreaks are extremely high, indicative of sufficient accumulation of AHLs, leading to the expression of virulence genes. Many studies have highlighted AHL-lactonases as potent enzymes to "paralyze" the quorum sensing systems of pathogenic VP strains, hence providing shrimps with enhanced resistance to VP infection.

The genus Bacillus has been found to contain several species which exhibit quorum quenching properties.

1.9 Bacilli and their known application as biocontrols

All bacilli fall under the genus of Bacillus, which are gram-positive, rod-shaped bacteria with well-studied endospore-forming abilities (Claus and Fritze 1989).

Bacilli are well known as potential biocontrol agents via inhibiting quorum sensing in pathogenic bacteria that are actively causing diseases in host animals, such as shark catfish (Gobi *et al*. 2016), catfish (Novita *et al*. 2015), tilapia (Villamil *et al*. 2014) and carp (Chu *et al*. 2010). Shrimp specific studies have shown that *Bacillus* sp. are common gut microbiota of healthy shrimp, many of which have been developed as potential probiotics for shrimp

(Ziaei-Nejad *et al*. 2006; Balcázar *et al*. 2007; Defoirdt *et al*. 2011; NavinChandran *et al*. 2014; Ramesh *et al*. 2014).

Several recent studies have covered a wide range of bacilli species as biocontrol agents and their colonisation abilities in an aquaculture system (Gullian *et al*. 2004; Hauville *et al*. 2015; Sreenivasulu *et al*. 2015; Guidoli *et al*. 2016).

1.10 *Bacillus* sp. as AiiA producers

Defoirdt *et al*. (2011) suggested Gram-positive spore-forming bacteria show good AHL degradation abilities. Several studies have identified gram-positive bacteria, *Bacillus* as AHL lactonases producers and have successfully cloned and characterized the genes that produce them (Dong *et al*. 2000; Dong *et al*. 2002; Pan *et al*. 2008; Yin *et al*. 2010; Zhang *et al*. 2015).

1.11 Bacilli biofilms

Master regulators of bacteria govern the transition between planktonic (free-swimming) and sessile (adhere and assemble into multicellular communities) states (Kearns *et al*. 2005). Bacteria transition from planktonic to the sessile state in response to many favourable environmental factors; this occurs as a way to “anchor themselves” (colonisation).

Biofilm formation of bacilli, especially marine bacilli have been widely studied as they play important roles to their survival (Davey and O’Toole 2000; Fux *et al*. 2005; Asadishad *et al*. 2014; Checinska *et al*. 2015). The reason behind bacilli biofilm formation on surfaces is mainly for nutrient absorption (Wolfaardt *et al*. 1998) as well as protection from abiotic and biotic stress factors via their extracellular polymeric matrix (Davey and O’Toole 2000). Angelini *et al*. (2009) also suggested that community associations in the form of bacterial biofilms even enable digestion of a broader range of nutrient sources other than protection from environmental changes. Several studies demonstrate that the bacilli form biofilm in order to protect themselves as well as provide a symbiotic relationship with their host (Kloepper *et al*. 2004; Rinaudi and Giordano 2010; Rao *et al*. 2015).

A survey of currently available literature showed that there has been a lack of research into utilization of coral mucus-associated bacteria for the purpose of biocontrol agents. The

possibility of coral mucus-associated bacilli as a bioprospecting pool in the search for bacteria with quorum quenching abilities was explored in this study.

1.12 Coral and Coral reefs

One of the most biologically diverse ecosystems on earth are coral reefs. The coastal waters of Sarawak, Malaysia are not particularly renowned for their coral reefs; however, several reef sites have been subject of research in recent years (Kuek *et al*. 2015; Tan *et al*. 2015; Lee XL 2012) (see Figure 10). Those research undertaken focused more on the diversity of organisms found in reefs; however, there is still very limited work done in terms of bioprospecting of industrially relevant organisms.

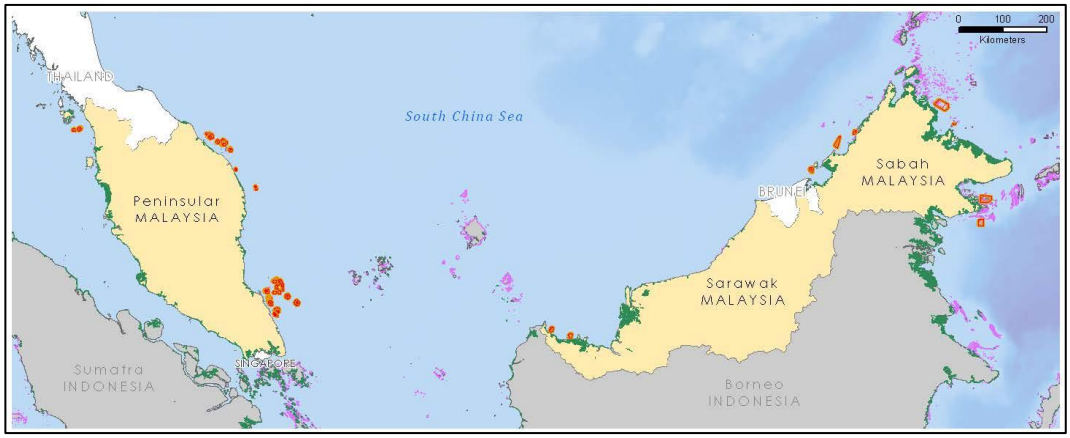


Figure 10. Map of coral reef distribution around the coast of Malaysia (UNEP-WCMC 2010). Highlighted pink regions indicate coral reefs and orange regions indicate marine protected areas of coral reefs.

1.13 Corals and Coral-associated bacteria

Coral reefs are areas whereby coral polyps are prevalent; there are several factors which can help or hinder an area to be suitable for establishment and growth of a coral reef, chiefly temperature, pH, depth, and sedimentation rates (Montaggioni 2005; Nzali 1998).

In a long term study by Rosenberg and Falkowitz (2004), the coral species *Oculina patagonica*, initially easily infected by a bleaching disease caused by *Vibrio shiloi*, was found to have developed a resistance against the pathogen over time. The development of coral resistance against pathogens has not been studied in detail up to that point as evidence pointed to corals having no adaptive immune system. The findings of Rosenberg and Falkowitz were further reviewed by Reshef *et al*. (2006), leading to an observation that a

symbiotic relationship exists between certain species of corals and bacteria associated with the corals.

The Coral Probiotic Hypothesis was proposed in order to explain how corals can eventually develop resistance to pathogens. This paradigm explains how corals can adapt to both biotic and abiotic factors by allowing the altered parameters to select for the most advantageous holobiont. This confers protection to the coral against harmful pathogens and environmental stresses, simply by associating itself with microorganisms which are able to rapidly diminish any of the negativity it experiences (Reshef *et al*. 2006). The coral holobiont typically refers to the ecological unit comprising of the coral polyps, symbiotic zooxanthellae, tissue associated microorganisms, and mucus associated microorganisms. (Sweet *et al*. 2011)

Mucus-associated bacteria represent the biological barrier between the external environment and the rest of the coral holobiont. The protection it confers to the coral as a physicochemical barrier (Hayes and Goreau 1998; Santavy and Peters 1997; Sutherland *et al*. 2004) as well as a barrier for potential marine pathogens (Brown and Bythell 2005) has been discussed in several studies. Although there are many other obvious places in the search for pathogen-inhibiting isolates, many recent studies such as Fatima *et al*. (2016) and Santhakumari *et al*. (2016) have come across coral-associated strains displaying antimicrobial activities against vibriosis.

Potential strains isolated to overcome pathogens can theoretically perform even better *in vitro* via artificially inducing evolution. In the case of this study, it was proposed that directed selection via co-cultivation with targeted *V. parahaemolyticus* strains can be applied to achieve strain improvement. Thus existing bacteria with potential as biocontrol agents may be further improved upon.

1.14 Bacterial evolution

Evolution is defined as the change of a biological population's heritable characteristics over successive generations, thereby giving rise to biodiversity (Abalaka and Abbey 2014). The theory of evolution, proposed by Charles Darwin, describes a common ancestry for all biological species and evolution occurs over time due to natural selection (Goldschmidt 1940).

Evolutionary relationships among bacteria were first studied via their differences in DNA sequences by Carl Woese in the year 1970 (Lenski 2012). The great diversity of bacteria found today can be contributed to selection pressures both biotic and abiotic which has selected for the most successful mutants in their respective niches. In nature, there is a continuous natural selection which enhances survivability of the individuals which were able to obtain resources and replicate faster than its competitors. In the laboratory, directional selection of mutants can be artificially induced in response to a competitor or physicochemical changes.

1.15 Coevolution

Janzen (1979) defined coevolution as "*an evolutionary change in trait of individuals in one population in response to a trait of individuals of a second population, followed by an evolutionary response by the second population to the change in the first*".

Previous studies have prioritized the use of adaptive evolution to stimulate secondary metabolite production by co-culturing two or more organisms (Perry *et al*. 1984; Kurosawa *et al*. 2008; Bills *et al*. 2009; Garbeva *et al*. 2011). In this study, coevolution was performed by subjecting selected biocontrol agents against a specific pathogen which will act as a driver for directional selection.

1.16 Basic mechanism of coevolution

The method of serially passing pathogen over time in media containing increased concentrations of antibiotics has long been genetically and molecularly studied (Levin *et al*. 2000; Hancock 2005; Martinez *et al*. 2011; Lázár *et al*. 2013). This evolution-based strategy allowed mutants with higher levels of resistance to arise. Therefore, Charusanti *et al*. (2012) proposed a coevolution strategy in which to make use of adaptive (competition-based laboratory) evolution in the presence of selection pressure (directional selection).

In the most accurate definition of the word, "coevolution" is a process which occurs when two organisms are constantly exposed to each other over a period of time, any observable phenotypic and/or genotypic changes would have occurred for both organisms. In the context of this work, the same term "coevolution" is used in a slightly looser manner as the objective was to confer permanent phenotypic advantages in only one type organism, in

this case the bacilli, by constantly co-cultivating the improved strains with one constant partner – the wild type *Vibrio parahaemolyticus*.

Evidence of successful phenotypic change or any form of strain improvement can typically be assessed via normal plate-based methods and applied when most variables are controlled. However, a complicated experimental environment will require better visualization of host-pathogen interaction, even more so during assessment of potential of a coevolved organism as a biocontrol agent.

Therefore, the development of a fluorescent indicator organism will aid in the observation of organisms of interest in their complex environment *in vitro* as well as *in vivo*.

1.17 Green fluorescent protein (GFP) and its Applications

Ever since the discovery and development of green fluorescent protein (GFP) by Roger Tsien in 1994, fluorescent labelling (particularly green fluorescent) has come a long way primarily used as in situ fluorescent hybridization probes, hence enabling easy visualization of specific biological entities upon exposure to UV (Heim *et al*. 1994).

Many studies have utilized GFP as detectable markers for gene expression and determine localization of bacteria and/or protein within cells (Lemon and Grossman 1998; Brazelton *et al*. 2000; Sawabe 2006; Boroviak *et al*. 2014; Reichmann *et al*. 2014). In this study, VP is tagged with a GFP and its application as an assessment of anti-VP activity of potential biocontrol agents in a simple liquid microcosm was explored and discussed.

1.18 Thesis Aim

The main aim of this study is to control the spread of Early Mortality Syndrome, without an over-reliance on antibiotics, by using directed selection to improve verified quorum quenching Gram-positive bacilli from marine sources. This preliminary *in vitro* investigation serves to highlight the potential of selected bacilli strains to be potentially utilized as biocontrol agents in shrimp aquaculture.

1.19 Thesis Objectives

- Isolation and characterization of bacteria, especially bacilli from shrimp gut and coral mucus, which express quorum quenching potential against *Vibrio parahaemolyticus*
- Quantitative phenotypic characterization of quorum quenching bacilli, especially regarding their biofilm forming potential, followed by strain identification
- Apply co-cultivation method on bacilli strains in liquid microcosms with *Vibrio parahaemolyticus* and evaluate any strain improvement via selected post-evolution characteristics
- Generate a stable and expressing GFP-tagged *Vibrio parahaemolyticus* and assess its efficacy as an accurate measure of anti-VP activity in simple liquid-based microcosms

2. Chapter 1: Isolation and preliminary characterisation of shrimp- and coral-associated gram-positive bacilli which exhibit quorum quenching potential against *Vibrio parahaemolyticus*

2.1 Introduction

This chapter describes the preliminary steps in isolating potential quorum quenching strains from two separate sources, which were shrimp intestine and coral mucus. Quorum quenching abilities were screened via plate-based assays as well as molecular screening for presence of quorum quenching gene. Preliminary characterisation of isolated strains was also carried out to assess the extent of antagonistic activity against VP. Flowchart in Figure 11 describes summary of work done in this chapter.

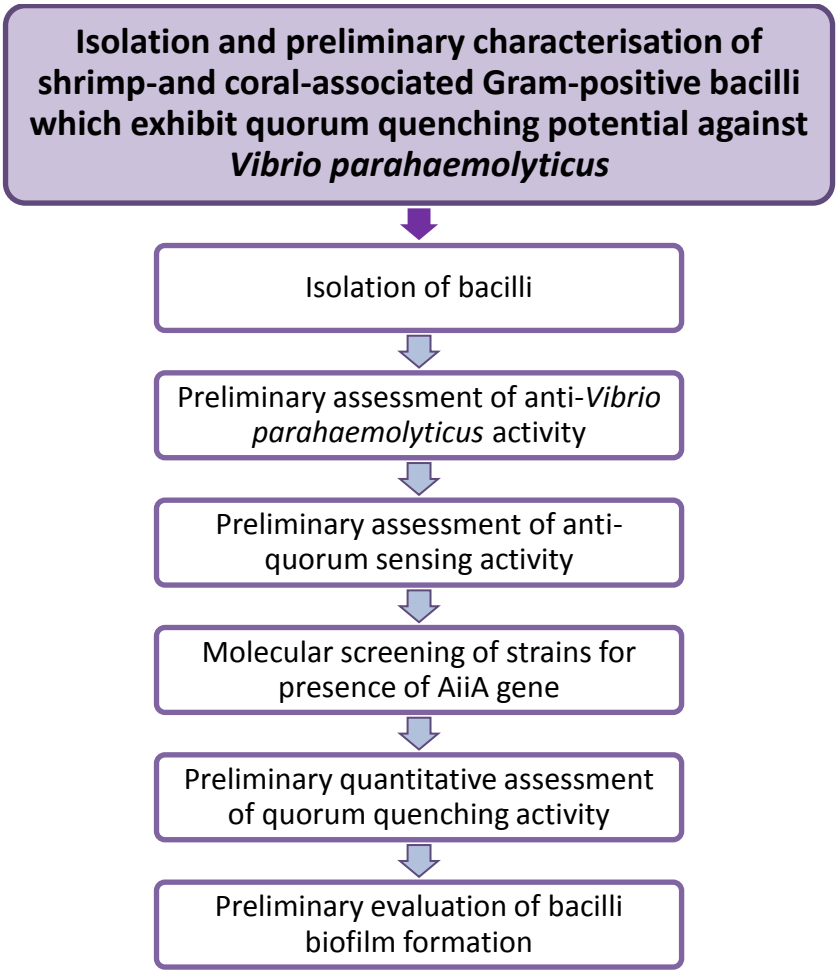


Figure 11. Flowchart which summarises the work done in this Chapter 1.

2.2 Methodology

2.2.1 Isolation of bacilli

2.2.1.1 Isolates from shrimps

Litopenaeus vannamei shrimp samples were obtained from brackish water, immersed in ethanol (2 min) for surface sterilisation, and dissected to obtain the hepatopancreas (HP) portion of shrimp digestive system. HP samples were then homogenized under aseptic conditions in sterile saline (0.9% NaCl). Serial dilutions of samples were carried out before plating onto half strength Zobell marine agar and incubated (24 hours, 30°C) (composition of media listed in Table 1).

Table 1. Composition of Zobell Marine agar in grams/L.

Composition	Grams/Litre (g/L)
Peptic digest of animal tissue	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.450
Magnesium chloride	8.8
Sodium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Bacteriological agar	15.0
Final pH 7.6 at 25°C	

To selectively isolate members of the *Bacillus* genus, a selective media (HiCrome Bacillus® agar, components of media listed in Table 2) was used for subsequent differentiation and selection from a mixed culture by applying a chromogenic method (24 hours, 30°C).

Table 2. Composition of HiCrome Bacillus ® agar in grams/L.

Composition	Grams/Litre (g/L)
Peptic digest of animal tissue	10.0
Meat extract	1.0
D-Mannitol	10.0
Sodium chloride	10.0
Chromogenic mixture	3.20
Phenol red	0.025
Bacteriological agar	15.00
Final pH 7.1 at 25°C	

2.2.1.2 Isolates from coral mucus

Coral mucus-associated bacteria were isolated from corals obtained from the South China Sea region. Corals were inverted to allow mucus layers to drip for collection before invasive collection was carried out (culturing original residents of coral mucus layer). Isolation from coral mucus to individual isolates was carried out as previously describe by Ritchie KB (2006) and Kuek *et al*. (2015). Fresh undiluted coral mucus (50 µL) were collected from each coral sample, then spread evenly onto Zobell marine agar plates and allowed to dry (10 min). All mucus-treated plates (in duplicates) were UV-sterilized (10 min, 320 nm) before another layer of mucus (50 µL) was applied on top evenly and left to incubate (24 hours, 30°C). For each double layer mucus plate, an uninoculated single layer mucus plate was left as a control. Isolates were then plated onto selective media, HiCrome Bacillus ® agar.

2.2.1.3 Purification of isolates

All single isolates obtained from both sources were gram stained as a check for purity.

2.2.1.4 Long term storage of pure strains

From overnight cultures, all bacilli isolates were stored in 15% v/v glycerol at -80°C respectively for the purpose of long term storage before proceeding to antagonistic assays.

2.2.2 Preliminary assessment of anti-*Vibrio parahaemolyticus* activity

2.2.2.1 Test organisms used

A virulent strain of *Vibrio parahaemolyticus* (VP_{EMS}) was obtained from a local shrimp farm of Seahorse Corporation Sdn. Bhd. The strain was isolated from the hepatopancreas of a shrimp with EMS/AHPNS, confirmed via a commercial PCR kit for detection of EMS-causing VP strain (IQ Plus AHPND/EMS Plasmid Kit, GeneReach Biotechnology Corp.) and cultivated in alkaline peptone water for enrichment. The composition of alkaline peptone water is listed out in Table 3.

Table 3. Composition of Alkaline Peptone Water in grams/L.

Composition	Grams/Litre (g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	10.0
Final pH 8.4 at 25°C	

2.2.2.2 Bacilli-*V. parahaemolyticus* interactions in dual culture

Pure bacilli cultures were subjected to a qualitative anti-*Vibrio parahaemolyticus* assay in a dual culture. Overnight cultures of both *Vibrio parahaemolyticus* and isolates from either shrimp or coral mucus were dropped-inoculated (25 µL) onto each end of a modified HiMedia Soybean Casein Digest agar (TSA, composition of media listed out in Table 4) agar plate, in replicates (n=3), then incubated (48 hours, 30°C). A zone of clearance or VP growth inhibition will be observed in the presence of anti-*Vibrio* activity.

Table 4. Composition of modified HiMedia Soyabean Casein Digest agar in grams/L.

Composition	Grams/Litre (g/L)
Pancreatic digest of casein	15.0
Papaic digest of soyabean meal	5.0
Sodium chloride	20.0
Bacteriological agar	15.0
Final pH 7.3 at 25°C	

2.2.3 Preliminary assessment of anti-quorum sensing activity

2.2.3.1 Test organism used

Indicator organism to access quorum quenching activity, *Chromobacterium violaceum* DSM 30191, was obtained from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DSMZ). Indicator bacterium, *C. violaceum*, regulates its pigment production by quorum sensing (C6-HSL) and become readily inhibited in the presence of AHL lactonases.

2.2.3.2 Bacilli-*C. violaceum* interactions in parallel proximity streak

Anti-quorum sensing assay was qualitatively screened via parallel arrow streaking method (parallel proximity streak). Isolates were streaked as an aero line onto TSA plates and incubated (24 hours, 30°C). Then, indicator organism (*C. violaceum*) was streaked 0.5 cm away from each isolate and incubated (24 hours, 30°C). Presence of pigment inhibition of *C. violaceum* was observed.

2.2.4 Molecular screening of strains for presence of AiiA gene

AHL-lactonases are able to hydrolyze lactone rings and amide linkages of N-acyl-homoserine lactones (AHLs), thus inactivating these autoinducer molecules and blocking quorum sensing systems of pathogens. The gene encoding this autoinducer inactivation (AiiA, protein of 250 amino acids), is often cloned and used for heterologous gene expression (Dong *et al*. 2000; Lee *et al*. 2002; Wang *et al*. 2007). In this study, PCR-based screening of the AiiA gene was carried out in order to molecularly determine the strains' ability to quorum quench and therefore, narrow down the selection for potential probionts.

2.2.4.1 Genomic DNA extraction

Overnight cultures of bacterial isolates which confirmed positive for both antagonistic assays were prepared in marine broth (prepared with the same composition as Zobell marine agar shown in Table 1 without bacteriological agar). Then, DNA was extracted from all cultures using freeze and thaw method (Tsai and Olson 1991), four cycles of 5 minutes in -80ºC followed by 3 minutes in 85ºC. Presence of extracted genomic DNA was verified using agarose gel electrophoresis (100 V, 30 min).

2.2.4.2 AiiA gene PCR

DNA was PCR amplified with a Mastercycler gradient thermocycler (Eppendorf), using specific AiiA gene primers (Pan *et al*. 2008). PCR was carried out using the protocol given in the Bioline Red Taq mix, the reaction mixture in each tube is given in Table 5 as follows:

Table 5. Composition of PCR reaction mixture for AiiA gene screening.

Components	**Volume (µL)**
2x Bioline Red Taq Mix	12.5
Forward primer, AiiA1 5'-ATGACAGTAAARAARCTTTATTTC-3'	1.0
Reverse primer, AiiA2 5'-TCACTATATATAYTCMGGGAACTC-3'	1.0
DNA template	2.0
dH_2O	8.5
Final volume	25.0

Amplification reactions were performed in the thermocycler programmed as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min 30 sec, 72°C for 2 min, and final extension at 72 °C for 10 min. PCR reaction results were checked using agarose gel electrophoresis (100V, 30 min).

2.2.5 Preliminary quantitative assessment of quorum quenching activity

2.2.5.1 Induction of extracellular production of AiiA

Further work was carried out on strains' quorum quenching abilities by subjecting strains to starvation, exposed only to substrate C6-HSL as sole carbon and nitrogen source. Mahdhi *et al*. (2012) determined that strains under stress conditions, such as starvation induce expression of potential probiotic enzymes.

Each strain was inoculated into microcosms of minimal salt media containing only C6-HSL (M9-C6-HSL; composition of media listed out in Table 6) and incubated (10 mL, 24 hrs, 30°C).

Table 6. Composition of M9-C6-HSL agar in grams/L.

Composition	Grams/Litre (g/L)
Sodium chloride	1.0
Potassium chloride	0.5
Magnesium chloride hexahydrate	0.4
Calcium chloride dihydrate	0.1
Potassium dihydrogen phosphate	0.2
Sodium sulphate	0.15
MES (2-(N-morpholino)ethanesulfonic acid)	1.0
Trace elements and vitamins (Artificial sea salt)	0.01×10^{-9}
C6-HSL	24 nM / 5×10^{-3}
Final pH 7.5 at 25°C	

2.2.5.2 Modified agar well diffusion assay

A modified well diffusion assay, modified from Vinoj *et al*. (2014), Chen *et al*. (2010) and McLean *et al*. (2004), was performed on modified HiMedia Luria Bertani agar plates (composition of media listed in Table 7) (20 mL each plate) inoculated with *C. violaceum* (indicator cultures) (300 µL) and a 5 mm diameter plug removed from the centre of the agar plate.

After incubation, a sample (20 µL) was removed from each microcosm, filtered through 0.22 µm membrane filter to obtain cell-free supernatant (CFS) and transferred to plate wells respectively. Replicates were carried out for each bacilli strain tested (n=8). Plates were then further incubated (48 hrs, 30°C). This was repeated for strains with initial substrate incubation at 48 and 72 hours.

Table 7. Composition of LB agar in grams/L.

Composition	Grams/Litre (g/L)
Casein enzymic hydrolysate	10.0
Yeast extract	5.0
Sodium chloride	10.0
Bacteriological agar	12.0
Final pH 7.5 at 25°C	

The area (mm^2) of *C. violaceum* non-pigmented zones was measured with ImageJ (Image Processing and Analysis in Java). *C. violaceum* will form decolourized pigments in the presence of quorum quenching enzymes, in this case AHL-lactonase enzyme, as is observed in Figure 12 below when compared to a negative control or negative result.

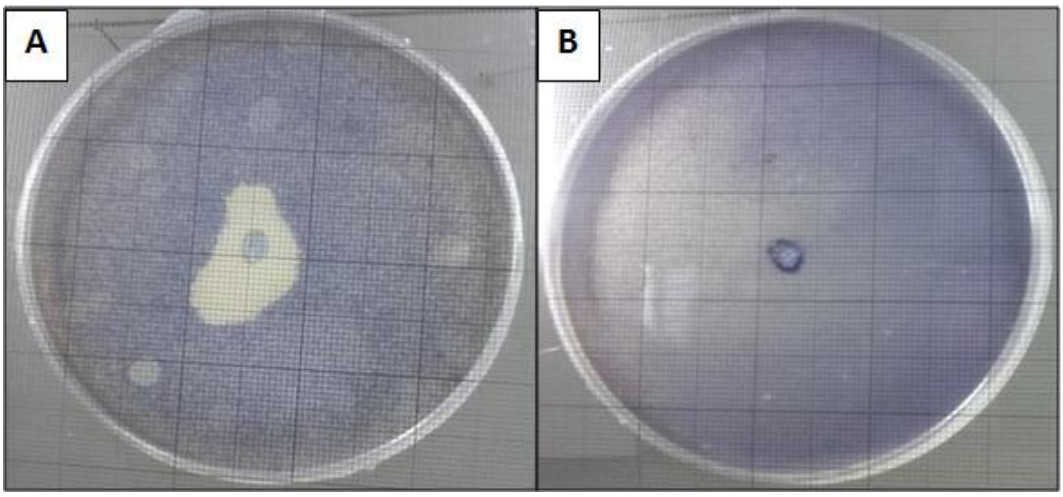


Figure 12. Quorum sensing inhibition demonstrated by the lack of pigment of indicator culture surrounding the well (A) as compared to negative control (B).

This characterization of AHL-lactonase protocol allows us to access the approximate amount of lactonase produced by the Bacillus sp. in the time span given. An assumption made in this study is that the area of non-pigmented *C. violaceum* growth is directly affected only by amount of extracellular enzyme secreted.

2.2.6 Preliminary evaluation of bacilli biofilm formation

2.2.6.1 Microtiter Plate Assay

A simple microtiter plate assay was modified from O'Toole (2011) and Mahdhi (2012) to access the ability of the bacilli strains to disrupt biofilm-forming properties of VP_{EMS} and/or presence of potential antifouling compounds produced by the bacilli strains. It is assumed that the direct disruption of biofilm-forming ability is caused by the disruption of quorum sensing system of the test pathogen, hence defecting virulence gene expression.

Overnight culture of *V. parahaemolyticus* (VP_{EMS}) was prepared. The bacilli strains are grown on plates of M9-C6-HSL. From the plates, each strain was further inoculated into Soyabean casein digest medium (TSB; prepared with the same composition as TSA as shown in Table 4 without bacteriological agar).

A 96-well polystyrene cell culture treated plate was inoculated as in Table 8 below. For each treatment, replicate wells were used (n=6).

Table 8: Experimental set up of 96-well polystyrene cell culture treated Plate 1.

Treatment	Components	Amount (microLitre, µL)
Blank	TSB	160
Negative Control	TSB	110
	V. parahaemolyticus (EMS)	10
Positive Control	TSB	100
	V. parahaemolyticus (EMS)	10
	Ampicillin	50 (0.07 g/L)
Sample	TSB (100µL) + VP (10µL) + bacilli culture (50µL)	100
	V. parahaemolyticus (EMS)	10
	Bacilli culture	50

Another 96-well plate was inoculated. Plate 2 was treated with bacilli cultures only without the VP_{EMS}.

After incubation, all liquid cultures were decanted and gently rinsed. Crystal violet (0.1%, 125 µL) was added to each well of the microtiter plate and left to incubate (25°C, 15 min). Plates were rinsed again, to rid plates of excess cells and dye, before adding acetic acid (30%, 125 µL) to each well to solubilize crystal violet stains and incubated (25°C, 15min). Absorbance was read with a BioRad ELISA plate reader at 550 nm.

2.2.7 Statistical analyses and modelling

All experiments and assays were conducted in replicates as stated, and the means ± SE provided where necessary. JMP Statistical Discovery Software (JMP v12, SAS Institute Inc., USA), and Microsoft Excel (version 2010) were used to analyse all data obtained. Differences between means were tested by ANOVA (*F*).

2.3 Results and Discussion

2.3.1 Isolation of bacilli

Litopenaeus vannamei or *Penaeus vannamei* samples, commonly known as whiteleg shrimp, were locally obtained. Following sterile extraction of shrimp hepatopancreas, the bacilli found among in HP microbiota was isolated aseptically onto HiCrome Bacillus agar, observed in Figure 13 below. Upon purity check, a total of 40 Gram-positive bacterial strains were successfully isolated from the samples obtained.

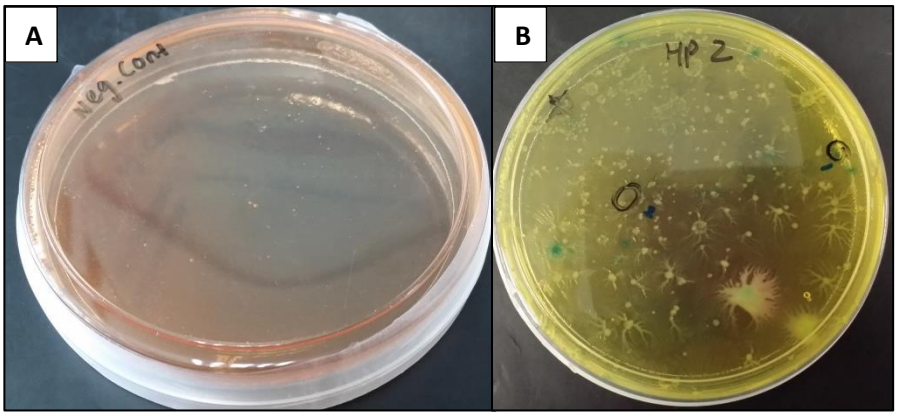


Figure 13. Selective and differential media for the isolation of bacilli from shrimp gut microbiota (B) as compared to negative control (A). Media, originally light red in colour (A) is observed to turn yellow in the presence of sugar utilisation, therefore acidic, as observed in (B).

From the coral mucus samples, a total of 55 Gram-positive coral mucus-associated bacteria were successfully obtained and immediately kept in 20% glycerol at -80°C long term storage, prior to subsequent antagonistic assays.

2.3.2 Preliminary assessment of anti-*Vibrio parahaemolyticus* activity

Anti-VP activity of all Gram-positive isolates was first assessed via a dual culture method as observed in Figure 14 below. The presence of a zone of clearance in between swarming VP and the isolate would suggest the sensitivity of VP towards the isolate, as observed in Figure 14(B). However, it is not confirmed if sensitivity was due to antibiosis or anti-quorum sensing.

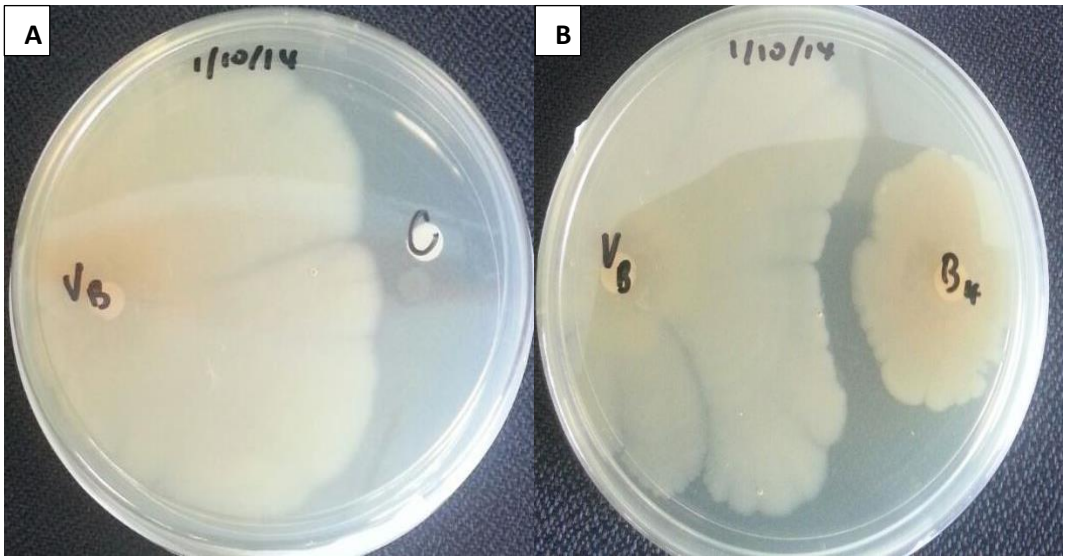


Figure 14. Bacilli isolate, as indicated in (B) expressing anti-*Vibrio parahaemolyticus* activity via dual culture method, as compared to negative control in (A) (with distilled water).

2.3.3 Preliminary assessment of anti-quorum sensing activity

As previously mentioned in Section 2.2.3.1, indicator bacterium, *C. violaceum* pigment is readily inhibited by the presence of enzyme AHL-lactonase. Bacilli isolates were first streaked onto plate and incubated overnight; this allowed for diffusion of any extracellular AHL-lactonase produced into the media. Presence of diffused quorum sensing inhibitors will halt pigment production of *C. violaceum* which was streaked onto the agar plate, as observed in Figure 15(B) below as compared to a negative result in (A).

At this point, it was observed that only 30 Gram-positive strains from coral mucus successfully inhibited the pigment production of *C. violaceum*, suggesting anti-quorum sensing activity. No isolates from shrimp microbiota yielded positive results.

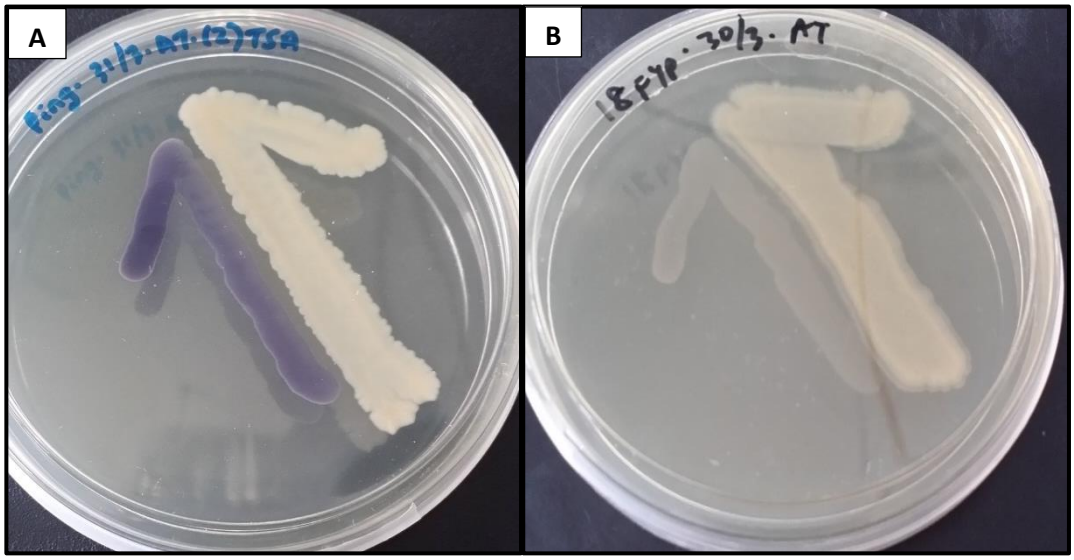


Figure 15. Assessment of quorum quenching activity via parallel proximity streak method; (A): Negative result, (B): Positive result.

2.3.4 Molecular screening of strains for presence of AiiA gene

Confirmation of the presence of the AiiA gene (genes encoding for the expression of AHL-lactonases) was carried out via PCR-based screening with primers described by Pan *et al*. (2008). This was to identify functional AiiA gene from the isolates which yielded positive results in the previous preliminary assessment. By carrying out PCR, isolates which possess the gene but do not express the gene as readily can also be identified.

From the 30 coral-mucus associated bacteria that inhibited pigment production of *C. violaceum* previously, gel image analysis of the PCR products showed that there were 14 bright bands, 6 faint bands and the rest did not possess the AiiA gene, as shown in Figure 16 below. When DNA from shrimp microbiota isolates were PCR-screened, none of the isolates possess the AiiA gene. From the twenty strains that possess the AiiA gene, the 14 that exhibited bright bands were selected to undergo further quorum quenching characterization.

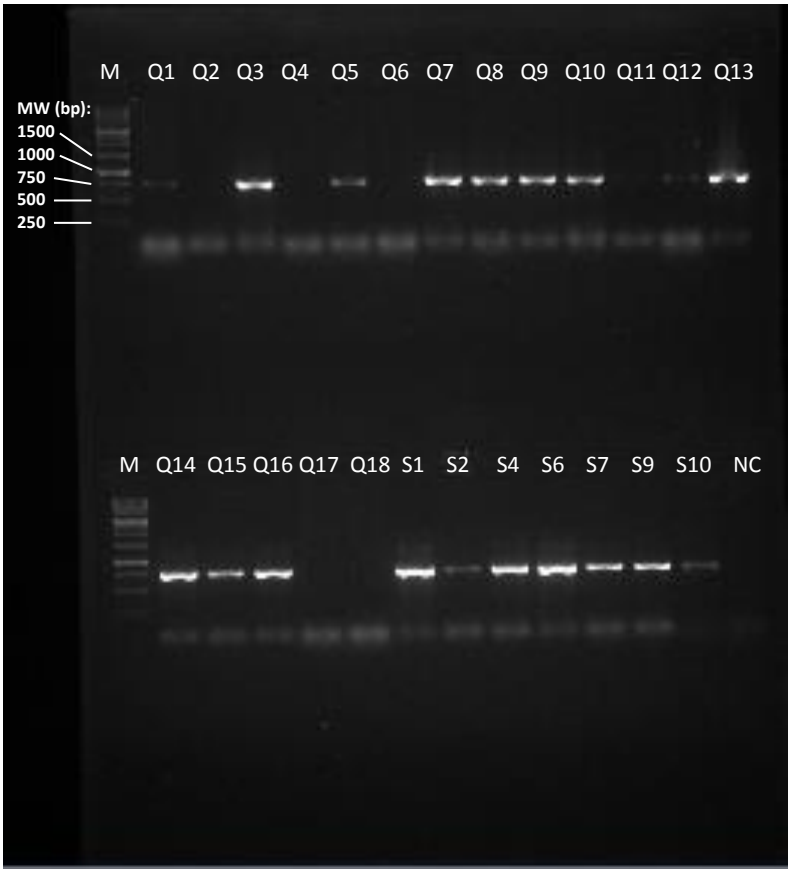


Figure 16. Gel image of PCR products using primers for the AiiA gene. Lane M – 1kb DNA marker (Fermentas), Lane NC – Negative control (no DNA template), other lanes consist of DNA of bacterial isolates.

2.3.5 Preliminary quantitative assessment of quorum quenching activity

Further expression and characterization of the AHL-lactonase enzyme expressed by the AiiA gene was carried out in vitro on the identified strains. These steps allowed selection of potential top strains for further characterization work as biocontrol agents.

Upon starvation to induce consistent expression of AHL-lactonase as suggested by Mahdhi *et al*. (2012), it was found that five out of the fourteen bacterial isolates were not able to grow on the M9-C6-HSL agar plates, although they possess the ability to produce the enzyme (AHL-lactonase) that was able to break-down and utilize C6-HSL as a carbon source. Hence, modified agar well diffusion assay was carried out with only nine strains. The area of non-pigmented *C. violaceum* growth was obtained and measured for each strain for 24, 48 and 72 hours as demonstrated in the bar chart in Figure 17 below.

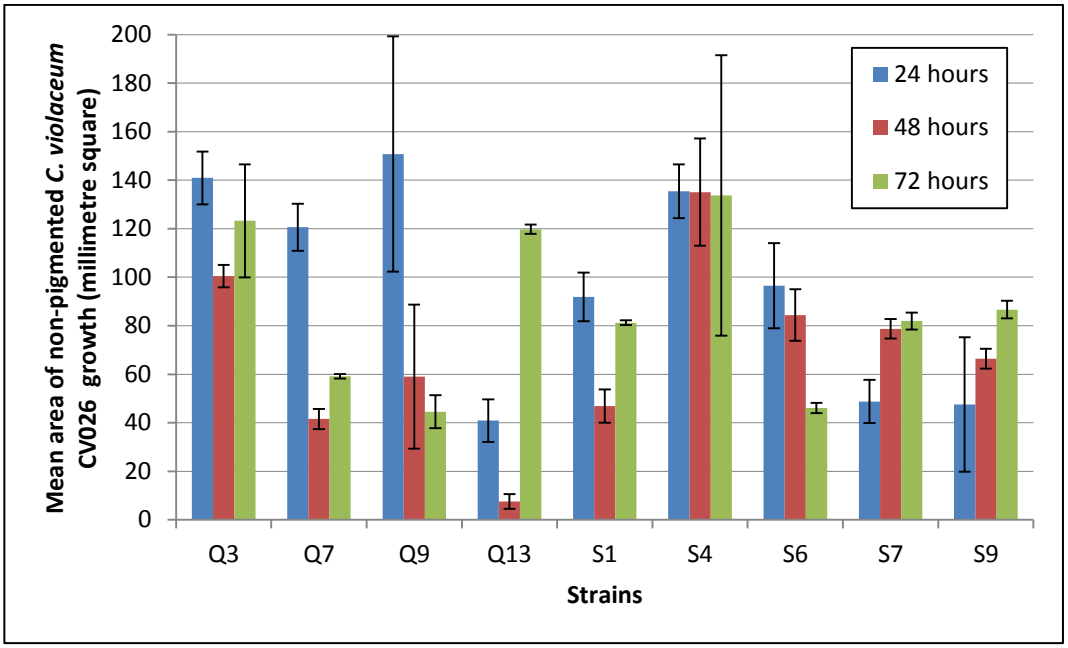


Figure 17. Bar chart comparison of mean area of non-pigmented *C. violaceum* growth (Mean±SEM) surrounding cell-free supernatant of each strain exposed to C6-HSL substrate for 24, 48 and 72 hours.

Cell-free supernatant of strain Q9 gave the highest mean area of non-pigmented *C. violaceum* growth (150.758 ± 48.0 mm^2) within the first 24 hours. However, S4 performed third best, after Q3, with a mean area of 135.432 ± 11.0 mm^2 but most consistently over the 72 hours period (ANOVA, p=0.0015).

Five out of nine of the bacterial strains, which are Q3, Q7, Q9, S1 and S6, show a general decreasing trend over the 72-hour period, which is an expected trend as nutrients available in the microcosm are limited and depleting by the day. As a result, smaller amounts of enzyme were needed for extracellular secretion to obtain food. It is possible for the strains to even "eat" the enzymes they produced previously (AiiA proteins) as carbon source.

On the other hand, strains Q13, S7 and S9 show a general increasing trend of AHL-lactonase produced. At first, these strains show a slow rate of enzyme secretion within the first 24hours. In time, the amount of enzyme produced increased significantly in order to degrade more C6-HSL from the media. Although Q13 gave the smallest mean area within the first 24 hours, there was a significant increase of enzyme produced, giving the third highest mean area after 72 hours (119.759 ± 2.0 mm^2).

However, S4 was able to produce enzyme consistently, or metabolize and fully utilize the carbon source slowly over the 72 hours, such that there is almost no evidence of nutrient depletion or the need to degrade its own enzyme for carbon source.

A good trait of a biocontrol agent in efforts to combat EMS is to possess quorum quenching abilities which can reach each strain's maximum potential within the first 48 hours of treatment, as that is the time taken to achieve 100% mortality in juveniles stocking ponds. S4 fulfils the criteria for an acceptable biocontrol agent in the case of secreting extracellular quorum quenching compounds.

2.3.6 Preliminary evaluation of bacilli biofilm formation

A simple microtiter plate assay was carried out in the search for bacilli strains to produce compounds that disrupt biofilms formed by VP. However the results obtained was quite different from what was expected.

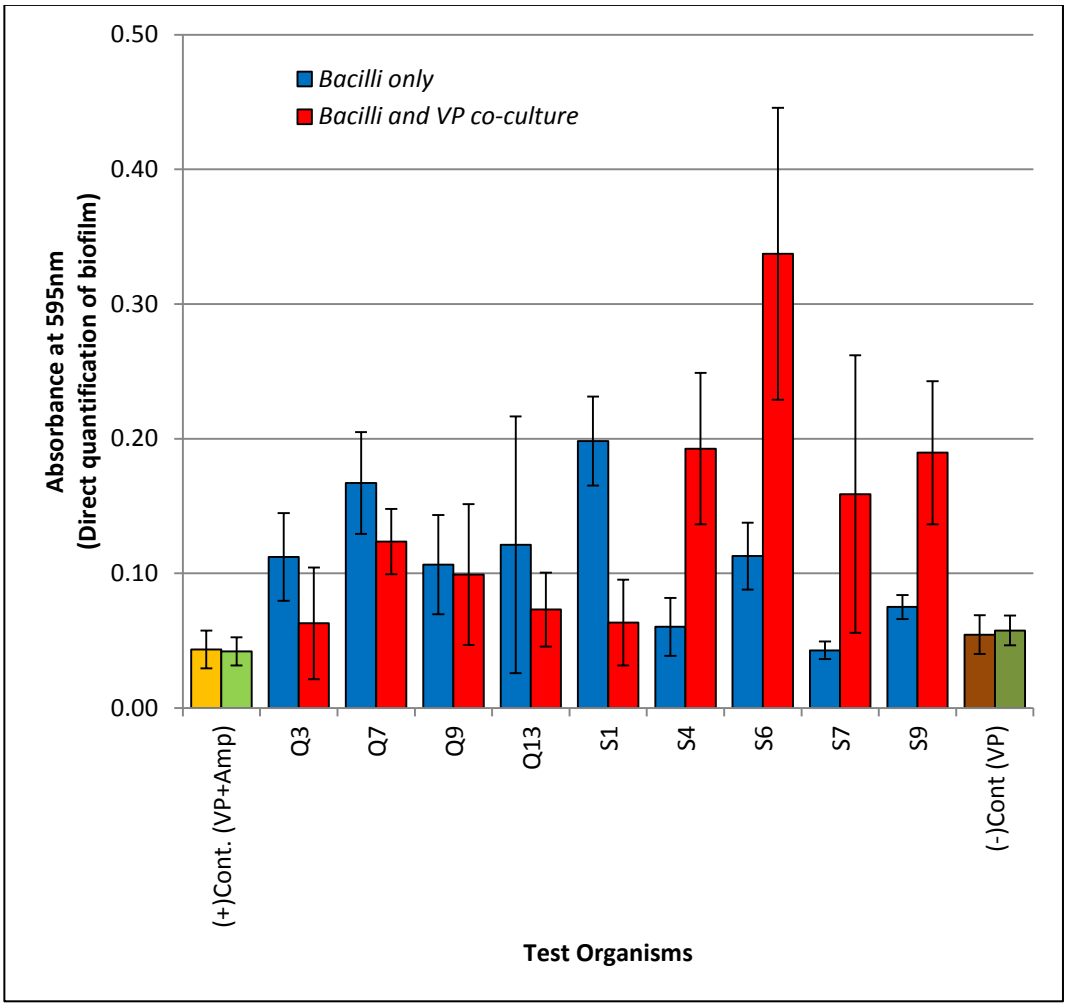


Figure 18. Comparison of biofilm quantity obtained when different bacilli and *Vibrio* were subjected to Microtiter Plate Assay. (Negative control: VP_{EMS} alone; Positive control: VP_{EMS} with ampicillin).

Bar chart in Figure 18 suggests that instead of inhibiting VP biofilms, the bacilli strains themselves form biofilms with or without the presence of VP on the polystyrene surfaces of the 96-well plates. Neither VP cultures in the negative control (VP alone) nor positive control (VP with ampicillin) show appreciable amounts of biofilm formation (low CV absorbance). Biofilm formation was only significant (high CV absorbance) when bacilli culture was present.

To some extent, VP cells did adhere to polystyrene of 96-well plates, binding of CV to cells on wall, hence some absorbance was detected. However, the negative control was only marginally higher than the positive control.

The assumption made in this experiment was that VP was able to form biofilm on the walls of the 96-well plates and that this biofilm-forming ability would be inhibited by the presence of bacilli strains. However, the bacilli themselves were more prolific than VP in adhering to the polystyrene surface. A theoretical suggestion would be that this method could still be used to assess VP biofilm disruption, but replacing the 96-well polystyrene plates with an alternative substrate that would allow VP to attach well, in turn that the presence of bacilli may inhibit the attachment.

From this experiment, it can therefore be concluded that there is evidence supporting bacilli being prudent biofilm formers. Hence, the need to quantify and characterize bacilli biofilms becomes significant as with good colonization abilities of bacilli, physical inhibition of VP_{EMS} colonization in animal host can be achieved. bacilli become the physical and biological barrier or first line of defence in the animal host.

2.4 General summary

Current literature available on coral associated bacteria mostly discusses the population dynamics of bacteria inhabiting the coral tissue, the impacts of ocean acidification, or other environmental impacts on coral health. This study explores the possibility of applying novel or non-novel isolates of coral mucus-associated bacteria as biocontrol agents. Due to the Coral probiotic hypothesis explained earlier (proposed by Reshef *et al*. 2016), these coral associated bacteria act as a first barrier for the coral's immune system, eventually leading to conferring long term adaptive immunity against pathogens. That means that these diverse population of bacteria are constantly exposed to great changes in marine conditions as well as common marine microorganisms such as *Vibrio parahaemolyticus* and the compounds they secrete (e.g. AHLs', etc.). Therefore, the chances of any one of the coral mucus-associated isolates possessing quorum quenching abilities are high.

Many studies have applied gut microbial flora from abalone, mussels, or shrimps as biocontrol agents and supplementing artificial diets to aquaculture animals in attempts to change gut bacterial composition to some extent. But since these are aquaculture-reared animals, these gut microflora are not constantly exposed to vast changes in both abiotic and biotic factors. This could be one of the reasons why none of the bacilli isolated from shrimps in this study possessed the AiiA gene.

The quorum quenching bacilli strains isolated from coral mucus may give rise to many novel phenotypic characteristics of note, some of which may aid to combatting against EMS.

3. Chapter 2: Quantitative phenotypic characterisation of quorum quenching bacilli strains

3.1 Introduction

This part of the study demonstrates the abilities of isolated quorum quenching bacilli to form biofilms as a way of niche/surface colonization, hence out-compete VP. Phenotypic differentiation was also carried out via several characterization assays to distinguish between strains via a hierarchical cluster analysis. Phenotypically distinct bacilli strains were selected for molecular identification as well as further quantitative characterization. Flowchart in Figure 19 summarises the work carried out in this chapter.

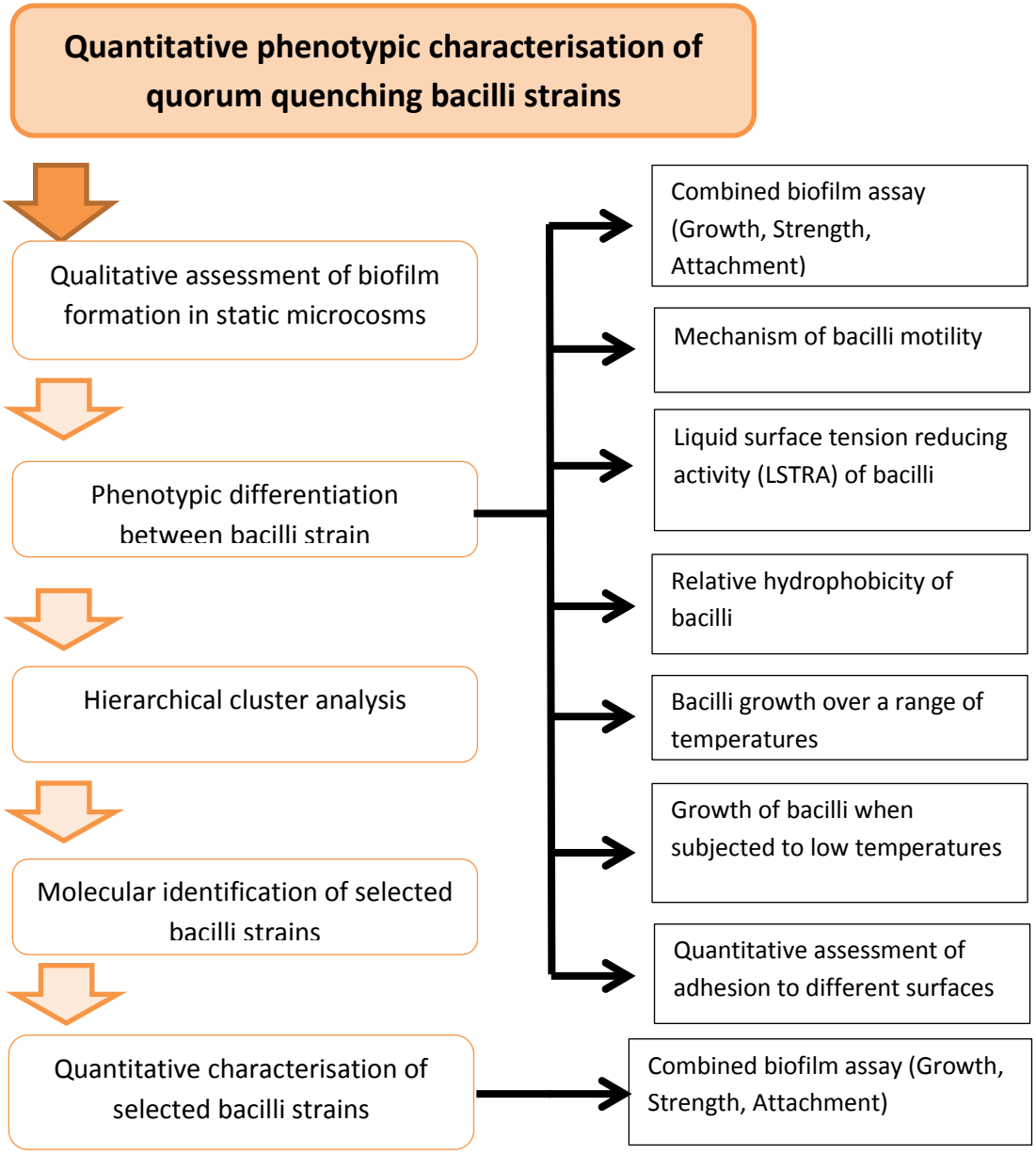


Figure 19. Flowchart which summarises the work done in this Chapter 2.

3.2 Methodology

3.2.1 Qualitative assessment of biofilm formation in static microcosms

A qualitative preliminary analysis was carried out on the biofilms formed on the air-liquid interface (A-L) after static incubation (20°C, 7 days) of ten bacilli strains in five different full strength liquid microcosms (6 mL) prepared according to compositions given in Tables 9 – 13.

3.2.1.1 Media used in experiment

Table 9. Composition of King's B (KB) (King *et al*. 1954) microcosm in grams/L.

Composition	Grams/Litre (g/L)
Protease peptone	10.0
Magnesium sulphate	0.75
Dibasic potassium phosphate	0.75
Glycerol	5.0
Final pH 7.5 at 25°C	

Table 10. Composition of HiMedia Luria Bertani (LB) microcosm in grams/L.

Composition	Grams/Litre (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0
Final pH 7.5 at 25°C	

Table 11. Composition of HiMedia Brain Heart Infusion (BHI) microcosm in grams/L.

Composition	Grams/Litre (g/L)
Calf brain, infusion from	200.0
Beef heart, infusion from	250.0
Proteose peptone	10.0
Disodium phosphate	2.5
Sodium chloride	5.0
Dextrose	2.0
Final pH 7.4 at 25°C	

Table 12. Composition of HiMedia Nutrient (NB) microcosm in grams/L.

Composition	Grams/Litre (g/L)
Peptone	10.0
Beef extract	10.0
Sodium chloride	5.0
Final pH 7.3 at 25°C	

Table 13. Composition of Glucose-supplemented Minimal salt media (M9-Gluc) (Sambrook *et al*. 1989) microcosm in grams/L.

Composition	Grams/Litre (g/L)
Sodium chloride	1.0
Potassium chloride	0.5
Magnesium sulphate	0.05
Calcium chloride	0.06
Potassium dihydrogen phosphate	0.2
Glucose	20 mM
Final pH 7.5 at 25°C	

3.2.1.2 Classification of distinct biofilm phenotypes

Visual determination of biofilm phenotypes *in situ*, categorized from Spiers *et al*. (2006), was noted down for each bacilli strain. The biofilms were then tipped into petri dishes to examine the characteristics of the component materials which form the biofilms, as previously carried out by Ude *et al*. (2006).

3.2.2 Phenotypic differentiation between bacilli strains

Quantitative characterization of ten bacilli strains was performed using several assays, inclusive of a combined biofilm assay (growth at OD_{600}, relative biofilm strength and biofilm attachment levels), swimming motility, liquid surface tension reducing activity, and relative hydrophobicity as outlined by Robertson *et al*. (2013) and Spiers *et al*. (2003).

3.2.2.1 Combined biofilm assay

Prior to carrying out the combined biofilm assay, all tested strains were inoculated in King's B microcosms and incubated (22°C, 7 days, static) with lids loosely fixed to ensure good aeration. Air-liquid interface biofilms of bacilli strains were observed to be supported well by King's B microcosms during qualitative assessment in Section 3.2.1. Moreover, a study carried out by Shemesh and Chai (2013) determined that the presence of glycerol (main carbon source in KB microcosms, previously shown in Table 9) promotes biofilm formation in bacilli. Replicates for each strain were carried out for each assay performed (n=8). The combined biofilm assay consists of three parameters: Growth, relative biofilm strength, and biofilm attachment.

3.2.2.1.1 Growth of biofilm-forming bacilli

Corresponding growth was measured by optical density measurements at 600 nm (OD_{600}) (turbidimetric assay).

3.2.2.1.2 Relative biofilm strength

Quantification of relative biofilm strength was achieved by determining the Maximum Deformation Mass (MDM) assay. Glass beads, having a mean weight of 0.015 g and diameter of approximately 1 mm, were placed at the centre of the air-liquid (A-L) interface biofilms formed by the corresponding strains. The number of beads required to deform the biofilm was recorded in grams.

3.2.2.1.3 Biofilm attachment level

Microcosm glass vials containing adherent biofilms were stained with crystal violet (2 minutes), then gently rinsed with water. Elution of the crystal violet stain was sequentially carried out with 95% ethanol (2 hours, shaking). Attachment of A-L biofilms was measured by the crystal violet content of eluent detected when absorbance was read at 570 nm (A_{570}).

3.2.2.2 Mechanism of bacilli motility

Strain swimming and twitching type motilities were assessed as a direct measurement of flagella-dependent motion as carried out by Robertson *et al*. (2013).

3.2.2.2.1 Assessment of swimming type motility

Overnight KB cultures of strains were prepared. KB-agar plates (0.1 times strength KB, 0.3% w/v agar) were stabbed-inoculated with 10µL of overnight cultures. A ring or sphere of bacterial expansion through the soft agar (rather than across the surface) after incubation (24hours, 22°C) was noted as positive results and will then have its diameter measured. Replicates were performed (n=8). Negative result would yield a growth restricted to the stab mark area as is observed when sterile KB is stabbed-inoculated into the agar plates.

3.2.2.2.2 Assessment of twitching type motility

Overnight KB cultures were prepared. KB agar plates (full strength KB, 1.0% w/v agar) were stabbed-inoculated with 10µL overnight cultures, in replicates (n=8), and incubated (24hours, 22°C). An expanding zone of growth between petri dish and agar interface would indicate a positive result when observed then its diameter measured.

3.2.2.3 Liquid surface tension reducing activity (LSTRA) of bacilli

3.2.2.3.1 Qualitative assessment of surfactant production

Overnight KB cultures of strains were prepared in replicates (n=3). Expression of biosurfactants was first assessed by a drop-collapse assay, whereby 10µL of overnight cultures were dropped onto petri dish lids as described by Chen *et al*. (2007). Positive results would yield a flattened drop with irregular edges, as opposed to a rounded drop as is produced by sterile KB.

3.2.2.3.2 Quantitative determination of liquid surface tension modulation

Cell-free supernatant were obtained from overnight KB cultures and their liquid surface tension (γ) was measured with a KRÜS K 100 Mk 2 Tensiometer (Krüss, Germany) using a standard rod method (22°C) as described by Fechtner *et al*. (2011).

3.2.2.4 Relative hydrophobicity of bacilli

Overnight KB cultures of strains were prepared (n=3). As outlined by Rosenberg *et al*. (1980), the microbial adhesion to hydrocarbons (MATH) assay was carried out to determine the relative hydrophobicity (H_r) of the bacilli cells. Hexadecane (3 mL) was added into the overnight bacilli cultures and vortexed (5 seconds), then allowed to stand (20 minutes) before OD_{600} of the aqueous phase was measured (OD_{600i}). Mixture was vortexed again (1 minute) and allowed to stand (20 minutes) before OD_{600} was re-measured (OD_{600f}). Relative hydrophobicity was obtained from the ratio of OD_{600f}/OD_{600i} of each bacilli strain tested.

3.2.2.5 Bacilli growth over a range of temperatures

A temperature-growth profile for each of the strains in replicates (n=3) were obtained. Relative change in cell density (growth) was measured by applying the ratio between OD_{600} readings before and after 24 hour period incubation at 4.5°C, 15°C, 20°C, 28°C, 35°C, and 40°C. With the ranging temperatures, strain activity in terms of growth can be estimated at the approximate temperature, e.g. lower cell activity at 4.5°C and 40°C, whereas optimal between 28°C to 35°C. An uninoculated microcosm was included for each temperature tested as a negative control.

$$Relative\ change\ in\ growth = \frac{OD_{600i}}{OD_{600f}}$$

3.2.2.6 Growth of bacilli when subjected to low temperatures

Overnight KB cultures were prepared. Before inoculation into KB microcosms, amount of inoculum was determined such that initial readings were set to approximately 0.2 AU at 600 nm to ensure consistency amongst strains and also to rule out cell lysis after set incubation period.

A long term assessment of individual strain viability at 4.0°C over 168 hours in shaking conditions was carried out via obtaining relative change in OD_{600} readings at intervals of 24, 48, 72, 120 and 168 hours, in replicates (n=3) upon inoculation of strains into KB liquid microcosms. Sampling was carried out via removing 1 mL of culture from microcosm at each time interval for measurements of cell densities.

3.2.2.7 Quantitative assessment of bacilli adhesion to different surfaces

Attachment assays were also conducted independently of the combined biofilm assays described in Section 3.2.2.1 and Spiers *et al*. (2003). Overnight cultures were prepared and used to inoculate replicate microcosms (n=8) of glass, polypropylene and polystyrene respectively prior to static incubation (7 days, 22°C). Attachment levels were determined via staining the adherent biofilms on each substrate with crystal violet and absorbance (A_{570}) read upon elution with ethanol, as previously described in Section 3.2.2.1.3.

3.2.3 Hierarchical cluster analysis

By applying all the means from the above strain phenotype characterization assays, a hierarchical cluster analysis, using the Ward method, on JMP Statistical Discovery Software (JMP 7.0, SAS Institute Inc, USA) was carried out in order to identify five key bacilli species.

3.2.4 Molecular identification of selected bacilli strains

From the constellation plot generated, five phenotypically different species were selected for molecular identification and further characterization assays.

3.2.4.1 Genomic DNA extraction

Total genomic DNA extraction was carried out using freeze and thaw method of overnight cultures, as previously described in Section 2.2.4.1.

3.2.4.2 16S rDNA gene PCR

PCR was carried out using universal 16S rDNA gene primers for bacteria, namely primer pair 8F and 519R (Turner *et al*. 1999) and the protocol given in the Bioline Red Taq mix. The reaction mixture in each tube is given in Table 14 as follows:

Table 14: Components of 16S rDNA PCR reaction per PCR tube. Distilled water was used in the place of DNA template for the control.

Components	Volume (μL)
2x Bioline Red Taq Mix	12.5
Forward primer, 8F 5'-AGAGTTTGATCCTGGCTCAG-3'	1.0
Reverse primer, 519R 5'-GWATTACCGCGGCKGCTG-3'	1.0
DNA template	2.0
dH_2O	8.5
Final volume	25.0

Amplification reactions were performed in the thermocycler programmed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, and final extension at 72 °C for 10 min.

PCR reaction results were checked using agarose gel electrophoresis (100V, 30 min).

3.2.4.3 Sequencing and sequence analysis

PCR products were then sent for Sanger sequencing to BGI Tech, Beijing prior to phylogenetic analysis using Chromas 2.22 (Technelysium Pty Ltd) for sequence processing followed by nucleotide sequence search using Basic Local Alignment Search Tool software (NCBI).

3.2.5 Quantitative characterisation of selected bacilli strains

3.2.5.1 Media used in experiment

Table 15. Composition of Iron (III)-supplemented King's B (KB-Fe(III)) microcosm (Koza *et al*. 2009) in grams/L.

Composition	Grams/Litre (g/L)
Protease peptone	10.0
Magnesium sulphate	0.75
Dibasic potassium phosphate	0.75
Glycerol	5.0
Iron (III) sulphate	0.1
Final pH 7.5 at 25°C	

Table 16. Composition of Luria Bertani with 4.0% (w/v) NaCl (LB-NaCl) microcosm in grams/L.

Composition	Grams/Litre (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	40.0
Final pH 7.5 at 25°C	

Table 17. Composition of Casamino acid-supplemented Minimal salt media (M9-CAA) microcosm in grams/L.

Composition	Grams/Litre (g/L)
Sodium chloride	1.0
Potassium chloride	0.5
Magnesium sulphate	0.05
Calcium chloride	0.06
Potassium dihydrogen phosphate	0.2
Casamino acid	4.0
Final pH 7.5 at 25°C	

3.2.5.2 Combined biofilm assay

Overnight cultures of strains selected were prepared. Each of the five key strains were inoculated in replicates (n=8) and incubated in microcosms with five different media, which were KB, iron-supplemented KB (KB-Fe(III)), Luria-Bertani with 4.0% NaCl (LB-NaCl), Brain Heart Infusion (BHI), and Casamino acid-supplemented Minimal salt media (M9-CAA). O'Toole (2011) suggested the use of either glucose or casamino acids to support bacterial biofilm formation. The media components are listed in Tables 9, 11, and 15 – 17 as shown above. These five strains were statically incubated (22°C, 7 days), then quantitatively assayed for growth (cell density at OD_{600}), biofilm strength, and attachment, as previously described in Section 3.2.2.1.

3.2.6 Statistical analyses and modelling

All experiments and assays were conducted in replicates as stated, and the means ± SE provided where necessary. JMP Statistical Discovery Software (JMP v12, SAS Institute Inc., USA), SPSS Statistics (SPSS 22.0, IBM, USA) and Microsoft Excel (version 2010) were used to analyse all data obtained.

Differences between means were tested by ANOVA (*F*) and *post hoc* multiple comparison tests including Dunnett's method (|*d*|) with a control and Tukey-Kramer HSD (TK-HSD, *q**) tests were carried out. A general linear model (GLM) approach was used to model strains, attachment and growth respectively as response with various effects and their significant effects were examined by LSMeans Differences Tukey HSD (Q) tests and associations examined by pairwise correlations (*r*).

3.3 Results and Discussion

Following the assessment of quorum quenching performance, all bacilli strains were subjected to several assays to provide evidence of phenotypic differences between them, thus narrowing the selection of strains down.

3.3.1 Qualitative assessment of biofilm formation in static microcosms

Bacterial biofilms, in terms of fixed colonisation onto animal hosts, can be beneficial to the hosts, aiding in resisting pathogens. Examples of successful protective effects of biofilms on marine macrofauna include *Vibrio midae* in abalone, *Haliotis midae* (Macey and Coyne, 2006), *Carnobacterium inhibens* K1 in Atlantic salmon, *Salmo salar* (Jöborn *et al*., 1999) and *Kocuria* SM1 in rainbow trout against *Vibrio anguillarum* (Sharifuzzaman and Austin, 2010). This study highlights the importance of protective fixed colonisation onto shrimp hosts.

Fourteen bacterial strains (see Section 3.1.4 for details on selection) were assessed for their biofilm formation in five different types of growth media in this preliminary optimisation for biofilm-promoting microcosms.

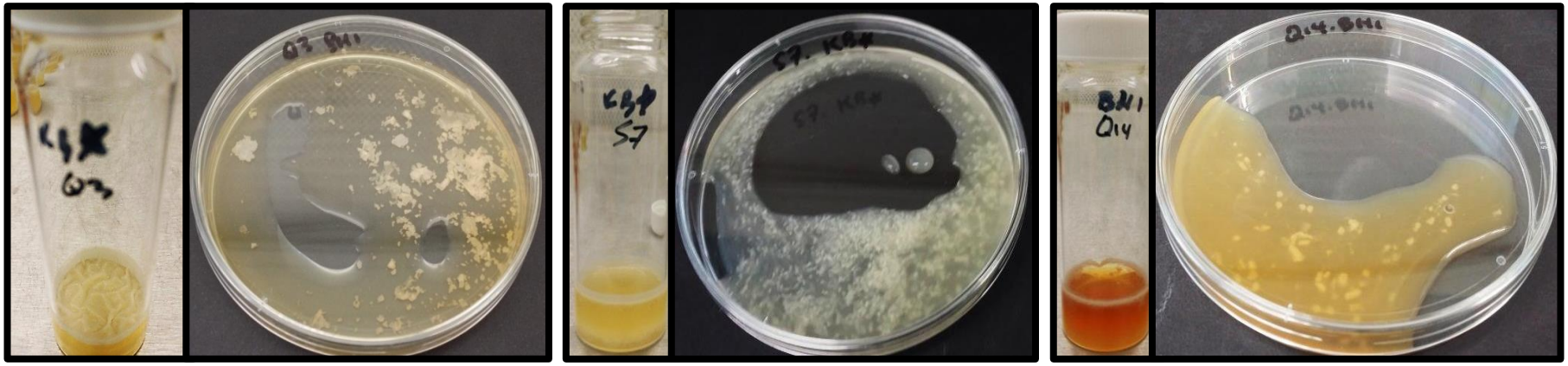
Upon visual determination of media turbidity, it was observed that four strains were not able to grow in the KB microcosms and were therefore excluded from further biofilm characterization assays. As for the remaining ten bacterial strains, it was noted that King's B medium was the best microcosm to support Bacillus sp. A-L interface biofilm formation. KB medium was consistent in inducing formation of biofilms which were visually distinct and relatively strong. As previously highlighted in Section 2.2.2.1, biofilm formation is promoted in the presence of glycerol as primary carbon source in KB media.

Visual determination of biofilm phenotypes were noted down for each bacteria strain. The phenotypes were categorized as one of the following four forms: waxy aggregation (WA), viscous mass (VM), floccular material (FM), or physically cohesive (PC) biofilm types, as described in Spiers *et al*. (2006) (see Figure 20). Results of this qualitative assessment were summarized in Table 18 constructed below; parameters include visible growth, visually detectable biofilm in microcosm and their respective phenotypes.

It was also noted that S1 may be further explored for its apparent phenotypic plasticity as it formed three distinct phenotypes in different microcosms; i.e. floccular mass (FM) in LB microcosm, physically cohesive in KB microcosm, and viscous mass (VM) in BHI microcosm.

Table 18. Summary of qualitative assessment of biofilm formed by the 10 bacilli strains and their respective biofilm phenotypes. Positive biofilm forming strains are denoted by '+', whereas no biofilm formed is denoted as '-'. In the absence of a biofilm in the microcosm, the phenotype is denoted as Not Applicable (N/A).

Strains	Media									
	Luria Bertani		King's B		Brain Heart Infusion		Nutrient		M9-Glucose	
	Biofilm	Phenotype	Biofilm	Phenotype	Biofilm	Phenotype	Biofilm	Phenotype	Biofilm	Phenotype
Q3	+	PC	+	PC	+	PC	+	WA	-	N/A
Q7	-	N/A	+	VM	+	FM	+	FM	-	N/A
Q9	-	N/A	+	VM	-	N/A	-	N/A	-	N/A
Q14	+	VM	+	PC	+	VM	-	N/A	-	N/A
Q15	-	N/A	+	VM	-	N/A	-	N/A	-	N/A
S1	+	FM	+	PC	+	VM	+	VM	-	N/A
S4	+	FM	+	VM	+	FM	-	N/A	-	N/A
S6	-	N/A	+	PC	+	VM	-	N/A	-	N/A
S7	-	N/A	+	FM	+	FM	-	N/A	-	N/A
S9	+	N/A	+	PC	+	VM	-	N/A	-	N/A



(A) **(B)** **(C)**

Figure 20: The phenotypes of A-L biofilms produced by the bacilli after 7 days static incubation.
(A) Strain Q3 and the physically cohesive (PC) type of biofilm; (B) Strain S7 and the floccular mass (FM) biofilm; (C) Strain Q14 and the viscous mass (VM) biofilm.

As can be observed in Figure 21 below, only strain Q3 developed a climbing film at the border of the pellicle. This observation was noteworthy as Angelini *et al*. (2009) reported this climbing film characteristic to be very sensitive to environmental conditions. This could explain why this characteristic was only significantly expressed by Q3 in the BHI liquid microcosm, lesser when grown in KB and LB media, and not observed at all in NB or M9-Glu microcosms. Angelini *et al*. (2009) determined that coordinated surfactin expression by bacterial community drives this spreading, hence the screening and characterisation of biosurfactant expression as a possible phenotypic characterization assay.

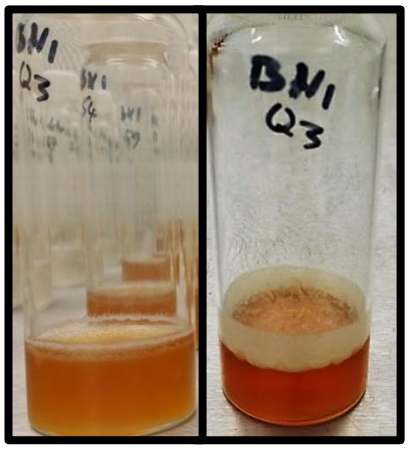


Figure 21. Strain Q3 in BHI microcosms;
A: Day 3 of A-L interface biofilm, B: Day 4 of climbing characteristic of only strain Q3.

3.3.2 Phenotypic differentiation between bacilli strains

Quantitative characterization of all 10 bacilli strains was performed using several assays, inclusive of biofilm formation, combined growth measurements, as well as assays for biofilm strength and attachment. For each assay, an ANOVA was performed in order to objectively determine significant differences between strains.

3.3.2.1 Combined biofilm assay

Figures 22, 24, and 26 contain bar charts which were constructed using values of means and standard error of the means (mean ± SEM) in descending order of mean values. Visual inspection of the three graphs shows variation between individual bacilli strains in terms of growth over seven days, biofilm strength and extent of attachment onto microcosms.

3.3.2.1.1 Growth of biofilm-forming bacilli

Turbidimetric assay by measurement of optical density at 600nm (OD_{600}) is an indirect measurement of bacteria cell number and indicator of growth. When KB media was used, S7 was found to have grown the most over the 7 days incubation period, with a mean of 2.081 ± 0.07 AU; this is shown in Figure 22. ANOVA carried out for the growth data proved significant variation between the strains as well as data robustness (R^2=0.963, ANOVA $F_{9,60}$=172.553, p<0.0001).

Despite Q3 was the best performing strain in terms of biofilm strength (Figure 24), it was found that its growth was second lowest (1.423 ± 0.2 AU) among the 10 strains tested, with only Q14 (1.173 ± 0.01 AU) experienced poorer growth. Means comparisons by Tukey-Kramer HSD for growth model placed the growth profile of Q3 as being significantly different from other strains except for Q14, indicated by the alphabetical labels in Figure 22 (Categories A, B, C, and D).

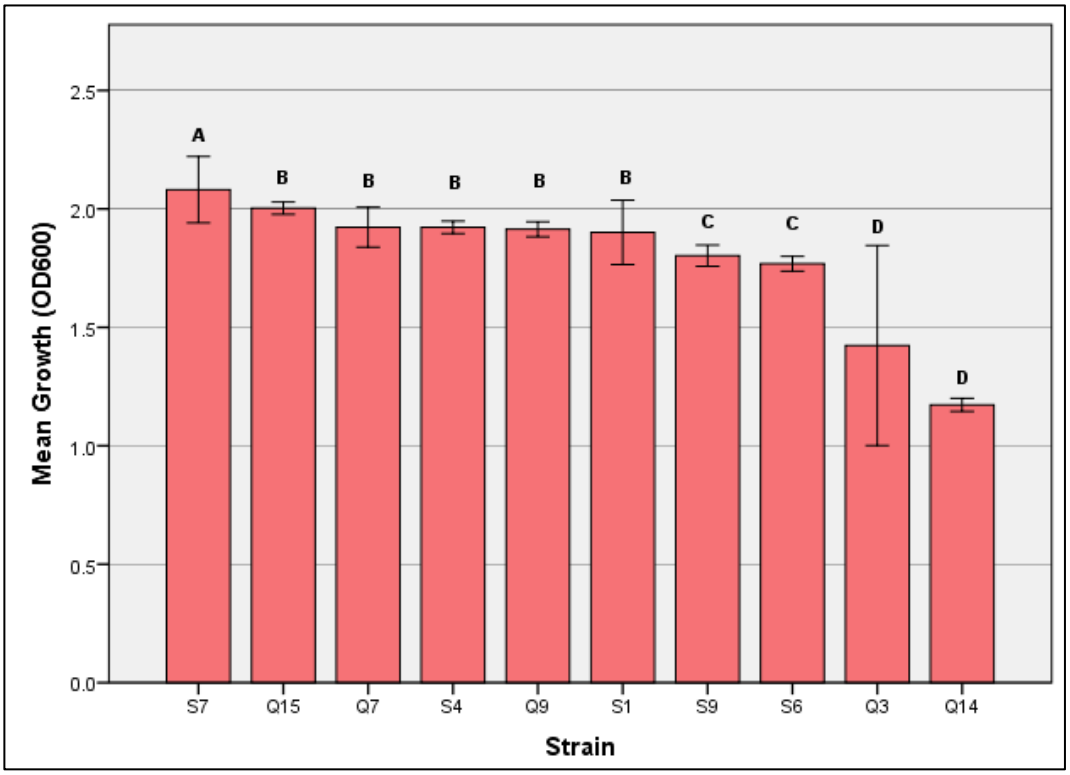


Figure 22. Comparison of mean growth of individual bacilli strains grown in the KB microcosms over 7 days. Bar chart of means ± SEM of 10 bacilli strains and their corresponding growth measured by optical density at 600nm (OD_{600}) in static microcosms incubated for 7 days in replicates (n=8). As reported from the Tukey-Kramer HSD test, bars which are not connected by a common letter are significantly different (q*=3.285, α=0.05).

3.3.2.1.2 Relative biofilm strength

The quantification was achieved by measuring the maximum mass (measured in grams) an A-L interface biofilm can withstand before undergoing deformation as is observed in Figure 23 below. Bar chart in Figure 24 shows mean biofilm strength (means ± SEM) for each bacilli strain in replicates (n=8).

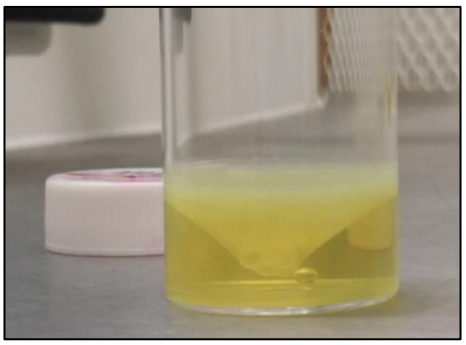


Figure 23. Photograph taken at the exact moment of biofilm collapse due to combined weight of glass beads, as observed during Maximum Deformation Mass (MDM) assay.

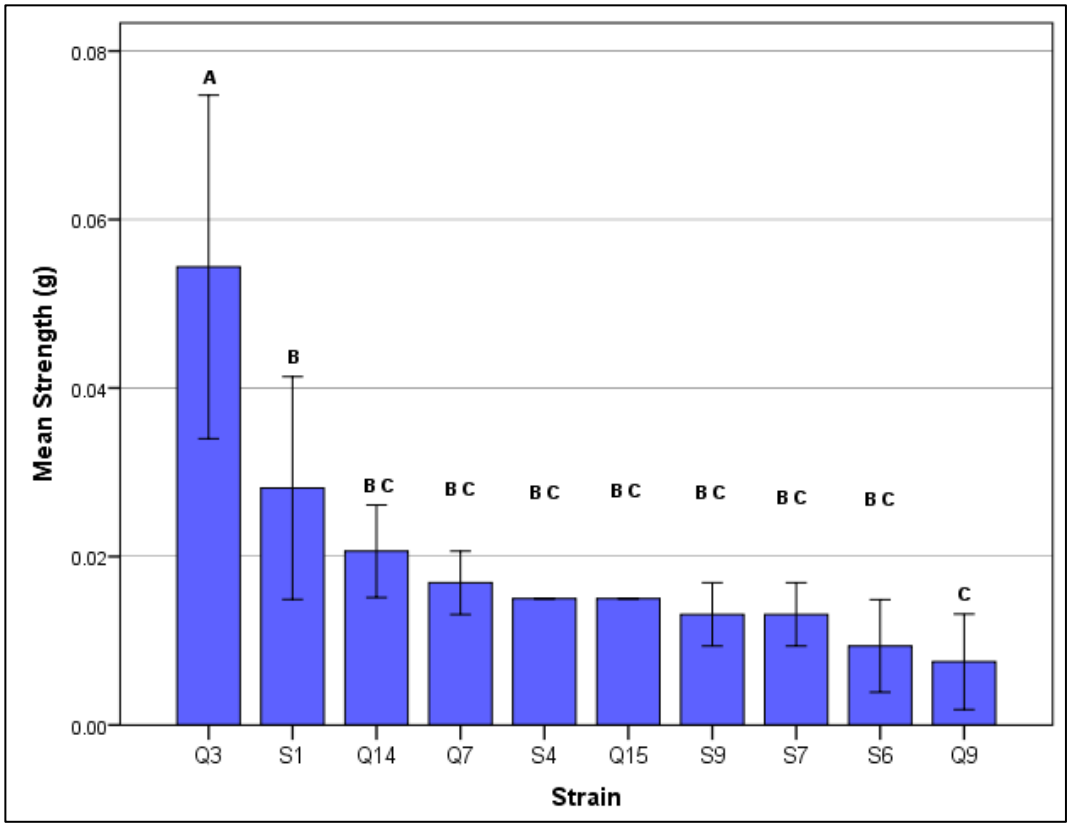


Figure 24. Comparison between individual strains and mean biofilm strength measured using the MDM assay method. The Tukey-Kramer HSD test indicates that individual bars not connected by a common letter are significantly different from each other (q*=3.268, α=0.05).

Based on Figure 24, the A-L interface biofilm formed by strain Q3 has the highest maximum deformation mass (MDM) value, able to withstand a mean mass of 0.054 ± 0.01 g before collapsing. A means comparisons analysis conducted using the Tukey-Kramer HSD test grouped the types of biofilm to three categories of A, B, and C. From the chart, it is possible to conclude that biofilm strength of Q3 is significantly different from the other 9 bacilli.

The category with the most members was found to be BC, consisting of seven strains (Figure 24). Category BC has upper limit mean value of 0.0206 ± 0.003 g (Q14) to lower limit mean of 0.0094 ± 0.003 g (S6) to which suggests some degree of significant variation within the spread, as annotated by the p-value of the ANOVA carried out (R^2=0.568, ANOVA $F_{9,70}$=10.215, p<0.0001).

3.3.2.1.3 Biofilm attachment level

Vials containing adherent biofilms were stained with crystal violet, then gently rinsed. Upon elution using technical grade EtOH, the crystal violet content of eluent was measured using absorbance at 570nm (A_{570}), as is observed in Figure 25 below. The amount of crystal violet detected is directly proportional to biofilm attachment levels.

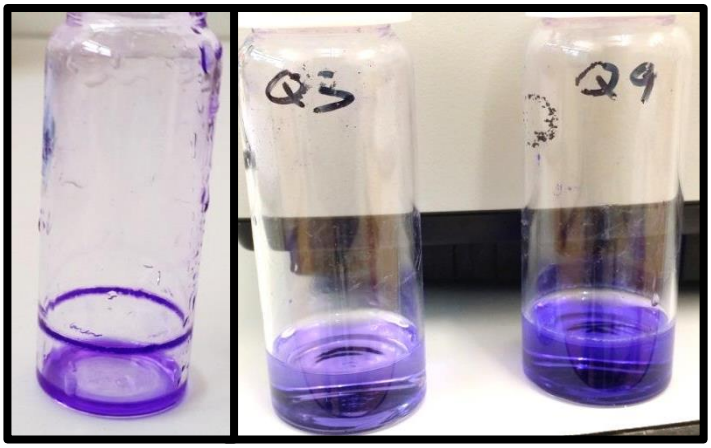


Figure 25. Assessment of strain attachment via staining vials with crystal violet;
Left: Crystal violet stain adherent biofilms, Right: Amount of crystal violet eluted from biofilms of Q3 and Q9.

A bar chart of means ± SEM of 10 bacilli strains, in replicates (n=8) and their corresponding mean attachment levels of A-L interface biofilms to microcosm glass vials was constructed. Amount of bacterial attachment to microcosm glass vials were observed to have significant variations, as shown in Figure 26 as well as supported by a robust data and significant p-value (R^2=0.956, ANOVA, $F_{9,66}$=158.804, p<0.0001).

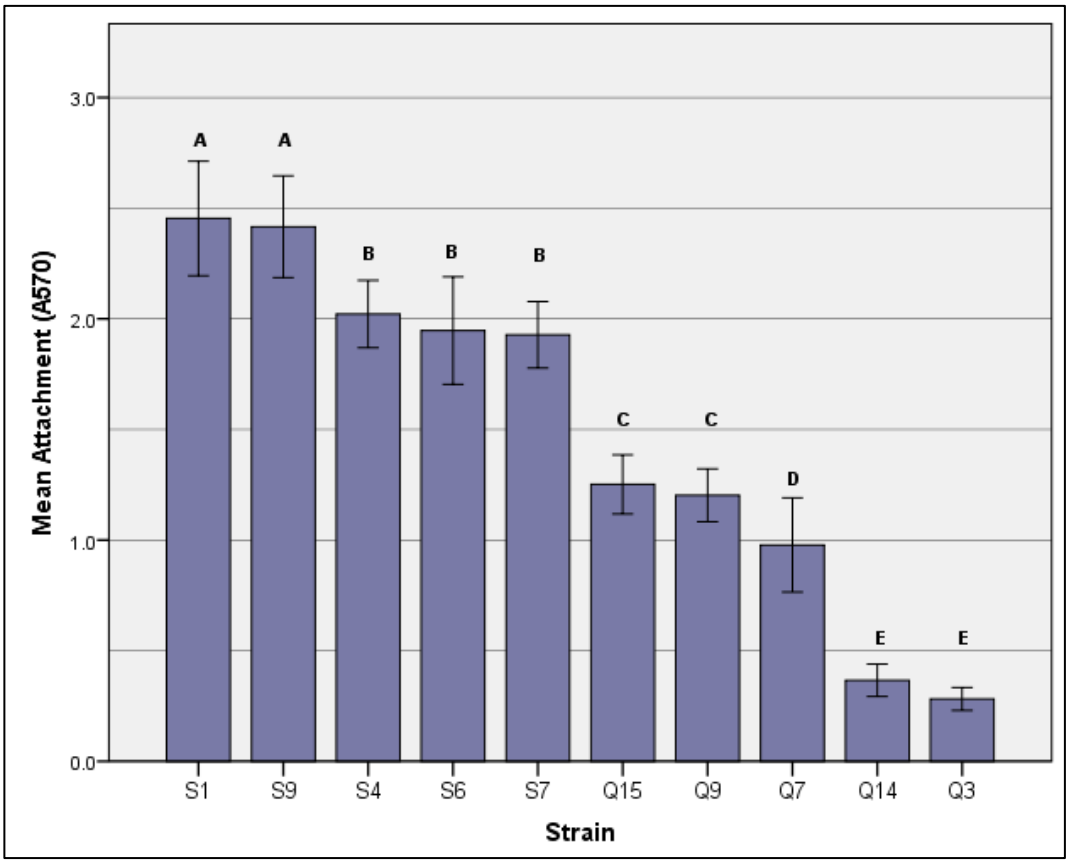


Figure 26. Comparison of individual bacilli strains and corresponding mean biofilm attachment onto glass surface. Using the Tukey-Kramer HSD test, bars which are not connected by a common letter are deemed to be significantly different (q*=3.274, α=0.05) from each other.

The strain which showed the highest level of attachment was S1 with a mean attachment of 2.454 ± 0.1 AU, whereas Q3 performed the lowest in terms of attachment (0.282 ± 0.03 AU) despite exhibiting the highest MDM value among the strains tested. Mean comparisons of attachment using the Tukey-Kramer HSD test conducted is indicative of significant variation between in strains' attachment levels (Figure 26). It was also observed that high similarity exists between strains Q3 and Q14, just as observed in the growth response of Figure 22.

3.3.2.2 Mechanism of bacilli motility

Bacteria motility occurs for many reasons, such as to colonize host tissues, obtain nutrients or spore spreading. Moist surface colonization responses of the bacilli include different kinds of actions including flagella-dependent motility termed swimming, and flagella-independent motility termed twitching (Harshey RM 2003; Mattick JS 2002).

Hence, swimming and twitching abilities of all strains were assessed. A bar chart was constructed to demonstrate the 2 kinds of bacilli translocation abilities, expressed in diameter (mm) of visible bacteria displacement (Means ± SEM), as observed in Figure 27.

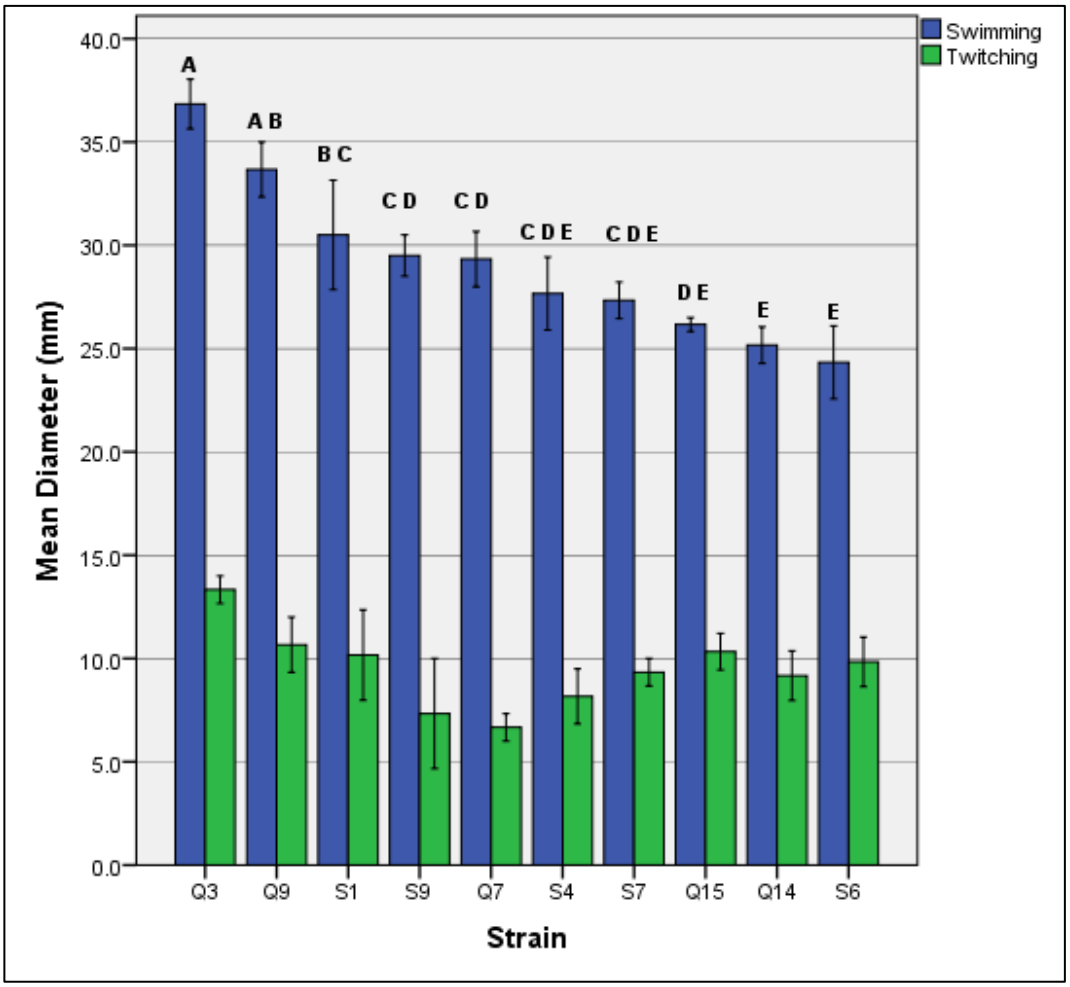


Figure 27. Bar chart comparison of swimming and twitching motility of 10 bacilli strains. *Post hoc* Tukey-Kramer HSD test conducted for swimming test reveals that bars not connected by the same letter are significantly different (q*=3.541, α=0.05).

For the swimming motility, significant variation exist between strains tested via ANOVA (R^2=0.928, ANOVA $F_{9,20}$=28.677, p<0.0001).

ANOVA on twitching motility revealed a good fit model and significant differences between strains' ability as well (R^2=0.895, ANOVA $F_{9,27}$=17.133, p<0.0001).

Strain Q3 showed the largest swimming and twitching motility areas, at diameters of 36.8 ± 0.6 mm for swimming and 13.3±0.3 mm for twitching respectively. Poorest swimming motility is observed by S6, diameter of 24.3 ± 0.9 mm, whereas Q7 had the smallest twitching diameter of 6.7 ± 0.3 mm. Generally, the bacilli strains tested preferred to swim although most had the ability to twitch, suggesting flagella-dependent motility was preferred.

3.3.2.3 Liquid surface tension reducing activity (LSTRA) of bacilli

Many species of biofilm-forming bacilli are known to produce biosurfactant (surfactin) as they enhance the spreading of multicellular colonies on media by lowering the surface tension of surrounding fluid. In the case of *B. subtilis*, the surfactin they produce aid in the formation of aerial structures for biofilm (Angelini *et al*. 2009). They further reported that biosurfactant production was a result of antagonistic interaction between different bacterial species and was necessary for the spread of colonies in the absence of external fluid flows.

As mentioned previously in the preliminary qualitative assessment (Section 3.2.1), further characterization was performed for the screening for liquid surface tension reducing activity (LSTRA) of the 10 bacilli strains in KB media. All bacilli strains were allowed to grow over periods of 24, 48 and 72 hours before use in the test, thus each strain contributed to 3 different samples, each of which was measured in triplicates. Lowest mean liquid surface tension (LST) was determined for each time interval.

Comparisons of means and standard error means (Mean ± SEM) of liquid surface tension for all 10 bacilli strains to determine the presence of liquid surface tension reducing activity (LSTRA) within the set period of time was carried out via bar charts in Figure 28 below.

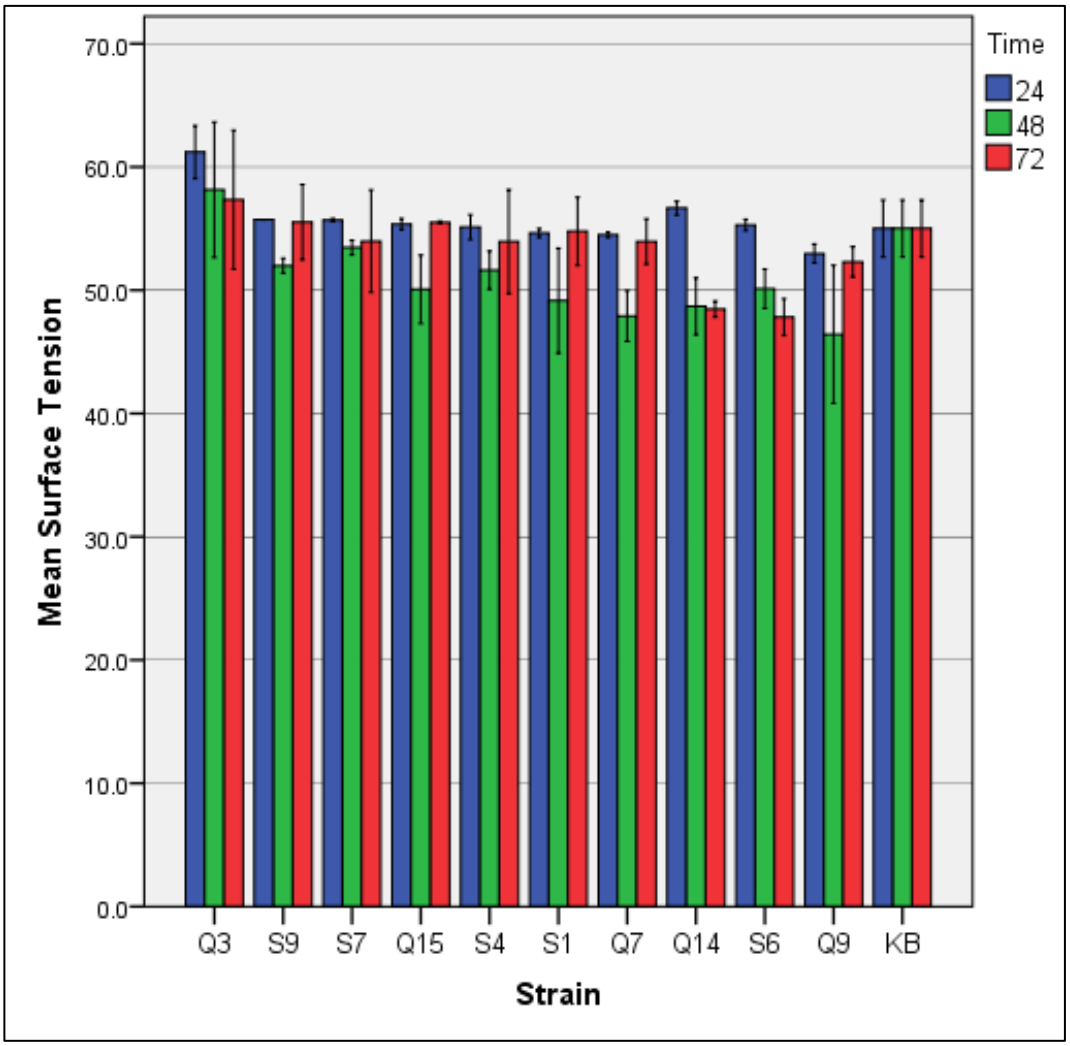


Figure 28. Liquid surface tension measurements for 10 bacilli strains when grown in KB broth; measurements were taken at every time 24 hrs time interval, with total 3 intervals taken (t_1=24, blue, t_2=48, green, t_3=72, red); uninoculated KB broth was used as the negative control.

The lowest mean LST measured at 24 hrs was obtained from strain Q9 at 52.976 ± 0.4 mN/m; whereas at 48 hrs, the lowest mean LST was also from Q9 at 46.422 ± 2.0 mN/m. At 72hrs incubation, it was strain S6 which exhibited the lowest mean LST of 47.820 ± 0.7 mN/m. Mean liquid surface tension for uninoculated KB media (experimental control) was 55.014 ± 1.0 mN/m. When compared to the negative control, a general trend observed was that at 24 hrs, the growth of strains resulted in increased liquid surface tension. At 48 hrs, the measured LST decreased below control mean values, with exception of strain Q3.

A general decrease in measured LST over time was to be expected, as seen with strains Q3, Q14 and S6. However the growth of other 7 bacilli strains resulted in increased liquid surface tension at 72 hrs from the initially observed minima at 48 hrs.

It was observed that Q3 gave increased LST regardless of the duration of incubation. Growth of strain Q3 yielded the highest recorded LST values for each of the 3 time periods – 61.203 ± 1.0 mN/m at 24hrs, 58.154 ± 2.7 mN/m at 48hrs, 57.334 ± 2.0 mN/m at 72hrs.

Preliminary analysis suggested that the presence of LSTRA be tested using the dataset containing the lowest overall LST measurement for each strain. This meant that the LST values at 48 hrs be chosen; except for S6, for which the LST at 72 hrs was selected. This dataset was presented in a histogram in Figure 29 below.

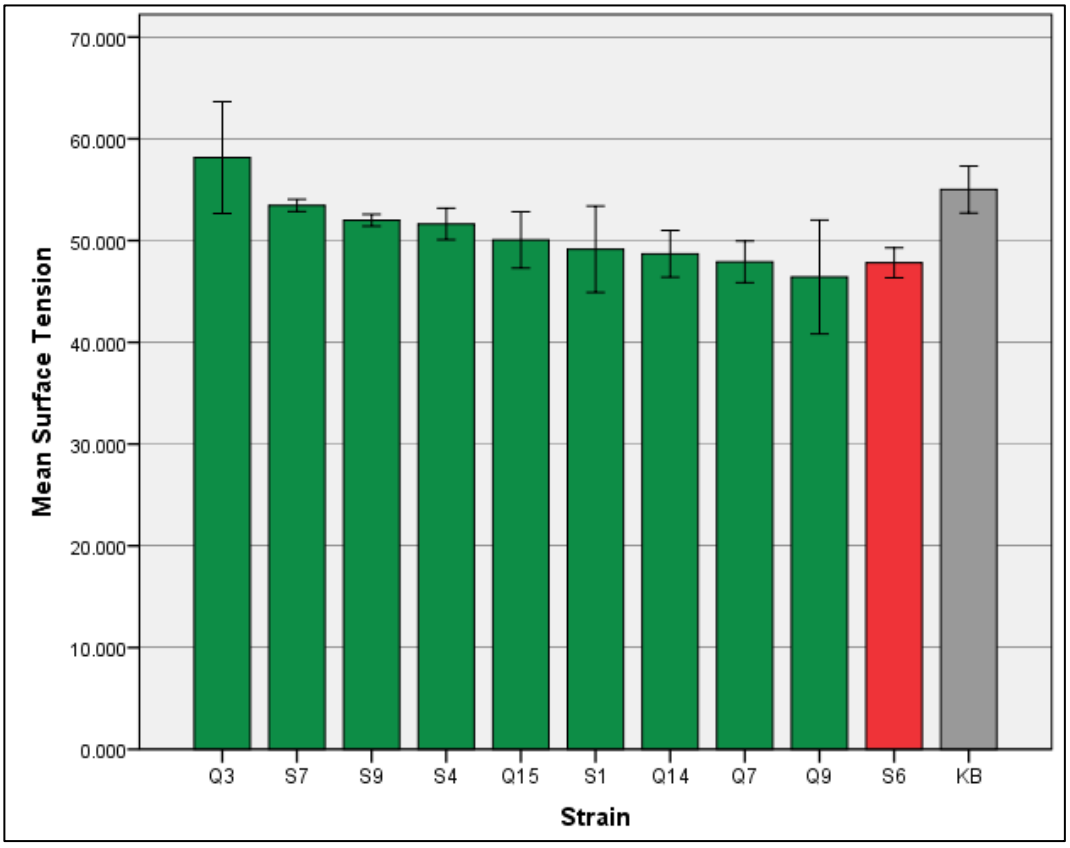


Figure 29. Comparison of means with control for all strains at specific time interval with lowest liquid surface tension, all of which were at 48 hr except strain S6 at 72 hr.
Histogram of liquid surface tension means of all strains at 48hours (green bars) except for strain S6 at 72hours (red bar) compared against uninoculated control (grey bar).

A one-way ANOVA carried out using the dataset in Figure 29 above proved that there were significant differences between the mean liquid surface tension of KB media in which each strain was inoculated into (R^2=0.751, ANOVA $F_{10,20}$=6.044, p=0.0003). Then, a *post-hoc* comparison by means with KB media control (using the Dunnett's Method) revealed that 5 strains are statistically different from the control (whereby p≤0.05), as shown in Appendix 1.

Dunnett's test revealed that strains with positive values are significantly different ($p \leq 0.012$) from the control. Hence, it was suggested that strains Q7, Q9, Q14, S1 and S6 were likely to have expressed weak surfactants ($|d|=2.994$, $\alpha=0.05$).

From this assay, it was concluded that within the tested intervals of 48-72 hrs there were significant changes to LST caused by the inoculated strains and hence, the strains were likely expressing either weak surfactants or surfactant-like compounds.

3.3.2.4 Relative hydrophobicity of bacilli

The concept of the MATH assay is to measure the relative amounts of bacterial cells removed by hydrocarbons. Hence, by measuring the relative difference in OD_{600}, a smaller ratio (OD_f/OD_i) will indicate that microbial cells are more hydrophobic.

Figure 30 reveals a robust dataset of the mean relative hydrophobicity and standard error of means as well as significant variation between bacilli strains tested ($R^2=0.986$, ANOVA $F_{9,20}=158.337$, $p<0.0001$). Strain S4 (0.626 ± 0.02) gave the smallest ratio, indicative of good removal of microbial cells by hydrocarbons did occur. Strain Q3 showed the largest ratio value of 1.287 ± 0.02, indicating that this strain's bacterial cells were the least hydrophobic as compared to the rest. A comparison of means was conducted via Tukey-Kramer HSD test, which determined that strains S4 and Q3 are significantly different from the rest of the 8 other bacilli strains, represented by the letters as seen in Figure 30 below.

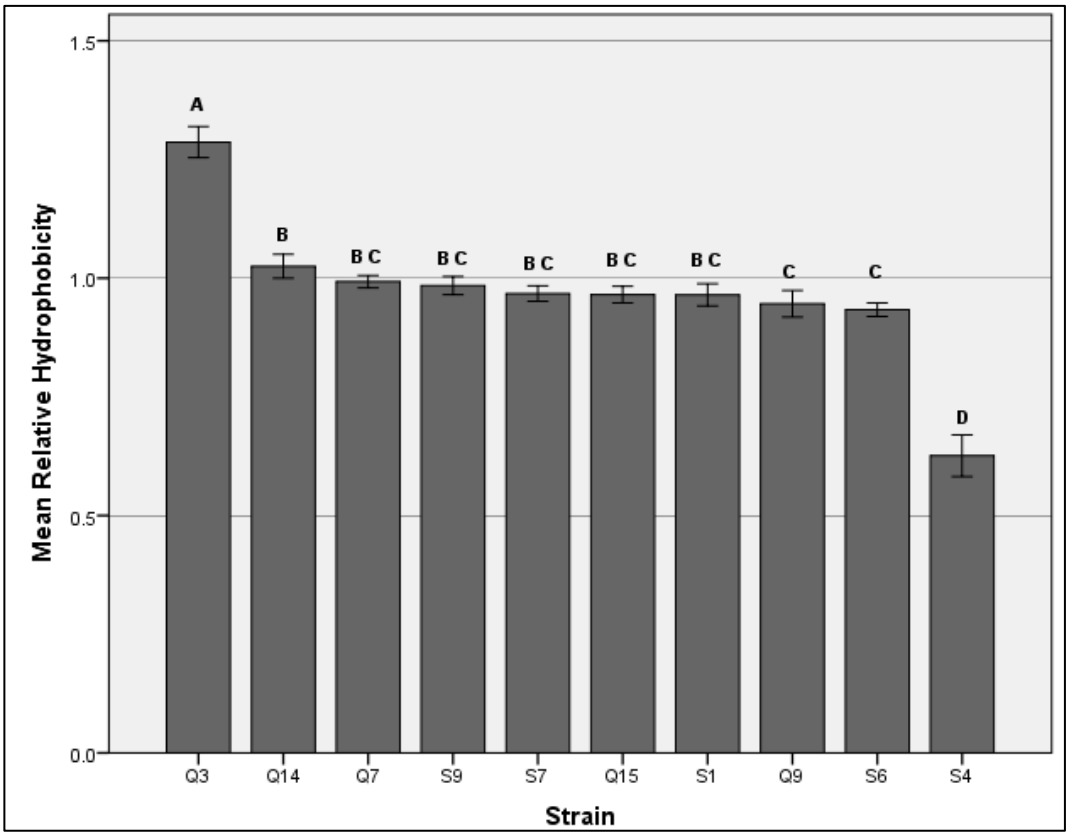


Figure 30. Mean relative hydrophobicity comparison between 10 bacilli species.
Bar chart of mean relative hydrophobicity and standard error means (Mean ± SEM) of 10 individual bacilli strains in replicates (n=3). Tukey-Kramer HSD test reveals that bars not connected by the same letter are significantly different ($q^*=3.541$, $\alpha=0.05$).

3.3.2.5 Bacilli growth over a range of temperatures

Growth (OD_{600}) of the ten bacilli strains over a 24 hour period of incubation was observed at selected temperatures, i.e. 4.5°C, 15°C, 20°C, 28°C, 35°C, 40°C. Standardization of initial inoculum amount was carried out prior to incubation.

For the purpose of better inter-species comparison, bar charts in Figure 31 and 32 compare relative change in growth of all 10 bacilli species at the temperatures tested (Relative change = $OD_{final}/OD_{initial}$).

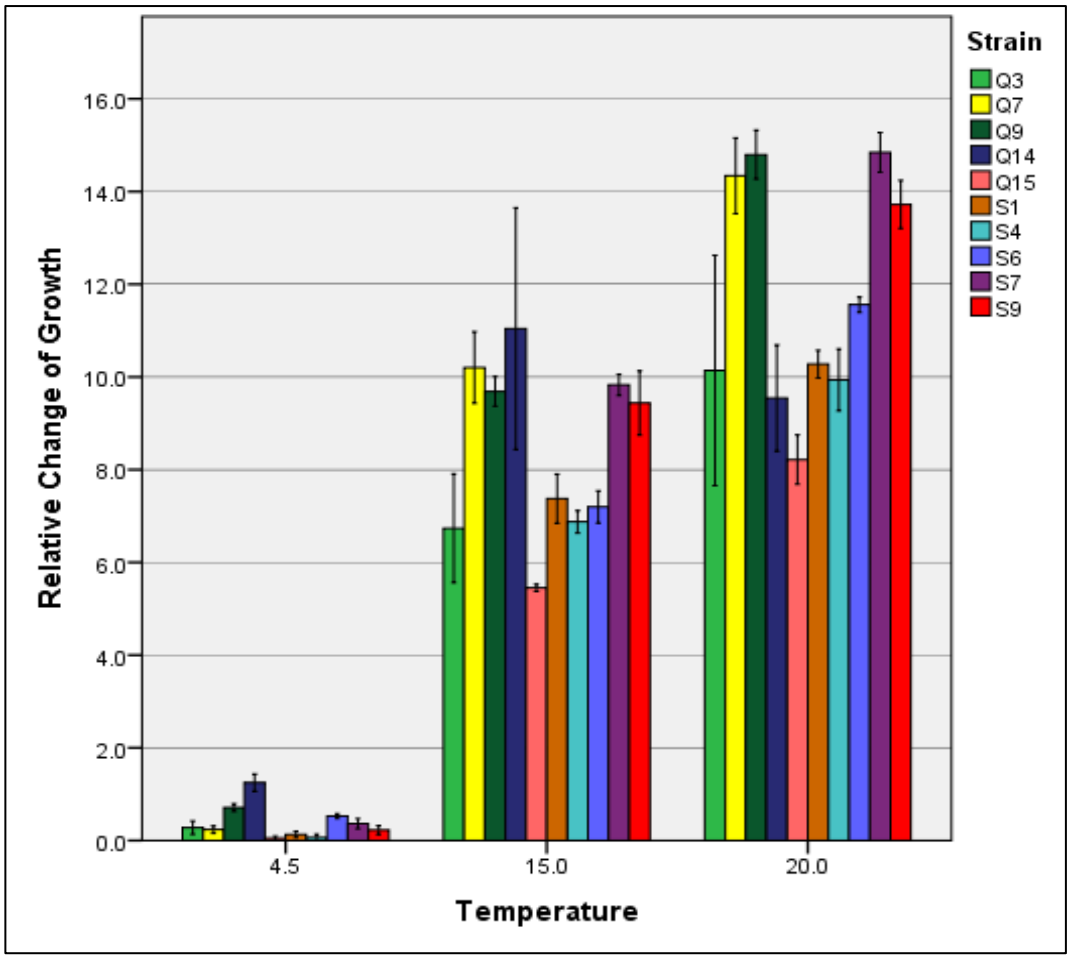


Figure 31. Relative change of growth (Mean ± SEM) comparison between species of 10 bacilli strains in replicates (n=3) at 4.5°C, 15.0°C and 20.0°C.

At the lowest temperature tested of 4.5°C, a slow progression in growth of the bacilli strains was observed, with Q14 as the highest growth increase by 1.250 ± 0.09 fold. At an increased temperature of 10.5°C, all strains showed increase in growth especially Q7 by 10.204 ± 0.4 fold. At a moderate temperature of 20°C, strain Q9 overtook Q14, showing growth increase by 14.795 ± 0.3 times.

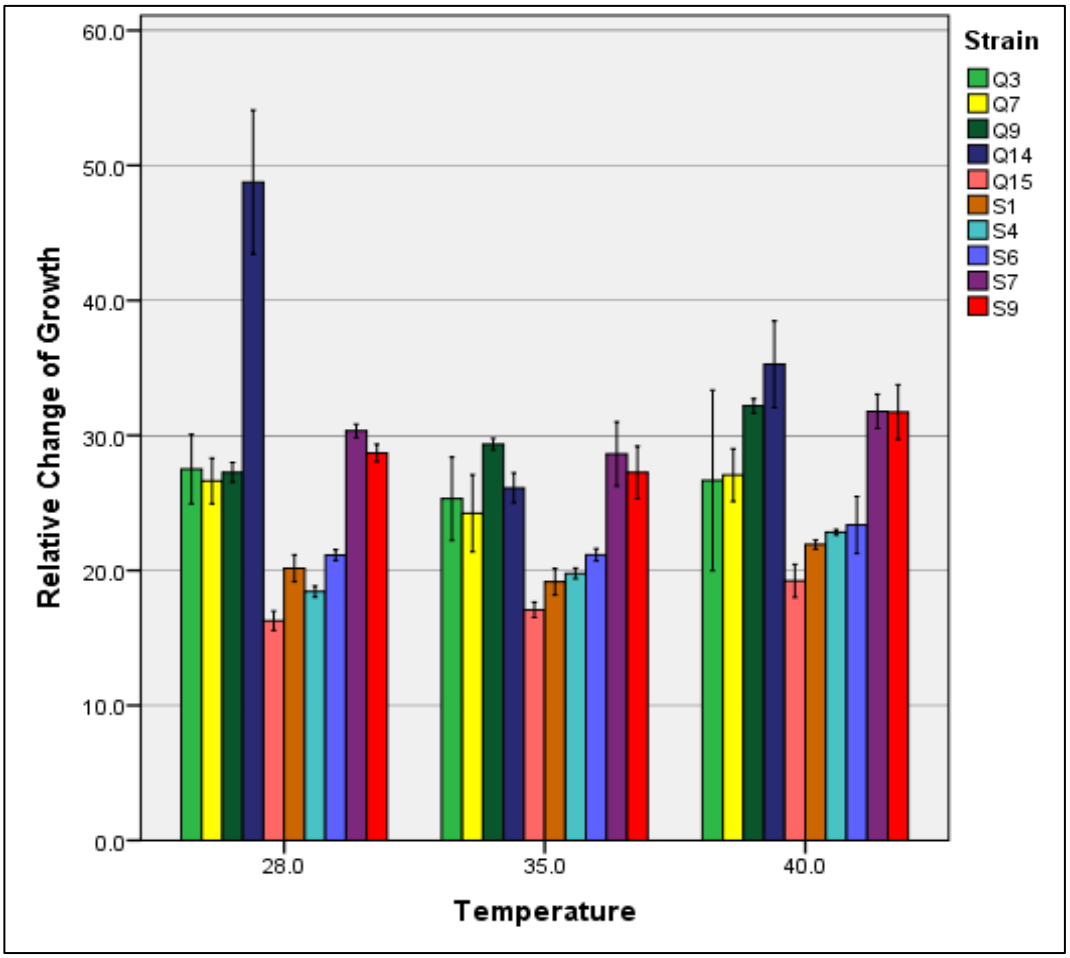


Figure 32. Relative change of growth (Mean ± SEM) comparison between species of 10 bacilli strains in replicates (n=3) at 28°C, 35.0°C and 40.0°C.

However, when temperature was increased to 28°C, all strains in general experienced good growth, with Q14 experiencing the highest amount of growth increase by 48.760 ± 2.0 times, while the strain experiencing the lowest growth increase was strain Q15 by 16.273 ± 0.4 times. This suggested that 28°C is the optimum temperature for best growth. Upon incubation at higher temperatures of 35°C and 40°C, all strains continue to exhibit steady to slight increase in rate of growth, especially Q15 having the lowest extent of growth but still increasing, even at 40°C.

Significant variation between the strains was observed at each temperature tested and this was supported by one-way ANOVA carried out for relative change of strain growth by temperature (refer to Appendix 2A for test statistic values as well as R^2 values).

Figure 33 shows a summary of growth response of each strain when grown in the temperature range of 4.5 to 45°C.

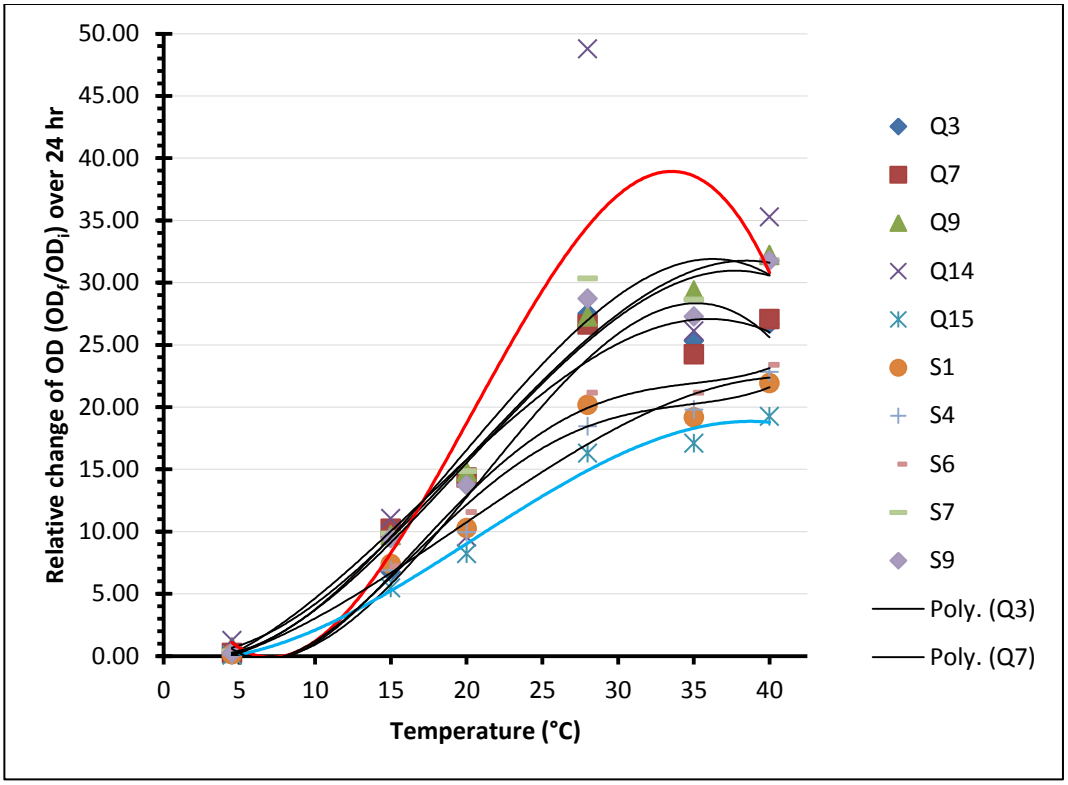


Figure 33. Spline-fit graph of relative change of strain optical density over temperature range of 4.5 to 40°C. Curve-fitting was solely for simplified visual presentation.

Strain Q14 outperformed other strains at most temperatures (i.e. 4.5°C, 15°C, 28°C, 40°C). A post hoc analysis, Tukey-Kramer HSD revealed that at the selected temperatures, Q14 had a significant growth advantage as compared to the other strains (refer to Appendix 2B for test statistic values (q*) of Tukey HSD test). The overall performances of strains S7 and Q9 across six temperatures was comparable to that of Q14, with S7 outperforming Q14 at temperatures 20°C and 35°C.

It was noticed that Q14, S7, Q9, Q7, and S9 were consistently in the top 50% best growing strains for each tested temperature. The slowest growth was always Q15 for all tested temperatures, but was most likely due to poor adaptation to the KB media as opposed to unfavourable temperature range.

Bacilli generally grew optimally at 28°C, although high temperatures do not deter their growth, even up to 40°C. These findings suggest that the bacilli tend to favour ambient to warm temperatures.

3.3.2.6 Growth of bacilli when subjected to low temperatures

Survivability of the ten bacilli strains were assessed at 4.0°C over a period of 7 days (168 hours) in shaking condition. Standardization of initial inoculum amount was carried out and growth (OD_{600}) was observed at intervals via destructive sampling of each replicate upon reaching targeted incubation times.

All strains tested were able to survive at 4.0°C for 168 hours. Cell density of Q3 decreased during the initial 72 hours, whereas all the other strains show slight increase in cell density, as is observed in Figure 34 below.

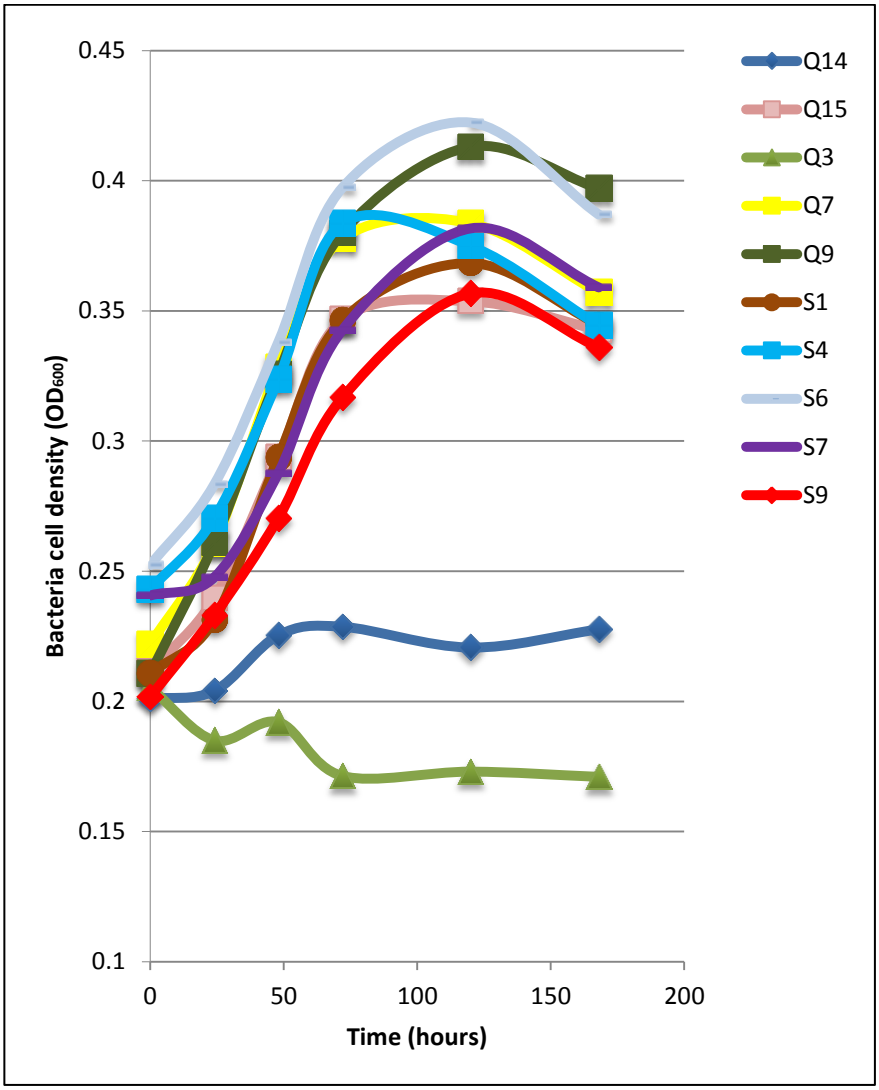


Figure 34. Line graph comparison of bacterial cell density (OD_{600}) of 10 bacilli strains incubated in shaking liquid KB microcosms over a period of 168 hours.

One-way ANOVA tested revealed that cell density of all strains at the 120th hour (5th day) varied significantly from one another (R^2=0.976, ANOVA $F_{9,20}$= 90.535, p<0.0001), whereby *post hoc* analysis (TK HSD) supported this claim by identifying strains Q14 and Q3 as significantly different from the other strains (q*=3.541, α=0.05). No difference was observed when the same analysis was carried out for neither the OD_{600} nor the relative change of OD_{600} at the 168th hour (see Figure 35, Refer to Appendix 3 for detailed results of strains at the 168th hour).

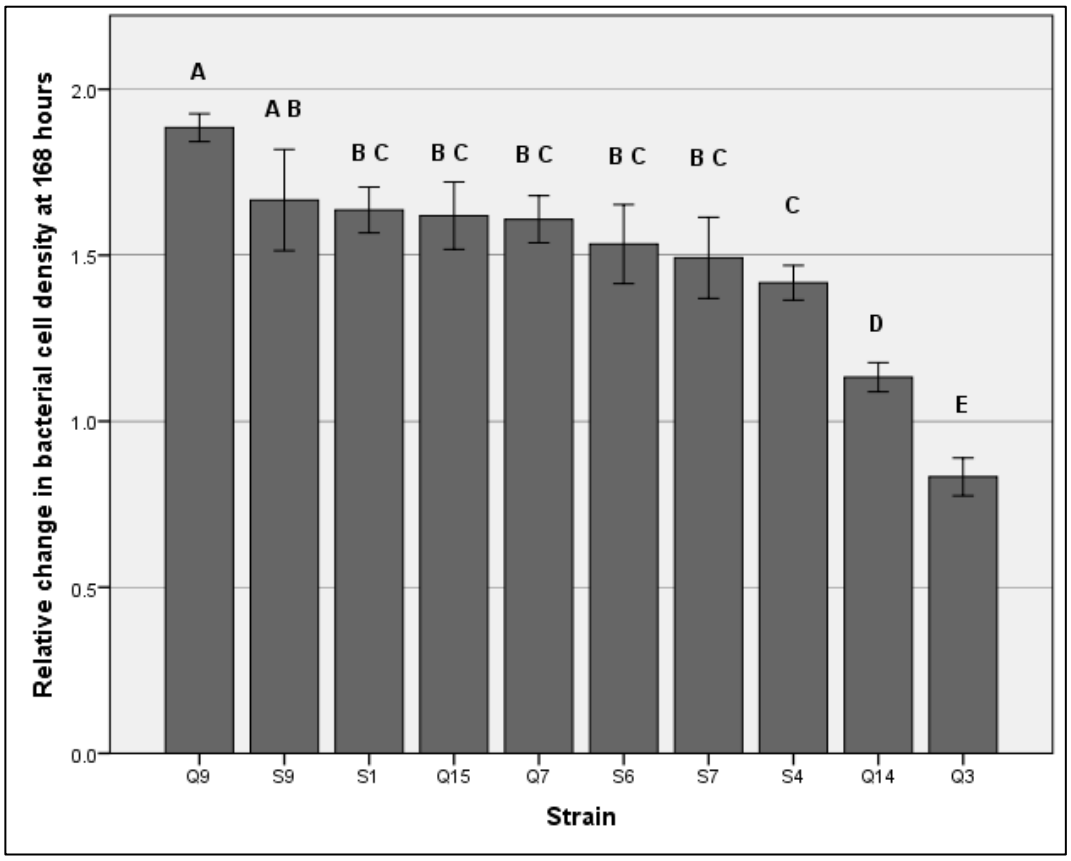


Figure 35. Histogram of relative change of bacterial cell density at 168 hours in 4°C. Results of the Tukey HSD analysis are represented by the letters, whereby strains that are not connected by the same letter are significantly different (q*=3.541, α=0.05).

All bacilli strains tested except Q14 and Q3 were able to grow and consume nutrients in 4°C, indicating that they were psychrotolerant in nature but not psychrophilic. Strains Q14 and Q3 show minimal growth but are still viable after the 168th hour. In Figure 34, it is observed that Q14 reached steady plateau in terms of growth and can be stored at 4°C for a longer period of time compared to the other strains.

Strain Q3 was the only strain that was observed to have decreased in optical density over the 168 hours of incubation in the cold. A subsequent streak onto solid agar microcosm

upon taking the final reading in the 168th hour revealed the survivability of strain in spite of observed decrease in optical density. Insufficient work was done to verify this, but it was suspected that strain Q3 formed spores upon prolonged exposure to low temperatures; this would account for lower cell counts and liquid media turbidity. The cold temperature could have easily led to bacilli endospore formation as the bacteria would have struggled to adapt to its new physical conditions, forming spores under stressful conditions which were dense, and sunk to the bottom of the cuvette. This suggestion was made in the knowledge that bacilli are known to produce endospores (Nicholson 2002).

3.3.2.7 Quantitative assessment of bacilli adhesion to different surfaces

Attachment of the ten bacilli strains was tested on three different substrates, i.e. glass, polypropylene and polystyrene Attachment was assessed by staining attached biofilms with crystal violet (see Figure 36).

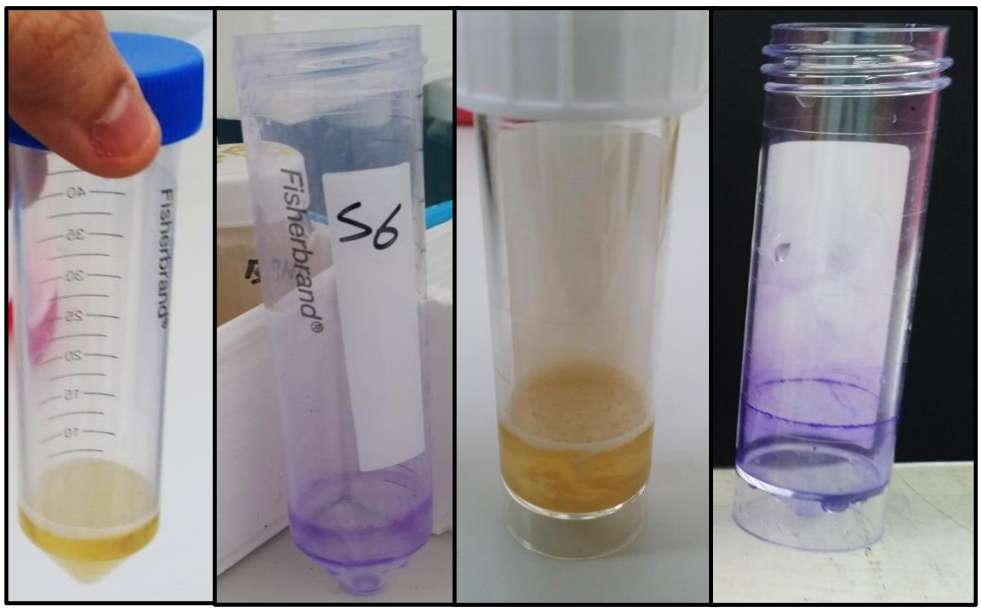


Figure 36. Attachment of bacilli biofilm onto plastic surfaces. Left to Right: Biofilm on polypropylene; attached biofilm on polypropylene stained with crystal violet; biofilm on polystyrene; attached biofilm on polystyrene stained with crystal violet.

Each of the attachment tests were carried out with a control, which was blank media inoculated with sterile water into the microcosms respectively. This control was then used to correct the attachment data obtained from the tested strains. Figure 37 shows the means ± SEM, which allows the comparison of strain to substrate interactions. A GLM approach was also used to model attachment responses of the strains tested.

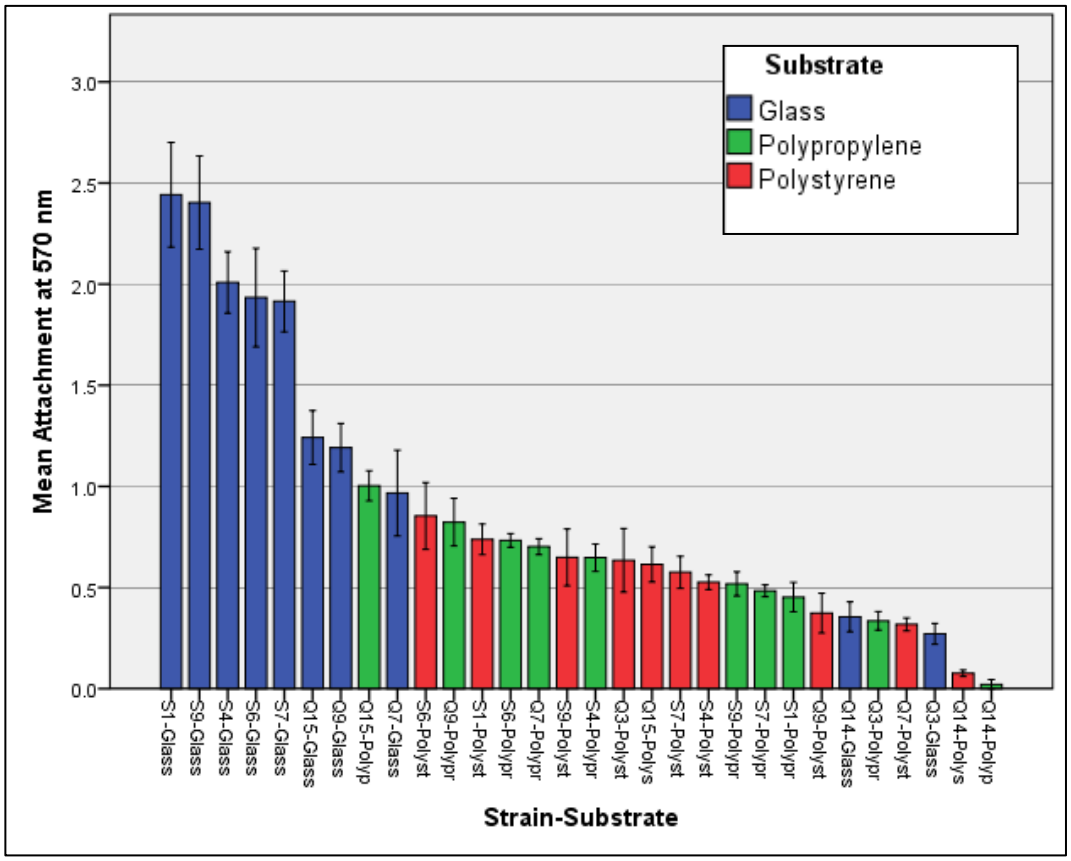


Figure 37. Histogram comparing mean attachment levels (A_{570}), post blank correction of the ten bacilli strains on 3 separate substrates; i.e. Glass, polypropylene and polystyrene.

ANOVA showed significant variation in attachment responses ($p<0.0001$). Best attachment was observed on glass microcosms by strains S1 and S9 having mean attachment of 2.442±0.1 AU and 2.404±0.1 AU respectively. Poorest performing strain in terms of attachment response to all substrates tested was Q14; 0.021±0.01 AU on polypropylene microcosm. Strain Q3 was considered to have poor attachment (threshold level: bottom 50% of the total number of cases), but observed to attach better to both the plastics than the glass microcosm vials (polystyrene first, then polypropylene). Overall, the GLM suggests that glass as a substrate for attachment was preferred when compared to the other two plastics (refer to Appendix 4 for pairwise comparisons of least square means using Tukey-Kramer HSD method).

Effects tests showed that strain ($Q=3.198$, $\alpha=0.05$), substrate ($Q=2.361$, $\alpha=0.05$) as well as strain to substrate interaction were significant ($Q=3.802$, $\alpha=0.05$; Table 19). Significant strain to substrate interaction indicates that the strains responded to the different surface characteristics of glass or plastics differently.

Table19. Effect tests of attachment difference response. Bolded p-values indicate that the effect is significant to attachment response (R^2=0.940, ANOVA $F_{30,209}$=109.489, p<0.0001). Abbreviations: NP, Number of parameters; DF, Degrees of freedom; F, f-statistic; P, p-value.

Source	NP	DF	F	P
Strain	9	9	101.593	**<0.0001**
Replicate	1	1	1.018	0.3141
Substrate	2	2	748.438	**<0.0001**
Strain*Substrate	18	18	48.469	**<0.0001**

Biofilms are made of extracellular polymeric substances (EPS) or exopolysaccharides. These are composed of polymers of simple sugars (monosaccharides) and non-carbohydrate constituents, e.g. acetate, pyruvate, succinate, etc. (Flemming and Wingender 2010). These non-carbohydrate constituents are all polar in nature, hence resulting in strong permanent dipole interactions with the polar oxygen component of silica in glass, as well as forming weak hydrogen bonds with the lone pairs of oxygen (Figure 38, left).

This is in contrast with constituents of polystyrene and polypropylene (Figure 38, right) as they are non-polar and therefore result in a lack of interaction between polar constituents with non-polar plastic structures.

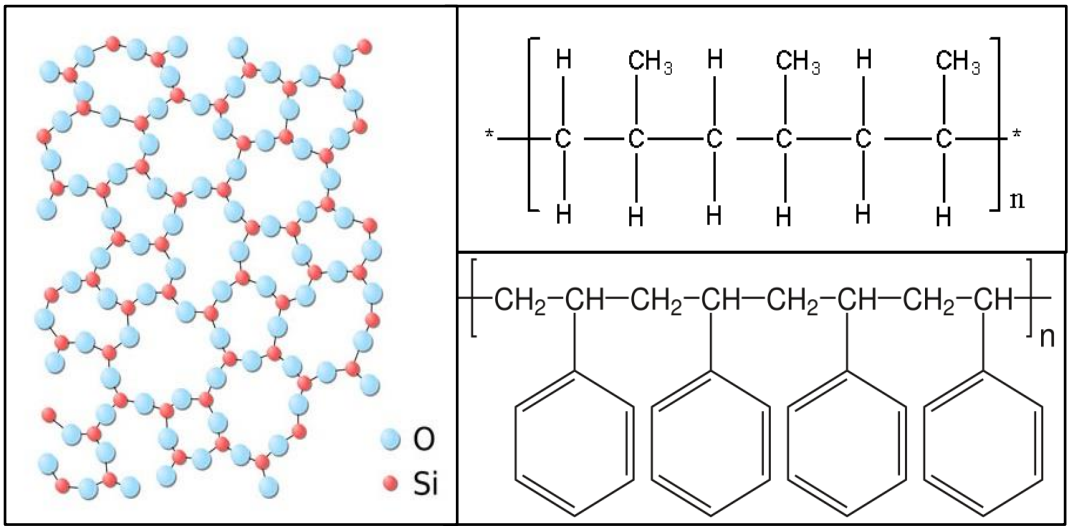


Figure 38: The detailed structures of materials used in the adhesion tests. Left: The structure for glass (Drewitt 2010); Right: The structures of polymeric polypropylene (top) (Diloné *et al*. 2012), and polystyrene (bottom) (Leyo 2010), which are very non-polar by nature.

The statistical analysis carried out supports the conclusion that strain and substrate have an effect on attachment levels. Interaction between strain and substrate is statistically significant (effect of strain depends on the substrate).

3.3.3 Hierarchical cluster analysis

By applying all the means from all the strain characterization assays from Section 3.2.2.1 to 3.2.2.7, a hierarchical cluster analysis was carried out in order to separate out bacilli key species from the ten tested strains. A constellation plot, shown below in Figure 39, was constructed based on data from hierarchical clustering.

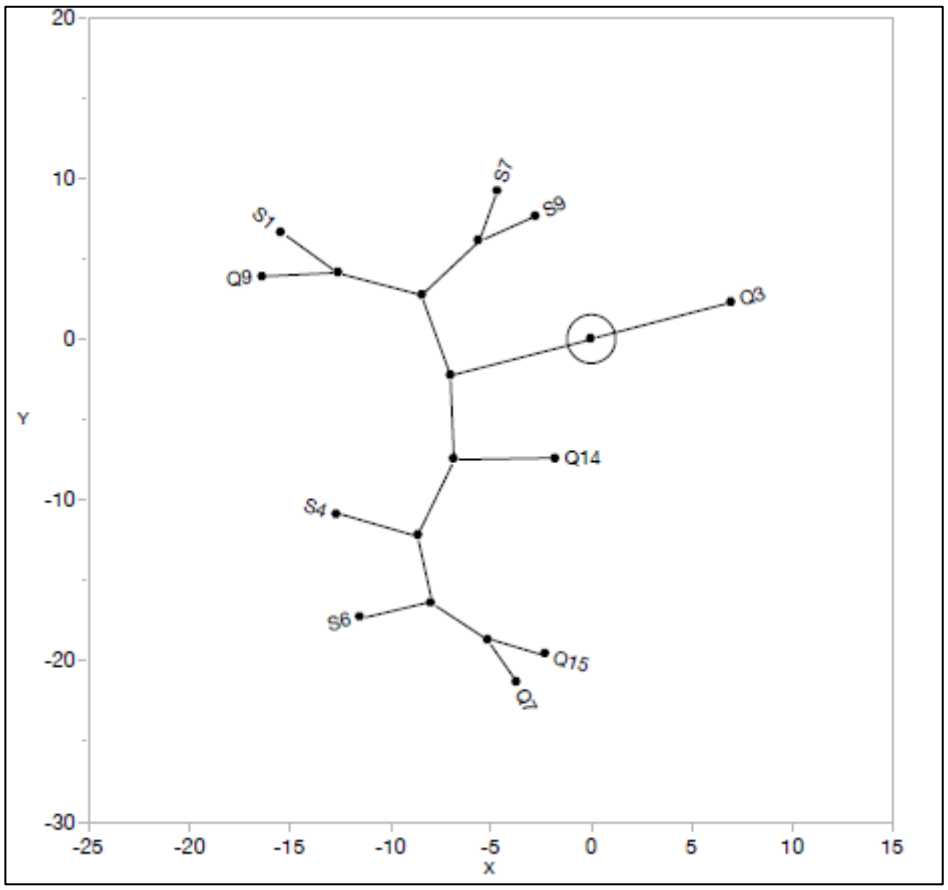


Figure 39. Constellation plot generated from all strains' characterization data means.

As can be observed in Figure 39 above, strain Q3 was most phenotypically distinct from the other strains, hence was first to be selected. From the first fork to the left of the central node, two clusters were observed. From the upper cluster, one strain was selected from the left (S1) and right (S9) branches; from the lower cluster, one strain was selected for greatest distance downwards from the fork (Q7), and a second strain was selected for its median properties (S4) in relation to the central node and the bottom-most strain. In cases of two strains placed at the end of a branch, selection was guided by desired results observed during strain characterization assays.

3.3.4 Molecular identification of selected bacilli strains

From the constellation plot generated in Figure 39, five phenotypically distinct strains were selected for molecular identification using universal 16S rDNA gene primers for bacteria. Phylogenetic analysis was carried out on sequences obtained from Beijing Genomic Institute, and subjected to Basic Local Alignment Search Tool analysis (NCBI). Based on the closest match, the most likely identities of the five selected strains are listed in Table 20 below.

Table 20. Identities of five selected strains from Hierarchical cluster based on closest match when subjected to Basic Local Alignment Search Tool analysis (NCBI).

Strain	Identity (based on closest match)	Accession	E-Value	Base pair match ratio (Percentage match)
Q3	*Bacillus subtilis*	HQ684005	0.0	485/488 (99%)
Q7	*Bacillus thuringiensis*	KY003095	0.0	467/480 (97%)
S1	*Bacillus anthracis*	AB506122	0.0	474/474 (100%)
S4	*Lysinibacillus sphaericus*	KT232321	0.0	474/475 (99%)
S9	*Bacillus cereus*	JQ311944	0.0	452/476 (95%)

3.3.5 Quantitative characterization of selected bacilli strains

3.3.5.1 Combined Biofilm Assay with Different Media for Five Strains

Five key bacilli strains were selected from the constellation plot from Figure 39, which were strains Q3, Q7, S1, S4 and S9. Each of these five strains were incubated in five different media, i.e. KB, KB-Fe(III), LB-NaCl, BHI, and M9-CAA. After incubation, the strains were assayed for growth, biofilm strength, and attachment.

Figure 40 compares the mean growth (mean ± SEM) of each of the selected strain in their respective microcosms by means of bacterial cell density at 600 nm. A general trend observed visually from the bar chart is that KB media best supports the growth of all of the strains except for strain Q3, followed by KB-Fe(III). The media with the poorest growth support for all the strains is M9-CAA.

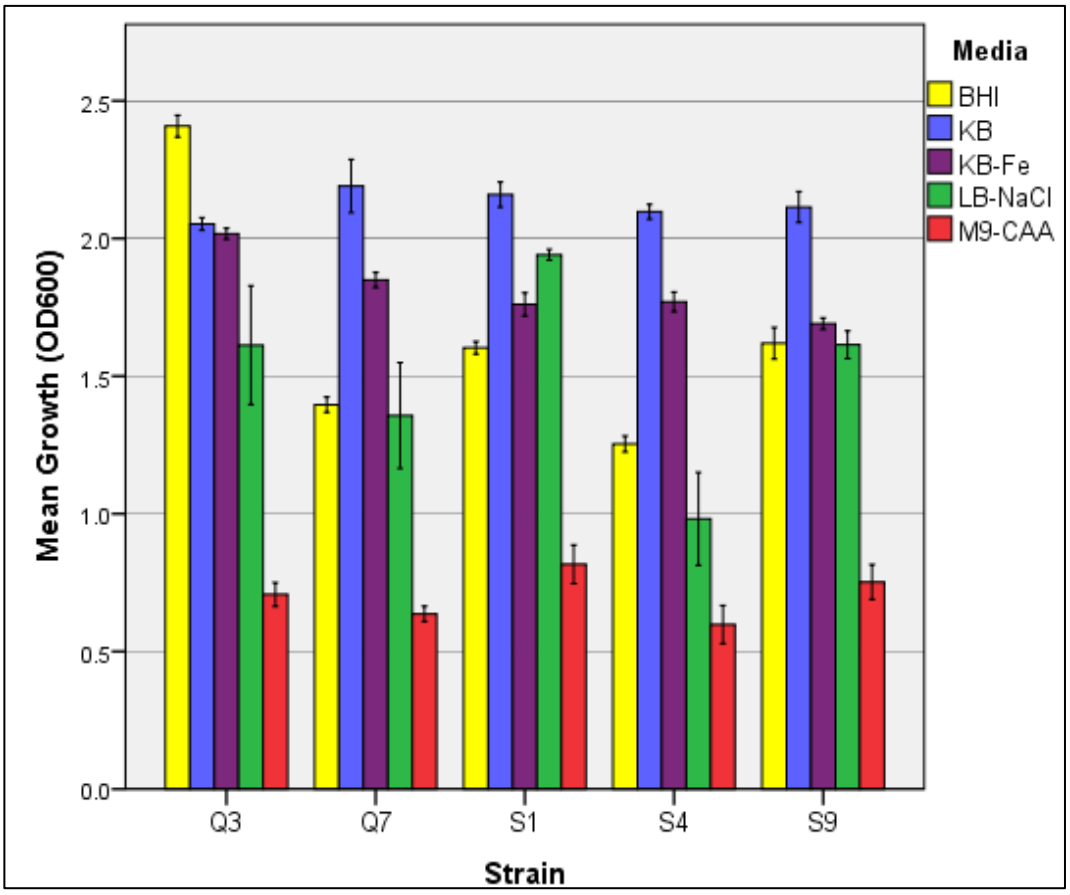


Figure 40. Bar chart comparisons of bacterial cell density between 5 key bacilli **strains grown in 5 different media respectively.**

Significant variation in growth between the strains grown in their respective microcosms were supported by a one-way preliminary ANOVA conducted (R^2=0.962, ANOVA $F_{24,175}$=185.451, p<0.0001). Although KB media promotes good growth for all the five strains tested, the overall best growth was observed by Q3 grown in BHI media, giving a final OD600 of 2.408 ± 0.02 AU. However, Q7 yielded the best growth in KB microcosms at a mean OD600 of 2.190 ± 0.05 AU. Despite LB-NaCl microcosms being the second poorest growth support media for most of the strains tested, it was also noted that S1 grew relatively well in LB-NaCl, yielding an OD600 of 1.941 ± 0.009 AU.

The biofilm strength of each of the five strains incubated in their respective media was determined via the MDM assay. Their means and standard error means were compared in Figure 41 below.

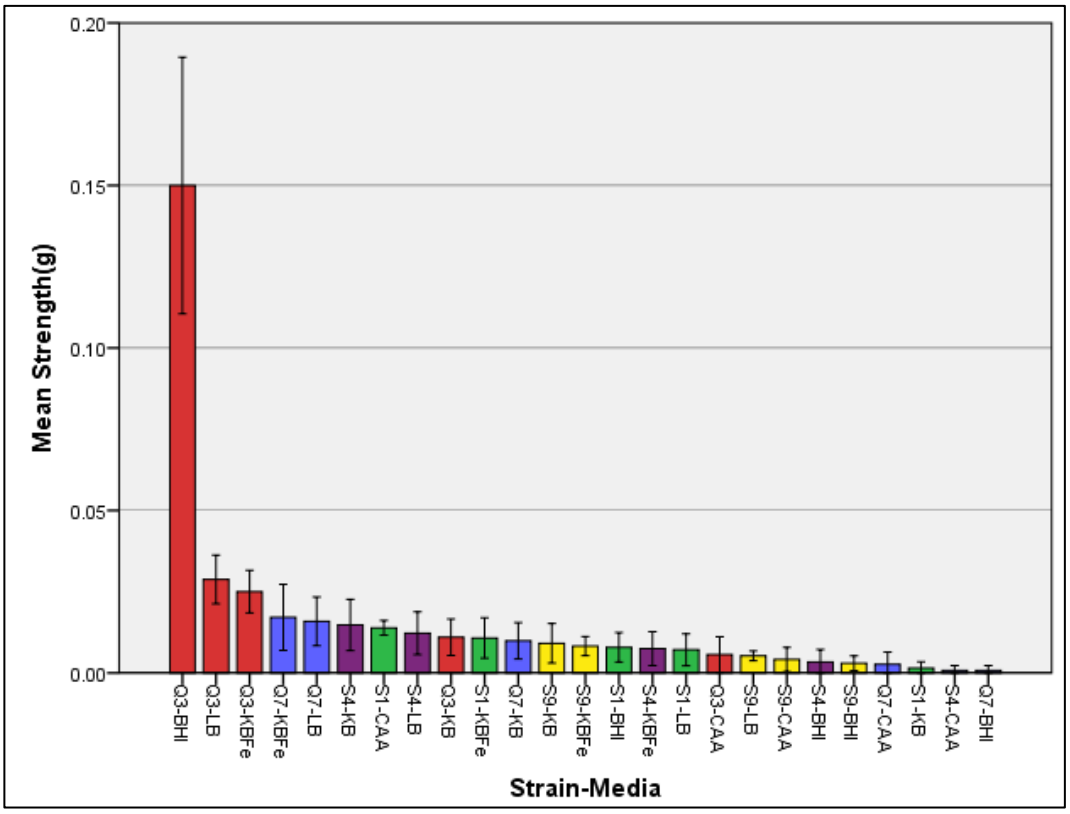


Figure 41. Histogram comparison of five individual strains incubated in their respective media in the order of descending mean biofilm strength (g) determined by the MDM assay.

A one-way preliminary ANOVA conducted determined the significant variation amongst the strength of A-L biofilms formed by each strain incubated in their respective media (R^2=0.841, ANOVA $F_{24,175}$=38.656, p<0.0001). MDM assay determined that Q3 formed the strongest A-L biofilm over the course of seven days in BHI media, resisting deformation up to the weight of 0.150 ± 0.04 g, as observed on the far left of the bar chart in Figure 41. It is also observed that Q3 was able to form a strong biofilm not only in BHI media, but also in LB-NaCl, KB-Fe(III) and KB, falling within the top 50% of the mean strength data.

Apart from Q3, it was found that Q7 is the second other strain exhibiting substantial biofilm strength but this strength was only observed when grown in LB-NaCl and KB-Fe(III). However, biofilm of S4 grown in KB microcosm was observed to be able to withstand the weight of 0.0148±0.01 g, which is the best performer amongst the strains in KB microcosm.

Biofilm attachment around the meniscus region of each microcosm was quantified by obtaining the absorbance of eluted crystal violet stain at 570 nm. The means and standard error means data were used to plot a histogram in Figure 42 for comparison between the strains and their respective media.

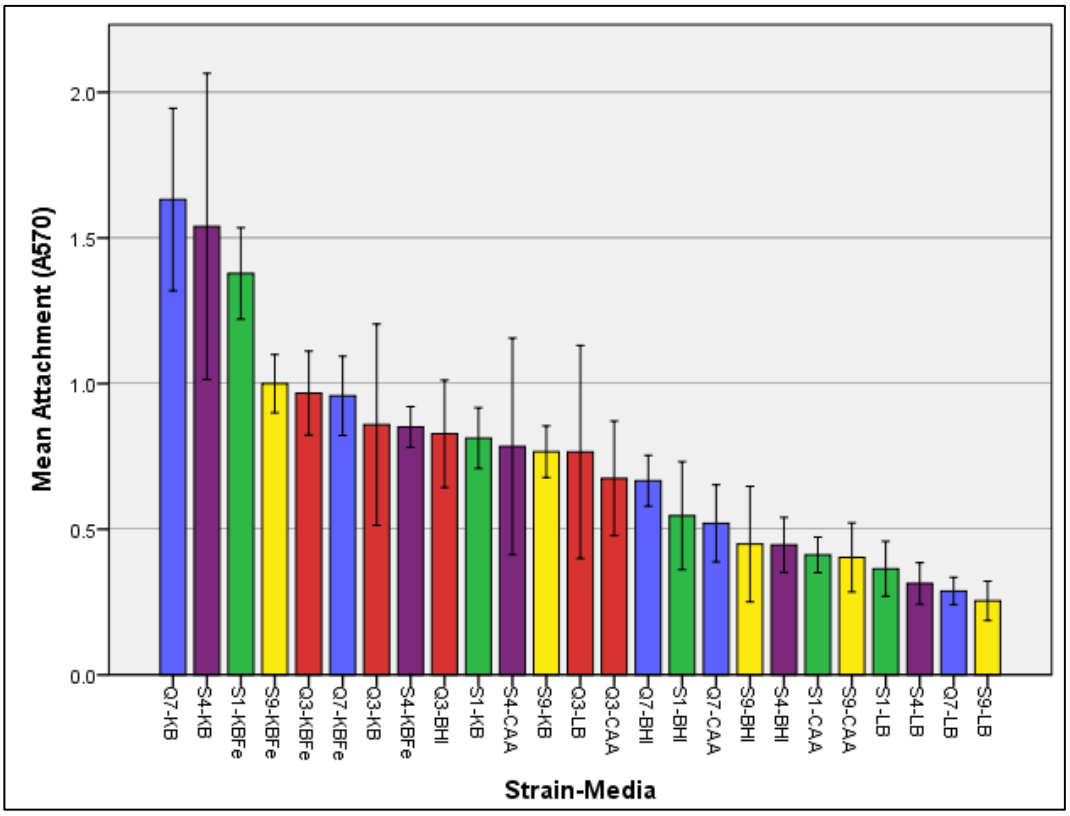


Figure 42. Comparison of key species incubated in their respective media and their mean biofilm attachment levels in descending order.

Although the dataset showed a moderate fit, the preliminary ANOVA conducted showed significant differences between biofilm attachment levels and strains grown in their respective media (R^2=0.665, ANOVA $F_{24,174}$=14.396, p<0.0001).

The strains which exhibit the three highest attachment levels are indicated on the left-side of Figure 42. The attachment levels were neither significantly correlated to media type nor strain type, thus no clear trend was observed. The highest mean attachment level observed was when Q7 was grown in KB to give A_{570} of 1.632 ± 0.157 AU, closely followed by S4 grown in KB as well, yielding an A_{570} of 1.539 ± 0.7 AU.

The effects of media to strain-specific interaction was not visually distinct in Figure 42 above, but it was noted that the attachment levels appear to be higher for all 5 strains when either KB or KB-Fe(III) was used. However, the attachment factor will also be modelled with media as an effect to statistically test visual claim. Attachment levels for most of the strains in chosen microcosms were generally moderate (A_{570} ≤ 1.0 AU).

Preliminary ANOVA conducted suggested significant variation of means across their respective grouping variables (growth, strength and attachment). A modelling approach (GLM) was then selected as a means to carry out a multivariate ANOVA, or MANOVA.

A nominal logistic fit test was first conducted to compare the fit of the specified models (Whole Model Test, DF=32, Chi-square=163.155, p<0.0001). The effects likelihood ratio tests, summarized in Table 21 below, determine the significance of the effects in the model and how good the fit would be without certain effects in the model.

Table 21: Effects Test to distinguish between strains. (Abbreviations: NP, number of parameters; DF, degrees of freedom; X^2, chi-square statistic; P, p-value)

Effects	**NP**	**DF**	**X^2**	**P**
Growth (OD_{600})	4	4	80.749	**<0.0001**
Media	16	16	82.519	**<0.0001**
Strength	4	4	48.561	**<0.0001**
Attachment (A_{570})	4	4	12.164	**0.016**
Replicate	4	4	0.474	0.976

Effects tests indicated clearly that factors of strain growth, biofilm strength, biofilm attachment, and media types were significantly affecting strain-specific interactions. Since the model works well (p-value was significant), this justified the need for a higher order model for growth, biofilm strength and attachment.

Factors contributing to biofilm strength, growth, and attachment levels were modelled using a general linear model which allowed for detailed comparison between these three factors and their corresponding effects. Results of tests conducted were summarized in Table 22 below.

Table 22. Summary of whole model analysis of variance (ANOVA) testing the effects of growth rate, attachment and biofilm strength. (Abbreviations: NP, number of parameters; DF, degrees of freedom; F, f-statistic; P, p-value)

ANOVA Parameters	**Factors**											
	Strength				Growth				Attachment			
R^2	0.540				0.889				0.475			
DF (Model, Error)	11, 187				11, 187				11, 187			
F	19.918				135.852				15.367			
P	<0.0001				<0.0001				<0.0001			
	Effect Tests											
Source	NP	DF	F	P	NP	DF	F	P	NP	DF	F	P
Media	4	4	25.396	**<0.0001**	4	4	247.825	**<0.0001**	4	4	23.168	**<0.0001**
Strength					1	1	79.805	**<0.0001**	1	1	4.526	**0.035**
Growth (OD600)	1	1	79.805	**<0.0001**					1	1	0.030	0.862
Attachment (A570)	1	1	4.526	**0.0347**	1	1	0.030	0.862				
Strain	4	4	10.460	**<0.0001**	4	4	17.166	**<0.0001**	4	4	2.692	**0.033**
Replicate	1	1	5.246	0.284	1	1	0.549	0.460	1	1	0.434	0.511

From these statistical analyses, it is clearly determined that factors growth, biofilm strength and attachment are dependent on the media type as well as strain tested. Media was established as a significant factor in affecting growth and attachment when the two factors were modelled with media as an effect. Moreover, there is evidence of significant distinguishable strain interaction between media used, be it KB, KB-Fe, LB-NaCl, BHI, or M9-CAA. Therefore, these findings suggest that chemical composition of shrimp feed is important as a prebiotic determinant of probiotic success.

When growth (OD_{600}) response was modelled, effects media, strength and strain were significant. *Post hoc* pairwise comparison via Tukey HSD method of least square means differences of strains revealed that S4 was significantly different from the other four strains tested (Q=2.755, α=0.05). Difference in media used also affected growth of this strain significantly, i.e. good in KB, poor in M9-CAA. (Refer to Appendix 5A for least square means of media and strain differences)

The biofilm strength model revealed that effects growth, attachment, strain and media were statistically significant. *Post hoc* analysis of pairwise comparison of least square means via Tukey HSD method determined that S1 and S9 significantly varied from Q3 and S4 (Q=2.755, α=0.05), whereas all media could be differentiated from one another with exception of BHI and LB-NaCl (Q=2.755, α=0.05). As can be observed in Figure 40, strains tend to respond accordingly to the media in which they were subjected to, e.g. Q3 formed strong biofilm in BHI, whereas S4 performs just as well but in only within KB microcosms. (Refer to Appendix 5B for least square means of media and strain differences)

When attachment was modelled, only effects biofilm strength, media and strain were significant. By applying attachment as a factor, KB and KB-Fe(III) could be differentiated from the other three media types via Tukey HSD test (Q=2.755, α=0.05). As a result, this supports the earlier claim that some common property of KB and KB-Fe(III) likely played a significant role in inducing high attachment levels. Pairwise comparison also determined that strains Q7 and S9 were significantly different from each other, whereas Q3, S1, and S4 behave similarly to each other (Q=2.755, α=0.05). This could explain visually indistinct strain-specific interaction to media based on histogram plotted in Figure 42. (Refer to Appendix 5C for least square means of media and strain differences).

A correlation coefficient analysis was conducted in order to test for dependent relationships between factors (growth, biofilm strength and attachment), and hence, suggesting links between the factors. A summary of the correlations between the basic microcosm measurements is listed in Table 23 below.

Table 23. Summary of correlation analysis between factors. (* Correlation estimated by REML (restricted maximum likelihood) method)

Strains	Q3		Q7		S1		S4		S9	
	Correlation statistic	p-value	Correlation statistic	p-value	Correlation statistic	p-value	Correlation statistic*	p-value	Correlation statistic	p-value
Growth to Strength	**0.528**	**0.0005**	0.255	0.113	**-0.461**	**0.0027**	0.250	0.119	0.227	0.159
Growth to Attachment	0.145	0.372	**0.693**	**<0.0001**	0.291	0.069	**0.484**	**0.0018**	**0.383**	**0.015**
Strength to Attachment	0.113	0.486	0.049	0.766	0.063	0.701	0.170	0.301	0.195	0.229

Test statistic and bold p-values indicate significant correlation (refer to Appendix 5D for detailed Scatterplot Matrix). The growth of strain Q3 shows significant positive correlated to its biofilm strength, whereas strain S1 has a negative test statistic value, which indicates a negative correlation between the growth and biofilm strength. However, there is a significant correlation between the growth and biofilm attachment of strains Q7, S4 and S9 respectively. This analysis strongly suggests that good attachment is associated with good growth of strains in corresponding media. Since S4 performed generally well in terms of factors biofilm strength and growth in KB microcosms (just as Q3 in BHI microcosms), it was interesting to note that both factors do not correlate to one another (p-value was not significant).

Data collected from the assays suggests no significant correlation between factors biofilm strength and attachment for any of the strains tested.

3.3.6 Significant highlights of strain characterization

Based on strain characterization assays carried out, relevant highlights of each species according to their most likely identities are summarized in the following Table 24.

Table 24. Relevant highlights of each of the five selected key species based on the strain characterization assays carried out from Sections 3.3.1 to 3.3.5.

Strain Q3: *Bacillus subtilis* [HQ684005]
Strain Q3 was able to produce good amounts of AHL-lactonases within the first 24 hours of being subjected to quorum signalling molecules, C6-HSL. Recent studies have reported *B. subtilis* to possess the AiiA gene (Koul and Kalia 2016, Chen *et al*. 2013, Pan *et al*. 2008) as well as AHL-degrading potential (Wang *et al*. 2004, Dong *et al*. 2000). *B. subtilis* is commonly isolated from upper layers (1 -3 cm) of soil samples and adapts quickly to unfavourable conditions via endospore formation (Mongkolthanaruk 2012). Plant-associated species often biofilm formers to attach and colonize plant surfaces, e.g. endophytic *B. subtilis* from plant stem showed strong inhibition of phytopathogens (Li *et al*. 2012). Although mechanism of entry to plant cell is not demonstrated, it was believed that surface motility plays a role in stable biofilm formation within the bacterial populations (Bais *et al*. 2004, Kinsinger *et al*. 2003). The "climbing" characteristic, as mentioned by Angelini *et al*. (2012), was demonstrated by this strain, giving more credence to conclusion of its most likely identity. However, Q3's inability to significantly alter liquid surface tension post incubation suggested a lack of biosurfactant or surfactant-like compounds produced. Strain also exhibits high biofilm strength although its attachment is low in comparison to the other bacilli strains tested. Twitching and swimming are types of collective motility for bacterial movement. Wilkins *et al*. (2016) established a link between the physicochemical properties of bacterial excretions (amphiphilic molecules) and their cooperative motility. When a nutrient concentration gradient exist within an A-L interface of the biofilm formed, a stratification of gene expression occurs, resulting in an increase in expression of these amphiphilic molecules around the edges of the biofilm. Although physical consequences of this occurrence within a biofilm are not well understood, it was suggested that such shifts in metabolic activities may accompany significant increases in biofilm production (White *et al*. 2010). This theory was postulated to explain the preference for Q3 to spread upwards and hence, create a "climbing" effect. This may also explain why the biofilm was formed at the side of the glass

was strong albeit of weak attachment. Moreover, a robust performance shown by this strain during tests of cooperative motility as compared to other bacilli strains tested further supports this suggestion.

Intriguingly, Q3 grows well in BHI as compared to KB media and its growth in KB-Fe(III) is not significantly different from that of KB, indicating that this strain responds better to iron as compared to the other bacilli strains tested. A study by Hasan *et al*. (1997) hinted that BHI is a medium with significant amounts of iron. Several studies have demonstrated that presence of iron exerts a direct influence on cellular functions of B. subtilis (Dertz *et al*. 2006, Gaballa *et al*. 2008, Pelchovich *et al*. 2013).

Strain Q7: *Bacillus thuringiensis* [KY003095]

Bacillus thuringiensis is distinctively known for its crystals during sporulation and is widely applied as a pesticide to overcome the reliance on chemical means to control insect pests (Azmi *et al*. 2015, Khan *et al*. 2016). Gillis and Mahillon (2014) believed that being ubiquitously distributed, in terms of environmental habitats, *B. thuringiensis* might play undiscovered yet significant ecological roles beyond bacterium-insect interactions.

A survey of latest literature revealed that *B. thuringiensis* is well known for its ability to quorum quench via production of AHL-lactonase. Many studies have acknowledged the potential of this promising strain as a safe broad spectrum biocontrol agent (Raddadi *et al*. 2008, Dong *et al*. 2004). This particular bacilli species do not interfere with the growth of their host, but rather eliminates the accumulation of AHL signals, hence providing significant protection against host infection (Dong *et al*. 2004). Park *et al*. (2008) suggested the symbiotic relationship occurs because AiiA is involved in the cell metabolism or survival mechanism during bacteria cell growth. *B. thuringiensis* are also known for species-conserved gene for AHL-degrading enzymes (Dong *et al*. 2002, Lee *et al*. 2002), which was observed phenotypically as well in this study.

Studies have acknowledged *B. thuringiensis* as an efficient biofilm producer, even to the extent of producing biofilms in high concentrations of heavy metal (Dash *et al*. 2014, Rathnayake *et al*. 2013) which can only be good for application in shrimp guts as it will be able to colonise the surfaces, leading to competitive exclusion of VP_{EMS}, while not being known to be pathogenic towards the animal host.

Strain was found to express weak surfactants or surfactant-like compounds. In recent studies, biosurfactants produced by *B. thuringiensis* have shown to exhibit biologically active compounds, with varying properties of fungicidal and insecticidal activity (Kim *et al*. 2004), organo-tin biodegradation (Tang *et al*. 2016), polycyclic aromatic hydrocarbon biodegradation (Ferreira *et al*. 2016). It could be possible that the antagonism it showed against VP_{EMS} could be due to production of either AHL-lactonases or biosurfactants.

Strain S1: *Bacillus anthracis* [AB506122]

Strain was suggested to express phenotypic plasticity as it exhibits distinctly different biofilm phenotypes in different media as shown in preliminary qualitative biofilm assessment.

Just like strain Q7, this strain was also tested to produce weak surfactants or surfactant-like compounds. It was found that coral associated strain of *B. anthracis* was able to produce biosurfactants, which were able to express antibiofilm activities against biofilm forming *Psuedomonas aeruginosa* (Padmavathi and Pandian 2014).

There is very little research into the beneficial usage for *B. anthracis* due to historical association with high bio-risk level and bioterrorism. Therefore any potential consumer applications of this strain should be approached with awareness of public perception as well as undergo strict testing to ensure that the end-users will not be harmed in case of mutations, i.e. toxicity gene knock-out mutants (Pomerantsev et al. 2011).

Strain S4: *Lysinibacillus sphaericus* [KT232321]

The potential of S4 being a suitable candidate as a biocontrol agent was further enhanced upon 16S rDNA phylogenetic identification which gave a most likely identity as *Lysinibacillus sphaericus*.

L. sphaericus was first described by Neide (1904) and was originally named *Bacillus sphaericus*. Initially noted as an endospore forming environmental isolate, this species has been found ubiquitously as a member of soil microbiota. It was in more recent times that Ahmed *et al* (2007) proposed a name change to its current name of *L. sphaericus* based on distinct cell wall phenotype and phylogenetic analysis.

It was long established as an antagonist of insects (Priest 1992), similar to that of *B.*

thuringiensis. Survey of recent literature revealed that *Lysinibacillus sphaericus* are potent isolates with insecticidal activity against mosquito larvae (Berry 2012, Wirth *et al*. 2014, El-Bendary *et al*. 2016). The known application of *L. sphaericus* as a larvicide hints at potential as a viable strain for the purpose of inhibiting the growth and/or toxicity of VP_{EMS}, a simple modification of established methods should be sufficient for tank trials using this strain in the future.

This strain produces a consistent high amount of AHL-lactonase over 72 hours, as seen in section 2.3.5. Such consistency in production of desired enzyme will be useful for its intended purpose.

When inoculated in KB liquid microcosms, S4 proved to exhibit the strongest biofilm formed amongst all other bacilli strains tested in KB medium. This biofilm formed was also the second best in terms of attachment. Which only furthers the argument that S4 would show good potential in preventing quorum sensing between VP_{EMS} cells, while itself forming a physical barrier within the shrimp gut to confer protection.

Strain S9: *Bacillus cereus* [JQ311944]

The use of *Bacillus cereus* as a reliable biocontrol agent has been well discussed in literature, mainly for its antifungal activity (Huang *et al*. 2005, Xu *et al*. 2014, Lozano *et al*. 2016).

Strain S9 was found to be slow in secretion of AHL-lactonases within the first 24 hours but showed increased production over the subsequent 48 hours of incubation with C6-HSL as sole carbon source. While this is a positive sign that the AHL-lactonase production may be sustained across a period of three days, it was not as efficient as the other bacilli strains tested. Many studies have often found *Bacillus cereus* as the predominant species to possess the quorum quenching AiiA gene when screened amongst other bacilli species (Huma *et al*. 2011, Zhao *et al*. 2008, Dong and Zhang 2005), but the natural expression rates are not well characterised.

Correlation analysis did pick up on positive correlation between strain growth and attachment in each growth media, suggesting that growth rate may be dependent on attachment. Biofilm formation could be a critical requirement for the good growth of this strain.

3.4 General Summary

Based on the analyses carried out, the differences in strain performances were used to pick out candidates to undergo a coevolution study with VP_{EMS}.

Q3 showed the best biofilm strength in three different media, and was the best performing strain for twitching motility response. Q3 also performed moderately well when subjected to elevated temperatures associated with *Vibrio* blooms, defined as rapid *Vibrio* proliferation on sporadic nutrient pulses in natural resources (Thompson and Polz 2006). In terms of phenotypic variation, this strain proves to be the most interesting and distinct as compared to the other four strains tested. Hence, it was selected as one of the 3 strains for the following coevolution studies.

Q7 showed best attachment level and above average growth at elevated temperatures in KB microcosms. Strain biofilm attachment is an important factor as it is an indicator of probiotic colonisation ability when exposed to gastrointestinal tract of host animal. Therefore, it was chosen as one of the 3 strains for coevolution.

S4 was chosen because it showed good biofilm strength as well as good attachment even though it was not the best strain for either of those two parameters/variables. S4 also performed above average under moderate to elevated temperatures. The overall performances of S4, especially in terms of biofilm strength, attachment level, and growth at warm temperatures, were consistently above average, suggesting favourable response as a possible candidate in coevolution studies leading to enhanced overall probiotic potential.

In selecting the best growth microcosm for coevolution studies, KB media was chosen from a list of five media due to its high nitrogen content, which resulted in good growth across all bacilli strains tested. A comparison microcosm was KB supplemented with Fe(III) at 100 µM concentration. The strains tested responded well to the nitrogen in KB but experienced slightly retarded growth due to presence of Fe(III) ions. This relationship between media and enhanced growth of probiotic bacteria led us to postulate that shrimp feed content should be of high nitrogen levels and low Fe(III) levels as a crucial prebiotic factor.

The microcosm vessel material chosen was glass as it was found in Section 3.2.2.7 that bacilli mean attachment response to glass was high (1.473 AU) compared to polystyrene (0.570 AU) and polypropylene (0.536 AU). It was shown that strain to glass interaction was significantly different from the other two plastics (Tukey HSD, α=0.05).

4. Chapter 3: Coevolution of chosen bacilli strains with *Vibrio parahaemolyticus* and evaluation of selected post-evolution characteristics

4.1 Introduction

Based on the performances of the strains in the previous chapter, bacilli strains were chosen to undergo co-cultivation as a method for strain improvement. This co-cultivation technique applies the theory of directed selection against *Vibrio parahaemolyticus* and was quantitatively assessed via competitive fitness. Growths in microaerobic environments as well as plate-based assays were used to assess evidence of strain improvement compared to their wild type. Flowchart in Figure 43 below summaries the work carried out in this chapter.

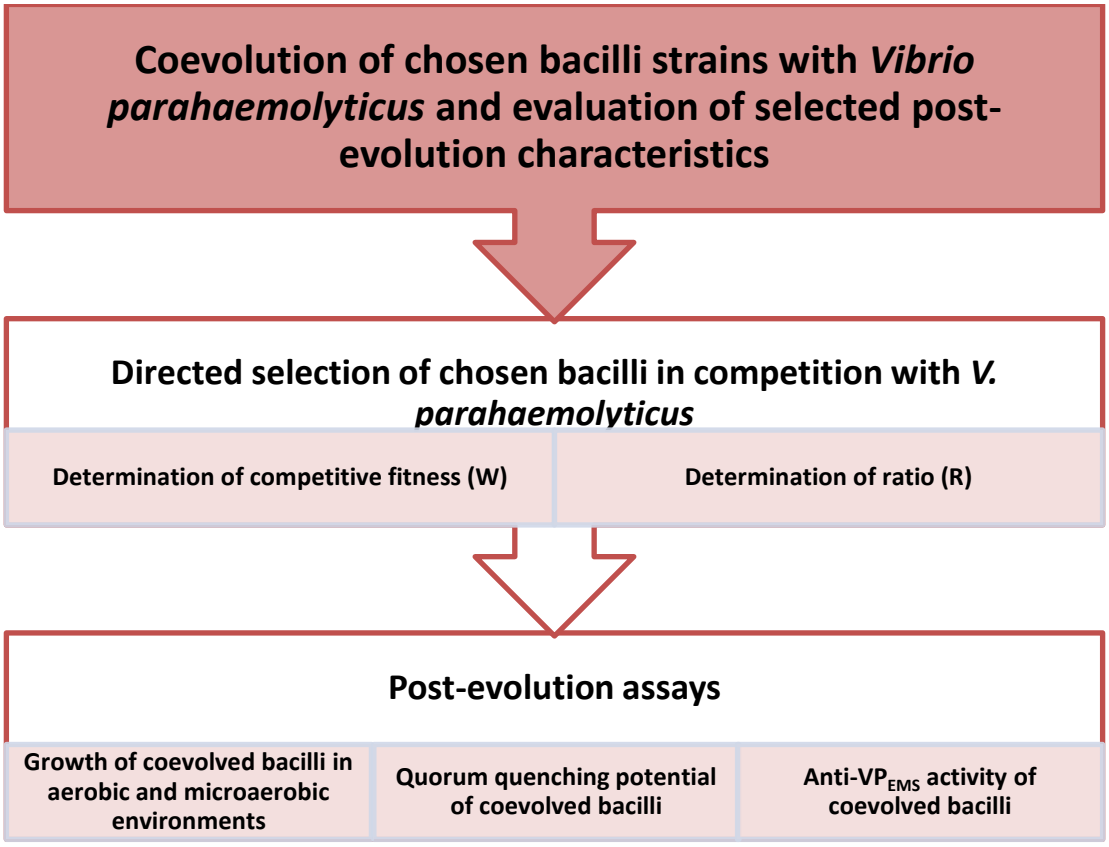


Figure 43. Flowchart which summarises the work done in this Chapter 3.

4.2 Methodology

4.2.1 Directed selection of chosen bacilli in competition with *V. parahaemolyticus*

From the five key bacilli species selected, three strains were chosen based on their overall colonization performance in characterization assays to undergo a combined coevolution and fitness experiment, modified from Charusanti *et al*. (2012) and Koza *et al*. (2011). Their ability to coevolve over a period of 6 weeks would be monitored via weekly assessment of fitness (W) against VP_{EMS} prior to further post-evolution assays.

4.2.1.1 Determination of competitive fitness (W)

Two sets of one mixture of overnight Bacillus and VP_{EMS} cultures (100µL, 1:1) were inoculated in replicates (n=3) and incubated into a 6mL KB microcosm (96 hrs, 22°C); one set under shaken condition and the other under static condition. Serial dilutions of mixtures were prepared, with sterile saline as diluent used, and aliquots spread onto a differential media used to differentiate VP_{EMS} and bacilli strains (composition of media used for differentiation and enumeration listed in Table 25 below). Plates were incubated for 48 hours before enumeration of the initial numbers.

Table 25. Composition of media used for differentiation between VP and bacilli strains in grams/L.

Composition	Grams/Litre (g/L)
Tryptone	5.0
Proteose peptone	5.0
Yeast extract	1.0
Sucrose	10.0
Sodium chloride	50.0
Phenol Red	0.025
Bacteriological agar	17.0
Final pH 8.2 at 25°C	

After 96 hours of incubation, bacteria were destructively recovered from microcosms by vigorous vortexing prior to spreading aliquots of serial dilutions onto differential plates (Table 25). Final numbers were enumerated using standard plate count methods after 48 hour incubation period. Bacilli colonies were isolated from respective plates after enumeration and mixed with wild type VP_{EMS} to be used as inoculum for subsequent weekly transfers up to five weeks.

Fitness (W) was determined as the ratio of the Malthusian parameters after Lenski *et al*. (1991) as follows:

$$Competitive\ Fitness, W = \frac{\ln\left[\frac{Bacillus_{final}}{Bacillus_{initial}}\right]}{\ln\left[\frac{V.parahaemolyticus - EMS_{final}}{V.parahaemolyticus - EMS_{initial}}\right]}$$

4.2.1.2 Determination of ratio (R)

Weekly transfers of the three chosen bacilli species were also carried out in the absence of VP_{EMS} to serve as the control, in which any evolution is purely due to adaptation to media and mutations acquired over time. The bacilli strains were inoculated in two sets of 6 mL replicate KB microcosms (n=3) and incubated (96 hrs, 22°C); one set under shaken condition and the other under static condition. Aliquots were spread onto differential plates (Table 25) and incubated for 48 hours before enumeration of initial and final numbers using standard plate count methods.

Improved growth due to adaptation to media was noted as Ratio (R), given by the formula as follows:

$$Ratio, R = \ln\frac{Bacillus_{final}}{Bacillus_{initial}}$$

4.2.2 Post-evolution assays

Following this co-evolution study, growth in micro-aerobic conditions, antagonistic assays against VP_{EMS} and anti-quorum sensing assays were carried out to compare competitive inhibition shown by coevolved strains and the wild type strains.

4.2.2.1 Growth of coevolved bacilli in aerobic and microaerobic environments

Growths of successfully coevolved strains, their wild types, and VP_{EMS} were assessed using cell density OD_{600} in order to determine growth response when subjected to micro-aerobic conditions. Inoculation of each overnight culture prepared were carried out in replicate KB microcosms (n=3) and incubated (48hours, 22°C). Micro-aerobic conditions were achieved

using AnaeroGen Compact system (Oxoid). All strains were also inoculated in replicate KB microcosms (n=3), incubated in aerobic conditions (48hours, 22°C) and OD_{600} measured to serve as control.

4.2.2.2 Quorum quenching potential of co-evolved bacilli

An agar well diffusion assay, as carried out by Holder and Boyce (1994), was used to assess any strain improvement post coevolution in competitively inhibiting VP_{EMS}. Overnight KB cultures of the three chosen bacilli strains post and pre-coevolution were prepared. KB plates were spread with aliquots of overnight indicator cultures (*C. violaceum*) before 5 mm-diameter plugs were removed. Bacilli cultures were inoculated in replicates (n=8) and incubated (24 hours, 22°C). Areas of pigment inhibition (mm^2) were measured via ImageJ.

4.2.2.3 Anti-VP_{EMS} activity of coevolved bacilli

Strain improvement on quorum sensing inhibition post coevolution was assessed via agar well diffusion method. Overnight KB cultures of the three chosen bacilli strains post and pre-coevolution were prepared. KB plates were spread with aliquots of overnight VP_{EMS} cultures before 5 mm-diameter plugs were removed. Bacilli cultures were inoculated in replicates (n=8) and incubated (24 hours, 22°C). Area of inhibition (mm^2) observed were measured via ImageJ.

4.2.3 Statistical analyses and modelling

All experiments and assays were conducted in replicates as stated, and the means ± SE provided where necessary. JMP Statistical Discovery Software (JMP v12, SAS Institute Inc., USA), SPSS Statistics (SPSS 22.0, IBM, USA) and Microsoft Excel (version 2010) were used to analyse all data obtained.

Differences between means were tested by ANOVA (*F*) and *post hoc* multiple comparison tests including Dunnett's method with a control and Tukey-Kramer HSD (TK HSD, *q**) tests were carried out. T-tests (*t*) were used to determine whether competitive fitness was significantly different to one ($W \neq 1$). Independent-samples t-tests (*t*) were used to determine whether competitive fitness was significantly different to one ($W \neq 1$).

4.3 Results and Discussion

The most common mode of action of probiotics studied is the competitive exclusion of pathogenic bacteria (Verschuere *et al*. 2000, Gullian *et al*. 2004, Callaway *et al*. 2008), leading to its elimination from ecological niche by one or more other non-pathogenic bacteria species competing for identical resources (Mayfield and Levine 2010). The fitness of the three chosen bacilli species, based on the rationale as stated in Section 3.4 (page 78) was studied, growing them under two separate conditions in the presence of the *V. parahaemolyticus*-EMS.

Ongoing mutations and/or selection constantly shape the genetic diversity of a population. Selection events that occur within a population induce novel mutations which augment existing variation and hence, give rise to more advantageous "mutants" (Beaudry and Joyce 1992). The term 'directed selection' comes to mind when generating desired phenotypic diversity by biased manipulation of artificial selection events.

4.3.1 Directed selection of chosen bacilli in competition with *Vibrio parahaemolyticus*

The competitive fitness performance (W) of the three chosen strains against VP_{EMS} was calculated as the ratio of Malthusian parameters after Lenski *et al*. (1991) and plotted as a combined bar chart in Figure 45 below.

Although a mixture of Q3 with wild type VP_{EMS} were initially inoculated into fresh microcosms during weeks 1 and 2, by the third week, only VP_{EMS} growth was present in all post incubation differential plates (see Figure 44). This suggests the dying out of Q3 after the second transfer in both conditions.

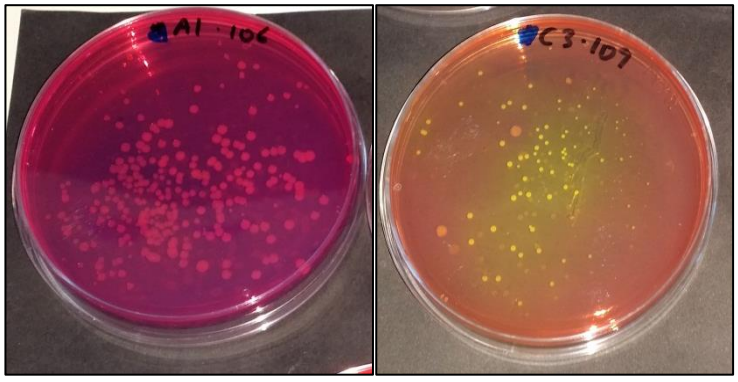


Figure 44. Left: Plating done after week 3 of Q3 transfers indicated only VP remained. (Right): Plating done for S4 plus VP mixture gave two different colony types on differential agar.

Figure 45 below suggests that strains Q7 (in static) and S4 (in shaken) both experienced initial "spike" in competitive fitness against VP for the first transfer (2.383 ± 0.009 and 3.227 ± 0.07 respectively). However, subsequent transfers have shown that stabilization in fitness had occurred. For Q7 (in shaken) and S4 (in static), it was observed that a dynamic equilibrium was successfully established between the bacilli and VP population.

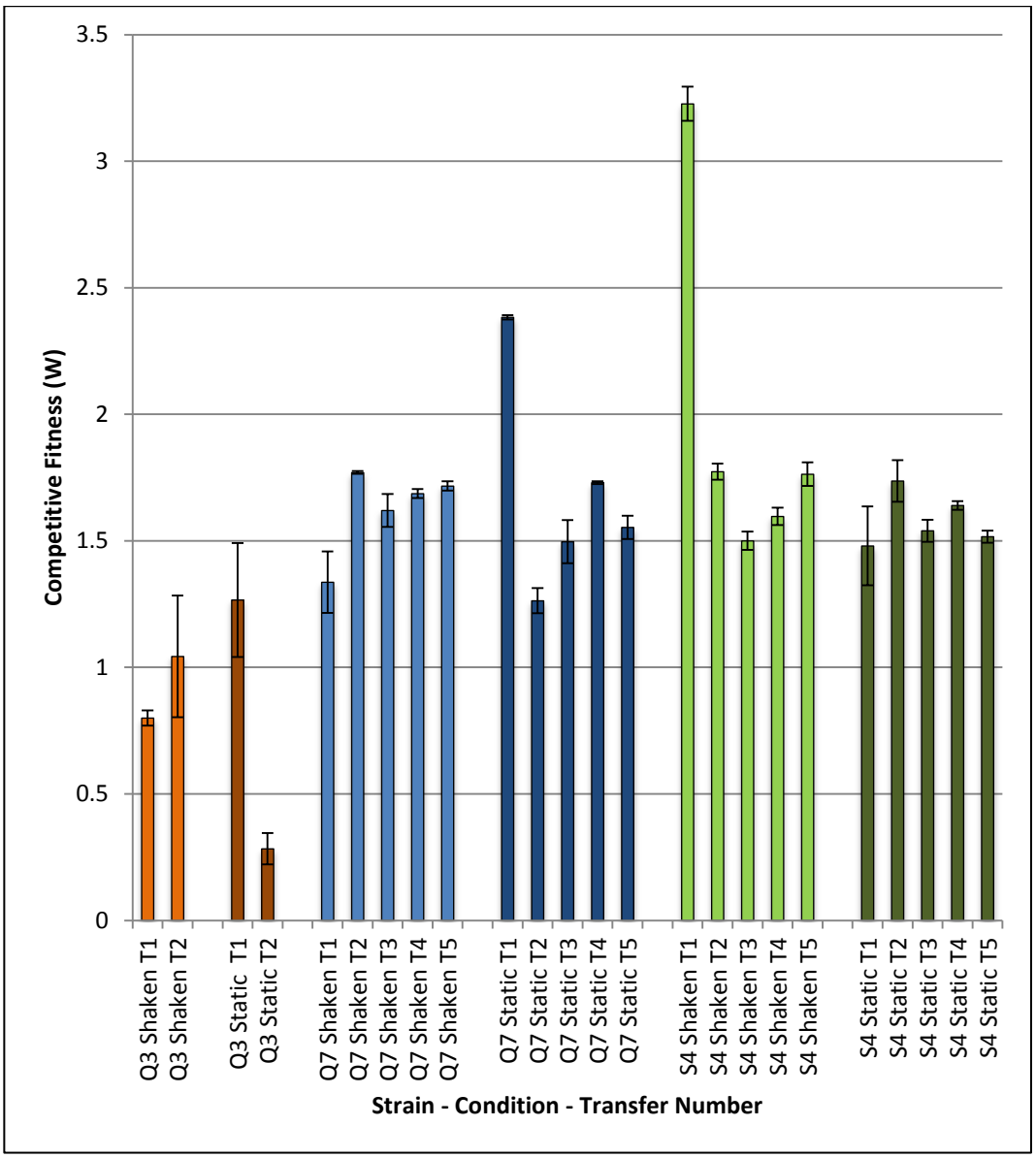


Figure 45. Combined bar chart of competitive fitness (W) (Means ± SEM) of three chosen bacilli strains incubated in their respective conditions with VP_{EMS} over five transfers (two transfers for strain Q3).

Weekly transfers of the three chosen bacilli strains were also carried out in the absence of VP_{EMS}. The growth of each strain was noted as Ratio (R) and plotted as a combined bar chart in Figure 46 below.

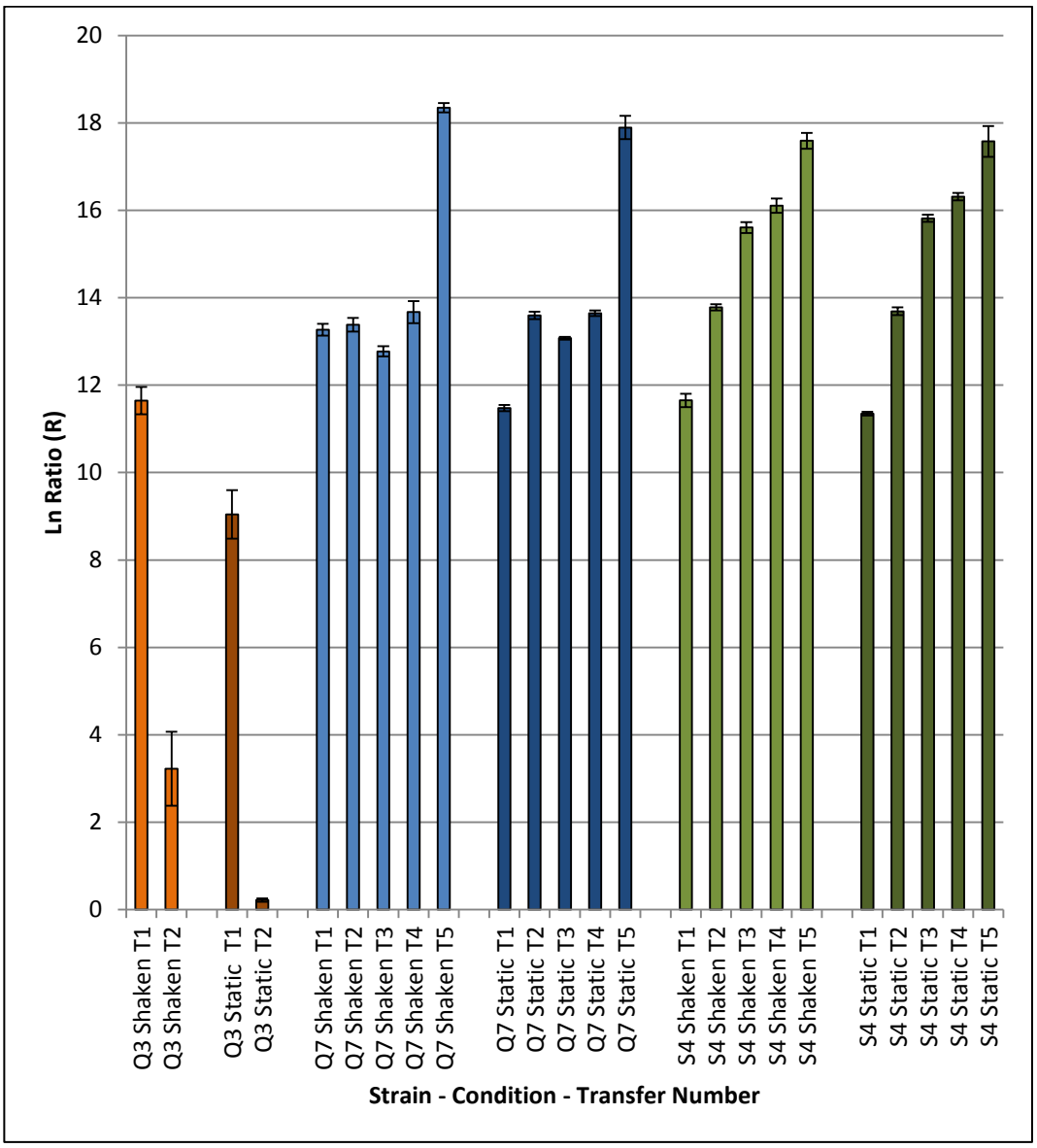


Figure 46. Combined bar chart of ln ratio (R) (Means ± SEM) of three chosen bacilli strains incubated in their respective conditions without VP_{EMS} over five transfers (two transfers for strain Q3).

As observed in Figure 46, the improved performance of strains Q7 and S4 is due to the adaptation to media over time without any interference.

After the second transfer, all post incubation plates show that Q3 is no longer viable (causing drop in Ratio as well as Fitness); this suggests that the earlier observations that Q3 performed well in KB was not sustained throughout the duration of study. While the exact cause for Q3's sudden drop in performance warrants further investigation, it is clear that

further coevolution work on Q3 will be redundant as its erratic behaviour *in vitro* would only lead to more problems even if successfully used as a biocontrol agent.

A bar chart of mean competitive fitness for each strain under each condition across the transfers was plotted for better strain-specific comparison (Figure 47). Competitive fitness across the transfers for strains Q7 and S4 were found to be significantly different as compared to Q3 (Tukey-Kramer HSD, $q^*=2.935$, $\alpha=0.05$).

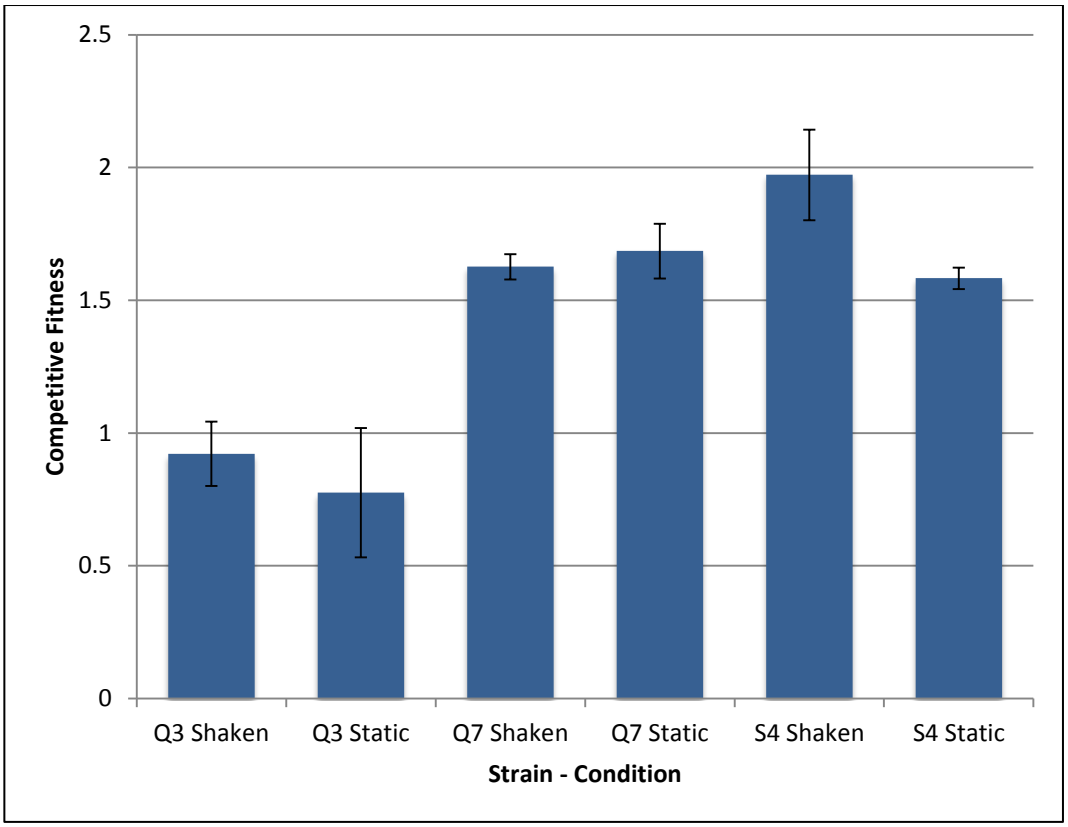


Figure 47. Bar chart comparing the three bacilli strains and their mean competitive fitness (Means ± SEM) against VP_{EMS} across five transfers.

Figure 47 suggests that S4 and Q7 have fitness advantages over VP_{EMS}, competitive fitness ranged from 1.583 ± 0.04 to 1.972 ± 0.2. In order to test the significance of this claim an independent-samples t-test was carried out. Results of the t-test are summarized in Table 26 below.

Table 26. Independent-samples t-test results for three tested bacilli strains and their competitive fitness across the five transfers against VP_{EMS} (Hypothesized value at 1).

Test Mean	Q3 Shaken	Q3 Static	Q7 Shaken	Q7 Static	S4 Shaken	S4 Static
Actual estimate	0.922	0.775	1.626	1.685	1.972	1.583
DF	5	5	14	14	14	14
Test statistic	-0.646	-0.924	13.255	6.646	5.692	14.604
p-value	0.547	0.398	<0.0001	<0.0001	<0.0001	<0.0001

Independent-samples t-test carried out hinted that Q3 cell count is at a disadvantage to VP_{EMS} (indicated by actual estimate value being larger than hypothesized value). It is inconclusive whether VP_{EMS} is significantly out-competing Q3 ($p<0.05$). However, strains Q7 and S4 indicated having significant fitness advantages over VP_{EMS} ($p<0.05$).

This demonstrates that strains Q7 and S4 were co-evolved strains with significant advantages over VP_{EMS} (i.e. $W>1$) regardless of the conditions in which they were grown together ($p<0.0001$). However, Q3 did not survive past the second transfer and its overall fitness was poor (i.e. $W\leq1$).

4.3.2 Post-evolution assays

Contrary to standard behaviour when shaken, a thick layer of biofilm was observed to have developed on the meniscus regions of microcosms for strains Q7 and S4 incubated under shaking conditions (200 rpm, see Figure 48). This occurred after the fourth transfer suggesting that these two bacilli strains were found to be responding to disturbance (or oxygen availability) better.

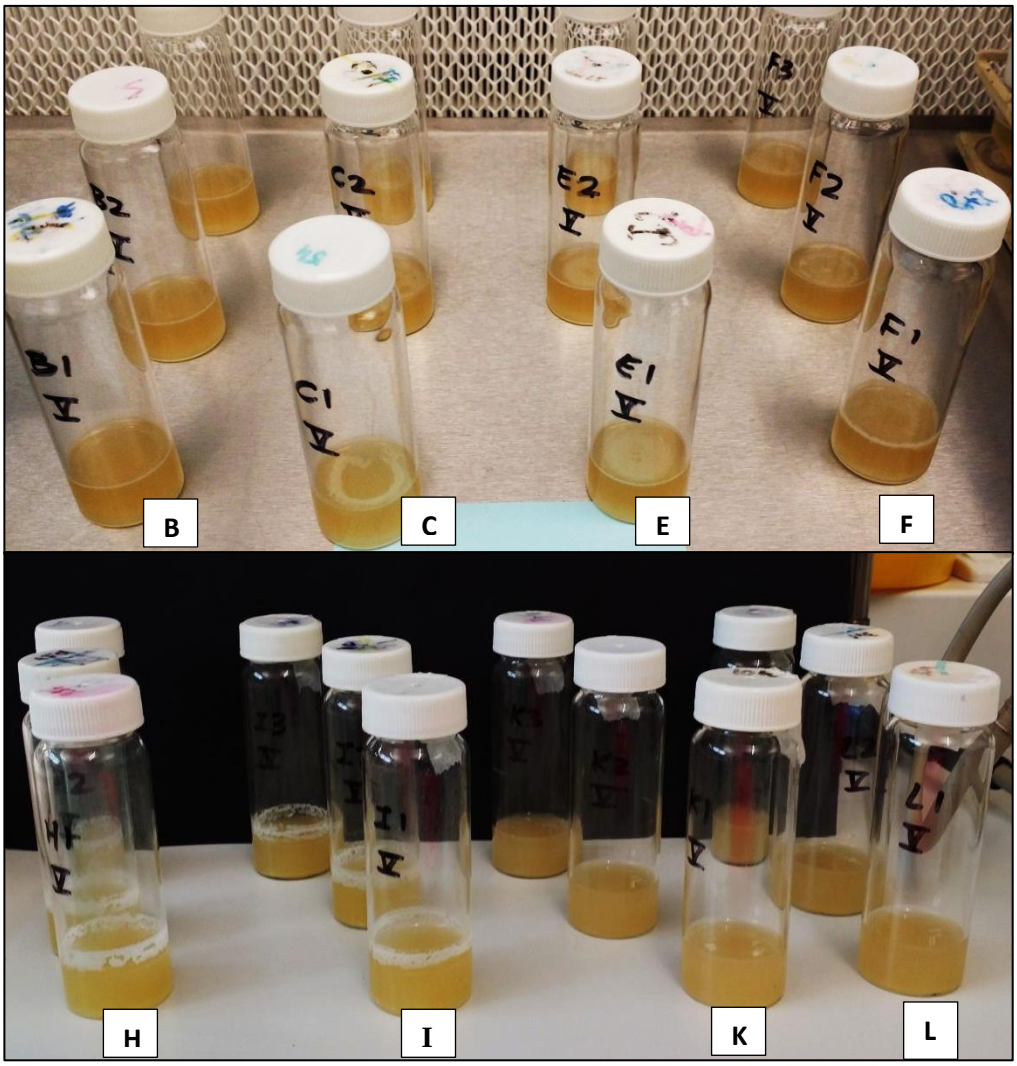


Figure 48. Photographs of comparison between statically coevolved strains (Top photograph) and shaken coevolved strains (Bottom photograph) after four days of incubation at the fifth transfer; (Top): Coevolved strain Q7 (B) and its wild type (E), Coevolved strain S4 (C) and its wild type (F). (Bottom): Coevolved strain Q7 (H) and its wild type (K), Coevolved strain S4 (I) and its wild type (L).

In a shaking condition, oxygen availability was believed to be consistent and high within the liquid microcosms.

This thick biofilm that was formed in spite of the constant disturbance would imply that biofilm was a direct consequence of colonization of most favoured region of the microcosm. It appeared that there was a distinct preference for the physical niche which has: a) high oxygen availability, b) constant availability of nutrients, and c) experiences least disturbances

However in the context of this study, the ideal biocontrol agent is required to thrive in a niche of low oxygen availability. Therefore, growths of all successfully coevolved strains need to be tested at low oxygen levels at microaerobic or anaerobic conditions.

Following up on the coevolution study previously, oxygen stress responses were evaluated, followed by antagonistic assays against VP_{EMS} and anti-quorum sensing assays were carried out to compare coevolved strains against the wild type strains.

4.3.2.1 Growth of coevolved bacilli in aerobic and microaerobic environments

Growth of coevolved bacilli was measured as relative change of final over initial OD_{600} readings. One-way ANOVA and a means comparison with a control via Dunnett's Method was carried out to test significance of relative bacilli growth as compared to that of VP_{EMS} under experimental conditions.

It was found that the relative growth of strains that were coevolved under the shaking conditions were neither comparable to that of VP_{EMS} nor their corresponding wild types. Under microaerobic conditions, all bacilli strains (including wild types) were found to not significantly differ from that of VP_{EMS} (ANOVA, $p=0.116$; $|d|=2.935$, $\alpha=0.05$), whereas in aerobic conditions, all bacilli strains (including wild types) were found to significantly differ from that of VP_{EMS} (ANOVA, $p=0.0028$; $|d|=2.935$, $\alpha=0.05$). This implies that there are no significant changes made to bacilli strains coevolved under shaking conditions.

However, bacilli strains that were coevolved under static conditions responded differently in terms of relative growth in both microaerobic and aerobic conditions.

Figure 49 compares VP_{EMS} to both coevolved and wild types of bacilli strains Q7 and S4 in a simulated microaerobic condition.

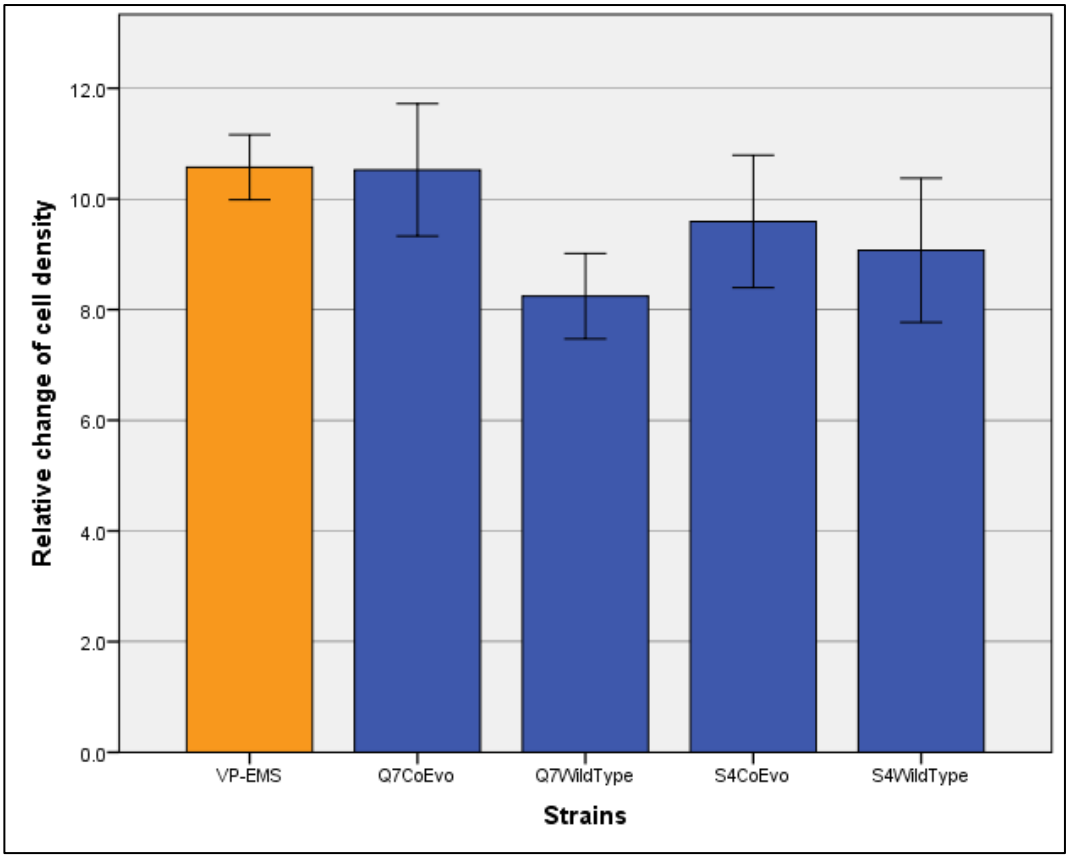


Figure 49. Relative change of bacteria cell density as measured by OD_{600} (Mean ± SEM) compares two successfully co-evolved strains under static conditions (Q7 & S4) with their wild types, and contrasted against VP_{EMS}, when all five were grown under microaerobic conditions.

Both visual observation of bar chart in Figure 49 and one-way ANOVA conducted determined that coevolved strains were found to cope better than wild types in microaerobic condition (ANOVA, p=0.0471). A means comparisons by applying Dunnett's method concluded that growth of coevolved strains under microaerobic conditions were comparable to that of VP_{EMS} ($|d|=2.890$, $\alpha=0.05$).

A separate comparison of relative change between the bacilli strains and VP_{EMS} was made under aerobic conditions. One-way ANOVA (p=0.307) and Dunnett's test ($|d|=2.890$, $\alpha=0.05$) conducted determined that both coevolved and wild type bacilli strains did not differ significantly from VP_{EMS}.

This suggests that bacilli strains, commonly described as aerobic bacteria, have gained enhanced ability to survive in microaerobic conditions after undergoing coevolution under static conditions, hence able to compete with VP_{EMS}, which are well known facultative anaerobes.

Bacilli strains that were coevolved under shaking conditions were consistently exposed to disturbance as a form of stress. The thick liquid-solid interface biofilms produced by the strains were suggested to be a way of them overcoming this stress, implying that physicochemical stress is more significant than that of biotic stress.

In order to adapt and survive in stressful environments, their normal metabolic functions would be affected. This is a form of directed selection for strains that can thrive under disturbed conditions as well as compete with the VP, as greater expenditure of nutrients would be required to overcome the stresses which bacilli strains were exposed to. Over time, the bacilli strains coevolved under shaking conditions might adapt better upon selection and proliferation of beneficial mutants which can tolerate and overcome the stress due to disturbance. It is further expected that these strains will further evolve due to presence of VP_{EMS} in favour of the motion-tolerant trait as well as being competitively superior to *V. parahaemolyticus*.

In contrast, bacilli strains that were coevolved under static conditions were only subjected to competition with VP. The lack of disturbance as a stress factor may have facilitated in faster mutation or adaptation to VP. Moreover in a static condition, within 5 - 10 mm from the surface of the liquid to solid interface, the oxygen concentration drops quickly (oxygen gradient), hence the better adaptation to compete with VP for niche colonization in a microaerobic condition.

It was hypothesised that the conditions in shrimp gastrointestinal tract will be oxygen deficient. The above test has shown that our potential probiotic bacilli should be able to keep pace with growth of VP_{EMS} in an environment with stress of low oxygen, the probability that *in vivo* survivability of strains in shrimp gut is high.

Therefore, it can be concluded that both bacilli strains coevolved under static conditions are able to compete with VP_{EMS} to get to their niches in either microaerobic or aerobic conditions and will be used to assess improvements in antagonistic activity.

4.3.2.2 Quorum quenching potential of coevolved bacilli

As can be observed in Figure 50, both Q7 wild type and coevolved strain showed no significant difference in their abilities (t-test, p=0.150). However, coevolved S4 has improved quorum quenching abilities as compared to its wild type. A t-test conducted confirmed significant improvement in quorum quenching abilities (p<0.0001) for coevolved S4.

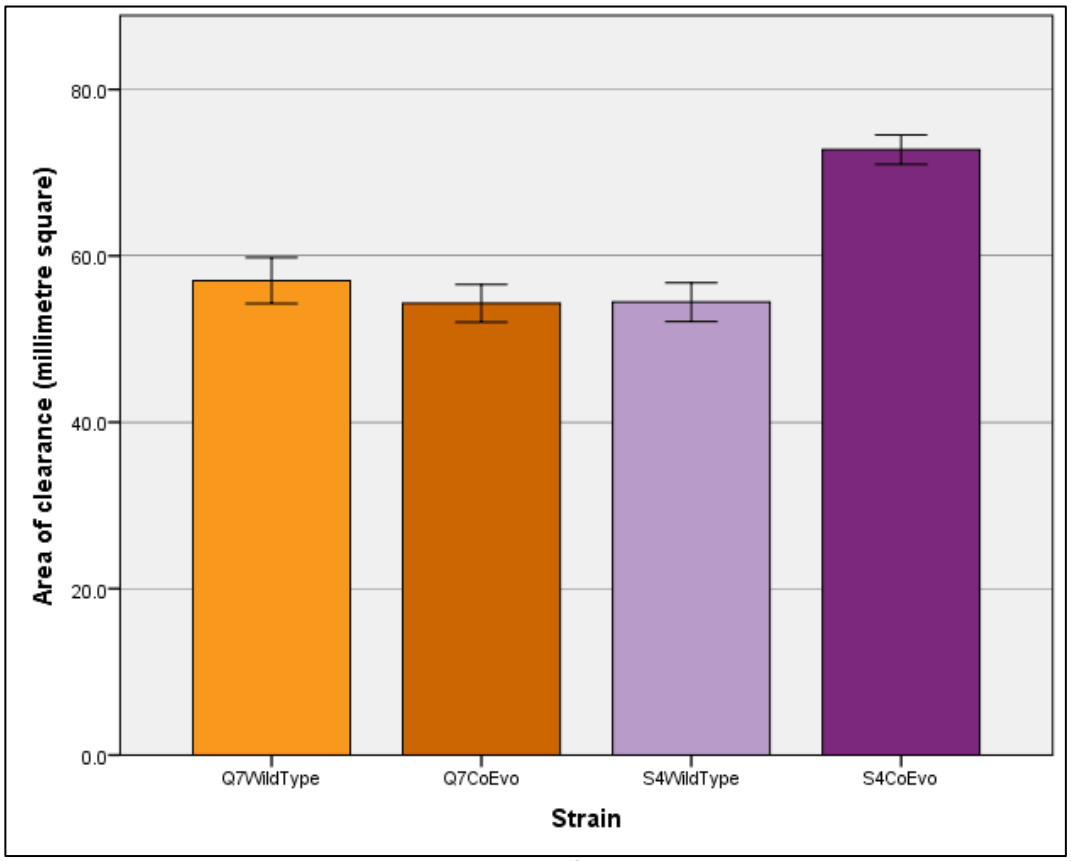


Figure 50. Bar chart comparison of area of clearance (mm^2) (Mean ± SEM) exhibited by two coevolved bacilli strains and their wild types against quorum quenching indicator bacteria, *C. violaceum*.

4.3.2.3 Anti-VP_{EMS} activity of coevolved bacilli

Significant inhibition of VP_{EMS} was observed by successfully coevolved strains Q7 ($p<0.0001$) and S4 ($p=0.0002$), yielding an improvement by 49.0% and 65.9% respectively as compared to their respective wild types. Comparison of inhibitory effects when wild type was used is shown in Figure 51, proving that co-cultivation did bring about enhancement in VP_{EMS} antibiosis.

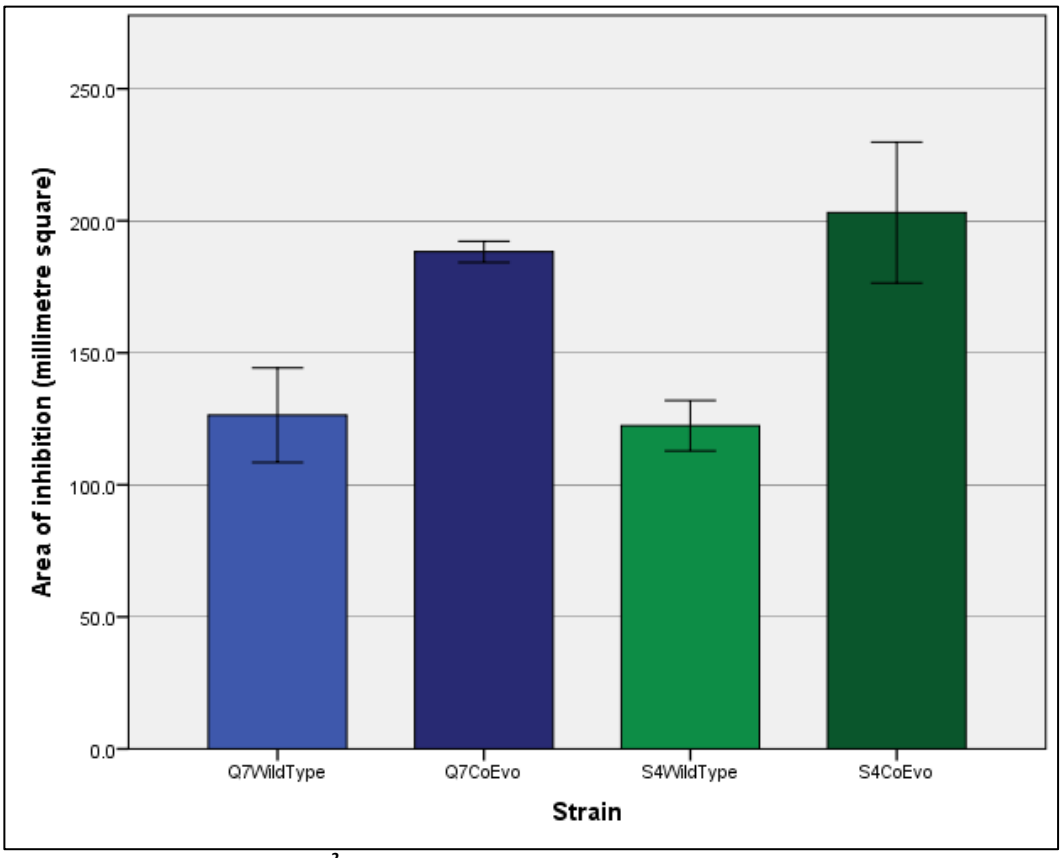


Figure 51. Area of inhibition (mm^2) (Mean ± SEM) comparison of successfully coevolved strains and their wild type against VP_{EMS}.

4.4 General Summary

Current methods used by the shrimp aquaculture industry for control and prevention of EMS involve direct chemotherapy using competitive inhibitors of the aromatic class to disrupt extracellular quorum signalling between VP cells or excess production of AHL-lactonases via heterologous gene expression at an industrial scale. These direct methods of inhibiting quorum sensing could potentially lead to pathogenic VP adapting against these compounds. There will always be a risk of emergence of resistant populations of pathogens which could easily render current generation of anti-quorum sensing compounds ineffective.

Coevolution as a method for strain improvement allows for reciprocal coevolution of the selected biocontrol agents against the pathogenic wild type VP. Therefore, this method may provide a long term solution to EMS with less likelihood of VP_{EMS} developing resistance.

Naturally occurring coevolution is typically viewed as a problem due to continual adaptations of pathogens to better infect hosts. Only in recent times, more attention has been given to applying coevolution on an *in vitro* level to generate a superior organism which has adapted to the biotic stress (Charusanti *et al*. 2012). This study was designed based on the premise that wild type bacilli strains which possess desired traits could only get better upon repetitive exposure against a rival VP_{EMS} strain to the point that a significant improvement between coevolved and wild type strains may be observed.

Survey of recent literature showed that coevolution studies were almost always in host-pathogen interaction research (Thrall *et al*. 2012, Brunner *et al*. 2013, Karasov *et al*. 2014, Tian *et al*. 2015). A recent study by Masri *et al*. (2015) describes reciprocal coevolution as a tool for the continuous expression of bacterial toxin against a nematode host. There has not been any body of work detailing the use of co-cultivation to improve existing bacteria strains in the context of shrimp aquaculture diseases.

Asides plate-based assays, there may be other ways to visually assess the effects of antibiosis resulting from competing species, such as a quantitative liquid-based assay.

5. Chapter 4: Assessment of anti-VP activity of coevolved bacilli strains in liquid medium using a GFP-tagged *V. parahaemolyticus* constructed via triparental conjugation

5.1 Introduction

The construction of a stable and expressing GFP-tagged *Vibrio parahaemolyticus* strain was carried out via triparental conjugation technique for the purpose of improved *in vitro* visualization of VP, which may also be applied in future *in vivo* studies. Upon successful tagging of VP, the fluorescent transformant was then co-cultured with both coevolved bacilli strains and their respective wild types in order to quantitatively assess the effects of antibiosis resulting from competing species. Flowchart in Figure 52 summarizes the work carried out for this chapter.

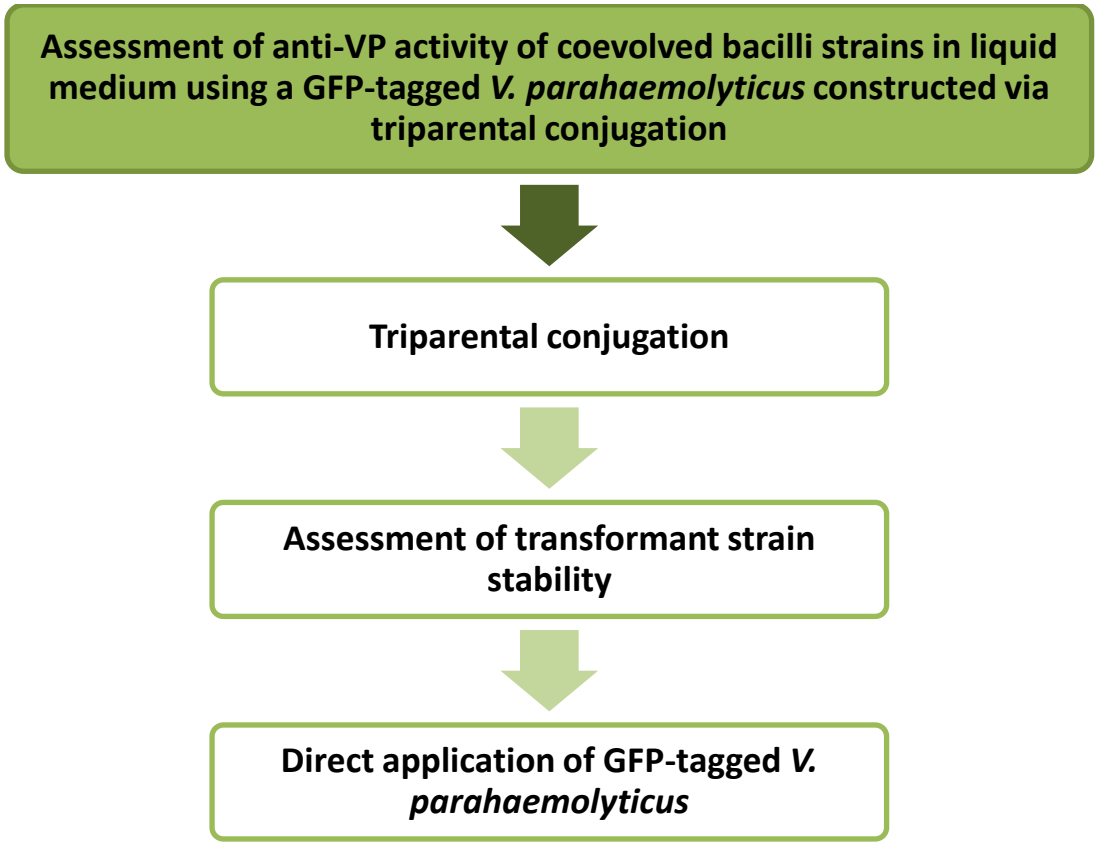


Figure 52. Flowchart which summaries the work done in this Chapter 4.

5.2 Methodology

5.2.1 Triparental conjugation

Triparental mating or conjugation is as a conjugal horizontal gene transfer process, involving three bacterial strains, i.e. donor strain (contains mobilisable plasmid of interest), helper strain (contains a self-transmissible conjugative plasmid) and recipient strain (Stabb and Ruby 2002). This study aims to generate a stable recipient strain (VP_{EMS}) which expresses the green fluorescent protein gene readily for better in vivo or in vitro visualization. The donor strain, *E. coli* DH5α-pVSV102 lacks the ability to initiate self-transfer of GFP-expressing plasmid into said recipient strain, and therefore require the help of the conjugative plasmid of the helper strain, *E. coli* λpir-pEVS104.

Triparental conjugation consists generally of a two-main steps process, as described previously by Trevors *et al*. (1987) and Shaw CH (1995). After the first conjugation process between the donor and helper strains, the donor strain will bear both the self-transmissible plasmid as well as the mobilisable plasmid of interest. Recipient strain will then be able to receive the plasmid of interest from the donor strain during the second conjugation process via the self-transmissible plasmid facilitating plasmid of interest mobilisation.

5.2.1.1 Strains used in experiment

***Escherichia coli* DH5α-pVSV102** - Donor strain, *Escherichia coli* DH5α-pVSV102, was obtained from Professor Eric Stabb from the University of Georgia, USA. This strain contains the GFP-expression/kanamycin-resistance plasmid pVSV102. 'VSV' is short for *Vibrio* shuttle vector, indicating that the plasmid is suitable for *Vibrio* recipients and expression by recipients (Dunn *et al*. 2006).

***Escherichia coli* CC118 λpir-pEVS104** - Helper strain, *Escherichia coli* CC118 λpir-pEVS104, was obtained from Professor Eric Stabb from the University of Georgia, USA. This strain carries the conjugative helper/kanamycin-resistance plasmid pEVS104.

***V. parahaemolyticus* ATCC and EMS strains** - A standard *Vibrio parahaemolyticus* strain DSM 10027 (VP_{ATCC}) was obtained from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DSMZ).

Virulent strain VP_{EMS} was previously described in Section 2.2.2.1.

Both strains of *V. parahaemolyticus*, VP_{ATCC} and VP_{EMS} were used in this study (as recipient strains) as a comparison to whether a laboratory strain is able to take-up, express and retain the GFP plasmid as compared to a wild type strain. Both strains were revived from -80°C glycerol stocks into saline Luria Bertani (sLB) media (components of media listed in Table 27 below).

5.2.1.2 Media used in experiment

Table 27. Composition of saline Luria Bertani (sLB) media in grams/L.

Composition	Grams/Litre (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	25.0
Final pH 7.5 at 25°C	

Table 28. Composition of Kanamycin-supplemented thiosulphate citrate bile salt (Kn-TCBS) media in grams/L.

Composition	Grams/Litre (g/L)
Proteose peptone	10.0
Yeast extract	5.0
Sodium thiosulphate	10.0
Sodium citrate	10.0
Oxgall	8.0
Sucrose	20.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Bacteriological agar	15.0
Kanamycin	0.1
Final pH 8.6 at 25°C	

Luria Bertani with 4.0% (w/v) NaCl (LB-NaCl) media used was previously described in Table 16, Section 3.2.5.1.

5.2.1.3 Experimental protocol

Overnight LB cultures of donor and helper strains were also prepared. Triparental conjugation was carried out via an agar-based method after Wise *et al*. (2006). The donor and helper strains were first streaked diagonally across an LB agar plate to form an 'X'. The recipient strain would then be smeared across the 'X'. Plates were incubated overnight (30°C) before picking up colonies from the regions of overlap (dubbed the mating zone), as highlighted by the red box in Figure 53 below. Colonies picked out from the regions of overlap were then streaked onto selective plates of Kanamycin-supplemented thiosulphate citrate bile salt (Kn-TCBS) agar (media components listed in Table 28 above) and incubated (24 hours, 30°C).

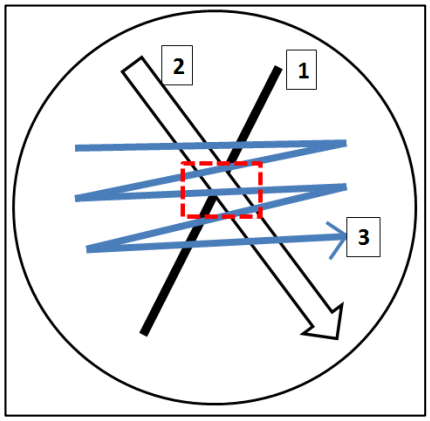


Figure 53. Triparental conjugation agar-based procedure; 1. Donor strain, 2. Helper strain, 3. Recipient strain. Red box region highlights the mating zone, whereby colonies were picked up and transferred to selective media after incubation.

5.2.1.4 UV visualization of transformed *V. parahaemolyticus*

Colonies that were able to grow on selective media Kn-TCBS plates were transferred onto LB-NaCl agar plates (Table 16) and incubated (24 hours, 30°C) before observing for fluorescent colonies under UV light via a UV trans-illuminator at 365 nm.

5.2.2 Assessment of transformant strain stability

Successfully transformed VP_{ATCC} and VP_{EMS} were assessed for their ability to retain and express the GFP plasmid. This was achieved by monitoring the level of fluorescence over an approximate 45 generations via a Cary Eclipse fluorescence spectrophotometer (excitation wavelength: 473 nm; emission wavelength peak: 508 nm). Optical density of transformants was measured at 600 nm (OD_{600}) using spectrophotometer in Absorbance mode.

5.2.2.1 Ratio of fluorescence intensity over cell density measurements

Overnight cultures of transformants were subjected to measurements of fluorescence intensity and optical density and the ratio was obtained for each day over 10 days, using the following equation:

$$Ratio = \frac{Fluorescence\ intensity\ (AU)}{Optical\ density\ (AU)}$$

5.2.2.2 Percentage retention of fluorescence over time

$$Percentage\ of\ fluorescence\ (\%) = \frac{Ratio\ obtained\ on\ day\ X}{Ratio\ obtained\ on\ day\ 1} \times 100$$

5.2.3 Direct application of GFP-tagged *V. parahaemolyticus*

5.2.3.1 Comparison of anti-VP_{EMS} activity of coevolved bacilli

Actively growing VP-GFP was inoculated together with coevolved bacilli strains and their wild type counterparts. Equivalent volumes (100 µL) of VP-GFP and bacilli were mixed together in a fresh NB liquid microcosm (10 mL). Negative control of a 100 µL inoculum of the same VP-GFP culture was prepared. All inoculated microcosms were incubated (24 hours, 30°C) under shaken conditions as a simulation of their most likely interaction in the external environment.

Post incubation the fluorescence intensity in each microcosm was assessed. A Cary-Eclipse fluorospectrometer was used, excitation wavelength was 473 nm; emission spectrum was obtained for the range of 480 nm to 550 nm with emission maxima observed consistently at 507-508 nm.

From this experiment, it was expected that VP-GFP grown alone will yield a high emission peak, whereas in the presence of bacilli, a significantly lower emission peak may be observed.

5.2.4 Statistical analyses and modelling

All experiments and assays were conducted in replicates as stated, and the means ± SEM provided where necessary. JMP Statistical Discovery Software (JMP v12, SAS Institute Inc., USA), and Microsoft Excel (version 2010) were used to analyse all data obtained.

Differences between means were tested by ANOVA (*F*) and *post hoc* multiple mean comparison tests including Tukey-Kramer HSD (TK-HSD, *q**) tests were carried out. A paired-samples t-test (*t*) was used to determine whether bacilli strains differed from one another.

5.3 Results and Discussion

Two reporter strains were constructed by tagging both the VP_{EMS} and VP_{ATCC} strains with GFP in order to facilitate fluorescent-based visualization of *V. parahaemolyticus*. VP_{ATCC} was used as a standard strain for comparison purposes in this experiment.

5.3.1 Triparental conjugation

Based on Figure 54 below, the wild type VP strains neither grow nor fluoresce under UV light at 365 nm, whereas transformed both VP_{ATCC} and VP_{EMS} do. This visual observation proves that the transformed VP strains have successfully taken up the expression plasmid with the GFP gene, resulting in fluorescent colonies.

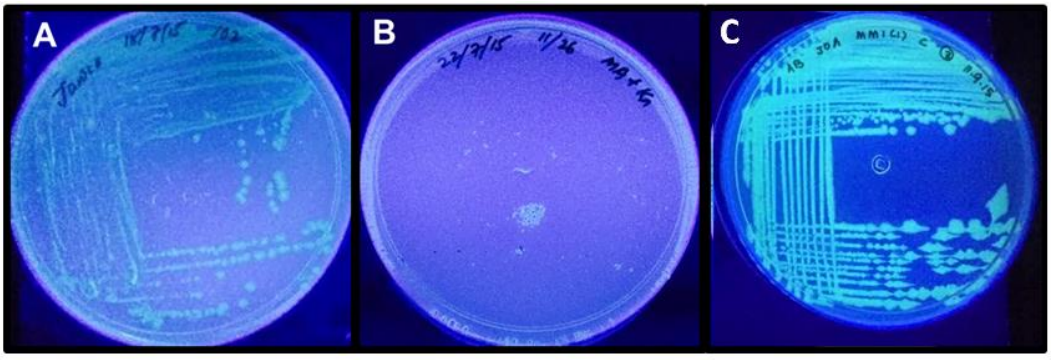


Figure 54. UV visualization of post triparental conjugation on marine agar plates supplemented with 0.1 mg/mL Kanamycin. A: *E. coli* harbouring pVSV102 (GFP-Kan^R); B: Wild type V. parahaemolyticus does not grow in presence of kanamycin; C: *V. parahaemolyticus* post-transformation with pVSV102 acquires GFP and kanamycin resistance.

Pair-wise combinations were set up as negative controls in order to verify that GFP-expressing *Vibrio* strains were produced only when three different bacteria were grown together (i.e. donor, helper and recipient).

GFP was chosen as a suitable detectable marker because it neither requires any external substrates for fluorescence activation nor does it affect the growth of any recipient strains (Vinoj *et al*. 2014). Several studies have utilized GFP-tagged *Vibrio*s for an in depth study of their interactions with marine animals, such as *Vibrio aestuarianus* in Pacific oyster infection (Aboubaker *et al*. 2013), *Vibrio harveyi* in abalone infection (Travers *et al*. 2008), and also *Vibrio fischeri* as the symbiotic partner of the Hawaiian bobtail squid (Lupp *et al*. 2003).

A survey of recent literature showed other techniques which used GFP-tagging, e.g. a recent study being chromosomal GFP labelling of *Vibrio coralliilyticus* for the visualization and tracking of coral pathogens (Pollock *et al*. 2015). However, this triparental conjugation technique was applied as it allows a rapid and inexpensive method of fluorescent tagging a specific bacterium without harming its growth or pathogenicity.

5.3.2 Assessment of transformant strain stability

The ability to retain and express the GFP plasmid was assessed by monitoring fluorescence intensity in corresponds to the OD_{600} of both strains over a period of ten days. Contrary to the standard practice of applying antibiotics to maintain plasmid retention, kanamycin was not added to the liquid microcosms during this experiment as a simulation of actual *in vitro* application of fluorescent VP (microcosms not containing kanamycin). Furthermore, the GFP-tagged strains must be able to retain stability during *in situ* testing.

Based on Figures 55 and 56 below, both GFP-tagged VP strains generated via triparental conjugation were observed to be stable as percentage of fluorescence did not fall below a retention threshold of 50% over approximately 45 generations. This retention threshold was set as a criterion in the event that *in vivo* visualization was required even after ten days without having to maintain GFP-tagged strains in a liquid microcosm containing kanamycin.

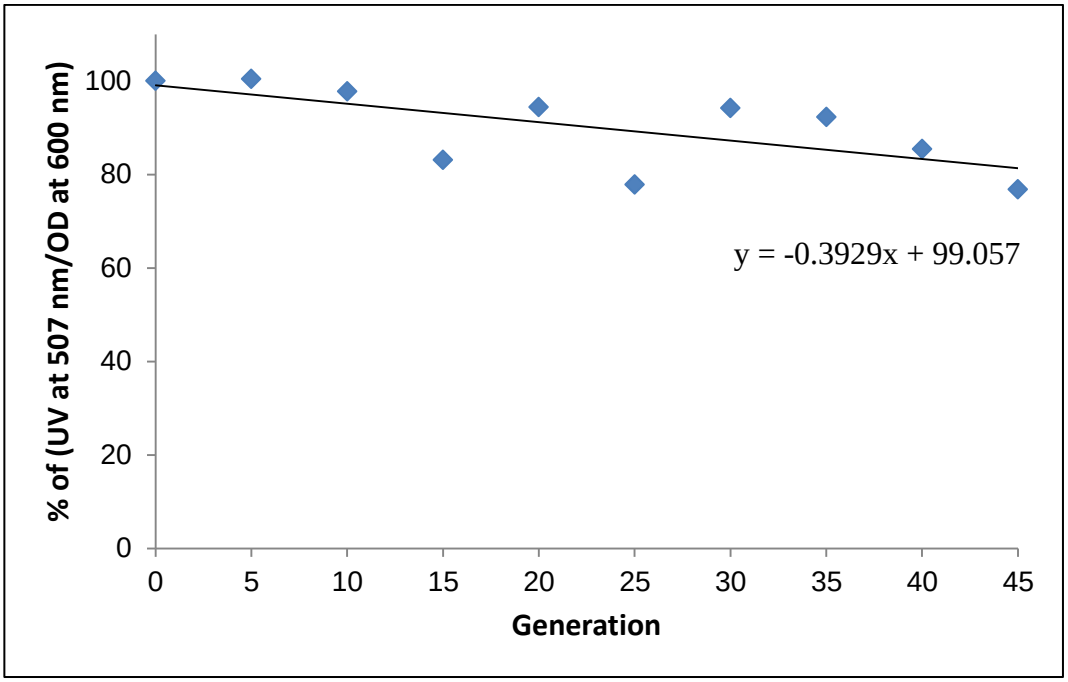


Figure 55. The ability to retain and express strain fluorescence of GFP-tagged VP_{ATCC} over 10 days (approx.45 generations).

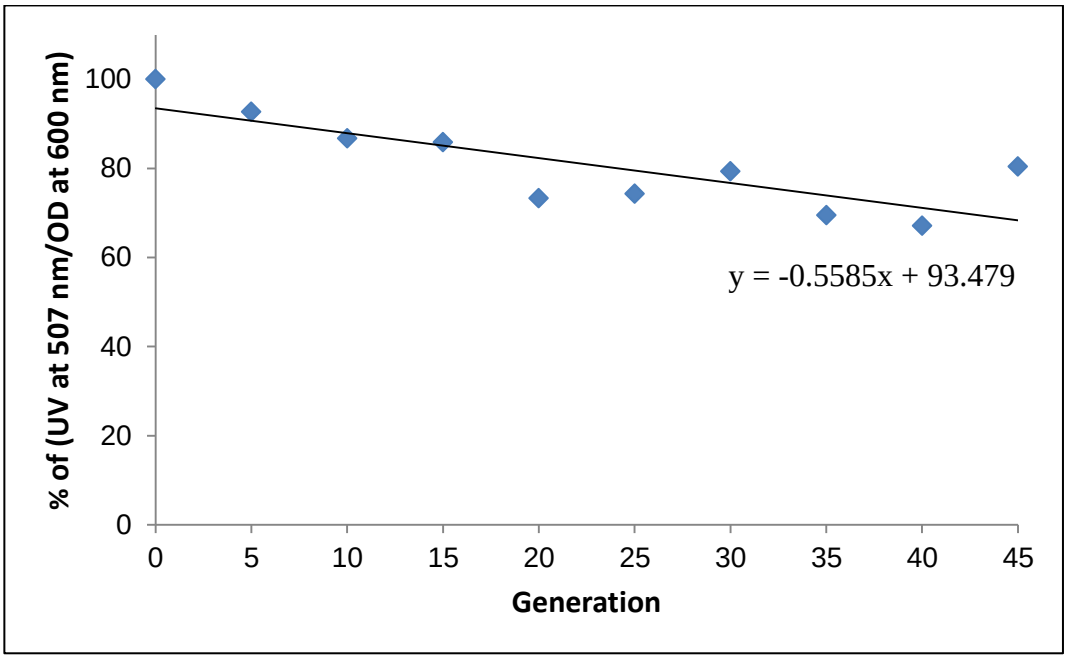


Figure 56. The ability to retain and express strain fluorescence of GFP-tagged VP_{EMS} over 10 days (approx.45 generations).

Strain VP_{ATCC} was able to maintain strain fluorescence of up to 77%, whereas VP_{EMS} maintained strain fluorescence of up to 67%. VP_{ATCC} was observed to be able to retain GFP-plasmid better, hence a more stable strain as compared to VP_{EMS} (indicated by steeper gradient of VP_{EMS}).

5.3.3 Direct application of GFP-tagged *V. parahaemolyticus*

GFP-tagged VP_{EMS} gives off a peak at 508 nm at an intensity of 201.477 a.u. (arbitrary units) as detected by Cary Eclipse fluorometer (see Figure 57 below) in overnight liquid microcosm. We explore the possibility of directly applying this GFP-tagged VP_{EMS} as a test organism to be used to assess anti-*Vibrio* activity in an *in vitro* liquid-based assay.

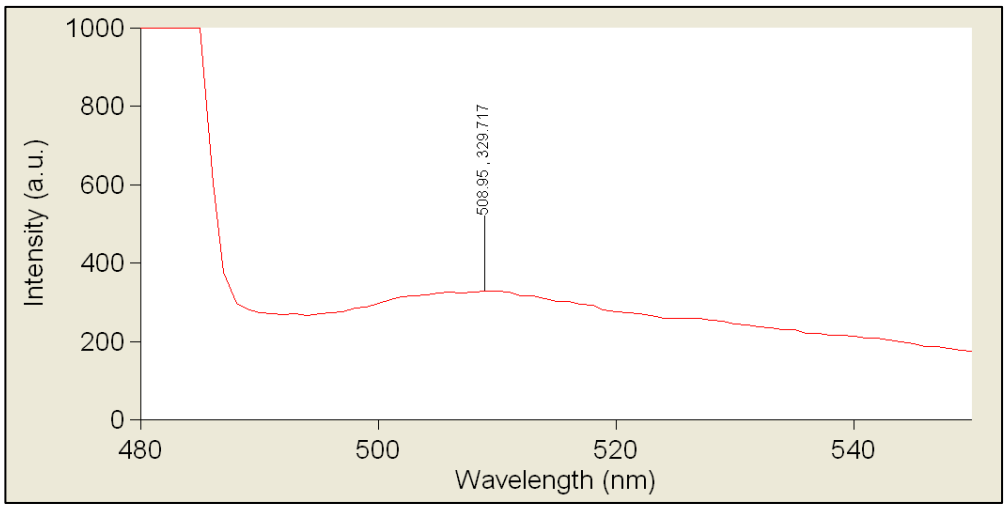


Figure 57. Print screen image of detection of peak at 508 nm by fluorometer in overnight liquid microcosm inoculated with GFP-tagged VP_{EMS}. Detection of peak threshold was set to 50 by default.

In this particularly study, we tested the efficacy of this assay by applying two bacilli strains (Q7 and S4) that were successfully coevolved to further enhance anti-*Vibrio* activity (see Section 4.3.2.3) and compared to their wild types (pre-evolved forms).

When either bacilli strains were co-cultured or present in liquid microcosms with the GFP-tagged VP_{EMS}, the fluorescence intensity was detected at a peak threshold of below 50; i.e. peak threshold of 25 for co-culture with wild type Q7, peak threshold of 12.5 for co-culture with coevolved Q7 and wild type S4, and peak threshold of 6.25 for co-culture with coevolved S4. This was directly compared to GFP-tagged VP_{EMS} which was inoculated with pure liquid microcosm in place of bacilli inoculum (peak threshold of 50). Relative efficacy was assessed by comparing ability to reduce fluorescence intensity (at 508 nm) of GFP-tagged VP_{EMS} at set peak threshold.

Raw data of fluorescence intensity to detection wavelengths were corrected for background. Line graphs constructed below demonstrate the fluorescence intensity detected via the fluorometer when GFP-tagged VP_{EMS} were co-cultured overnight with Q7 (see Figure 58) and S4 (see Figure 59) respectively.

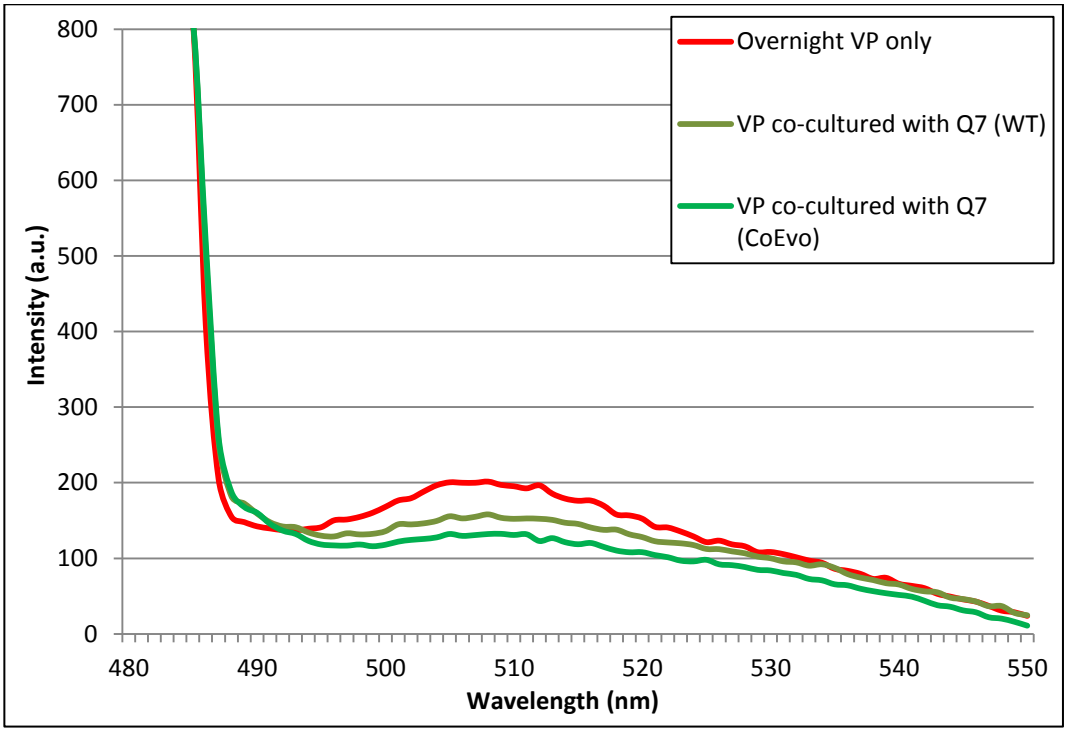


Figure 58. Line graph showing decrease in fluorescence intensity of GFP-tagged VP_{EMS} when co-cultured with coevolved Q7 and its wild type respectively.

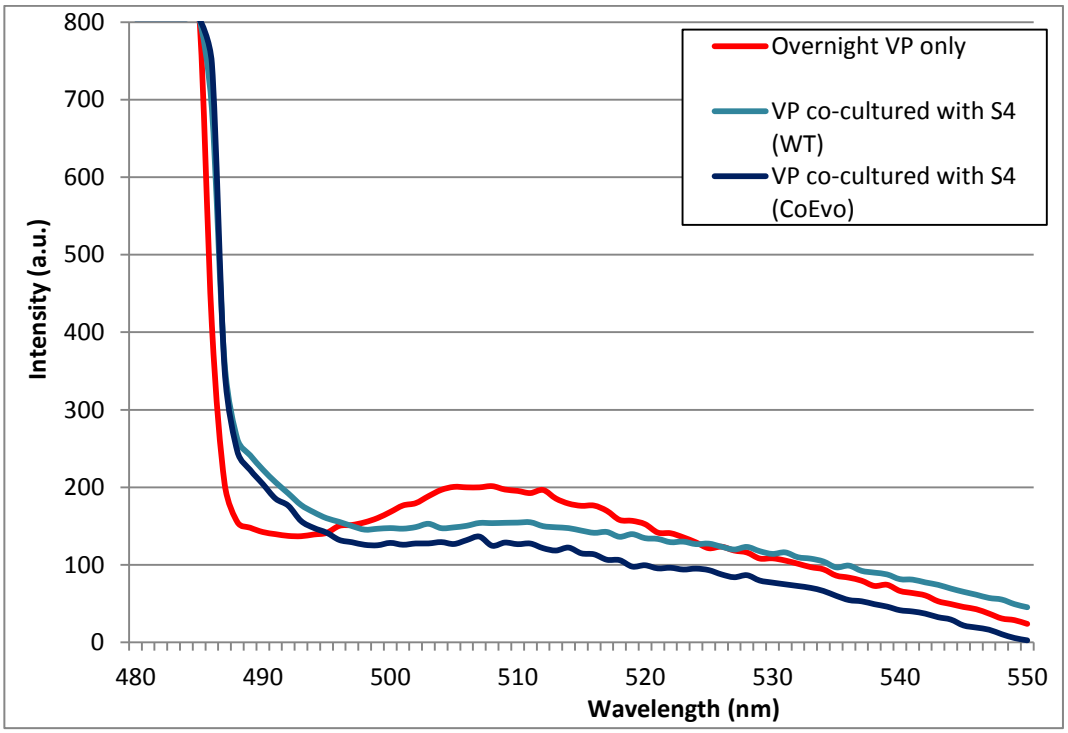


Figure 59. Line graph showing decrease in fluorescence intensity of GFP-tagged VP_{EMS} when co-cultured with coevolved S4 and its wild type respectively.

Means comparison via one-way ANOVA proved significant differences ($p<0.0001$), and *post hoc* analysis Tukey-Kramer HSD test revealed that fluorescence intensity of VP_{EMS} was indeed significantly different as compared to the two bacilli strains ($q^*=4.012$, $\alpha=0.05$). Paired-samples *t*-tests were carried out to compare means equal between the wild types and their coevolved forms when co-cultured with VP_{EMS}. One-tailed t-test ($\alpha=0.05$) carried out determined the following results: (a) co-culture with coevolved Q7 emitted a significantly lower fluorescence intensity compared to with Q7 wild type ($p=0.0047$), and (b) co-culture with coevolved S4 emitted a significantly lower fluorescence intensity compared to with S4 wild type ($p=0.0043$).

The wild types of both strain were not significantly different from each other in terms of lowering the fluorescence intensity ($p=0.155$). A similar result was observed when the pair of coevolved strains were compared to each other ($p=0.189$).

Overnight bacilli liquid microcosms without the presence of VP_{EMS} were also scanned as negative control for rejection of potentially interfering fluorescence peaks. No significant peaks were detected near the GFP emission wavelength of 508 nm. However, there was a slight naturally occurring, non-fluorescent peak observed at 530 nm when axenic cultures of bacilli Q7 and S4 were assessed.

An assumption was made when carrying out this assay, i.e. peaks detected at 508 nm can be directly correlated to growth of GFP-tagged VP_{EMS} in liquid microcosm without any hindrance. The limitation to this test is that it cannot prove whether the bacilli inhibited VP_{EMS} growth or merely inhibited VP_{EMS} expression of the GFP plasmid. In spite of this limitation, it is assumed that the decrease in fluorescence intensity of GFP-tagged VP_{EMS} is directly related to the inhibition of VP_{EMS} growth.

Regardless of the mechanism of growth inhibition of VP_{EMS}, bacilli were able to limit the growth of VP_{EMS} and hence, prevent VP_{EMS} from achieving the threshold density required for quorum sensing. As a result, VP expression of pathogenicity would be inhibited. It can also be concluded that the presence of the coevolved bacilli strains are most likely able to prevent colonization of host tissue by VP. This assay proves that this GFP-tagged VP strain can be a suitable model organism for liquid-based assay to assess anti-*Vibrio* activity. The application of this model organism can be used as a relatively quick efficacy test against any bioactive or chemical compounds to inhibit VP growth in a liquid microcosm as well as comparing relative efficacy between different compounds.

5.4 General Summary

In order for easy visualisation of pathogenicity *in vitro* and *in vivo*, GFP tagging of *V. parahaemolyticus* strain was carried out. Successful tagging was accomplished using the triparental conjugation method on: **a**) a wild type strain isolated from an affected local shrimp farm, as well as **b**) a standard strain for comparison (ATCC).

Retention and expression of the GFP plasmid was assessed via measuring the fluorescence intensity when subjected to UV irradiation. In the absence of antibiotic selection pressures, transformed ATCC strain retained 77% of initial fluorescence levels, while the EMS-causing strain retained 67%.

The GFP-tagged *V. parahaemolyticus* were then co-cultivated *in vitro* with two verified antagonistic bacilli species to visually assess the effects of antibiosis resulting from competing species.

This GFP-tagged EMS-causing *V. parahaemolyticus* can be applied and developed as a visualisation tool for assessing efficacy of *in vivo* treatments against EMS.

6. Outlook

Most of the findings in this study are based on *in vitro* assessments. We are still a long way off from obtaining a guaranteed effective biocontrol agent for safe introduction into aquaculture ponds. Based on results obtained, some further work can be done to develop the most ideal treatment and prevention of EMS. Further works leading from this project could be divided into two connected parts: a) additional *in vitro* assessments, and b) testing of ideal probiont for *in situ* applications.

In vitro assessments – Biofilm attachments of good potential bacilli were assessed on inorganic surfaces (i.e. glass, polypropylene and polystyrene). It will be interesting to study their attachment levels on the chitinous shell of shrimps. Crystal violet stains the biofilm cells and is easily washed away by water from the three inorganic surfaces studied on so as to not interfere with the attachment tests (background reading). A coated chitin material could be used to explore this idea, e.g. coat glass microcosms with chitinous components or apply chitin alginate beads on column microcosms. Column microcosms were adapted from Udall *et al*. (2015), whereby drip-fed glass bead microcosms were used to test attachment levels of a certain mutant form of *Pseudomonas fluorescens*.

The coevolved strains of Q7 and S4 can be extensively subjected to modified co-cultivation techniques in order to amplify its current anti-VP activity as well as “training up” its quorum quenching ability. It is believed that microcosms used for future coevolution experiments should contain not just VP but also a significant amount of quorum sensing molecules (C6-HSL). The constant exposure to quorum sensing molecules may be necessary to trigger expression of quorum quenching genes on a consistent basis. It is believed that if the techniques used in this study yielded improved anti-VP activity; then theoretically an extension of the technique can greatly enhance abilities for quorum quenching as well.

In vivo applications – Enhanced visualization of inter-species interactions on surfaces using the GFP-tagged VP model should be explored. The possibility of tagging bacilli strains with a distinctly different colour (e.g. red fluorescent protein tagging) should also be considered to visualize their interactions with the VP on such surfaces. If successful, the ratios of green-red intensity could be obtained to give data sets similar to microarray analyses.

The above suggestion allows for visualisation of fluorescent-tagged pathogens and probionts for direct *in situ* applications. The use of a confocal laser scanning microscopy was described in some papers (Godin and Touitou 2004, Yeh *et al*. 2011) to enable quick visualization of infection routes and/or mechanisms. This method could be used in conjunction with cell cytometry for quantification purposes. Changes to *Vibrio* attachment to surfaces due to the presence of bacilli could also be monitored using the said techniques.

Every *in situ* application must be backed up by shrimp mortality test data. This could be carried out via isolated tanks with replicates, whereby the effects of probiotic strain inoculation into water prior to VP infection will be assessed by recording the percentage of shrimp mortalities in each test tank. The set-up for each tank should simulate a typical shrimp pond environment in terms of physicochemical parameters to test environmental effects on the coevolved strains.

There were many noteworthy observations made throughout the period of this study which could potentially be further assessed.

In the coevolution experiment, it was unexpected that the strains from coevolution under shaken conditions responded poorly in microaerobic conditions. However over time, these strains might adapt better upon further directed selection. Theoretically, further transfers would select for motion-tolerant phenotypes of the strains, as well as acquiring competitive superiority against VP. Moreover, the thick biofilms formed on liquid-to-solid interface whilst under shaking conditions were suggested to be a consequence of being physically agitated. If the strains were left to incubate statically and allowed to form air-liquid interface biofilms, there is a possibility that these strains may show improvements in key biofilm properties such as attachment levels when subjected to combined biofilm assays.

Strain Q3 was quite noteworthy throughout this study, not because of its antagonistic abilities, but its distinct climbing phenotype amongst the other bacilli strains tested. Further tests should be conducted to further elucidate the mechanism of its wall-climbing characteristic, and any relevance it may have for survival in the external environment. Further work is also required in order to explain why Q3 was found to increase the liquid surface tension of its growth medium.

Furthermore, Q3 was suspected to have undergone adaptive radiation in BHI microcosm because the time taken for biofilm formation shortened from one week to only three days. This could be caused by stochastic events associated with genetic bottleneck, whereby selection pressures and/or genetic drift resulted in adaptive diversity which differs significantly from the wild type strain. Bouzat (2010) emphasized on the necessity to explore relationships between population bottlenecks and variable responses of said bottlenecks (e.g. fitness and phenotypic variation). A relative strength of biofilm versus time study could be carried out; distinct changes in colony morphology should also be scrutinised for presence of phenotypic variation between possible mutant Q3 and its wild type.

7. Conclusion

Shrimp aquaculture has come a long way since the 1980's when it was first introduced into the world. It has helped many countries meet needs (food source) and is a substantial source of revenue due to international exports. Disease is a natural progression of life but irresponsible application of modern medicine to open aquaculture systems will not only be detrimental to sustainable shrimp farming, but may also lead to development of superbugs.

This study applies the knowledge of symbiosis, and the establishment of bacteria-shrimp mutualism in a similar manner as the bacteria once had for the protection of corals. Assessments carried out *in vitro* successfully identified a few bacilli strains from coral mucus that were able to quorum quench communication molecules of a known shrimp pathogen, *V. parahaemolyticus*, and deter it from reaching threshold for pathogenicity activation.

Further assessments were carried out to identify three phenotypically distinct bacilli strains to undergo coevolution as a method for strain improvement. Through a series of *in vitro* assessments, it was found that two successfully coevolved strains (Q7 and S4) were the two most promising candidates for further *in situ* and *in vivo* studies.

Assessment of strains' phenotypic characterization whilst simulating *in situ* conditions shows favourable to average growths across both strains, which is expected of promising probionts. Q7 and S4 were able to survive both low and elevated temperatures, grew well in saline environment, and are motile. Air-liquid interface biofilms of both strains were found to have good attachment levels on inorganic surfaces. However, further attachment assays on organic surfaces or chitin-coated inorganic surfaces should be carried out. Moreover, it was also found that prebiotic nutritional factors will significantly influence outcome of probiont screening, as well as any conclusions made regarding probiont's *in situ* application.

Two VP strains (ATCC and EMS) that were able to pick up the mobilisable GFP plasmid and express it stably were successfully generated via triparental conjugation. GFP-tagged VP_{EMS} was successfully applied as a model organism in this study to test the efficacy of the two coevolved bacilli strains. In view of this success, further work can be done to deepen understanding of VP virulence and its mechanism of pathogenicity in shrimps.

This study has achieved the main aim, which is to isolate potential quorum quenching candidates for EMS biocontrol as well as apply directed selection to improve strain abilities.

Although potential candidates are still in undergoing *in vitro* assessments, this study highlights the potential of selected bacilli strains from a unique marine source as biocontrol agents in shrimp aquaculture. Therefore, further work should still be carried out for the purpose of *in situ* application and enable sustainable shrimp farming in Sarawak without the over-reliance on antibiotics as a disease control method.

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9. Appendix

Appendix 1

Based on Section 3.3.2.3: Liquid surface tension reducing activity (LSTRA) of bacilli

Post hoc means comparisons with control (sterile KB media) via Dunnett's method. Strains likely to express weak surfactants indicated by bolded p-values

Strain	Absolute value of Difference	p-value
Q3	-5.39	1.000
Q7	**1.92**	**0.004**
Q9	**3.406**	**0.001**
Q14	**1.129**	**0.012**
Q15	-0.24	0.067
S1	**2.168**	**0.004**
S4	-1.8	0.340
S6	**2.008**	**0.004**
S7	-3.63	0.956
S9	-2.16	0.460
Control	-5.19	1.000

Appendix 2A

Based on Section 3.3.2.5: Bacilli growth over a range of temperatures

One-way ANOVA for relative change of strain growth by temperature

Growth at temperature	R^2	df	F Ratio	p-value
4.5°C	0.967	9, 19	61.572	<0.0001
15.0°C	0.955	9, 18	42.652	<0.0001
20.0°C	0.978	9, 18	88.148	<0.0001
28.0°C	0.991	9, 19	221.130	<0.0001
35.0°C	0.966	9, 17	54.351	<0.0001
40.0°C	0.969	9, 18	63.406	<0.0001

Appendix 2B

Based on Section 3.3.2.5: Bacilli growth over a range of temperatures

Tukey-Kramer HSD test statistic values (q*) at α=0.05 for relative change of strain growth by temperature

Growth at temperature	Test statistic (q*)
4.5°C	3.562
15.0°C	3.585
20.0°C	3.585
28.0°C	3.562
35.0°C	3.612
40.0°C	3.585

Appendix 3

Based on Section 3.3.2.6: Growth of bacilli when subjected to low temperatures

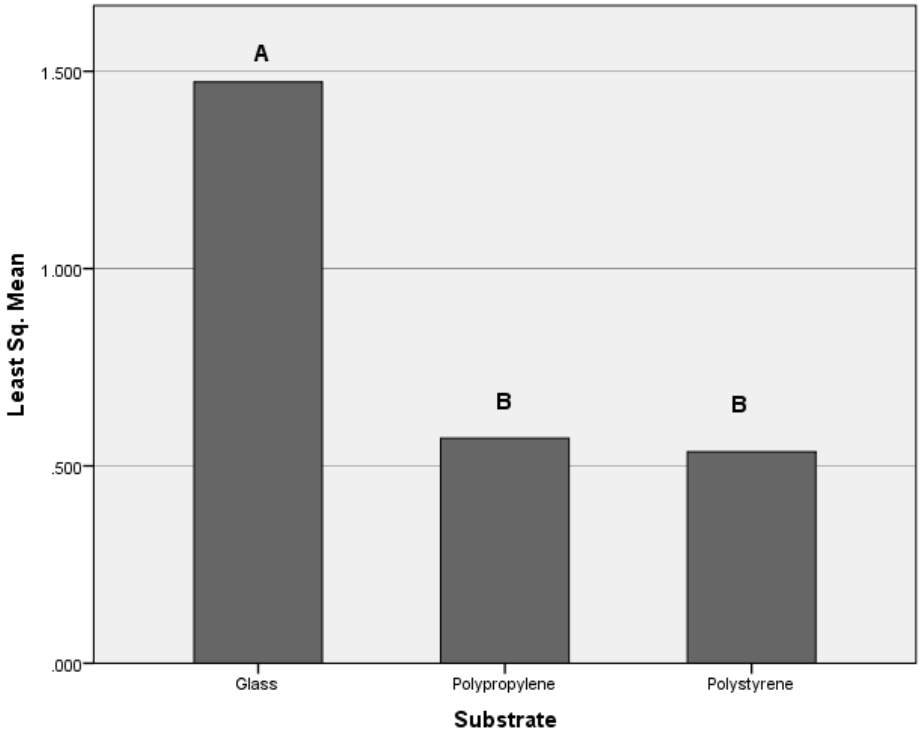
One-way ANOVA of strain growth at 168th hour

Parameter	R^2	df	F Ratio	p-value
Bacterial cell density (OD_{600})	0.958	9, 20	51.047	<0.0001
Relative change in OD_{600}	0.951	9, 20	43.546	<0.0001

Appendix 4

Based on Section 3.3.2.7: Quantitative assessment of bacilli adhesion to different surfaces

Pairwise comparison of least square means differences (using Tukey-Kramer HSD method) of effect of substrate to attachment (Q=2.361, α=0.05). Substrates not connected by the same letter are significantly different.



Appendix 5A

Based on Section 3.3.5.1: Combined biofilm assay with different media for five strains Pairwise comparisons of least square means of media and strain differences via Tukey HSD method for growth response (OD_{600}). Strains not connected by the same category are significantly different.

Strain	Least Square Mean	Category
S1	1.689	A
Q3	1.625	A, B
S9	1.601	A, B
Q7	1.512	B
S4	1.367	C

Appendix 5B

Based on Section 3.3.5.1: Combined biofilm assay with different media for five strains Pairwise comparisons of least square means of media and strain differences via Tukey HSD method for biofilm strength. Strains not connected by the same category are significantly different.

Strain	Least Square Mean	Category
Q3	2.620	A
S4	1.845	A, B
Q7	0.980	B, C
S9	0.384	C
S1	-0.136	C

Appendix 5C

Based on Section 3.3.5.1: Combined biofilm assay with different media for five strains

Pairwise comparisons of least square means of media and strain differences via Tukey HSD method for biofilm attachment. Strains not connected by the same category are significantly different.

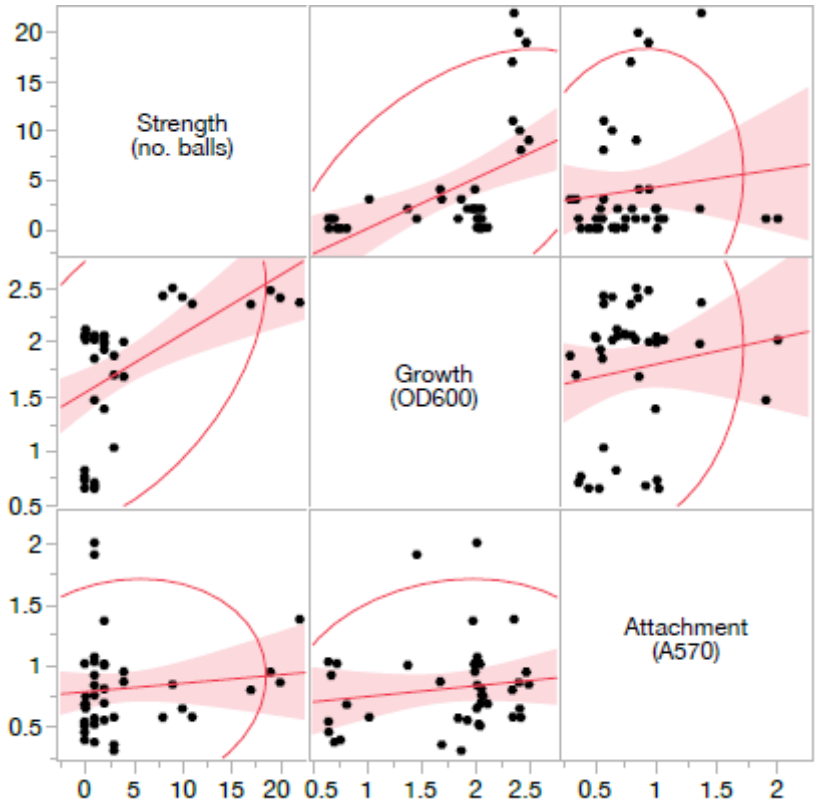
Strain	Least Square Mean	Category
Q7	0.823	A
Q3	0.759	A, B
S4	0.744	A, B
S1	0.721	A, B
S9	0.595	B

Appendix 5D

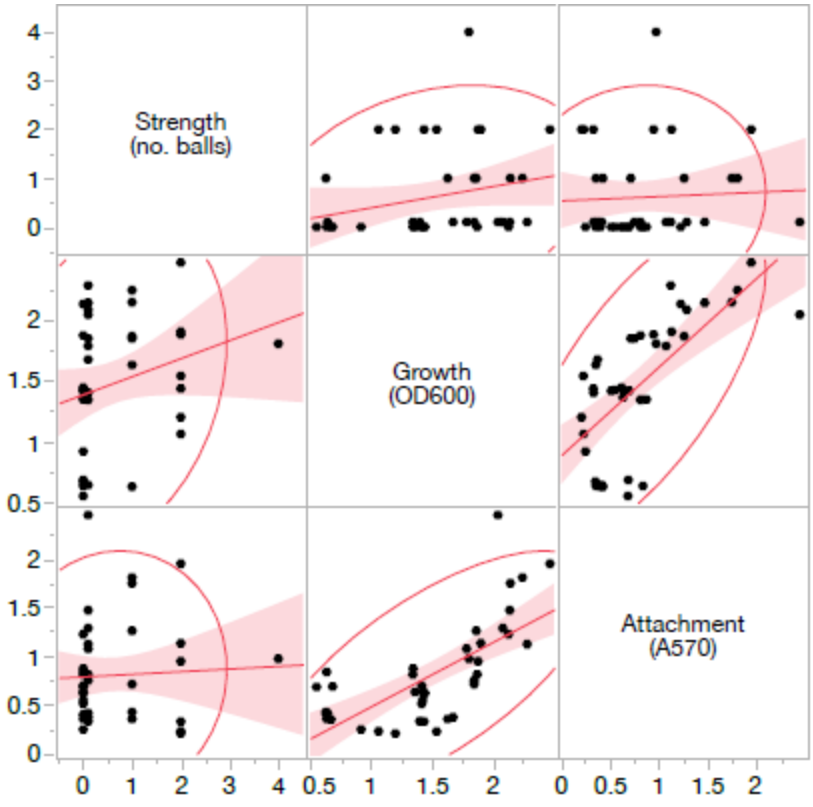
Based on Section 3.3.5.1: Combined biofilm assay with different media for five strains

Scatterplot Matrix for correlation coefficient analysis between factors for each strain

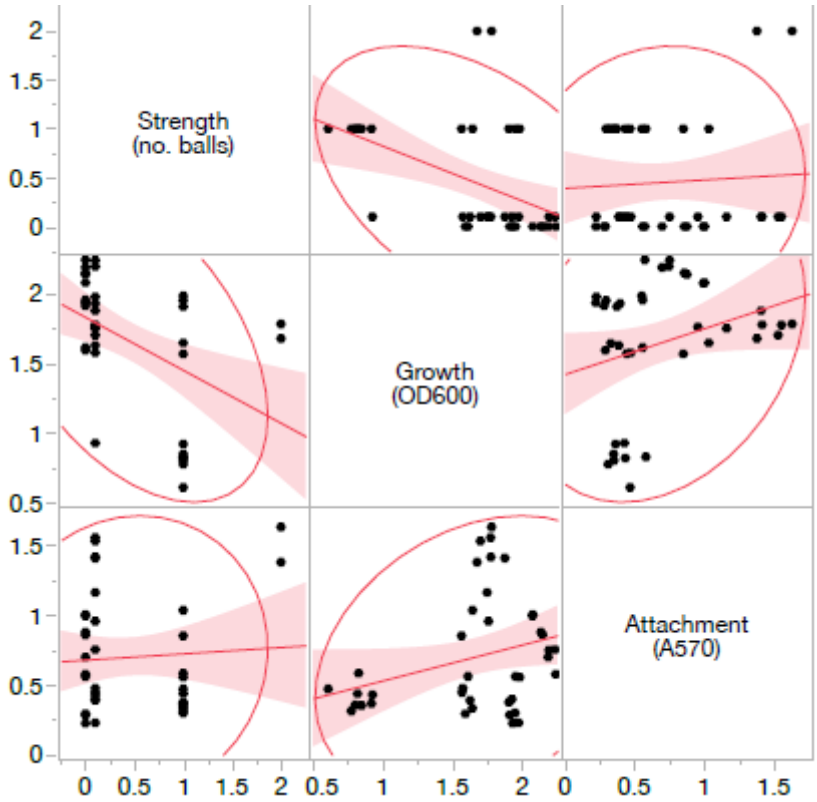
Strain Q3



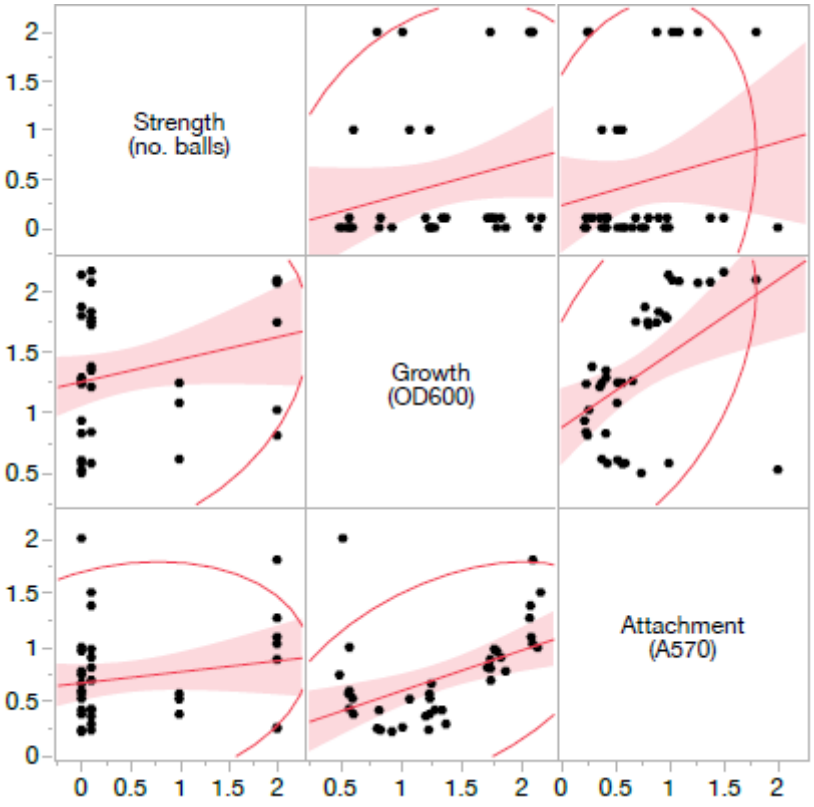
Strain Q7



Strain S1



Strain S4



Strain S9