ISOLATION AND MOLECULAR IDENTICAFICATION OF BACTERIA FROM SPICES FOR ANALYZING THEIR PROBIOTIC POTENTIAL

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Research Thesis submitted in the Partial Fulfillment for the

Degree of Master of Sciences in Plant Biotechnology

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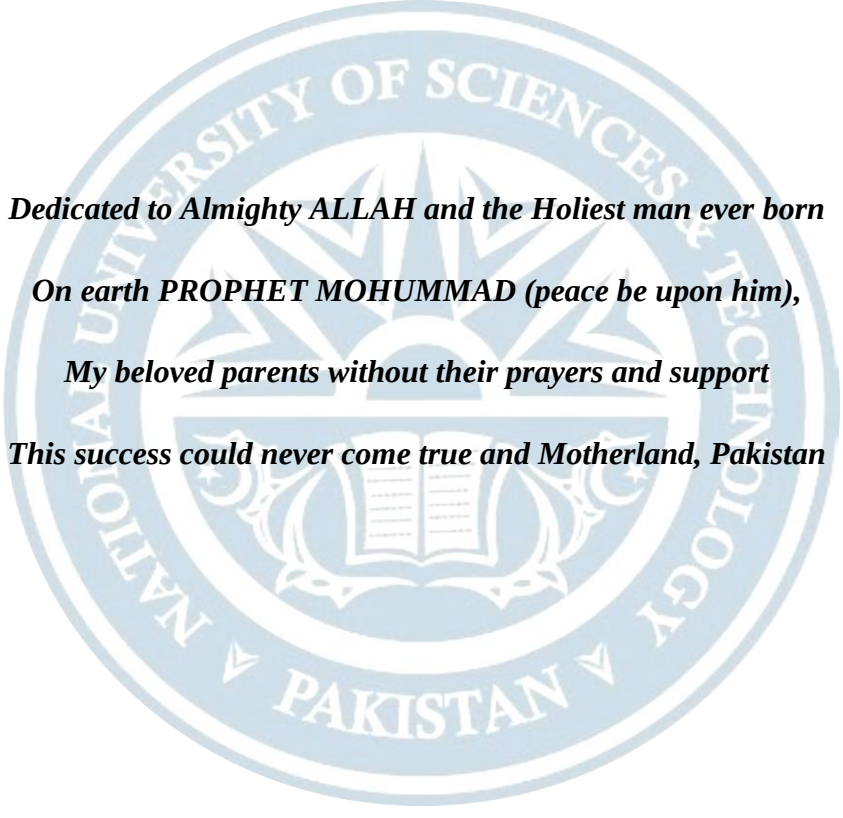
2017

“He has made subject to you the Night and the Day, the Sun and the Moon and the Stars are in subjection by his command: Verily in this are signs for men who are wise”.

(Surah An-Nahl (16); Ayah 12)



Dedicated to Almighty ALLAH and the Holiest man ever born

On earth PROPHET MOHUMMAD (peace be upon him),

My beloved parents without their prayers and support

This success could never come true and Motherland, Pakistan

DECLARATION

I hereby declare that this project, neither as a whole nor as a part thereof, has been copied out from any source. In case of using any references material, proper references have been provided. It is further declared that I have developed this research and report entirely on the basis of my personal effort, made under the sincere guidance of my research supervisor.

No portion of the work presented in this report has been submitted in support of any application for any other degree or qualification of this or any other university or institute of learning.

HAFSA TANVEER _____

ACKNOWLEDGEMENTS

All praises for ALLAH ALMIGHTY, the Most Merciful, the Most Gracious, Who helped and blessed me all the way along till the completion of the research work. Without His grace and mercy, I am nothing.

In the first place with great pleasure, I express my most cordial and humble thanks to my eminent, respected guide Dr. Nasar um Minullah for providing his valuable guidance, inspiration, supervision, keen interest and moral support from the very early stage of my research work till the end of my dissertation work. His encouraging words were source of constant motivation to act with quality and precision and they enriched my growth as a student. I am indebted to him for his constant support.

I express my deep gratitude and special thanks to Dr. Muhammad Faraz Bhatti for his valuable guidance and enduring support during my research work. I am thankful to my G.E.C member Dr. Abdur Rahman and external supervisor Dr. Atif Jamal for reviewing my thesis and guiding me about research skills.

I am also very thankful to Dr. Stephan Wawra from University of Cologne, Köln Germany. He visited NUST for international conference on "Bio-approaches for environment and sustainability". He visited our laboratory, gave a lecture "how to make presentation" and his short interactive session with all the students was very useful. Personally I learned a lot from him, he has been very innovative and inspiring with his suggestions.

It is my great privilege to express my sincere bunch of thanks to Principal ASAB Dr. Peter John and HoD Plant Biotechnology Dr. Muhammad Tahir for his timely valuable suggestions, co-operations and help.

I have been very blessed in my life, particularly in my friendships. In all of the ups and downs that came my way during the dissertation years. In particular, I would like to thanks my Lab fellows Memoona Javaid, Wajiha Shamsi, Aqeel Ahmad, Atif Shafique, Hira Iftikhar, Syed Hamza, Roohi Aslam, Miss Rabia Zaman Bhatti, Miss Shakira for their friendship, and for being

part of wonderful company in many memorable events like birthday parties, cafe parties and for their moral support in difficult times during my research work. The completion of this project could not have been possible without the assistance my seniors Hafsa Khan, Midhat Mustafa, Danish Gul, Azam Fareed, Ayesha Javaid, Mohsin Ali, Samia and Ahsan.

In the end, my deepest gratitude goes to my parents for their unflagging love and support throughout my life, this dissertation is simply impossible without them. I am indebted to my father, for his care and love. I have no suitable word that can fully describe his everlasting love to me. It is his dream that I get excellence in my studies.

I cannot ask for more from my mother as she is simply perfect. I have no suitable word that can fully describe her everlasting love to me. I remember many sleepless nights with her accompanying me, giving me strength and praying for my success. I remember her constant support when I encountered difficulties. Mother, I love you. I would like to thank my beloved brothers Hassan and Hasnain, their prayers and support in any way.

It may not be enough to contain the words of thanks giving, it may not capture the endearing thanks that I have for Allah but now I am making this compilation to let the world know that Islam is a religion of love, generosity, and peace as it promote learning by saying ” Read! With the name of thy Lord, Who created you” (Al Alaq; 1).

I am proud of being a Muslim.

HAFSA TANVEER

Contents

Abbreviations

B. subtilis	*Bacillus subtilis*
CTAB	Cetyl trimethylammonium bromide
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribo nucleic acid
dNTP	Dinucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal Tracts
IL-6	Interlukin-6
Kb	Kilobase pair
L. monocytogenes	*Listeria monocytogenes*
MIC	Minimum inhibition concentration
ml	Microliter
mm	Milimeter
NaCl	Sodium chloride
P. syringae	*Pseudomonas syringae*
PCR	Polymerase chain reaction
pM	Pico molar
Rpm	Revolution per meter
rRNA	Ribosomal ribose nucleic acid
SCAN	Scientific Committee on Animal Nutrition
SDS	Sodium dodecyl sulphate
SGF	Stimulated Gastric Juice
SGF	Stimulated Gastric Juice
TAE	Tris acetate ethylene diamine tetra acetic acid
TE	Tris ethylene diamine tetra acetic acid
Th1/ Th2	T helper 1/ T helper 2

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Abstract

Bacteria are the oldest, structurally simplest and the most abundant form of life on earth. They infect a wide range of organisms including plants, animals and invertebrates. Spices constitute an important group of agriculture commodities and they are not free from microbial association like fungi, bacteria and viruses. They may acquire microorganisms during growth and development or during postharvest stages like collection, processing, storage and marketing. The present work was aimed to identify different types of bacteria associated with common spices to understand their physiological characteristics and to find out the potential probiotic *Bacillus* strains. Total 65 isolates were isolated from spices; only ten strains were characterized by using biochemical tests and molecular techniques. Eight selected strains were accessed for tolerance to acidic pH, high osmotic concentrations of NaCl and different concentrations of bile salts, as a pre-requisite for potential probiotic candidate. Theses isolates were subjected to pH of 2.0 and 3.0, NaCl concentration of 3.5 and 6.5 percent and bile salts concentration of 0.037, 0.15, 0.3 percent with nutrient broth at 37°C. A varied tolerance capacity for acid, NaCl and bile was observed for these isolates. Bacterial strains were identified by sequencing using 16s rRNA primers. Phylogenetic analysis was conducted using Geneious software. Among the tested strains, one strain KY986416 showed high level of tolerance at pH 2 and 3, different bile concentrations and this strain was found susceptible to all 4 antibiotics CN-10 (Gentamicin), AMC-30 (Amoxycillin), TE-30 (tetracycline) and PB-300 (Polymyxin) tested for safety assessment of antibiotic resistance gene. It is confirmed by biochemical and molecular identification that bacteria associated with spices are mostly from Genus Bacillus. Total 12 different bacterial species have been identified through sequencing in this study and their data is submitted in the Genbank. Few of the tested *Bacillus* species are showing tolerance towards the acidic conditions and different salt concentrations so they might have potential to be used as probiotic candidates. They can be further analyzed by using *in-vivo* studies.

CHAPTER 1: INTRODUCTION

1.1. Probiotics:

Probiosis has recently got importance and it has started to receive the appraisal in the scientific world as well. Probiotics are defined as “live microorganisms that improve the health of the host by interacting with intestinal microflora” (Fuller, 1989).

The probiotics are used for the broader range of hosts but two main categories in classification of Probiotics are for human and animals. Probiotics are used as alternative of antibiotics (therefore used as the growth promoters) in the raw feed of animals. Currently, the most commonly available bacteria are lactic acid bacteria (e.g., *Lactobacillus* spp.) which are being used as probiotics but probiotic bacterium does not fall in any universal class of bacteria. Naturally these lactic acid bacteria are found in the gastrointestinal tract (GIT) as indigenous or commensal microorganisms but there is no visible notion of their use for enhancing the growth of gut microorganisms or restoring the natural gut microflora. A second class comprise of those bacteria which are not naturally found in the GIT. For example, pseudomembranous colitis induced by *Clostridium difficile* as well as *Escherichia coli* showing antagonistic action can be prevented effectively by *Saccharomyces boulardii* (Czerucka et al., 2000).

Within the group of allochthonous fall the spore-forming bacteria which are being used as probiotic microbes, normally it includes genus *Bacillus* members. The spores products used here can be stored for unlimited time period. The usage of spore-based products is a controversial issue and there are many questions which need to be answered regarding bacterial use as probiotics how do bacterial species to be used as probiotics exert beneficial effects on health of living organisms. Since they are not the permanent members of gastrointestinal microflora?

As the natural life cycle of the spore forming bacteria include the germination of spores, proliferation, then re-sporulation when the conditions is not favorable for the growth of bacteria, Although they are not true commensals of the gut, a case was made that spore forming bacteria exhibit a very unique bimodal life cycle showing microbes survive in the environment as well as the gut of the living organisms; it could be the one important factor towards their probiotic effect.

1.2. Introduction to Spices:

Spices are used to give better taste, flavor to food due to its aromatic properties. (Banerjee and Sarkar, 2003). The term spices are applied to the substances of plant origin that can be sold in the dried powder form or in small pieces. Most of the spices do not have any nutritional value but organoleptic properties help in the digestion of food by stimulating the production of gastric juice or by increasing the motility of the digestive organs (Garcia et al., 2001).

Synthetic preservatives are always questioned by the people, that's why being of plant origin people are contended to use spices (Sağdıç et al., 2002). Some of the spices are consumed in raw form like onion/capsicum. So, we can say that spices can be part of our food in cooked, semi cooked or even in raw form like as salads, sauces. (Shobana and Akhilender Naidu). Spices include important group of plant families which are essential in the food and medical science. The spices are grown in the humid areas which are usually warm. The roots, leaves, barks, rhizome, bulbs, stems, flowers and seeds of plants are sources of spices (Khan, 2012.).

1.2.1. Antioxidants Properties of Spices:

The principal properties of the spices is to give taste and zest to food and it's antioxidant and antimicrobial properties increase its importance to be used as medicine in pharmaceutical industry (de Souza et al., 2005). Spices give strong oxidant stability to all the synthetic products, that's why they are used in the food industry as the antioxidants (Martínez-Tomé et al., 2001).

1.2.2. Antimicrobial Properties of Spices:

Owing to have antimicrobial properties different plants are used to fight against various diseases. Different kinds of spices like garlic, ajwain, black pepper, clove, ginger, cumin and caraway are already being used in daily medicines. Garlic is used to treat infectious disease as it shows strong antibacterial activities. Mostly pickles and chutneys are preserved in the presence of spices due to its antimicrobial strength (Arora and Kaur, 1999). Clove is rich source of Eugenol, which is used to treat toothache, possess local anesthetic activity and it is also used as antiseptic (Suresh et al., 1992).

In animal production, the use of antibiotics is reduced and it is replaced by the herbs and spices. They contain strong antimicrobial compounds used in vitro against microbial contaminations,

including fungi (Dorman and Deans, 2000). The health status of animals can be improved by using the plant extracts or spices extracts as separate compounds or mixture of these two can for better performance. (Guo et al., 2006) observed in their study that the number of beneficial bacteria can be increased and number of harmful bacteria can be reduced in caecum by using the extracts of plants (Hosseinzadeh et al.,2014).

1.3. Traditional Fermentation by *Bacillus* species*:*

Bacillus involved in the food fermentation can give us insight of used microorganisms which have potential to be used in the industries like many other microorganisms which are associated with fermented foods. There are many traditional fermented foods which contain number of microorganisms associated with them; hence, the study of fermented food would give an easy access to study more about the *Bacillus* species, genera with potential industrial properties. the best example here is to study the natto used in japan, it is produced by fermenting soybeans food in the presence of *Bacillus subtilis* (natto) (Yin et al., 2010).

Another most aged product produced by *Bacillus* fermentation of steamed soybean is Itihiki-natto. These kind of fermented products are also used in China and Thailand. The stringy characteristics of the natto are due to the production of slime consist of a levan-type fructan and γ-D-polyglutamic acid (γ-PGA). Mixing the string fermented food with sucrose made it more viscous. *B. subtilis* (natto) usually produce many different enzymes, two commonly produced enzymes including cellulases and amylases. While the most useful enzymes are proteases enzymes in the production of natto, one with the pH optima of 8.5 and the second one with 10.3 – 10.8 have been characterized (Yoshimoto et al., 1971).

The proteases are involved in the hydrolysis of protein and it is basically giving the taste by hydrolyzing the soybean protein. Many beneficial health effects are associated with the natto or *B. subtilis* (Yin et al., 2010). The antibacterial activity shown by the natto is due to the heat unstable bacterial lytic activity and dipicolinic acid. The level of vitamin K in breast milk increases by the use of natto during lactation period. The pathogenic bacteria have been removed from the intestinal tracts by the use of *Bacillus subtilis* (natto) cultures. In specific laboratory trials the natto has been known to show anticancer properties as well as it reduce the blood pressure (BP).

1.4. Antimicrobial Properties of *Bacillus* Species:

There is a lot of quest going on to find out more bacteria with antimicrobial properties so that these bacteria could be used as

1) starter culture
2) As new probiotics or to use them in place of antibiotics
3) To use them against pathogenic bacteria (Guo et al. 2006).

The microorganism which plays the most important role in the fermentation of H. sabdariffa seeds into Bikalga is the *Bacillus subtilis* group species (Ouoba et al., 2008). *Bacillus* species have been playing major role in the microbiology and industrial biotechnology, since the production of natto thousand years ago by the fermentation of the solid state soybeans using *Bacillus subtilis* (natto) it was first time experimented in Japan (Hara and Ueda, 1982). These excellent properties of *Bacillus* strains have been used and expanded more over the past few years. The molecular biology techniques actually helped the scientist to explore more about the development and production strategies of these *Bacillus* species, it was not possible in early days due to the lack of facilities. *Bacillus* species are powerful workhorse in the industrial microbiology for many reasons including:

1) High growth rate leading to short fermentation cycle
2) Ability to produce proteins in the extracellular environment
3) Generally possess GRAS Status with the Food and Drug Administration for species.

The *B. subtilis* isolated from different African alkaline fermented food items for example Maari, Netetu, and Soumbala are able to exhibit many antimicrobial properties against harmful bacteria and fungi, although the identified antimicrobial compounds are very few. (Kaboré et al., 2012). Foodborne illnesses are mostly treated with antibiotics of various origins but now days several cases have been reported to develop the multiple drug resistance microbes' worldwide so, alternative ways are being searched to solve the problem. One of the growing area is to treat the food borne infections is the use of probiotics. Probiotic bacteria are useful which confer beneficial effects on health and help in controlling the infectious pathogenic strains; they are providing clear solution for the prevention and treatment of such pathogenic strains (Moore, 2013).

CHAPTER 2: REVIEW OF LITERATUE

Spices such as garlic, onion, ginger, peppermint are known to have anti-microbial properties still the high amount of spices can increase the chances of microbial contamination. More than this it can also change the organoleptic properties of food. Spices have greater chance to be contaminated during harvest and post-harvest processes as they are exposed to open environment for drying and as they are direct in contact with soil and polluted air. In addition, the spices can be contaminated with dust particles, faeces of birds, rodents and insects during storage and marketing due to poor hygienic conditions (Moreira et al., 2009).

The spices may be polluted with different microbes as they are not collected, stored and processed under rigorous hygienic conditions ultimately it affects food which is consumed by the human beings. Spices, herbs, vegetal condiments took high name because they impart specific color, flavor and aroma to the food. But the presence of microbes that could be pathogenic and non-pathogenic bacteria, yeast, molds can cause rapid deterioration to the food. Predominant flora belongs to the Bacillus, Clostridium, Pseudomonas genera, the enterobacteriaceae family, fungal spores and yeast associated with spices can definitely grow if food is not cooked under specific thermal and pressure conditions. It may lead to food deterioration that will be cause of many diseases (Aguilera et al., 2005).

Under these happenings, spices may cause serious threats to the health of the people who are using spices in cooked or uncooked food. Use of spices in ready to cook food may cause alarming situation if they are not properly processed. (Moreira et al., 2009)

Many scientists have worked on the microbial contamination of spices. Satchell *et al*. selected four spices *viz.* black and white pepper, coriander and fennel seeds for analysis of microbial community with them. Antai reported the *Bacillus* flora of Nigerian spices (Khan, 2012.).

The processed canned food that contains herbs and spices are matter of huge concern because they are used unprocessed or may be eaten raw so they may pose serious threats to public health. Soups, casseroles, stews, and gravies produced by catering establishments are principal source of spore forming bacteria as these large volume food dissolve variety of spices in it. They provide favorable environment to these bacteria to multiply and cause food toxicities. Herbs and spices can be the cause of introducing potential food spoilage microbes to range of food (Pafumi, 1986).

The aerobic and anaerobic bacteria are major cause of contamination; at the time of collection they are associated with spices originating from the soil. They are mostly sources of deterioration in the tropical and sub-tropical countries where spices are collected, handled and stored in the very unhygienic conditions. As spices are dried they have usually low active water value which is not favorable for microbes. Because the way of handling when spices are rehydrated by the addition of moist ingredients the end products are more susceptible to spoilage.

Different techniques and methods are used to reduce the potential hazardous microbes, like decontamination techniques such as irradiation is of fundamental importance, to get rid of pathogenic strains. Bacterial pathogens (e.g. *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus*, etc.) are not associated with outbreaks or serious threats but they have been reported to have connection with food borne illness due to *E.coli* and *Salmonella* contamination. *Bacillus* which is usually present in the spices can cause food borne illnesses due to poorly stored leftovers for several days or improperly refrigerated spiced foods. Spices, herbs contain 3.0% high counts of *B. cereus* which is usually present and affect those dishes which are poorly processed for very short duration after the addition of spices. The spices imported from India have serious issues of being contaminated. At the time of export four unprocessed Indian spices were tested, they were shown to be highly contaminated with bacteria. Spore-forming aerobe bacteria *B. cereus* was detected in number of samples as compared to other bacteria (Saad et al,.2015).

Food borne pathogens have drastic effects on human population and it is very difficult to control them. Domestically acquired foodborne illnesses is most commonly caused by *Staphylococcus aureus*, it fall in top five pathogens associated with foodborne illnesses. Because of the flavor properties and aroma spices are used in all kinds of prepared foods all over the world. Quality of spices is being evaluated by its flavor properties and cleanliness. As we know spices harbor great number of environmental microbes which are pathogenic, they may contain useful microbes as well as they show antimicrobial activity (Garcia et al., 2001).

2.1. Spore-Forming *Bacillus* Spp. as Probiotics:

The Probiotic terminology is more closely related to lactic acid bacteria as *Lactobacillus* and *Bifidobacterium* and this term can also be expanded to add more microorganisms which are not

explored yet. Enterogermina® is an Italian commercialized product which contains the *Bacillus* species and it has been used as probiotic since 50 years. Among the *Bacillus* group few other species have been assessed are *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus, Bacillus coagulans,* and *Bacillus clausii.* (Cutting, 2011).

The spore forming bacteria known as *Bacillus* spp. show beneficial qualities both for the human and animal health hence, they are referred as Probiotics (Hong et al., 2005) . Due to its extended shelf life and stability without losing viability, the spores are preferred over the vegetative cells. *Bacillus* spp. are known to exist in diverse animals in their faeces and ileal biopsies, it suggests that these species may colonize intestinal tract rather than passing through it. The most prominent features of *Bacillus* spp. include

1) Their potential to survive and germinate in the gut.
2) To synthesize biofilms.
3) To secrete antimicrobials (Guo et al., 2006).

When Pigs were feed with the *Bacillus* spores, it was determined that the 70-90% spores were able to germinate in the close portion of the intestine while only small number of vegetative cells are able to grow here (Leser et al., 2008). European Food Safety Authority (EFSA) regulates the use of *Bacillus* cultures in the animal nutritional feed (Hazards, 2010). The species *B. subtilis, B. licheniformis and B. amyloliquefaciens* have received the Qualified Presumed Safety (QPS) status, which means they are safe to use as they are non-toxigenic. Many *Bacillus* species are known to be safe and used as probiotics for animals, have number of qualities as they promote the digestive system and growth, better feed utilization. (Gaggìa et al., 2010).

The pigs and broiler chicken were given the B. subtilis C- 3102 (Calsporin®) in their diets for the analysis of this commercial probiotic and it showed improvement in the growth and feed conversion. (Hazards, 2010). Toyocerin® containing (*B. cereus var. toyoi*) is also known to decrease the severity of the diarrhea and morbidity in piglets caused by *Eschericha coli*, and it also helps to increase the weight gain in piglets after 6 months duration.(Williams et al., 2009).

In 2000 the antibiotics started to be used as growth promoters were banned in the pig industry, in 2006, use of antibiotics was completely banned. Within Europe the use of antibiotics in animal feed was completely banned on proposed date 2006. The use of antibiotics alternatives would be

encouraging and it was the major reason to introduce and develop the industry of probiotics and to get the licensed probiotics products for animals use. However the point of concern while giving large number of bacteria to animals is the transfer of antibiotics resistance traits. In Europe the estimated price to get a license new probiotic for animal use is about 1.4 million Euros (Hong et al., 2005).

2.2. Natural Habitat of *Bacillus* Species:

Bacillus species are Gram-positive saprophytic bacteria, they are commonly found in water, dust soil, and air (Nicholson, 2002). They are also involved in food spoilage (e.g., spoilage of milk by *B. cereus* strains). Allochthonous bacteria are examined and they also show association with food and enter gut.

2.3. Gut as a Habitat of *Bacillus* Species:

The spores of *Bacillus* species are usually found in the soil, it is usually assumed that the habitat of *Bacillus* (vegetative) bacteria is soil. This is manifesting an unauthentic presumption as the bacterial spores can be spread through air, water means to different locations so they can be found everywhere. So, their presence anywhere on earth does not tell about their natural territory. By carefully reviewing the literature, it is confessed that the gut of animals and insects is also the habitat of spore forming *Bacillus* species. It is proved experimentally by demonstrating through fecal sampling. By ingesting the *Bacillus* bacteria present within soil whether in the form of spores or vegetative cells can give rise to bacteria within the gut. Nonetheless, more consolidated theory is appearing these days in which it is elaborated that *Bacillus* species have ability to temporarily survive and even proliferate within the GIT, and within the host these bacteria show an endosymbiotic relationship. In some cases though, pathogen are evolved from the endosymbionts, taking advantage of this relationship they get entry to gut of the host (*B. anthracis*) or they might use it as a site for secreting of enterotoxins (*B. cereus, B. thuringiensis*) (Jensen et al., 2003).

2.4.*Bacillus* Species as probiotic strain in Human Product:

Probiotic products to be used by human fall in two major groups, 1.Prophylactic use 2.those sold as novel foods or health food supplements. *Bacillus coagulans, Bacillus subtilis, Bacillus clausii, Bacillus pumilus, Bacillus cereus,* and *Bacillus licheniformis* species being used in human

products fall in *Bona fide Bacillus* category. *Paenibacillus polymyxa* and *Brevibacillus laterosporus* are the spores formers used, both of them now belong from the *Bacillus sensu lato* group but formerly they belong to *Bacillus* species.

2.4.1. Prophylactics:

Prophylaxes are sold in market for the gastrointestinal disorders particularly infant's diarrhea (mainly rotavirus infections) or they are used as a substitute of antibiotic. Very often these products are recommended by the physician but they are used over the counter (OTC), their use also relies on national and local culture of the region. For example, in UK probiotics are not available for human use against gastrointestinal disorders as prophylactics, on other side in Europe these products are commonly used and Italy is major user of it. Enterogermina® which is made up of the blend of four antibiotic-resistant strains; it is one of the major oldest products available in the market of Italy since 1950's. Four antibiotic-resistant strains of *B. clausii* are alkaliphilic species able to tolerate highest pH [7 to 14].

In South East Asia there is a history to use probiotics and extensive antibiotic as an adjunct and within this region it is tradition especially in underdeveloped nations. Consequently, there are now many products which carry poorly defined species and sold in the market e.g., Biosubtyl® 'Nha Trang' (*B. pumilus*), Biosubtyl 'Da Lat' (*B. cereus*), Subtyl (*B. cereus*), and Bibactyl® (*B. subtilis*), but with most of them carry temporary resistance to antibiotics. (Lee et al., 2001).

2.4.2. Health, Food and Dietary Supplements:

Many *Bacillus* products are known as enhancers, they enhance the wellbeing of users by restoring the gut microflora etc. Such products are available on the internet containing poorly defined or invalid species (e.g., *Bacillus laterosporus, Lactobacillus sporogenes*). Blend of *Bacillus* species are present in some commercially available Probiotic products carry (e.g. Nature's First Food listing 42 species including 4 spore-forming species) (Sanders et al., 2003).

Cooked soya beans are fermented to produce a Japanese product with *Bacillus subtilis* called natto or *B. subtilis* var. *natto*. It is justifiable to mention here the natto that is Japanese product has been known to have probiotic properties and the *B. subtilis* var. *natto* have anti-cancer properties, and stimulate the immune system, it is also thought to produce vitamin K_2, (Inooka et

al., 1986). The concept of probiosis is strongly supported by the widely held beneficial properties of product natto that is fermented food product in Japan.

2.5. *Bacillus* Species as probiotic strain in Animal products:

In Europe, after the medical profession, by 1997 the second largest consumer of antibiotics was farming. Of this, almost 1/3rd was used as supplements in animal feed and remaining 2/3rd were used for therapeutics functions. In 1997 avoparcin antibiotic was banned to be used for animals. The development of animal vaccines was emerged to be an interesting field after the complete absence of antibiotics usage in the animal feed husbandry. Few other approaches to limit the use of antibiotics are to use prebiotics, probiotics and synbiotics. Prebiotics can stimulate the metabolic activity and growth of bacteria present in the colon. Pre-biotics are basically non-digestible food particles present in our daily food intake (Gibson and Roberfroid, 1995) and synbiotics contain the blend of pre- and probiotics.

In Europe, BioPlus® 2B and Toyocerin® are the two licensed products contain *Bacillus* strains. Many of *Bacillus* products have not been registered or licensed or withdrawn completely. Similarly, Esporafeed Plus® was failed to satisfy SCAN that enterotoxins are not produced by the *B. cereus* and it is safe to use. In addition, tetracycline resistance gene *(tetB*) is present in the *B. cereus* strain; within *B. cereus* genome *tetB* is present on a transposon. Since its ability to transfer this gene can't be ignored.

2.6.*Bacillus* Species as probiotic strain in Aquaculture products:

Most researchers are unaware of the use of *Bacillus* species in the *Bacillus* community in aquaculture, but husbandry of fish and especially shellfish is flourishing in many different countries (e.g., South East Asia). Before the proper development of digestive tract and immune system the most of the larval forms of fish and shellfish are released into the surrounding atmosphere that's why these larvae are particularly attacked and diseased with gastrointestinal disorders (Timmermans, 1987). The growth and proliferation of pathogens in intensive farming is usually promoted by the detritus that build up in a breeding pond and the resulting harvest can be catastrophically being affected. Economical losses due to such diseases have vital devastating effect for those countries which relies on aquaculture mainly for income. In 1996 economical losses alone from aquaculture were more than US$ 3 billion. Therefore, Probiotic will have a

powerful impact on supplements that can treat and save larva and would reduce such huge losses. Vaccination can only provide (even if feasible) short-term protection against pathogens in Shrimps because they have a non-specific immune response. On other side Probiotic treatment would provide protection on large spectrum. Biostart® is a commercial product carrying *Bacillus* spores used as biocontrol agent. Bacterial supplements have three major uses in aquaculture, probiotics, and biocontrol and as bioremediation agents.

2.7.The Fate of Ingested Spores:

What is the fate of these spores if they are ingested? Bacterial spores might be battered by stomach and small intestine enzymes as when these spores would be treated as food particles. Spores are vigorous bio particles so, it is predicted that a large number of these spores will sustain in the stomach, pass through the GIT and finally removed from the body with faeces. Definitely, this presumption is established on the facts about most *Bacillus* species (i) being facultative aerobes: they are not able to spread in the GIT, and (ii) mostly *Bacillus* species does not interact with GIT as they are originated from soil.

2.8.Transit kinetics:

The fate of spores can be examined experimentally after the ingestion. Four human volunteers were used to conduct a study about the transit action of the spores. In this experiment a fixed dose of 10^5 *Bacillus stearothermophilus* was given to volunteers. Many of the *B. stearothermophilus* CFU g^{-1} removed from the body in the faeces was keep up at a consistent number after initial four days of post-dosing. After eight days the spores count dropped to pointless levels. In a related study, after 10 days of first dose *B. stearothermophilus* was present in the faeces (Vesa et al., 2000). Interestingly, by the experiment it was proved that the transit kinetics of *B. stearothermophilus* was similar to that of *L. plantarum* a *Lactobacillus* probiotic bacterium that showed the colonization or, at least temporary stay in the GIT. It should also be considered that number of spores was count by CFU g^{-1} only at the time of sampling but it did not show total number of counts in the faecal sample. Despite that, in the human GIT the temporary stay duration (or longevity) of *B. stearothermophilus* was 8 to 10 days; it is somehow lengthy than the experimentally estimated time duration of the marker in the gut (Graff et al., 2001).

2.9.Spore Germination and Proliferation:

The ligated ileal loops of rabbits were used to conduct the experimental studies of spore germination in the GIT. Murine model were used to thoroughly study the spore germination. *B. subtilis* strain PY79 (derived from the 168 type strain) was used and doses of spores (from 10^8 to 10^{10}) was given to groups of inbred (Balb/c) or outbred mice (Hoa et al., 2001). The mice were accommodated individually in the cages which have gridded floors and total number of faeces was collected after 1-2 days interval after administrating the spores. These experimental results showed that after 3 hours of post-dosing spores count was detectable, more interestingly after 18 hr. of post dosing spores had been released more than what was given initially. By 5 to 7 days the level of spore count was very low. An increase in total CFU was observed in total spores count by as much as 6-fold. Since the result clearly explains that the cumulative spore count is greater in number than what was initially administered, it means spore get chance to germinate, proliferate to some extent and then re-sporulation. These results seem improbable because no direct evidence has been provided yet that shows are *B. subtilis* spores able to germinate. More than this another fact is *B. subtilis* is a facultative aerobe how can it germinate in the GIT in such severe deficiency of oxygen? More advanced research has proved that *B. subtilis* has ability to live in "anaerobic" condition as well, if it can use nitrate or nitrite as an electron acceptor and through the process of fermentation when the electron acceptor is absent (Hoa et al., 2001).

The surprising findings were not that *B. subtilis* spores were able to germinate due to two major reasons, Firstly, as spore is inert life form and proximal part of small intestine would be full of nutrients so spores can germinate as they don't need *de novo* protein synthesis. Secondly, as described earlier, some spore forming *Bacillus* are already known to proliferate and germinate in the Gut, most noticeably *B. cereus*. The surprising fact was that these spores can germinate outgrow, multiply and re-sporulation. It is also explained that GIT is not surely oxygen deficient; it has complex microenvironment which allows the growth of aerobic strains as well such as *B. subtilis*. Advocating this fact, some microaerophilic bacteria such as *Helicobacter* and *Campylobacter* could easily live in the GIT. *B. subtilis* is not only spore forming species that has ability to germinate in GIT. Recent studies have also shown in poultry and in pigs the *B. cereus* var *toyoi* was capable to germinate. Toyocerin® is a commercial probiotic product containing the strain e *B. cereus* var *toyoi* (Jadamus et al., 2001).

In the crop of broiler chickens the spores were seen to show rapid germinated and reaching at the level of 90% of initially administered spore dose. Interestingly, sporulation process was observed in the piglets by dosing with 10^8 *vegetative* cells and after 22hr over this experiment, 10^7*spores* g^{-1} of digested could be retrieved; it showed that process of sporulation is quite fast in the small intestines. Results obtained in the similar study using broiler chickens were almost same. Few other studies also showed that *B. cereus* var *toyoi* vegetative cells have inherent characteristic to show high tolerance level to both to bile salts and gastric juice. Same experiments were conducted using *lactobacillus* (*l. plantarum* ncimb 8826, *l. fermentum kld)* which are probiotic strains described that, only 7 % of an oral dosage was surviving in small intestine juices like (both to bile salts and gastric juice (Vesa et al., 2000). In mice *B. subtilis* var. *natto* had been proved to transit and germinate gut (Hosoi et al., 1999).

2.10. Resistance to Bile Salts, Low pH and Gastric Juice:

The recent studies revealed that strains of *B. coagulans* is susceptible to stimulated gastric juice (SGF; pH2-3) but resistant to 0.3% bile salts concentration. In two in vitro studies *B. subtilis* has been examined. The 1st study showed that *B. subtilis* cells were susceptible and with an almost full debt of life in 1 h to the SGF and bile salts (0.2%). Another study showed *B. subtilis* MIC of bile salts to be 0.4% and for two probiotic strains, *B. cereus* IP5832 (Bactisubtil®) as 0.2% and *B. clausii* (Enterogermina®) <0.05%. Contrary to spores of *B. subtilis* have been shown to have full potential to tolerate SGF and bile salts despite the fact that the germination of *B. subtilis* spores was partly repressed by bile salts. Another surprising and unexpected result confirms that not all spores were showing tolerance to SGF and bile salts. Especially, spores of those strains that are used in one of the commercialized product Biosubtyl® containing the spores of one of the *B. cereus* were highly susceptible to SGF and also to bile salts. Considering *B. cereus* strains have spores which are highly tolerable. The activation of spore germination due to acid could be the one reason of such unpredictable results (against the germination activated by heat). A large number of vegetative cells could be produced by the acid induced germination of spores; such vegetative cells can be targeted by SGF. Same kinds of spores were less susceptible to bile salts in comparison to SGF (Duc le et al., 2004).

These researches showed that the vegetative *Bacillus* cells are susceptible to GIT and stomach secretions which are formidable barriers; on the other hand spores are unaffected. The tentative

predictions should be presented as the gastric physiology of mouse is different from the human beings (e.g., increased stomach pH). If spores germinate as sufficient evidence proved it, they need to survive and proliferate for that purpose they might need to find a way to disappear the acidic luminal fluids of GIT. The toxic effects of the bile salts would be diluted as the spores transit through GIT from small intestine and finally the cells would passed to the anaerobic atmosphere of the colon. Presumably, the protection provided by covering effect of food and clumping would be enough. Alternately, perhaps the formation of blend of biofilms with gut microbes and adhesion to the gut mucosal membrane would temporarily provide the niche to the bacteria. Basically, The most efficient techniques for surviving used by the spore formers is re-sporulation in the extreme conditions of the stress provided by the passage through GIT (Duc le et al., 2004).

2.11. Colonization:

Presently, the residence of non-pathogenic spore forming bacteria depends on the physiological, dietary factors of the host as well as the specific spore forming species. The *B. cereus* which is pathogenic strain, it also has temporary infection approx. 24 h and it cast off completely after 24 to 28 h. It should be reminded again that information about *Bacillus* is far from completion as these strains have not been tested in animal models, and most of the research is conducted on the specific strains or mostly pathogenic strains. It should not be rejected that new gut colonizing bacteria still need to be identified and characterized.

B. cereus strains show more hydrophobicity than other *Bacillus* spp. and different *B. cereus* strains spores show adherence to different kinds of surfaces. Another advance studies showed that the binding of human epithelial cells depends on hydrophobicity of spores, they are directly proportional to each other means the greater the hydrophobicity of spores, the greater would be the adhesive properties (Andersson et al., 1998).

(Duc le et al., 2004) studied that different probiotic strains have different transit times in the GIT and it depends on the hydrophobic ability of the spores to bind to mucosal epithelial cells. One more considerable point is the production of biofilm on the mucosal epithelium. Microorganisms in the gut usually exists in blended biofilms by attaching to mucosal membrane and food particles (Palestrant et al., 2004) and *B. subtilis* has been reported to synthesize multicellular

structures and as well as biofilms. The most preferred site for spore formation is the fruiting bodies as these are robust films with aerial structures (Branda et al., 2001). Very little is known about the spore formers and their natural habitat as many studies were conducted to study the interaction of *Bacillus* spp. with surfaces that mimic their natural environment.

2.12. Dissemination and Intracellular Fate:

While assessing the potential of probiotic bacteria even if they can cross the mucosal membranes and spread to reach other aimed tissues and organs or do it proliferate? Using laboratory strain of *B. subtilis* spores recent experiments were designed. Almost 10^9 spores were given to inbred mice single dose for 5 consecutive days. Low level spores still with viable count were observed in Peyer's Patches and mesenteric lymph nodes. The spores were not able to reach the deep organs like kidney and liver but they had potential to cross the mucosal barriers. The M cells are located in the mucosal epithelial cells of small intestine have ability to take up by recognizing 1.2 μm spore size and then passes it to Peyer's Patches before transporting them to efferent lymph nodes. Antigen presenting cells are present in greater number in the Peyer's Patches; specifically it is enriched with dendritic cells that are activated in response of Th1 and Th2 cellular response. Another in vitro experiment has shown that *B. subtilis* spores could be easily phagocytosed in the presence of cultured macrophages (a raw264.7 cell line) of murine model (Duc et al., 2004).

Unlike *B. subtilis* the *B. anthracis* which has another important aspect to escape the host toxic intracellular environment because their vegetative cells have capsule around them, it also protects them from host immune system. It was suggested that as a 1^{st} step in destroying the bacterium, phagocytic cell may help to induce the intracellular spore germination, and phagocytic cells may provide appropriate signals to spores to start germination (Duc et al., 2004). Interestingly, the germination process is same in many species as the genes involved in the germination process are remarkably conserved amongst *Bacillus* species (Setlow, 2003).

2.13. Mechanism of Probiosis:

2.13.1. Immune Stimulation:

Probiosis is supported by an important mechanism of immunomodulation or immune system activation. Immune system is activated by the oral administration of spores; it has been already proved by a number of studies in the humans and animals. This proposed the fact that spores are neither considered as innocuous gut passengers nor treated as a food. As explained earlier following oral inoculation of *B. subtilis* spores result in dissemination of spore's gut-associated lymphoid tissue GALT (Peyer's Patches and mesenteric lymph nodes). Few in vitro studies showed that phagocytosed spores can germinate but are unable to express vegetative genes, also not able to replicate. Anti-spore IgG responses level would be at significant level after oral dosage. If antigen is taken up by B cells normally Anti-spore IgG and secretory IgA are produced. Detailed analysis of the subclasses showed IgG2a to be the initial subclass produced and this is often seen as being indicative of a type 1 (Th1) T-cell response. The subclass IgG2a showed to be the initial subclass is produced and it is indicative of the type 1 (Th1) cellular response (Robinson et al., 1997).

It was confirmed by in vitro studies B. *subtilis* or *B. pumilus* spores cultured with macrophages induce the production of proinflammatory cytokine IL-6. Proinflammatory responses are not always necessarily beneficial probiotic feature. The proinflammatory response is linked with the autoimmune diseases such as inflammatory bowel diseases including Crohn's disease and ulcerative colitis (Sartor, 1996).

2.13.2. Synthesis of Antimicrobial compounds:

One of the principal mechanisms depicted by the probiotics is the production of antimicrobials (microbial interference therapy) that inhibit the growth of pathogenic microorganisms in the GIT. *Bacillus* species usually produce a large number of antimicrobials compounds (Hong et al., 2005).

A number of commercial Probiotic products contain strains of *B. coagulans* which are usually mislabeled as *Lactobacillus sporogenes*. Coagulin is produced by *B. coagulans*, a heat-stable, protease-sensitive BLIS with antimicrobial activity against Gram-positive bacteria. *Candida*

albicans growth in the intestinal tract is inhibited by the antimicrobials produced by the *B. subtilis* var. *natto* (Ozawa et al., 1979) and another antimicrobial compound a surfactin has been known with activity against yeast. The antimicrobials effect of such compounds is not clearly understood in vivo and to mimic the in vitro effects in the host GIT is quite complex. To demonstrate this Hosoi et al. (Hosoi et al., 1999) mice was dosed with *B. subtilis* var *natto* spores and explained that the growth of *Lactobacillus* was promoted under some dietary conditions but decreased viable counts under others. Formation of biofilms on the gut epithelium is influenced by the dietary and physiological conditions of the extremely complex GIT with microenvironment. The ingested spores of probiotic strains are subjected to number of different facts which influence its interaction with gut microbiota and in turn, its ability to secrete antimicrobial compounds and survive in the GIT.

2.14. Safety of *Bacillus* Products

Many questions arise regarding the safety of probiotic use whether for human or animal use, since the product is utilized in large quantities on a regular basis? For human use the primary concern is whether the bacterial species is safe to ingest or GMP (good manufacturing practices) conditions have been used in production. The concerns for probiotic use in animals feed is because of uncertainty of inter species transfer of antibiotic resistance genes. The Food and Drug Administration of the United States of America has not, as yet, granted any probiotic product GRAS (Generally Regarded As Safe) status although *Bacillus* species do carry GRAS status for specific industrial applications (e.g., enzyme production) (Sanders et al., 2003).

CHAPTER 3: MATERIALS & METHODS

3.1. Sample Collection:

The spices were collected from Lahore market. The eleven different kinds of spices were taken in the raw edible form and packed into zipper bags to store at room temperature. The spices used are as follow:

Sample No	Scientific Name	English Name	Urdu Name	Parts used	Place of Collection
1	*Piper nigrum L.*	Black pepper	kali mirch	dried fruits	Lahore
2	*Coriandrum sativum L.*	Coriander	Dhaniya	seeds	Lahore
3	*Allium sativum*	Garlic	Lehsan	cloves	Lahore
4	*Mentha × piperita L.*	Peppermint	Podina	leaves	Lahore
5	*Syzygium aromaticum*	Clove	Longue	flower buds	Lahore
6	*Zingiber officinale Rose.*	Ginger	Adrak	dry rhizomes	Lahore
7	*Trigonella foenum-graecum L.*	Fenugreek	Methe	seeds	Lahore
8	*TrachyspermumAmmi L.*	Ammi	Ajwain	seeds	Lahore
9	*Cuminum cyminum L.*	Cumin	Zeera	seed	Lahore
10	*Elettaria cardamomum*	Cardamom	Alaechee	seeds	Lahore
11	*Cinnamomum verum*	Cinnamon	Dar-cheene	Bark	Lahore

Table 1: Samples of Spices collected from Lahore city in Pakistan.

3.2. Isolation of Bacteria:

The spices were dried and grinded using autoclaved pestle and mortar. The 10g powder form of spices was added into 10ml of 0.1% peptone water (diluents) and put it in orbital shaker for almost about 20 minutes.

Serial dilution technique was used to isolate bacteria. 1ml of sample was serially diluted with 900 µl of double distilled water up to 10^{-8} dilutions. From last four dilutions, 50 ul were spread on nutrient agar having pH 7.4 and incubated at 37°C for overnight. Isolated strains were purified and grown in nutrient broth and then with 40% glycerol, stocks were made. These stocks were kept at -20°C and -80°C respectively.

3.3. Phenotypic Identification:

3.3.1. Morphology:

Pure cultures were platted on nutrient agar plates and incubated at 37°C for overnight. Colonies were observed for their shape and color.

3.3.2. Gram Staining:

Colonies were gram stained by using standard procedure. Single colony from nutrient agar plates was selected for this purpose. A drop of distilled water was dispensed on the slide and colony was transferred with the help of sterile loop from nutrient agar plate to prepare smear. The smear was then heat fixed and treated with crystal violet for 1 min. After washing with distilled water iodine solution was added for 40 sec and then again washed with distilled water. After washing, ethanol was added as decolorizing agent for 5 sec. Again washed with distilled water and safranin was added for 40 sec and washed with distilled water. After air drying, slides were observed under light microscope at 100X objective with immersion oil. Purple stained bacteria were gram positive. After staining of bacterial isolates, cells were observed to study their morphology.

3.4. Biochemical Tests:

3.4.1. Catalase Test:

This test is used to recognize microorganism that generate enzyme, catalase. This enzyme detoxifies hydrogen peroxide by separating it down under water and also oxygen gas. Standard procedure was used for catalase test. Single colony from nutrient agar was picked and transfer to a dry clean slide by sterile loop. Place a drop of 3% H_2O_2 on the colony, mix well.

3.4.2. Methyl red test:

This test was performed to test the ability of microorganism to produce and maintain stable acid end products to overcome the buffering capacity of system. Standard procedure was used methyl red test. Single colony from nutrient agar was picked and transferred to tubes containing MR-VP broth Incubate at 37 °C for up to 1 day. Add about 5 drops of the methyl red indicator solution to the test tubes. A positive reaction is indicated, if the medium show changes in color to red within a few minutes.

3.4.3. Oxidase Test:

The oxidase test is used to identify bacteria that produce cytochrome c oxidase. Standard procedure was used for oxidase test. Grow a fresh bacterial culture (24 hours) in 6 ml of nutrient broth. Add 1-2 drops of Oxidase reagent (Wurster's reagent) shake robustly to ensure mixing and thorough oxygenation of the culture. A positive test is shown by the development of dark purple color.

3.4.4. Indole Test:

Check for the presence of "tryptophanase" enzyme which split tryptophan into indole. Standard procedure was used for Indole Test. Inoculate the tryptophan broth with broth culture. Incubate at 37°C for 1 day. Add 0.5 ml of "Kovac's reagent" to the broth culture. Positive results are shown by the Development of bright red color at intermix of reagent and broth.

3.5.Genotypic identification:

3.5.1. Extraction of Genomic DNA:

Bacterial isolates were grown overnight in 10ml nutrient broth at 37° C. The 2ml of bacterial culture is taken out from overnight grown culture in to Eppendorf and centrifuged for 3 minutes. The supernatant was discarded and pellet was suspended in 570µl TE buffer. The mixture was incubated for 1 hour at 37°C after the addition of 30µl of 10% SDS with thorough mixing. In the next step 100µl of 5M NaCl was added followed by addition 80µl of CTAB/NACL and incubated at 65°C. About 700µl of chloroform/isoamyl alcohol was added and centrifuge 4 to 5 minutes at 1300 rpm. Transfer supernatant to fresh tube. Add 600µl of isopropanol and mix gently until the stringy white DNA precipitate appears. After centrifuge for 2minutes at room temperature discard supernatant. Add 70% ethanol to pellet microfuge for 5 minutes at room

temperature at 1100 rpm and air dry the pellet. Finally the pallet was suspended in 20µl TE buffer.

DNA analysis was done using the following two methods.

3.5.2. Agarose Gel Electrophoresis:

DNA was analyzed on 1% agarose gel. 0.5 grams (gm) agarose was melted in 50 ml of 1x TAE (25 mM Tris,5mM Glacial Acetic acid, 1mM EDTA, pH 8.0) in a flask in a microwave oven until a clear transparent solution was achieved. After cooling, 5µl of 0.01% ethidium bromide was added. The melted agarose was poured into a gel caster. An appropriate comb was selected when the gel set completely at room temperature the comb was carefully removed and placed the gel tray in an electrophoresis tank contained sufficient amount of 1X TAE. DNA samples were mixed with 6X loading buffer (0.4% bromophenol blue, 0.4% xylene cyanol and 25% Ficol). The DNA samples and DNA markers [10 kilobase pair (kb)] were loaded into the wells of solidified gel submerged in 1X TAE. Gel was allowed to run at 80 volts for 45 minutes. The DNA bands in the gel were visualized using Dolphin-Doc plus Image System.

3.5.3. Quantification of DNA:

The isolated DNA was quantified using NanoDrop- spectrophotometer (Thermo Scientific) as per user manual.

3.6.Polymerase Chain Reaction (PCR):

The fragment of 16s rDNA with size 1500bp was amplified using PCR (MultiGene) for all the isolates using primers FD1 and rp2 manufactured by Eurofins Scientific.

Table 2: Primers	Primers used for 16S rDNA Amplification Sequence 5‘ to 3‘
FD1 f	AGAGTTTGATCCTGGCTCAG
rp2 r	AAGTCGTAACAAGGTAGCCT

Total of 50µl Reaction (1µl taq polymerase, 6µl Buffer, 2.5µl MgCl2, 2µl dNTP’s, 1µl forward and reverse primers, 3µl template, 33.5µl PCR H2O) mixture was prepared for PCR reaction.

3.6.1. PCR Conditions:

Condition used for PCR were 94°C for 5 mins, 94°C for 30s, 58°C for 30s, 72°C for 2 mins and 72°C for 5 mins.

3.7.Phylogenetic Tree:

To conduct the phylogenetic study, sequences were retrieved from NCBI database. The sequences were aligned using Geneious alignment (Geneious v. 8.1.6) (Kearse *et al.*, 2012).

3.7.1. Inclusion & Exclusion Criteria:

There is an insertion and deletion criteria for the sequences;

- The sequences which are too small are deleted.
- The sequences which are large in size are edited from the respected side so that all the sequences are same in length.
- An outgroup sequence AM179883 Pseudomonas spp. was used.

3.7.2. Phylogenetic Analysis Using Neighbor-Joining Method:

Phylogenetic analysis was concluded by using the Neighbor-Joining (NJ) method and HKY algorithm model. Bootstrap value set for the analysis was 1,000.

3.8.Assessment of Probiotic Potential:

3.8.1. Acid Tolerance:

All the isolates were grown in nutrient broth for 24 hours. The 2ml of overnight grown bacterial culture was taken and centrifuged for 3 minutes. The pellet was suspended in 2ml of 0.9% saline. To check pH tolerance, pH of nutrient broth was adjusted to 2 and 3 with (1.0) molar of hydrochloric acid. 1ml of inoculum was added into the 10ml of pH adjusted nutrient broth and in control with normal pH. Samples were withdrawn at 3 different time intervals i.e. 0 minutes, 240 minutes and 480 minutes. Optical density (OD) was measured at 600nm in spectrophotometer.

3.8.2. NaCl Tolerance:

All the isolates were grown in nutrient broth for 24 hours. The 2ml of overnight grown bacterial culture was taken and centrifuged for 3 minutes. The pellet was suspended in 2ml of 0.9% saline. To check NaCl tolerance, the nutrient broth containing 3.5%, 6.5% was prepared. 1 ml of

inoculum was added into the 10ml of NaCl containing nutrient broth and in control without NaCl. Samples were withdrawn at 2 different time intervals i.e. 240 minutes and 480 minutes. Optical density (OD) was measured at 600nm in spectrophotometer.

3.8.3. Bile Tolerance:

To check ability of isolated strains to grow in presence of Bile salt, Oxbile of Oxide was added into nutrient broth. Each strain was being inoculated (2% v/v) into 10ml nutrient broth containing 0%, 0.037%, 0.15%, and 0.3% (w/v) of Oxbile along with a control i.e., without bile salt cultures were incubated at 37°C on shaking. Samples were drawn at 4 different time intervals i.e. 0min, 60min, 120min, 180min to measure optical density at 600nm.

3.9.Antibiotic Susceptibility Assay:

To analyze antibiotic resistance of isolates four different antibiotics were used. All the antibiotics were available in the form of disc. Isolates were grown in nutrient broth for 24 hours. 80ul of the inoculum was spread evenly on nutrient agar plates. Antibiotics used were CN-10 (Gentamicin), AMC-30 (Amoxycillin), TE-30 (tetracycline), PB-300 Polymyxin. Antibiotic discs were then placed on the nutrient agar plates and incubated at 37°C for 1 day. Appearance of inhibition zone was showing bacterial susceptibility to the antibiotics. The diameter of the inhibition zones was measured and the declarative zone sizes were presented.

CHAPTER 4: RESULTS

4.1.Sample Collection:

The eleven different kinds of spices were taken from Lahore city in Pakistan. Morphologically different bacteria were selected at varied dilutions of samples. The highest percentage of bacterial isolates was found in black pepper and peppermint. The probiotic strain *Bacillus subtilis* was found in few of the spices like ammi, cinnamon, cardamom.

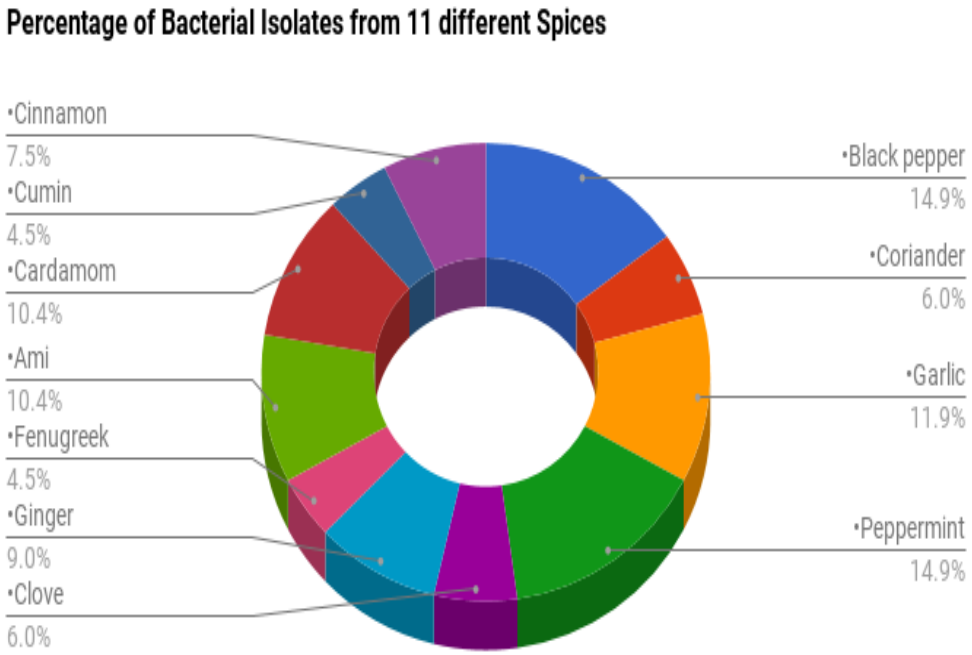

Figure 1: Percentage of different bacteria isolated from 11 different spices.

4.2.Isolation of Bacteria:

Stock samples of 11 different spices were prepared and spreading on nutrient agar media was executed to get the mother plates. Mother plates contain different number of bacteria associated with spices. Further dilutions were prepared to get the pure colonies separately.

Figure 2: Mother Plate: the spreading of stock spice ammi.

This plate is containing total number of bacteria associated with one of the spices sample.

4.3.Phenotypic Identification:

4.3.1. Colony Morphology:

The purified bacterial isolates were cultured on nutrient agar plates. These colonies were observed for their color and shape.

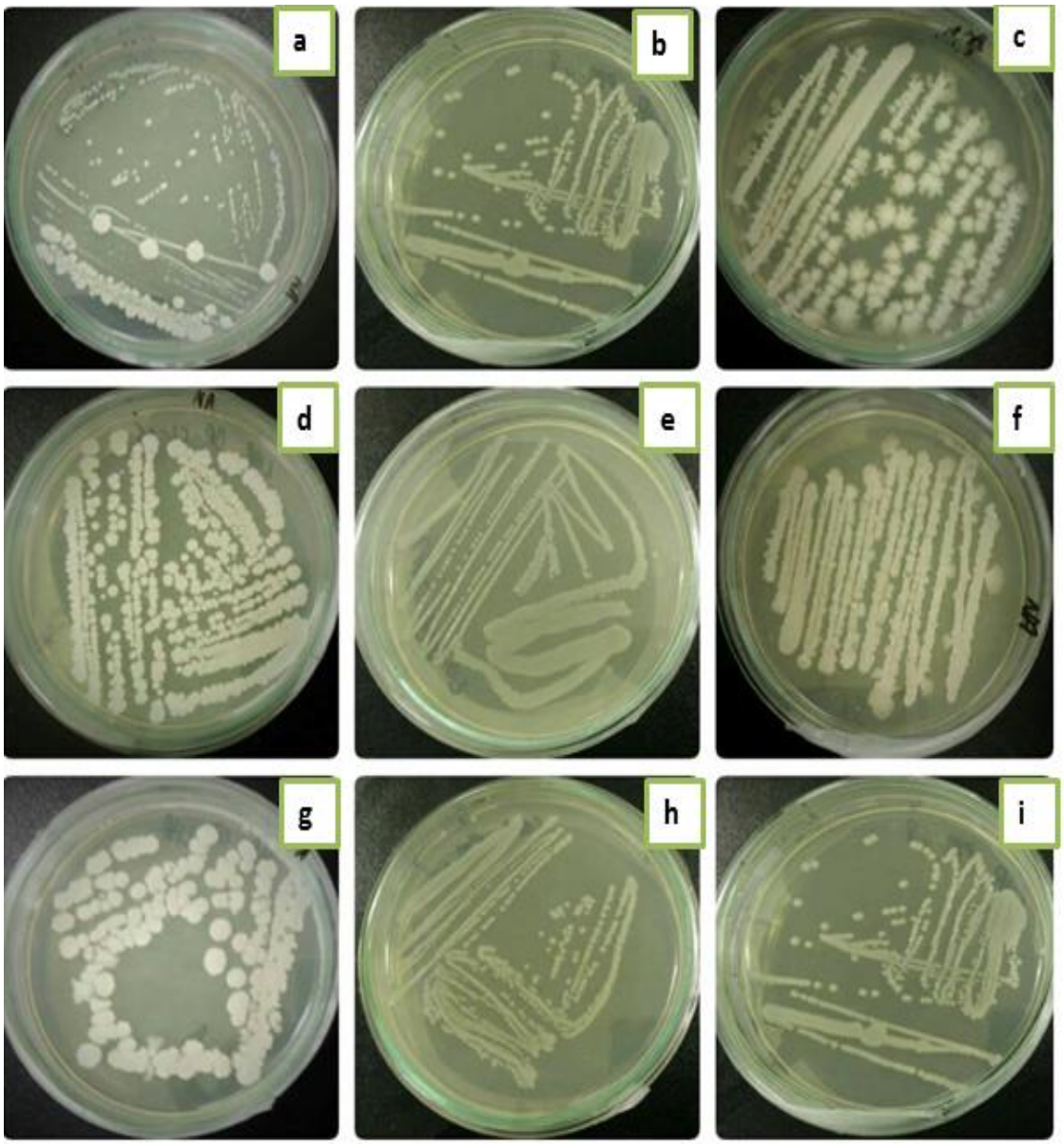

Figure 3: Different Bacterial Isolates from Spices grown on Nutrient agar media.

Bacterial isolates from different Spices were purified on nutrient agar by streaking to obtain single pure colony. These are the morphologically different bacteria found in association with a. Black pepper, b. Pepper mint, c. Fenugreek, d. Coriander, e. Cinnamon, f. Ginger, g. h. Ammi, i. Cumin, h. Cardamom.

By this procedure many different isolates were purified by streaking and total number of isolates from every sample was different and it is listed below.

No	Samples	Scientific Name	Bacterial Isolates
1	Black pepper	*Piper nigrum L.*	10
2	Coriander	*Coriandrum sativum L.*	4
3	Garlic	*Allium sativum*	8
4	Peppermint	*Mentha × piperita L.*	10
5	Clove	*Syzygium aromaticum*	4
6	Ginger	*Zingiber officinale Rose.*	6
7	Fenugreek	*Trigonella foenum-graecum L.*	3
8	Ami	*TrachyspermumAmmi L.*	7
9	Cardamom	*Cuminum cyminum L.*	7
10	Cumin	*Elettaria cardamomum*	3
11	Cinnamon	*Cinnamomum verum*	5

Table 3: The total number of bacteria isolated from different spices.

4.3.2. Gram Staining:

Gram staining was performed for all the selected isolates. The selected eight isolates were gram positive.

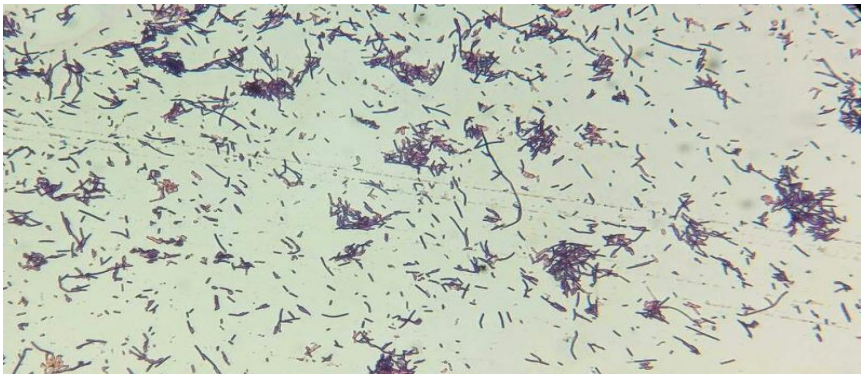

Figure 4: Morphology of one isolate as reveled by gram staining.

Gram staining was done to know whether the strains are gram positive or gram negative.

Sample name	Gram Staining
Bacillus subtilis	+
Bacillus spp.	+
Bacillus subtilis	+
Bacillus tequilensis	+
Bacillus subtilis	+
Bacillus siamensis	+
Bacillus subtilis	+
Bacillus velezensis	+
Lactobacillus plantarum	+
Staphylococcus aureus	+

Table 4: Showing the gram staining results of selective strains

4.4.Biochemical Tests:

4.4.1. Catalase test:

Catalase negative isolates does not produce bubbles when colonies are treated with 3% hydrogen per oxide. Catalase positive isolates produce small bubbles on interaction with 3% hydrogen per oxide. This test is basically conducted to differentiate aerobic and anaerobic bacteria.

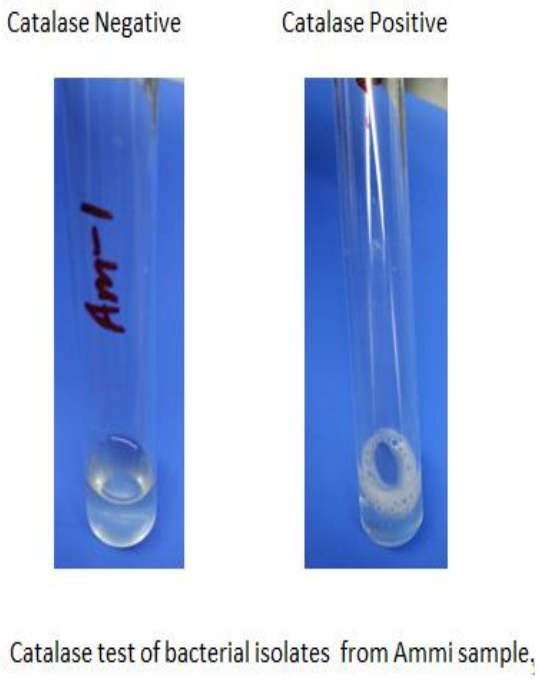

Figure 5: Catalase test of bacterial isolates from ammi sample.

4.4.2. Methyl red test:

Methyl red indicator test is used to test whether the bacterial isolates have acid fermentation capability when provided with glucose. A positive reaction is indicated, if the color of the medium changes.

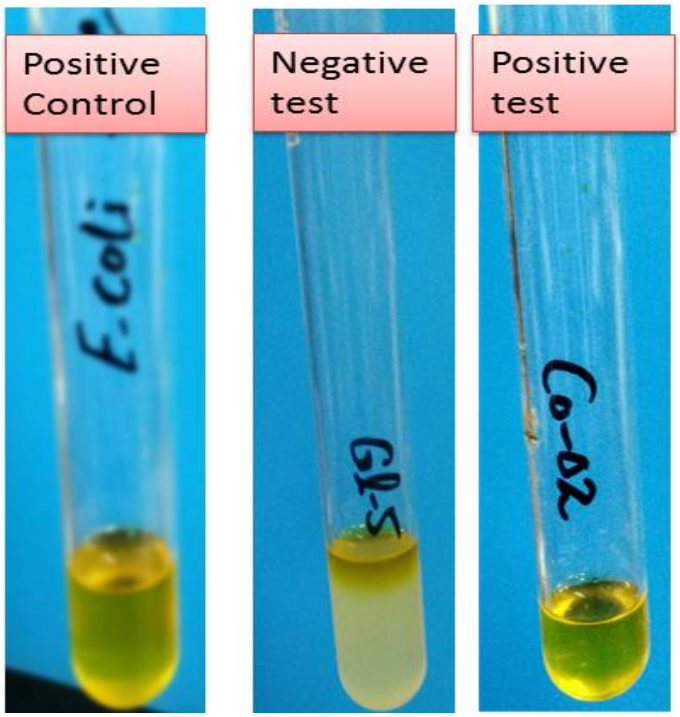

Figure 6: Methyl red test of E.coli positive control and bacterial isolates from garlic and coriander sample.

4.4.3. Indole Test:

Kovac's reagent is used to test bacterial culture if they have ability to convert the tryptophan amino acid into indole acetic acid. Positive results are shown by the development of bright red color at intermix of reagent and broth.

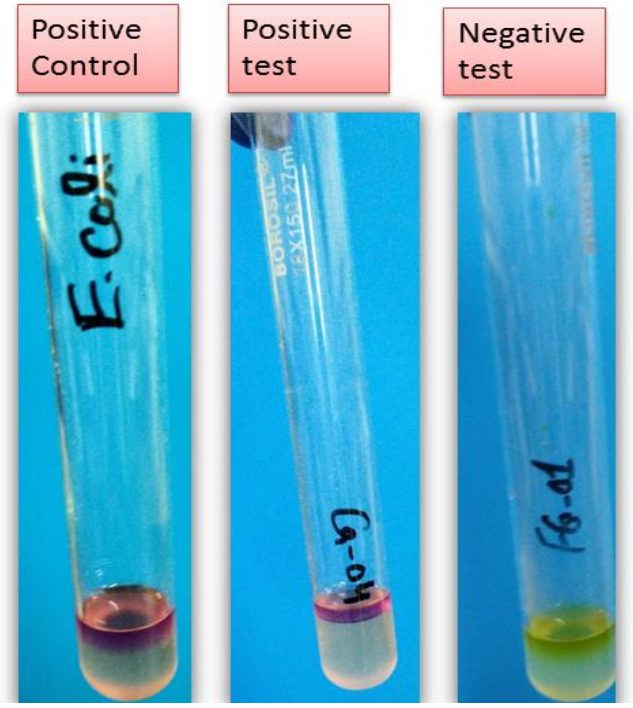

Figure 7: Indole test of E.coli positive control and bacterial isolates from cardamom and fenugreek sample.

4.4.4. Oxidase Test:

In oxidase test, Wurster's reagent is used to test the presence cytochrome c oxidase enzyme in electron transport chain. A positive test is shown by the development of dark purple color.

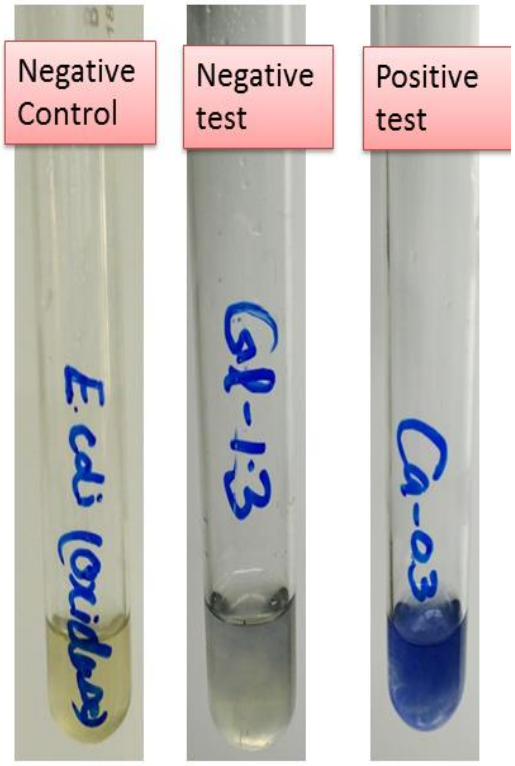

Figure 8: Oxidase test of E.coli positive control and bacterial isolates from garlic and cardamom sample.

These different biochemical tests were performed for all the strains involved in this study. This table is compromising of only those strains that assessed to have probiotic properties.

Sample name	Catalase Test	Oxidase Test	Indole Test	Methyl red Test
Bacillus subtilis	+	-	-	+
Bacillus siamensis	+	+	+	+
Bacillus velezensis	+	+	+	+
Bacillus subtilis	+	-	-	+
Bacillus tequilensis	+	-	-	-
Bacillus sp.	+	+	-	+
Bacillus subtilis	+	+	-	+
Bacillus subtilis	+	-	-	+

Table 5: Biochemical tests of different bacterial isolates.

4.5.Genotypic Identification:

4.5.1. Extraction of Genomic DNA:

Bacteria from a saturated liquid culture are lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides, and remaining proteins are removed by selective precipitation with Cetyl trimethylammonium bromide (CTAB), and high-molecular-weight DNA is recovered from resulting supernatant by isopropanol precipitation.

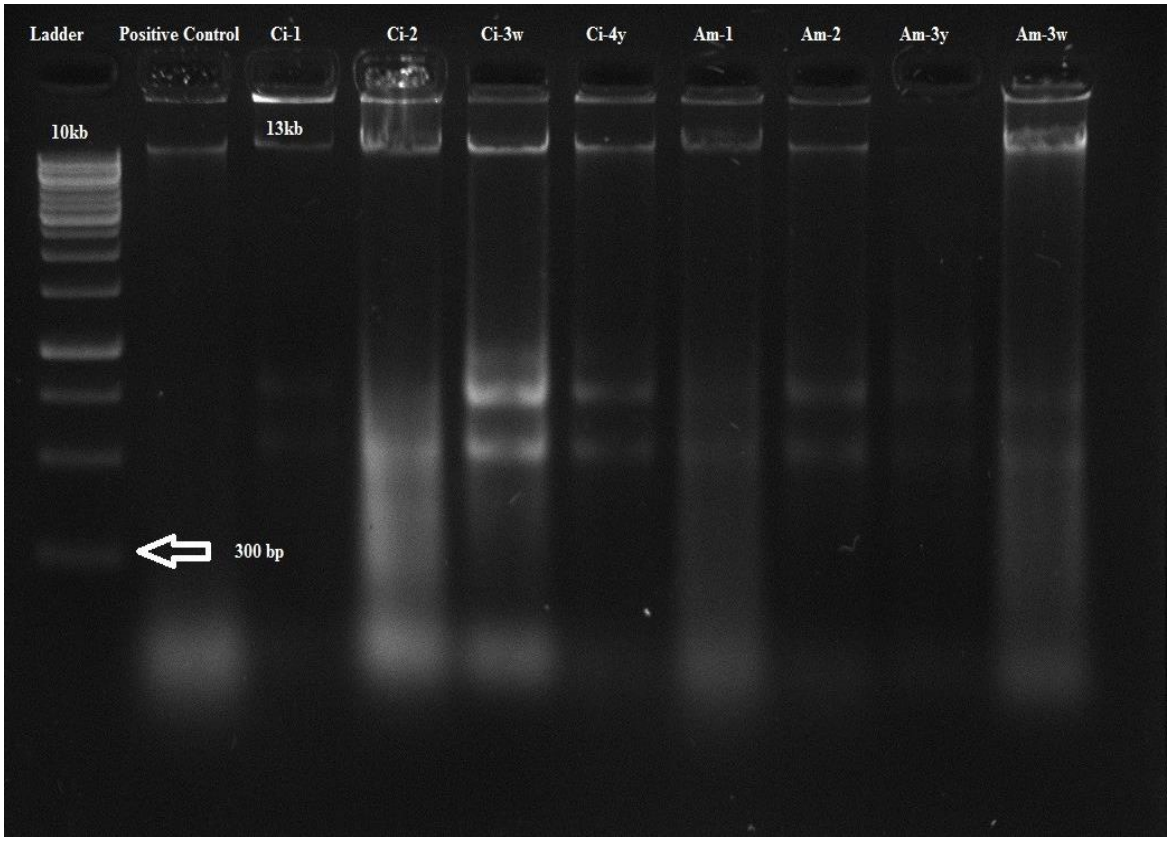

Figure 9: DNA Extraction of Garlic Bacterial Isolates by CTAB method

4.5.2. Quantification of genomic DNA:

Genomic DNA of all the isolated bacterial strains was quantified on NanoDrop spectrophotometer. 1ul of sample contains more than 100ng of DNA.

4.6.Polymerase chain reaction (PCR):

PCR product of 16S rDNA was 1500 bp. It was amplified by using FD1 and rp2 primers. PCR product was observed on agarose gel because of electrophoretic separation. A fragment of 1500bp was observed for all the samples.

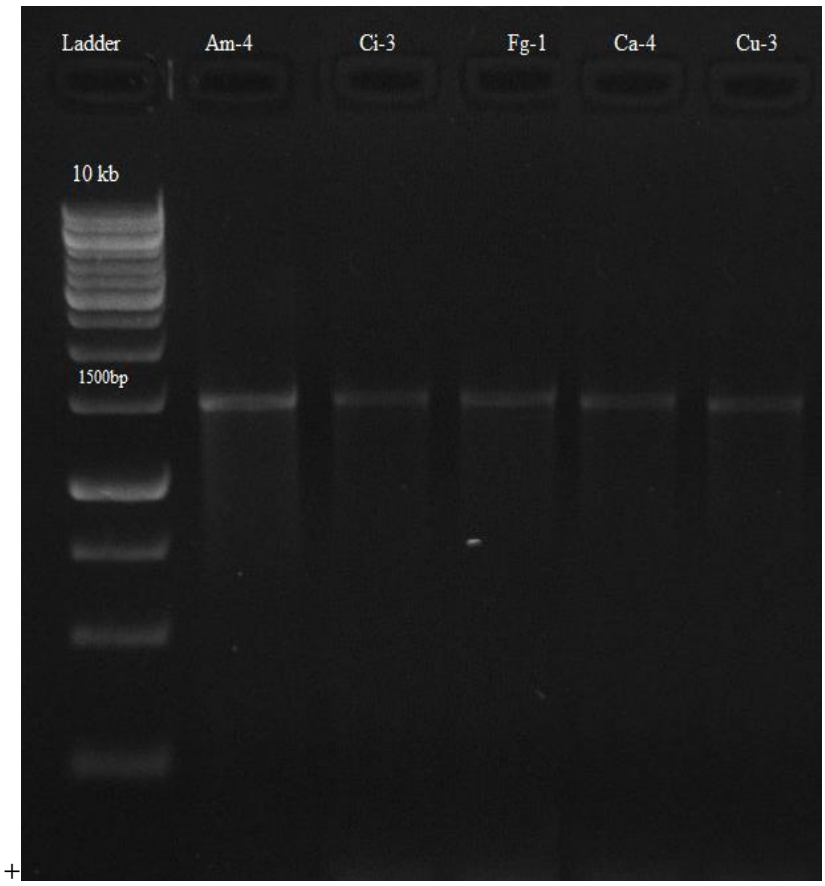

Figure 10: The confirmation of 16S rDNA amplification as reveled by the presence of 1500bp fragment on 1% agarose gel.

4.7.Submission of Ribosomal RNA (rRNA) Sequencing Results in Genbank:

PCR purified products of 18 isolates were sent for sequencing to Eurofin USA. Sequencing was done through Sanger DNA sequencing method. Out of these 18, results of only 8 strains were retrieved by making consensus sequence. Range of consensus sequence of all strains lies between 400- 914bp. Other sequences have unreliable sequence result of forward and reverse

primers in their chromatogram. All the consensus sequence was blast to get the similarity profile. Results showed 97%-100% similarity. The results received were showing presence of *Bacillus sp., Bacillus subtilis, Bacillus tequilensis, Bacillus velezensis, Bacillus siamensis, Bacillus subtilis, Bacillus subtilis, and Bacillus subtilis.* The sequences were submitted in GenBank to get the accession number.

Sample name	**Accession number**
Bacillus subtilis ca-5	MF359552
Bacillus sp.	KY986529
Bacillus subtilis ca-7	KY971625
Bacillus tequilensis	KY986446
Bacillus subtilis ci-4w	KY986416
Bacillus siamensis	KY986746
Bacillus subtilis am4	KY971638
Bacillus velezensis	KY971953
Lactobacillus plantarum	MF480392.1
Staphylococcus aureus	KY972266.1

Table 6: The accession numbers of 16S rDNA sequences submitted in GenBank for specie level identification.

4.8.Phylogenetic Analysis:

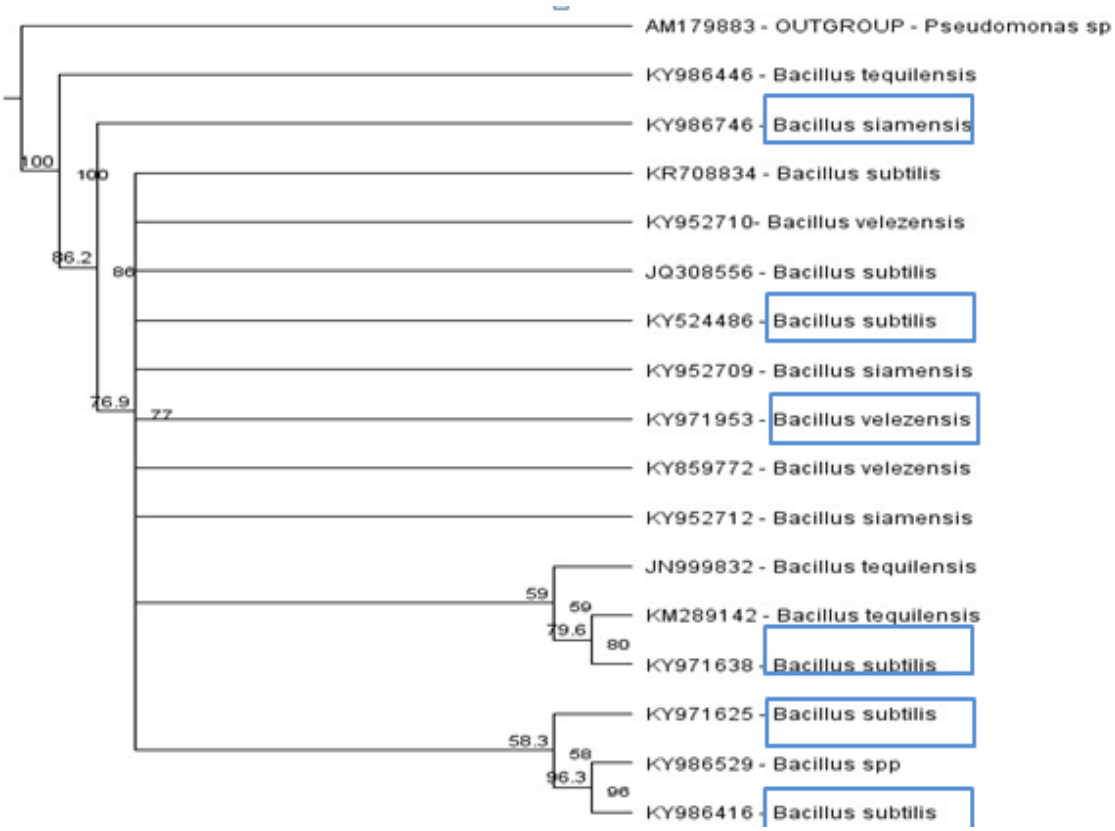

Figure 11: Phylogenetic tree constructed using Geneious software

This tree represents the evolutionary relationship of *Bacillus* species associated with Spices. The evolutionary distances were computed using Neighbor-Joining method and HKY Algorithm model. The confidence support value of nodes was estimated by 1,000 replicates of bootstrap.

4.9.Assessment of Probiotic Potential:

4.9.1. Acid Tolerance:

All the eight *Bacillus* isolates were showing high tolerance at pH of 2 till 120 minutes and then their growth decreases as their incubation time increases. Most of the strains showed normal growth pattern that is their growth was least in the start at 0 min and then start increasing till 120 minutes and then start decreasing at 240 minutes. But some of the strains showed very insignificant increase in their growth at 120 minutes. There were two strains which showed exceptionally well growth at 240 minutes.

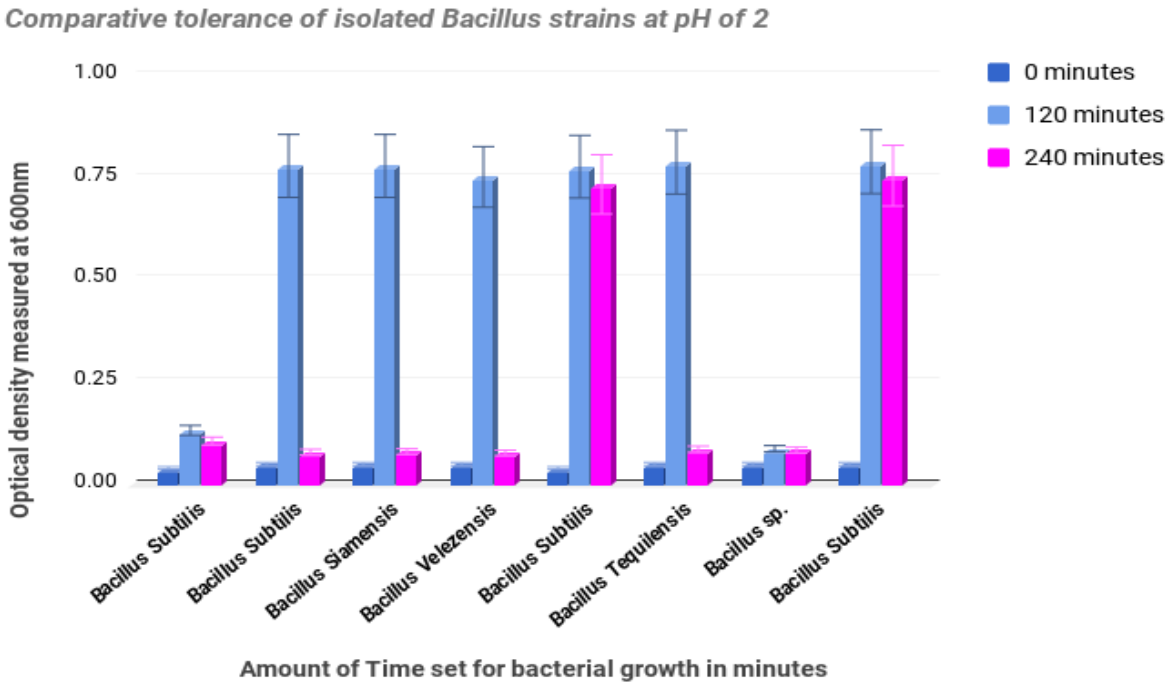

Figure 12: Bacterial isolates grown in media with pH of 2.

This graph shows that 8 isolates grown in media with pH of 2 and their OD was measured at 3 different time intervals. All the isolates were having normal behavior except two strains.

All the eight *Bacillus* isolates were showing high tolerance at pH of 3 till 120 minutes and then their growth decreases as their incubation time increases at 240 minutes. But two strains showed increased in growth rate even at 240 mins at pH of 3.

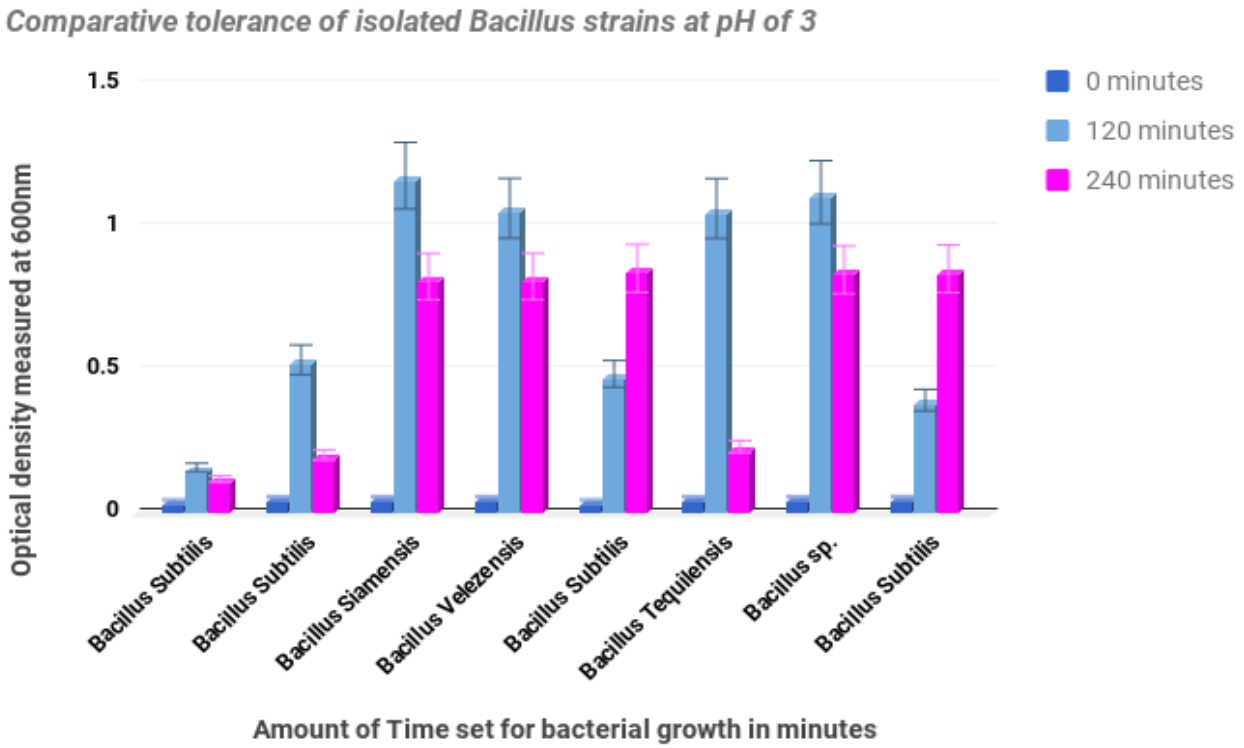

Figure 13: Bacterial isolates grown in media with pH of 3.

This graph shows 8 isolates grown in media with pH of 3 and their OD was measured at 3 different time intervals.

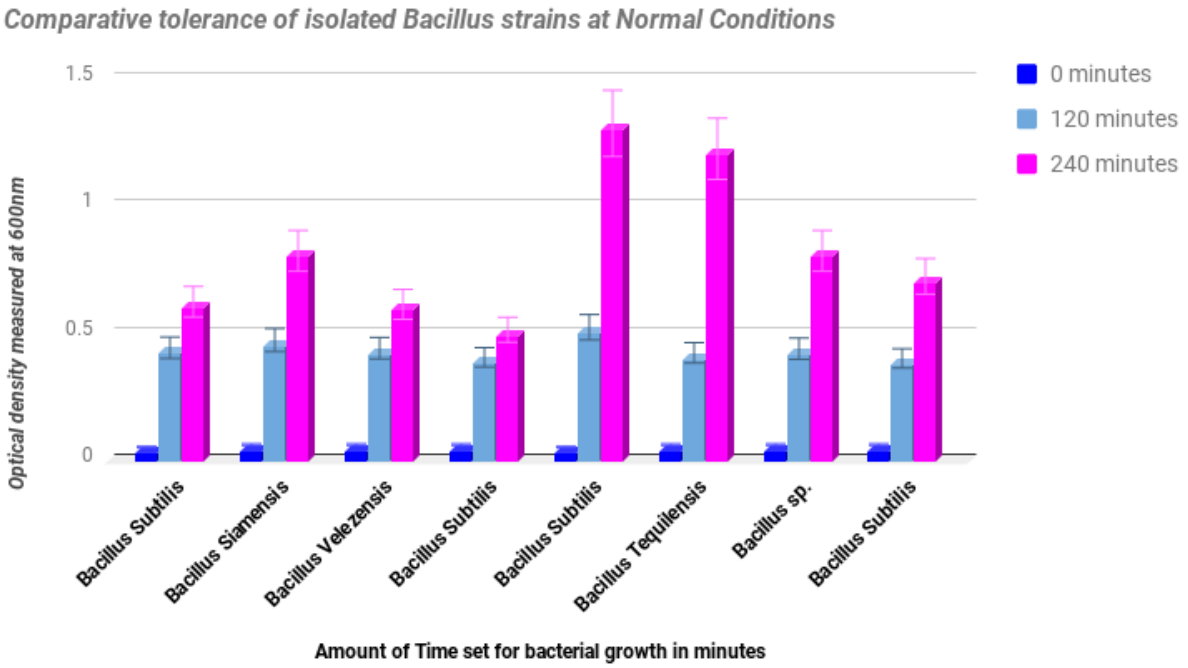

Figure 14: Growth of bacterial strains at normal conditions (Positive control).

4.9.2. NaCl Tolerance:

All the eight *Bacillus* isolates were showing high tolerance for 3.5% NaCl at 120 minutes and 240 minutes. This test explained the osmotolerance potential of all *Bacillus* spp. The physiology, metabolism, enzyme activity, and water activity could be affected if bacterial cells are cultured at high salt concentration because the cells might be able to lose their turgor pressure.

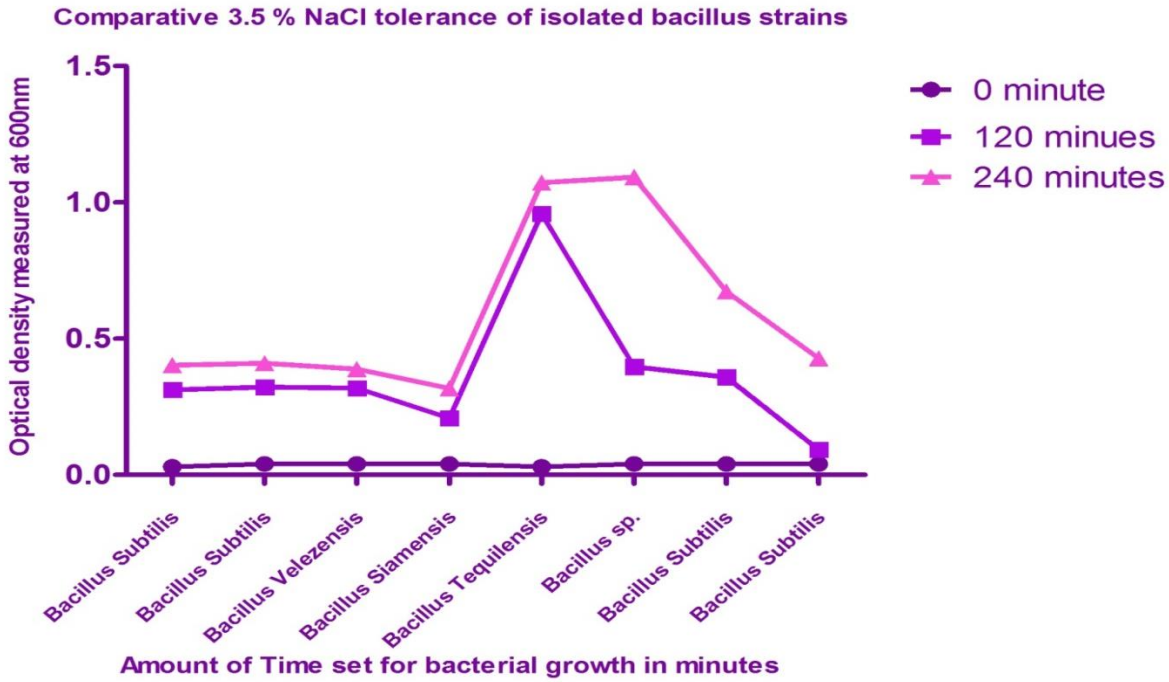

Figure 15: Bacterial isolates grown in media with 3.5% NaCl.

This graph is showing all the 8 isolates resist as they were grown in media with 3.5% NaCl and their OD was measured at 2 different time intervals.

All the eight *Bacillus* isolates were showing high tolerance for 6.5% NaCl at 120 minutes but at 240 minutes only three strains showed exceptionally high growth rate.

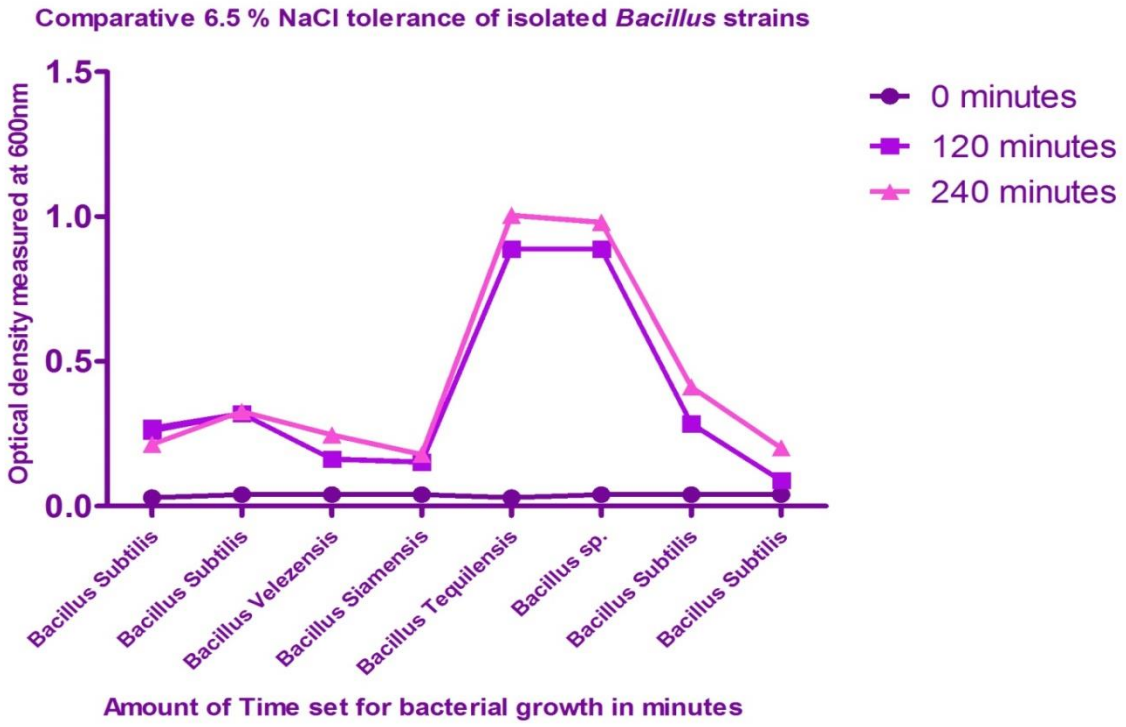

Figure 16: Bacterial isolates grown in media with 6.5% NaCl.

This line graph depicts all the 8 isolates resist as they are grown in media with 6.5% NaCl and their OD was measured at 2 different time intervals.

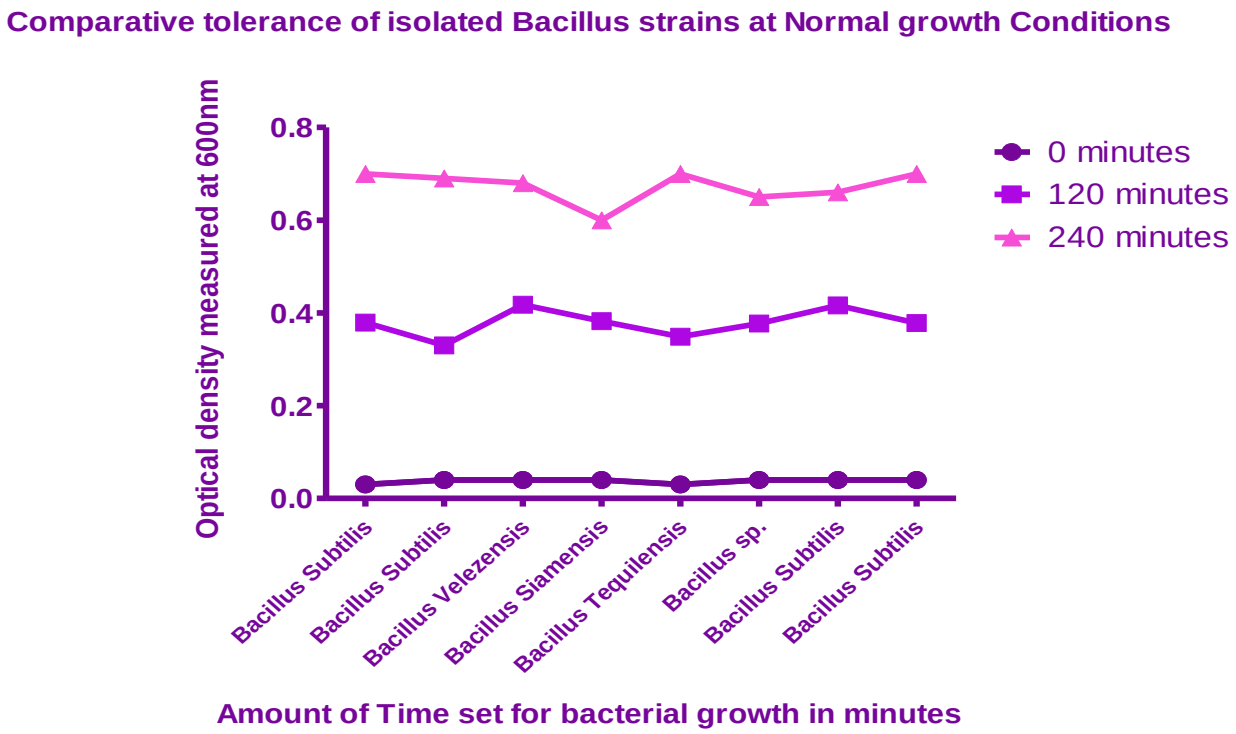

Figure 17: Growth of bacterial strains at normal conditions (Positive control).

4.9.3. Bile Tolerance:

Most of the isolated strains were tested against three different bile concentrations for examining their tolerance level to bile salts. The normal pattern of strains growth was that, it increases slowly for 60 minutes till 120 minutes after that tolerance level decreases and result in no significant growth.

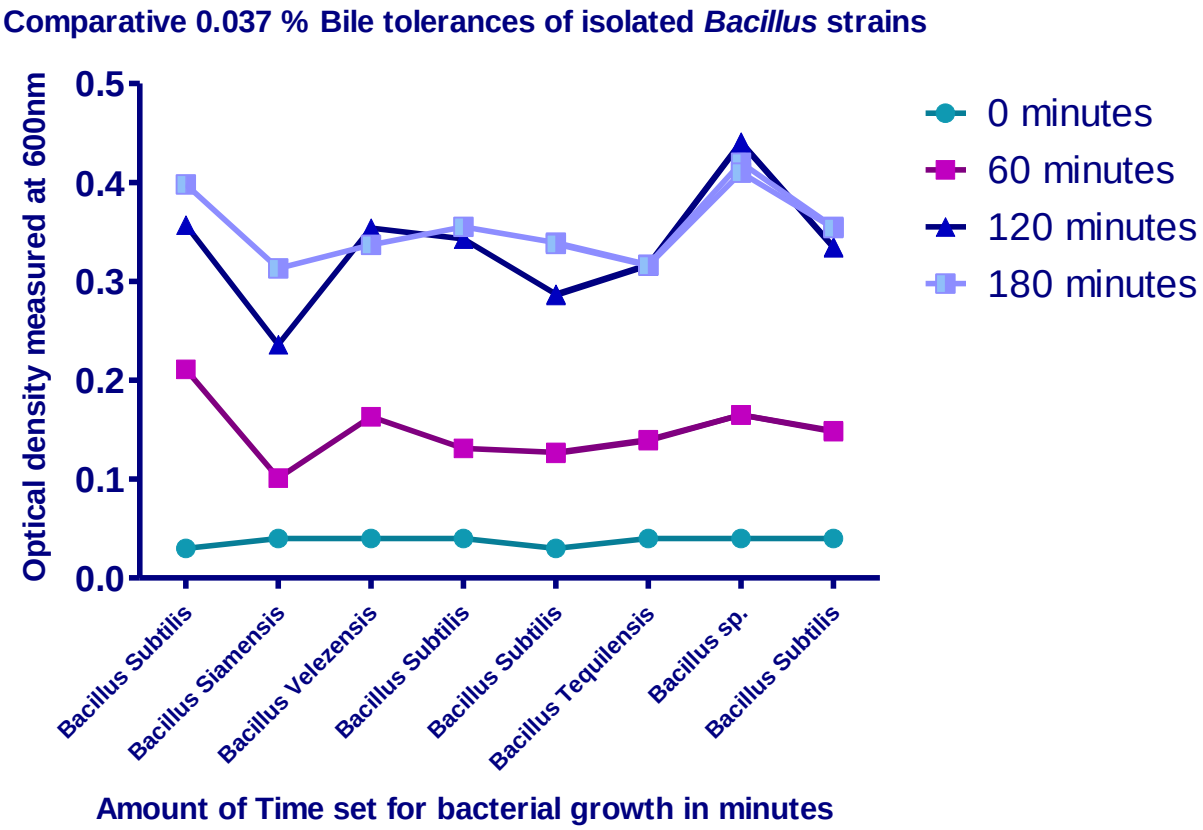

Figure 18: Tolerance level of bacterial isolates towards 0.037% bile salt concentration.

All the isolates were showing increase in growth in the start at 60 minutes and at 120 minutes while in the end at 180 minutes three strains showed very minor increase in growth. This showed higher tolerance in 3 strains against bile salt stress.

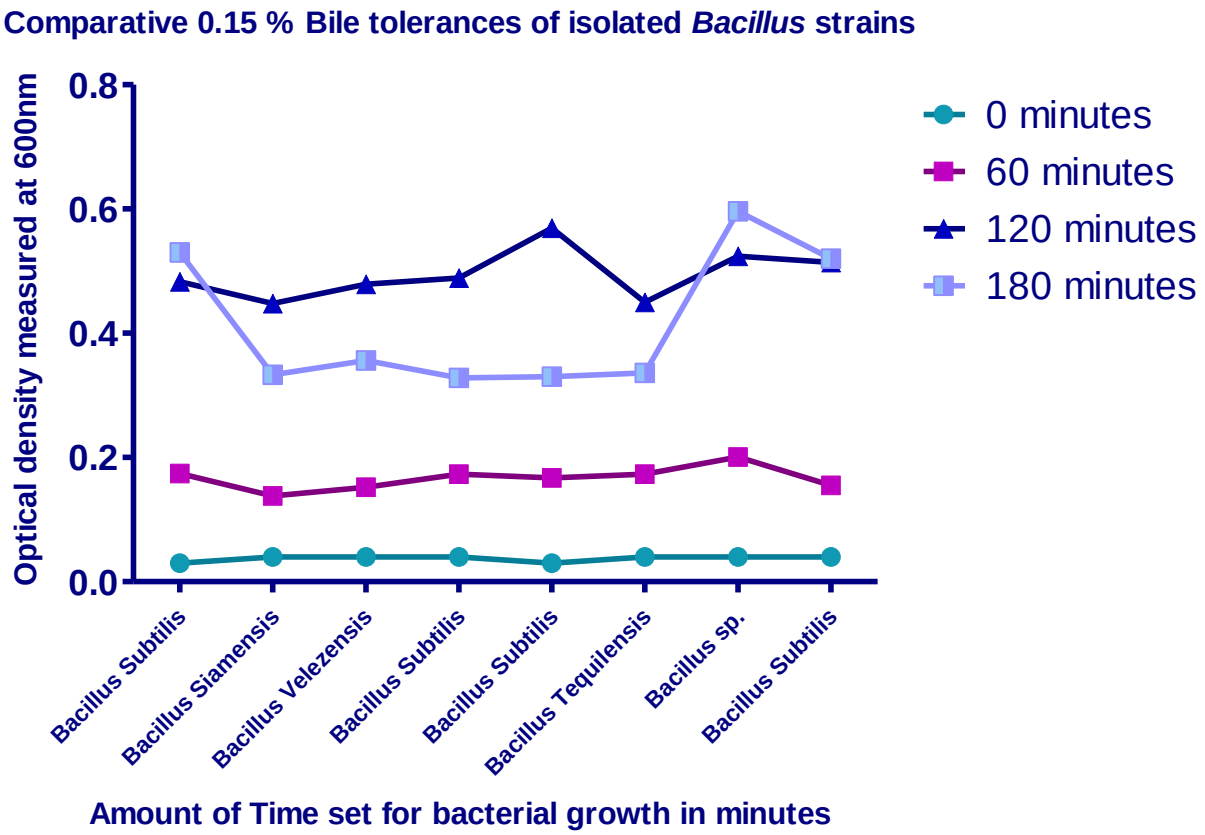

Figure 19: Tolerance level of bacterial isolates towards 0.15% bile salt concentration.

All the isolates were showing increase in growth in the start at 60 minutes and at 120 minutes while in the end at 180 minutes two strains showed very minor increase in growth. That means these two strains were resistant towards 0.075% bile salt stress.

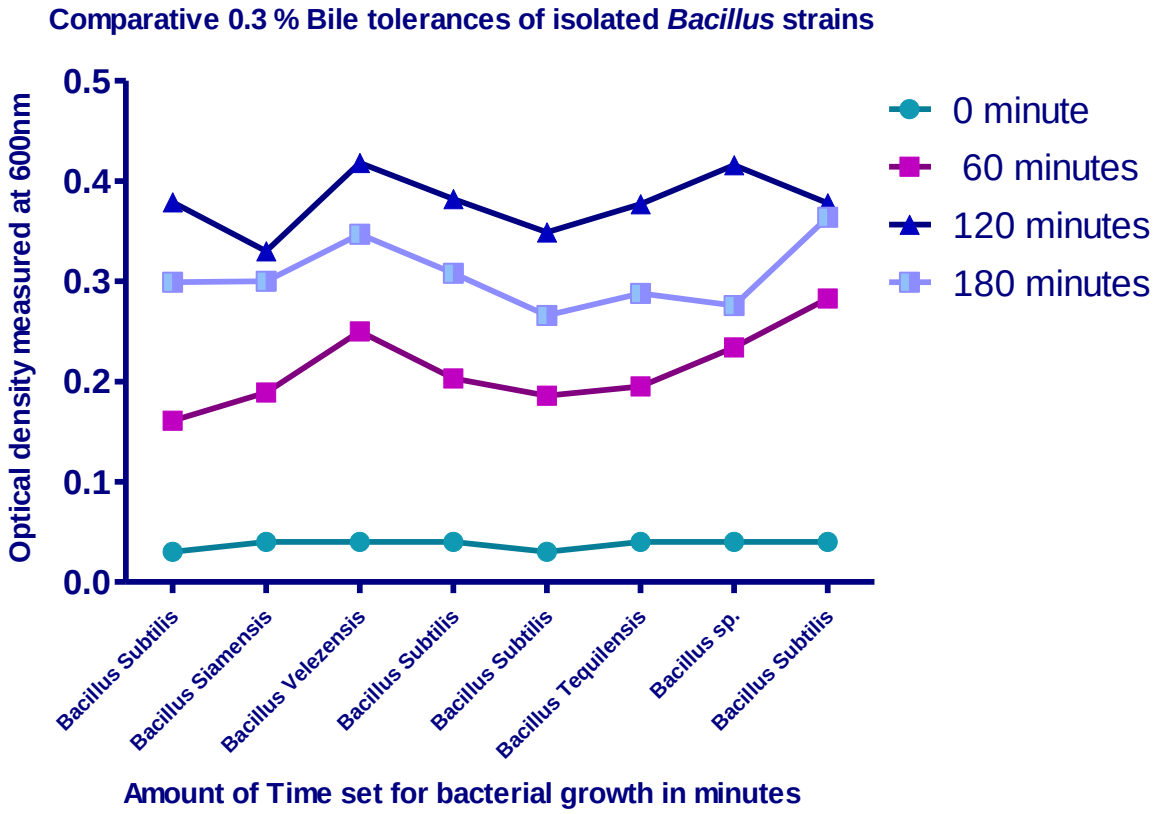

Figure 20: Tolerance level of bacterial isolates towards 0.3% bile salt concentration.

All the isolates were showing increase in growth in the start at 60 minutes and at 120 minutes while in the end at 180 minutes growth decreases. This showed end of tolerance in the strains against 1.5% bile salt stress. Only one strain did not show decrease in growth but its growth remains constant after 120 minutes.

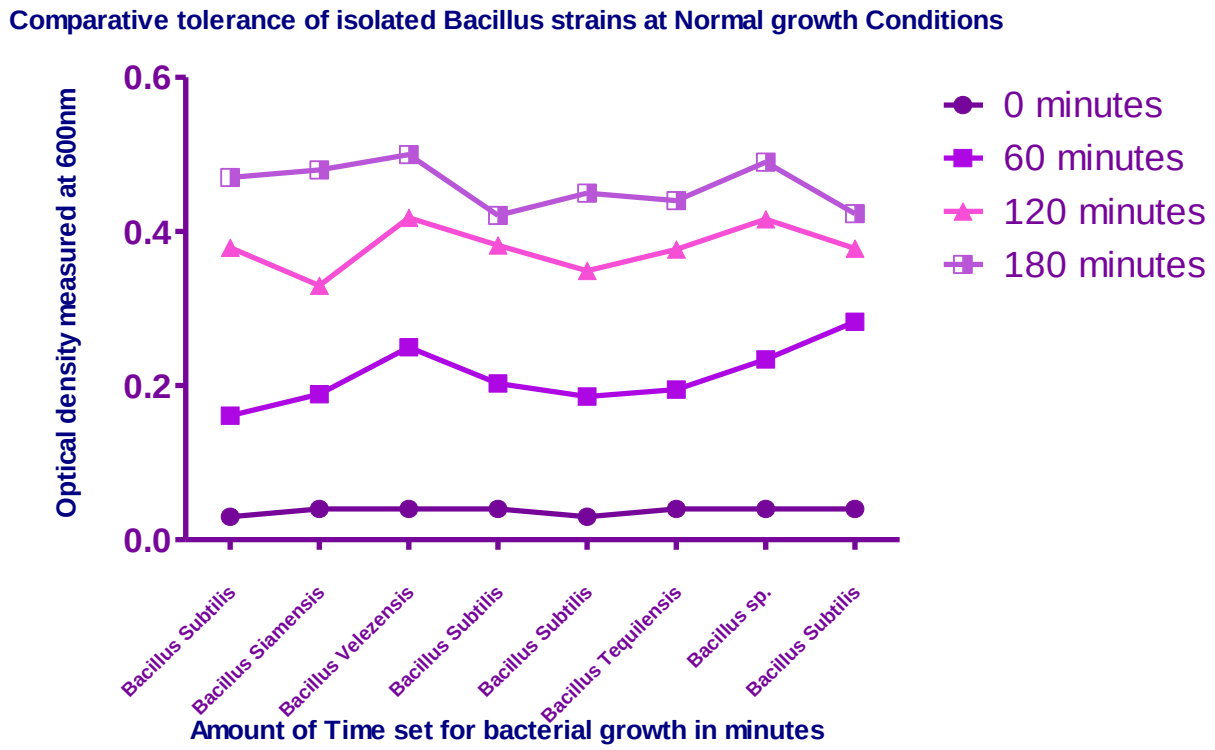

Figure 21: Growth of bacterial strains at normal conditions (Positive control).

4.10. Antibiotic Susceptibility Assay:

Four different antibiotics were used to analyze antibiotic resistance of isolates. Antibiotics used were CN-10 (Gentamicin), AMC-30 (Amoxycillin), TE-30 (tetracycline), PB-300 (Polymyxin). All of the 8 isolates were tested against all these antibiotics. Gentamycin and streptomycin belong to aminoglycosides, they work by binding to 30s ribosomal subunit and modifying enzymes. Both are effective against gram negative and gram positive bacteria. Amoxicillin is penicillin like antibiotic. It is effective against gram positive bacteria and acts by inhibiting the synthesis of bacterial cell wall. 6 strains are showing susceptibility while other 2 strains are highly resistant. Polymyxins belong to non-ribosomal peptides include Polymyxins B and E also known as colistin. This antibiotic is usually used to treat the Gram-negative bacterial infections. Those bacteria which are resistant to antibiotics will grow on the plat and will consider resistant while bacteria sensitive to antibiotics won‘t be able to show any growth pattern. There are strains which are resistant to some antibiotics while sensitive to others. These drugs work by breaking up the bacterial cell membrane. Only one strain was resistant to this antibiotic, all other strains were susceptible. Tetracycline antibiotics work by blocking the protein synthesis. These antibiotics do not allow the binding of aminoacyl-tRNA to the mRNA-ribosome complex.

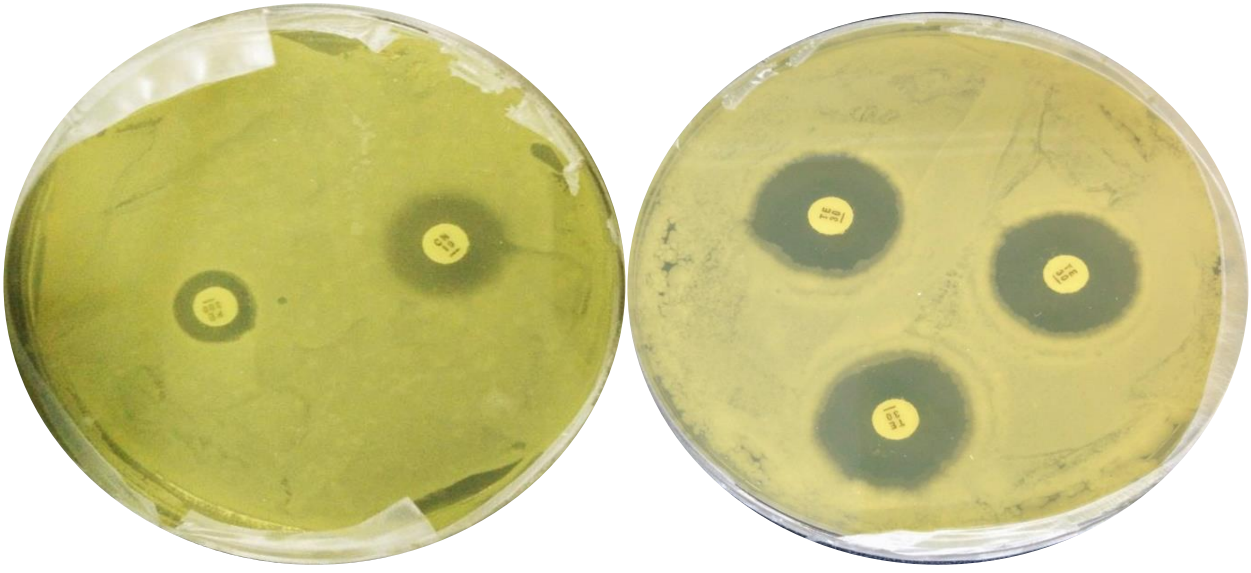

Figure 22: Minimum inhibition concentration of Bacterial strains Positive control *E.coli* for CN-10 (Gentamicin), AMC-30 (Amoxycillin), TE-30 (Tetracycline), PB-300 (Polymyxin).

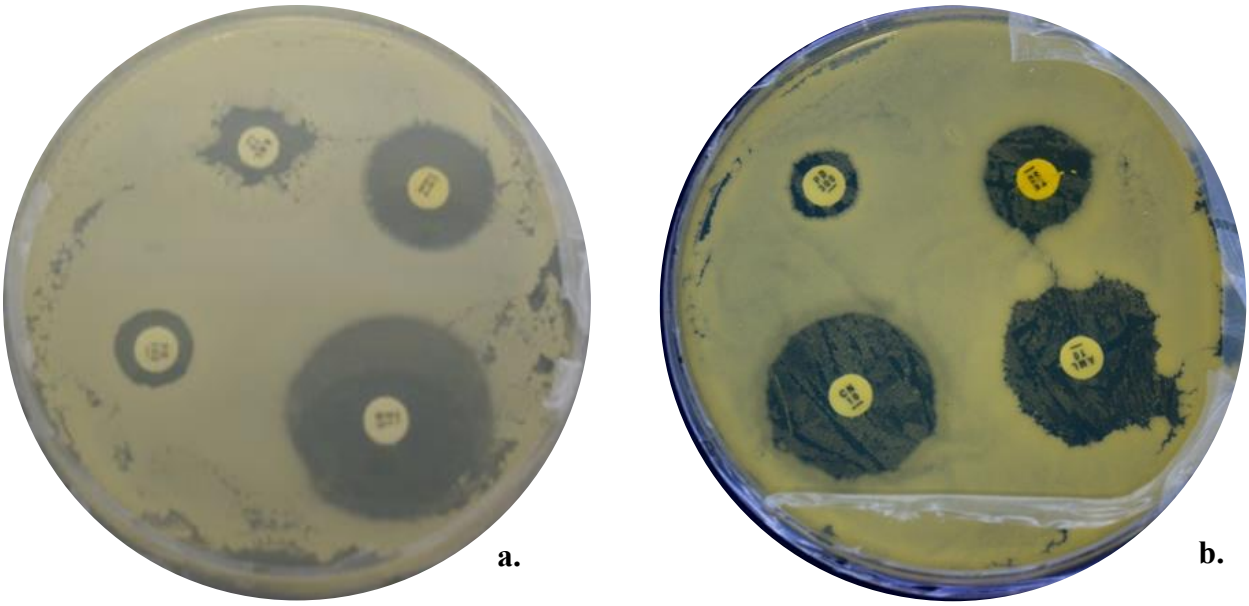

Figure 23: Minimum inhibition concentration of Bacterial strains for CN-10 (Gentamicin), AMC-30 (Amoxycillin), TE-30 (Tetracycline), PB-300 (Polymyxin) against (a) KY986416 and (b) KY971625.

Sample name	**CN-10 (Gentamicin)**	**AMC-30 (Amoxycillin)**	**TE-30 (tetracycline)**	**PB-300 (Polymyxin)**
Bacillus sp. KY986529	++	R	+++	R
Bacillus Subtilis KY971638	++	R	+++	++
Bacillus Tequilensis KY986446	++	R	++	+++
Bacillus Subtilis KY971625	++	+++	++	++
Bacillus Siamensis KY986746	++	R	++	++
Bacillus Subtilis KY986416	++	+++	++	++
Bacillus Subtilis MF359552	+++	+++	++	+

Table 7: The antibiotic resistance/susceptibility profile of isolates against 4 antibiotics CN-10 (Gentamicin), AMC-30 (Amoxycillin), TE-30 (tetracycline), PB-300 (Polymyxin), R = Resistant

Resistance (R): 0–5 mm

Sensitive (S+): 6–15 mm

Moderately Sensitive(S++): 16–25 mm

Extra Sensitive(S+++): 26–35 mm

CHAPTER 5: DISCUSSION

In the present work *Bacillus spp*. were isolated, identified and characterized from 11 different spices. The samples were collected from Lahore city of Pakistan (Table 1). Spices are used to give better taste, flavor to food due to its aromatic properties (Banerjee and Sarkar, 2003). Most of the spices do not have any nutritional value but organoleptic properties help in the digestion of food by stimulating the production of gastric juice or by increasing the motility of the digestive organs (Garcia et al., 2001).

The purpose of the present study was to identify the types of bacteria that are associated with spices to ascertain whether these bacteria are beneficial or harmful for us and to test the probiotic potential of *Bacillus* strains. The protocol was optimized for selective isolation of bacteria as was confirmed from phenotypic and genotypic characterization of isolates. Among 18 isolates 8 isolates were recognized as *Bacillus* showing ≥99 similarity through NCBI Basic Local Alignment Search Tool (Table 6). There were 4 strains identified *as Bacillus subtilis* is reported as potential probiotic candidates (Green et al., 1999). On the basis of colony morphology and biochemical characteristics, acid tolerance, bile tolerance and antimicrobial activity, isolates were identified as *Bacillus* species (Nithya and Halami, 2013). For molecular identification by 16S ribosomal DNA fragment of 1500bp was amplified and sequenced, 16S ribosomal DNA sequencing determined that these strains belong to *Bacillus spp.* (Sreekumar and Krishnan, 2010). Sequence compared with the genome data available at NCBI database directed presence of *Bacillus sp., Bacillus subtilis, Bacillus tequilensis, Bacillus velezensis, Bacillus siamensis, Bacillus subtilis, Bacillus subtilis, and Bacillus subtilis*. Eight strains have unreliable chromatogram results. Phylogenetic tree showed resemblance of these strains with reference to 16S rDNA sequence result.

Bacillus subtilis is generally regarded as safe (GRAS) and have important role in preservation of food and fermented products (Schallmey et al., 2004). Probiotics need to meet certain criteria such as acid and bile tolerance and survival in human gastrointestinal tract (Shanahan, 2003). All isolated *Bacillus* strains were tested for their acid and bile tolerance. The tolerance to acidic condition is required due to its survival during gastric stress. High concentration of acid reaches pH 1.5-2 during fasting (Fernández et al., 2003). In our study there is generally decrease in bacterial count as they were exposed to pH 2, 3 for 180 minutes. This study is conducted for 8 isolates to access their viability in acidic stress. Among 8 isolates, all the strains MF359552,

KY986529, KY971625, KY986446, KY986416, KY986746, KY971638 and KY971953 are showing increase in their growth at pH of 2 and 3 during time interval of 120 minutes But after 120 minutes there is visible decrease in growth rate when OD is measured (Figure 12). Though KY986416 and MF359552 are showing same growth rate even at 240 minutes with pH of 2 (Figure 12). But at pH of 3 these two strains are tolerant to acidic environment and their growth rate increases even at 240 minutes (Figure 13). This was also conformed in earlier finding by Hyronimus et al. (2000) carried out acid tolerance tests of *Lactobacillus sporogenes, Bacillus laevolacticus, and Bacillus racemilacticus* and demonstrated that only *Bacillus laevolacticus* showed a significant survival rate at pH 2.5 (Hyronimus et al., 2000). Some advantages of the bacterial spores are their resistance to heat, allowing the storage at room temperature in a dried form. Also, these bacteria are able to reach small intestine since they survive the gastric pH of the stomach (Barbosa et al., 2005). These different trends in low pH growth of *Bacillus* may be attributed to great genetic diversity as a consequence of differentiation at specie level.

All strains (vegetative cells) MF359552, KY986529, KY971625, KY986446, KY986416, KY986746, KY971638, and KY971953 are also able to tolerate high osmotic concentrations of NaCl 3.5 % and 6.5 % (Figure 14, 15). Two strains which showed exceptionally high tolerance level are KY986446, and KY986529. This result was in accordance with finding of Liu et al., 2009, *B. subtilis* E20 and *Bacillus* strains are able to grow at a broad range of temperatures (10 to 50 °C), pH values (5 to 10), and NaCl levels (0% to 9%) proposing that these characteristics can be very useful if these strains are used in aquaculture, such as probiotics for marine species both and freshwater species (Liu et al., 2009).

Before selection of probiotic bacteria for human consumption it should be tolerable to 0.3% - 0.5% bile concentration (Gilliland et al., 1984). Among 8 isolates all are showing increase in growth for initial 60 minutes and 120 minute at three different bile salt concentrations 0.037 %, 0.15%, 0.3 %. At 0.03 % bile concentration, three number of isolates survived were KY971625, KY971638, KY986416 and MF359552 (Figure 16). At 0.15 % bile only three strains were resistant KY986529, KY971638 and MF359552 (Figure 17). At 0.3 % only one strain was showing tolerance KY986416. KY986416 is the only strain that has potential to tolerate pH2 and pH3. While some isolates are highly tolerant to bile salts but are sensitive to acid. Hence, in the present study, *Bacillus* spp. were found in both the 'tolerant' and 'sensitive' groups. These results are in agreement with those observed with other cultures such as *B. subtilis and B. toyoi* (Cosson and Deschamps 1994) and *B. coagulans* strains that few strains are susceptible but few are resistant to different bile salt concentrations (Nithya and Halami, 2013). Probiotic bacterial strains should show high tolerance level against lysozyme, gastric juices, bile secretions, different enzymes and antibiotics used as growth enhancers in animal nutrition and different therapies.

These characteristics could be used as in vitro prerequisites to select the strains as probiotic. (Hyronimus et al., 2000).

The safety of probiotic strains is becoming prerequisite with antibiotic resistance as an emerging issue. All isolated *Bacillus spp.* were tested against seven available antibiotics to check their resistance. These antibiotics were CN-10 (Gentamicin), AMC-30 (Amoxycillin), TE-30 (tetracycline), PB-300 (Polymyxin) (Table 9). There are three visible trends observed on bacterial plates with antibiotic disc sensitive, moderately sensitive, extra sensitive. With CN-10 (Gentamicin) 7 strains are moderately sensitive, 1 is extra sensitive. With AMC-30 (Amoxycillin) 5 strains are resistant and 3 strains are extra sensitive. With TE-30 (tetracycline) 6 strains are moderately sensitive and 2 strains are extra sensitive. With PB-300 (Polymyxin) 2 strains are resistant, 4 strains are moderately sensitive and one strain is extra sensitive. Resistance profile of these strains may be attributed to increase use of antibiotics in our environment as these strains are isolated from environmental samples that is spices, this can be one reason of high resistance among strains. Sorokulova also reported antibiotic resistant probiotic *Bacillus subtilis* 3 and B. licheniformis 31 strains which are compounds of probiotic Biosporine®(Sorokulova et al., 2008). *Bacillus* isolates in the presence of plasmid DNA have usually been linked with antibiotic resistance determinants and toxins production (Bernhard et al., 1978). However, since three of our cultures KY986416, KY971625, and MF359552 are found to be sensitive to any of the antibiotics tested, they would not be responsible for transmission of drug resistance genes to other intestinal and/or food-borne pathogens, in the food matrix or, more importantly, in the gastrointestinal tract, if introduced as probiotics.

There were total 65 isolates that are isolated from raw form of spices, only eight strains that are characterized by biochemical tests and molecular techniques to analyze their probiotic potential. Four major prerequisites are tested to check strength of *Bacillus* strains. The assumption made in the beginning of research that spices may contain bacteria that are useful for us is proved as one strain KY986416 was showing high level of tolerance in pH2, pH3, different bile concentrations and this strain is susceptible to all the antibiotics used. It was also reported earlier that in animal production the antibiotics use is reduced and it is replaced by the herbs and spices. They contain strong antimicrobial compounds used in vitro against microbial contaminations, including fungus (Dorman and Deans, 2000). Another study that was proving the result of this research stated that Health status of animals could be improved by using the plants extract or spices extracts as separate compounds or mixture of these two can for better performance. Guo et al. observed in their study that the number of beneficial bacteria can be increased and number of harmful bacteria can be reduced in caecum by using the extracts of plants (Hosseinzadeh et al.,2014).

So, present study concluded by biochemical and molecular identification it is confirmed that bacteria associated with spices are mostly from Genus Bacillus. Total 12 different bacterial species have been identified through sequencing in this study and their data is submitted in the Genbank. Few of the tested *Bacillus* species are showing tolerance towards the acidic conditions and different salt concentrations so; they might have potential to be used as probiotic candidates. They can be further analyzed by using *in-vivo* studies. ***In-vivo*** experiments should be conducted to study mechanism of Pro-biosis by immune stimulation and synthesis of antimicrobial compounds.

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