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PREVALENCE, ANTIBIOTIC-RESISTANCE AND GROWTH PROFILE OF VIBRIO SPP., ISOLATED FROM IMPORTED SHELLFISH IN THE LOCAL MARKETS

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Food Science

Under the Supervision of Dr. Mutamed Ayyash

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Declaration of Original Work

I, Hind Obaid Mohammed Al Rumaithi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Prevalence, Antibiotic-Resistance and Growth Profile of Vibrio Spp., Isolated from Imported Shellfish in the Local Market"* hereby, solemnly declare that this dissertation is my original research work that has been done and prepared by me under the supervision of Dr. Mutamed Ayyash, in the College of Food and Agriculture at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been appropriately cited and acknowledged by appropriate academic conventions. I further declare that there is no potential conflict of interest concerning the research, data collection, authorship, presentation and publication of this thesis.

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Abstract

Seafood related human illness caused by *Vibrio* species is a major problem. Seafood are prone to contamination by pathogenic *Vibrio* bacteria especially, *Vibrio mimicus*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. The study on prevalence of these microorganisms in seafood of United Arab Emirates is vital due to the cultural background of the Emiratis as a coastal heritage. A study was conducted to assess the prevalence of *Vibrio* spp. in imported shellfish from local markets, identify the *Vibrio* spp, examine the antimicrobial resistance and profile growth conditions of the isolated *Vibrio*. In the present study, 200 shellfish samples were collected from four different main markets at four cities (Al-Ain, Dubai, Fujairah and Abu Dhabi) in United Arab Emirates. *Vibrio* spp. were isolated from the collected samples and identified by the standard culture method. DNA was extracted from all the isolates and used for molecular characterization by Polymerase Chain Reaction (PCR). The antibiotic study was also performed to find out the resistance and sensitivity of the *Vibrio* species. The factors affecting growth rate and survival of the isolated *Vibrio* spp. was studied by analyzing the effect of different parameters such as temperature, pH and salinity. Results showed that *Vibrio paraheamolyticus* was predominant in the isolates. The presence of *Vibrio* spp. was confirmed in 184 (92%) of the 200 isolates collected from different cities. The isolates from Al-Ain and Dubai showed an occurance of 12.24% and 23.80% for *Vibrio paraheamolyticus. Vibrio mimicus* was not detected in isolates from Al-Ain and Dubai. *Vibrio* isolates from Fujairah showed an occurrence of 15.5% for *Vibrio paraheamolyticus*, 11.11% for *Vibrio mimicus*. The prevalence of *Vibrio* in isolates from Abu Dhabi was 6.25% for *Vibrio paraheamolyticus* and 25% for *Vibrio mimicus.* Antibiotic sensitivity of the isolates were evaluated by measuring the zone of inhibition against 6 common antimicrobial agents. *Vibrio parahemolyticus* and *Vibrio mimicus* isolates were resistant to penicillin G, daptomycin, vancomycin, ampicillin and erythromycin while all the two *Vibrio* spp. were susceptible to sulfamethoxazole-trimethoprim. The effect of various parameters such as temperature, pH and salinity on growth and survival of *Vibrio* isolates showed *Vibrio parahemolyticus* and *Vibrio mimicus* isolates exhibited maximum growth rate at 37°C, while increasing the temperature to 47°C the growth percentage was decreased. The two *Vibrio* spp. were grown significantly at alkaline pH (pH 5 and 7). Increasing the

concentration of NaCl from 0.5% to 2%, the growth rate of *Vibrio* isolates were increased and optimum growth rate was showed in 1% NaCl. From the results, it can be concluded that the *Vibrio* isolates in shellfish from different cities of UAE showed antibiotic resistance and it is a threat to public health as the antibiotic resistant determinacies transferred to other bacteria of the clinical significance.

Keywords: *Vibro* spp., shellfish, antibiotic-resistance, growth profile, survival.

Title and Abstract (in Arabic)

انتشار ومقاومة المضادات الحيوية وملامح النمو لـ .*VIBRIO* SPP ، المعزولة من المحار المستورد في الأسواق المحلية

الملخص

تعتبر الأمراض المتعلقة بالمأكولات البحرية التي يتعرض لها الانسان والناجمة عن أنواع *Vibrio* مشكلة حقيقية. وتعتبر المأكولات البحرية عرضة للتلوث بواسطة بكتيريا *Vibrio* المسببة للأمراض وعلى وجه التحديد، *Vibrio mimicus*، *Vibrio parahaemolyticus* و *Vibrio vulnificus*. هذا وتعتبر دراسة انتشار أنواع *Vibrio* في المأكولات البحرية التي يتم بيعها في أسواق دولة الإمارات العربية المتحدة أمراً مهماً نظراً إلى الخلفية الثقافية للشعب الاماراتي كتراث ساحلي واستهلاك الاكل البحري. ولقد تم إجراء هذه الدراسة لتقييم مدى انتشار بكتيريا .*Vibrio* spp في المأكولات البحرية غير السمكية المستوردة والتي يتم بيعه في الأسواق المحلية بالإضافة إلى وتحديد بكتيريا .*Vibrio* spp وأيضاً دراسة مقاومة مضادات الميكروبات والظروف التي تنمو فيها بكتيريا *Vibrio* المعزولة. في الدراسة الحالية لقد تم العمل على جمع 200 عينة محار من أربعة أسواق رئيسية مختلفة في أربعة مدن (العين ودبي والفجيرة وأبو ظبي) في دولة الإمارات العربية المتحدة. لقد تم عزل بكتيريا .*Vibrio* spp من العينات التي قد تم جمعها ولقد تم تحديدها بواسطة طريقة الاستنبات القياسية. ولقد تم استخراج الحمض النووي من جميع العينات المعزولة واستخدامه للتوصيف الجزيئي بواسطة تفاعل البلمرة المتسلسل (PCR). كما تم إجراء دراسة على المضادات الحيوية لمعرفة مدى مقاومة وحساسية أنواع *Vibrio*. لقد تمت دراسة العوامل التي تؤثر على معدل نمو .*Vibrio* spp المعزولة وبقائها على قيد الحياة من خلال تحليل تأثير عوامل ومعطيات مختلفة مثل درجة الحرارة ودرجة الحموضة والملوحة. ولقد أظهرت النتائج أن *paraheamolyticus* كانت السائدة والمهيمنة في العينات المعزولة. فيما تم التأكيد على وجود .*Vibrio* spp في 184 (92%) من أصل 200 عينة معزولة تم جمعها من مدن مختلفة. هذا ولقد أظهرت العينات المعزولة من العين ودبي وجود 12.24% و 23.80% فيما يخص *Vibrio paraheamolyticus*، ولم يتم تحديد *Vibrio mimicus* في العينات المعزولة من العين ودبي. أظهرت العينات المعزولة من الفجيرة وجود 15.5% فيما يخص *Vibrio parahaemolyticus* و 11.11% فيما يخص *Vibrio mimicus*. ولقد كانت نسبة انتشار *Vibrio* في العينات المعزولة من أبوظبي 6.25% فيما يخص

Vibrio parahaemolyticus و 25% فيما يخص *Vibrio mimicus*. لقد تم تقييم حساسية مضادات الميكروبات للعينات المعزولة عن طريق قياس منطقة تثبيط ضد 6 عوامل مضادات حيوية المشتركة. قاومت كل من *Vibrio parahaemolyticus* و *Vibrio* mimicus البنسلين جي، الدابتوميسين، الفانكومايسين، الأمبيسلين والاريثروميسين، في حين أن كل من *Vibrio* spp. كانتا سريعات التأثر في سلفاميثوكسازول – تريميثوبريم. لقد أظهر تأثير العديد من العوامل مثل درجة الحرارة ودرجة الحموضة والملوحة على نمو عينات *Vibrio* المعزولة وبقائها على قيد الحياة، أن *Vibrio parahaemolyticus* و *Vibrio mimicus* أظهرت معدل نمو اقصى عند درجة حرارة 37 درجة مئوية، مع زيادة درجة الحرارة إلى 47 درجة مئوية، انخفضت نسبة النمو. ولقد نمت بكتيريا *Vibrio* spp. بشكل ملحوظ في درجة الحموضة القلوية درجة الحموضة 5 و7). مع زيادة تركيز كلوريد الصوديوم من 0.5% إلى 2%، تمت زيادة نسبة نمو عينات *Vibrio* المعزولة وأظهرت معدل النمو الأمثل عند (1%) من كلوريد الصوديوم. من النتائج التي توصلنا إليها يمكن أن نستنتج أن عينات *Vibrio* المعزولة من مدن مختلفة في دولة الامارات العربية المتحدة أظهرت مقاومة للمضادات الحيوية وأنها تشكل تهديداً للصحة العامة حيث تم تحديد محددات مقاومة المضادات الحيوية إلى البكتيريا الأخرى ذات الأهمية السريرية.

مفاهيم البحث الرئيسية: *Vibrio* spp.، المحار، مقاومة المضادات الحيوية، معدل النمو، البقاء على قيد الحياة.

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Dedication

To my beloved family

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Chapter 1: Introduction

Vibrio spp. causes serious disease in humans and animals. Numerous studies have shown that *Vibrio* spp., are extremely abundant in aquatic environments, including estuaries, marine coastal waters and sediments. Twelve *Vibrio* spp. have been acknowledged as imminent foodborne disease promoters in which, *Vibrio parahaemolyticus* is the most common (Adams & Moss, 2008). *Vibrio parahaemolyticus* is a halophilic and mesophilic bacterium, generally Gram-negative and found in estuarines (McCarter, 1999; Su & Liu, 2007). *Vibrio parahaemolyticus* are mostly inhabited in the aquatic environment and is also colonized in oysters, crayfish, fish, shellfish, shrimp and other aquatic organisms (Lee et al., 2008). The potential vectors for many *Vibrio* spp., are environmental bacteria accumulated in gills and digestive glands of bivalves (Potasman et al., 2002). The consumption of raw or undercooked seafood especially shellfish, *Vibrio parahaemolyticus* causes wound infections, septicemia and acute gastroenteritis (Letchumanan et al., 2015).

1.1 Prevalence of *Vibrio* spp., in GCC and MENA countries

Vibrio vulnificus isolates from clams (*Mercenaria mercenaria*) in Qatar were characterized by Multiple Antibiotic Resistance (MAR), plasmid profiles, and DNA Polymorphisms and results demonstrated the high MAR index and genomic heterogeneity of *Vibrio vulnificus* (MKurdi Al-Dulaimi et al., 2019). Alsalem et al. (2018) found that among the 234 isolates from the coastal areas in the Eastern Province of Saudi Arabia, 65 (17.9%) samples were positive for *Vibrio vulnificus* which were highly resistant to ampicillin (96%), cephalothin (73%), rifampicin (63%), and amoxicillin-clavulanic acid (56%). Ghenem and Elhadi (2017) confirmed the presence of *Vibrio parahaemolyticus* in the Eastern coast of Saudi Arabia. Elhadi (2018) used

genetic fingerprints patterns by ERIC-PCR method was used to study the genetic relationships of *Vibrio parahaemolyticus* isolated from coastal water in Saudi Arabia. Youssef et al. (2018) conducted a study for the molecular characterization of *Vibrio parahaemolyticus* isolated from shellfish harvested from Suez Canal area, Egypt and revealed that overall prevalence of *Vibrio parahaemolyticus* in shellfish was 9.27%. Al-Taee et al. (2017) investigated the occurrence of potentially pathogenic species of *Vibrio* in seven types of fish sampled from fish farms located in different districts in Basra governorate, Iraq and found that *Vibrio alginolyticus* was the predominant species, followed by *Vibrio cholerae, Vibrio furnisii, Vibrio diazotrophicus, Vibrio gazogenes and Vibrio costicola* The prevalence of *Vibrio* species was 37.1% in fish species; 47.1% in *Mulloidichthys vanicolensis*, 34.3% in *Lethrinus lentjan* and 30.6% in *Siganus rivulatus* collected from Red Sea in Egypt (Abdel-Azeem et al., 2016). Alaboudi et al. (2016) reported the prevalence rates of pathogenic *Vibrio parahaemolyticus* were 4%, 8%, and 12% in sediment, water, and fish samples collected from Gulf of Aqaba in Jordan. Ibrahim et al. (2016) identified *Vibrionaceae* (58.4%), followed by *Aeromonadaceae* (10.4%), *Shewanellaceae* (3.57%), *Pasteurellaceae* (2.9%), *Caulobacteriaceae* (2.0%), *Pseudomonadaceae* (1.56%), *Enterobacteriaceae* (1.56%) and *Burkholderiaceae* (1.33%) in seafood obtained from the Eastern Province of Saudi Arabia. Anand et al. (2016) isolated and identified pathogenic *Vibrio* species from Qatari coastal seawaters and found that *Vibrio alginolyticus* (50%) was the predominant species. Al-Sunaiher et al. (2010) identified severeal types of *Vibrio* as *Grimontia (=Vibrio) hollisae* (54.5%), *Vibrio. fluvialis* (20.5%), *Photobacterium (=Vibrio) damselae* (12.6%), *Vibrio alginolyticus* (6.8%) and *Vibrio vulnificus* (4.5%) in cultured fishes in the Kingdom of Saudi Arabia having multiple antibiotic resistance. In Eastern province of Saudi Arabia the prevalence of

Vibrio in coastal waters was 38% for *Vibrio alginolyticus,* 13.3% for *Vibrio parahemolyticus*, 7.6% for *Vibrio vulnificus*, 5.6% for *Vibrio cholera* non-O/non-O139 and 0.33% for *Vibrio mimicus* (Elhadi et al., 2013). Abd-Elghany and Sallam (2013) investigated the occurrence and molecular identification of *Vibrio parahaemolyticus* in retail shellfish in Mansoura, Egypt and found that 16.7% of shellfish samples were positive for *Vibrio parahaemolyticus*. Biochemical strips and 16s rDNA-based molecular methods confirmed the prevalence of *Vibrio* in market seafood samples of Kuwait and found that *Vibrio* occurance in the seafood samples was 77.9% (Al-Mouqati et al., 2012). Al-Sunaiher et al. (2010) identified the presence of *Grimontia (=Vibrio) hollisae (*54.5%*), Vibrio fluvialis (*20.5%*), Photobacterium (=Vibrio) damselae (*12.6%*), Vibrio alginolyticus (*6.8%*) and Vibrio vulnificus (*4.5%*)* in some cultured fishes in the Kingdom of Saudi Arabia. Kelly, (1982) found that *Vibrio vulnificus* is commonly found in Gulf Coast environments and that the occurrence of the organism was favored by warm temperatures and relatively low salinity.

Very little research has reported about the prevalence of *Vibrio* spp. in seafoods from UAE. Tarfa and Ayyash (2019) studied the prevalence, antibiotic-resistance and growth profile of *Vibrio* spp. isolated from imported fish in the local markets of UAE. The researchers found that *Vibrio parahaemolyticus* was predominant in the fish samples and the isolates were resistant to antibiotics except sulfamethoxazole-trimethoprim. To the best of knowledge, this is the first report about the prevalence of *Vibrio* spp. in shellfish in UAE. This study aims to determine the prevalence of *Vibrio* spp. isolated from imported shellfish in local markets of UAE, identify the *Vibrio* spp. and examine the antimicrobial resistance and growth profile of the isolated *Vibrio* spp.

Chapter 2: Literature Review

2.1 *Vibrio* spp. Definition

Many definitions of *Vibrio* spp. have been provided by different researchers so far. According to Paydar et al. (2013), the name *Vibrio* is a genus of bacteria that belong to the family of *Vibrionaceae*. Bisha et al. (2012) extend this definition; in their evaluation of *Vibrio*, they considered *Vibrio* as being "a marine micro-organism" that inhabits in the estuarine waters. Besides, they noted that such organisms were originally identified as being foodborne pathogen. Nonetheless, this definition was based on the *vibrio* that was viewed as a key reason for occurrence of diarrhea, in many parts of the world. Traditionally, *Vibrio* spp. are found in the alimentary canal of the shellfish that belong to the mollusk family and usually uses filter feeding. In an evaluation by Paydar et al. (2013), they cited oysters, mussels and clams as examples of such species. Hlady (1997) adds that despite the fact that effective cooking destroys the organisms, in majority of the countries, oysters is eaten raw, a practice that is associated with *Vibrio parahaemolyticus* infection.

Recent advances in this area indicate that the genus *Vibrio* are characterized by "ubiquitous heterotrophic bacteria" that stay in the marine environment and often accumulate within shellfish, which offer the source of food, including plankton and other organisms (WHO, 2019). The definitions above both state that *Vibrio* spp. are organisms found in waters across the world. It means that they are halophilic and thus need salt for survival. However, there are certain isolated cases when the bacterium can live in streams running inland in brackish waters. The number of bacteria in the waters are influenced by the level of temperature as well as salt (Oliver et al., 2013).

While developing definitions for this genus, it is essential to understand that different species are aerobic and gram-negative; besides, they are chemo-organotrophic.

So far, there are nearly 100 species of this kind of bacterium. Some types of this bacterium are saprophytes while others are parasitic in their mode of nutrition. There are still more discoveries to be made regarding the nature of this bacterium. Various species of the genus have adverse effects on human beings, although, based on the definitions, their primary goal is to ensure that they maintain the aquatic milieu (Oliver et al., 2013). The variability of the aquatic environment influences the fitness level of every species of *Vibrio*. There is a difference between the species in freshwater and those living in saline conditions. Some species of this bacterium invade and inhabit fresh waters. However, freshwaters have fewer sodium ions that affect their growth and survival. While the general definitions of *Vibrio* spp. are standard, the divergence arises more on what is the focus of the genus, for example, concerning the place of inhabitation (Austin & Zhang, 2006). When one looks critically at definitions and critical features of *Vibrio*, one can arrive at a comprehensive definition that *Vibrio* is a classical food-poisoning agent that is distributed globally, but its densities in the environment and seafood differ much based on the season, location, and the nature of the sample, as well as analytical methodology used for calculations

2.2 The History of *Vibrio* spp.

Vibrio spp. being a marine microorganism was initially found in the crustacean and was characterized and considered it to be secreted into the culture marine (WHO, 2019). Most people who have reported cases of *Vibrio*-related infections have usually indicated the possibility of consuming or being in contact with different types of seafood, including shellfish, crabs, oysters, or clams among other types of seafood.

However, the spread of infections are not limited to seafood or being in contact with seafood, some people get infections from contact with the brackish water inhabited by these sea creatures. People with a weak immune system, severe liver disease, and even poor storage of iron in the body, are likely to contract severe infections and their health deteriorates very fast.

There is no early sign of the disease. However, cases of steady increase in the wounds and even the development of septicemia are common. Accordingly, infecting with *Vibrio* spp. naturally occurs in salty and marine environments and has three common clinical symptoms. First, there is the common gastroenteritis, and then the development of wounds and the septicemia. The bacterium of *Vibrio* spp. was identified as the common cause of infections contracted from the seafood in Japan in the early 1950s. During this period, the scientists managed to isolate this kind of bacterium the first time (Letchumanan et al., 2015). This means that the species have more abundance for annual cycle within the estuaries as well as near shore marine.

There are recent developments when looking at the prevalence and characteristics of *Vibrio* spp. in shellfish (WHO, 2019). In the World Bank report, they noted that outbreak infection characterized by eating sardines has resulted in illness to 272 people with 20 people dying (WHO, 2019). Besides, past studies have found *Vibrio* spp. to cause foodborne diseases in humans; however, there are still limited evaluations on the prevalence and characteristics of *Vibrio* spp. in shellfish. This is important to develop remedies on the effects of *Vibrio* spp. in humans. Despite the growing number of infections caused by *Vibrio* spp. *Vibrio parahaemolyticus*, as well as other diseases caused by non-cholera *Vibrio* spp. have not been reported in many

states yet and so prevalence, as well as characterization of *Vibrio* spp. isolated from the shellfish, is lacking in the literature.

It can be noted that literature indicated that *Vibrio* spp. has been widely identified and successfully removed out of the environment. Nonetheless, Pacini, who was a medical student from Italy, was the first to describe *Vibrio* spp. In 1854, a primary argument on the germ theory vs. theory of miasma was developed (Farmer & Hickman-Brenner, 2006). In a few years, John Snow managed to isolate some of the bacteria. After that, the genus has started to attract significant attention of marine microbiologists. *Vibrio vulnificus* is the third kind of species that belong to the family. Bacterium *Vibrio* spp. was recognized in the 1970s as a disease-causing organism (Ceccarelli & Colwell, 2014). At the time, the infection caused by bacteria provokes the development of a syndrome known as the primary septicemia. Recent developments on *Vibrio* spp. imply that there is need to examine its prevalence and characteristics, more so for the shellfish that is limited in literature.

2.3 *Vibrio* spp. in Seafood

Tan et al. (2017) reported the density of *Vibrio parahaemolyticus* strains ranging from 3.6 to >105 most probable number/g and microbial loads of *Vibrio parahaemolyticus* strains positive ranging from 300 to 740 most probable number/g in short mackerels (*Rastrelliger brachysoma*) from different retail markets in Malaysia. Kang et al. (2016) studied the changes in the environmental parameters and occurrence of *Vibrio parahaemolyticus* in oyster aquaculture sites and found that 75% of the 44 isolates exhibited resistance to vancomycin. Yang et al. (2017) reported that the prevalence of *Vibrio parahaemolyticus* was more common in summer than winter among the 98 strains identified in seafood from South China with 8.16% and 12.2%

of prevalence to thermostable direct hemolysin and thermostable direct hemolysin-related hemolysin genes and 79.5% of isolates were resistant to ampicillin. Yaashikaa et al. (2016) isolated and identified *Vibrio cholerae* and *Vibrio parahaemolyticus* from prawn (*Penaeus monodon*) seafood using different enrichment and selective plating methods. Alaboudi et al. (2016) examined the prevalence of pathogenic strains of *Vibrio parahaemolyticus* in marketed fish and water and sediment samples from the Gulf of Aqaba and results showed that both 16S rRNA had same sensitivity and tested isolates had high nucleotide similarity irrespective of their sources. Xie et al. (2016) studied the features of *Vibrio parahaemolyticus* in ready-to-eat foods in China and found 39 strains of *Vibrio parahaemolyticus* with 33.3% isolates of serotype O_2 having negative results for genes which are resistant to streptomycin (89.7%), cefazolin (51.3%), and ampicillin (51.3%). Kang et al. (2016) found that *Vibrio parahaemolyticus* isolated from oysters in Korea exhibited resistance to cephalothin (52%), rifampin (50.7%), streptomycin (50.7%) and (53.5%) of the total 71 isolated strains showed the presence of gene confirmed by PCR analysis. Xie et al. (2015) investigated the prevalence of *Vibrio parahaemolyticus* in aquatic products of South China and found that among the 224 samples analysed, 150 isolates were negative for thermostable direct hemolysin, 61 strains were thermostable direct hemolysin-related hemolysin positive and 88.6% isolates were resistant to streptomycin. Letchumanan et al. (2015) investigated the antimicrobial resistance of *Vibrio parahaemolyticus* strains in shrimps from wet markets and supermarkets in Malaysia in which 57.8% isolates were positive for *Vibrio parahaemolyticus*. Lopatek et al. (2015) evaluated the occurrence of *Vibrio parahaemolyticus* in live bivalve molluscs in Polish market and *Vibrio parahaemolyticus* was identified in 70 (17.5%) of the 400 samples Yu et al. (2015) investigated the prevalence and drug resistance of *Vibrio parahaemolyticus*

isolated from retail shellfish in Shanghai. Oramadike and Ogunbanwo (2015) investigated prevalence of *Vibrio parahaemolyticus* in food samples prepared using croaker fish, shrimps, blue crab collected from landing sites along the Lagos Lagoon in Nigeria.

Xu et al. (2014) reported 37.7% of *Vibrio parahaemolyticus* with bacterial densities less than 100 most probable number/g in studied shrimp samples from Chinese retail markets. Yano et al. (2006) investigated the prevalence and antimicrobial resistance of pathogenic *Vibrio cholera* and *Vibrio parahaemolyticus* which are resistant to ampicillin and oxytetracycline and *Vibrio vulnificus* resistant to 20% nalidixic acid in shrimps cultured at inland ponds with low salinity in Thailand. Al-Othrubi et al. (2014) studied the antibiotic profile of *Vibrio parahaemolyticus* gastroenteritis associated with the consumption of contaminated shrimp and cockles marketed in Selangor Malaysia. Jones et al. (2012) investigated biochemical profiles, serotype, and the presence of potential virulence factors in *Vibrio parahaemolyticus* isolates from oyster and established that all isolates were positive for oxidase, indole, and glucose fermentation Koralage et al. (2012) investigated the prevalence and molecular characteristics of *Vibrio* spp. in 170 farmed shrimp (*Penaeus monodon*) samples in Sri Lanka and found that 98.1% of the farms and 95.1% of the ponds were positive for *Vibrio* spp. The *Vibrio parahaemolyticus* isolates were not positive for the virulence-associated genes. Rodriguez-Castro et al. (2010) reported that *Vibrio parahaemolyticus* was present in 35.3% and 535 strains were isolated in a study conducted in coastal waters of Galicia, Spain. Yang et al. (2008) identified 8 isolates of *Vibrio parahaemolyticus* positive in seafood samples from fishing farm, retail markets, restaurants and cooking rooms of hotels in Jiangsu province and Shanghai city of China. Jun et al. (2012) investigated the incidence, risk assessment, antibiotic

resistance, and genotyping of *Vibrio parahaemolyticus* in Korean seafood. Adebayo-Tayo et al. (2011) studied the occurrence of pathogenic *Vibrio* spp. in sea foods and water samples obtained from Oron creek and the results showed *Vibrio* spp. was recovered from 44.2% of samples, with 90% of fish, and in water *Vibrio cholerae* was the most predominant spp. Raghunath et al. (2008) studied levels of virulence genes in *Vibrio parahaemolyticus* which were estimated in 83 seafood samples from southwest coast of India by colony hybridization.

2.4 Species of *Vibrio*

2.4.1 *Vibrio cholera*

Vibrio cholera is the widely known species in the world. The species are described as being "gram-negative," "oxidase-positive," and "bean-shaped" (Drasar & Forrest, 1996). This species is freshly isolated and described as "phototrophic." The species often exhibit a faster rate of breeding and a possible maximum growth rate of about 30 min. An anaerobic environment facilitates this growth, even when they are facultative in nature (Finkelstein, 1996). The strain also survives well under alkaline conditions, but it is likely to be destroyed if the PH for the environment reduces to six (Drasar & Forrest, 1996). Besides, other areas such as intestines, stool or aquatic environment are areas in which *Vibrio* spp. can be found.

Vibrio spp. falls in two groups of cholerae 01 and cholera 0139. Both of the groups are characterized by cholera toxins, which are the cause of cholera. In addition, there are non-toxigenic of 01 and 0139 (Faruque et al., 2003). The two types of bacteria are the main causes of a number of diseases, including infections of wounds, isolated cases of diarrhea, skin infections, and even septicemia. Faruque et al. (2003) note that nontoxigenic strains in the environment are mainly found in the exoskeleton of

zooplankton and phytoplankton. It is a way in which the non-toxigenic strains get acquitted to the environment. Many of the structures for cholera species, for instance, pili, are active, thus allowing the bacteria to colonize the surface (Drasar & Forrest, 1996). Besides, the presence of *Vibrio* spp. in shellfish is a significant concern in literature. The reason for this is based on the disease being associated with *Vibrio* spp. and has an impact on the outer walls of the chitin surface (Pruzzo et al., 2008). This calls the need to examine *Vibrio* spp. as such species require biofilm information since it is vital to the ecological existence.

2.4.2 *Vibrio parahaemolyticus*

It is the most common type of *Vibrio* spp. that does not cause cholera that can be isolated. Just like *Vibrio cholera*, it inhabits the marine environment. This type of *Vibrio* is very common; a person gets infected when consuming poorly-cooked seafood. Earlier, experts believed that this bacterium produced the chemical Thermostable Direct Hemolysin which later caused the production of another compound, namely b-hemolysis, in the blood. Such hemolytic reaction is known as the Kanagawa phenomenon, named after the prefecture in Japan where it was discovered for the first time (Di Pinto et al., 2008). Nearly all the strains related to the clinical specimens were the Kanagawa-positive, while only 1-2% of the strains came from the environmental sources, which gave a positive reaction for the Kanagawa. While Thermostable Direct Hemolysin is well-identified in this study, it has been a long-standing contributor to *Vibrio parahaemolyticus* pathogenicity; the recent evidence indicated that the mechanism of virulence could be predicted on a more than single virulence factor (Su & Liu, 2007).

A common way to classify *Vibrio parahaemolyticus*, away from the presence of the Thermostable Direct Hemolysin is through lipopolysaccharide somatic O, as well as the capsular polysaccharide K antigens (Chowdhury et al., 2004). The large-scale production of the antisera is now going on in Japan and other countries worldwide. However, there is less association of serotype and virulence features; however, most isolated compounds are common in separation from clinical areas other than the food or the environment. All cells have two types of flagella, the many lateral flagella, and the single flagella with one polar. The polar form of flagella runs on the motive force from the sodium ions while the lateral type runs on the motive force from protons. In their turn, *Vibrio* spp. and the flagella system are qualified as the bacteria with two systems of flagella.

2.4.3 *Vibrio vulnificus*

This is considered as an opportunistic pathogen in humans that is associated with most of the seafood deaths across the United States (Chowdhury et al., 2004). Besides, it forms a part in the natural flora within the marine environments across the world (Froelich & Noble, 2016). This type of bacterium causes disease mainly identified by specific symptoms, including nausea, fever, and shock (Strom & Paranjpye, 2000). Other instances in which lesions could form in the patient. The lethal infection which comes out of *Vibrio vulnificus* is called septicemia. Ordinarily, the rate of deaths of these infections stands at 50%. In addition, this type of bacterium causes wound infections. These wounds are likely to form the ecchymoses, bullae, and even the cellulitis, which later may cause more infections in the affected site (Strom & Paranjpye, 2000).

There are two biotypes of *Vibrio* spp. This classification is majorly based on biochemical features of the species. Most of the infections that occur in the human beings form the biotype 1 (Strom & Paranjpye, 2000). The other ones that belong to biotype 2 are connected to pathogens *Vibrio* spp. (Osunla & Okoh, 2017). The third type is so far discovered and is related to both the type 1 and the type 2 (Di Pinto et al., 2008). In addition, there are more genes in the genomic island considered species of pathogenesis, as well (Strom & Paranjpye, 2000).

There are ecological requirements for *Vibrio vulnificus*. More often, the temperature of the water should not exceed 180°C, with the level of salinity being 15-25 parts of dissolved salt per one thousand parts of seawater (Blackwell & Oliver, 2008). In line, Blackwell and Oliver (2008) assert that this bacterium causes many incidents of infections in the tropical climate. Note that this species can bring diseases to a person, however, under specific body conditions for its survival such as inhospitable. In addition, the disease must first overcome the immune system of a person to make symptoms visible (Blackwell & Oliver, 2008). It can be noted the fact that natural virulence factors of a species that try to enhance its pathogenicity allow it to survive in the human body long enough to develop symptoms of the infection.

This disease is common in America and other parts of Europe such as Spain. The species is considered as one of the key causes of seafood fatalities across the United States. In 2001 and 2010, for instance, South Korea reported 588 cases of the disease. There were numerous fatal cases; in such a manner, 285 patients out of 588 died. Such outbreaks have been widely witnessed in different parts of the world.

2.5 *Vibrio* Classification and Taxonomy

Genomic taxonomy is based on the polyphasic appproach (Thompson et al., 2009). The Average Amino Acid Identity is capable of determining the nature and group of the species of *Vibrios* and this method is used in the identification of the connection between the gene content being shared and the material being considered. The findings are calculated by genes conserved between every pair of the genomes. Another type of algorithm called BLAST can be applied to solving the entire problem of the genome analysis in a pairwise manner.

On the other hand, the genome signature dissimilarity for *Vibrio* species has been discovered to be more similar between closely-related species as compared to the distantly-related species. This method is based on the assumption that there is a likelihood of the species belonging to the genus (Thompson et al., 2009). The relative dinucleotide presence is an important part, which is common in most genomic signatures. Although, there is diversity on the *Vibrio* species, there is limited differences among them, for instance, it lies at 50 kilos for a particular genome (Thompson et al., 2009). The main differences are determined by the level at which certain aspects recur. The genomes may differ by signatures and these differences show the extent of evolutionary connections. Significant deviations at the level of name are an indicator of horizontal transfer of the segment from other species. The methods may help in indicating the connection between the variant of *Vibrio* spp.

Furthermore, the Genome BLAST is a method used for depicting compositional differences between genomes of various *Vibrio* species. In the process of the analysis, the differences are observed based on the gene content and DNA features in every species. The technique is used as the measure that validates the results

of the methods used in identifying and classifying members of *Vibrio* species in scientific research (Thompson et al., 2009). The figure below demonstrates the Genome BLAST.

2.6 *Vibrio* Diseases

In most cases, infection associated with *Vibrio parahaemolyticus* causes gastroenteritis, usually accompanied by diarrhea; at times, patients experience hematochezia, fever, nausea, headache, vomiting, or abdominal cramps. At times, *Vibrio parahaemolyticus* boosts the development of wound infections. *Vibrio cholera* is another main cause of cholera (Drasar & Forrest, 1996). It has such sings as diarrhea and dehydration of the body. In most cases, this kind of disease causes death; there are other symptoms related to loss of skin elasticity that are well documented in literature (WHO, 2019; CDC, 2018). In addition, infections caused by *Vibrio* are a result of people eating contaminated seafood; these diseases have a higher prevalence.

2.6.1 Gastrointestinal Illness

Gastro intestinal tract are the ailments related to digestive system such as the throat, stomach or intestines (WHO, 2019). This disease would also include a more chronic diagnosis. This disease is characterized by diarrhea, pain in the abdomen, vomiting, fever, and nausea, as well as chills or cramping in the abdomen (CDC, 2018). Many other conditions caused by the disease are found only in people with a weak immune system. The key way of preventing this infection is by preparing food adequately. The raw seafood needs to be stored separately from other products. In addition, people should avoid exposing open wounds to seawater (WHO, 2019).

2.6.2 Cholera

This infection is considered to cause diarrhea that might provoke dehydration and likely death. The disease is mainly caused by consuming food or water contaminated with *Vibrio cholera* bacterium. There are signs and symptoms related to this disease for instance increase in the rate of the heart or diarrhea (CDC, 2018). Three critical methods to treat cholera. First, rehydration therapy is crucial. The process involves manipulations aimed at restoring the level of fluid and salts in the body. Oral rehydration with low-osmolality is effective in malnourished patients (CDC, 2018). Second, treatment with antibiotics seeks to reduce the need for fluids in the body and time of illness. Third, treating illness symptoms, especially in children, is crucial (CDC, 2018).

2.7 *Vibrio* spp. Outbreak

A few outbreaks of cholera caused by *Vibrio* bacteria occurred in the African continent during the period between 1991 and 1996. During this period, the number of reported cases ranged from 70,000-160,000 as according to official statistics provided by the WHO (2019). The outbreak of cholera in 1991 in Latin America was serious, as well. The outbreak had lasted for over two years; 75,000 cases were reported, out of which 65,000 were mortalities (WHO, 2019). Finally, another outbreak of *Vibrio*-related cholera occurred during the period between April and July of 2018.

Vibrio species accused of the outbreak was *Vibrio parahaemolyticus*. A recent outbreak in 2013 was considered to be caused by shellfish (CDC, 2018). It affected 13 states across the United States; 104 people were hospitalized. However, no cases of deaths were reported. It should be noted that cholera has been experienced in the African continent since 1971 (CDC, 2018). Yemen still reports incidences of cholera

outbreaks. It implies that the outbreak of infectious disease remains a threat to the health of the global community.

2.8 *Vibrio* spp. Prevalence in Food

Seafood is the main method of transmission of infections caused by *Vibrio* bacteria. Food pathogens, for instance, *Vibrio* spp., have been considered a significant cause of food-borne outbreaks across the world (WHO, 2019). *Vibrio parahaemolyticus* was initially reported in Asia in 1951 (Letchumanan et al., 2015). Later, species of the bacterium were isolated in the seafood, for example, shrimp and oysters, in markets located in southeastern regions of Asia (Su & Liu, 2007). It is important to note that there have been successful cases of isolation of *Vibrio* shrimps in Thailand and Malaysia. In addition, similar species were identified as a critical reason for foodborne infections in China (Letchumanan et al., 2015). Additionally, in 2001 and 2012, 13,607 cases of diarrhea were related to *Vibrio parahaemolyticus*, and a few instances were reported in India's Kolkata slums (Letchumanan et al., 2015). However, there are limited aspects of prevalence and characteristics of *Vibrio* spp. in shellfish.

The WHO (2019) adds that across Europe, isolated cases were associated with the seafood received from the Baltic Sea, Black Sea, and the Mediterranean Sea. According to the research done by the WHO, shellfish collected in the waters along the coast of Guadeloupe contained a considerable level of *Vibrio parahaemolyticus*. Similarly, other available studies suggest that in France, there was an outbreak in 1997, which affected 44 people. Nevertheless, in other parts of the world, food poisoning is as a result of bacterium strain. Similarly, other countries such as the U.S have reported

this disease due to consuming uncooked seafood have been reported in different American Coastal regions (WHO, 2019).

A number of investigations across the world have shown that there are cases of cholera associated with food poisoning. For instance, in 2016, about 132,121 cases were caused by *Vibrio cholera*. The evaluation of these reports shows that 17 of the cases originated from Africa, four were from Europe, 12 were from Asia, four were from the United States, and one was from Oceania (WHO, 2019). About eighty percent of the cases have occurred in the Democratic Republic of the Congo, Haiti, and Yemen, as well as Tanzania. Despite this fact, research indicates that the real number of cases associated with food contamination in children is higher than the reported figures (WHO, 2019). More frequently *Vibrio vulnificus* is present in oysters as compared to other kinds of seafood harvested across the world (Blackwell & Oliver, 2008).

Present studies have shown that environmental factors, for example, interaction with other hosts have a significant influence on the evolution of certain types of pathogens (Wilson & Salyers, 2003). In such a manner, the pandemic strains with show some biological features, for example, increase in the production of a toxin or ability to live in natural environments, gives more insights into the manner that underlie the emergence and spread of the strains of *Vibrio* spp. (Wong et al., 2002). The prevalence as well as characterization of *Vibrio parahaemolyticus* bacteria is viewed to be under effect from some of the environmental factors, such as temperature, water, salinity, and level of concentration of oxygen. While there are advances in the area of hygiene, treatment of food, and the method of processing worldwide, food-related pathogens create a significant threat to human health globally. Based on the level of food-related prevalence, studies show that *Vibrio parahaemolyticus* bacteria have been the main

one among the three species identified so far with *Vibrio vulnificus* bacterium being second and *Vibrio cholera* coming third. There is limited research on the prevalence of *Vibrio* in shellfish across the world. Therefore, this is an area that needs further investigations, more so on the prevalence and characteristics of *Vibrio* spp. in shellfish.

2.9 *Vibrio* spp. in Shellfish

The existence of *Vibrio* species in the shellfish cause a considerable health risk and thus is a primary problem for consumers of shellfish and the global economy at large (Lee et al., 2008). Besides, contamination of shellfish with *Vibrio* species brings about an increased burden associated with the global healthcare system because of the possible disease outbreak. While shellfish is considered a part of the healthy diet, it is the cause of many foodborne diseases globally. Shellfish are often associated with *Vibrio vulnificus* during warm seasons, thus increasing chances of people being infected by this strain. Other than the existence of *Vibrio vulnificus* species in shellfish, *Vibrio parahaemolyticuis* bacterium has also been associated with most diseases caused by seafood in China and Malaysia (Malcolm et al., 2015). It means that there are significant changes in the majority of fish products imported from China being contaminated with different strains of *Vibrio* bacterium. From the economic perspective, China is the primary producer of shellfish in the world with growing incidences of fish poisoning caused by *Vibrio* species around the world. Halpern and Izhaki (2017) note that there are chances that shellfish could be a reservoir of *Vibrio* species, particularly *Vibrio cholerae*.

Consumption of shellfish is associated with a high occurrence of diseases caused by *Vibrio* species, for example, rare species of *Vibrio harveyi* and least documented species that targeted Latin America and the United States in the 1960s. It

implies that such species of fish could cause foodborne diseases and disease outbreaks (Whitaker et al., 2012). Tetrodotoxin, a harmful toxin produced by *Vibrio* species, is isolated in some species of fish. Theoretically, *Vibrio* species and shellfish share the same ecological niche. *Vibrio* species are good swimmers; in addition, they can attach to other organisms living in the water and move with them (Di Pinto et al., 2008). Hence, shellfish is not an exception, implying that in the contaminated water environment, it is most likely that any fish species would be *Vibrio*-contaminated, thus spreading infections when consumed raw or undercooked. Malcolm et al. (2015) recommended routine screening for fish products as a way to control Vibriosis infections. Nonetheless, nothing much has been done in literature in characterizing the *Vibrio* spp. in shellfish to inform further treatments for the diseases caused by *Vibrio* spp.

2.10 Shellfish and Shellfish Products

Globally, the production of shellfish attained an all-time high with about 109 million tons. Out of the total production, 88% is consumed directly by human beings. In 2016, the per capita consumption reached 15.6%. Recently, the aquaculture sector has experienced considerable economic growth because of the contribution of Africa and Asia. The value of the global export of shellfish increased to reach $105,067 billion in 2018. Across the world, France is the primary consumer of shellfish (mussels, scallops, and oysters). There is not enough supply in the domestic market, which makes the exportation of shellfish attractive in the global market. According to the Global Trade Tracker, France imported seafood worth € 2425.41 million (it was a growth of 25% as compared to 2015); in the United Arab Emirates , the exports of shellfish increased by a six-figure digit and now amounts to € 1.3 million, or 2% of

exports to the United Arab Emirates in 2016. In terms of value, the United Kingdom with the market share of shellfish of 16.2% is among the leading suppliers of shellfish products followed by France and the United Arab Emirates (with a market share of 4.8%).

On average, leading shellfish producers in the world such as France and the UK produce an average of 200,000 metric tons for the shellfish every year. However, it is not enough to satisfy the consumer market for shellfish products. Mussels and oyster have about 39.1% and 38.3% respectively in the global demand for seafood with scallops, clams, and abalones having the rest. Canada is the fifth supplier of shellfish products in the world with an annual oyster production of 76,714 metric tons. China, the UK, North Korea, Japan, and the United States of America. Followed by the United Kingdom.

Mussels have a market of more than 181,000 metric tons, which relies on local production and imports and is widely consumed globally (Euromonitor International, 2016a). Large volumes are imported as fresh products. For instance, in 2016, 14,941 metric tons of fresh mussels were exported by Spain; similarly, the Netherlands exported 13,829 metric tons to the global market (Euromonitor International, 2016a). The fresh mussels make up much of sales for retail fishmongers and supermarkets, as well as the catering industry, in which they are among the favorite dishes.

Many countries across the world produce scallops. Peru and Argentina are the leading producers with France being a significant importer providing about 13,197 metric tons (Euromonitor International, 2016b). Canada has been a critical traditional and essential supplier of scallop products that have been always associated with conviviality, luxury, and festivity. Just like oysters, the scallop consumption is mainly

influenced by seasons with much growth in sales during Christmas time and the New Year festive.

2.11 Shellfish Economy in the UAE

In 2014, shellfish sales in the UAE reached the growth rate of more than 5% in terms of volume, thus reaching a market high of 106,040 tones (Euromonitor International, 2016b). According to the reports provided by Euromonitor International (2016a), the growth was facilitated by various factors, including increased availability of shellfish in retail outlets. Predictions indicate that the shellfish consumption is likely to exceed 900,000 tones, with the entire GCC fishing industry producing only 392.000 tones yearly at the moment. The Ministry of Economy of the United Arab Emirates states that 75,000 tones (19%) of the regional production of shellfish are from the United Arab Emirates (Euromonitor International, 2016b). The implication of this fact is a substantial deficit that needs to be filled with the help of importation. Oman is a significant producer in the region, although bulk imports are made from such nations as China, India, and Thailand.

The growth in the modern grocery outlets associated with sizeable fresh shellfish at the counters contributes to the growth of shellfish economy in the United Arab Emirates, simultaneously with the increase in population. Medical experts emphasize the health benefits associated with the consumption of shellfish three times a week. In addition, it is considered a healthy alternative to pork, lamb, and beef (Euromonitor International, 2016a). A vast majority of the fish and shellfish in the United Arab Emirates with organic products represented 3% of the total sales volume in 2016. The natural fish and shellfish products are mainly imported for high-income expatriates through premium retailers. The number of foodservice outlets that

specialize in shellfish has recently increased. This fact not only contributes to the growth of the sales of shellfish but also boosts the economy.

The United Arab Emirates is the second after Oman in terms of the per capita shellfish consumption. The growth in population results in the increased consumption of shellfish by the young protein-demanding community (Environmental Agency-Abu Dhabi, 2017). With the increased shellfish consumption, there is a need to establish food security measures in the UAE due to the risk associated with undercooked or raw shellfish.

2.12 Incidence of *Vibrio* spp. in Shellfish in the UAE

The incidences of the presence of *Vibrio* spp. in shellfish, for instance, cholera and the wound infections, have been examined in the literature across the world (Oliver et al., 2013; Osunla & Okoh, 2017). Many countries around the globe have reported incidences of Vibriosis infections. For example, note that in India, *Vibrio parahaemolyticus* gets isolated from the clinical as well as environmental samples (Pazhani et al., 2014). In most of the countries in Europe, *Vibrio parahaemolyticus* gets isolated in Baltic Sea, the North Sea, as well as Mediterranean Sea, the sample examined was 53 out of 100. There were some of the cases, which were detected and included *Vibrio parahaemolyticus* gastroenteritis. Similar evaluations were done in Spain, Greece, the UK, and Turkey. Despite this fact, there have been limited or no study on the prevalence and characteristics of shellfish *Vibrio* spp. in the United Arab Emirates.

With fishing having a significant contribution to the growth of the economy of the United Arab Emirates, the fishing sector relies on consumers to continue its

production. Despite that fact, the United Arab Emirates performance in the fishing sector is threatened by the existence of pathogenic species in shellfish. Four species of *Vibrio* about foodborne illnesses that have been examined in the literature; three of them are considered to prevail in shellfish products. With limited investigations done on shellfish in United Arab Emirates, the need to investigate prevalence and characteristics of *Vibrio* spp. in shellfish is essential to fill the research gaps, in this area.

It is likely to be a significant threat to the public health, thus implying the need to examine *Vibrio* spp. in this area and provide recommendations on the reduction of the species' prevalence and characterization of *Vibrio* spp. in shellfish products in the United Arab Emirates. In the recent years, concerns on *Vibrio* spp. have been raised across the world; the effects of climate change, the adaptation of pathogens to cooler waters, the emergence of new strains, and distribution through ballast water have been well-documented in the literature in developed countries.

No documented study attempts to address the area of food security and food microbiology in the United Arab Emirates despite the increased consumption of shellfish and its role in the growth of the local economy. Pathogenic bacteria cause superficial gastrointestinal infections associated with diarrhea, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. The organisms secrete a number of toxins that enhance pathogenicity and generate a non-essential target that attracts host defense systems, while bacteria themselves remain unharmed. Much has not been learned regarding *Vibrio* spp. in shellfish imported and sold in the United Arab Emirates markets, on the molecular mechanisms, which underlie the superficial gastrointestinal infections. Availability of insights is crucial to the development of improved control

and prevention strategies against pathogens with the view to improving food security and food microbiology.

Chapter 3. Materials and Methods

3.1 Study Area and Sample Collection

Fresh local shellfish samples (n=200) were imported from four different main markets at different cities (Al-Ain, Dubai, Fujairah and Abu Dhabi) in United Arab Emirates. Samples were collected at one time period during summer extended from June to September, 2017 at early morning. The samples were placed in individually labeled and sealed in plastic bags and transported in sealed containers with dry ice to UAEU laboratory for microbial analysis**.**

- Experimental Layout

The details of the experiments conducted in the study are shown in Figure 3.1.

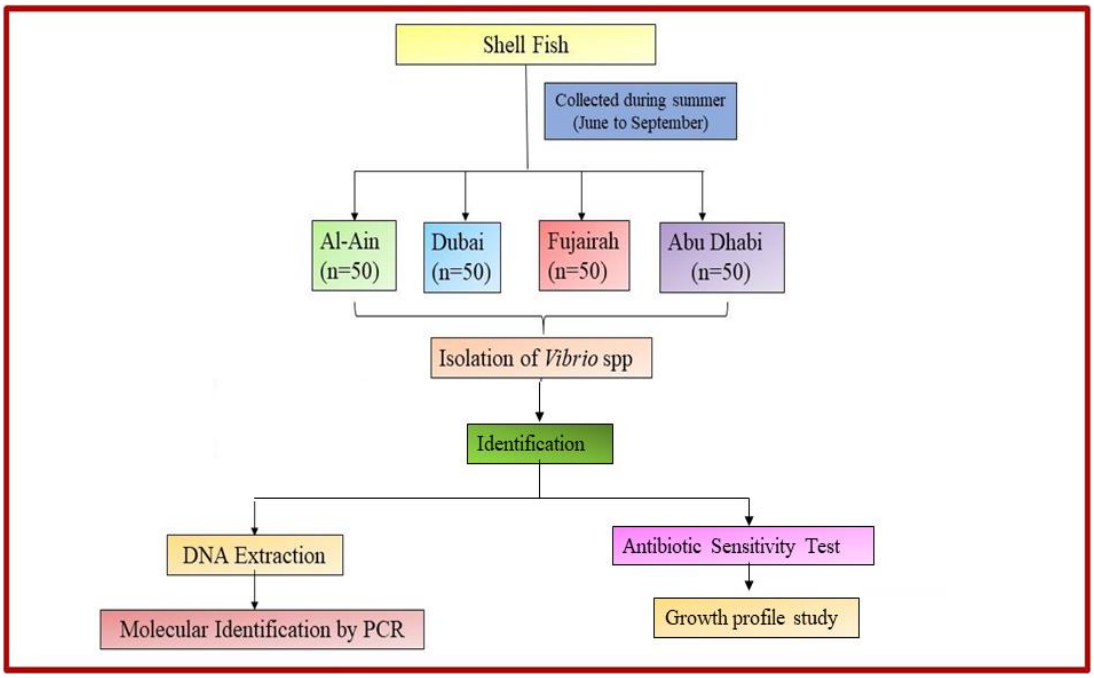


Figure 3.1: Schematic representation of experimental layout

3.2 Isolation of *Vibrio* spp.

Vibrio spp. was isolated and identified by the standard culture method according to Sujeewa et al. (2009).

3.2.1 Reagents

1. Thiosulfate-citrate-bile salts-sucrose agar (TCBS Agar)

Table 3.1: Composition of TCBS Agar

Ingredients	**Gms/Litre**
Proteose peptone	10
Yeast extract	5
Sodium thiosulphate	10
Sodium citrate	10
Bile	8
Sucrose	20
Sodium chloride	10
Ferric citrate	1
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.

Note: Preparation of TCBS Agar Plates

TCBS (89.08 g) was suspended in 1000 ml distilled water and the pH of the solution was adjusted to 8.6±0.2. The medium was completely dissolved by heating up to boiling and then cooled to 45-50°C. Mixed well and poured into sterile petri plates (Table 3.1).

2. Modified Cellobiose-Polymyxin B-Colistin Agar (mCPC Agar)

Table 3.2: Composition of mCPC Agar

Ingredients	**Gms/Litre**
Peptone	10
Peptone Beef Extract	5
Cellobiose	10
Sodium chloride	20
Bromo thymol blue	0.040
Cresol red	0.040
Agar	15

Note: Preparation of mCPC Agar Plates

mCPC agar (60.08 g) was suspended in 1000 ml of distilled water and the pH of the solution was adjusted to 7.6±0.2. The medium was completely dissolved by heating at 100°C. Sterilized the medium by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled to 45-50°C and aseptically added 1 vial of modified colistin supplement. Mixed well and poured into sterile petri plates (Table 3.2).

3.2.2 Procedure

Twenty-five gram of imported shell fish flesh samples were homogenized in 225 mL alkaline peptone saline water (APSW, Hi Media, Bombay, India). The homogenate was mixed thoroughly for 1 min at 260 rpm using Stomacher Circular Unit 400 (Seward Ltd., London, UK), and incubated at 42°C for 8 h. Then 10 ml of the incubated homogenate was streaked in duplicate on TCBS and mCPC agar plates. The inoculated plates were incubated at 37°C for 18 to 24 h (Figure 3.2).

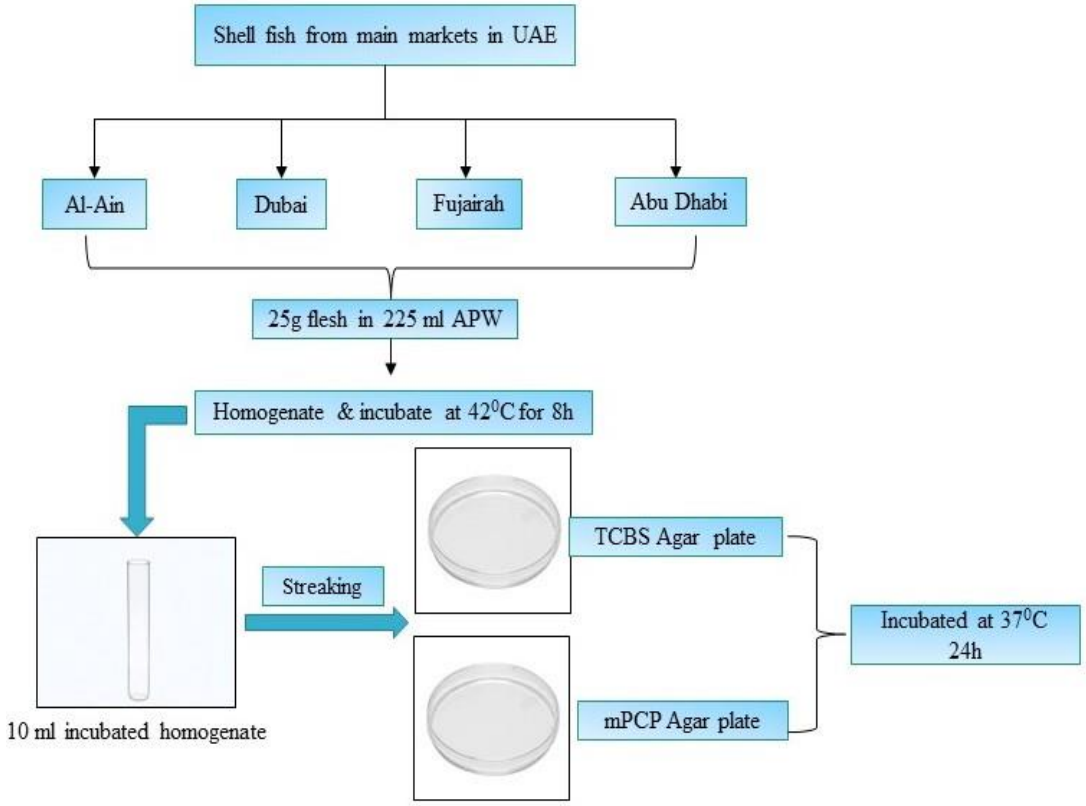


Figure 3.2: Schematic representation of isolation of *Vibrio* spp.

3.3 Molecular Identification of *Vibrio* spp.

3.3.1 DNA Extraction from Shellfish

Tissue homogenate of shellfish (10 ml) incubated at 37°C was streaked in duplicate on TCBS agar (Hi Media) and tryptone soy agar (TSA, Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 3% Sodium chloride (NaCl). The inoculated plates were incubated at 37°C for 18 to 24 h. Suspected colonies were streaked again on TSA + 3% NaCl to obtain a pure isolate.

3.3.1.1 Reagents

Solution CB1: an ethanol-based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes

residues of salt, and other contaminants while allowing the DNA to stay bound to the silica membrane

Solution IRS: IRS solution contains a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

Solution SB: Solution SB is a highly concentrated salt solution. It sets up the high salt condition necessary to bind DNA to the Spin Filter membrane

3.3.1.2 Procedure

DNA was extracted by QIAGEN DNA extraction kit. Briefly, 1.8 ml of bacteria culture was added to a 2 ml collection tube and centrifuge at 10,000 xg for 30 s at room temperature. Decanted the supernatant and spin the tubes again at 10,000 xg for 30 s at room temperature. Supernatant was removed, the cell pellet was resuspended in 300 µl of Power Bead Solution and vortexed gently. Resuspended cells were then transferred to Power Bead Tube and 50 µl of CB1 solution was added and vortexed for 10 min. The tubes were centrifuged at a maximum of 10,000 xg for 30 s at room temperature and the supernatant was transferred to 2 ml collection tube. 100 µl of IRS Solution was added to the supernatant, vortexed for 5 s and incubated at 4°C for 5 min. The tubes were then centrifuged at 10,000 xg for 1 min at room temperature. 900 µl of SB solution was added to the supernatant and vortexed for 5 s. In the next step, 700 µl of supernatant with SB solution was loaded into a MB Spin Column and centrifuge at 10,000 xg for 30 s at room temperature. Then, 300 µl of CB solution was added and centrifuged at 10,000 xg for 30 s at room temperature. The MB Spin Column

was placed in a new 2 ml collection tube, 50 µl of elution buffer was added in the centre of white membrane. Centrifuged at 10,000 xg for 30 s at room temperature. The MB Spin Column was discarded and DNA was collected.

3.3.2 Confirmation of *Vibrio* spp. by PCR

Polymerase Chain Reaction (PCR) assay was performed for general (*Vibrio* spp.) genes of the suspected *Vibrio* isolates. The amplification conditions were 35 cycles of amplification, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The reaction mixtures were resolved by electrophoresis in 2% agarose gel and visualized under UV light. The presence of the gel bands compared with the DNA molecular weight standard (100 bp marker) was recorded. Table 3.3 shows the primers used for confirmation of *Vibrio* spp.

Table 3.3: Primers used for confirmation of *Vibrio* spp.

Primer code	**Sequences (5' to 3')**
Vibrio spp. F	CGGTGAAATGCGTAGAGAT
Vibrio spp. R	TTACATGCGATTCCGAGTTC

3.4. Antibiotic Sensitivity of *Vibrio* spp.

Antibiotic sensitivity was studied by the method of Yaashikaa et al. (2016). The test culture was transferred into a sterilized broth. The broth is then incubated at 35°C till it becomes slightly turbid. By using a sterile cotton swab the standardized bacterial test suspension was inoculated evenly on the entire surface of sterile Muller Hinton Agar plates. Antimicrobial susceptibility test discs (Oxoid, Thermofischer scientific) were placed on the surface of the medium and plates were incubated on

37°C for 24 h. The antimicrobial activity was interpreted from the diameter of zone of inhibition which was measured in millimeter (Table 3.4).

Table 3.4: Antimicrobials used for antibiotic sensitivity study

Antibiotics	**Concentration/disc**	**MIC break point (mm)**		
		S	**I**	**R**
Penicillin G	10 IU	10	11-19	20
Vancomycin	2 mcg	12	-	13
Daptomycin	30 mcg	14	20	15
Ampicillin	10 mcg	14		15
Erythromycin	15 mcg	13	18	16
Sulphamethoxazole/Trimethoprim	25 mcg	13	14-16	17

Breakpoints as recommended by the CLSI M45-A (2010). IU- international unit, mcg-microgram, mm- milli meter. S, I and R stand for susceptible, intermediate and resistant, respectively**.**

3.5 Species Identification by PCR

PCR assay was performed separately for specific (16 S rRNA) genes of the suspected *Vibrio* isolates. The amplification conditions were 35 cycles of amplification, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The reaction mixtures were resolved by electrophoresis in 2% agarose gel and visualized under UV light. The presence of the gel bands compared with the DNA molecular weight standard (100 bp marker) was recorded. Table 3.5 shows the primers used for species identification.

Table 3.5: Primers used for species identification

Primer code	**Sequences (5' to 3')**
*V.*16S-700F	CGG TGA AAT GCG TAG AGA T
*V.*16S-1325R	TTA CTA GCG ATT CCG AGT TC

3.6 Factors Affecting Growth Rate of *Vibrio* spp.

The effect of temperature, pH and salinity on the growth and survival rate of *Vibrio* spp. were studied by the method of Yaashikaa et al. (2016)

3.6.1 Reagents

1. Nutrient Broth

The composition of nutrient broth is given in Table 3.6.

Table 3.6: Composition of Nutrient Broth

Ingredients	**Gms/Litre**
Gelatin Peptone	5.0
Beef Extract	3.0

3.6.2 Procedure

Nutrient broth medium (8 g) was added in one liter of distilled water. Mixed well and dissolved by heating with frequent agitation. Boiled for one minute until complete dissolution. Sterilized in autoclave at 121°C for 15 minutes and stored at 2-8°C.

3.6.3 Effect of Temperature on Growth of *Vibrio* spp.

The nutrient broth was taken in a boiling tube and sterilized. All the tubes were inoculated with 0.1 ml of *Vibrio* isolates and incubated for 22 h at different temperatures (25°C, 37°C and 45°C). Then serial of tenth fold dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and10^{-6}) were used in sterile distilled water for each tube and incubated for 24 h at 37°C. After the period of incubation, the viable count of bacteria was

determined by measuring the absorbance at regular intervals of time with spectrophotometer at 620 nm (Packiavathy et al., 2013).

3.6.4 Effect of pH on Growth Rate of *Vibrio* spp.

The effect of pH on the growth rate of *Vibrio* isolates were determined by preparing a series of pH values ranged from 3, 5 and 7 in nutrient broth. All the tubes were autoclaved and inoculated with 0.1 ml of *Vibrio* isolates and incubated for 22 h at 37°C. Then serial of tenth fold dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and10^{-6}) were used in sterile distilled water for each tube and incubated for 24 h at 37°C. After the period of incubation the viable count of bacteria was determined by measuring the absorbance at regular intervals of time with spectrophotometer at 620 nm (Packiavathy et al., 2013).

3.6.5 Effect of Salinity on Growth Rate of *Vibrio* spp.

Nutrient broth was taken in boiling tubes (10 ml for each tube) and NaCl was added to each tube at various concentrations (0.5%, 1.0% and 2.0%). The pH was adjusted to 8.5 by using Sodium hydroxide (NaOH, 0.1N) and then autoclaved. The tubes were inoculated with 0.1ml of *Vibrio* isolates and incubated for 20 h at 37°C, then serial of tenth fold dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) were used in sterile distilled water for each concentration. Growth of isolates were observed by measuring the absorbance at regular intervals of time with spectrophotometer at 620 nm (Packiavathy et al., 2013).

3.7 Statistical Analysis

Growth profile data in triplicate were subjected to the analysis of variance using general linear model and mean comparisons were performed using Duncan's

multiple range test to compare significant differences between means for all analyses. Statistical analysis was carried out using the Statistical Analysis System. Values are expressed as average of 3 samples ± standard error

Chapter 4: Results and Discussion

4.1 Isolation of *Vibrio* spp. in Shellfish

A total of 200 fresh local shellfish samples were imported from four different main markets at different cities (Al-Ain, Dubai, Fujairah and Abu Dhabi) in United Arab Emirates.

A total of 184 (92%) isolates imported from local markets were *Vibrio* positive in which 49 samples in Al-Ain were *Vibrio* positive. The number of *Vibrio* positive isolates in other cities were Abu Dhabi (48) > Fujairah (45) > Dubai (42). The percentage occurrence of *Vibrio* in Al-Ain was 98% while in Abu Dhabi, Fujairah and Dubai the percentage was 96, 90 and 84% respectively (Figure 4.1 & Figure 4.2). The present study confirmed that the prevalence of *Vibrio* was higher in shellfish imported from different local markets in UAE. Several reports revealed that *Vibrio* spp. is a major cause of bacterial infections due to the consumption of imported shellfish and other fish products from local markets (Tan et al., 2017). Elhadi (2018) reported that the prevalence of *Vibrio* spp. was 90% in samples collected from eastern coast of Saudi Arabia. The overall prevalence of *Vibrio parahaemolyticus* in shellfish was 9.27% in shellfish (164 clams, 86 mussels, and 160 shrimps) collected from the three Governorates of the Suez Canal area (Youssef et al., 2018). Asgarpoor et al. (2018) found that prevalence of *Vibrio* spp. was 22.8% in studied shrimp samples from retail outlets in Zanjan, Iran. Letchumanan et al. (2015) found a high level of *Vibrio* in fish samples purchased from wet markets compared to supermarkets. Raissy et al. (2014) revealed that 29.3% of the examined fish samples were *Vibrio* positive. In the present study, *Vibrio paraheamolyticus* was predominant in shellfish samples among the *Vibrio* spp. isolated. The prevalence of *Vibrio* isolates (33%) detected in shellfish

imported from retail markets in Mansoura, Egypt was also reported to be less than that observed in this study (Abd-Elghany and Sallam, 2013).

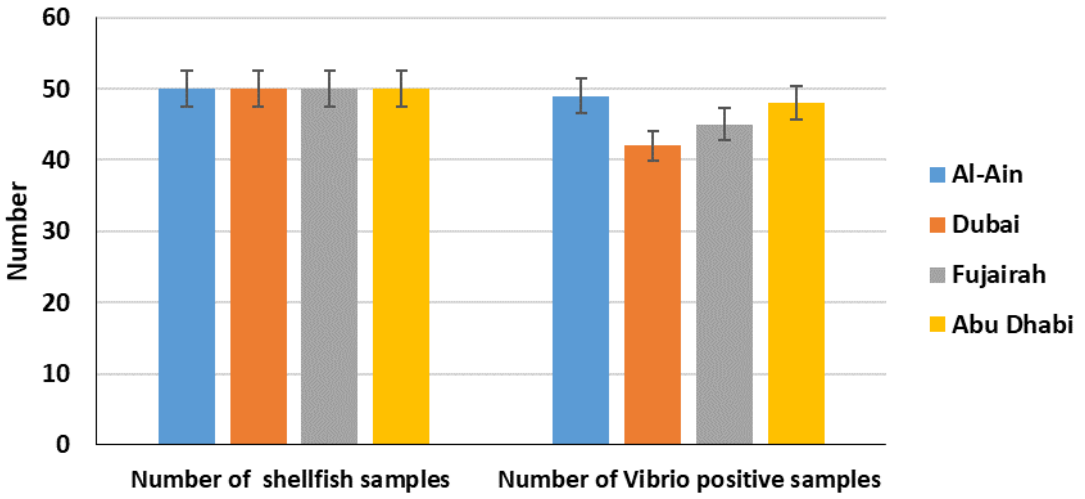


Figure 4.1: Occurrence of *Vibrio* spp., in shellfish

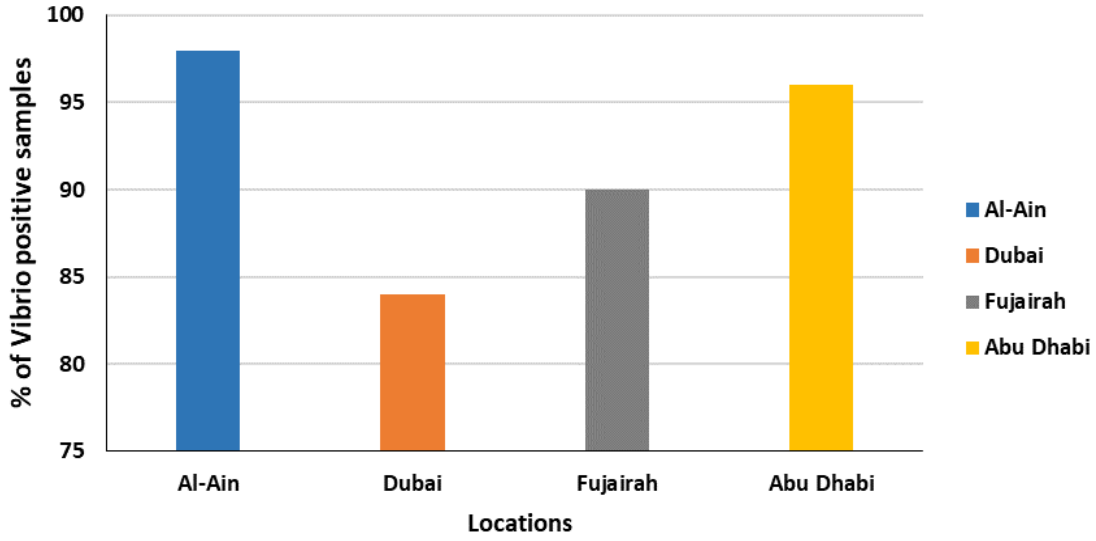


Figure 4.2: Percentage prevalence of *Vibrio* spp., in shellfish

4.2 Molecular Identification of *Vibrio* spp.

Results showed that among the *Vibrio* spp. the prevalence of *Vibrio parahemolyticus* was higher in shellfish samples when compared to *Vibrio mimicus*. *Vibrio vulnificus* was not present in the studied shellfish samples (Table 4.1). An

incidence of 14.12% for *Vibrio paraheamolyticus* was observed in isolates from different cities while for *Vibrio mimicus* the prevalence was only 9.23%. Ghenem and Elhadi (2018) reported that 90% of studied samples from coastal water in the Eastern Province of Saudi Arabia were positive for *Vibrio* spp. and the predominant *Vibrio* spp. in the identified species was *Vibrio parahaemolyticus*. This data is in agreement with the present study. Some studies reported lower infection rates of *Vibrio parahaemolyticus* in seafood. The percentage of *Vibrio parahaemolyticus* in shrimps harvested from Dardanelles Market in Turkey was zero (Colakoglu et al., 2006). Most studies demonstrated a predominance of *Vibrio alginolyticus* in shrimp or seafood samples (Chitov et al., 2009). Chen et al. (2011) found that *Vibrio parahaemolyticus* was the predominant *Vibrio* spp., which is similar to the data in this research. Similar results were reported by Yucel and Balci (2010). *Vibrio parahaemolyticus* were present in the gills, skin and intestine of shellfish as well as other fish samples and overlying water (Amiromazafari et al., 2005). Youssef et al. (2018) reported that overall prevalence of *Vibrio parahaemolyticus* in shellfish was collected from Suez Canal area, Egypt was (9.27%), whereas in water an occurance rate of 12/48 (25%) was observed. The study by Gopal et al. (2005) revealed the dominance of *Vibrio alginolyticus*, followed by *Vibrio parahaemolyticus* in east and west coast of seafood samples from India.

Table 4.1: Prevalence of *Vibrio* spp., in shellfish

***Vibrio* spp.**	**Prevalence of *Vibrio* spp. in different cities**				**% Prevalence**
	Al-Ain	Dubai	Fujairah	Abu Dhabi	
V.paraheamolyticus	6	10	7	3	14.13
V.mimicus	0	0	5	12	9.26
Others	43	32	33	33	76.6

PCR was used for the molecular identification of the *Vibrio* positive isolates. The presence of *Vibrio* spp. was confirmed by using both general and *Vibrio* specific sequences. Recently, many PCR assays have been reported for the identification of the major pathogenic *Vibrio* species (Izumiya et al., 2011). *V16.S* rRNA gene is present in all of the *Vibrio* isolates and could be used as marker genes for specific detection of this bacterium (Zhang and Orth, 2013). Panicker et al. (2004) developed a gene-specific DNA microarray coupled with multiplex PCR for the comprehensive detection of pathogenic *Vibrios* of warm coastal waters and shellfish. A multiplexed real-time PCR assay using four sets of gene-specific oligonucleotide primers and four TaqMan probes labeled with four different fluorophores for detection of total and pathogenic *Vibrio parahaemolyticus*, including the pandemic O3:K6 serotype in oysters were developed. Kim et al. (2006) characterized *V16.S* involved in regulation of gene expression in *Vibrio*. Cluster D. 16S rDNA-based identification was used for the confirmation of *Vibrio paraheamolyticus* present in mussels in Qatar using a specific primer set for *V.*16S, target bands of 370 bp (Alaboudi et al., 2016). Atypical strains of *Vibrio* spp. was identified using 387-bp fragment of chromosomal region with PCR. Occurrence of *Vibrio* spp. has been confirmed using multiplex PCR and *V16.S* rRNA gene in other sea food samples including cockles (50%) from Indonesia (Zulkifli et al., 2009), oysters (44%) from Alaska (Zimmerman, 2007), shellfish (85%) from Chile (Fuenzalida et al., 2007). In this study, presence of *Vibrio* spp. in shellfish samples were atypical in different location. The result also support that the *V.16S*-based approach is a reasonable method to identify the presence of *Vibrio* cluster (Figure 4.3).

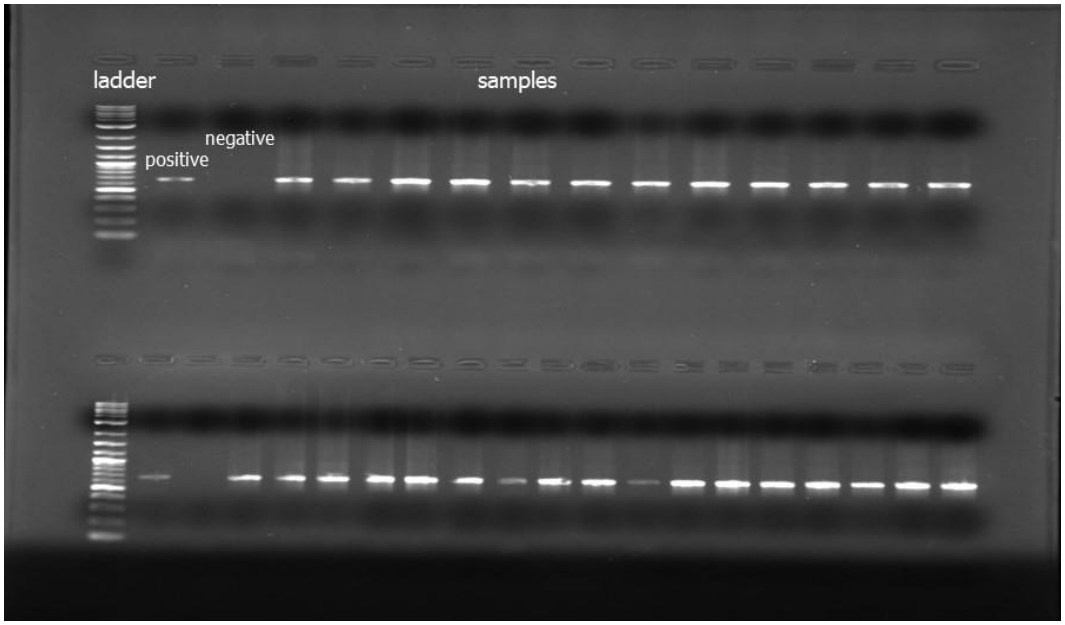


Figure 4.3: Gene amplification profile of *Vibrio* spp.

4.3 Antimicrobial Resistance of *Vibrio* spp.

Vibrio parahemolyticus isolates from shell fish were resistant (100%) to most of the studied antibiotics especially penicillin G, daptomycin and vancomycin. Among the isolates, 26.9% were resistant to ampicillin, 61.53% were resistant to erythromycin while 2 (7.6%) of *Vibrio parahemolyticus* were resistant to Sulfamethoxazole-trimethoprim. Results showed that the *Vibrio mimicus* isolates were 100% resistant to penicillin G, daptomycin and vancomycin. *Vibrio mimicus* isolates showed 5.8% resistance to ampicillin, 94.11% resistance to erythromycin while only 2 *Vibrio mimicus* isolates (11.76%) were resistant to Sulfamethoxazole-trimethoprim (Table 4.2). This finding is in agreement with the results reported by Letchumanan et al. (2015) where 92% of the *Vibrio* isolates from shrimp samples were resistant to penicillin, erythromycin, daptomycin and ampicillin. *Vibrio* isolates in mussels of Qatar showed resistance to antibiotics with the most common resistances were demonstrated towards penicillin (93%), ampicillin (70%), cephalothin (65%),

clindamycin (66%), vancomycin (64%), and erythromycin (51%) (MKurdi Al-Dulaimi et al., 2019). The susceptibility of *Vibrio parahaemolyticus* isolates in oysters from the United States for ampicillin showed decreased exposure (Han et al., 2015). Assessment of antimicrobial susceptibility profile of *Vibrio parahaemolyticus* isolated from short mackerels *(Rastrelliger brachysoma)* in Malaysia revealed majority of the isolates were highly susceptible to ampicillin sulbactam, meropenem, ceftazidime, and imipenem, but resistant to penicillin G and ampicillin (Tan et al., 2017). In cultured seafood products, the *Vibrio parahaemolyticus* isolated were resistant to penicillin G, vancomycin and ampicillin (Elexson et al., 2014). In microbes mainly Gram-negative bacteria, due to the intricacy of their outer membrane which inhibits the passage of antibiotic compounds through the outer membrane. Antimicrobials like penicillin G, vancomycin, daptomycin and erythromycin are ineffectual against *Vibrio* isolates. Sulfamethoxazole-trimethoprim was more effective against the *Vibrio parahemolyticus* isolates while ampicillin was more effective against *Vibrio mimicus* isolates as evidenced by the antibiotic resistance results

Table 4.2: Antimicrobial resistance of *Vibrio* spp.

Antimicrobial Agents	**Disc concentration**	**MIC break point (mm)**			**Number of isolates resistant to antibiotics (%)**		
		S	**I**	**R**	***Vibrio. parahemolyticus***	***Vibrio mimicus***	**Others**
Penicillin G	1 IU	10	11-19	20	26 (100)	17(100)	58(41)
Daptomycin	2 mcg	12	-	13	26 (100)	17(100)	125(88.6)
Vancomycin	30 mcg	14	20	15	26 (100)	17(100)	105(74.46)
Ampicillin	10 mcg	14		15	7 (26.9)	1(5.88)	14(9.9)
Erythromycin	15 mcg	13	18	16	16 (61.53)	16(94.11)	30(21.2)
SXT	25 mcg	13	14-16	17	2(7.6)	2(11.76)	3(2.1)

Results expressed as the number of positive samples; the numbers in bracket indicate the percentage. Disc conc: - Disc concentration, SXT- Sulfamethoxazole-trimethoprim, IU-international units, mcg-microgram. MIC- Minimum Inhibitory Concentration. Breakpoints as recommended by the CLSI M45-A (2010). S, I and R stand for susceptible, intermediate and resistant.

Lee et al. (2019) studied the antibiotic resistance profiles of *Vibrio* isolates of seafood in South Korea from fishery auction markets, fish markets as well as online markets and found that among the twenty-eight samples, three samples were *V parahaemolyticus* positive and were pathogenic and also resistant to ampicillin. Multiple antibiotic resistance was exhibited by *Vibrio* spp. isolated from cultured marine fishes in Malaysia and in all strains showed resistance against ampicillin, penicillin, polypeptides, cephems and streptomycin (Mohamad et al., 2019). Seventy-one *Vibrio* isolates from oysters in Korea during different season showed resistance against 16 antibiotics in which all isolates were resistant to ampicillin and vancomycin, and 52.2%, 50.7%, and 50.7% of isolates exhibited resistance to cephalothin, penicillin and streptomycin (Kang et al., 2016). The *Vibrio parahemolyticus* isolates from Shellfish in Selangor, Malaysia demonstrated 88% resistant to ampicillin, 81% to amikacin, 70.5% to sulphamethoxazole, 73% to cefotaxime, and 51.5% to ceftazidime (Letchumanan et al., 2015). Antibiotic profiling of *Vibrio parahaemolyticus* isolated from raw shellfish in Poland revealed that most isolates were resistant to ampicillin (87.5%) and to streptomycin (70.3%), but all of them were susceptible to tetracycline and chloramphenicol (Lopatek et al., 2015). Jun et al. (2012) reported the anti-microbial resistance of *Vibrio* isolates in Korean sea food which showed resistance against twenty-two commercial antibiotics and all the strains showed resistance to more than four antibiotics. The occurrence of multi-resistance of *Vibrio* to collective antimicrobial agents has been documented from developing countries (Kitaoka et al., 2011).

Daptomycin is an antibiotic with rapid killing, excellent clinical activity and very potent against *S. aureus* with low minimum inhibitory concentrations (Steenbergen et al., 2005). Boss et al. (2016) studied the antimicrobial resistance of

Escherichia coli, Enterococci, Pseudomonas aeruginosa, and *Staphylococcus aureus* from raw fish and seafood imported into Switzerland and the result revealed the highest rates of resistance in *E. coli* to ciprofloxacin (22%), and in *Staphylococus. aureus* to daptomycin (56%). Susceptibility profiles of *Vibrios* to antibiotics such as cefotaxime, imipenem and daptomycin were studied and found some isolates were sensitive to these antimicrobials, which are first-line drugs used in clinical treatment (Akins et al., 2000). The antibiotic resistance patterns of *Vibrio parahaemolyticus* isolates from marine and freshwater fish in Selangor showed the resistance range in the order ampicillin (88%)> daptomycin (64%)> kanamycin (50%) (Lee et al., 2018). Of the 254 isolates of *Vibrio* in Papua New Guinea tested against erythromycin, 97 (38.2%) were resistant while 139 (54.7%) demonstrated intermediate resistance (Murhekar et al., 2013). Similar antimicrobial resistance profiles were also reported in studies using large numbers of *Vibrio parahaemolyticus* isolates from coastal environments (Baker-Austin et al., 2009).

Trimethoprim-sulfamethoxazole acts synergistically against a wide variety of *Vibrio* spp. This antibiotic is a combination of two antimicrobial agents also known as co-trimoxazole. Results showed that *Vibrio* isolates in shellfish imported from different locations were susceptible to sulfamethoxazole-trimethoprim. Susceptibility results of isolates to sulfamethoxazole-trimethoprim was similar with other studies reported in different seafood sources from several countries (Ottaviani et al., 2013). Obaidat et al. (2017) studied the virulence and antibiotic resistance of *Vibrio parahaemolyticus* isolates from seafood of three developing countries and of worldwide environmental, seafood and clinical isolates from 2000 to 2017 and revealed that *Vibrio* isolates showed limited resistance to sulfamethoxazole-trimethoprim. Baker-Austin et al. (2010) reported higher percent intermediate

susceptibility among *Vibrio* isolates against sulfamethoxazole-trimethoprim compared to that of the isolates reported in this study. *Vibrio parahaemolyticus* isolated from shellfish in the Coastal water and sediment of Georgia and South Carolina, USA were susceptible to antibiotics like ampicillin, erythromycin and sulfamethoxazole-trimethoprim (Baker-Austin et al., 2010). Determinations of the minimal inhibitory concentration in liquid media and by agar dilution method showed that classical *Vibrio* strains were uniformly more resistant to sulfamethoxazole than were El Tor strains (Northrup et al., 1972).

4.4 Factors Affecting Growth Rate of *Vibrio* spp.

The more antibiotic resistant *Vibrio* isolates from different locations of UAE were used to study the effect of different factors such as temperature, salinity and pH on survival and growth rate of the bacterium.

4.4.1 Effect of Temperature on Growth Rate of *Vibrio* spp.

Vibrio isolates were incubated at different temperature (25 to 45°C) and the growth rate was determined.

4.4.1.1 Growth Rate of *Vibrio* spp. at 25°C

During the incubation period (0 to 16 h) a gradual increase in growth rate was observed in *Vibrio* isolates. Among the *Vibrio parahemolyticus* isolates, *Vibrio parahemolyticus* 1,2 and 24 attained a maximum growth rate of 80% at 25°C (Figure 4.4 a & d). The growth rate of other *Vibrio parahemolyticus* was in the range of 30% and 68% (Figure 4.4 b & c). *Vibrio mimicus* isolates attained a maximum growth rate of 78% which was showed by *Vibrio mimicus* 3 (Figure 4.4 e) while other *Vibrio*

mimicus isolates showed growth rate between 60% and 70% at 25°C (Figure 4.4 f, g & h).

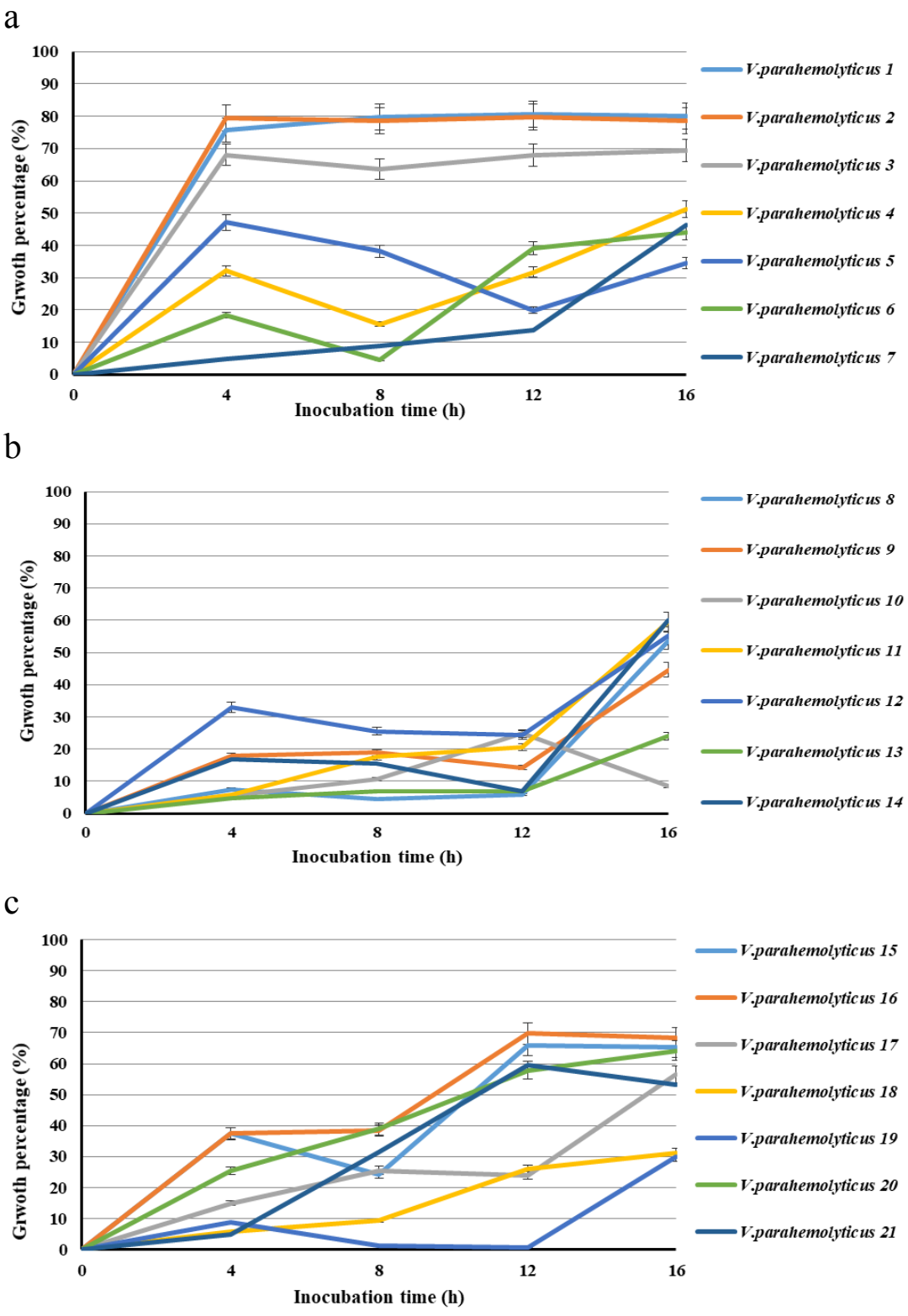
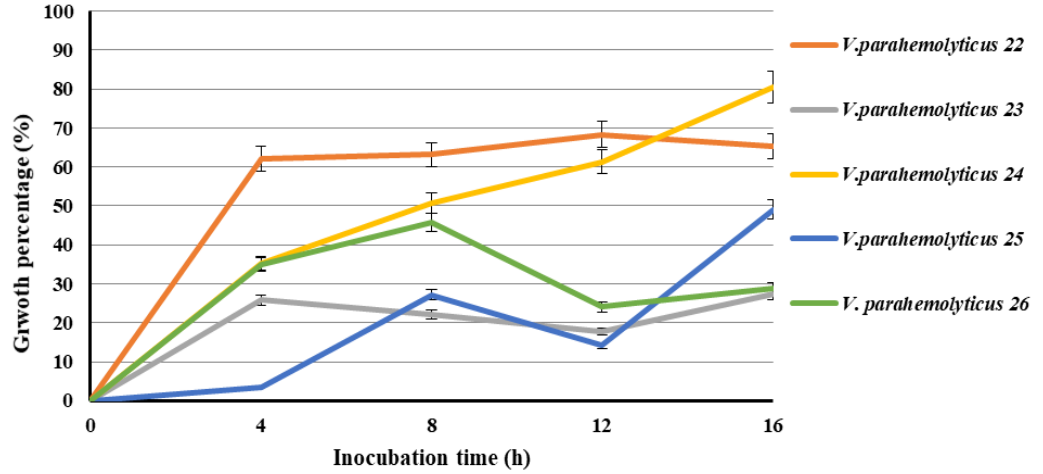


Figure 4.4: Growth rate of *Vibrio parahemolyticus* at 25°C

d



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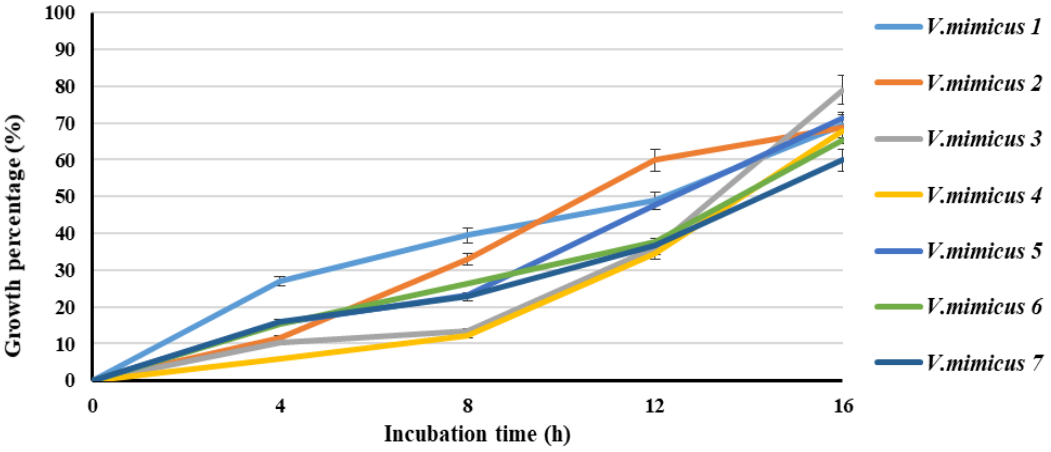

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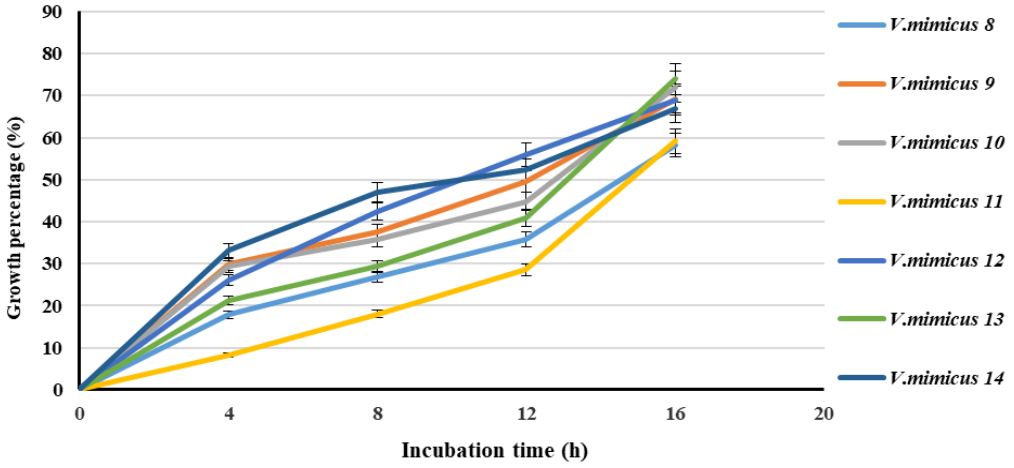


Figure 4.4: Growth rate of *Vibrio mimicus* at 25°C (Continued)

g

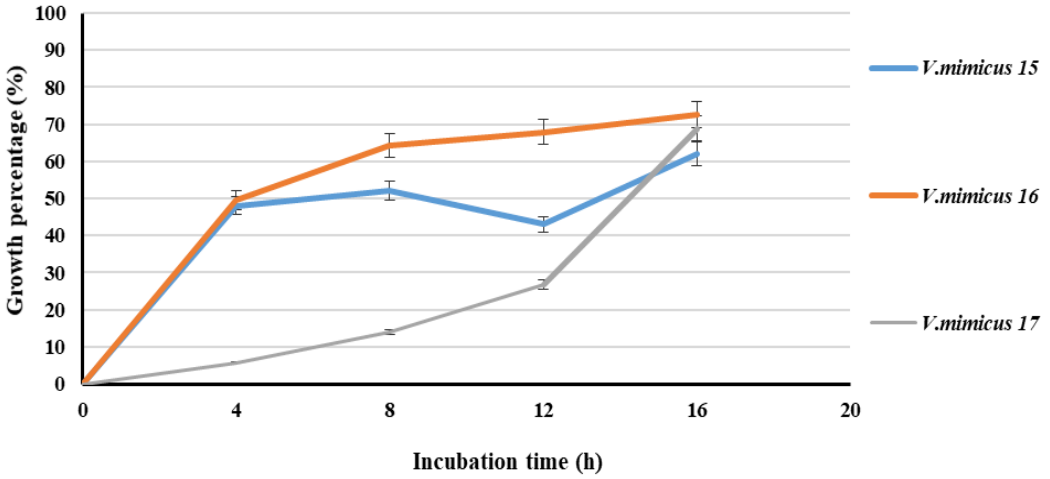
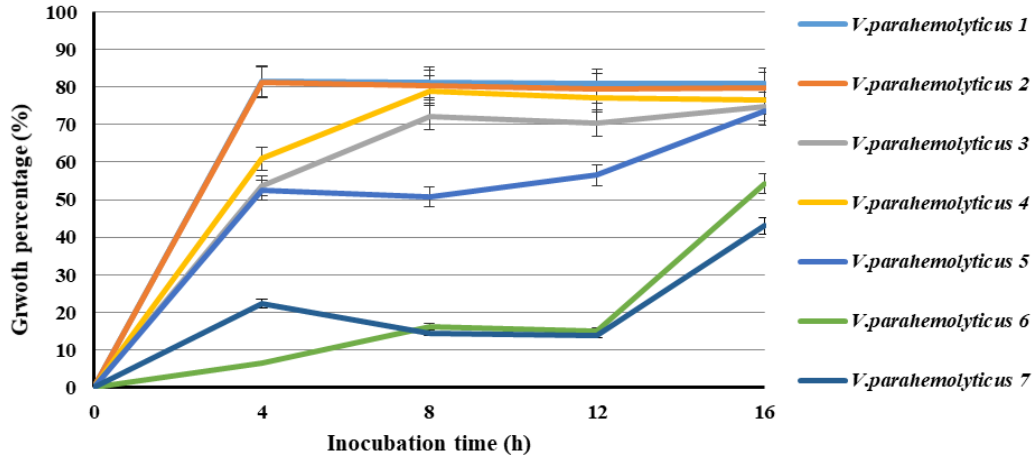


Figure 4.4: Growth rate of *Vibrio mimicus* at 25°C (Continued)
Values are expressed as average of 3 samples ± standard error

4.4.1.2 Growth Rate of *Vibrio* spp. at 37°C

In the present study, results showed that all the two types of isolated *Vibrio* spp. attained maximum growth rate at 37°C. Among the isolates, *Vibrio parahemolyticus* 1, 2, 13, 14 and 24 attained 80% growth rate (Figure 4.5 a, b & d) while 50% of other *Vibrio parahemolyticus* isolates attained a growth rate of above 75% at 37°C (Figure 4.5 a, b, c & d). Among the *Vibrio mimicus* isolates, *Vibrio mimicus* 8 and 12 (Figure 4.5 f) attained maximum growth rate of 86% and 83% respectively while *Vibrio mimicus* 1, 2 (Figure 4.5 e) and 16 (Figure 4.5 g) attained 80% growth rate at 37°C.

a



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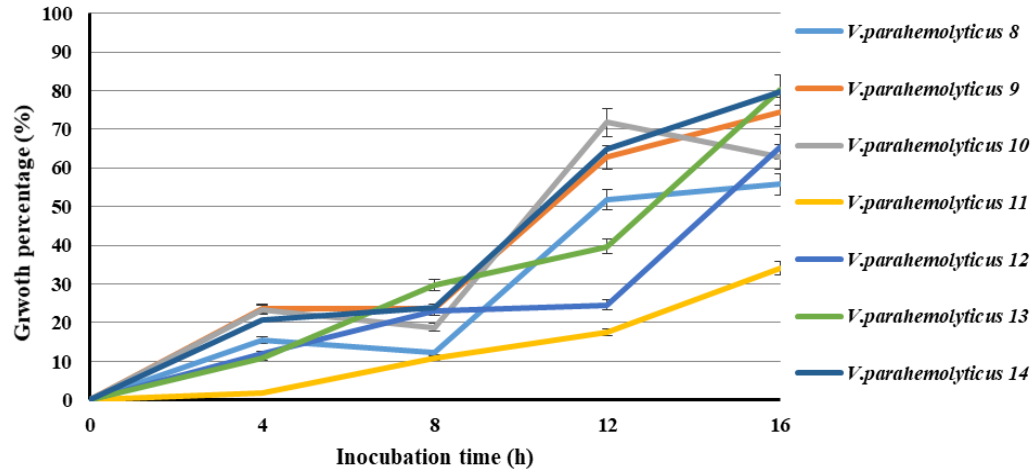

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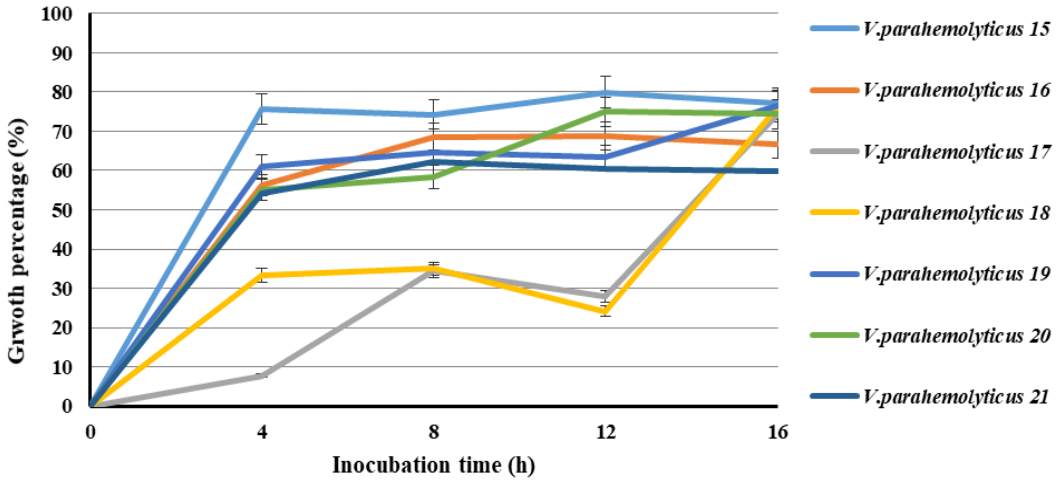
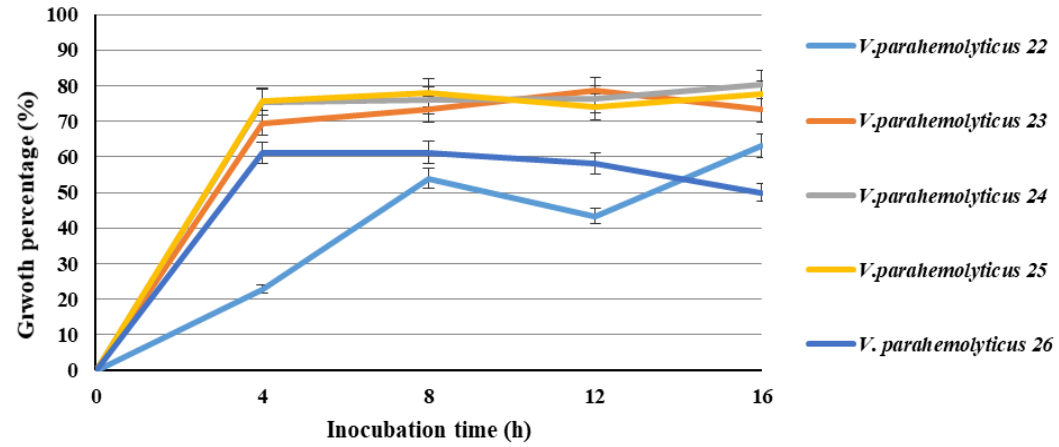


Figure 4.5: Growth rate of *Vibrio parahemolyticus* isolates at 37°C

d



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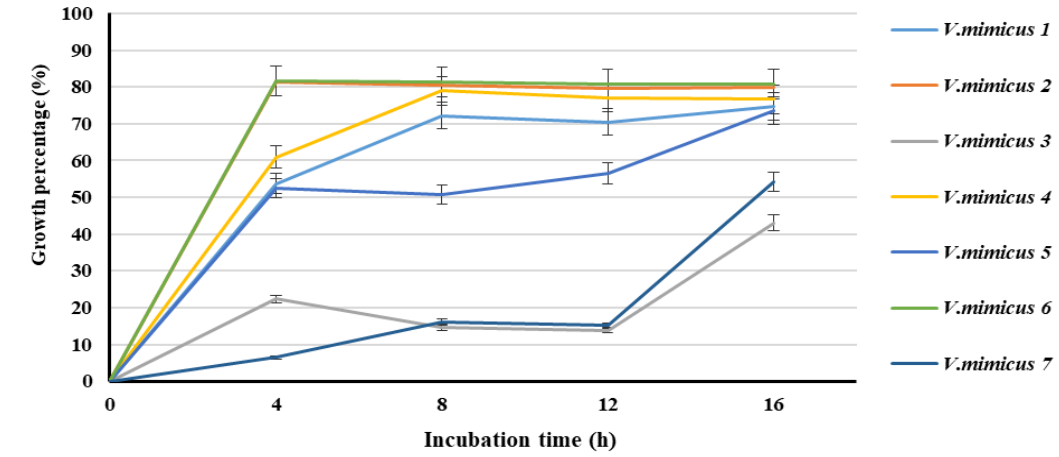

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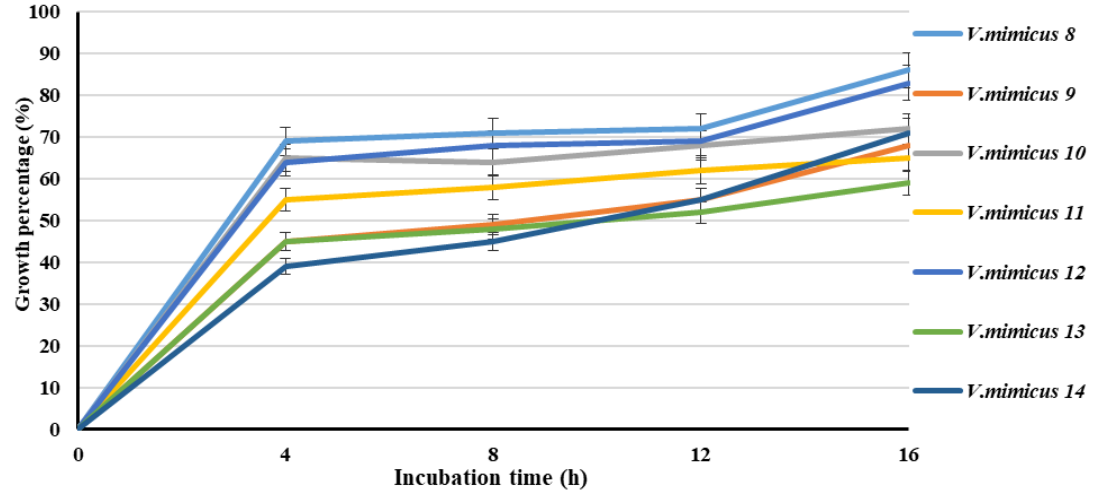


Figure 4.5: Growth rate of *Vibrio mimicus* at 37°C (Continued)

g

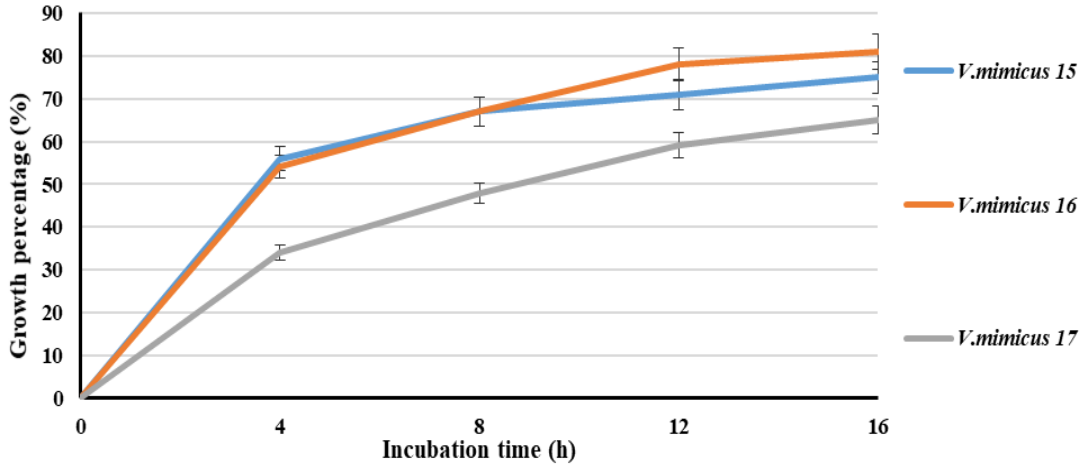
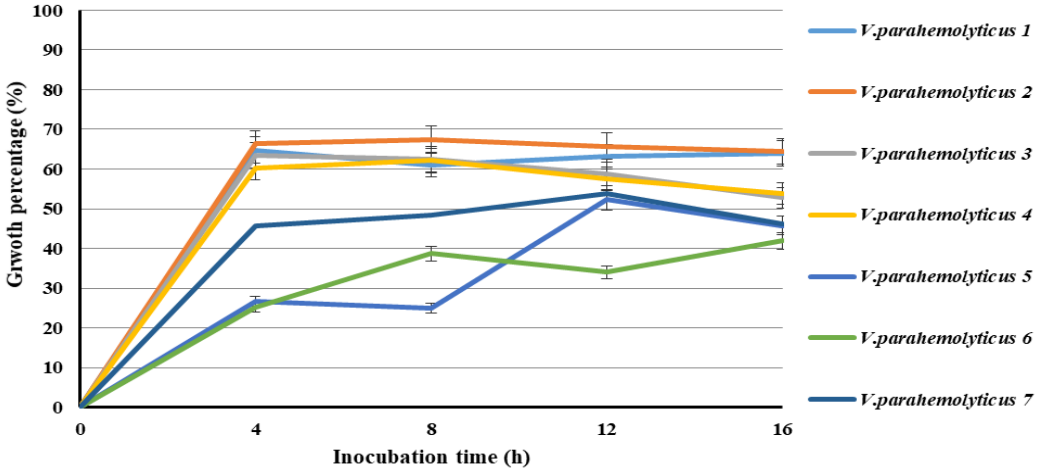


Figure 4.5: Growth rate of *Vibrio mimicus* at 37°C (Continued)
Values are expressed as average of 3 samples ± standard error

4.4.1.3 Growth Rate of *Vibrio* spp. at 45° C

Results showed that *Vibrio* isolates attained decreased growth rate at 45°C when compared to the growth rates at 25°C and 37°C. Among the *Vibrio parahemolyticus* isolates, a growth rate of 74% at 16h was observed in *Vibrio parahaemolyticus* 18 (Figure 4.6 b) and *Vibrio parahaemolyticus* 19 attained 72% growth rate at 45°C (Figure 4.6 d) while *Vibrio parahemolyticus* 14, 23, 24 and 25 attained a growth rate of 70% at 47°C (Figure 4.6 b & d). The maximum growth rate attained by *Vibrio mimicus* at 45°C was 75% by *Vibrio mimicus* 9 and 14 (Figure 4.6 f) and 72% by *Vibrio mimicus* 12 and 16 (Figure 4.6 f & g).

a



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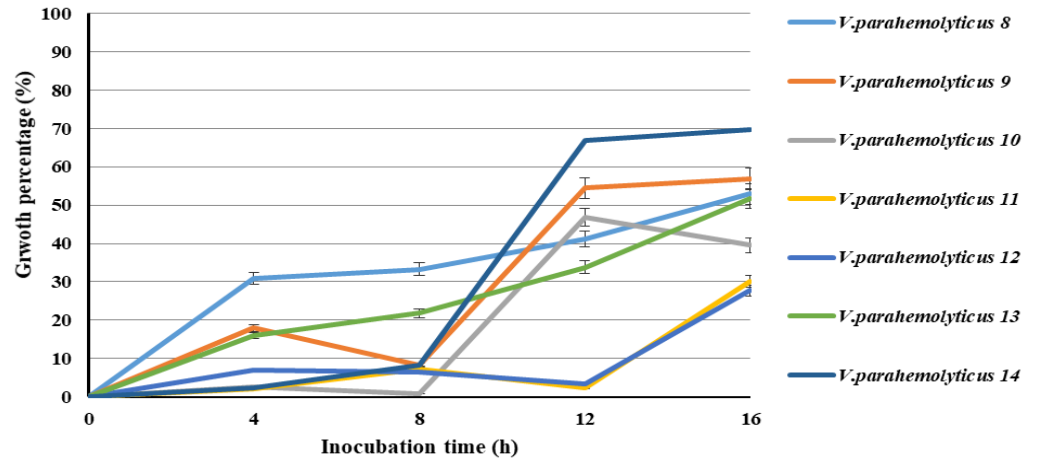

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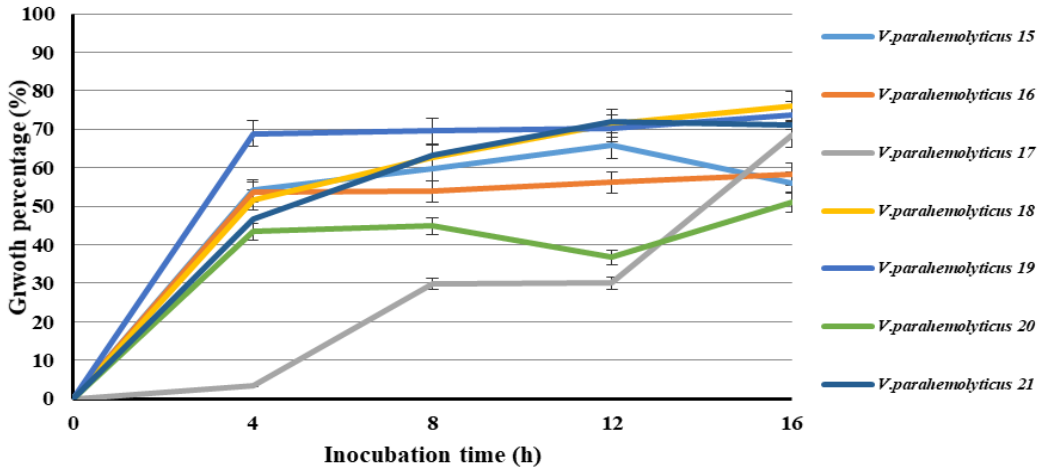
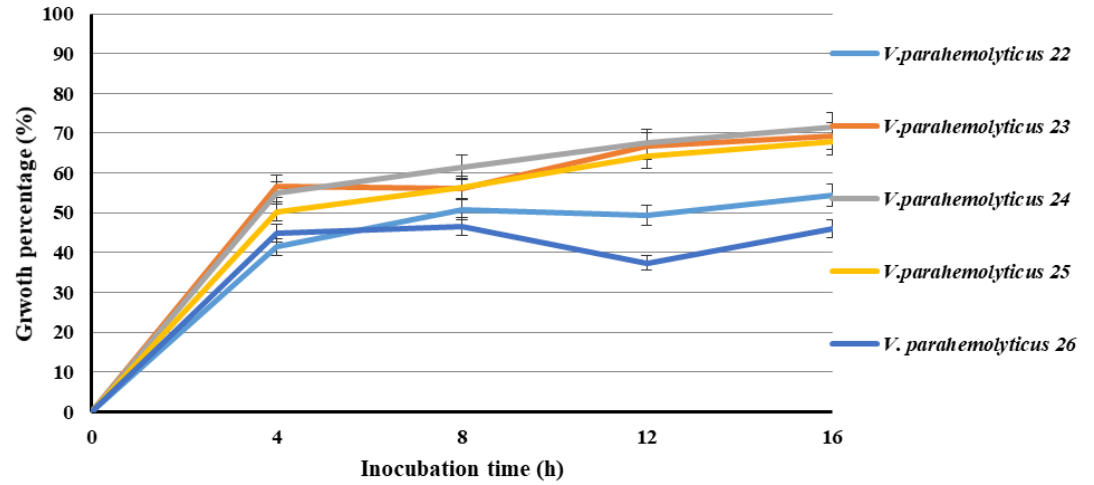


Figure 4.6: Growth rate of *Vibrio parahemolyticus* at 45°C

d



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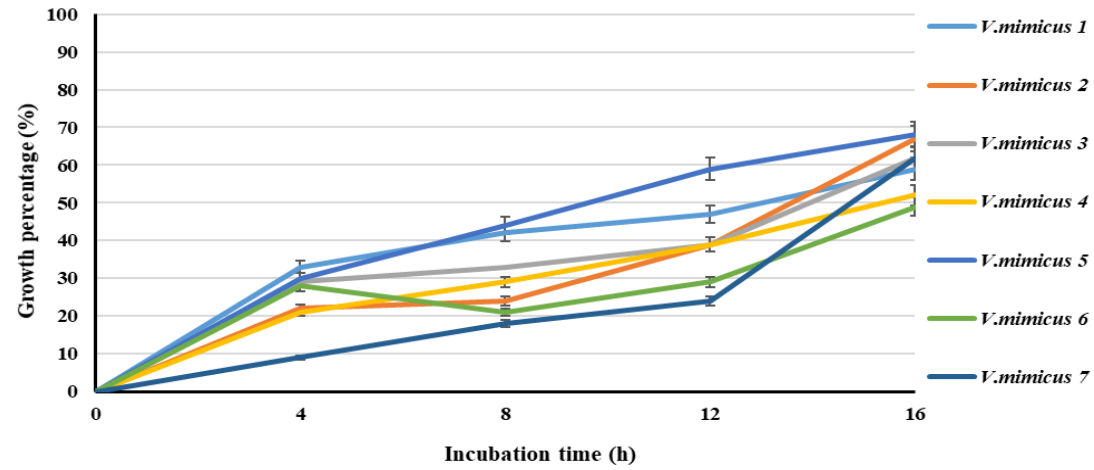

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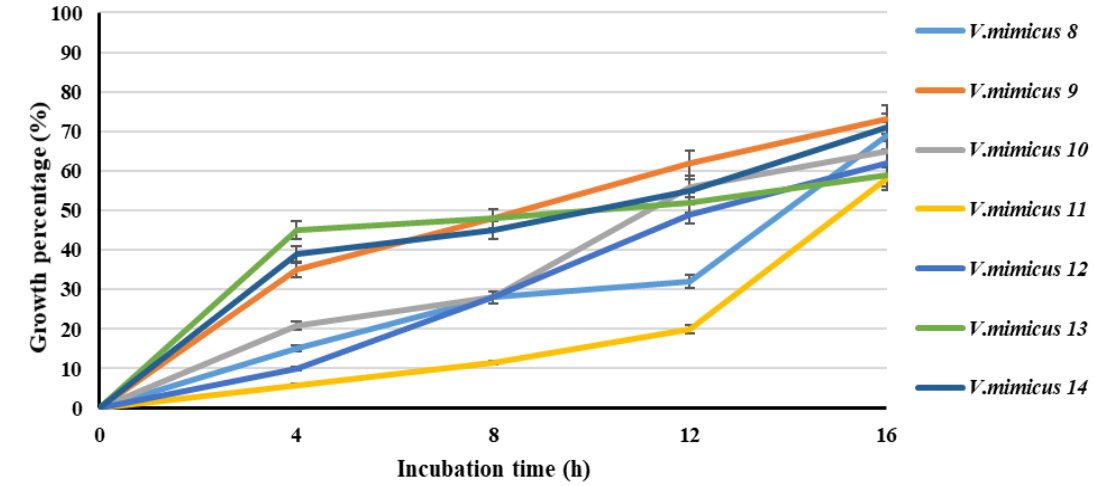


Figure 4.6: Growth rate of *Vibrio mimicus* at 45°C (Continued)

g

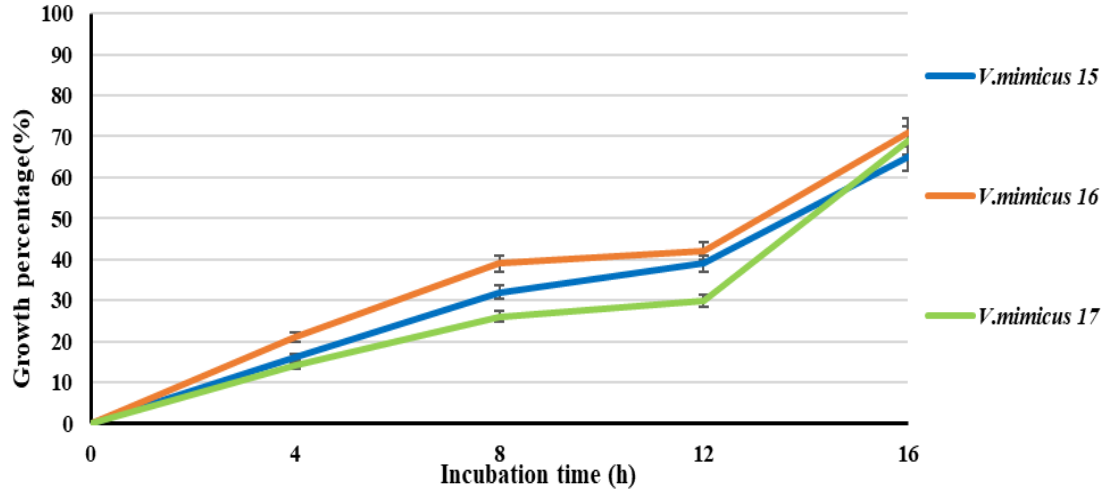


Figure 4.6: Growth rate of *Vibrio mimicus* at 45°C (Continued)
Values are expressed as average of 3 samples ± standard error

Vibrio parahaemolyticus in alkaline peptone water, salt broth increased rapidly when temperature was higher than 15°C, while decreased gradually at 0 and 5°C (Zhang et al., 2014). Yang et al. (2009) inoculated *Vibrio parahaemolyticus* on salmon meat over a temperature range from 0°C to 35°C for studying the growth and survival curves of the *Vibrio* spp. The pathogenic *Vibrio parahaemolyticus* showed continuous growth under 15, 25, and 35°C, while a decline in growth was found under 5°C (Wang et al., 2007). Lopez-Joven et al. (2018) determined if there were any differences in growth and survival of potentially pathogenic *Vibrio parahaemolyticus* and found that *Vibro parahaemolyticus* multiplied rapidly in live clams held at 28°C. Kim et al. (2006) evaluated the growth and survival of *Vibrio* spp. in ready-to-eat seafood such as sashimi and raw oyster meat and found that specific growth rate values between flounder and salmon sashimi were at temperatures ranging from 13°C to 30°C. Research regarding the effect of temperature on the growth of *Vibrio* spp. were also reported by Miles et al. (1997); Yoon et al. (2008) and Fernandez-Piquer et al. (2011).

4.4.2 Effect of pH on Growth Rate of *Vibrio* spp.

The effect of different pH level on growth rate and survival of *Vibrio* spp. was studied. *Vibrio* isolates were incubated at different pH (3- 5) at different time period (0 to 16 h).

4.4.2.1 Growth Rate of *Vibrio* spp. at pH 3.0

At pH 3.0 most of the *Vibrio parahemolyticus* isolates showed decreased growth rate in which *Vibrio parahemolyticus* 19 attained a highest growth rate of 60% (Figure 4.7 c). The growth rate of other *Vibrio parahemolyticus* isolates are less than 50% at pH 3.0. *Vibrio mimicus* also attained decreased growth rate at pH 3.0 which was 62% by *Vibrio mim*icus 2, 8 and 16 (Figure 4.7 f, g & h). Other *Vibrio mimicus* isolates exhibited a growth rate of less than 50%.

a

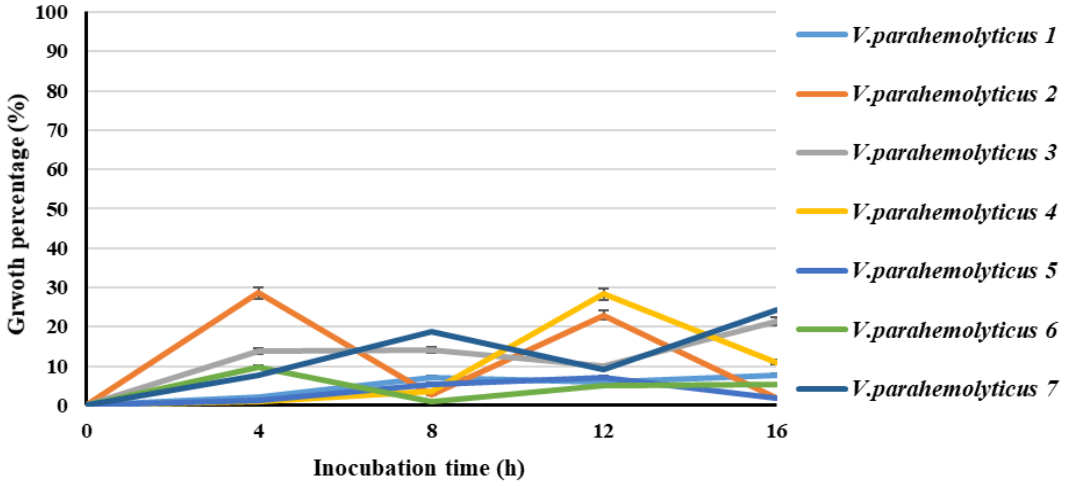
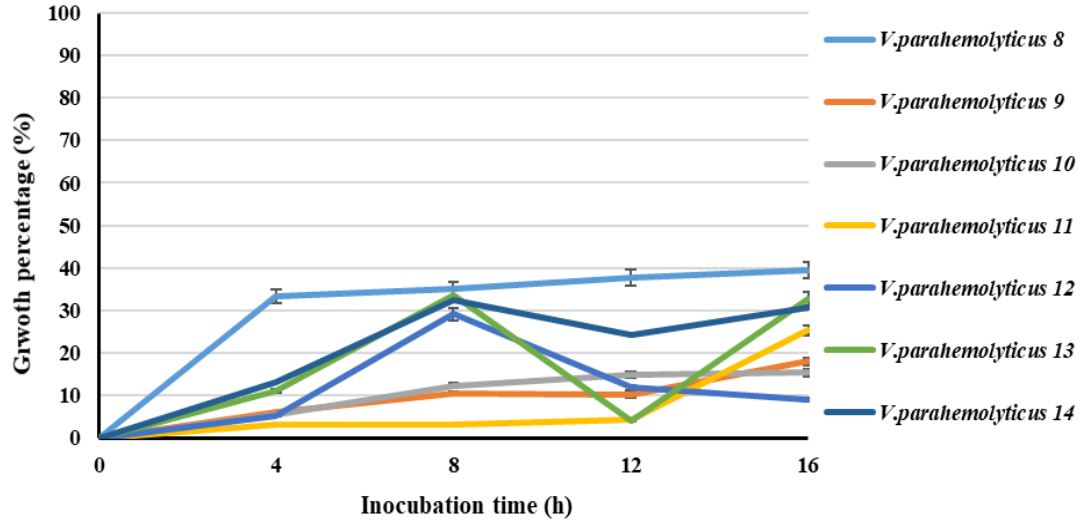


Figure 4.7: Growth rate of *Vibrio parahemolyticus* at pH 3.0

b



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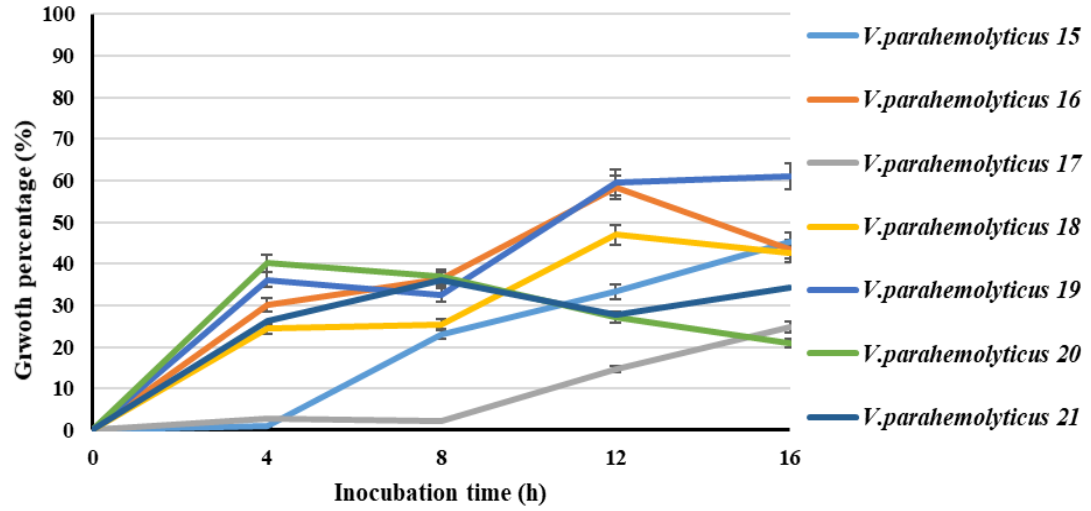

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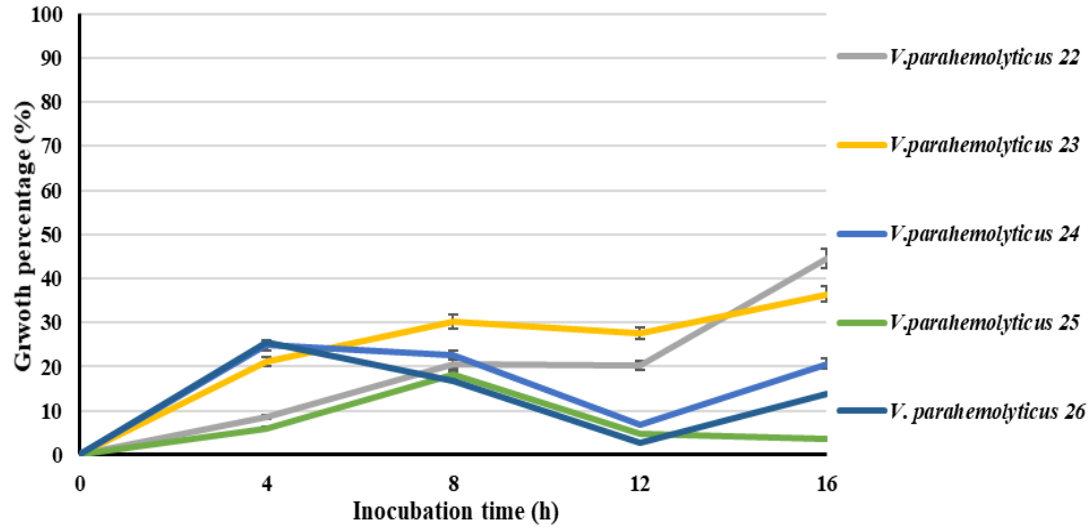
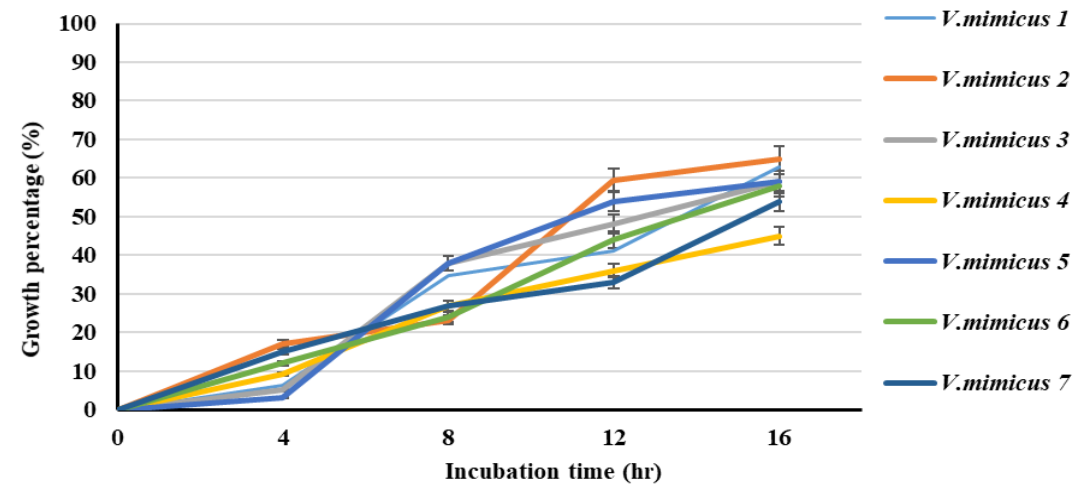


Figure 4.7: Growth rate of Vibrio mimicus at pH 3.0 (Continued)

e



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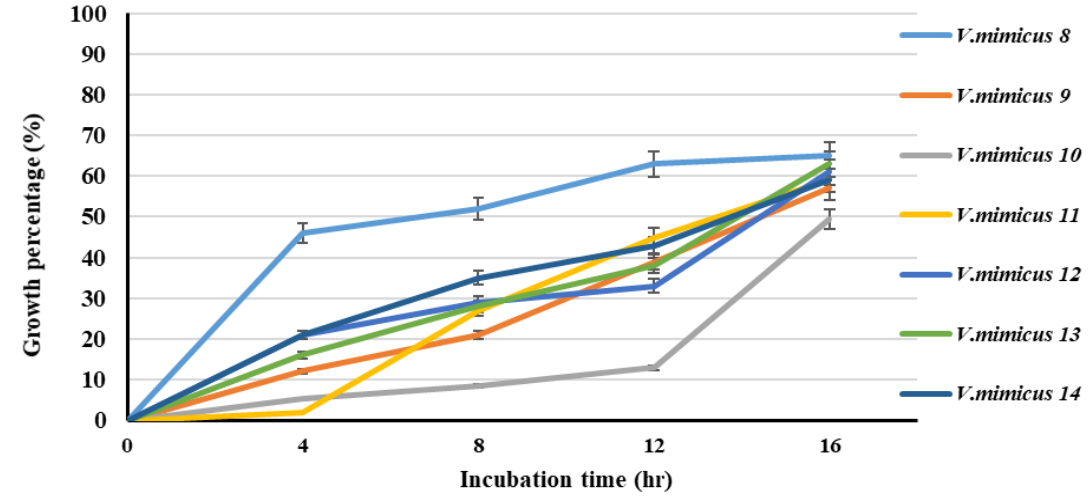

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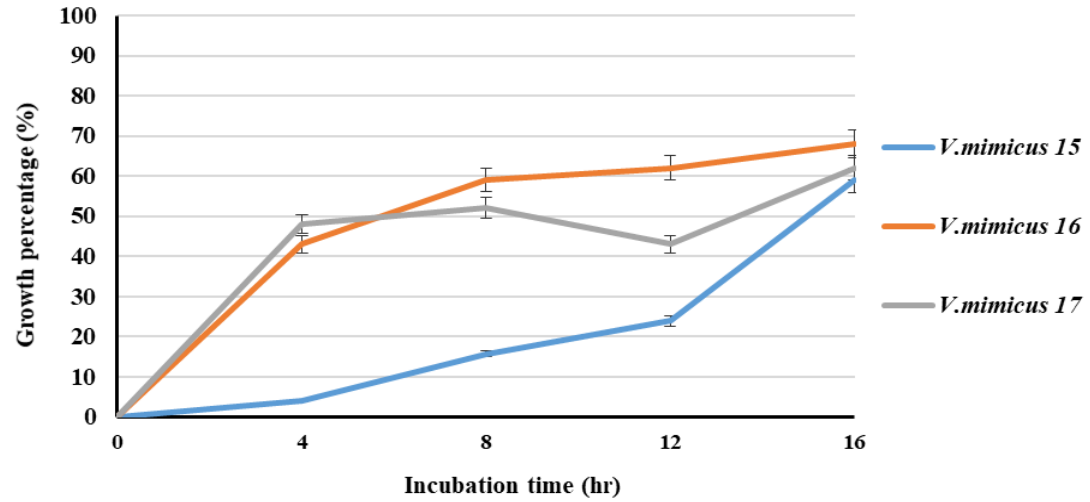


Figure 4.7: Growth rate of *Vibrio mimicus* at pH 3.0 (Continued)
Values are expressed as average of 3 samples ± standard error

4.4.2.2 Growth Rate of *Vibrio* spp. at pH 5.0

Results showed that there was a slight increase in growth percentage of *Vibrio* isolates when the pH of the system was increased from pH 3.0 to pH 5.0. Among the *Vibrio parahemolyticus* isolates, *Vibrio parahemolyticus* 2 (Figure 4.8 a), *Vibrio parahemolyticus* 12 (Figure 4.8 b) and *Vibrio parahemolyticus* 22 (Figure 4.8 d) showed maximum growth rate of 65% at pH 5.0. At pH 5.0 *Vibrio mimimicus* isolate 1 (Figure 4.8 e) attained 60% growth rate, *Vibrio mimimicus* 12 (Figure 4.8 f) attained 65% growth rate while *Vibrio mimicus* 15, 16 & 17 (Figure 4.8 g) exhibited maximum growth rate of 60%.

a

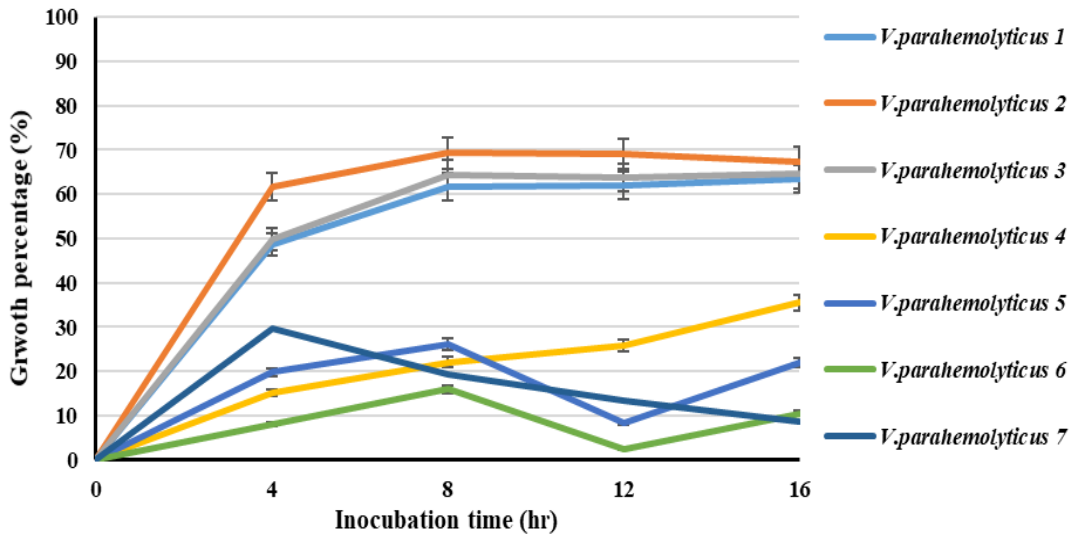
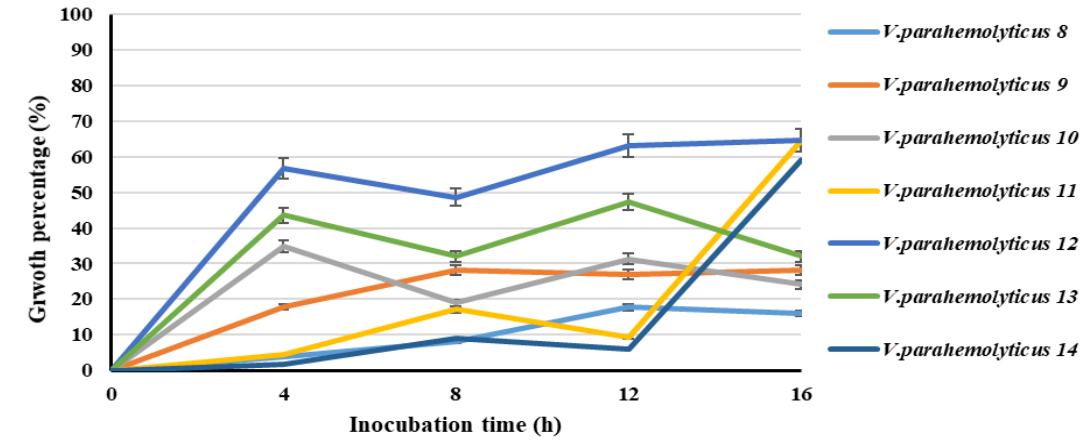


Figure 4.8: Growth rate of Vibrio parahemolyticus at pH 5.0

b



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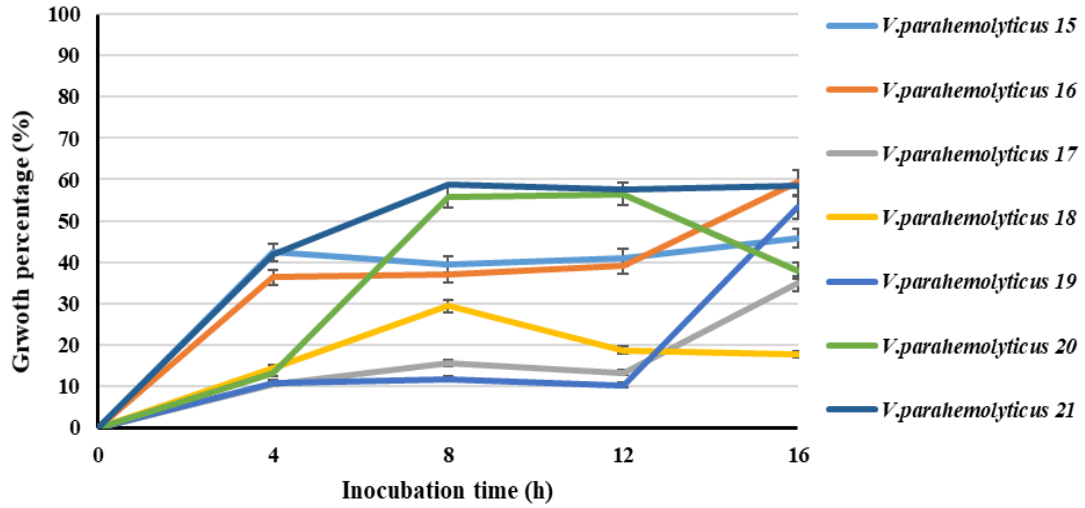

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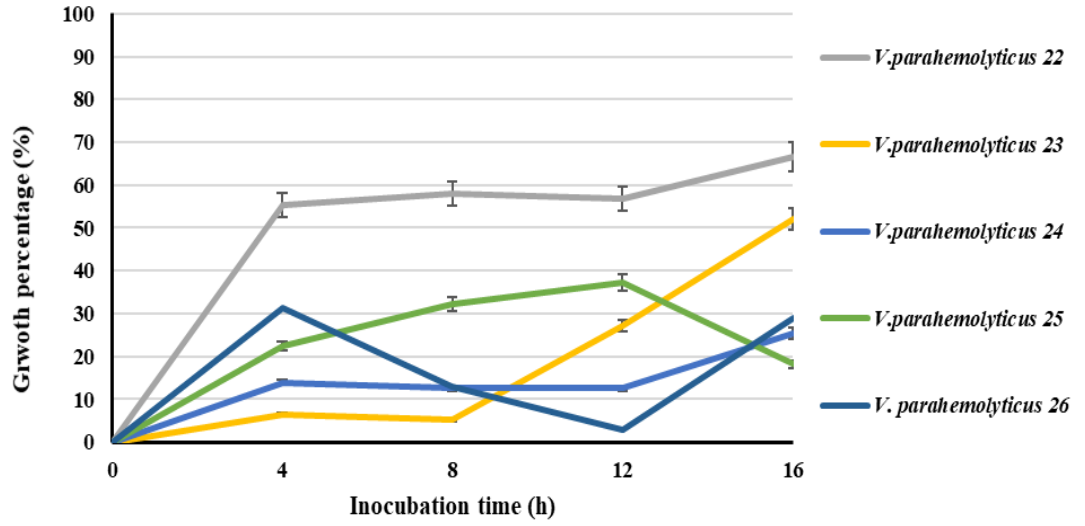
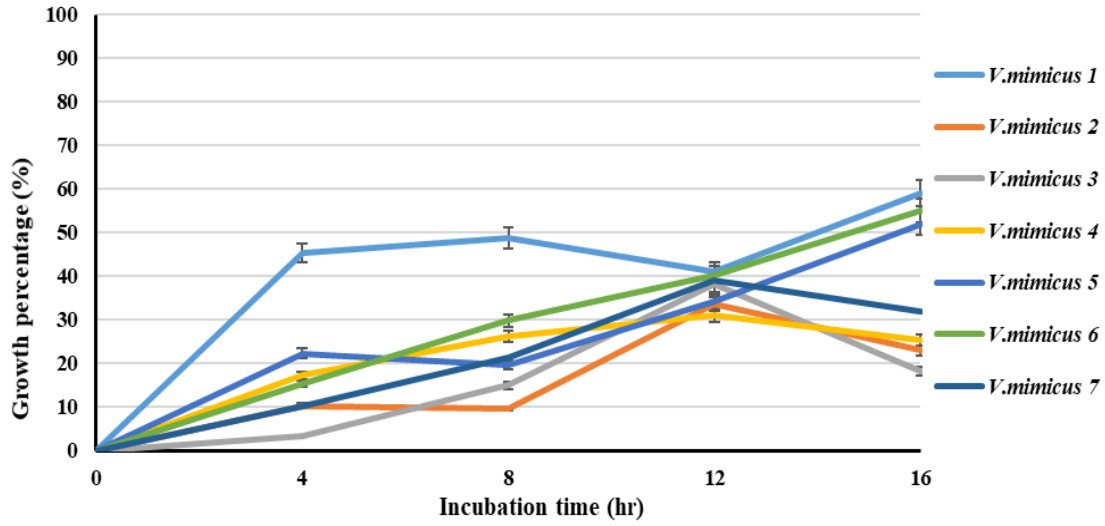


Figure 4.8: Growth rate of *Vibrio parahemolyticus* at pH 5.0 (Continued)

e



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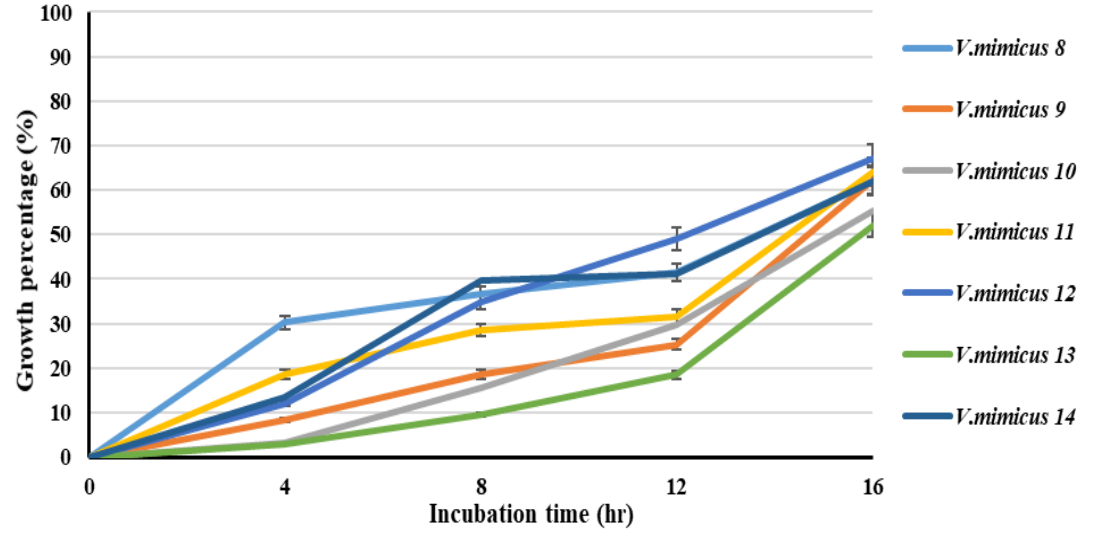

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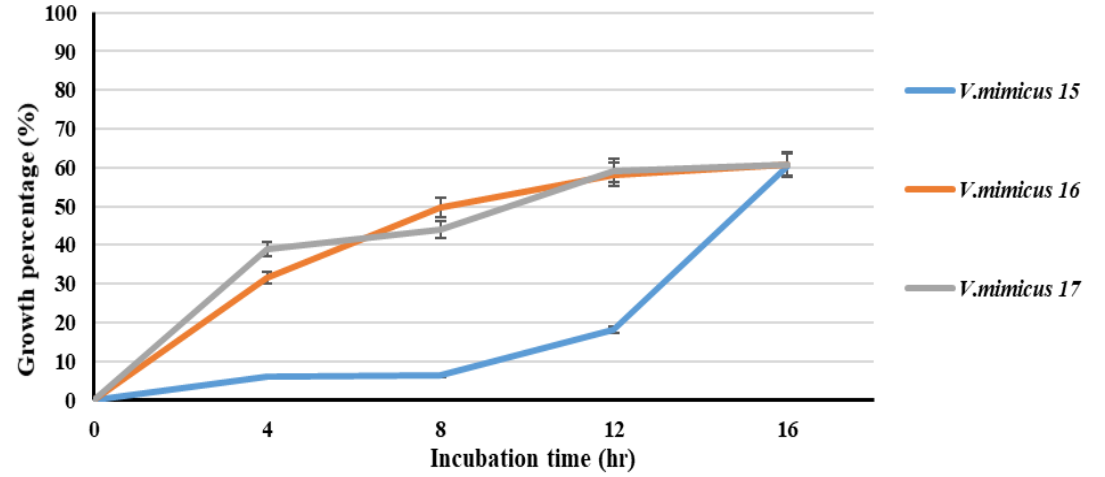
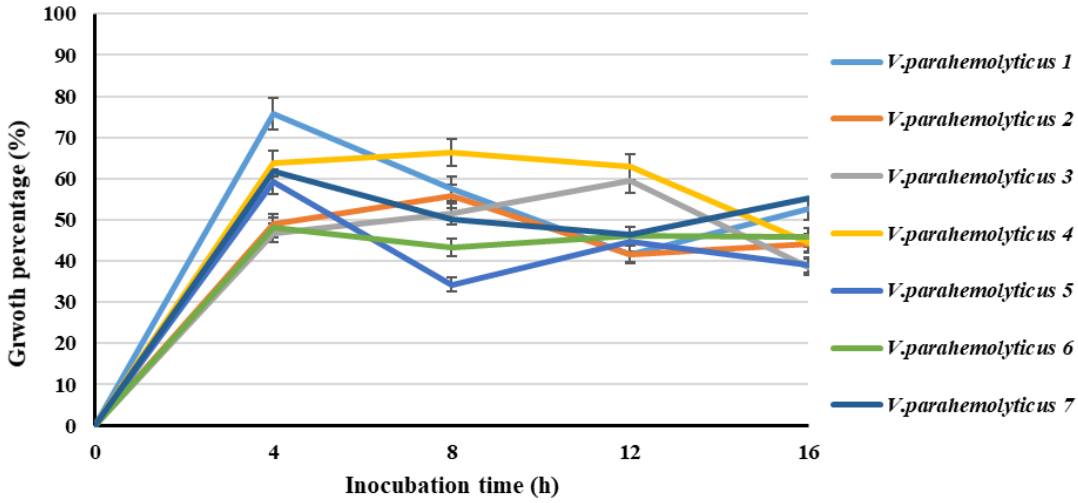


Figure 4.8: Growth rate of *Vibrio mimicus* at pH 5.0 (Continued)
Values are expressed as average of 3 samples ± standard error

4.4.2.3 Growth Rate of *Vibrio* spp. at pH 7.0

At pH 7.0, *Vibrio parahemolyticus* attained a maximum growth rate of 78%. *Vibrio parahaemolyticus* 19, 23 (Figure 4.9 c & d) showed 78% growth rate on 16 h of incubation. The growth rate of other *Vibrio parahemolyticus* isolates were in the range of 40 to 60%. *Vibrio mimicus* 12, 13 (Figure 4.9 f & g) showed a growth rate of 74% while *Vibrio mimicus* 16 attained 72% growth rate which were the highest growth rate of *Vibrio mimicus* isolates.

a



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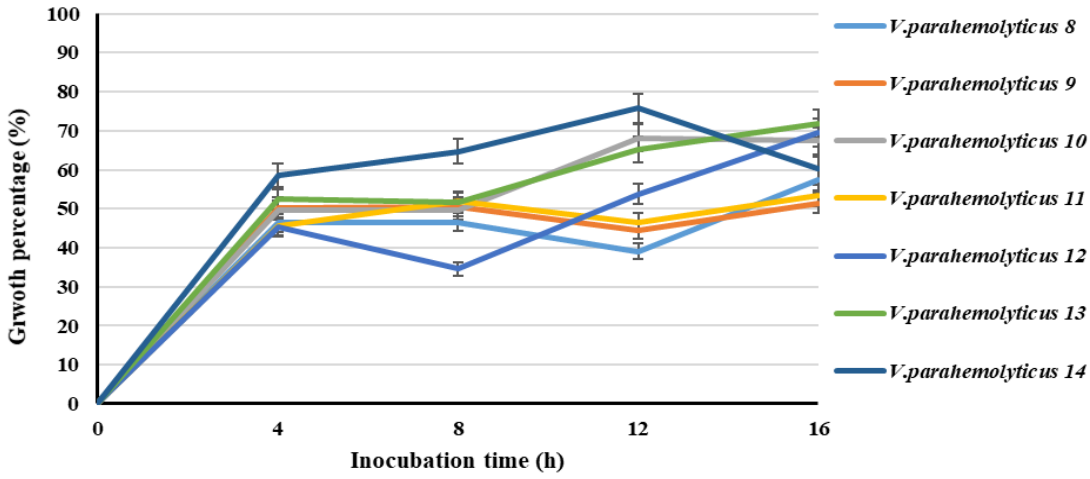
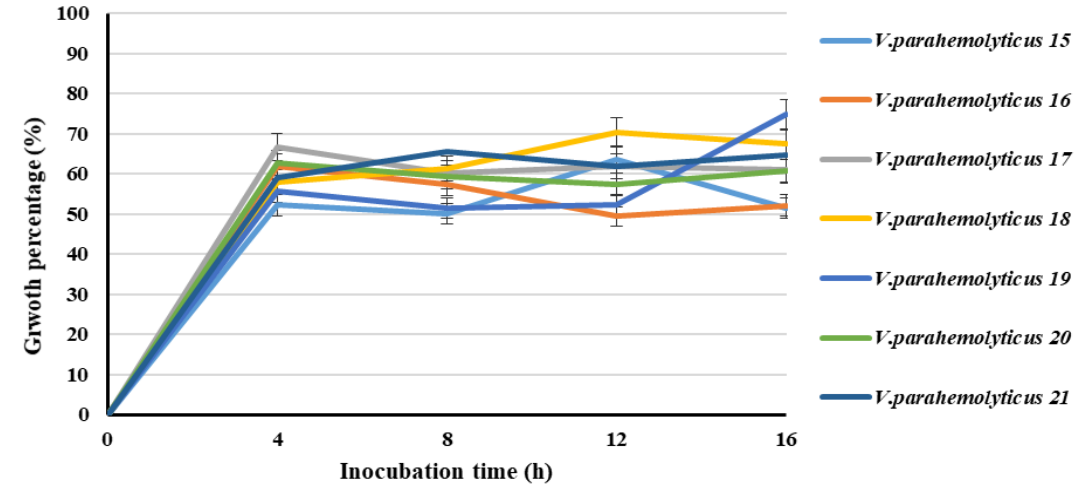


Figure 4.9: Growth rate of *Vibrio parahemolyticus* at pH 7.0

c



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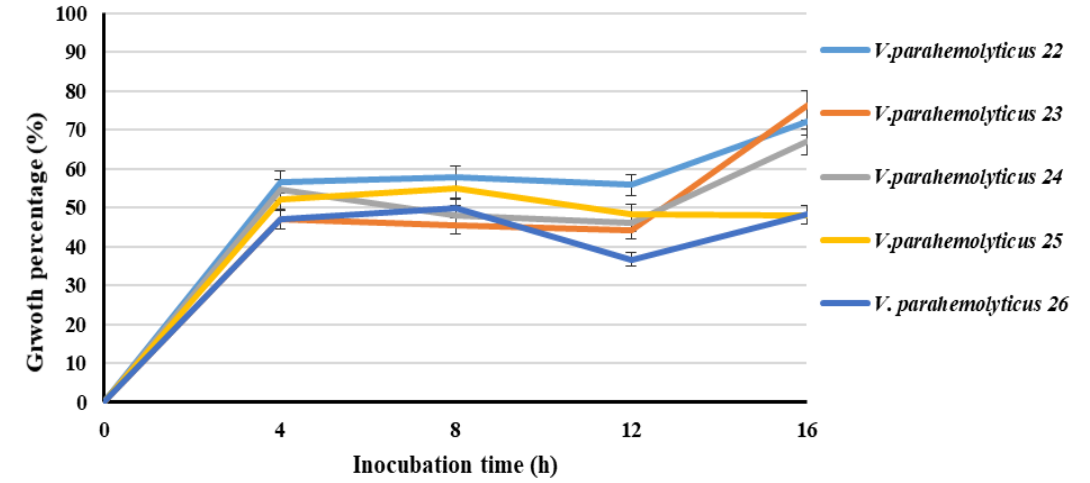

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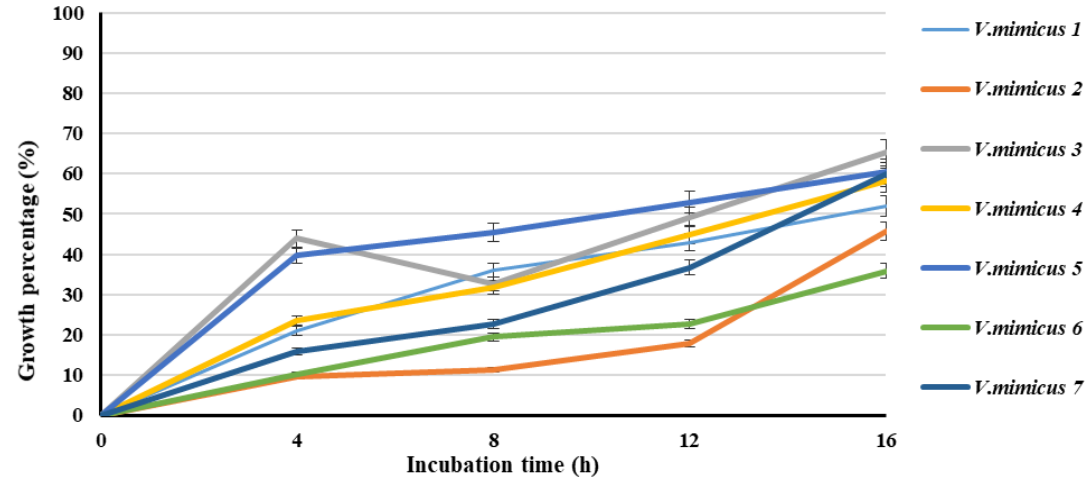
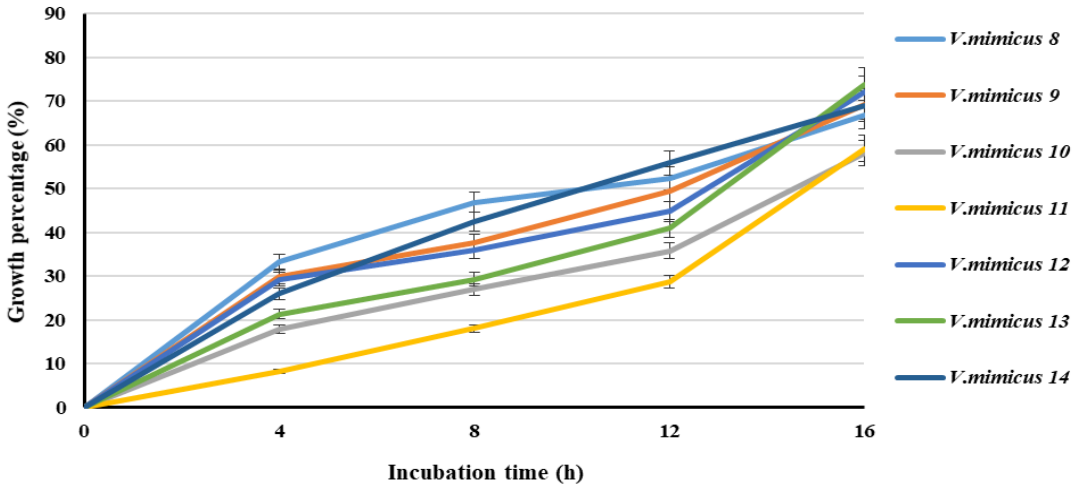


Figure 4.9: Growth rate of Vibrio mimicus at pH 7.0 (continued)

f



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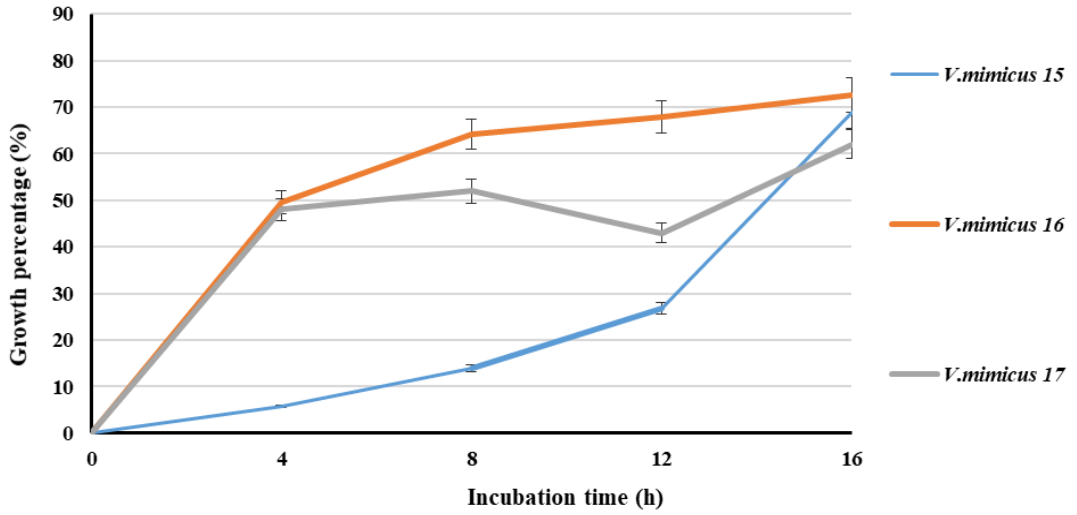


Figure 4.9: Growth rate of *Vibrio mimicus* at pH 7.0 (continued)
Values are expressed as average of 3 samples ± standard error

The *Vibrio* spp. isolated from prawn (*Penaeus monodon*) seafood grows best at alkaline pH and the maximum growth rate was observed at pH 9 (Yaashikaa et al., 2016). *Vibrio parahaemolyticus* was able to grow at pH 5.0 to pH 11, and at NaCl concentrations of 1 to 7% (Twedt, 1969). Beuchat (1973) studied the influence of growth conditions on survival rate of six strains of *Vibrios* and found that the lowest pH permitting growth was pH 7.3.

4.4.3 Effect of NaCl on Growth Rate of *Vibrio* spp.

The growth rate and survival of *Vibrio* spp. was studied at different salinity level. *Vibrio* isolates were incubated with different concentration of NaCl (0.5% to 2%) at different time period (0 to 16 h)

4.4.3.1 Growth Rate of *Vibrio* spp. at 0.5% NaCl

Vibrio parahaemolyticus isolates *Vibrio parahemolyticus* 15 and 17 (Figure 4.10 c) showed growth rate of 80% while *Vibrio parahemolyticus* 4, 5 (Figure 4.10 a) attained a growth rate of 70% at 0.5% NaCl which were the highest growth rate of *Vibrio parahemolyticus* isolates. *Vibrio mimi*cus 5 (Figure 4.10 e) and *Vibrio mimicus* 7 showed highest growth rate of 83% at 0.5% NaCl concentration (Figure 4.10 e). The growth rate of other *Vibrio mimicus* isolates were less than 75%.

a

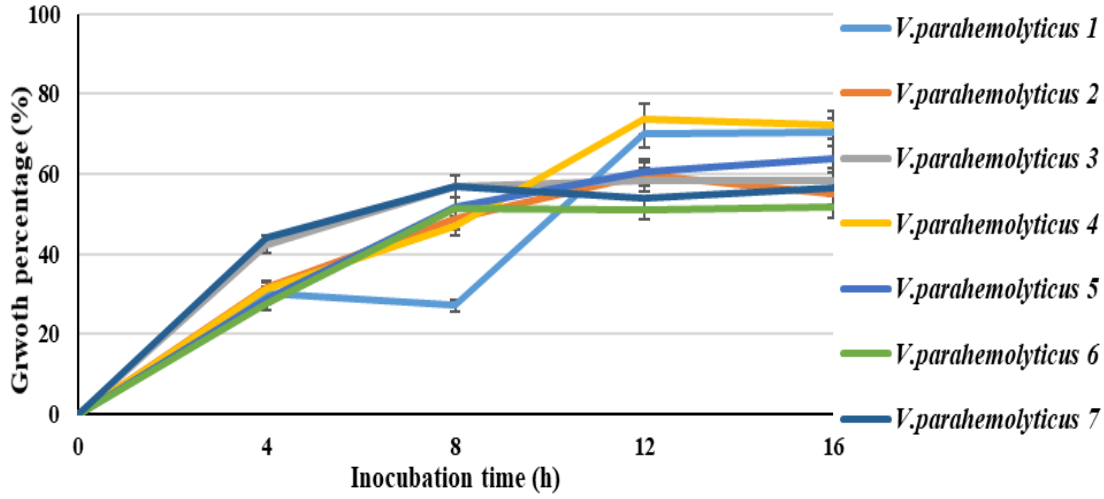
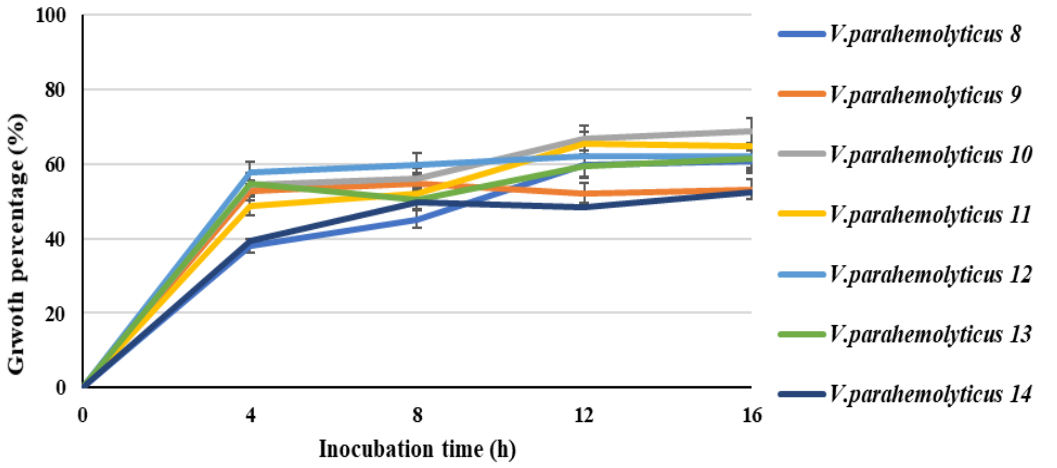


Figure 4.10: Growth rate of Vibrio parahemolyticus at 0.5% NaCl

b



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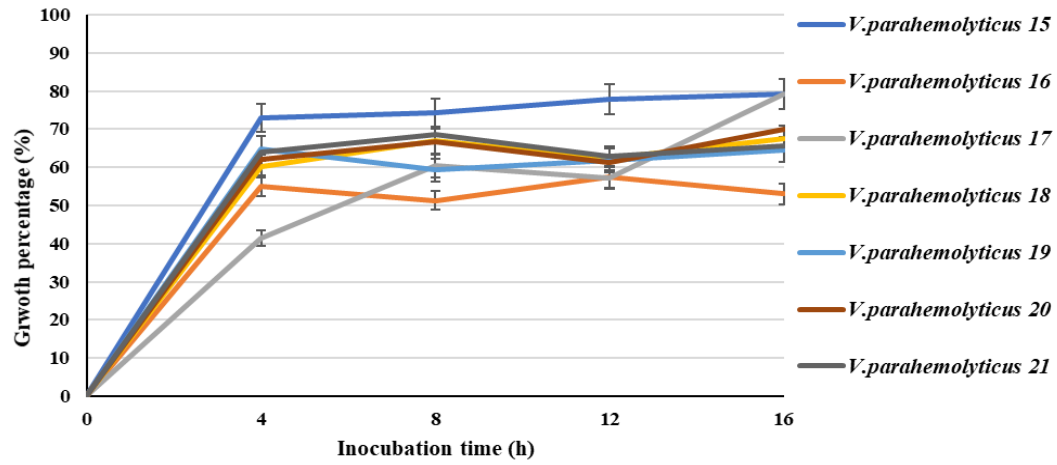

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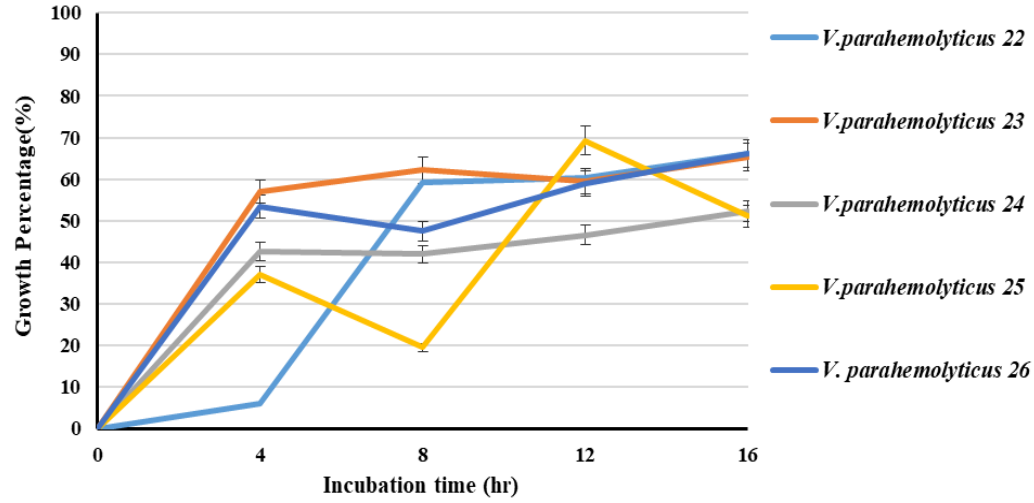
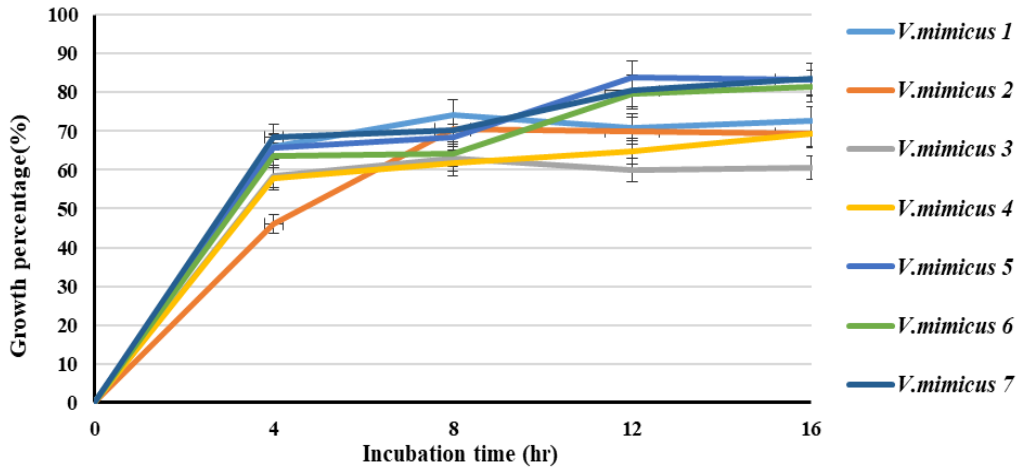


Figure 4.10: Growth rate of Vibrio mimicus at 0.5% NaCl (Continued)

e



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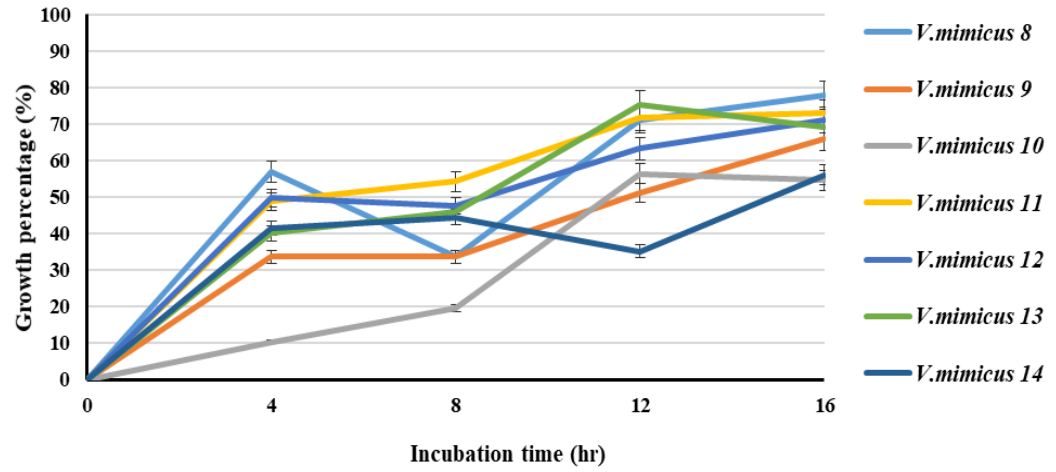

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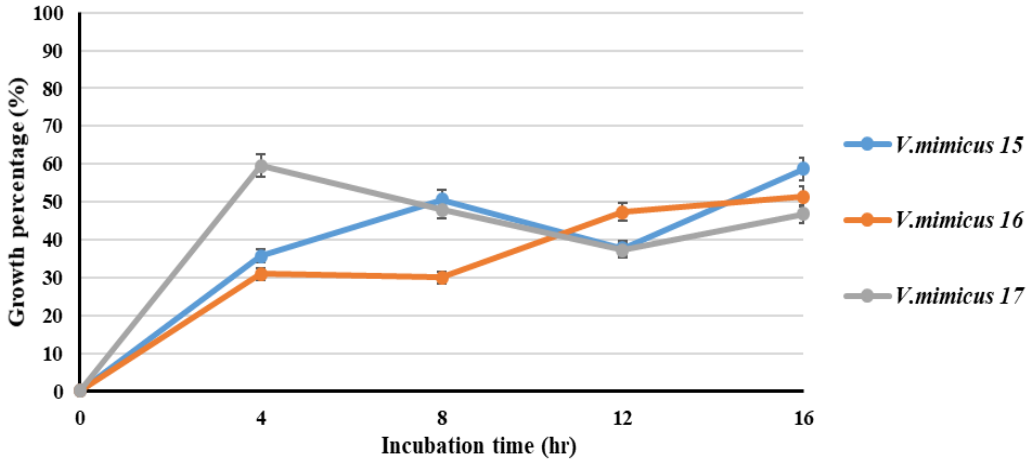


Figure 4.10: Growth rate of *Vibrio mimicus* at 0.5% NaCl (Continued)
Values are expressed as average of 3 samples ± standard error

4.4.3.2 Growth Rate of *Vibrio* spp. at 1% NaCl

At 1% NaCl, *Vibrio* isolate *Vibrio parahemolyticus* 11, 14, 18, 19, 23 and 26 (Figure 4.11 b, c & d) showed highest growth rate of 82%. The growth rate of other *Vibrio parahemolyticus* isolates were in between 60% and 75%. *Vibrio mimicus* 5, 6, 7 and 17 (Figure 4.11 e & g) showed a growth rate of 87% when compared to other *Vibrio mimicus* isolates. The growth rate of other *Vibrio mimicus* isolates were between 65% and 84% respectively (Figure 4.11 e, f & g)

a

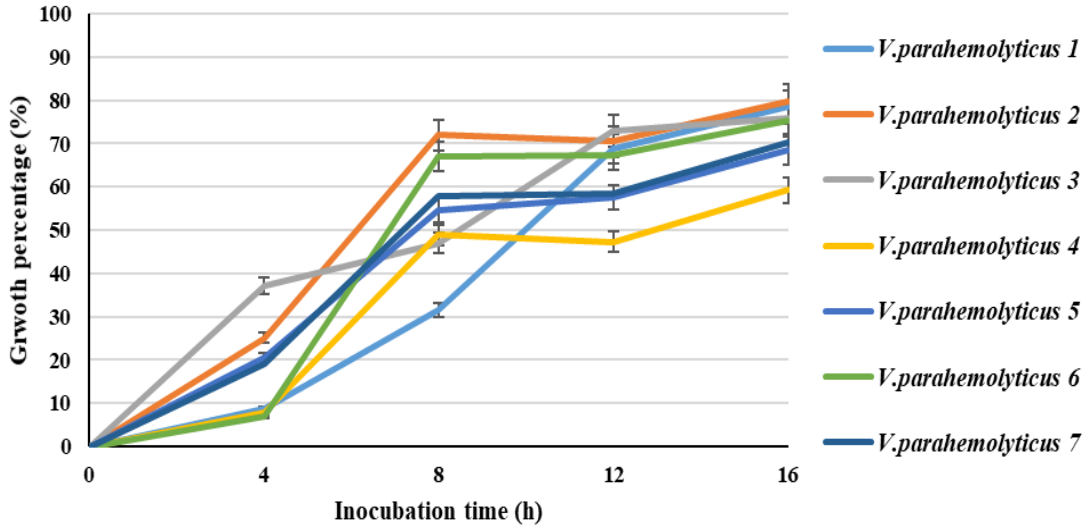
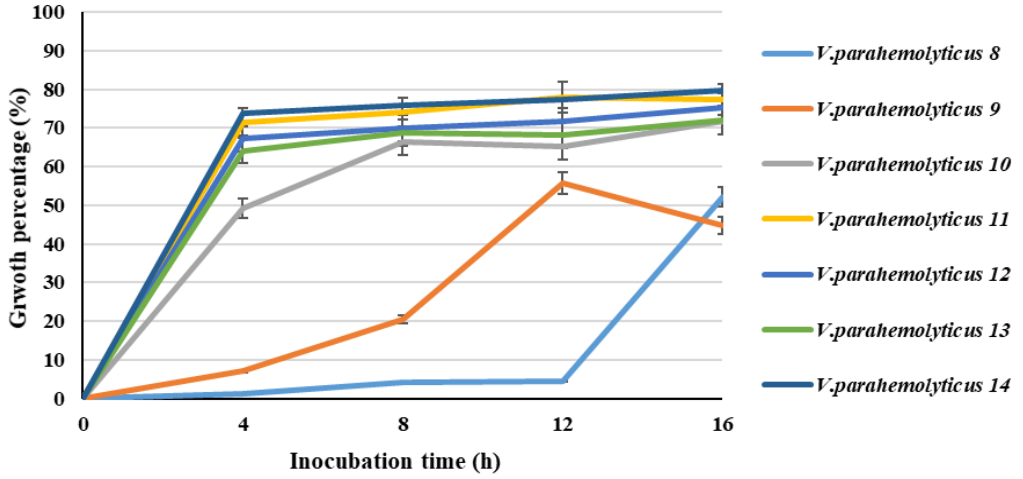


Figure 4.11: Growth rate of Vibrio parahemolyticus at 1.0% NaCl

b



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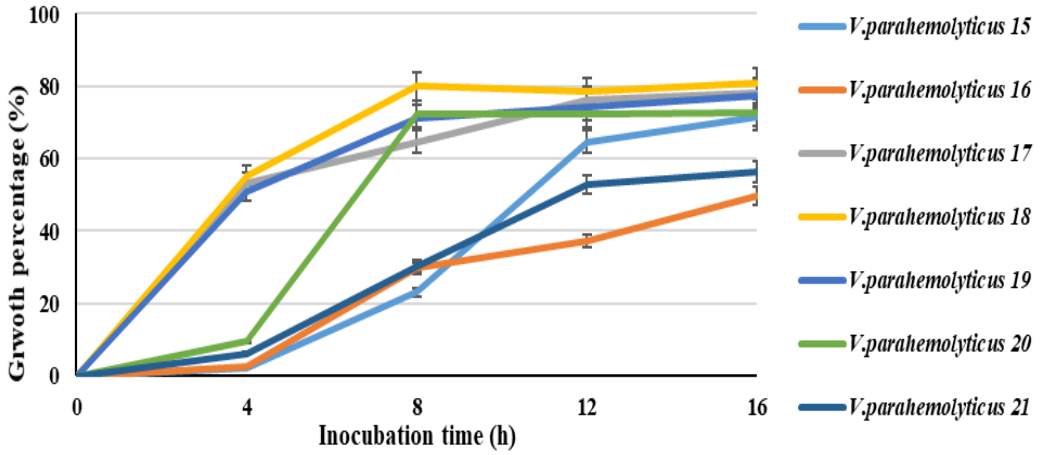


d

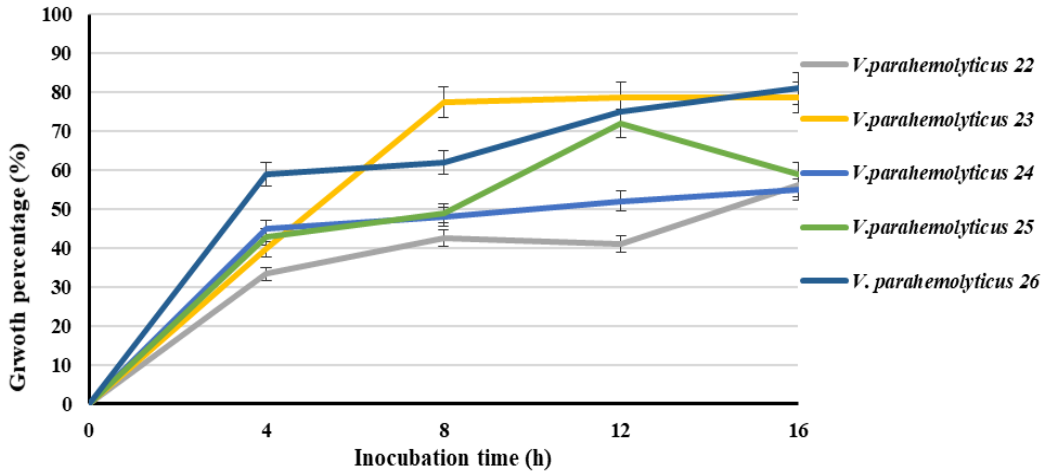
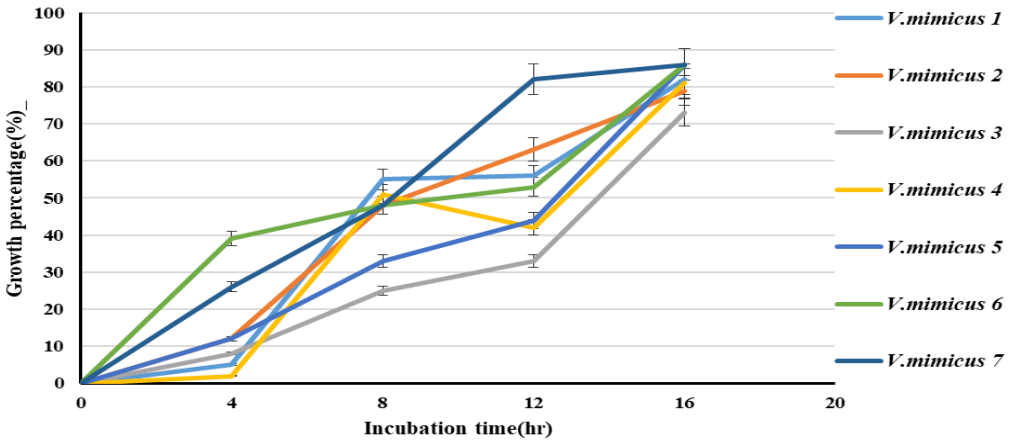
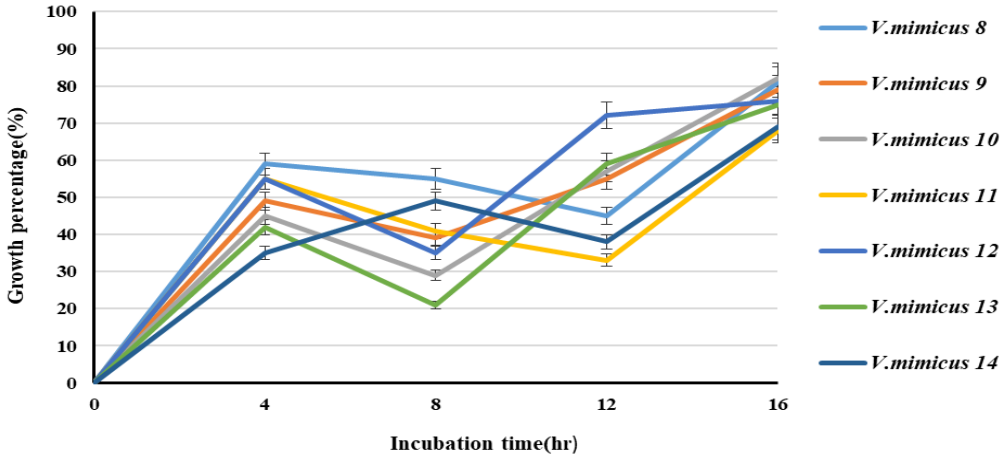


Figure 4.11: Growth rate of *Vibrio parahemolyticus* at 1.0% NaCl (Continued)

e



f



g

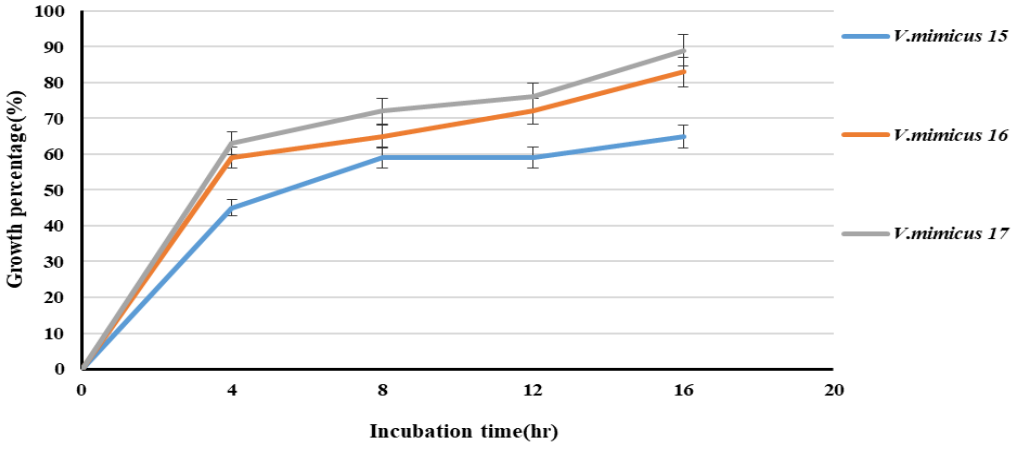


Figure 4.11: Growth rate of *Vibrio mimicus* at 1.0% NaCl (Continued)
Values are expressed as average of 3 samples ± standard error

4.4.3.3 Growth Rate of *Vibrio* spp. at 2.0% NaCl

At 2.0% NaCl, *Vibrio parahemolyticus* 26 (Figure 4.11 d) attained a growth rate of 88%, while *Vibrio parahemolyticus* 18 and 19 (Figure 4.12 c) showed 80% of growth rate on 16 h of incubation. Among the *Vibrio mimicus* isolates, the growth rate of *Vibrio mimicus* 17 (Figure 4.12 g) was 83% at 2.0% NaCl and *Vibrio mimicus* 4, 5 and 6 attained a maximum growth rate of 80% (Figure 4.12 e).

a

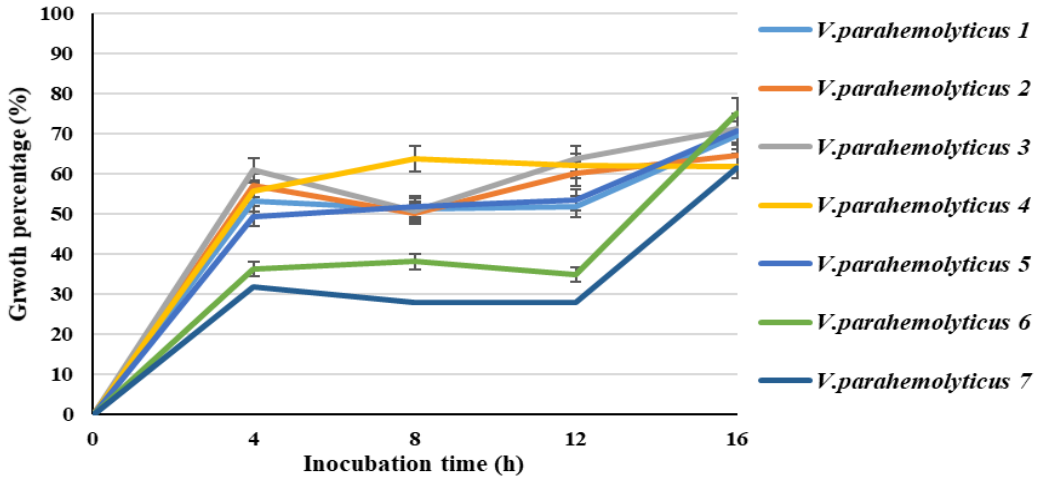
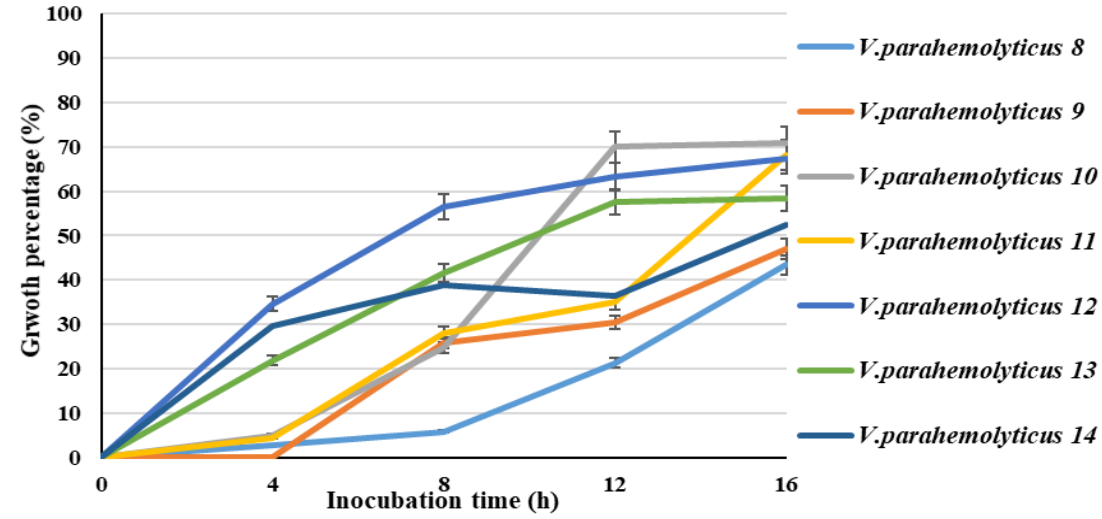
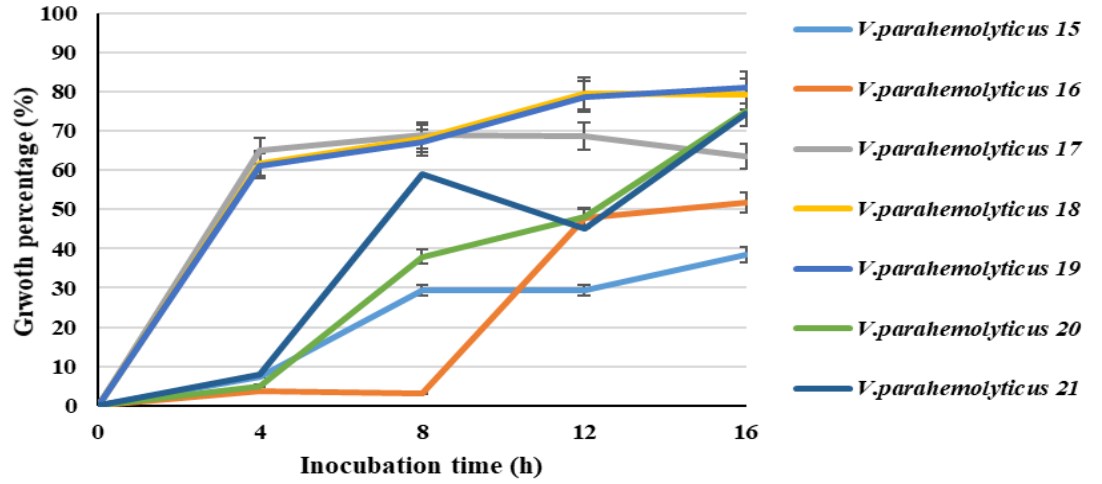


Figure 4.12: Growth rate of *Vibrio parahemolyticus* at 2.0% NaCl

b



c



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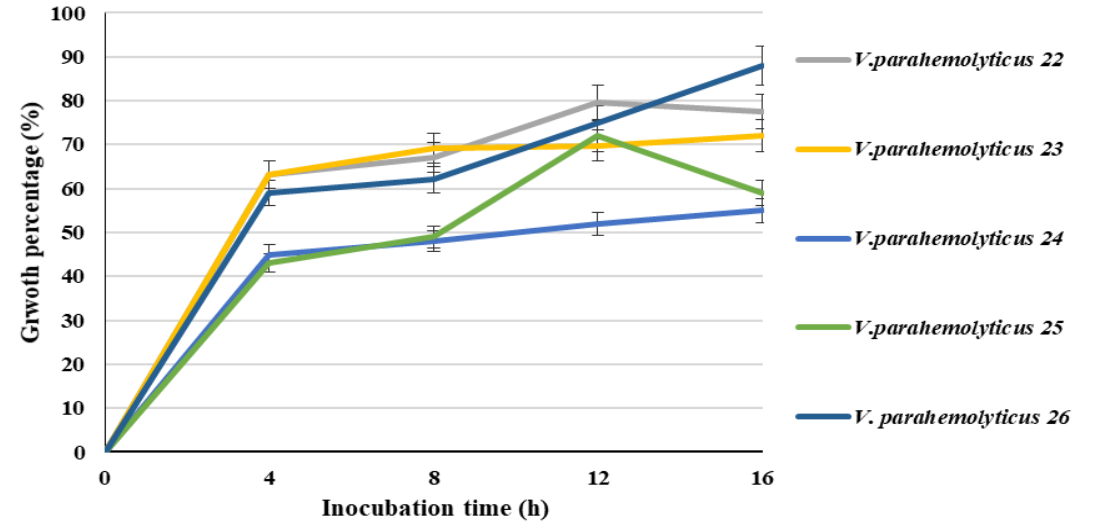
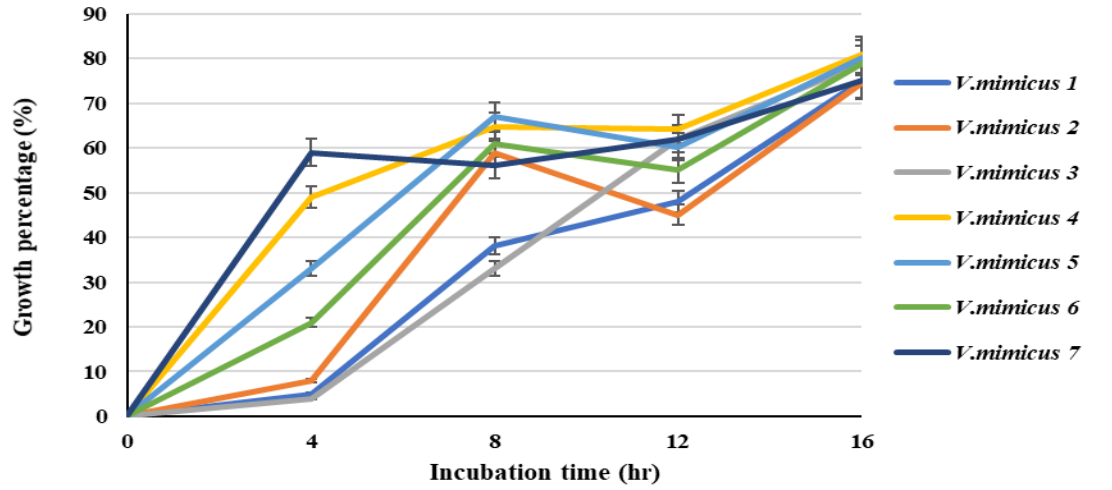


Figure 4.12: Growth rate of Vibrio mimicus at 2.0% NaCl (Continued)

e



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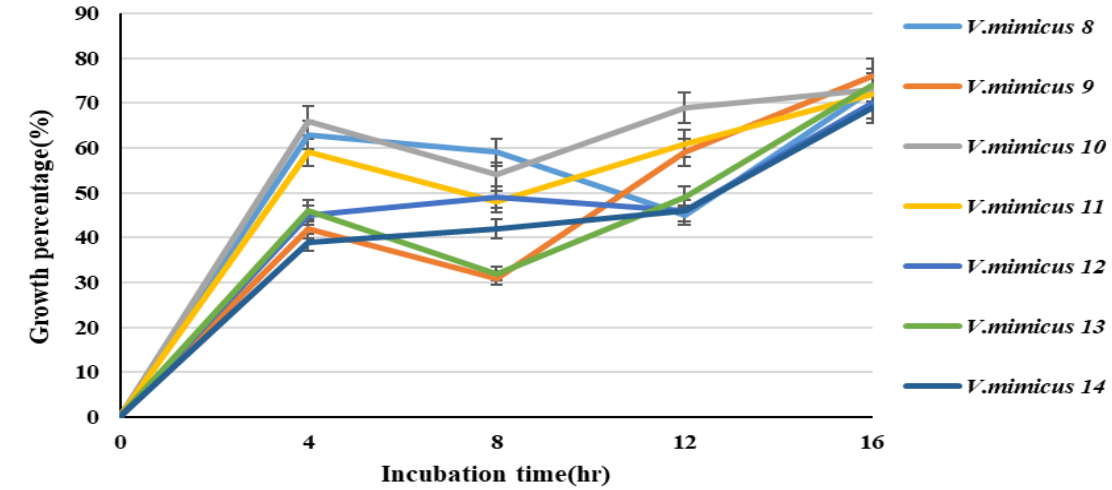

g

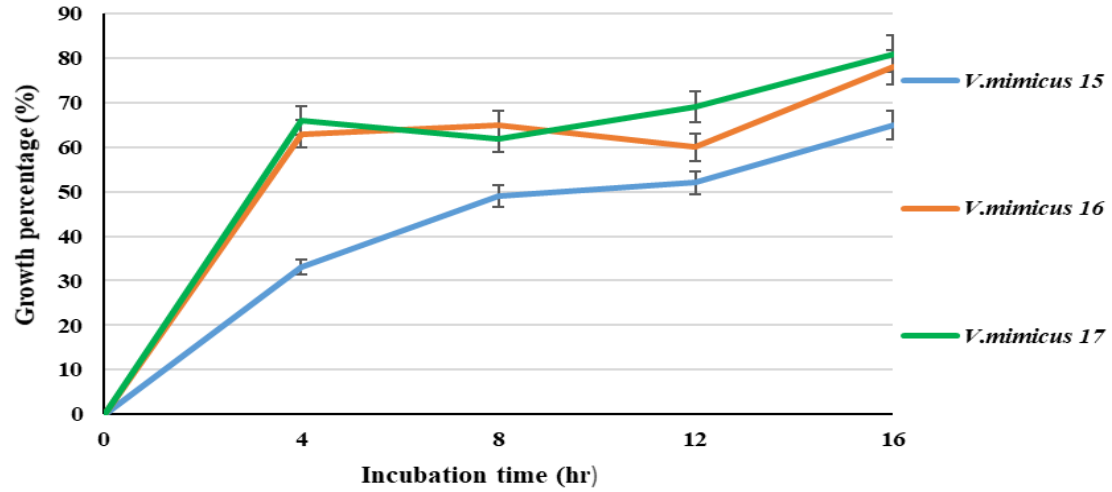

Figure 4.12: Growth rate of *Vibrio mimicus* at 2.0% NaCl (Continued)
Values are expressed as average of 3 samples ± standard errorr

The present study confirmed that increasing the concentration of NaCl results in an increase in the growth rate of *Vibrio* spp. which was in agreement with the results of study conducted by Yoon et al. (2017) who established that *Vibrio parahaemolyticus* and *Vibrio vulnificus* were rapidly reached the viable-but-nonculturable state with increasing levels (≤30%) of NaCl. Numbers of endogenous *Vibrio vulnificus* in oyster shellstock increased by more than 100-fold in shell stock stored at 30°C but were reduced approximately 10- and 100-fold after 14 days at 2 to 4°C and 0°C (Kaspar and Tamplin, 1993). Whitaker et al. (2012) reported that growth of *Vibrio parahaemolyticus* in 1% NaCl was significantly less when compared to growth in 3% NaCl. *Vibrio parahaemolyticus* is moderately halophilic in nature and requires a minimum of 0.086 M (0.5%) NaCl for growth (Palasuntheram, 1981). High-salt preadaptation of *Vibrio parahaemolyticus* cross-protected the organism and significantly increase its survival under lethal acid stress at salt concentrations of 3.5% NaCl and cold temperature stress conditions (Kalburge et al., 2014).

Chapter 5: Summary

The present study assessed the prevalence of *Vibrio* spp. in shellfish sold in UAE. The shellfish samples from different cities showed the presence of *Vibrio*. The prevalence of *Vibrio* spp. in shellfish collected from Al-Ain showed the incidence of only *Vibrio paraheamolyticus* (12.24%). The *Vibrio* isolates from Dubai showed the presence of 23.80% of *Vibrio paraheamolyticus* while in Fujairah an incidence of 15.5% for *Vibrio paraheamolyticus and* 11.11% for *Vibrio mimicus*. The prevalence of *Vibrio* isolates in Abu Dhabi was 6.25% for *Vibrio paraheamolyticus* and 25% for *Vibrio mimicus*.

The *Vibrio* isolates *Vibrio parahemolyticus* were resistant (100%) to penicillin G, daptomycin, and vancomycin as evidenced by the results. Among the isolates, 26.9% were resistant to ampicillin, 61.53% were resistant to erythromycin while 2 (7.6%) of *Vibrio parahemolyticus* were resistant to Sulfamethoxazole-trimethoprim. *Vibrio mimicus* isolates were 100% resistant to penicillin G, daptomycin and vancomycin. The antibiotic resistance of *Vibrio mimicus* isolates to other antibiotics was in the order erythromycin > sulfamethoxazole-trimethoprim >ampicillin (94.11% > 11.67% > 5.8%).

During the incubation period at different temperature a gradual increase in growth rate was observed in *Vibrio parahemolyticus*, and *Vibrio mimicus* isolates and the growth rate attained maximum at 37°C. In the present study, results showed that when the pH of the system was increased from pH 3.0 to pH 5.0, the growth percentage was also increased. Most of the *Vibrio parahemolyticus* and *Vibrio mimicus* attained a maximum growth rate of 80% at pH 5.0 on 16 h of incubation. At different salinity level, the growth rate and survival of *Vibrio* spp. was studied and results showed that

the growth rate of *Vibrio parahemolyticus* and *Vibrio mimicus* isolates were increased while increasing NaCl concentration from 0.5% to 2.0%.

Chapter 6: Conclusion and Recommendations

6.1. Conclusion

Rapid development of antibiotic resistance in bacteria and emergence of drug resistant microbial disease possess serious problems in environmental, economic and management and in addition create human health hazards.

The present study found that 14.13% of isolates showed the presence of *Vibrio paraheamolyticus* among the 129 *Vibrio* positive isolate in shell fish imported from different locations *Vibrio mimicus* was present in 9.26% of isolates.

The coastal zones of United Arab Emirates and water reservoirs around the main cities especially Dubai and Abu Dhabi have traditionally been popular recreational zones. The number of international visitors to the country has drastically increased in the last decade. Al Ain city is a part of Abu Dhabi and the prevalence of *Vibrio* in Al Ain was also very high. Fujairah is a developing industrial area now a day. The combination of climate change in particular, elevated air and surface water temperatures and the increasing anthropogenic effects of tourism may increase the risk of emergence and spread of *Vibrio* spp. which will lead to water-borne and food-borne infections. Salinity level and water temperature at all sampling sites was positively correlated with the abundance of clinically important *Vibrio* spp. Water temperature of The Arabian Gulf reaches a maximum of 35^{o}C during summer and drop to 15^{o}C during winter. Salinity levels in the water reaches up to 70 parts per million (ppm) in shallower areas, twice the average seawater rate.

The identified *Vibrio* isolates were more resistant to pencillin G, daptomycin, vancomycin, ampicillin and erythromycin. The *Vibrio* isolates were susceptible to

sulfamethoxazole-trimethoprim. At 37°C, all the identified *Vibrio* spp. attained 80% growth rate. Incubation temperature of above 37°C is recommended. At higher temperature, the survival rate of *Vibrio* spp. will be reduced. Alkaline pH (pH 5 to pH 7.0) promotes the growth of *Vibrio* isolates. So acidic pH is suggested by this study, at acidic pH the survival rate of *Vibrio* spp. will be less. The effect of different salt concentration on growth and survival of *Vibrio* spp. confirmed that higher salt content increased the survival rate as evidenced by the study. NaCl concentration of less than 0.5% is recommended.

6.2 Recommendations

The shellfish samples from different cities of UAE showed the presence of *Vibrio parahaemolyticus* and *Vibrio mimicus* spp. All *Vibrio* isolates are highly pathogenic showing multiple antibiotic resistance and are being potential to cause serious food borne illness thus posing risk to human consumers. The occurrence of pathogenic *Vibrio* isolates in shellfish samples requires extended surveillance across the UAE. Hence, continuous monitoring of *Vibrio* strains in food samples and their antibiotic susceptibility by food control authorities in UAE is necessary to ensure the best treatment for consumers to avoid diseases like gastroenteritis and thereby ensuring seafood safety. The simple and effective control of the pathogen by using effective antimicrobials is recommended as a better choice for avoiding *Vibrio* contamination in future risk assessment. Indeed, further investigations are required to explore the presence of *Vibrio* spp. in seafoods more extensively. The limitation of this study was the low number of samples and genes related to the antibiotic resistance in *Vibrio* species. These two limitations are required to be addressed in the future studies.

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Appendices

Appendix 1

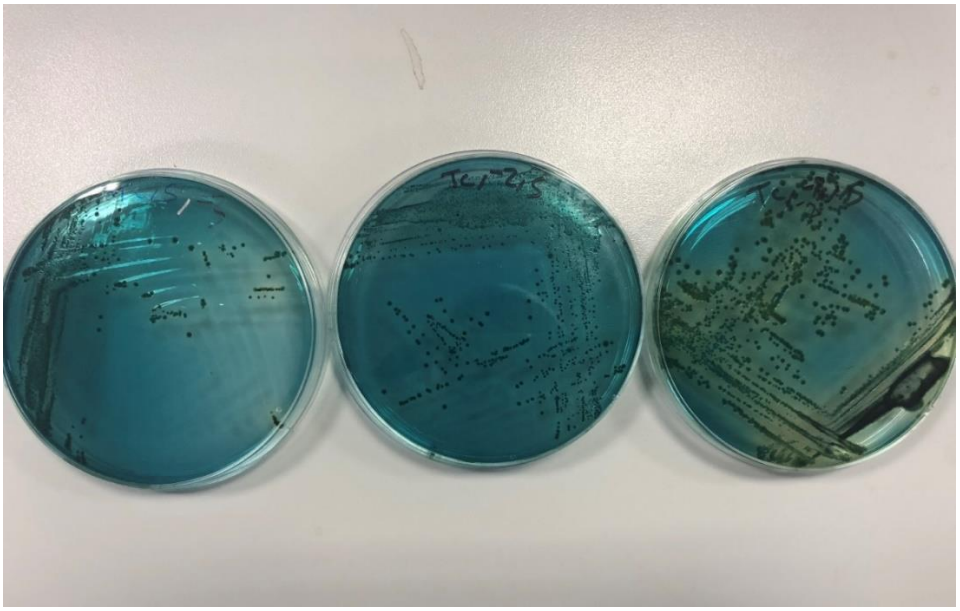

Figure A1.1: Culture plate of isolated *Vibrio* spp. in Thiosulfate-citrate-bile salts-sucrose agar (TCBS Agar)

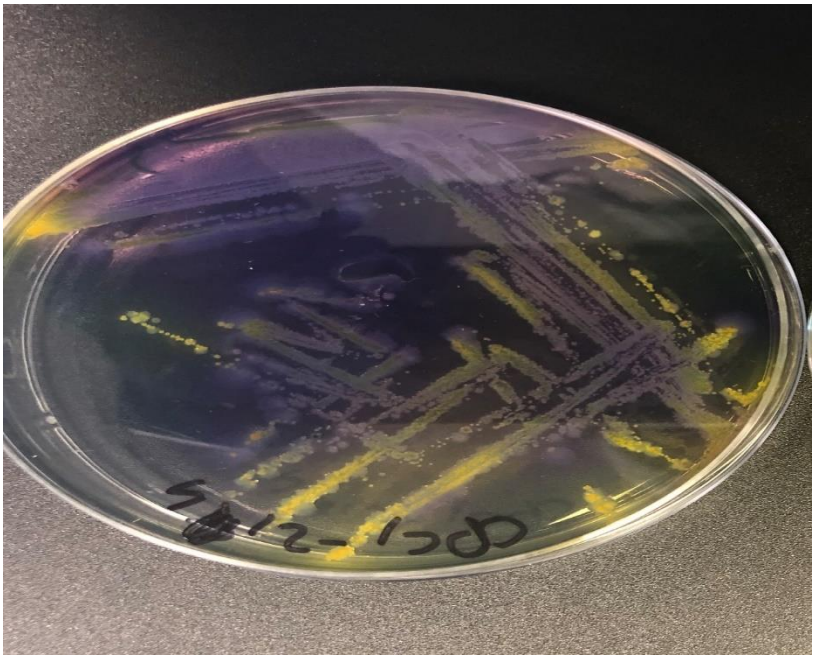

Figure A1.2: Culture plate of isolated *Vibrio* spp. in Cellobiose polymyxin B colistin agar (CPC Agar)

Appendix 2

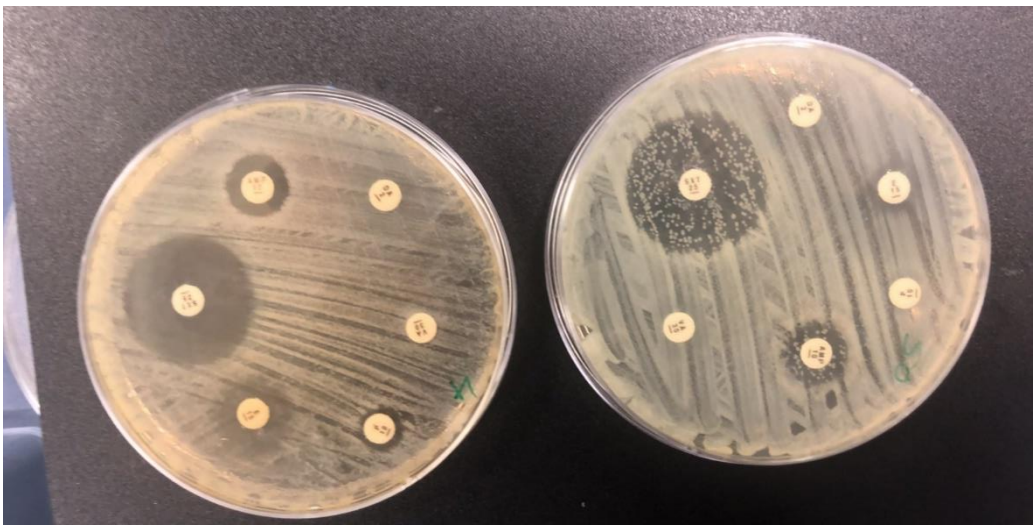

Figure A2.1: Antibiotic sensitivity of *Vibrio* isolates for six different antibiotics

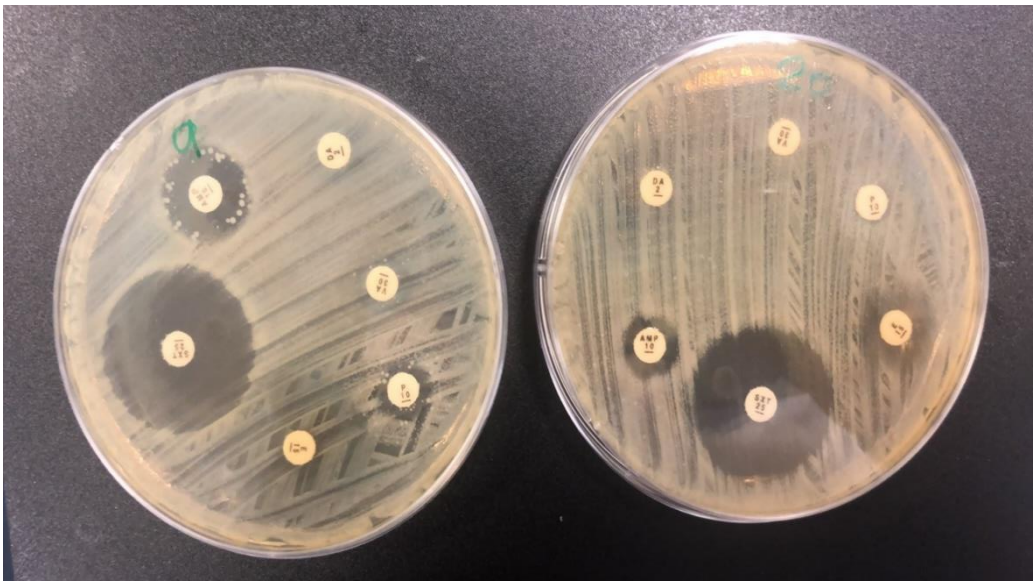

Figure A2.2: Antibiotic sensitivity of *Vibrio* isolates for six different antibiotics

Appendix 3

Experienced User Protocol for DNA Isolation Kit Sample; DNeasy UltraClean Microbial Kit (Qiagen, Carlsbad, CA, USA)

1. Added 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuged at 10,000 xg for 30 seconds at room temperature. Decanted the supernatant and spin the tubes at 10,000 xg for 30 seconds at room temperature and completely removed the media supernatant with a pipette tip.

2. Resuspended the cell pellet in 300 µl of microbead Solution and gently vortexed to mix. Transferred the resuspended cells to microbead Tube.

3. If solution MD1 is precipitated, heat the solution at 60°C until the precipitate has dissolved. Added 50 µl of solution MD1 to the glass micro bead tube.

4. Secure micro bead tubes horizontally using the vortex adapter tube holder or secure tubes horizontally on a flat-bed vortex pad with tape. Vortexed at maximum speed for 10 min.

5. Centrifuged the 2 ml micro bead tubes at 10,000 xg for 30 s at room temperature.

6. Transfered the supernatant to a clean 2 ml collection tube.

7. Added 100 µl of solution MD2, to the supernatant. Vortexed for 5 s. Then incubated at 4°C for 5 min.

8. Centrifuged the tubes at room temperature for 1 min at 10,000 xg.

9. Transferred the entire volume of supernatant to a clean 2 ml collection tube

10. Shook to mix the solution MD3 before use. Added 900 µl of solution MD3 to the supernatant and vortexed for 5 s.

11. Loaded 700 µl into the spin filter and centrifuged at 10,000 xg for 30 s at room temperature. Discarded the flow through, added the remaining supernatant to the spin filter, and centrifuged at 10,000 xg for 30 s at room temperature.

12. Added 300 µl of solution MD4 and centrifuged at room temperature for 30 s at 10,000 xg.

13. Discarded the flow through and centrifuged at room temperature for 1 minute at 10,000 xg.

14. Added 50 µl of solution MD5 to the center of the white filter membrane.

15. Centrifuged at room temperature for 30 s at 10,000 xg.

16. Discarded spin filter column.

17. DNA was stored at (-20°C).

Appendix 4

Gel electrophoresis images for virulence genes detection

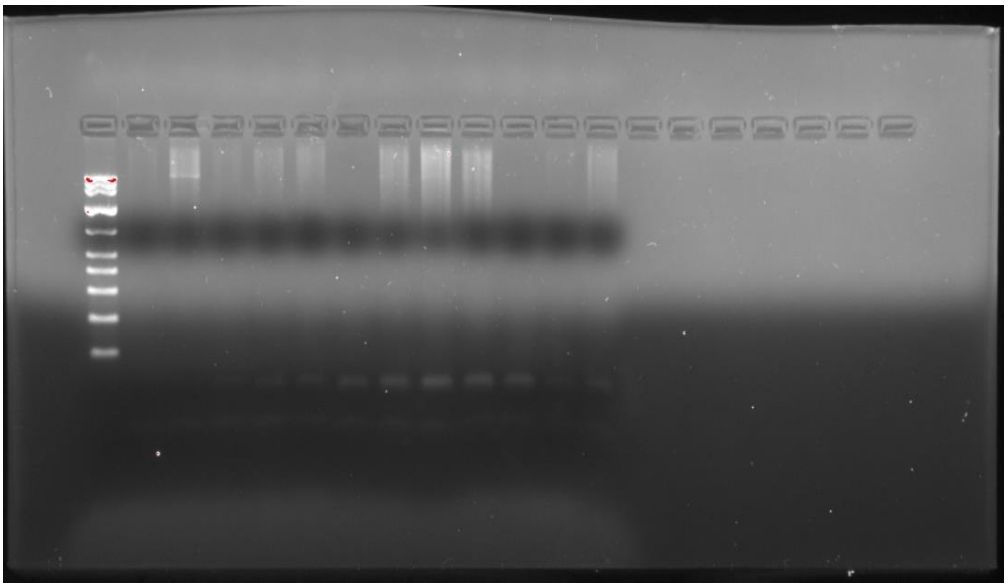

Figure A4.1: PCR product of amplified virulence gene of *Vibrio parahaemolyticus*

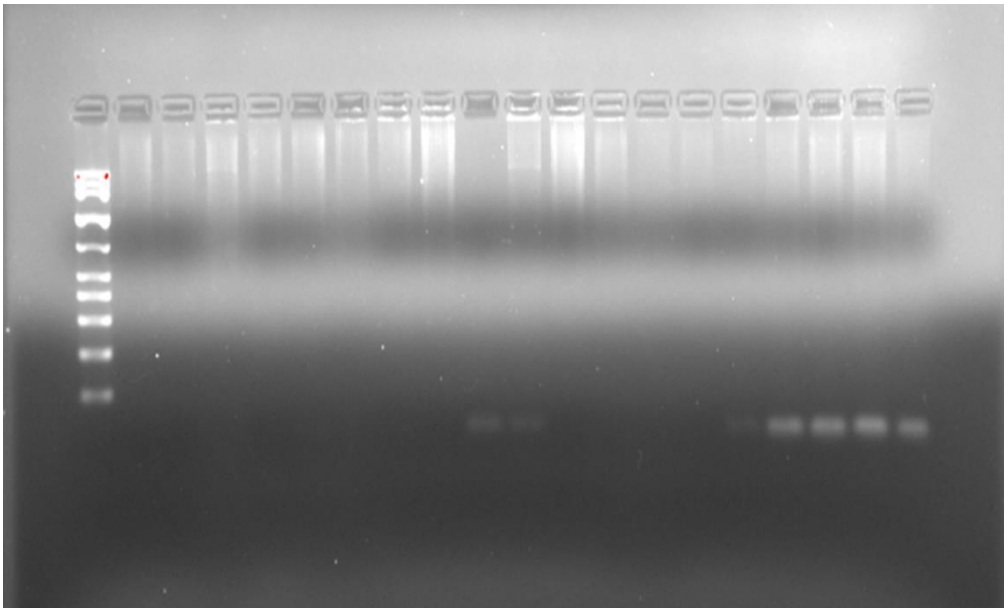

Figure A4.2: PCR product of amplified virulence gene of *Vibrio mimicus*