Evaluation of 16S rRNA gene sequencing for the analysis of bacterial isolates

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

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By

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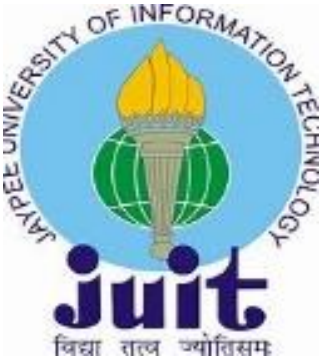
Under the Guidance of

Dr. Jitendraa Vashistt



JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

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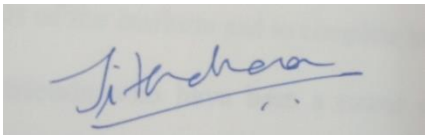
CERTIFICATE

This is to certify that the work titled "**Evaluation of 16S rRNA gene sequencing for analysis of bacterial isolates**" submitted by "Ms**. Rhea Singh**" in partial fulfillment for the award of the degree of B. Tech Biotechnology of Jaypee University of Information Technology, Waknaghat has been out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor 

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Date 30th May 20222

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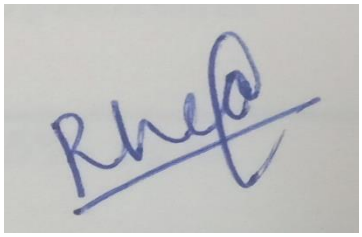
Candidate's Declaration

I hereby declare that the work presented in this report entitled "**Evaluation of 16S rRNA gene sequencing for analysis of bacterial isolates**" in partial fulfilment of the requirements for the award of the degree of the **Bachelor of Technology in Biotechnology** submitted in the department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, H.P. is an authentic record of my own work carried out over a period from July 2021 to May 2022 under the supervision of **Dr. Jitendraa Vashistt**, Associate Professor in the department of Biotechnology.

The matter embedded in the report has not been submitted for the award of any other degree or diploma.

This is to certify that the above statement made by the candidate is true in the best of their knowledge.



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B. tech Biotechnology

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PLACE: JUIT, Solan

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LE	Levofloxacin
NA	Nalidixic acid
AK	Amikacin
HLG	High level Gentamicin
IPM	Imipenem
LB	Luria Broth
NB	Nutrient Broth
rRNA	Ribosomal RNA
MHA	Mueller Hinton Agar

Abstract

The 1500 bp 16S rRNA gene comprises nine variable regions interspersed throughout the highly conserved 16S sequence. In this study, *Bacillus clausii* was analyzed by various methods including gram staining, antibiotic susceptibility testing by disk diffusion method, and agarose gel electrophoresis. The results of this study include gram staining which depicted rod-shaped purple-colored colonies and indicated the presence of a gram-positive bacteria. AST was done to determine susceptibility of *B.clausii* against certain antibiotics. The greatest zone of inhibition was formed by Imipenem followed by High Level Gentamycin, Amikacin, and Levofloxacin. Lastly, the PCR products of 16S rRNA gene separated on 1.5% agarose. PCR product of universal 16S rRNA gene and E.coli specific 16S rRNA gene was amplified and was checked for the presence in both isolates, namely *Bacillus clausii* and *E. coli* ATCC 25922. Both 1.5 kb was conserved in all species but species specific 16S rRNA E.coli did not show any bands in *Bacillus clausii* as it was specific of *E.coli* only and was unable to amplify *B.clausii.*

Chapter 1: Introduction

Screening of 16S rRNA sequences from various organisms has showed that some parts of the subunit undergo fast genetic changes, allowing different species in same genus to be distinguished. Other locations change relatively slowly, allowing for the distinction of far larger taxonomic levels. The data collected of 16s rRNA gene sequences between species is based on a set of assumptions [1]. It seems doubtful that the pace at which base alterations arise and thus are established inside one species is constant. Environmental changes are predicted to change the ecological niches or selection pressures that affects the level of recombinationas well as the rate at which diverse species can develop. The rRNA sequences represent the fundamental distinctions between Archaea and Bacteria, which are obvious in the structure of their lipids and cell walls, as well as the usage of various biosynthetic processes, enzymes, and enzyme cofactors. Bacterial and Archaeal rRNAs are as distinct from one another as eukaryotic rRNA is. This implies that the archaeal lineages split from a common ancestor before eukaryotic cells evolved [1] [2].

During the 1980s, another norm for distinguishing microorganisms started to be created. In research centers of Woese and more researchers, there was a depiction of phylogenetic connections with microbes, as well as, for sure, other living things, is controlled by contrasting steady piece of that hereditary code [3]. Possibility of the hereditary region in microbes incorporated the qualities that code for the 5S, the 16S, and the 23S rRNA. The piece of the DNA that is generally utilized for an ordered reason for microorganisms is the 16S rRNA quality. The 16S rRNA quality is likewise assigned 16S rDNA, these terms have been used synonymously,: the ASM strategy involves "16S rRNA quality" in being utilized [4] [5]. The 16S rRNA quality can measure up among all microorganisms as well as with the 16S rRNA quality of archeobacteria and the 18S rRNA quality of eucaryotes. Microscopic organisms (prokaryotes), and Eucarya, just as the significant branches inside the procaryotes dependent on these quality successions [6].

Chapter 2: Review of Literature

2.1 16S rRNA gene

Ribosomal RNA (rRNA) is a component present in cell which is a constituent of the protein-synthesis organelle known as the ribosome and is thus transferred to the cytoskeleton to assist in the translation of messenger RNA (mRNA) into protein. The three basic types of RNA present in cells are rRNA, mRNA, and transfer RNA [7]. rRNA is produced in the nucleolus, a specialised portion of the cell nucleus that appears as a highly populated region within the nucleus and contains the rRNA-encoding genes. Based on their size, encoded rRNAs are classed as big or tiny [8]. Each ribosome contains at least one large and one tiny rRNA. The big and small rRNAs combine with ribosomal proteins in the nucleolus to form the large and small subunits of the ribosome. In general, these subunits are called for their rates of sedimentation, which is estimated in Svedberg units [S]. Ribosomal proteins are produced in the cytosol and transferred to the nucleus for nucleolus subassembly. After that, the subunits are reintroduced to the cytoskeleton for assembly [9].

The rRNAs build large secondary structures and actively recognize conserved mRNA and tRNA segments. In eukaryotes (i.e the organisms that have a well-defined nucleus), there may be Fifty to 5,000 sets of rRNA genes and up to one million ribosomes in just one cell [10] [11].

Prokaryotes (organisms without a nucleus) often include lesser sets of gene sequences and ribosomes for every cell. In the bacteria *Escherichia coli*, for example, 7 copies of the rRNA genes manufacture around 15,000 ribosomes for each cell [12].

In bacteria, the most valuable gene for studying phylogenetic linkage is 16S rRNA, a DNA sequence that codes the RNA element of the bacterial ribosome's smaller subunit. The 16S rRNA gene is found in all bacteria, and a comparable version can be found throughout all cells, including eukaryotes [13]. Analysis of 16S rRNA sequences from various organisms hasshowed that some parts of the molecule undergo fast genetic changes, allowing different species within that genus to be distinguished. Other locations change relatively slowly, allowing for the distinction of far larger taxonomic levels [13].

The capacity of rRNA to catalyse the peptidyl transferase process during protein synthesis has other evolutionary consequences [14].

The most conserved (least variable) DNA in all cells is the rRNA gene. The rDNA sequences of distantly related species are very similar. This suggests that sequences from distantly related species may be appropriately matched, allowing genuine differences to be assessed.
As a result, genes encoding rRNA (rDNA) have been widely used to determine taxonomy, phylogeny (evolutionary relationships), and forecast rates of species divergence among bacteria [15]. Thus, comparing 16s rDNA sequences can show evolutionary relationships between bacteria. Carl Woese pioneered this study by proposing the three Domain system of classification based on such sequencing information: Archaea, Bacteria, and Eucarya [15] [16].

16S rRNA is a DNA sequence that encodes the RNA component of the bacterial ribosome's smaller subunit. The 16S rRNA gene is found in all bacteria, and a comparable version can be found in all cells, including eukaryotes [17].

16S rRNA gene is conserved in bacteria and contains hypervariable sections that can offer species-specific signature sequences, it is commonly employed in bacterial identification and phylogenetic investigations. 16S rRNA sequencing is distinguished by its rapidity, low cost, and excellent accuracy [18].

16S rRNA gene sequencing is extensively used to identify, classify, and quantify microorganisms in complex biological mixes such as environmental samples (for example, seawater) and gut samples (eg human gut microbiome) [19] [20].

The 16S rRNA gene is employed for phylogenetic investigations because it is highly conserved among bacteria and archaea because 16S rRNA sequences from distantly related bacterial lineages exhibit comparable functions, it is argued that the 16S rRNA gene can be employed as a trustworthy molecular clock [21].

Bacteriophage and viruses isolated from wastewater treatment systems have even been shown to include host 16S rRNA genes. The source of 16S rRNA genes discovered in environmental viral samples is assumed to be a generic transducing phage [22].

The sequencing of 16S rRNA, the gene that encodes the RNA component of the bacterial ribosome's smaller subunit, may also be used to determine evolutionary relatedness. The 16S rRNA gene is found throughout all bacteria, and a similar version is found in all cells. The *E. coli* 16S rRNA gene has a length of 1,542 nucleotides, with some parts double-stranded and others single-stranded. Because the opposite strand lacks complementary bases, single- stranded

regions frequently form loops [23]. The rate over which uncontrolled random mutation may alter the sequencing of the nucleotides in the rRNA is extremely slow because 16S rRNA has extremely precise connections with many distinct ribosomal proteins including other portions of itself. Any mutation in structure at one place must be adjusted for by another alteration to somewhere else in the rRNA or even in a ribosomal protein, otherwise the ribosome will unable to assemble properly or function in protein synthesis, resulting in cell death [24] [25].

Screening of 16S rRNA sequences from various organisms has showed that some parts of the subunit undergo fast genetic changes, allowing different species in same genus to be distinguished. Other locations change relatively slowly, allowing for the distinction of far larger taxonomic levels. The data collected of 16s rrna gene sequences between species is based on a set of assumptions. It seems doubtful that the pace at which base alterations arise and thus are established inside one species is constant. Environmental changes are predicted to change the ecological niches or selection pressures that affects the level of recombinationas well as the rate at which diverse species can develop [26].

The rRNA sequences represent the fundamental distinctions between Archaea and Bacteria, which are obvious in the structure of their lipids and cell walls, as well as the usage of various biosynthetic processes, enzymes, and enzyme cofactors. Bacterial and Archaeal rRNAs are as distinct from one another as eukaryotic rRNA is. This implies that the archaeal lineages split from a common ancestor before eukaryotic cells evolved. That theory also suggests that now the eukaryotic line is relatively old and did not evolve from just about any currently known bacterium [27]. Previously, it was thought that eukaryotic cells formed when certain bacterial cells absorbed other variety of bacterium. The bacteria could well have created a symbiotic connection in which the swallowed cell survived but progressively relinquished its freedom and gained on the characteristics of an organelle [28]. Although the initial eukaryotic cell may or may not have been generated from bacteria, it is highly probable, that eukaryotic cells (e.g., mitochondria and chloroplasts) are progeny of bacteria acquired by eukaryotic cells through symbiotic parasitism [29].

Early theories regarding the beginnings of life indicated that the earliest cells got initial vitality from the decomposition of minerals in a rich organic liquid environment that emerged in the early oceans as a result of the effect of light and strong radiation from the sun on the early, arid atmosphere. Photosynthesis may have originated much later in reaction to the progressive loss

of those abundant nutrition sources. rRNA sequence analysis, on the other hand, reveals photosynthetic capacity in practically all of the main bacterial divisions and demonstrates that physiological genera are closely linked to nonphotosynthetic genera.

Because photosynthesis is indeed an evolutionarily conserved and mechanistically complicated process, it seems improbable that the capacity to perform photosynthesis could have developed at various times in so many distinct organisms. Lithotrophy (from the Greek word lithos, meaning "rock"), the capacity to receive energy by electron donation from hydrogen and oxygen to inorganic recipients, is much more common among prokaryotes. It has been hypothesised that the first life forms on Earth utilised lithotrophic metabolism, with photosynthesis coming later. Nonlithotrophic and nonphotosynthetic Bacteria evolved from the earliest types of Bacteria, albeit they have lost their lithotrophy and photosynthesis capacities [30].

The hypothesis that lithotrophy was widespread among bacterial species before photosynthesis evolved shows that Archaea descended from a distinct line of ancestry than Bacteria. Halobacterium, the sole photosynthetic archaeon, uses a fundamentally different sort of photosynthesis than plants and bacteria, which does not employ chlorophyll in massive associated proteins to excite an electron. It instead involves just one protein, bacteriorhodopsin, wherein sunlight radiation is captured by retinal, a kind of vitamin A, and used to activate a proton pump (hydrogen ion) [31].

In 1965, Dubnau observed preservation during 16S rRNA quality arrangement connections in Bacillus spp. Broad utilization of quality arrangement for microbial distinguishing proof and scientific classification ensuing a collection of spearheading work completed by Woese, who characterized significant properties. Level of preservation is expected to be observed from the significance of the 16S rRNA as a basic part of cell work [32]. This is as opposed to the qualities expected to make compounds. Transformations in these qualities can as a rule be endured all the more every now and again since they might influence structures not generally so interesting and fundamental as rRNA (in case a bacterium doesn't have the quality to make the catalysts expected to use lactose [33] [34].

Subsequently, barely any different qualities are just about as exceptionally saved as the 16S rRNA quality. Albeit the outright pace of progress during the 16S rRNA quality succession isn't known, it marks developmental distance and relatedness of life forms. Issues in allocating a mathematical worth to this pace of progress incorporate the likelihood that this pace of progress of 16S rRNA quality may not be indistinguishable for all life forms (diverse scientific categorizations could have various paces of progress), the rates could shift now and again during

advancement, and the rates could be distinctive at various locales all through the 16S rRNA quality [35]. There are supposed "problem areas" which show bigger quantities of changes; these regions are not the equivalent for all species. 16S rRNA is likewise the objective for a long time specialists. Accordingly, changes during the 16S rRNA quality can influence the vulnerability of the organic entity to these specialists and the 16S rRNA quality succession can recognize phenotypic protection from antimicrobial specialists. Be that as it may, these attributes don't block or influence the utilization of 16S rRNA quality succession for bacterial ID or task of cozy connections at the family and species level, as utilized in clinical microbial science. They can greaterly affect the task of connections of the more profound (all the more remotely related) branches [36].

Finally, the 16S rRNA quality is widespread in microbes, thus connections can be estimated among all microorganisms [37]. As a rule, the examination of the 16S rRNA quality arrangements permits separation between living beings at the family level across all significant phyla of microbes, as well as grouping endure numerous levels [38] [39]. A periodic exemption for the value of 16S rRNA quality sequencing normally identify with more than one notable animal varieties having something similar or fundamentally the same as arrangements [40].

2.2 Bacillus species

Bacillus species, that produce spores, have indeed been employed as probiotics for over half a century. Spore producing probiotics have an advantage over non-spore forming probiotics such as Lactobacillus spp. in that they really are stable at high temperatures and might even be incubated at room temperature without losing vitality. Spore-forming microorganisms are however resilient to the acidic conditions of the stomach (low pH), allowing them to endure the transport to the gut.

Bacillus (genus Bacillus), even one of a genus of rod-shaped, gram-positive, aerobic

or anaerobic bacteria that are common in soil and water. Bacillus is a broad word that refers to any and all cylindrical or rodlike microbes. B. megaterium, the biggest recorded Bacillus species, is about 1.5 m (micrometres; 1 m = 10-6 m) across and 4 m long. Bacillus is commonly found in groups [41].

In 1877, German botanist Ferdinand Cohn published an authorised description of two types of hay bacillus (currently known as Bacillus subtilis): one that can be destroyed by heat and one that was heat resistant. He dubbed the heat-resistant forms "spores" (endospores), and observed how these latent structures could be transformed into vegetative, or actively developing, forms [42]. Under severe environmental circumstances, most Bacillus species can produce dormant spores. These endospores can survive for extended periods of time.
Endospores are heat, chemical, and sunlight resistant and are found in abundance in nature, particularly in soil, from where they penetrate dust particles [43].

Some Bacillus bacteria are toxic to people, plants, and other species. B. cereus, for example, can cause spoiling in canned goods and short-term food poisoning. B. subtilis is a typical laboratory culture contaminant (it troubled Louis Pasteur in several of his studies) and is frequently discovered on human skin. Most Bacillus strains are not harmful to people, although as soil organisms, they may inadvertently infect humans. B. anthracis, that generates anthrax in people and domestic animals, is a rare exception. B. thuringiensis secretes a toxin (Bt toxin) which induces insect illness [44].

B. subtilis produces antibiotics that are valuable in medicine (bacitracin). Furthermore, strains of B. amyloliquefaciens bacteria found in combination with some plants are known to produce a variety of antibiotics, namely bacillaene, macrolactin, and difficidin. These chemicals shield the plant tissues against fungus and other microorganisms and have been explored for their potential application as biological pest control agents [45].

The most attention has been paid to *Bacillus subtilis, Bacillus clausii, Bacillus cereus, Bacillus coagulans, and Bacillus licheniformis*. It is clear that little scientific study has been undertaken on the viability of these species in probiotics, particularly in contrast to the usage of lactic acid bacteria [46]. Bacillus has a clear advantage over other potential probiotic strains in that they'll be produced efficiently and inexpensively by dehydration and last a long period. It would also withstand production of stomach acid and carry it all to the rumoured region of action, the colon. Some strains have the ability to germinate and spread all through the human gastrointestinal system [47].

A variety of products are registered for both human and animal usage (pigs, poultry, calves, aquaculture). The majority of products available lack comprehensive clinical trial evidence. Claims for extragastrointestinal benefits, such as relief for allergies and rheumatoid arthritis symptoms, are common, but some medications also make claims for gastrointestinal

effectiveness. It is unknown what processes bacilli use to be effective as probiotics.

Extracellular proteases have been detected in certain strains. Natto is a native Japanese B. subtilis–fermented soy foodstuff with several health advantages. It's a major staple item.

Natto has a pungent odour and flavour, as well as a thick, stringy texture. This is attributed, in part, to the development of a poly—glutamic acid cell capsule in stationary phase. Several B. cereus probiotic materials are sourced from strains that generate or possess enterotoxin genes [48] [49].

2.3 Bacillus clausii

Bacillus clausii (B. clausii) spore & cells can attach to and colonise the mucosa of the gut. Because *B. clausii* is inherently resistant to acidic environments, the whole amount of bacteria swallowed reaches the small intestine unscathed. *Bacillus subtilis, B. clausii, B. cereus, B. coagulans, and B. licheniformis* are the Bacillus probiotics that have received the most attention. *B. clausii* is a prophylactic that has been used in Italy since the 1960s to treat viral dysentery in infants as well as antibiotic-related adverse effects. *B. clausii* can inhibit pathogen growth in the digestive tract through 3 separate mechanisms [50]: colonialization of unrestricted niches, that are no longer accessible for the development of other microbes; challenge for epithelial adhesion, which is incredibly significant for spores in the preliminary or moderate germination phase; and development of antibiotics metabolized into the gastrointestinal system, particularly peptide antibiotics. Antimicrobial compounds were discovered to be released by *B. clausii* strains in the medium [51].

Bacillus clausii is a popular Bacillus spp. probiotic. Clinical evidence supports being used in the management and prophylaxis of gut barrier dysfunction [52].

Spore-forming Bacillus spp. were being used for generations in byproducts or microbe probiotic pills; nevertheless, very little Bacillus strains are considered safe and are commercially accessible. Ghelardi (2015) and Upadrasta (2016) B. clausii is among the most often employed Bacillus spp. bacteria in medicine. 2016 Lopetuso Bacillus spp. bacteria, formerly assumed toward being soil microbes, may need to be regarded as stomach commensals because their frequency in animal faeces is now discovered to be larger than previously thought.

Probiotic medicines are often used for centuries. In a Persian translation of something like the Old Testament, Abraham's vitality was attributed to drinking sour milk. Plinius, a Roman

historian, mentioned fermented milk around 76 BC for treating gastroenteritis [53] [54].

Revised descriptions have emerged in the literature to cover processes involved and stimuli in systems other than bacteria. "A composition or product including live, defined bacteria in more than enough numbers to affect the microbiota (via inoculation or colonialism) in some type of such a section of the organism and therefore have beneficial health benefits on this host," according to one group [55].

2.3.1 Description and Significance

Nielsen et al. identified DSM 8716 as a novel Bacillus species with unusual cell structure and metabolism features [56]. KSM-K16 was identified using 16S rRNA sequencing, which compares the rRNA sequences of two or more strains to discover sequence homology. Two further classification processes are fatty acid analysis, which detects fatty acids in the membrane, and carbohydrate intake assays, which characterize the metabolic features of the organism [57].

2.3.2 Cell Structure and Metabolism

Bacillus clausii is a gram-positive bacterium with a rod-like form and a thick cell wall. The peptidoglycan murien makes up the cell wall. *B. clausii* cells prefer to create a chain-like configuration, which may be seen as a long rod cell. *B. clausii* is an endospore-producing bacterium that produces ellipsoidal sporangium spored situated subterminally or paracentrally. Many medications, including erythromycin, lincomycin, cephalosporins, and cycloserine, are resistant to *B. clausii* spores [58].

2.3.3 Pathology

Due to its antibiotic tolerance and capacity to boost immunological activity, *Bacillus clausii* sporulated strains are employed in the treatment of gastrointestinal diseases to restore gut flora- a type of bacteria known as probiotics [58].

2.3.4 Dosing

The study of bacteria in the human Digestive system and their impact on illness is an essential component from the US National Institutes of Health's ongoing Human Microbiome Project. In clinical trials, 2 x 109 spores were usually consumed as a tablet or suspension two to three times daily for 10 days to 3 months [57].

Product details from the manufacturer:
Adults: 4–6 x 10^9 spores per day (2–3 ampoules per day or 2–3 capsules/day). 2 to 4 × 10^9 spores/day for children and breast-fed newborns. It is only indicated for short-term use.

Preterm neonates (births less than 34 weeks): 2.4 × 10^9 spores/day administered till the age of six weeks.

Nasal allergies in children: 3 sachets per day (2 × 10^9 spores/vial) usually consumed for three weeks to minimise nasal symptoms [58].

2.3.5 Pregnancy and Lactation

According to the product safety regulation, *B. clausii* has been proven to cause no issue during pregnancy and lactation as well as while breast-feeding infants [58].

2.3.6 Uses and Pharmacology

Probiotics are used to supplement the natural immune gut bacteria. 2016 Gastroenterology Probiotics' possible modes of action include the generation of pathogen-inhibitory chemicals, the suppression of pathogen attachment, the suppression of the activity of microbial pathogens, the activation of antibody A, and therapeutic effects on gastrointestinal system.
Each drug or preparations may well have a different mechanism of action, with certain probiotic pathogens being somewhat potent than others. Experiments included within meta-

analyses are frequently varied, particularly in terms of the probiotic strain utilized [57], [58].

B. clausii spores have been studied for their utility mostly in management and prophylaxis of gut barrier damage. Unlike lactic acid probiotics like lactobacilli and bifidobacteria, endophyte Bacillus spp. probiotics are particularly acid and heat resistant. *B. clausii* is notable for its resilience to bile & gastric acids, as well as its capacity to grow at large levels of salt concentration, attach to the intestinal epithelium, induce epithelial development, and sustain transport through to the digestive tract. As just a retail venture, these special properties enabled *B. clausii* to still be maintained without cooling or in a dried state without compromising its viability [58].

Experiments show that *B. clausii* influences intestinal mucosa homeostasis through gene expression modulation. Up- and down-regulation of genes associated with immune reaction as well as swelling, death, cell expansion & cell signaling, cell adhesion, transcription, cell communication, and defence response functions were observed in the small bowel mucosa [58].

2.3.7 Commercial Application as Probiotics

B. clausii as well as other similar Bacilli spores are utilised as probiotics to enhance intestinal microbial equilibrium during antibiotic use, modulate GI immune system performance, and serve as anti-microbial agents altogether. There are probiotic-containing therapies for health and nutrition, livestock feed supplementation, and fisheries. Enterogermina, an antibiotic-resistant probiotic, is made up of four Bacillus microbe strains (O/C, N/R, SIN, and T), which were all lately reassigned from B. subtilis to *B. clausii*. Enterogermina is most frequently used to diagnose diarrhoea and prevent infectious gastrointestinal illnesses. Though perhaps not comprehended, *B. clausii* enzyme releases during sporulation are thought to be responsible for these beneficial effects on the gastrointestinal system; during sporulation. Enterogermina strains were discovered to produce antimicrobial chemicals and alter immunological function by boosting the synthesis of antibody A. With a better understanding of *B. clausii's* role as an Enterogermina, the use of this microbe in therapeutics may be maximised and executed more effectively [58].

2.4 Probiotics

Probiotics are said to be living microorganisms that show a positive effect on human health when consumed. They aid by improving the conditions of the gut. They are mostly found in fermented foods such as yoghurts, kombucha, sauerkraut, miso, pickles. They are also known as healthy “bacteria” [59].

There are a total of 7 microbial organism genera that are used in the making of probiotics:

1. Lactobacillus,
2. Bifidobacterium,
3. Saccharomyces,
4. Streptococcus,
5. Enterococcus,
6. Escherichia, and
7. Bacillus

The most commonly used ones are Lactobacillus and Bifidobacterium. Every probiotics have a different role in our bodies. For instance, if a particular strain of Lactobacillus is used for preventing a particular kind of disease it does not mean that the other strains would do that too i.e. they would have a completely different role.

Probiotics are said to have three main roles:

3 They help the body to produce substances to show specific effects.

4 Improve the immunity of the human body

5 Lastly, they aid the body by significantly improving the number of good bacteria in the system which helps us to remain healthy and fight of diseases [59].

2.4.1 Mechanism of action of Probiotics

The human gut is heavily colonized by various microorganisms like bacteria, virus, archae, fungus, etc. The changes caused in the activity of this gut microbiota or microflora can lead to various disorders affecting the health of the human populations [60].

When the probiotics are consumed they influence the microflora by colonizing the gastrointestinal tract depending on the strain and the region of the gut.

There are 3 mechanisms by which probiotics do that:

3 nonspecific,

4 species-specific, and

5 strain-specific

a) NON-SPECIFIC MECHANISM

This mechanism works by inhibiting the growth of disease causing microorganisms of the gut. This improves the conditions of the gut microbiota by normalizing the number and activity of the harmful microbes.

b) SPECIES-SPECIFIC MECHANISM

Through this mechanism the probiotics neutralize the toxicity of the microbes by producing vitamins and bile salts. This mechanism is valid for almost all strains of a particular species.

c) STRAIN-SPECIFIC MECHANISM

This is only used for a few strains of a particular species. It effects the nervous system and the brain. It works by producing cytokines [60], [61].

Probiotics are usually measured in CFUs (colony forming units). This indicates the numbers of live cells. Probiotics can be of different CFUs including $1*10^9$ or $5*10^{10}$. However, more number of CFUs does not mean that the health benefits will increase or vice versa [61]

2.4.2 Risks of Consuming Probiotics

People suffering with auto-immune disorders like rheumatoid arthritis, multiple sclerosis, psoriasis, etc. should not consume probiotics. The reason behind this is quite simple, people having auto-immune disorders are given medicines to suppress their immune systems so their cells do not harm their organs. On the other hand, probiotics enhance the immune systems. Hence, they have a completely opposite effect to the auto immune disorder medicines. The enhanced immune effects can lead to the patients suffering from stronger effects of the disease [61].

There have been very few studies to judge the frequency or extremity of the side effects involved. The risk of side effect increases in people suffering from severe or chronic disorders or having compromised immunes [62].

The side effects of the probiotics also include infections, transferring of resistance genes from probiotic microbes to the human gut [62].

Moreover, there are times when there is a presence of microbes other than those listed on the label of the product. These are generally contaminants and can cause serious health issues [62].

Many times people consume antibiotics as an excuse to not visit their doctors. This behaviour should be strictly avoided. Also, people with compromised immune systems or other serious illnesses should be carefully noticed after they consume probiotics [62].

2.5 Polymerase Chain Reaction

Kary Mullis invented the Polymerase Chain Reaction (PCR) in 1983 as an in vitro technique for enzymatic production of a particular DNA fragment. It's a straightforward method for identifying, analyzing, and generating DNA from nearly any living thing (plant, animal, virus, bacteria). PCR is a technique for amplifying a specific DNA segment from a complicated combination of starting materials known as template DNA [63].

The main components are needed for a simple PCR:
Two primers complementary to the 3' ends of every one of the sense and anti-sense strands ofthe

DNA template containing the area to be expanded. Deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), are the basic components through which the DNA polymerase reconstructs a new DNA strand. The buffer solution creates an ideal microenvironment for DNA polymerase activity and stability. Bivalent magnesium/manganese ions are required for optimum Taq polymerase activity and have an impact on the annealing efficiency of primers to templates. A PCR is a technique for amplifying a particular DNA or RNA segment [63]

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Chapter 3: Methodology

3.1 Preparation of Bacterial Culture

In this work, *B.clausii* was extracted from a commercial probiotic vial containing 4 kinds of strains each of which are resistant against the antibiotics, namely novobiocin and rifampicin (strain N/R), chloramphenicol (strain O/C), streptomycin and neomycin (strain SIN) and tetracycline (strain T). The vial was added to a test tube containing 10 ml nutrient broth (Appendix). The culture was inoculated at 37 degree Celsius for 24 hours. This culture was then further used for subsequent experiments [64].

Figure 1. Pouring agar media into plates inside the laminar air flow chamber to avoid contamination.

3.2 Streaking

A non-contaminated MHA petri plate was taken and labelled (Appendix). The inoculum was taken from the bacterial culture. It was then streaked on the solid agar petri plate inside the LAF to avoid contamination. The plate was placed inside the incubator at 37 degree Celsius in static conditions for 24 hours to obtain isolated colonies [65].

3.3 Gram Staining

An isolated colony was picked up from the plate with the help of a sterilized inoculum loop. The colony was placed on a clean glass slide containing a drop of distilled water. A smear was made with the loop and was later air dried.

Further, crystal violet was poured on the slide for around 45 seconds and then washed with water. The slide was then flooded with gram's iodine for 60 seconds and once again washed with water. Additionally, the slide was washed with 95% alcohol for 20 seconds. Safranin was added for 45-50 seconds and washed off with water.

The slide was air dried and observed under the microscope at 10X, 40X and 100X [66].

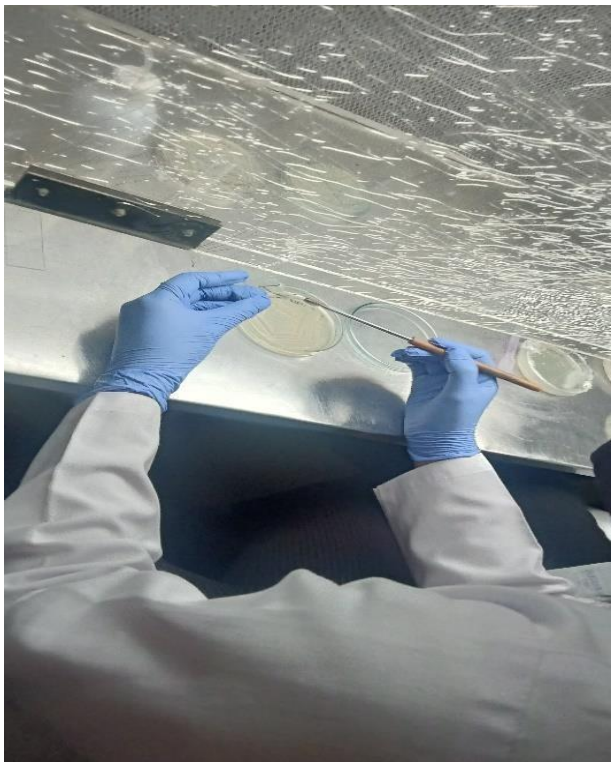


Figure 2. Depicts making of culture suspension for gram staining inside the laminar air flow to avoid contamination.

3.4 Antibiotic Susceptibility Testing

A non-contaminated MHA petri plate containing the isolated colonies of *B.clausi* was taken to perform antibiotic susceptibility testing (Appendix). A sterile cotton swab was taken and dipped in the bacterial culture. The bacterial culture was spread on the solidified agar plate

5 antibiotics were taken, namely:

1. LE 5 ug/disk
2. NA 30 ug/disk
3. AK 10 ug/disk
4. HLG 120 ug/disk
5. IPM 10 ug/disk

European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were followed [67].

3.5 DNA Extraction by Heat Boiling Method

3-4 colonies from overnight grown culture were taken. The colonies were placed in a 1 ml centrifuge tube containing 50 µl of nucleus free water. A cloudy suspension was formed with the aid of a sterilised inoculation loop. The tube was boiled in water bath at 95 degree Celsius for 10 mins, and then were centrifuged at 10,000 rpm for 3 mins. 20 µl of supernatant was extracted for PCR and agarose gel electrophoresis [68].

3.6 Polymerase Chain Reaction

The following components were added:

Table 1. Represents the components involved in the Polymerase Chain Reaction.

S.No.	Components	Amount(µl)
1	Nuclease free water	4.1
2	Template DNA	0.5
3	Primer (Universal primer and species specific 16S *E.coli* primer)	0.2(F) + 0.2(R)
4	Master mix	5

Total: 10 µl

All the above components were added to a centrifuge tube with the components completely and carefully mixed. Both the primers were added to *B.clausii and E.coli*. The tubes were placed inside the thermocycler to obtain amplified DNA. A centrifuge tube containing all components except primer was also placed in the thermocycler and used as a negative control.

3.7 Agarose Gel Electrophoresis

3.7.1 Preparation of 1 litre of 50X stock of TAE

242 g of Tris-base was added to a flask. Then, 57.1 ml of acetate (100% acetic acid) and 100 ml 0.5M sodium EDTA were added to the same flask. Add dH2O up to one litre.

To make 1x TAE from 50X TAE stock, dilute 20ml of stock into 980 ml of distilled water [70].

3.7.2 Preparation of 1.5% of Agarose Gel

1.5% agarose was made in 40ml of TAE i.e. 0.6g of agarose was weighed and added into a microwavable flask containing 40ml of TAE. The mixture was boiled for approximately 5-7 minutes with constant swirling after every 30 seconds. After the mixture became clear, which was originally foggy, the heating was stopped to avoid over-heating of solution to avoid evaporation of the buffer leading to change in the final percentage of agarose. The mixture was slightly cooled and EtBr (Ethidium Bromide) was added to a concentration of 0.5 ug/ml [70].

3.7.3 Loading of Sample and Running on Agarose Gel

The agarose was poured into a gel tray with the comb in place. It was left for 15-20 mins to solidify. The gel was then placed in the running tank and was filled with 1X TAE. The 3 µl of 100 base pair DNA ladder was loaded. Loading buffer was added to the samples and were loaded into the gel. The electrodes were attached (black for negative and red for positive). run at 50 V till the dye line reached the bottom of the gel. It took about 1-1.5 hours. After that, the PCR products were analyzed under UV light and compared with the ladder. The figure 3 shows the general apparatus of an Agarose Gel Electrophoresis [71].

Figure 3. Agarose Gel Electrophoresis Apparatus in Laboratory.

Chapter 4: Results and Discussion

Quadrant streaking was done to achieve pure isolated colonies to perform further activities. Figure 4 depicts the results of the quadrant streaking. It was incubated for 24 hours in an incubator at 37 degree Celsius. At the end, the inoculum was so diluted that single colonies of the bacterial growth were visible. These colonies made it easier to perform further experiments. Though the inoculating loop had to be sterilized repeatedly to avoid any contamination.

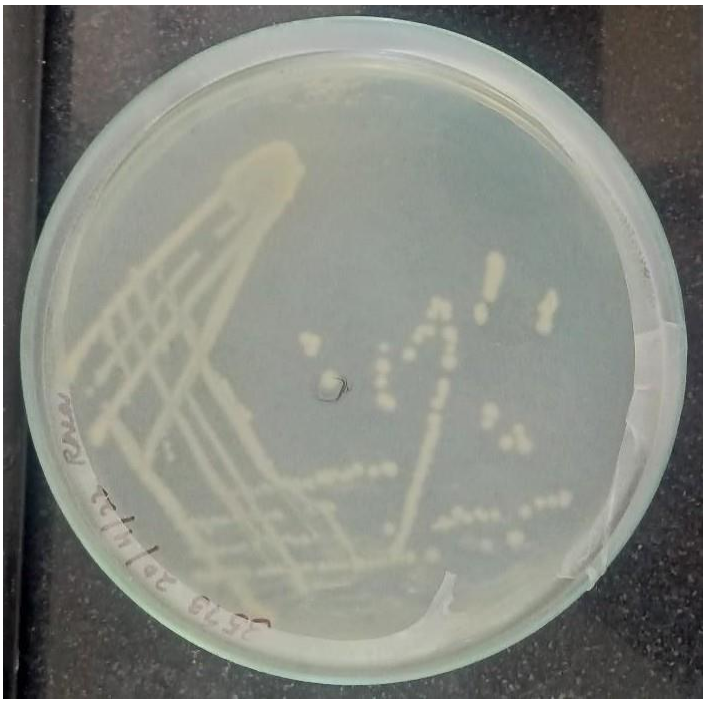


Figure 4. The result of quadrant streaking. Pure isolated colonies were obtained at the end of streaking.

The results of gram staining were observed under the microscope at different magnifications i.e 10X, 40X and 100X. The number of bacteria present were pretty high. The bacteria was rod shaped and its morphology is clearly present in fig. 5b. Also, they are purple coloured meaning that it is a gram positive bacteria proving the presence of Bacillus. For viewing under 100X, oil immersion was used to increase resolution.

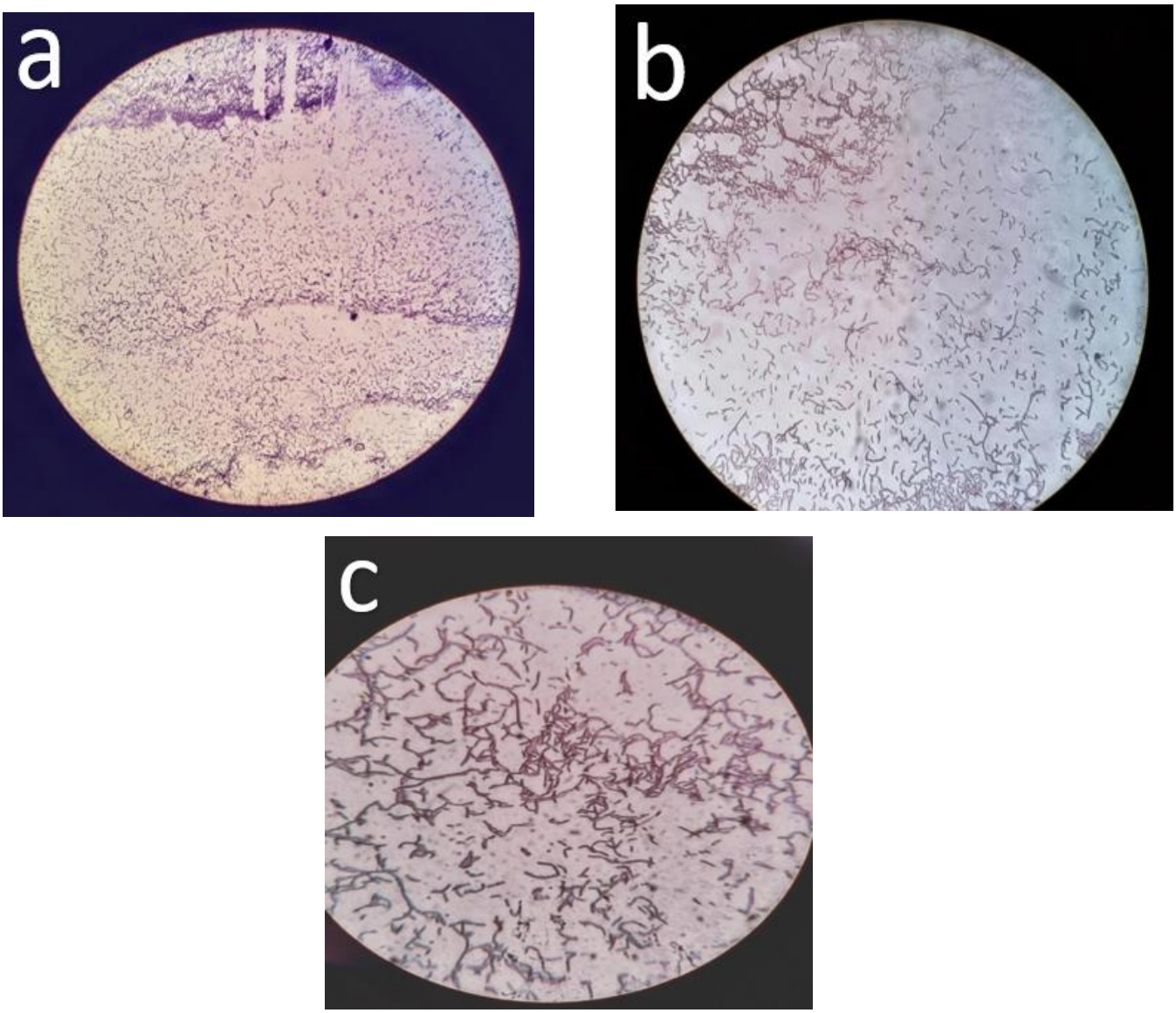


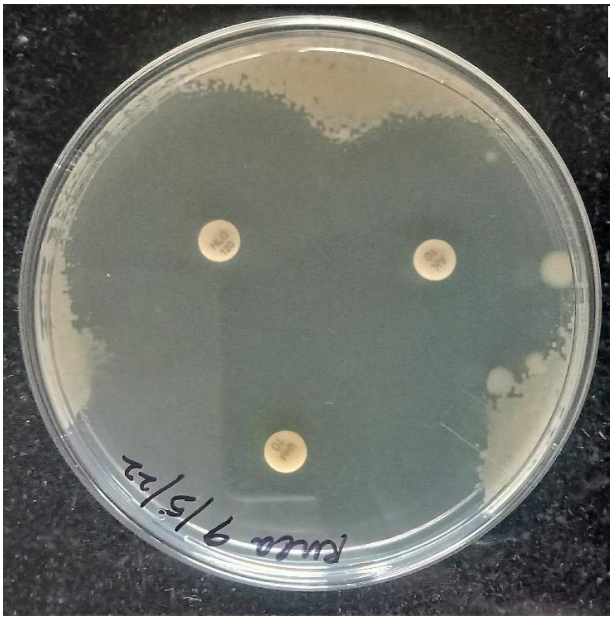
Figure 5. Light microscopy results of gram staining of *Bacillus clausii.* a) Magnification of microscope at 10X. b) Magnification of microscope at 40X. c) Magnification of microscope at 100X.

Further, AST was performed by adding 5 antibiotics and the bacteria was sensitive to almost all of the antibiotics added. By adding antibiotics to the growth media, the sensitivity and resistance of the inoculated organism became known. Figure 6a and 6b show the zone of inhibitions formed. Imipenem had the biggest zone of inhibition followed by Levofloxacin, whereas, Nalidixc Acid gave no zone of inhibition.

Table 2. Results got after performing Antibiotic Susceptibility Testing

S. no.	Antibiotics Used	ug/disk	Zone of Inhibition (mm)
1	LE	5	41.5
2	NA	30	No Zone
3	AK	10	46.7
4	HLG	120	48
5	IPM	10	59.3

a)



b)

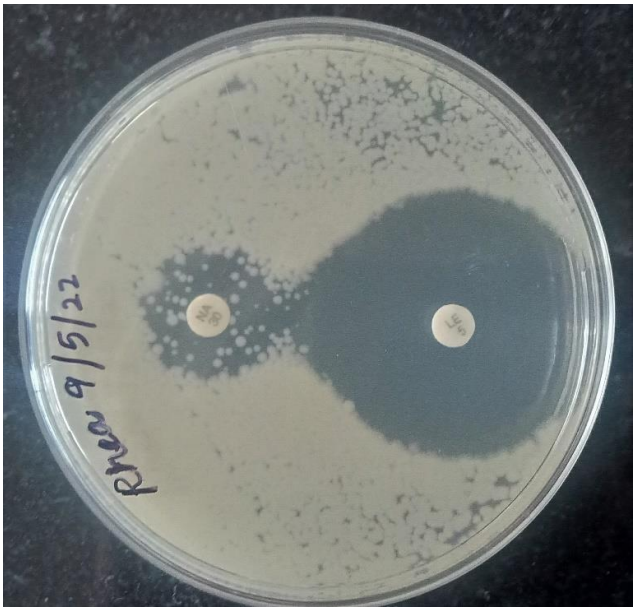


Figure 6. Zone of Inhibitions formed after performing AST. (a) Starting from left top corner: High level Gentamicin, Amikacin, and Imipenem. (b) Starting from left to right: Nalidixic acid and Levofloxacin

The DNA was extracted successfully using the heat boiling method. The centrifuge tubes contained the supernatant at the top and pellet at the bottom. After centrifugation, the pellet which has a higher density settled at the bottom whereas, the supernatant containing lighter particles remained at the top. The DNA is found in the supernatant. The supernatant was extracted for further experiments involving PCR and AGE whose results have been mentioned below.

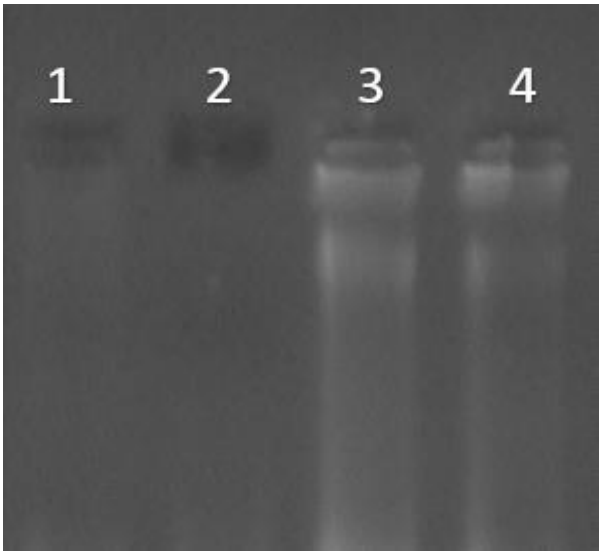


Figure 7. The DNA bands obtained by Agarose Gel Electrophoresis on 0.8% agarose. The DNA bands obtained in wells 1 and 2 were faded because only 2 µl of *B.clausii* was used. Whereas, the wells 3 and 4 were comparatively visible as 4 µl of *B.clausii*.

In the results of Agarose gel electrophoresis, the bands of bacillus were formed only with universal primer and not with species specific 16S rRNA *E.coli* primer. DNA is negatively charges hence it ran from black electrode to red electrode. The DNA ladder worked as a guide to help me in inferring the size of the PCR product in the sample lanes.

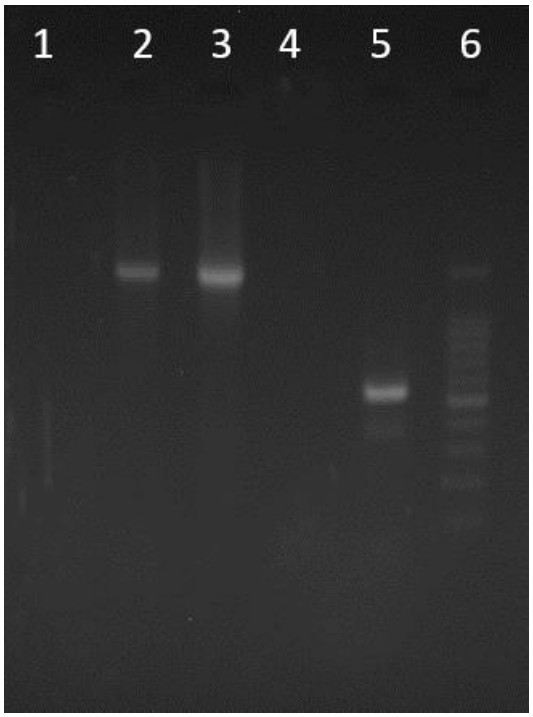


Figure 8. PCR products of 16S rRNA gene separated on 1.5% agarose. Lane 1: Negative control. Lane 2 and 3: PCR product of universal 16S rRNA gene in *Bacillus clausii* and *E. coli* respectively. A PCR product of 1.5kb is amplified in both bacterial isolates. Lane 4 and 5: PCR product of species specific 16S rRNA *E.coli* primer in *Bacillus clausii* and E.coli. The band is not visible in lane 4 but visible in lane 5 with a size of 508bp.

Chapter 5: Conclusion

This study made an attempt to determine the role of 16S rRNA gene for identification of bacterial isolates. The commercialized probiotic was susceptible to most of the antibiotics used. Rod shaped purple-colored bacteria were observed that indicated the presence of gram-positive bacteria. Amplification of universal 16S rRNA and *E.coli* specific 16S rRNA gene was determined in *Bacillus clausii* and *E. coli* isolates. The presence of 1.5 kb PCR products in both of the bacterial isolates indicate that 16S rRNA is conserved among bacterial isolates. However, *E.coli* specific 16S rRNA gene was not amplified in *Bacillus clausii* proving that the 16S rRNA gene have species specific conserved regions that can be utilized for molecular identification of bacterial isolates. All these steps taken were helpful in confirming that the commercialized probiotic contained the organism mentioned.

Chapter 6: References

1. Clayton, R. A., G. Sutton, P. S. Hinkle, Jr., C. Bult, and C. Fields. Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. Int. J. Syst. Bacteriol. Vol. 45, pp. 595-599, 1995.
2. Bansal, A. K., and T. E. Meyer. Evolutionary analysis by whole- genome comparisons. J. Bacteriol. Vol. 184, pp. 2260-2272. 2002.
3. Celard, M., G. Gevigney, S. Mosnier, P. Buttard, Y. Benito, J. Etienne, and F. Vandenesch. Polymerase chain reaction analysis for diagnosis of Tropheryma whippelii infective endocarditis in two patients with no previous evidence of Whipple's disease. Clin. Infect. Dis. Vol. 29, pp. 1348-1349. 1999.
4. Pfister, P., M. Risch, D. E. Brodersen, and E. C. Bottger. Role of 16S rRNA helix 44 in ribosomal resistance to hygromycin B. Antimicrob. Agents Chemother. Vol. 47, pp. 1496-1502. 2003.
5. Callahan, B. J. et al. High-throughput amplicon sequencing of the full-length 16S rRNA gene with single-nucleotide resolution. Nucleic Acids Res. Vol. 47, pp. 103 2019.
6. The Human Microbiome Project C,. et al. Structure, function and diversity of the healthy human microbiome. Nature. Vol. 486, pp. 207. 2012.
7. Acinas, S. G., Marcelino, L. A., Klepac-Ceraj, V. & Polz, M. F. Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. J. Bacteriol. Vol. 186, pp. 2629. 2004.
8. Mikhailov, I. S. et al. Similarity of structure of taxonomic bacterial communities in the photic layer of Lake Baikal's three basins differing in spring phytoplankton composition and abundance. Doklady Biochemistry and Biophysics. Vol. 465, pp. 413–419. 2015.
9. Newton, R. J., Jones, S. E., Eiler, A., McMahon, K. D. & Bertilsson, S. A Guide to the natural history of freshwater lake bacteria. Microbiology and Molecular Biology Reviews. Vol. 75, pp. 14–49. 2011.
10. Rossi-Tamisier, M., Benamar, S., Raoult, D. & Fournier, P. E. Cautionary tale of using 16S rRNA gene sequence similarity values in identification of human-associated bacterial species. International Journal of Systematic and Evolutionary Microbiology. Vol. 65, pp. 1929–1934. 2015.
11. Edgar, R. C. Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics. Vol. 1, pp. 5. 2018).

12. Gutell, R. R., Larsen, N. & Woese, C. R. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiological Reviews. Vol. 58, pp. 10–26. 1994.
13. Yu, Z., García-González, R., Schanbacher, F. L. & Morrison, M. Evaluations of different hypervariable regions of archaeal 16S rRNA genes in profiling of methanogens by Archaea-specific PCR and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology. Vol. 74, pp. 889–893. 2008.
14. Woo, P. C., K. H. Ng, S. K. Lau, K. T. Yip, A. M. Fung, K. W. Leung, D. M. Tam, T. L. Que, and K. Y. Yuen. Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J. Clin. Microbiol. Vol. 41, pp. 1996-2001. 2003.
15. Woese, C. R. Bacterial evolution. Microbiol. Rev. Vol. 51, pp. 221-271. 1987.
16. Wilson, R. W., V. A. Steingrube, E. C. Bottger, B. Springer, B. A. Brown- Elliott, V. Vincent, K. C. Jost, Jr., Y. Zhang, M. J. Garcia, S. H. Chiu, G. O. Onyi, H. Rossmoore, D. R. Nash, and R. J. Wallace, Jr. Mycobacterium immunogenum sp. nov., a novel species related to Mycobacterium abscessus and associated with clinical disease, pseudo- outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. Int. J. Syst. Evol. Microbiol. Vol. 51, pp. 1751-1764. 2001.
17. Teske, A., J. Wolters, and E. C. Bottger. The 16S rRNA nucleotide sequence of Mycobacterium leprae: phylogenetic position and development of DNA probes. FEMS Microbiol. Lett. Vol. 64, pp. 31-237. 1991.
18. Thorne, J. L., H. Kishino, and I. S. Painter. Estimating the rate of evolution of the rate of molecular evolution. Mol. Biol. Evol. Vol. 15, pp. 1647-1657. 1998.
19. Sultana, F., Y. Kawamura, X. G. Hou, S. E. Shu, and T. Ezaki. Determination of 23S rRNA sequences from members of the genus Streptococcus and characterization of genetically distinct organisms previously identified as members of the Streptococcus anginosus group. FEMS Microbiol. Lett. Vol. 158, pp. 223-230. 1998.
20. Schmalenberger, A., F. Schwieger, and C. C. Tebbe. Effect of primers hybridizing to different evolutionarily conserved regions of the small- subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl. Environ. Microbiol. Vol. 67, pp. 3557-3563. 2001.
21. Roth, A., M. Fischer, M. E. Hamid, S. Michalke, W. Ludwig, and H. Mauch.

Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. J. Clin. Microbiol. Vol. 36, pp 139-147. 1998.

22. Patel, J. B. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol. Diagn. Vol. 6, pp. 313-321. 2001.
23. Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F. C. Bange, and E. C. Bottger. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J. Clin. Microbiol. Vol. 31, pp. 2882-2889. 1993.
24. Kattar, M. M., J. F. Chavez, A. P. Limaye, S. L. Rassoulian-Barrett, S. L. Yarfitz, L. C. Carlson, Y. Houze, S. Swanzy, B. L. Wood, and B. T. Cookson. Application of 16S rRNA gene sequencing to identify Bordetella hinzii as the causative agent of fatal septicemia. J. Clin. Microbiol. Vol. 38, pp. 789-794. 2001.
25. Gee, J. E., C. T. Sacchi, M. B. Glass, B. K. De, R. S. Weyant, P. N. Levett, A. M. Whitney, A. R. Hoffmaster, and T. Popovic. Use of 16S rRNA gene sequencing for rapid identification and differentiation of Burkholderia pseudomallei and B. mallei. J. Clin. Microbiol. Vol. 41, pp. 4647-4654. 2003.
26. Dewhirst, F. E., B. J. Paster, N. Tzellas, B. Coleman, J. Downes, D. A. Spratt, and W. G. Wade. Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family Coriobacteriaceae: description of Olsenella gen., reclassification of Lactobacillus uli as Olsenella uli comb. Description of Olsenella profusa sp. Int. J. Syst. Bacteriol. Vol. 51, pp. 1797-1804. 2001.
27. Hall, L., K. A. Doerr, L. S. Wohlfiel, and G. D. Roberts. Evaluation of the MicroSeq system for Identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J. Clin. Microbiol. Vol. 41, pp. 1447-1453. 2003.
28. Gupta, S., and M. C. Maiden. Exploring the evolution of diversity in pathogen populations. Trends Microbiol. Vol. 9, pp. 181-185. 2001.
29. Cook, V. J., C. Y. Turenne, J. Wolfe, R. Pauls, and A. Kabani. Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. J. Clin. Microbiol. Vol. 41, pp. 1010-1015. 2003.
30. Drancourt, M., C. Bollet, A. Carlioz, R. Martelin, J. P. Gayral, and D. Raoult. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol. Vol. 38, pp. 3623-3630. 2000.

31. Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. C. Bottger. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. Vol. 17, pp. 7843-7853. 1989.
32. Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk, Jr. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. Vol. 42, pp. 166-170. 1992.
33. Principi N, Cozzali R, Farinelli E, Brusaferro A, Esposito S. Gut dysbiosis and irritable bowel syndrome: The potential role of probiotics. J Infect. Vol. 76, p. 111-20. 2018.
34. Ortiz-Lucas M, Tobias A, Saz P, Sebastian JJ. Effect of probiotic species on irritable bowel syndrome symptoms: A bring up to date meta-analysis. Rev Esp Enferm Dig. Vol. 105, pp. 19-36. 2013.
35. Tsai CC, Lin PP, Hsieh YM, Zhang ZY, Wu HC, Huang CC. Cholesterol-lowering potentials of lactic acid bacteria based on bile-salt hydrolase activity and effect of potent strains on cholesterol metabolism in vitro and in vivo. ScientificWorldJournal. 2014.
36. Hill C, Sanders ME. Rethinking "probiotics." Gut Microbes. Vol. 4, Issue 4, 269-270. 2013
37. Hooijmans CR, de Vries RB, Rovers MM, Gooszen HG, Ritskes-Hoitinga M. The effects of probiotic supplementation on experimental acute pancreatitis: a systematic review and meta-analysis. PLoS One. Vol. 7, Issue 11. 2012
38. Lopetuso LR, Scaldaferri F, Franceschi F, Gasbarrini A. Bacillus clausii and gut homeostasis: state of the art and future perspectives. Expert Rev Gastroenterol Hepatol. Vol. 10, Issue 8, pp. 943-948. 2016
39. Marseglia GL, Tosca M, Cirillo I, et al. Efficacy of Bacillus clausii spores in the prevention of recurrent respiratory infections in children: a pilot study. Ther Clin Risk Manag. Vol. 3, issue 1, pp. 13-17. 2007

40. McFarland LV. Use of probiotics to correct dysbiosis of normal microbiota following disease or disruptive events: a systematic review. BMJ Open. Vol. 4, issue 8. 2014
41. Nista EC, Candelli M, Cremonini F, et al. Bacillus clausii therapy to reduce side-effects of anti-Helicobacter pylori treatment: randomized, double-blind, placebo controlled trial. Aliment Pharmacol Ther. Vol. 20, Issue 10. 2004
42. Oelschlaeger TA. Mechanisms of probiotic actions—A review. Int J Med Microbiol. Vol. 300, Issue 1, pp. 57-62. 2010
43. Ripert G, Racedo SM, Elie AM, Jacquot C, Bressollier P, Urdaci MC. Secreted compounds of the probiotic Bacillus clausii strain O/C inhibit the cytotoxic effects induced by Clostridium difficile and Bacillus cereus toxins. Antimicrob Agents Chemother. Vol. 60, Isuue 6, pp. 3445-3454. 2016
44. Schrezenmeir J, de Vrese M. Probiotics, prebiotics, and synbiotics—approaching a definition. Am J Clin Nutr. Vol. 73, Issue 2, pp. 361S-364S. 2001
45. Susannah G Tringe, Philip Hugenholtz, A renaissance for the pioneering 16S rRNA gene, Current Opinion in Microbiology, Volume 11, Issue 5, 2008, Pages 442-446
46. Hamady M, Knight R. 2009. Microbial community profiling for human microbiome projects: tools, techniques, and challenges. Genome Res. Vol. 19, pp. 1141–1152.
47. Bahram M, Anslan S, Hildebrand F, Bork P, Tedersoo L. 2018. Newly designed 16S rRNA metabarcoding primers amplify diverse and novel archaeal taxa from the environment. Environ Microbiol Rep. Vol. 11, pp. 487–494.
48. Teng F, Darveekaran Nair SS, Zhu P, Li S, Huang S, Li X, Xu J, Yang F. 2018. Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. Sci Rep. Vol. 8.
49. Yeh Y-C, Needham DM, Sieradzki ET, Fuhrman JA. Taxon disappearance from microbiome analysis reinforces the value of mock communities as a standard in every sequencing run. mSystems. 2018.
50. De Filippis F, Parente E, Zotta T, Ercolini D. A comparison of bioinformatic approaches for 16S rRNA gene profiling of food bacterial microbiota. Int J Food Microbiol. vol. 265, pp. 9–17. 2018
51. Brandt J, Albertsen M. Investigation of detection limits and the influence of DNA extraction and primer choice on the observed microbial communities in drinking water samples using 16S rRNA gene amplicon sequencing. Front Microbiol. Vol. 9, pp. 2140. 2018.

52. Almeida A, Mitchell AL, Tarkowska A, Finn RD. Benchmarking taxonomic assignments based on 16S rRNA gene profiling of the microbiota from commonly sampled environments. Gigascience 7: 2018.
53. Park S-C, Won S. Evaluation of 16S rRNA databases for taxonomic assignments using mock community. Genomics Inform 16: 2018.
54. Reitmeier S, Kiessling S, Clavel T, List M, Almeida EL, Ghosh TS, Neuhaus K, Grallert H, Linseisen J, Skurk T, Brandl B, Breuninger TA, Troll M, Rathmann W, Linkohr B, Hauner H, Laudes M, Franke A, Le Roy CI, Bell JT, Spector T, Baumbach J, O'Toole PW, Peters A, Haller D. Arrhythmic gut microbiome signatures predict risk of type 2 diabetes. Cell Host Microbe. Vol. 28, pp. 258–272. 2020.
55. Prodan A, Tremaroli V, Brolin H, Zwinderman AH, Nieuwdorp M, Levin E. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. PLoS One. Vol. 15, 2020.
56. Kurylo CM, Parks MM, Juette MF, Zinshteyn B, Altman RB, Thibado JK, Vincent CT, Blanchard SC. Endogenous rRNA sequence variation can regulate stress response gene expression and phenotype. Cell Rep. Vol. 25, pp. 236–248. 2018.
57. Khairnar Y, Krishna VK, Borast A., Gupta , Trivedi S, Patli P, Gupta G, Gupta M, Jhadav A, Mujapara A, Joshi B, Mishra D, Study of Pectinase Production in Submerged Fermentation Using Different Strains of Aspergillus niger. International Journal of Microbiology Research, Vol. 1, pp. 13-17. 2009.
58. Kavuthodi B, Sebastian D, Review on Bacterial Production of Alkaline Pectinase with Special Emphasis on Bacillus Species. Bioscience Biotechnology Research Communications, Vol. 11, pp. 18-30. 2018.
59. Li Z, Bai Z, Zhang B, Li B, Jin B, Zhang , Lin F, Zhang H, Purification and Characterization of Alkaline Pectin Lyase from a Newly Isolated *Bacillus clausii* and Its Application in Elicitation of Plant Disease Resistance. Applied Biochemistry and Biotechnology. Vol. 167, pp. 2241-2256. 2012.
60. Pedrolli DB, Monteiro AC, Gomes E, Carmona EC, Pectin and Pectinases: Production, Characterization and Industrial Application of Microbial Pectinolytic Enzymes. The Open Biotechnology Journal, Vol. 3, pp. 9-18. 2009.
61. Prathyusha K, Suneetha V, Bacterial pectinases and their potent biotechnological application in fruit processing/ juice production industry: a review. Journal of Phytological Research, Vol. 3, pp. 16–19. 2011.

62. Priya V, Sashi V, Pectinase producing microorganisms. International Journal of Scientific and Research Publications, Vol. 4, pp. 1–4. 2014.
63. Urena IC, A Review of natural and engineered enzymes involved in bioethanol production. Graduate Student Theses, Dissertations, & Professional Papers. 2016.
64. Zhou C, Xue Y, Ma Y, 2017. Cloning, Evaluation, and High-Level Expression of a Thermo-Alkaline Pectate Lyase from Alkaliphilic Bacillus clausii with Potential in Ramie Degumming. Applied Microbiology and Biotechnology, vol. 101, pp. 3663-3676.
65. Ellerbrok H, Nattermann H, Ozel M, Beutin L, Appel B, Pauli G. 2002. Rapid and sensitive identification of pathogenic and apathogenic Bacillus anthracis by real-time PCR. FEMS Microbiol Lett. Vol. 214, pp. 51–59.
66. Abbrescia A, Palese LL, Papa S, Gaballo A, Alifano P, et al. Antibiotic sensitivity of Bacillus clausii strains in commercial preparation. Clin Immunol Endocr Metab Drugs. Vol. 1, pp. 102–110. 2015.
67. Joshi S, Udani S, Sen S, Kirolikar S, Shetty A. Bacillus Clausii septicemia in a pediatric patient after treatment with probiotics. Pediatr Infect Dis J. 2019.
68. Gargar JD, Divinagracia RM. When good things go bad: a case series of bacteremia from probiotics. Chest. Pp.155. 2019.
69. Enterogermina (Spores of Poly-antibiotic resistant Bacillus Clausii Origgio (VA) – Italy: Sanofi S.p.A. - Viale Europa. pp. 11–21040. 2020.
70. Bozdogan B, Galopin S, Leclercq R. Characterization of a new erm-related macrolide resistance gene present in probiotic strains of Bacillus clausii. Appl Environ Microbiol. Vol. 70, pp. 280-284.2004.
71. Abbrescia A, Palese LL, Papa S, Gaballo A, Alifano P, et al. Antibiotic sensitivity of Bacillus clausii strains in commercial preparation. Clin Immunol Endocr Metab Drugs. Vol. 1, pp. 102–110. 2015.

Appendix

Preparation of 10 test tubes of Nutrient Broth

1) 10 clean test tubes were taken and placed in a test tube stand.
2) Every test tube contained 10 ml of broth, therefore, 100ml of broth was made.
3) 7.5g of nutrient broth powder was added to a clean flask.
4) 100ml of distilled water was added and mixed well to dissolve all lumps.
5) The flask was sterilized by autoclaving it at 121 degree Celsius for 15-20 mins
6) The flask was carried to the laminar air flow chamber with the help of a tray to avoid burns.
7) 10 ml was measured by using a measuring cylinder and poured into each test tube.

Figure 9. Test tubes containing Nutrient Broth

Preparation of 250 ml of Nutrient Agar

1) 7g of nutrient agar was added to a clean flask.
2) 250 ml of distilled water was added to the same flask.
3) The flask was sterilized by autoclaving it at 121 degree Celsius for 15-20 mins

Figure 10. Flask containing Agar mixture ready to be autoclaved

4) The flask was carried to the laminar air flow chamber with the help of a tray to avoid burns.
5) Cool the mixture to 45-50 degree Celsius.

Preparation of 10 Nutrient Agar Plates

1) The cooled mixture was placed inside the laminar air flow chamber.
2) The mixture was poured into 10 glass plates.
3) The plates were incubated in the incubator at 37 degree Celsius overnight to check for contamination.
4) The non-contaminated plates were placed in 4 degree Celsius for further use.

Components of Master Mix for PCR

Table 3. Components of Master Mix for PCR

S. No.	Ingredients for PCR	Volume in µl
1	Molecular Biology Grade Water 30.5 µl	30.5 µl
2	10X Assay Buffer 5 µl	5 µl
3	Template DNA 2µl	2µl
4	Forward Primer (10 nM) 1 µl	1 µl
5	Reverse Primer (10 nM) 1 µl	1 µl
6	25 mM Mg Cl2 5 µl	5 µl
7	2.5 mM dNTP Mix	5 µl
8	Taq DNA Polymerase	0.5 µl
	Total volume 50µl	

Components of Nutrient Broth

Table 4. Components of Nutrient Broth

Components	**250 ml**
dH_2O	250 ml
Beef extract	0.25 g
Yeast extract	0.5 g
Peptone	1.25 g
Sodium chloride (NaCl)	1.25 g