CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Exploring Probiotic Bacteria from Different Food Sources.

by

Sadaf ul Hasnat

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my family life. To my mother and father, who never stopped believing in me and their prayers have always enlightened my way throughout my life To my mother and father, who never stopped believing in me and this can't be possible without their unwavering support, endless love and encouragement throughout my pursuit for education. My parents taught me that the best kind of knowledge to have is that which is learnt for its own sake. I hope this achievement will fulfill the dream they envisioned for me.

CERTIFICATE OF APPROVAL

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(MBS221015)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Uzma Abdullah	PMAS AAUR, Rawalpindi
(b)	Internal Examiner	Dr. Arshia Amin Butt	CUST, Islamabad
(c)	Supervisor	Dr. Sahar Fazal	CUST, Islamabad

Dr. Sahar Fazal
Thesis Supervisor
March, 2024

Dr. Marriam Bakhtiar
Head
Dept. of Bioinfo. and Biosciences
March, 2024

Dr. Sahar Fazal
Dean
Faculty of Health and Life Sciences
March, 2024

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Abstract

Probiotics are nonpathogenic and have been shown to improve host health and also help in disease prevention and as a therapy. They can be used as food supplements or additives. Some probiotic strains can grow and survive during food fermentations. Different food sources (yogurt, Dahi, Apple cider vinegar, Brine curd Olives and coca powder) were used for the isolation of probiotic bacteria. Samples were processed and cultured on nutrient agar and MRS media. Colonies were observed for Morphological characterization and Biochemical characterization was performed including various tests such as Gram staining, Catalase, Oxidase, Indole, Methyl Red, Voges-Proskauer, Simmons Citrate, and Motility-Ornithine tests. Isolated strains were also evaluated for probiotic potential. Antibiotic sensitivity was also checked for the isolated bacterial strains. Molecular characterization was done using 16S RNA and blast was performed to check the similarity.Morphological characterization of six isolates revealed that 2 strains retrieved from yogurt were gram positive, whereas 2 strains from dahi and vinegar and 1 strain from olives and coco powder were gram negative and these six bacterial colonies were characterized by rod-shaped morphology. Strains isolated from yogurt showed all biochemical test negative except motility ornithine test. In the case of Dahi, strains were positive for catalase test while negative for all other tests. Brine cured olives isolated strains were positive for Catalase, Oxidase test, Methyl red test and negative for rest of tests. Apple cider vinegar bacterial strain was positive for 4 tests (Catalase, Voges Proseur test, Simmons citrate test and Lactic acid test) and negative for rest of tests. For last food source coco powder, was positive for Catalase test, Simmons citrate test and Motility Ornithine test other all test were negative. Probiotic activity was negative for apple cider, yogurt, dahi and brine cured olives and only one of the strain isolated from yougrt was lactic acid producing All bacterial strains isolated were non-hemolytic. The strains isolated from the commercial yogurt were highly susceptible to all the antibiotics tested whereas strains isolated from olives were highly susceptible to Trimethoprim/Sulfamethoxazole and no susceptibility to Cefotaxime, and from Dahi was less susceptibility against Trimethoprim/Sulfamethoxazole. Molecular

characterization using 16 sRNA and Blast reveled maximum range of similarity and Identity percentage from 97% to 100% .Accession number of the isolated strains are [Y1- *Lactiplantibacillus plantarum* -OR484908], [Y2- *Lactiplantibacillus plantarum* -OR484910], [D3- *Kurthia gibsonii* -OR484904], [O2- *Bacillus pumilus* -OR484905], [C1- *Acinetobacter baumannii* -PP098451], [V3- *Bacillus subtilis* -PP098412]. These strains can be use for the nutritional purpose as well as for therapeutic purposes.

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Abbreviations

ACE	Angiotensin-converting enzyme
AK	Amikacin
AMC	Ampicillin
BSH	Bile salt hydrolases
CaCO3	Calcium carbonate
CFU	Colony-forming unit
CTX	Cefotaxime
DO	Doxycycline
EcN	Escherichia coli Nissle
GALT	Gut-associated lymphoid tissue
GRAS	Generally recognized as safe
IBD	Inflammatory bowel disease
IgA	Immunoglobulin A
IL-8	Interleukin 8
MHA	Mueller Hinton Agar
MIO	Motility-indole-ornithine
MRS	De Man–Rogosa–Sharpe
MR-VP	Methyl Red-Voges Proskeur
NEC	Necrotic inflammation of the distal small intestine
OCD	Obsessive-compulsive disorder
P	Penciline
PCR	Polymerase chain reaction
PH	Potential hydrogen
rRNA	Ribosomal ribonucleic acid

Th1	T helper 1 cells
Th2	T helper 2 cells
TLRs	Toll-like receptors
TMPD	N, N, N',N'-tetramethyl-p-phenylenediamine
TMPD	N, N, N',N'-tetramethyl-p-phenylenediamine dihydrochloride
TNF	Tumor necrosis factor
TS	Trimethoprim/Sulfamethoxazole
TSA	Tryptic Soya Agar
VEGFR	Vascular endothelial growth factor receptors

Chapter 1

Introduction

Probiotics are living microbes that naturally occur in human as well as animal bodies. They include bacteria and yeast. They establish mutually advantageous interactions, particularly in the gut environment. Probiotics have health benefits as long as they are taken at recommended dosages. Live bacteria, yeast, or a combination of the two can be found in probiotics; all provide benefits and safety for the host organisms. These microbes aid in better digestion, bolster the immune system and reduce the chance of certain illnesses, including acute infectious diarrhea and respiratory tract infections [1].

The words "probiotic" and "bios" (which mean "*life*" and "*for*," respectively) have their roots in Latin and Greek, respectively. The linguistic origin of probiotic goods emphasizes that they are made of live microorganisms that support a healthy lifestyle and aid in the body's efficient operation. Probiotics were first scientifically defined in the early 1990s [2], when specialists described probiotics as a change in the variety of bacteria or flora in the human body, where good microbes take the place of harmful ones. Probiotics are currently defined as an effective culture of bacteria, either mixed or solitary, as explained in [3]. These cultures enhance the properties of the natural flora, which is advantageous to the host, when fed to people or animals.

Probiotics, prebiotics, and synbiotic have all been characterized in different ways, but a broader description sees them as bacteria or groups of microbes that live in

the digestive system and function inside to benefit the host body [7]. These are commonly eaten as supplements containing live, active cultures of bacteria derived from their natural habitats, such as *Lactobacilli*, *laccocci*, or *Bifidobacteria* [8].

The wide range of microbial species that are recognized for their probiotic properties primarily in relation to nutrition, microorganisms included in the lactic acid bacterial category. In terms of practical applications, genera *Lactococcus* and *Bifidobacterium* are particularly important [9]. The ability of both Gram-positive and Catalase-negative bacteria to create lactic acid has led to their classification as lactic acid bacteria, which is the major byproduct of the fermentation of carbohydrates. The classification of *Bifidobacterium* within this family is more conventional than entirely phylogenetic due to its utilization of a different metabolic pathway. Notably, two important lactic acid bacteria that are essential to the food business are *Streptococcus thermophilus* and *Lactococcus lactis*. These bacteria are known to be crucial for their respective industries' manufacture of dairy products [10].

The many health-promoting qualities of probiotics are widely acknowledged. Comprehending the variables that impact the viability of probiotic strains while preparation and preservation has been the principal objective of recent research. Their resilience to substances such as bile, stomach fluid, low pH, pancreatic and intestinal fluids, interactions with respiratory or intestinal mucus, and isolated cells or cell cultures are all examples of this. Not only must a probiotic strain reach its intended active location, but it must also flourish there in order to perform at its best. It should not be harmful, allergic, mutagenic, or carcinogenic, and it should be immune system friendly [11].

The phrase "generally regarded as safe" should appear on probiotics intended for human use. Which denotes a low likelihood of sickness formation or worsening of existing conditions. For best incorporation into food items, the selected probiotic must also be compatible with the food production process. Furthermore, Foods containing probiotics must maintain the natural qualities of the dish [12].

It is recommended that probiotics be added to one's diet on a regular basis to assist re-establish a healthy gut microbiome. Research indicates that those dealing with anxiety, sadness, and obsessive compulsive disorder (OCD), and other illnesses may benefit from consuming supplements containing *Bifidobacterium* and *Lactobacillus* strains. Probiotics have been demonstrated to be useful in resolving immune regulatory disorders, resulting in improved recovery. Probiotics not only enhance immune cells such as Not only do they activate the body's built-in immune system and natural killer cells, but they also aid in the production of antibodies [12].

Probiotics function through a variety of pathways, while it's still unclear exactly how they accomplish their goals. These pathways include immunological regulation, lowering of gut pH, bacteriocin and short-chain fatty acid synthesis, nutritional competition, and activation of the mucosal barrier function. Interestingly, numerous studies have concentrated on the immunological regulatory component in particular, and compelling evidence suggests that probiotics impact multiple facets of the immune system, including both innate and acquired responses. Th2 responses are weakened, T-cell responses are changed, phagocytosis and IgA syn thesis are stimulated, and Th1 responses are reinforced as a result of this influence [13–15].

Probiotics function by triggering particular genes in neighboring host cells, which in turn activates, modifies, and regulates the host's immunological response. They influence the secretion of gastrointestinal hormones and regulate brain function via a bidirectional communication system in which neurons play a part in the relationship between the digestive tract and the nervous system [16]. Probiotics are essential because they activate the vascular endothelial growth factor receptor (VEGFR), promotes the formation of blood vessels in the stomach. As a result, this controls the intestinal mucosa's acute and chronic inflammation brought on by the onset of inflammatory bowel disease, also known as IBD [17]. Probiotics are also useful in the fight against obesity and excess weight because they have biophysical characteristics that support the bacterial environment that regulates the host and help to keep it healthy [18].

Probiotics' advantages for both human and animal health are contributing to their growing popularity. Even though probiotics have a lot of potential for use in nutrition and therapeutic settings, more study is needed to properly include them into diets, improve human health, and treat a range of illnesses.

Probiotic strains that have been used for medicinal purposes have been the subject of much investigation. The discovery of microbial strain imbalances "in vivo" has helped to understand disease states and has sparked the creation of a number of probiotic-based therapies. Probiotics are currently the subject of intensive ongoing study to learn more about their potential to treat a wide variety of illnesses and conditions.

Identifying probiotic cultures accurately has become essential due to the substantial consequences for establishing their commercial value, notably in the expanding functional and nutritious food industries. This is necessary to settle disagreements about the validity of probiotic products and to remove the possibility of deceptive claims [19]. Molecular biology techniques have been made possible by the genetic information that the 16S RNA gene provides, allowing for a comprehensive analysis of the human gut microbiota [20]. These technologies have proven invaluable in closely monitoring certain strains and using a range of molecular techniques to find possible probiotic indicators.

1.1 Problem Statement

Exploration of multiple food sources and their expanded use in potential probiotics for the improvement of health conditions in different disorders is the need of current era. Examining and assessing the specific and efficient application of the diverse range of probiotic strains in different metabolic illnesses might enhance the general well-being of the organism.

1.2 Aim

This study aims to determine the exploration of multiple food sources for their utility as potential probiotics.

1.3 Objectives

The study aims to achieve the following objectives:

1. To isolate bacterial strains from food sources (yogurt, dark chocolate, brine cured olives, and apple cider vinegar) that are Probiotic in nature.
2. To perform biochemical characterization of the isolated strains.
3. To identify and classify the isolated strains using 16sRNA.

1.4 Scope of Study

Using probiotic strains from fermented foods and drinks as a starting culture is beneficial when producing traditional goods on a large scale. Moreover, some probiotics have functional characteristics that improve their ability to fight food-borne infections.

1.5 Impact on Society

Probiotic bacteria are beneficial in treating a range of illnesses brought on by pathogenic microbes that are resistant to therapy. These strains of probiotics aid in growth, the synthesis of enzymes, the suppression of pathogens, the provision of nutrients, and the improvement of immunological responses.

Chapter 2

Literature Review

Probiotics have their origins in history, where the Greeks and Romans encouraged the consumption of cheese and fermented milk due to their belief in the special health benefits they offered, particularly for digestive health. Individuals consumed particular amounts of probiotic supplements, such as cheese and fermented milk, in order to achieve beneficial health outcomes. Probiotics include both bacteria and yeast, with the latter occasionally contributing to their beneficial properties. Probiotics, which are made up of bacterial cell components, have since been shown to improve overall health [21].

2.1 Probiotics, Postbiotics, Prebiotics and Synbiotics

There are other definitions for probiotics, prebiotics, and Synbiotics; however, the most accurate one is that they are microorganisms, or colonies of microorganisms, that reside in the gut and supply internal nourishment to the host body [22, 23]. Most commonly, they are consumed as preparations created from live, active cultures of bacteria obtained from the natural environments of the microbial organisms such as *lactobacilli*, *lactococci*, or *Bifidobacteria* [24]. Known for their many qualities as significant health enhancers, most of the research conducted in

the modern era has concentrated on examining the culture parameters and stability of probiotic strains all over their production and preservation. This covers their susceptibility to low pH levels, bile, pancreatic and gastrointestinal fluids, stomach fluid, connections to potentially hazardous pathogens and isolated or cultured cell populations.

2.1.1 Probiotics

Supplements containing alive populations of beneficial bacteria, specifically strains of *Lactobacillus* and *Bifidobacterium*, are now referred to as probiotics. Probiotics' core traits and importance originate from their capacity to support the immune system's normal growth, gastrointestinal microbes and intestinal ecosystem. Because of their susceptibility to varied environments, particularly temperature and pH levels, these specific probiotic strains require careful isolation, processing, and storage. Probiotic strains have been shown to bind to mucous and epithelial membranes in humans and animals, exhibiting enhanced tolerance to bile salts, resistance against bile acids, and resilience to stomach acidity. Probiotics aid in the suppression of pathogenic bacteria through their antimicrobial properties. Probiotics' primary goal is to increase and supply nutrient levels, especially in the stomach.

Probiotic supplements are typically seen to be safe when used, although there is sometimes a chance of unfavorable side effects or interactions between the bacteria and the hosts. In essence, probiotics are the gut flora itself, and they have been associated with enhancing immunity and exhibiting antibacterial, immune-regulating, and anti-inflammatory qualities, among other good aspects of microbial ecology [25].

TABLE 2.1: List of microbes currently used as probiotics [25].

Sr. No.	Probiotic Bacterial Genera	Species Involved
1	*Lactobacillus*	*Plantarum lactam, Paracasei lactam, Acidophilus lactam, Casei, Rhamnosus lactam, Crispatus lactam, Gasseri, reuteri, and Bulgaricus lactam*
2	*Propionibacterium*	*P. jensenii, P. freudenreichii*
3	*Peptostreptococcus*	*P. productus*
4	*Bacillus*	*B. coagulans, B. subtilis, B. laterosporus*
5	*Lactococcus*	*L. lactis, L. reuteri, L. rhamnosus, L. casei, L. acidophilus, L. curvatus, L. plantarum*
6	*Enterococcus*	*E. faecium*
7	*Pediococcus*	*P. acidilactici, P. pentosaceus*
8	*Streptococcus*	*S. sanguis, S. oralis, S. mitis, S. thermophilus, S. salivarius*
9	*Bifidobacterium*	*B. longum, B. catenulatum, B. breve, B. animalis, B. bifidum*
10	*Bacteroides*	*B. uniformis*
11	*Akkermansia*	*A. muciniphila*
12	*Saccharomyces*	*S. boulardii*

2.1.2 Prebiotics

which is why they are so important for maintaining the overall health of the microbes in the intestinal tract. These substances play a crucial part in promoting the growth and activity of beneficial gut flora, which in turn affects the microbiome's composition. Important prebiotic foods are oligo fructose, bifidogenic insulin, sucrose fructo-oligosaccharide extracts, and oligosaccharides that contain xylose and galactose. Prebiotics concentrate on fostering the growth of advantageous germs, as opposed to probiotics, which occasionally unintentionally encourage the growth of dangerous bacteria. The gut microbiome's *Bifidobacterium* ferments carbohydrates to provide colon epithelial cells with energy. Furthermore, non-digestible oligosaccharides are fermented by certain bacteria found in the gut microbiota.

Fresh fruits, veggies, and whole grains are common foods high in prebiotics that we eat on a daily basis. These foods are vital because they are critical providers of vitamins and nutrients, in addition to their prebiotic qualities. Prebiotics do more

for health than just provide you food. They also help prevent and treat a number of diseases include diarrhea, intestinal tract issues, colon cancer, and inflammation. Prebiotics also help prevent obesity and improve heart health by reducing risk factors and improving the body's absorption of nutrients and minerals [27,28].

TABLE 2.2: Natural sources of bioactive compounds [27,28].

Postbiotics and Prebiotics and Their Natural Sources		
	Bioactive Compounds	**Natural Sources**
	Postbiotics	
1.	Bacteriocins	*Lactobacillus plantarum I-UL4*
2.	Heat-killed LGG	*Lactobacillus rhamnosus*
3.	Soluble mediator	*Lactobacillus paracasei*
4.	Butyrate	*Faecalibacterium prausnitzii*
5.	Polyphosphate	*Lactobacillus brevis*
6.	Exopolysaccharides	*Lactobacillus pentosus*
7.	Short-chain fatty acids	*Lactobacillus gasser*
	Prebiotics	
1.	Fructo-oligosaccharides	Garlic, Wheat, Oats, Jerusalem artichokes, Onion, Leek, and Asparagus
2.	Inulin	Garlic, Jerusalem artichokes, agate, burdock camas, chicory, coneflower, Costus, dandelion, and elecampane
3.	Isomalto-oligosaccharides	Sauce, Sake, Honey, Miso, and Soy
4.	Lactulose	Skim milk
5.	Lactosucrose	Milk sugar
6.	Galacto-oligosaccharides	Human milk, kidney bean, green pea, chickpea/hummus, lentil, and lima bean
7.	Soybean oligosaccharides	Soybean
8.	Xylo-oligosaccharides	Bamboo shoot, Fruits, Vegetables, Milk, Honey
9.	Fructo-oligosaccharides	Onion, Chicory, Garlic, Asparagus, Banana, Artichoke
10.	Arabinoxylan	Cereals
11.	Resistant starch-1,2,3,4	Beans/legumes, Starchy fruits and vegetables (e.g. bananas), Whole grains

TABLE 2.3: Microbial sources of synbiotics [27,28].

Common synbiotics and their microbial sources.	
Synbiotics	
Prebiotics	**Probiotics**

Common synbiotics and their microbial sources.	
1. Citrus-based polymers	Bifidobacteria, Bacteroides fragilis, Peptostreptococcaceae, Klebsiellae
2. Aspartame	Bifidobacterium animalis, Lactobacillus acidophilus, Lactobacillus paracasei
3. Dodecyl-oligosaccharides	Bifidobacteria, Bacteroides fragilis group
4. Cellulose	Bifidobacteria lactis, Lactobacillus bulgaricus, L. acidophilus, L. rhamnosus
5. Lactosucrose	Zymomonas mobilis
6. Lipopolysaccharides	Bifidobacterium adolescentis, L. plantarum
7. Dimeric oligosaccharides	Bifidobacterium longum, B. catenulatum
8. Oligo-fructosaccharides	Bifidobacterium bifidum, B. lactis
9. Arabicinoxylan and its oligosaccharides	Bifidobacterium sp.
10. Resistant starch-1,2,3,4	Bacteroides, Eubacterium rectal

2.1.3 Postbiotics

Postbiotics are what are left over after probiotics and prebiotics are broken down and metabolized. These byproducts are the result of probiotic activity in the gastrointestinal tract [29]. Postbiotics are basically the leftovers that live on and have functions and metabolic pathways in common with the parent bacteria. Postbiotics, which are generally derived from probiotic microbes that have fulfilled their function, are mostly composed of vital nutrients including vitamins K and B, important amino acids for protein synthesis, and antimicrobial peptides that prevent the growth of pathogenic germs [30].

Postbiotics contain these components as well as antibiotics, naturally occurring acids (Lactic acid, ethanol, diacetyl, and acetaldehydes), and trace levels of hydrogen peroxide. Remarkably, some bacterial structures survive the destruction of specific probiotics, for instance, because of heat, and they yet operate as postbiotics in a similar way to their probiotic counter parts [31, 32]. Numerous specialists suggest that these metabolic byproducts show promise as antibiotic substitutes because of their effectiveness against dangerous microbes. Importantly, neither the

human health nor any pathogenic activity is harmed by these metabolic byproducts. Postbiotics fall within a particular category of probiotic metabolites that come from non-viable bacteria [33].

2.1.4 Synbiotics

When probiotic and prebiotic materials are joined or blended, they generate what is known as "synbiotic" components, depicting a scenario where probiotics and prebiotics create a mutually beneficial interaction. Probiotics, particularly those found in the gut microbiota, proliferate more quickly and sustainably as a result of this synergy. Synbiotics exhibit greater efficacy and efficiency when compared to conventional probiotics and prebiotics [34]. Among the many health benefits of synbiotics are improved digestion and strong immune system support. Because of its inherent ability to improve gut health, prevent a variety of ailments, and generally improve human well-being, the concept of synbiotics has captured the interest of many experts. Often used medicinally, scientists are always looking for new ways to increase and colonize the probiotic population in the human stomach through the use of these substances. The search is on for novel food sources that are able to spontaneously isolate and grow synbiotic strains [35].

2.2 Probiotic Microorganisms

Different probiotic microorganisms carry out particular functions, with each strain displaying unique characteristics that differentiate it from the others. Furthermore, variables including species variances, ambient conditions, and local microbiota affect these strains' probiotic ability. Some strains exhibit traits including adherence specific to a particular place and differences in their immune responses. Different effects can also be observed in different age groups and health circumstances, for example, between healthy adults and frail babies. Different parts of the intestine contain different strains with varied concentrations and compositions. Different results might arise from interactions between host-microbe and microbe-microbe

in the stomach and gut microbiome. Probiotics from genera like *Saccharomyces*, *Lactobacillus*, *Bacillus*, *Escherichia*, *Enterococcus*, and *Streptococcus* are thought to be very promising. Some microorganisms are selected for their critical roles in site-specific processes, which are especially pertinent in the context of diseases [36].

2.2.1 *Bifidobacterium*

Bifidobacterium is a genus of just 30 species, which is distinguished by its wide range of sizes and shapes, from curved and club-shaped to short rods and bifurcated Y-shaped rods. Interestingly, ten of these species originate from human habitats, including the vagina, teeth cavities, and feces. There are seventeen distinct species of *Bifidobacterium* found in animal intestines, including the rumen. Certain *Bifidobacterium* strains are discovered in fermented milk, others are shown to flourish in freshwater environments. These robust microbes exhibit adaptability in the challenging conditions of human as well as animal gastrointestinal systems. The specific ratio and concentration of these bacteria are affected by the host's age and dietary habits. When it comes to infants, Early on after birth, *Bifidobacterium* colonizes the gut flora. Both colostrum and the k-casein found in human milk contain glycoprotein components that both encourage and regulate the growth of these bacteria [37].

2.2.2 *Lactobacillus*

The genus *Lactobacillus* was first identified in 1990 with the isolation of the strain *Lactobacillus acidophilus*, which is a member of the widely recognized class of gut bacteria. These bacteria are gram-positive, flagella-negative, and sometimes classified as coccobacilli. They have unique properties. Without the ability to produce spores, they can only be fermentative or anaerobic. The genus *Lactobacillus* contains 56 recognized and approved species. They are categorized according to their unique ecological preferences since they are found in large quantities in the

digestive and vaginal systems of different animals as well as humans in a variety of ecological environments. The makeup of microbial flora within these animals is modified and regulated by external factors such as the conditional availability of oxygen, body pH levels, and concentrations of specific substrates, particular secretions, and especially, interactions among different bacteria. The majority of *Lactobacillus* strains in this genus are thought to be harmless and non-disease causing to humans. Rarely and under particular circumstances do cases of their connection to gastrointestinal and other intestinal disorders arise. Probiotic strains that have a reputation for improving health, especially in the digestive system and genitalia of humans, are these ones [38].

2.2.3 *Saccharomyces*

A wide variety of species can be found in the yeast genus *Saccharomyces*; *Saccharomyces cerevisiae*, for example, is widely used in the brewing of wine, bread, and beer. *Saccharomyces boulardii* is a member that is also used in wine making, and it is used as a probiotic in medical applications. *Saccharomyces* yeasts and bacteria develop symbiotic relationships, building matrices utilized in kefir production [39]. They are sometimes added to kombucha formulations as well [40]. Being a probiotic with a strong safety record, preserved *S. boulardii* is frequently marketed as a treatment for diarrhea[41]. Whatever the etiology of diarrhea, a plethora of research consistently demonstrate the clinical efficacy of *S. boulardii* in shortening its duration. Shorter hospital stays and beneficial social and economic consequences result from this reduction [42, 43, 44, 45, and 46]. Irritable bowel syndrome patients [47], relapse prevention and therapy [48, 49], and mild ulcerative colitis symptoms have all shown positive responses to *S. boulardii*. Interestingly, co-administration of *S. boulardii* with traditional antibiotics on a regular basis considerably decreases the recurrence of pseudomembranous colitis caused by Clostridium difficile [50]. No anomalies were reported in a review that examined the safety of probiotics during pregnancy [51]. But it's important to recognize that *S. boulardii* might cause localized infections or fungemia in immunocompromised or susceptible people [52, 53].

2.2.4 Genus *Lactococcus*

The Lactococcus genus, which includes lactic acid bacteria that are Gram-positive, is necessary for the milk industry to produce fermented goods. It plays a crucial part in reducing the acidity of dairy products, which prevents bacteria from spoiling them. Moreover, nisin synthesis (discovered in Subspecies *Lactococcus lactis* CV56) and adherence to vaginal epithelial cells are two probiotic traits shared by a number of *Lactococcus lactis* subsp. lactis strains [54, 55]. When treating diarrhea brought on by antibiotics, it is recommended to use these particular strains in addition to other probiotics because they function as probiotics [56].

2.2.5 Enterococcus Genera *Enterococcus* & *Streptococcus*

The lactic acid bacteria genera *Streptococcus* and *Enterococcus* include strains linked to serious medical conditions such as vancomycin-resistant *Enterococcus faecium*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* [57]. They also include other strains. The commensal human microbiome, which is found in the mouth, skin, and intestine, is vital to the functioning of some strains from these genera, including *Enterococcus faecium* PC4.1 [58] and others. Probiotics of note from these genera are *Enterococcus durans* [59] and Streptococcus thermophilus [60], whose latter is combined with *Lactobacillus delbrueckii subsp. Bulgaricus* in the yogurt-making process. The fact that *Enterococcus faecium* has been used in probiotics for a very long time is noteworthy, especially in the prevention of drug-associated diarrhea [61]. However, animal investigations have revealed certain strains of these bacteria as opportunistic pathogens, potentially containing virulence genes and antibiotic resistance [62]. As such, even though these strains are usually thought to be advantageous probiotics for animals, they are not regarded as safe (GRAS) for use in humans [63, 64].

2.2.6 Genus *Bacillus*

Bacillus is a facultative, aerobic, spore-producing, Gram-positive genus that comprises species like B. cereus, *B. coagulans*, and *B. subtilis*, all of which may have probiotic properties. *Coagulans Bacillus*, notably, has proven remarkable success in the treatment or prevention of antibiotic induced diarrhea [65, 66]. Applications for Bacillus subtilis spores are used to cure diarrhea and eradicate the human *H. pylori* infection; probiotic usage in animals has also been proposed [67, 68]. However, giving *Bacillus subtilis* spores as probiotics to immunodeficient individuals carries a high risk. A number of cases demonstrate the risks: four cases of invasive bacteraemia following an oral medication containing *B. subtilis* spores [70] and one in which a patient with compromised immune system experienced recurrent infection following ingestion of probiotic strain *B. subtilis* spores [69]. It is important to note that eating *Bacillus subtilis* spore is usually thought to be safe for human consumption [71].

2.2.7 Genus *Escherichia*

A probiotic strain of *Escherichia coli* has been discovered, called EcN 1917. Although the Gram-negative *Enterobacteriaceae* family includes the genus *Escherichia*, which is frequently linked to highly pathogenic serotypes like *E. coli* O157:H7, *Escherichia coli* is a typical lower intestinal inhabitant. As previously noted, when combined with other probiotics, *Escherichia coli* Nissle 1917 has shown potential in treating inflammatory bowel illness [72] and constipation [73]. This specific strain has shown potential in addressing indications for gastrointestinal disorders, ulcerative colitis, Crohn's disease, and potentially colon cancer [72, 74]. To increase our understanding of its medicinal potential, more research is necessary.

2.3 Properties of Probiotic

A viable probiotic strain needs to meet certain requirements to guarantee the best health benefits for people. Specific in vitro experiments, such as the following, are used to evaluate these properties:

i. Tolerance for oral delivery of acid and bile.
ii. It is important to examine probiotic microorganisms for their ability to stick to mucosal and epithelial layers, as they stop the colonization and growth of proteolytic bacteria by doing so.
iii. Restricting and inhibiting harmful microorganisms to assess antibacterial efficacy.
iv. Assessing the activity of bile salt hydrolase.
v. Need the probiotic strain to possess antibacterial qualities.
vi. Creating an immune system simulation for a stronger reaction.
vii. Showing tolerance for both alkaline and acidic pH values.

To ensure optimal efficiency, the proper amount of probiotics must be consumed, leading to the creation of precise formulations with precise counts of colony-forming units (CFUs). Probiotic products typically need at least 106 CFU/ml, with 108 to 109 probiotic germs needed to produce the probiotic effect, while some details may vary for individual bacteria [75,76].

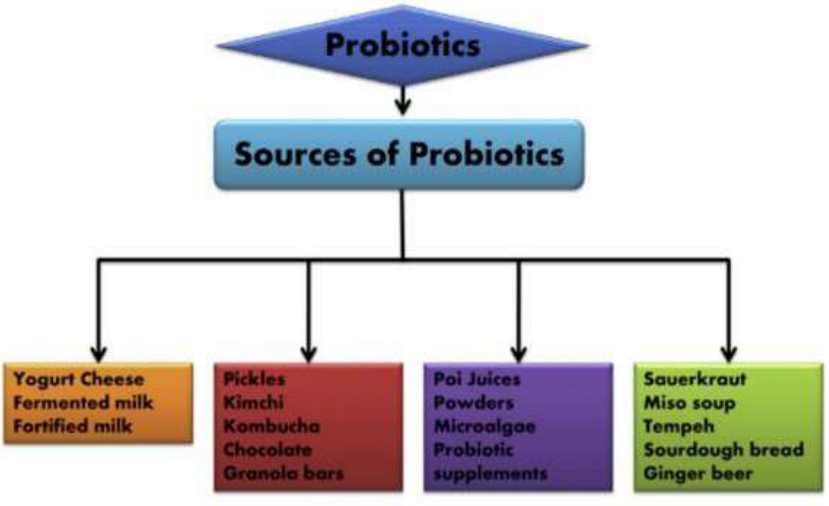

FIGURE 2.1: Different sources of probiotics [77]

2.4 Probiotics' Health Benefits

The native microbial communities are known to be host-specific, geographically restricted, highly complex in composition, and to have advantageous qualities for the host.

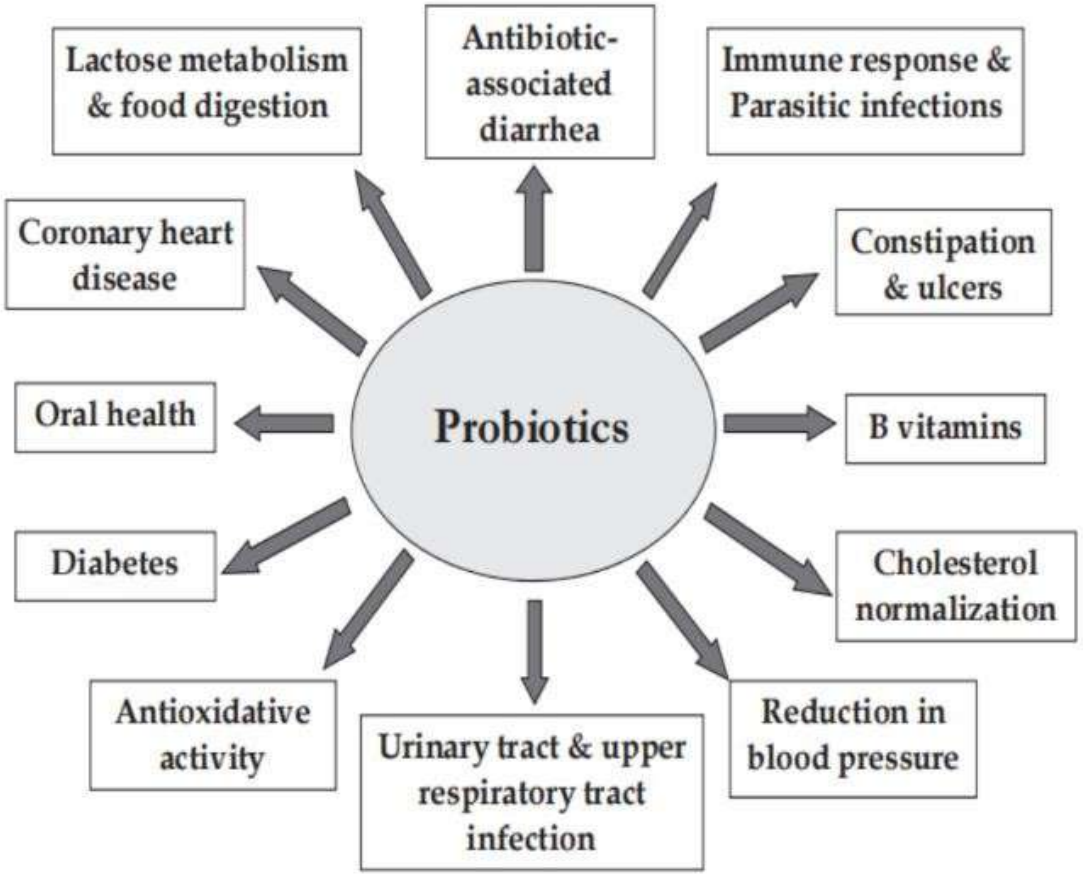

FIGURE 2.2: Projected prostective health attributes of probiotics [78]

2.4.1 Enhancement of Epithelial Barrier Function

As a vital component of the body's protective barrier, the intestinal epithelial cells use a number of simultaneous processes to preserve the integrity of this barrier. Mucus secretion within the intestinal wall, as well as the control of water balance and chlorine levels, are some of these activities. Mucins specifically, *MU2* and *MU3* are produced by goblet cells, which are engaged in immunological control and responsible for producing mucus. This mucus provides resistance against

microbial toxins, protein-degrading enzymes, and abrasion. Specific strains of probiotic lactobacillus, such *L. orhamnosus* GG and *L. oplantarum* 299v, increase the mucin concentration. These bacteria not only shield intestinal epithelial cells from harmful infections but also hinder *E. coli* adhesion and colonization [79]. Moreover, the amount of chlorine secreted by *E. coli* in the intestinal environment is influenced by particular strains of L. acidophilus and Streptococcus thermophilus [80].

Through tight junction signaling, the expression of particular genes is essential for improving the intestinal barrier's resistance and functionality. These genes are regulated by *Lactobacilli*, which also affect the adherence proteins in epithelial cells, which decreases the adhesion of proteolytic bacteria. Notably, important proteins like *E-cadherin* and catenin are phosphorylated by *Lactobacilli* in the intestines. Probiotics also help the barrier become more effective again after being damaged by some chemicals and pathogenic microbes. In situations where pathogenic bacteria have damaged the mucus layer, T84 epithelial cells, and *Caco-2* cells in the gut environment, *E. coli* appears to be a restorer. In order to restore the damaged components, this entails overexpressing tight junction proteins and inhibiting protein kinase signaling [81].

2.4.2 Enhanced Absorption by Intestinal Epithelial Cells

The colonization and adhesion of microorganisms, particularly certain strains of bacteria found in the gut ecosystem, are essential to the immune system's operation. Stronger adherence of probiotics is associated with improved immune responses. Notably, *Lactobacilli* strains, illustrated by *L. plantarum 299v*, demonstrate a heightened adhesion property to human colonic cells, boosting several activities, including the considerable release of chemokines and cytokines. Through their stimulation of mucosal immunity and reinforcement of the barrier, these substances play a crucial role in boosting the host immune system [82].

2.4.3 Competitive Exclusion of Pathogenic Microorganisms

Probiotic microorganisms protect the body by limiting the growth of dangerous bacteria. By generating acetic and lactic acids, probiotic bacteria aid in reducing the pH of the colon, disturbing the formation of toxins. The harmful bacterial population, which includes strains like *Salmonella* and *E. coli*, is reduced by these organic acids, especially acetic and lactic acids, which effectively stops their growth. Furthermore, probiotic bacteria compete with proteolytic bacteria for vital nutrients by attaching to and inhibiting different receptor sites. This complex strategy leads to the release of certain metabolites and antibacterial compounds, which in turn protect the gut in addition to trace levels of particular conjugated linoleic acids [83].

2.4.4 Production of Antimicrobial Peptides

Some probiotic strains have the ability to generate toxins called *Bacteriocins*, which are antimicrobial substances that have the power to affect, disturb, and kill bacteria that consume them. As an example, the strains of *Lactococcus lactis* and *Lactobacillus acidophilus* can produce nisin and plantaricin, respectively, and *lactacin* B. Gram-positive bacteria secrete these bacteriocins, which are antibacterial substances having a molecular weight greater than 1000. They are essential in encouraging the bacterial cell wall's pores to develop, which eventually causes the bacteria to perish [84]. Additionally, another antimicrobial compound produced by *L. oreuteri* demonstrates the ability to halt and eliminate various pathogens, including bacteria, viruses, and fungi. Defensins, another class of antimicrobial compounds, contribute to the host's barrier function by disrupting and destroying the cell wall of specific pathogenic microbes. Notably, the *E. coli nissle* strain can enhance the mucosal barrier in the intestines by secreting certain amounts of beta-defensin 2 in the epithelial cells, thereby limiting the entry of proteolytic bacteria into the intestine [85].

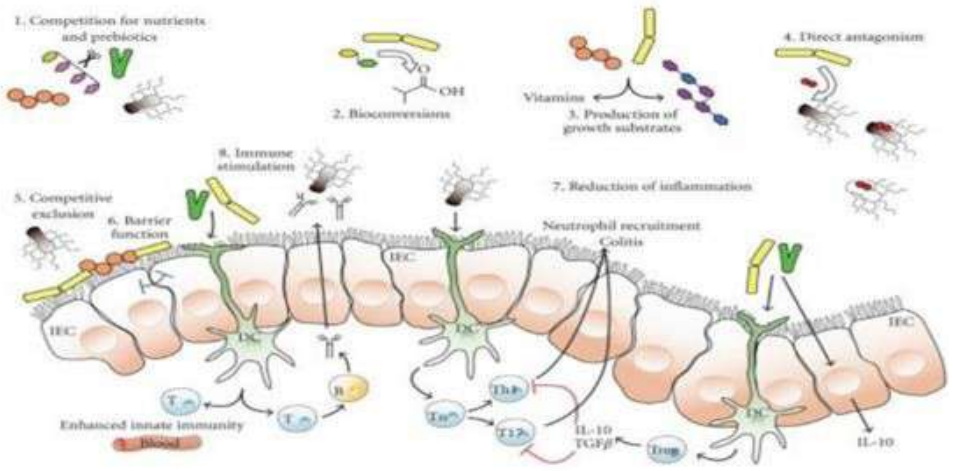

FIGURE 2.3: Mechanism of probiotic action [86].

For the purpose of using growth components as growth substrates, a competitive environment is first formed. After then, the sugars start to ferment, which results in the formation of fermented byproducts with inhibitory effects. Proteolytic bacteria are eradicated by these by-products by direct antagonistic action via bacteriocins. Proteolytic bacterial growth is inhibited by the competitive occupation of binding sites, which binds to the epithelium to improve barrier function. Proteolytic bacteria are suppressed and their colonization capabilities are altered as a result of this process, which reduces inflammation and eradicates any potential hazards. To ensure a thorough and efficient response, this system also activates innate immune responses [87].

2.4.5 Regulation of the Immune System

It has been found that probiotics imparts certain immune modulatory properties. Some of these properties are described as:

2.4.5.1 Increasing the Phagocytic Capacity of Macrophages

A multitude of probiotic microbes can increase the activity of phagocytosis, this is one of the primary defense mechanisms of cells. *L. acidophilus* strain, in particular, has the ability to affect the function of the immune system's function by increasing

the phagocytic process of specific leukocytes. These findings were noted in subjects who were taking probiotic supplements. This property's effectiveness depends on the bacterial strain's ability to adhere to surfaces and colonize new areas. Notably, even with somewhat diminished adhesion properties, *B. lactis* exhibited a modulation of increased phagocytic activity [88]. Some probiotic strains have the ability to increase natural killer cell activity, which helps to stimulate the immune system more quickly. An indigestible cell wall is left behind by these probiotic bacteria when they are phagocytosed by certain monocytes. The lingering cell wall increases the synthesis of interleukin-12O (IL-12), which in turn boosts natural killer cell activity [89].

2.4.5.2 Stimulating IgA Production

Various probiotic microbes support humoral immunity by activating memory B cells, which results in increased IgA synthesis. Because of the easier binding of this elevated IgA synthesis, antigens have less contact with epithelial cells. Particularly, strains of bacteria like *L. acidophilus* and *B. bifidium*. Which are frequently present in fermented milk, show increased production of IgA against harmful bacteria like Salmonella. Moreover, it have been observed that the strain *L. rhamnosus* raises IgA production, particularly following a child's rotavirus vaccination. These findings demonstrate how probiotic strains can stimulate and improve the host's humoral and systemic immune responses [90].

2.4.5.3 Modulation of Cytokine Production

Probiotic strains with immune-modulatory action also have the ability to modulate cytokine production. Probiotic strains target specific areas such as interleukin-10 (IL-10) which has been connected with the stimulation of specific helper and regulatory T cells. Only the host's ability to reduce inflammation is the reason interlukin-10 is being targeted. With the aid of heterodimer, such as p70 IL-1, natural killer cells generate interferon-γ. These particles are pro-inflammatory

response markers and threat eliminators that also stimulate the generation of regulatory T cells in addition to natural killer cells [91].

On the basis of immune modulatory response probiotics are categorized into two types:

i. Immuno-stimulatory which enhances Natural Killer cells activity by promoting defense by stimulating Interlukin-12..

ii. Immuno-regulators which regulate the pathway of regulatory T cells by in ducing Interlukin-10.

Lactobacilli belongs to immuno-stimulatory categories and *Bifidobacterium* belongs to the immuno-regulatory category [92].

2.4.6 Disruption of Quorum Sensing Signal Molecules

Numerous microorganisms, mainly microbes, possess the ability to interact with one another and their surroundings using chemical stimulus signals known as auto inducers, a phenomenon referred to as quorum sensing. In order to alter biological activity, quorum sensing can affect and control gene expression in nearby species. Specific pathogenic bacterial strains can be disrupted and obstructed from communicating by probiotic strains such as *Bifidobacterium*, *Lactobacillus*, and *B. cereus*. To do this, they secrete certain enzymes and antibacterial chemicals. In certain situations, these probiotic bacteria can create auto inducer antagonists that successfully regulate the expression of virulence genes in pathogenic strains like as *L. acidophilus*. Certain *E. coli* strains experience a disturbance in the transcription of their genes due to the inhibition and interpretation of quorum sensing signaling caused by this chemical compound release. Both colonization and adhesion in the intestines depend on these genes. This disruption highlights the probiotic strains' beneficial function by reducing bacterial toxicity in the gut [93].

2.4.7 Antimicrobial Properties

A complex ecology exists in the gut microbiota, and it is challenging to introduce new species into this fiercely competing setting. Therefore, living things possess a distinct advantage if they can generate a substance that prevents other living things from growing. The ability of probiotics to eliminate competitors aids in their colonization of the gastrointestinal system.

TABLE 2.4: Bioactive compounds synthesized by probiotic bacteria with antimicrobial properties [94].

Sr. No.	**Probiotic**	**Compound**
1.	*Lactobacillus GG*	*Wide spectrum antibiotic*
2.	*L.acidophillus*	*Acidolin, Acidophilin, Lactodin, Lactocin b,*
3.	*L.delbreukii ssp. bulgaricus*	*Bulgarican*
4.	*L.plantarum*	*Lactolin*
5.	*L.brevis*	*Lactobacillin, Lactobrevin*
6.	*L.reuteri*	*Reuterin*
7.	*L.sake[45, L.sake LB 706]*	*Lactocin S, Sakacin A*
8.	*L.jhonsonii*	*Lactocin F*
9.	*L.helveticus*	*Helveticin J*
10.	*L.cremoris*	*Diplococcin*
11.	*Lactococcus lactis*	*Nisin, Lactostrepsin, Lactocin, Lacticin*
12.	*Pediococcus*	*Pediocin*
13.	*Penteosaceous,P.acidilatis*	*Streptophillin*
14.	*Enterococuss faecium DPC1146*	*Enterocin 1146*

2.5 Probiotics in Health

Probiotics are essential for the management of many illnesses and inflammatory conditions, including *Clostridium difficile colitis*, *Helicobacter pylori infections*, inflammatory bowel diseases, cancer, necrotizing enterocolitis, and diarrhea brought on by antibiotics. They are especially useful in treating surgical infections and female urogenital infections [95]. Probiotics also have relevance in the animal realm

where they improve overall digestive processes in a variety of animal species by aiding in improved digestion and greater absorption capacities [96].

2.5.1 Probiotics in Animal Health

Probiotics, living microorganisms recognized for their health advantages, have been shown to improve the health of animals [97]. Numerous studies conducted on chicken farms show that the use of probiotics increases the absorption of important amino acids in hens by 5%, adding to their overall body weight gain [98]. Furthermore, probiotics have been shown to enhance calcium absorption due to their influence on gut metabolism via bacteria such as *Bacillus amyloliquefaciens* [99].

2.5.2 Nutritional Impact

Probiotics are important nutritional bacteria that provide a wide range of health advantages when consumed. Its favorable impact on a variety of diseases and health problems emphasizes its importance. Probiotics can help reduce weight, control chronic kidney damage, and cure illnesses like osteoporosis. Their impact is not confined to particular diseases; it extends to an array of disorders, involving developmental disabilities like autism and the healing of wounds. Furthermore, they aid to strengthen the immune system.

Probiotics have a positive impact on agricultural in addition to human health. They help to cultivate crops, fruits, and vegetables by fermenting them, supporting the development of healthy food [100]. Probiotics also influence the flavor character of specific items, such as rice bran oil, sprouted mung beans, and whole grains like buckwheat. Furthermore, they produce substances that help with conditions like low immunity, variable glucose levels, tiredness, and inflammation [101].

2.5.3 Dental Cares

Probiotic microorganisms have several health benefits, including their ability to treat and prevent infectious diarrhea. Rotavirus, the largest cause of acute infantile diarrhea worldwide, has a considerable influence on infant mortality because it replicates in the small intestine's specialized absorptive columnar cells. The presence of healthy microflora appears to be necessary for the host to fight off illness, as indicated by studies demonstrating that germ-free mice absorb antigens more readily than their normal counterparts [106]. Acute rotavirus diarrhea can be treated shorter with probiotics similar to *Lactobacillus reuteri*, *Bacillus animalis Bb12*, *Lactobacillus rhamnosus GG*, and *Lactobacillus casei Shirota*. This has been demonstrated in carefully monitored clinical studies. The most convincing evidence is notably provided by *B. animalis Bb12* and *L. rhamnosus GG* [107–109].

The reason for this effectiveness, as well as the improvement of immune response and competitive inhibition of receptor loci governing secretion and propulsive defenses, is the discovery of materials that actively degrade virus particles. Beyond rotavirus infection, data sug gests that specific probiotic strains, whether in food or non-food forms, can limit the growth and adherence of numerous diarrheal disorders. For children having acute diarrheal signs, a daily intake of probiotics like *Lactobacillus reuteri*, *Lactobacillus rhamnosus GG*, *Lactobacillus casei*, and *S. boulardii* has been associated with shorter durations of symptoms [107,109]. In some trials, the mean duration of diarrhea was much shorter when using L. casei than when using a traditional probiotic yogurt product [110]. One such study had participants who took prescription drugs.

Research on probiotics' efficacy in preventing adult travelers' diarrhea has also been conducted; however, the results have varied depending on research demographics, probiotic kinds, dosages, travel destinations, and traveler compliance. Notably, *L. rhamnosus GG*, *S. boulardii*, *B. bifidum*, and *L. acidophilus* seem to be very useful in this situation [111–113]. Furthermore, numerous investigations

conducted on animals have confirmed the probiotics' ability to suppress entero pathogens, mainly by forming bacteriocins [114].

2.5.4 Antibiotic Associated Diarrhea

Diarrhea episodes, ranging in severity from moderate to severe, are frequently a side effect of antibiotic treatment. This is because the suppression of normal microflora allows pathogenic or opportunistic strains to proliferate. The spectrum includes diarrhea with normal mucosal tissue and pseudomembranous colitis, this is a severe type of diarrhea linked to antibiotic use brought on by *Clostridium difficile cytotoxic* strains. The disease is named after the fibrin purulent material forms a plaque-like attachment to the damaged mucosal layer. If untreated, this condition can result in toxic mega colon and perforation

Recommendations for treatment include stopping the offending medicine, treating electrolyte abnormalities, and, in more serious situations, starting metronidazole or vancomycin therapy. Probiotics, particularly *L. rhamnosus* and *S. boulardii*, have been used in clinical settings. Probiotic usage may help lower the prevalence of diarrhea linked to antibiotics, according to several studies [103,104]. Probiotic therapy is linked to a lower incidence of the syndrome, as per the findings of a recent meta-analysis that concentrated on *Saccharomyces boulardii*, the yeast often found in most studies, *Lactobacillus rhamnosus*, and *Lactobacillus casei*. Future studies should go into finding appropriate probiotic dosages and comparing the efficiency of various probiotic treatments [105].

2.5.5 Infectious Diarrhea

Evidence suggests that some probiotic strains, found in food and non-food forms, can prevent the growth and adhesion of several diarrheal syndromes, in addition to rotavirus infection. Children's acute diarrheal symptoms have been demonstrated to be shorter when probiotics such like *Lactobacillus rhamnosus GG*, *S.*

boulardii, *Lactobacillus reuteri*, *Lactobacillus casei* and *Lactobacillus rhamnosus GG* are taken [107,109].

The ability of probiotics to prevent adult travelers' diarrhea has also been investigated, however the results have varied depending on the study demographics, probiotic kinds, dosages, travel locations, and traveler compliance. Among them, *L. acidophilus*, *S. boulardii*, *B. bifidum* and *L. rhamnosus GG* appear to be especially successful [111–113]. Numerous investigations conducted on animals have demonstrated that probiotics have the ability to suppress enteropathogens, mostly by means of bacteriocin production [114].

2.5.6 Lactose Intolerance

Lactose intolerance is brought on by a genetically caused beta-galactosidase deficiency, which prevents lactose from hydrolyzing into glucose and galactose. Osmotic diarrhea is caused by bacterial enzymes breaking down undigested lactose in the large bowel. Short bowel syndrome, rotavirus infection that affects lactase-producing cells, and pelvic radiation therapy that causes mucosal injury are examples of acquired causes of beta-galactosidase insufficiency, which is typically reversible. Lactose intolerance causes flatulence, diarrhea, and abdominal discomfort after drinking milk or dairy products.

Probiotics have been linked to better lactose metabolism; nevertheless, specific strains and amounts may be important. While traditional yogurt preparations containing *L. delbrueckii ssp.* and *S. thermophilus. Bulgaricus* are more successful due to their increased beta-galactosidase activity, probiotics are also recognized for their potential advantages. Probiotic supplementation has been proven to have favorable effects in certain individuals, leading physicians to view it as a potential therapy option [115,116].

2.5.7 Probiotics and Allergy

Probiotics may be a safe substitute for strengthening a baby's immature immune system and possibly guarding against allergies, according to recent studies. Probiotics have been demonstrated to enhance the mucosal barrier's performance, which may help lower the incidence of allergic responses. The importance of gut microbiota in the development of allergies is highlighted by comparative research that show quantitative and qualitative differences between adults without allergies and those who have them [117–120]. These probiotic effects appear to be especially beneficial for ailments like atopic dermatitis, a prevalent chronic skin illness that recurs frequently in infancy and children, and food allergies.

Many research have examined the efficacy of specific probiotic species in treating and preventing allergies in newborns. Surprisingly, a recent study revealed that *B. lactis* and *L. rhamnosus GG* can reduce a newborn's atopic eczema severity when the infant is breastfed. Additionally, it was discovered that prenatal administration of *L. rhamnosus GG* to mothers with a medical history of allergies and asthma, allergy rhinitis, or allergic eczema was helpful in reducing the occurrence of allergic eczema in risk offspring [121]. While using probiotics has been demonstrated to successfully lessen asthma symptoms, there is not enough data to support this claim. [122].

The mechanisms underpinning *L. rhamnosus GG's* immune-modulating function, particularly in the context of food allergies, are not entirely known. Food allergies are characterized by an adverse immune-mediated reactivity to food antigens, which causes intestinal inflammation. Although the precise mechanisms are unknown, there appears to be a link between *L. rhamnosus GG* activity and antigen transit through the intestinal mucosa [123]. Recent research on probiotic supplements in people with milk hypersensitivity, which is distinct from lactose intolerance, suggests that particular bacterial strains may reduce inflammatory reactions caused by milk and ease allergic symptoms. However, more research in this area is considered important [124,125].

2.6 Anti Carcinogenic Properties

According to studies, both nutrition and antibiotics can reduce carcinogen generation in the colon, hence lowering the risk of chemically generated malignancies. These effects appear to be affected by the composition of gut microbial communities. *L. acidophilus* consumption has been linked to fewer mice developing artificially produced carcinoma of the colon [126].

Suppression of intestinal bacterial enzymes responsible for transforming pro carcinogens into more potent carcinogens is one possible mechanism underlying these anticancer effects [127]. To investigate this, testing probiotics for their capacity to prevent nitrosation and the growth of high-enzyme flora species like b-glucuronidase, azoreductase, nitro reductase, and b-glycosidase is advised by researchers. [128].

Liver cancer can also result from eating food tainted with aflatoxin. Aflatoxin B1 (AFB1) causes unique genetic changes in the proto-oncogenes Ras and the tumor suppressor gene p53. AFB1 can be bound and neutralized in vivo by some probiotic bacterial strains, which decreases the amount of toxin absorbed from the stomach [129, 130].

Probiotic Bifidobacterium longum has also been given to rats, and the intestinal mucosa of these animals has shown strongly anticancer effects. Downregulated are both the expression of Ras-p21 and cell proliferation [131] genes for transduction, integrins, intracellular adhesion molecules, and cell adhesion (cadherins) have all been shown to be modulated by the administration of *Lactobacillus GG*, suggesting that it may have an impact on a number of different cellular processes [132].

2.6.1 Antiatherogenic and Cholesterol-Lowering

Cardiovascular disease (CVD) patients or those at high risk of developing it are treated with a variety of medicines. These treatments aim to decrease levels of triacylglycerol (TAG), low-density lipoprotein (LDL), and high-density lipoprotein cholesterol. It is unknown whether fermented milk products can function as

low cholesterol levels agents in human nutrition. Existing research vary in quality, with disadvantages such as insufficient documentation and varied statistical analysis [133]. A thorough analysis that included research from both human and animal experiments found that eating fermented foods containing probiotic bacteria may help lower cholesterol somewhat. Moreover, research by [134] showed that *Bifidobacterium spp.* and *L. acidophilus* could lower cholesterol.

Some of the postulated processes that account for probiotics' antiatherogenic and cholesterol-lowering actions include bacterial cholesterol assimilation, bile salt deconjugation, cholesterol production is reduced and cholesterol binds to the cell walls of bacteria [135].

2.6.2 Probiotics in Diabetes and Obesity

Gut flora play an essential part in the pathogenesis of obesity and resistance to insulin (type II diabetes), according to substantial research [136]. Gut flora has been shown in studies on both humans and animals to enhance resistance to insulin and body weight. When microbiota is transplanted in obese mice, these features are transferable to the gut flora, as opposed to normal or bacteria-free mice. The processes related with gut flora-mediated pathophysiology of both diabetes and obesity are through

i. Higher energy harvesting

ii. Elevated levels of toxic substances (blood LPS)

iii. Minimal inflammation [137]

Therefore, the alteration of gut flora is being researched as a potential treatment for obesity and diabetes. Probiotics are known to be novel gut microbiota modulators, and recent studies have focused on them as possible medications for the management and avoidance of diabetes and obesity [138,139]. Research has indicated that dahi, or yogurt, a fermented milk product enriched with probiotics,

considerably lowers insulin resistance brought on by food and shields animal models against streptozotocin-induced diabetes. Moreover, probiotic dahi application showed a suppressive effect on diabetes progression and associated consequences, which was linked to the antioxidant system being enhanced [140,141].

Nevertheless, discussions over the exact relationship between probiotics and the pathophysiology of diabetes and obesity continue, primarily driven by research derived from farm animals [142, 143, 144]. Importantly, compared to women of normal weight, *Bifidobacteria*, a vital probiotic class, have been linked to weight loss in overweight women [145].Studies conducted recently indicate that strains of *Bifidobacteria* and *Lactobacilli* selected for their probiotic qualities have positive effects on type II diabetes and obesity [146]. In human individuals, *L. acidophilus*, for example, has been connected to a decrease in insulin resistance and inflammatory markers [147]. Further research has shown that feeding particular strains of *Bifidobacteria* and *Lactobacilli* can slow the growth of obesity and diabetes [148-152]. This highlights how probiotics may be used as a therapeutic technique to manipulate the gut microbiota in order to treat obesity and diabetes. There are still few human trials showing meaningful impacts, despite encouraging results in animal research.

2.7 Sources

Probiotics provide nutritional and physiological benefits to host organisms since they are made up of live bacteria and yeast. A healthy dose of probiotics from reputable sources promotes development, growth, and nutritional absorption while preventing the growth of infections. Probiotics are in high demand as supplements for human and animal health due to the growing understanding of the complex relationship between diet and health, which has sparked a great deal of study into the precise functions of these supplements. Numerous investigations have examined the origins of probiotics, novel formulations, and their frequency in fermented dairy products such as cheese, yogurt, and cultured buttermilk. The

comparatively low pH of these fermented dairy products creates an ideal habitat for probiotic bacteria to thrive.

Probiotic bacteria, including *Lactobacillus* and *Bifidobacterium* species, and the yeast *Saccharomyces cerevisiae*, are frequently used in animal and human nutrition. Although dairy products are a great source of probiotics for humans, probiotics are also frequently obtained by animals through their own digestive systems. Probiotic options can now include fermented nondairy foods such grains, beans, maize, and sorghum, according to recent studies. The nutritional and medicinal qualities of yogurt, a dairy product that is widely consumed worldwide, have attracted interest. Regular yogurt gains additional functional qualities when probiotic cultures are added, making it a self-care food with supportive therapeutic advantages. Rich in essential nutrients, probiotic yogurt fights germs, promotes better health, and helps avoid sickness. It is advised to regularly consume probiotic yogurt in order to maintain a healthy life style.

Probiotics were previously linked to dairy-based goods that included lactose, which had to be broken down by lactase enzymes. Research and development on non-dairy probiotic products, however, has shifted due to concerns about lactose intolerance, allergic reactions to milk proteins, and cholesterol levels. These other sources include probiotic-rich options with extra antioxidant phytochemicals. Examples of these sources are veggies, fresh fruits, and even food waste. It has shown to be successful in preserving their viability to add probiotics to non-traditional items like chocolate. Rich in cocoa content, dark cocoa powder promotes blood pressure and vascular health while also being a nutritional choice. It also functions as an antioxidant. Known for its possible health advantages, apple cider vinegar has natural probiotic strains that strengthen the immune system, promote gastrointestinal health, and help people lose weight, and contribute to lowering blood pressure.

Probiotics need to fulfill a number of requirements in order to be an effective and healthy supplement, such as being able to withstand the effects of bile and gastric acid, adhering to the mucosa of the gastrointestinal tract, and competing with pathogens for clearance. The screening process is guided by traditionally

given criteria for appropriate probiotics, which guarantee their positive effects traditionally, the proposed criteria for useful probiotic are:

i. Be beneficial effect to host organism

ii. Be non-toxic, non-pathogenic, and no adverse effects to host

iii. To withstand the gastrointestinal tract conditions

iv. Be present in probiotic product in a required number of viable cells to have health benefit

TABLE 2.5: Health benefits of probiotic bacteria to the host, and speculated mechanisms involved [163].

HEALTH BENEFITS	**PROPOSED MECHANISMS INVOLVED**
Resistance to enteric pathogens	Engagement in conflict. The adjuvant action boosts the synthesis of antibodies influence of the systemic immune. Resistance to colonization limiting the presence of intestinal pathogens (pH, bacteriocins/defensins, antimicrobial peptides, formation of lactic acid, and harmful oxygen metabolites).
Aid in lactose digestion, Small bowel bacterial Overgrowth	In the small intestine, bacterial lactase digests lactose. The activity of flora in overgrowth is influenced by *Lactobacilli*, which reduces the formation of harmful metabolites. Normalization of a microbial population in the small intestine properties of antibacterial.
Immune system Modulation	Strengthening of antigen-specific and non-specific immunity to infection and malignancies Adjuvant impact in immunological reactions to a specific antigen regulating/influencing Th1/Th2 cells and cytokine production that fights inflammation reduced release of dangerous N-metabolites
Anticolon cancer effect	Antimutagenic properties. Carcinogenic metabolite detoxification. Changes in the colonic bacteria' pro-cancerous enzymatic activity. Immune function stimulation influence on the concentration of bile salt

HEALTH BENEFITS	**PROPOSED MECHANISMS INVOLVED**
Decreased detoxification / excretion of toxic microbial metabolites Allergy	Improvements in hepatic encephalopathy following the injection of *Bifidobacteria* and lactulose include increased *Bifidobacterial* cell counts, a switch from a protein -to carbohydrate -metabolizing microbial population, less harmful and for putrefactive metabolites, and less poisonous putrefactive metabolites. Preventing the transfer of antigen into the blood Avoid overreacting immunologically to gut antigen stimulation that is more intense.
Blood lipids, heart disease	Bacterial cells assimilating cholesterol Modifications in BSH enzyme activity Antioxidant impact
Antihypertensive Effect	Antihypertensive tripeptides are produced from milk protein by bacterial peptidase. Components of the cell wall serve as ACE inhibitors.
Urogenital Infections	Adhesion to cells of the urinary and genital tracts. Exclusion due to competition. Production of inhibitors (H2O2, biosurfactants)
Infection caused by Helicobacter pylori	Colonization with competition. Growth and attachment to mucosal cells are inhibited, and the concentration of gastric H. pylori is reduced.
Hepatic encephalopathy, Neutralization of dietary, Carcinogens	Gut flora that produces urease is competitively excluded or inhibited. Carcinogens found in food are less active when butyric acid is produced.
NEC (Necrotic inflammation of the distal small intestine)	TLRs and signaling molecules are downregulated, and negative regulatory mechanisms are upregulated decreased IL-8 response
Rotaviral Gastroenteritis	Increased IgA response to the virus
Inflammatory bowel diseases, type I diabetes	Enhancement of mucosal barrier function
Crohn's disease	TNFa expression among intraepithelial lymphocytes, a decrease in the number of CD4 cells, and a reduction in the production of pro-inflammatory cytokines, including TNFa
Caries gingivitis	*L. reuteri* reduces gingivitis, *Streptococcus* mutants are affected, and *Lactobacilli* colonize the surface of teeth. Less carries following oral immunization with heat-killed *Lactobacilli* or consumption of live *Lactobacilli.*
Enhanced nutrient Value	Vitamin and cofactor production/

2.8 Gap Analysis

Most of studies have been reported with probiotic effect from dairy products. There are many other non-dairy food sources that are also rich in probiotic and have not been explored or little focus have been on this food as potential food source. There is a need to explore such source that are enriched in probiotic that can be used for different purposes.

2.9 Research Questions

Q1: Which type of probiotic bacterial strains are present in food sources?

Q2: What is the impact of probiotic activity in bacterial strains on their potential as probiotics?

Q3: Does isolated probiotic strains get effected with the different generations of antibiotics?

Chapter 3

Material and Methods

3.1 Methodology Chart

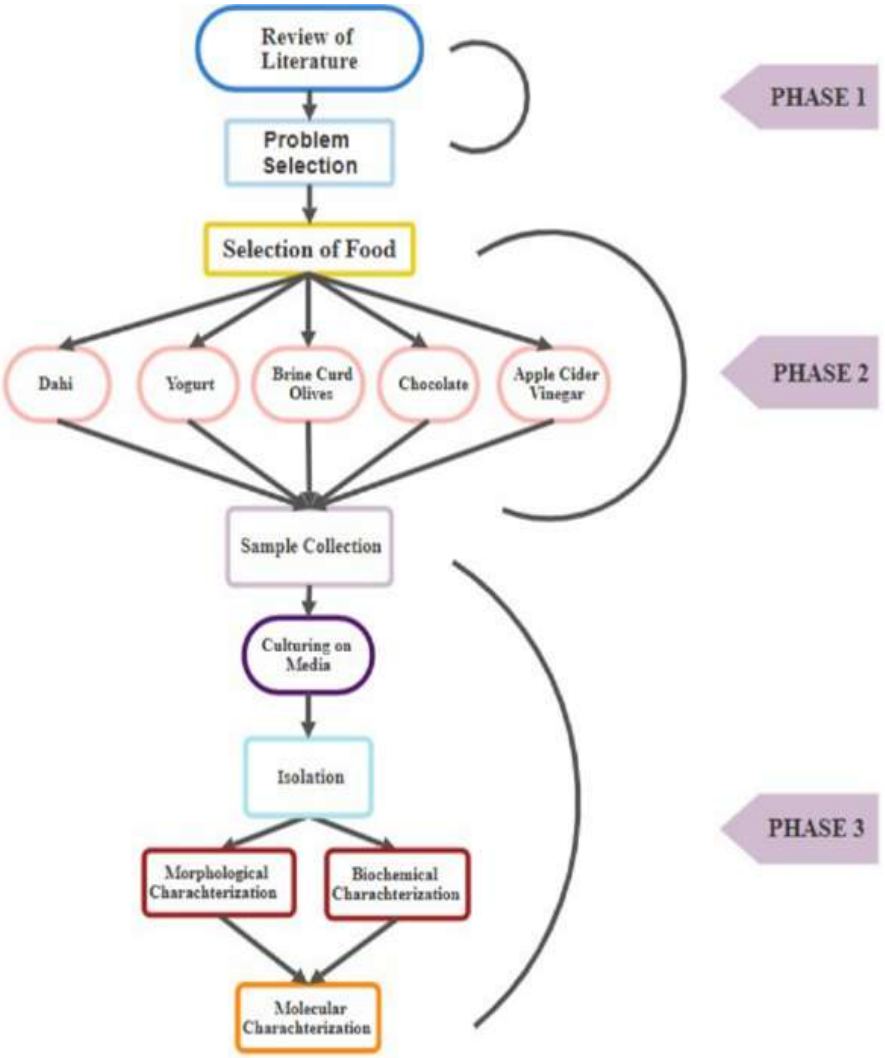

FIGURE 3.1: Flow Chart shows Major Steps of Methodology

3.2 List of Equipment

Weighing scale, Magnetic stirrer, Vortex, Centrifuge, Shaker, Grinder, PH meter, Sample storage box, Autoclave, Incubator, Laminar flow hood, PCR thermocycler, Microscope.

3.3 List of Apparatus

Spirit lamp, petri dishes(100mm x 15mm), beakers(5 ml to 1000 ml), conical flask (250ml, 500ml, and 1000ml), measuring cylinders (5ml to 4000ml), test tubes, Eppendorf tubes(0.5 ml to 2.0 ml), micro-pipettes, micro-pipette tips, dropper, gloves, spatula, mortar, pestle, filter paper, para-film tape, paper tape, sterile storage bags, inoculating loop, falcon tubes.

3.4 List of Chemicals

Nutrient agar, MRS (De Man, Rogosa and Sharpe) agar, MRS agar supplement, Crystal violet, Gram iodide, Decolorizing solution, Safranin, SIM (Sulfur, Indole, Motility) media, distilled water, Hydrochloric acid, Immersion oil, Sodium hydrochloride, Ethanol 70%, Ethanol 90%, Methanol, Acetone, Hydrogen peroxide 3%, Oxidase reagent and Indole Kovac's reagent, Methyl red, Methyl Red-Voges Proskeur (MR-VP) broth, Alpha-naphthol, Potassium hydroxide, Simmon citrate agar blood agar media Motility-indole-ornithine (MIO) media, Tryptic Soya Agar (TSA), 1% calcium carbonate, and Mueller Hinton Agar (MHA).

3.5 Sample Collection

Samples of food yogurt (Nestle), brine cured olive (Fermented pitted Spanish green olive samples (Figaro Company), apple cider vinegar (Key Grand) and coco powder (Mellow company) were collected from local supermarket of Islamabad. Samples were brought to the lab and were stored in refrigerator at 4^oC. Samples were labeled as mentioned in table 3.1.

TABLE 3.1: Tags used to label food samples.

No	Food Source	Sample ID
1	**Yogurt**	Y
2	**Dahi**	D
3	**Brine Curd Olives**	O
4	**Apple Cider Vinegar**	V
5	**Cocoa Powder**	C

3.6 Sample Processing and Culturing

3.6.1 Yogurt

A 1g portion of yogurt sample was introduced into a test tube containing 9 mL of saline. Subsequently, each sample group underwent sequential dilution with concentrations ranging from 10^1 to 10^5. Following this, 200 μl of the 10^3, 10^4, and 10^5 diluted bacterial solutions were applied to Nutrient agar solid plates and incubated for 24 hours at 37^oC.

3.6.2 Brine-cured Olives

The sealed bottles of fermented green olives were opened under sterile conditions, close to a flare to prevent contamination. By means of sterile forceps, a small number of olives were extracted from the flasks and subsequently pounded within

a mortar and pestle, which had been disinfected with a 70% ethanol swab. After that, using a sterile spatula, the minced olives taken from the mortar were transferred onto autoclaved falcon tubes & submerged in autoclaved distilled water. Also kept in sterile, autoclaved falcon tubes was the brine that was collected using the green olive samples. The refrigerator was used to hold all the autoclaved falcon tubes that contained samples of brine and olive oil for isolation. Spread plate method was used to inoculate nutritional agar with brine and crushed fermented green olives. The plates were then incubated for 24 hours at 37^{o}C. [164].

3.6.3 Coca Powder

A test tube was filled with 9 mL of distilled water, in addition 1 gram of cocoa powder sample remained added. Subsequently, the samples underwent serial dilution, with concentrations spanning from 10^1 to 10^5. Afterwards, 200 μL of the 10^4, 10^5, diluted bacterial solutions were spread onto solid plates of Nutrient agar, followed by a 24-hour incubation at 37^{o}C.

3.6.4 Apple Cider Vinegar

Sample apple cider vinegar was serially diluted with autoclaved distilled water under aseptic condition in laminar flow hood. 9 ml autoclaved distilled water was taken in 5 test tubes and 1 ml of each sample was added to it mix them thoroughly to make first dilution. Then 1 ml form first dilution was added to second test tube with 9 ml with autoclaved distilled water. Likewise all 3-4 dilutions were prepared and it was spread evenly over nutrient agar plate and plates were incubated for 24 hours (overnight) at 37^{o}C.

3.7 Isolation on MRS Agar Media

A sterilized inoculating loop was used to pick up colonies from nutrient agar media (4^{th} dilution) and streaked on MRS agar media. This process separated individual

colonies and prevent mixed cultures. The inoculated petri dishes were placed in an incubator set to the 37^{o}C temperature for 24 hours. After the appropriate incubation time, all plates were observed for signs of growth.

3.8 Characterization of Bacterial Strains

3.8.1 Morphological Analysis of Strains

After the incubation period, MRS agar plates were carefully examined to identify and quantify bacterial colonies. The agar plates were observed under appropriate lighting conditions, and distinct colonies were visually identified based on their morphology, color, and other observable characteristics. For morphological determination of the isolated strains, color, shape, size, and texture were observed with naked eye.

3.9 Morphological Characterization Using Gram Staining

Gram staining was achieved to distinguish bacteria based on their cell wall characteristics. A heat fixed bacterial smear was arranged on a slide by gently heating the slide with the bacterial sample. 02 drops of crystal violet dye were poured on smear for 2 minutes and after this smear was washed with distilled water to remove excess stain. After applying Gram's iodine to the slide, the smear was let to stand for one minute. After that, the slide was rinsed with water again. The slide was decolorized using decolorizing solution of ethanol or acetone till purple color of crystal violet rinsed off. The slide then was counterstained with safranin, and allowed to stand for 02 minute. The additional safranin was cleaned off with water, and the slide was allowed to air dry. The slides were then examined under microscope for the desired characteristics. Similar process was repeated for all isolates.

3.10 Biochemical Characterization

3.10.1 Catalase Test

The purpose of the test was to find out if the isolated strains could produce the enzyme catalase to break down hydrogen peroxide. On the spotless glass slide, the test was conducted. A clean glass slide with droplets of three percent hydrogen peroxide solution was rubbed with an inoculating loop comprising isolates, and the production of bubbles after 30 seconds was carefully monitored.

3.10.2 Oxidase Test

The oxidase test was performed to classify bacteria that yield cytochrome c oxidase, an enzyme involved in the electron transport chain. The filter paper soaked with oxidase reagent i.e., tetramethyl-p-phenylenediamine dihydrochloride was rubbed with inoculating loop containing isolates and the formation of a purple color was closely observed immediately.

3.10.3 Indole Test

The ability of separated microorganisms to generate tryptophanase, an enzyme that breaks down tryptophan in indole, pyruvic acid, plus ammonia, was assessed using the indole test. Sterilized test tubes were filled with 4 ml of tryptophan broth. The necessary broth was added to test tubes, which were then aseptically inoculated and incubated for 24 hours at 37^o degrees Celsius. The broth culture was then given 0.5 ml of Indole Kovac's reagent, and the presence or lack of a red ring was noted.

3.10.4 Methyl Red Test

The ability of an organism to create and sustain stable acidic end products via glucose fermentation was assessed using the Methyl Red (MR) test. The red dye known as methyl red, which is employed as an indicator, inspired the test's name. Methyl red turns yellow at pH levels over 6.2 and crimson at pH values under 4.4. Whenever methyl red is added, the pH of the culture medium drops due to bacteria that formed stable acidic byproducts from the fermentation of glucose. This causes the medium to turn red. A tiny portion of the isolated colony was put into a test tube, along with a few droplets of methyl red indicator, and it was cultured aerobically for 24 hours at 37 degrees Celsius.

3.10.5 Voges-Proskauer Test

The Voges-Proskauer (VP) test was performed to spot the production of acetoin, a neutral end product of glucose fermentation. The isolates were grown in a glucose-containing broth medium, such as MR-VP broth. After incubation aerobically at 37 ^{o}C for 24 hours, a small amount of the culture was transferred to a test tube. Two reagents alpha-naphthol and potassium hydroxide (KOH) were added sequentially: The tube was then gently mixed after each reagent addition.

The alpha-naphthol and KOH reagents react with the acetoin and produce red color indicates positive result for bacterial strains that produces acetoin whereas for non-acetoin producing strains remain colorless or turn light yellow, indicating a negative result for the VP test.

3.10.6 Simmons Citrate Test

A living can use citrate as its only source of carbon for growth was tested using the Simmons citrate test. It suggests that some bacteria are capable of producing an enzyme called citrate-permease, which enables citrate to be transported into the cell of the bacteria as the only carbon source.

The Simmons citrate agar surface was streaked with a loopful of the separated strain, and it was then incubated for 48 hours. Following that, cultures were carefully watched for changes in hue. Visible development on the slanted surface combined with a vivid Prussian blue color indicates citrate positive in the medium. The alkaline carbonates and bicarbonates, resulting from citrate catabolism, elevate the medium's pH to over 7.6. This shift causes the bromothymol blue to transition from its original green hue to a blue color. In the case of citrate negative, minimal or no visible growth will be observed. There will be no alteration in color; the medium will retain its original deep forest green shade, resembling the uninoculated agar. Since only bacteria capable of using citrate as their exclusive carbon and energy source can thrive on Simmons citrate medium, a culture testing negative for citrate will closely resemble an un inoculated slant, making them nearly indistinguishable.

3.10.7 Motility-Ornithine Test

The Motility-Ornithine test was used to determine the motility and ornithine decarboxylase activity of microorganisms. Sterile needle was used to pick an isolated colony in addition to being stabbed into the medium in test tubes. Inoculated medium was incubate at 37^{o}C until growth was evident. A red turbid region spreading beyond the line of inoculation signifies a positive motility test. Conversely, a negative test is characterized by red growth restricted to the inoculation line without further extension.

3.11 Probiotic Activity Test

3.11.1 Lactic Acid Test

The lactic acid test was performed for lactic acid bacteria to measure the creation of lactic acid by the isolated strains of bacteria. The MRS agar with 1% *CaCO3* concentration was prepared as per protocol and then autoclaved the medium at

121o for 15 min. It was left to cool and then poured in petri plates. Bacterial strain was streaked on the medium and incubated at 37^{o}C for 24 hours.

3.11.2 Hemolysis Test Using Blood Agar

The hemolysis test was used as a diagnostic assay determine the hemolytic action of bacteria, on blood agar plates. The test involves inoculating the microorganism onto a blood agar plate and observing for different patterns of hemolysis around the bacterial colonies. Hemolysis refers to the cessation or destruction of red blood cells, consequential in the release of hemoglobin.

Blood agar plates were prepared by adding human blood to Tryptic Soya agar medium, following the manufacturer's instructions and transferred on petri plates. The isolated strains were streaked onto the blood agar plate using a sterile inoculating loop or needle. Plates were incubated at 37^{o}C for 24 hours. After incubation, blood agar plate were observed for different patterns of hemolysis around the bacterial colonies.

3.12 Antimicrobial Sensitivity

A loop for inoculation or a needle that was sterile was used to prepare the inoculum. The organisms to be tested, isolated colonies, were suspended in two milliliters of normal saline. Suction was made smooth by vortexing saline tubes. The suspension's turbidity was then brought down to a 0.5 McFarland standard by either diluting the solution with sterile saline if it was too heavy or adding more organisms if it was too light. A cotton swab was touched to create a grass on petri plates after being immersed in inoculum. The 25 mm antibiotic discs were positioned on inoculum-containing plates. The usage of the following antibiotics (Table 3.2).

TABLE 3.2: Name of antibiotics tested for antibiotic sensitivity

No	**Generation**	**Antibiotic Name**	**Abbrevation**	**Concentration/ Dosage**
1	3^{rd}	Ampicillin	AMC	30 μg
2	3^{rd}	Amikacin	AK	30 μg
3	4^{th}	Trimethoprim/ Sulfamethoxazole	TS	10 μg
4	2^{nd}	Doxycycline	DO	30 μg
5	4^{th}	Penciline	P	30 μg
6	3^{rd}	Cefotaxime	CTX	30 μg

3.13 Molecular Characterization using 16S rRNA

The 16S rRNA sequencing procedure was used for identifying and classifying isolated strains at the molecular level. It involved the extraction of DNA, amplification and sequencing of the 16S rRNA gene, which is a conserved region found in the prokaryotic ribosome.

3.14 DNA Extraction

Microbial DNA from the sample of isolated strains were isolated using following protocol:

Loop full colony was added to 500 μl of Reagent A in Eppendorf tube. 2-3 μL of reagent B and 20 μL of Reaction C was added in it. It was incubate at 95^{o}C for 1Hour. After that 500µL of Reagent D was added in it. It was spun at 13000 rpm for 10 mins. Upper aqueous layer was removed and added in new. 500 μL ice chilled Reagent E was added in it and incubated for 20 mins at room temperature and then spun at 13000 rpm for 15 mins. Supernatant was discarded and add 500

μL of Reagent F to the pellet. The suspension was spun at 8000 rpm for 5 mins. Again the supernatant was discarded and pellet was air dried, 40 μL of Reagent G was added to the pellet and incubate at 600^{o}C for 30 mins. Purified extracted sample was stored at -20^{o}C.

3.14.1 Polymerase Chain Reaction (PCR) Amplification

Pre designed primers were selected from (reference) and ordered on Macrogen. Primers were optimized for annealing temperature through gradient PCR method.

Table 3.3: Primer selected

NAME	**FORWARD**	**REVERSE**	**T(a)**	**PRODUCT SIZE**
16S	CCTAYGGGRB GCASCAG	GGACTACNNGG GTATCTAAT	57^{o}C	465BP

The reaction mixture used and conditions for PCR was:

3.14.2 Reaction Mixture

Table 3.4: Reaction Mixture

S. No	**Reaction Mixture**	**Volume**
01	Master Mix	6.5 μL
02	Forward Primer	1 μL
03	Reverse Primer	1 μL
04	PCR Water	2 μL
05	Template	2.5 μL
	Total Volume	13 μL

Select primers were those that target the conserved regions of the 16S rRNA gene (e.g., universal primers like 27F and 1492R). PCR reaction with the extracted DNA

as the template, using the selected primers and a high-fidelity DNA polymerase was set, typically consisting of denaturation, annealing, and extension steps, for multiple cycles to amplify the 16S rRNA gene.

3.14.3 PCR Product Purification

PCR products were purified to remove primers, nucleotides, enzymes, and other contaminants using a purification kit or method (e.g., PCR purification columns).

3.15 Gel Electrophoresis

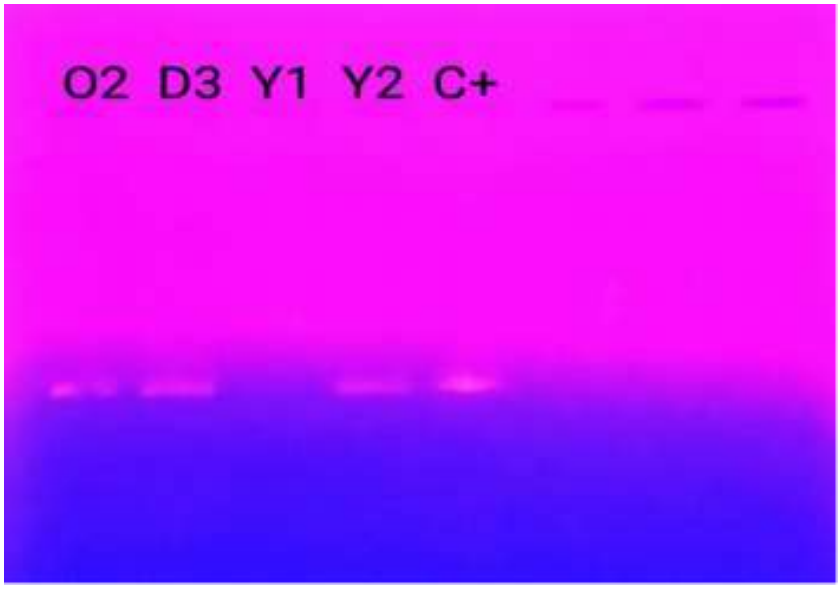

FIGURE 3.2: Gel Electrophoresis (bands of bacterial isolates)

Purified PCR products were run on 1.5% Agarose gel. 30 mL of 1.5% gel is prepared by adding 0.45g of Agarose in 30mL 1 x TBE buffer. Solution was boiled for 1 minute in microwave and cooled down a bit before adding 5 μL Ethidium Bromide. 5 μL PCR purified Samples are then loaded on the gel after adding 2μL loading dye in it.

3.16 16S rRNA Sequencing

The large volume the 16S rRNA sequence, which appears to be most effectively preserved, was used as the first method to investigate the ecology of microorganisms. It is an economical method for surveying bacteria in a population. The conserved, purified PCR strains have been sequenced using 16s rRNA to identify the microbiota linked to the probiotic bacteria.

3.17 BLAST

The NCBI offers a tool called Basic Local Alignment Search Tool (BLAST) that is used to align a sequence with a reference sequence and determine the Materials and Methods similarity index based on matches, mismatches, and gaps.

3.18 NCBI Submission

After the elimination of low-quality sequences, sequences were submitted in the NCBI. The National Center for Biotechnology Information (NCBI) serves as a comprehensive database, facilitating access to a vast collection of biological information. Operated by the National Library of Medicine, NCBI is a crucial resource for researchers, scientists, and the general public.

Chapter 4

Results

4.1 Culturing and Isolation Strains

4.1.1 Culturing on Nutrient Agar Media

Nutrient agar is a common solid growth medium used in microbiology to cultivate a wide variety of microorganisms, such as bacteria, yeast, and molds. It provides essential nutrients that support the growth and reproduction of these microorgan isms [165]. All the plates with serial dilutions (3 serial dilutions: each dilution was replicated thrice for each sample) showed varied growth of bacterial colonies ranging from high concentrations showed dense colonies where as the lowest serial dilution revealed very sparse growth of bacteria (Fig. 4.1). Brine sample collected from the brine cured olives showed no growth on the nutrient agar so it was not further processed.

4.1.2 Isolation on MRS Agar Media

MRS (de Man, Rogosa, Sharpe) media is a specialized culture medium designed for the isolation and cultivation of lactic acid bacteria, particularly species of Lacto bacillus. MRS agar and MRS broth are two common formulations of this medium, and they are used in microbiology laboratories for the enrichment, isolation, and

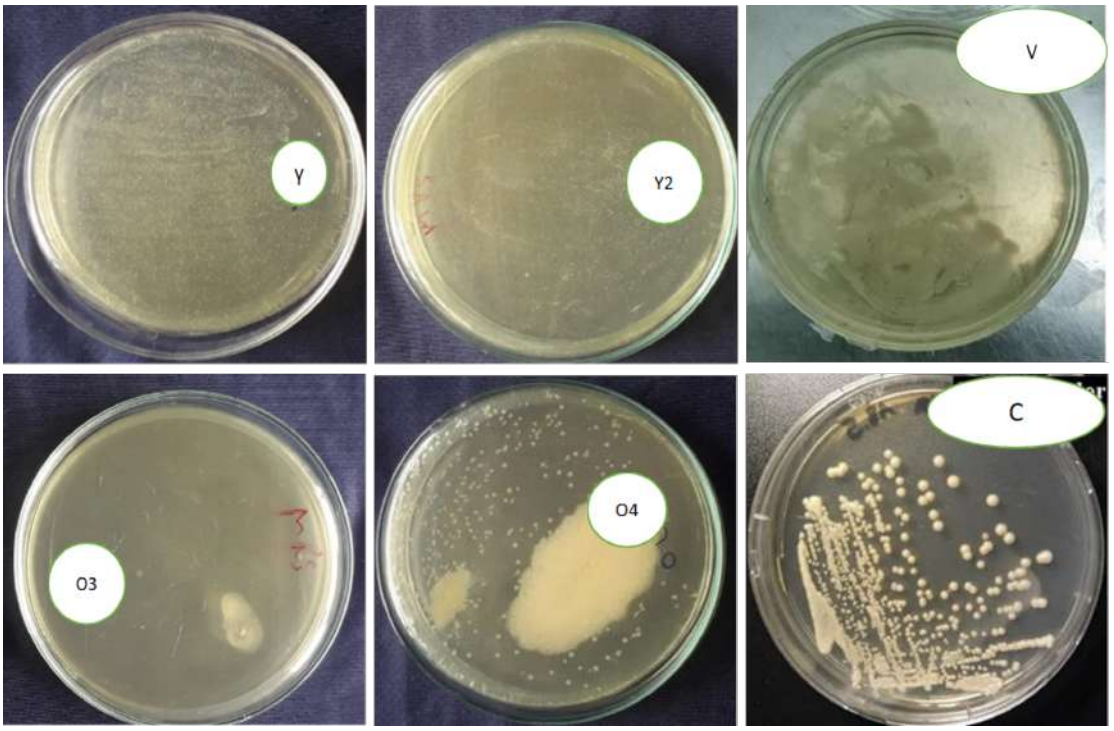

FIGURE 4.1: Growth of bacterial strains from different samples on nutrient agar media. Y = yogurt, O = olives, C = coco powder, V = apple cider vinegar.

Enumeration of lactic acid bacteria from various sources. Lactic acid bacteria, including various species of Lactobacillus, are commonly found in fermented foods, dairy products, and the gastrointestinal tract. MRS media provides an environment that encourages the growth of these bacteria while suppressing the growth of unwanted microorganisms. Different microbes from 4^{th} dilution of all samples were selected and purified by streaking on MSA agar showed the growth as shown in 4.2.

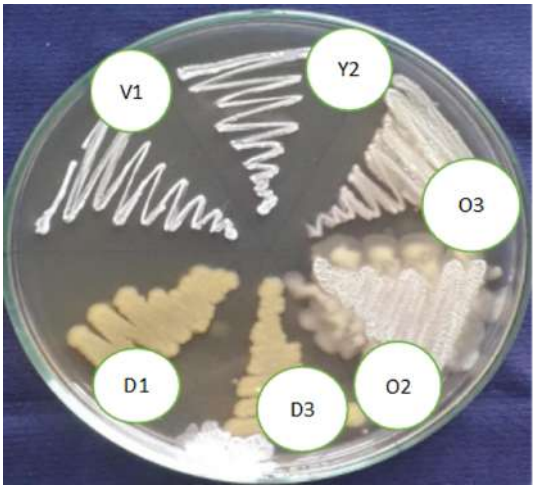

FIGURE 4.2: Different microbes purification

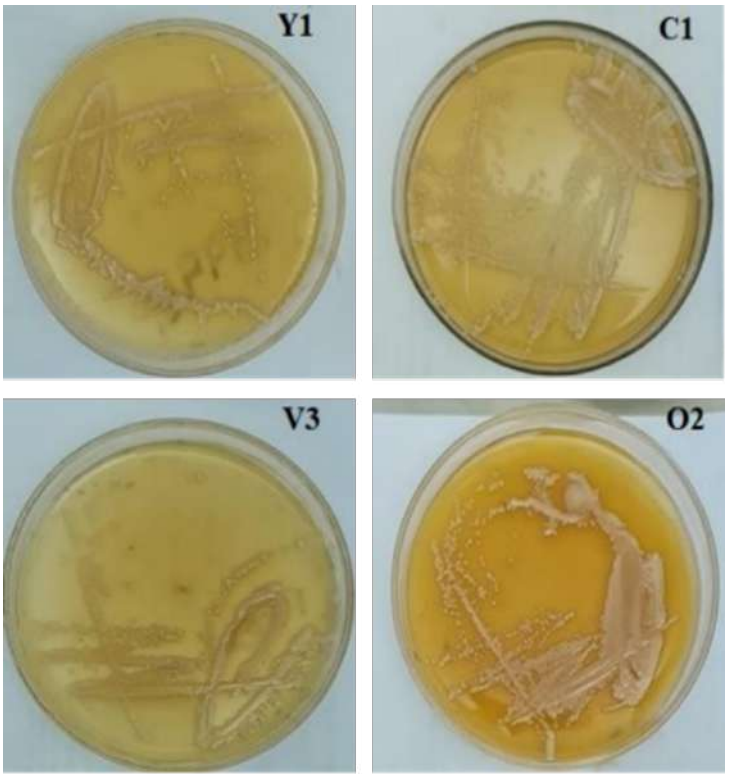

FIGURE 4.3: Isolated strains of bacteria from Commercial Yogurt, Dahi, Brine cured Olives, Apple Cider Vinegar and Coco Powder

4.2 Gram Staining

Gram staining performed for isolated strains indicated that out of 12 strains, 6 strains were gram positive and 8 were gram negative strains whereas among these 8 were rod shape and 06 were circular in shape irrespective of staining results. All 2 strains retrieved from yogurt were gram positive, whereas 2 strains from dahi and vinegar and 1 strain from olives and cocoa powder were gram negative (Table: 4.1).

TABLE 4.1: Gram staining of isolated strains from different foods

Sample	**Sample ID**	**Shape**	**Gram Stain**
Yogurt	Y1	Rods with rounded ends	+ive
	Y2	Rods with rounded ends	+ive
Dahi	D1	Circular	-ive
	D2	Rods	-ive
	D3	Rods	+ive

Sample	Sample ID	Shape	Gram Stain
Brine Cured Olives	O1	Circular	-ive
	O2	Rods	+ive
Apple Cider Vinegar	V1	Circular	-ive
	V2	Rods	-ive
	V3	Rods	+ive
Cocoa Powder	C1	Rods	+ive
	C2	Circular	-ive

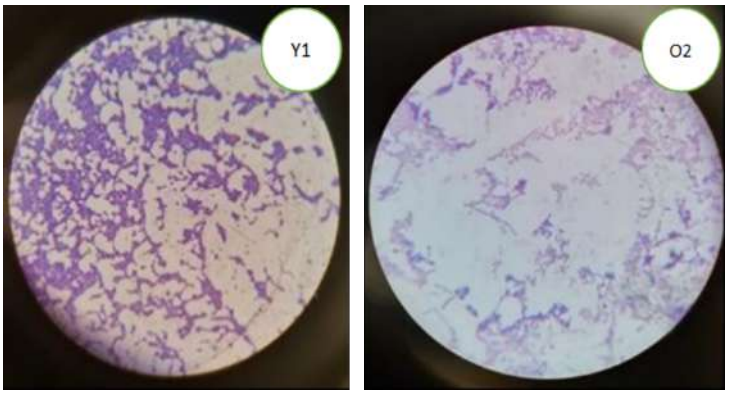

FIGURE 4.4: Gram negative strains of yogurt and olives

4.3 Morphological Characterization of Strains

The morphological inspection involved the examination of six bacterial colonies characterized by rod-shaped morphology and positive Gram staining. The colonies were of white, creamy white or off white in color whereas shape of colonies varies from circular, and filamentous texture was observed with naked eye shown in Table 4.2.

TABLE 4.2: Morphological examination of bacterial strains isolated from different food sources

Sample	Sample ID	Color	Elevation	Form
Yogurt	Y1	White	Flat	Circular
	Y2	Creamy white	Raised	Circular
Curd	D3	Yellow	Flat	Circular
Brine Cured Olives	O2	Creamy white	Flat	Roughly Circular
Apple Cider Vinegar	V3	White	Raised	Circular , Rough, Opaque

Sample	**Sample ID**	**Color**	**Elevation**	**Form**
Cocoa Powder	C1	White	Flat	Circular,Opaque

4.4 Biochemical Characterization

4.4.1 Catalase Test

The catalase test is employed to identify the existence of the catalase enzyme through the breakdown of hydrogen peroxide, resulting in the release of oxygen and water as demonstrated in the reaction: $2H_2O_2 \rightarrow 2H_2O + O_2$. The catalase reaction is recognizable by the swift generation of bubbles. Out of six strains only two strains isolated from commercial yogurt shows catalase negative while strains isolated from dahi (D), olives (O), Vinegar (V) and Cocoa powder (C) were catalase positive (Table 4.3).

TABLE 4.3: Biochemichal charachertization of bacterial strains. Isolated from different food sources.

ID	**Catalase**	**Oxidase**	**Indole**	**Methyl Red**	**VP Test**	**Simmon Citrate**	**Motility-Ornithine**	**Lactic Acid**	**Hemolysis Test**
Y1	-ive	-ive	-ive	-ive	-ive	-ive	+ive	+ive	-ive
Y2	-ive	-ive	-ive	-ive	-ive	-ive	+ive	+ive	-ive
D3	+ive	-ive	-ive	-ive	-ive	-ive	+ive	-ive	-ive
O2	+ive	+ive	-ive	+ive	-ive	+ive	-ive	-ive	-ive
V3	+ive	-ive	-ive	-ive	+ive	+ive	-ive	+ive	-ive
C1	+ive	-ive	-ive	-ive	-ive	+ive	+ive	-ive	-ive

4.4.2 Oxidase Test

The oxidase test is employed for identifying bacteria that produce cytochrome c oxidase, a key enzyme in the bacterial electron transport chain. Notably, all

bacteria with a positive oxidase test are aerobic, indicating their ability to use oxygen as the terminal electron acceptor in respiration. The purple or bluish appearance indicates the presences of cytochrome as a part of their respiratory chain labeled as the positive result. Whereas the reagent not oxidase, appeared colorless with in the test limits, it indicates the absence of cytochrome e as a part of their respiratory chain. All samples cultured on MRS media were oxidase negative as no color change was observed, only **O2** was oxidase positive. A clear colour change (purple) was noticed as shown in (Fig 4.5)

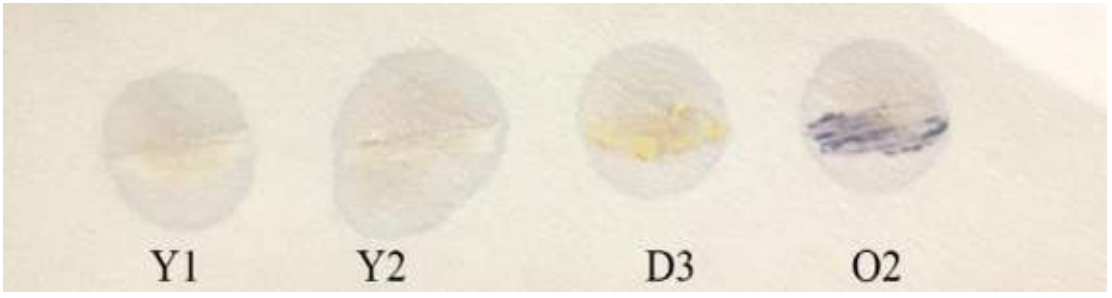

FIGURE 4.5: Oxidase negative bacteria except O2 that was oxidase positive

4.4.3 Indole Test

The indole test assesses an organism's capacity to generate indole through the breakdown of the amino acid tryptophan. Tryptophan undergoes hydrolysis facilitated by tryptophanase, yielding three potential end products. Among these, indole is one, while the remaining products consist of pyruvate and ammonium ion, as indicated in the subsequent reaction. No color change was observed after the addition of Kovac's reagent to the tryptone broth culture. All the six bacterial strains were indole-negative as shown in 4.6.

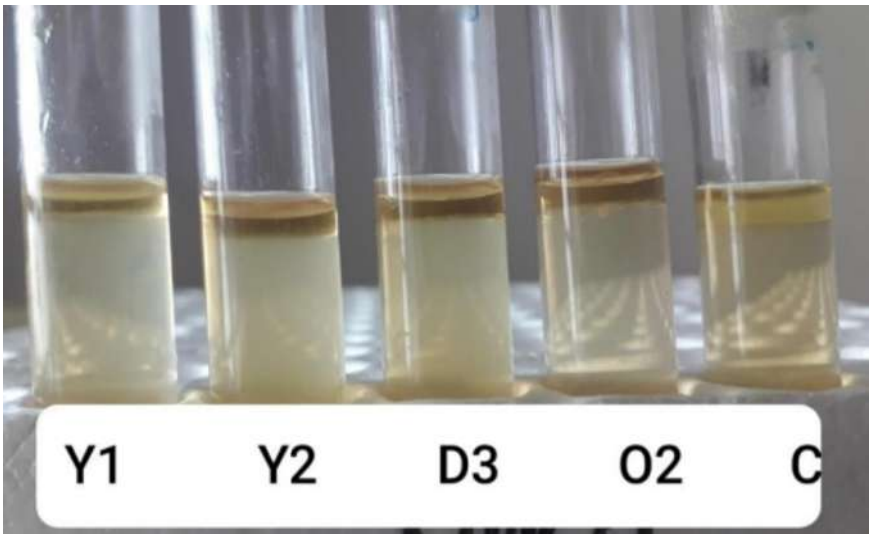

FIGURE 4.6: Indole test showing all strain indole negative

4.4.4 Methyl Red Test

The methyl red test is a diagnostic procedure employed to assess the ability of an organism to perform mixed acid fermentation. During this test, a pH indicator, methyl red, is added to the culture medium after bacterial growth. If the bacteria produce stable acids, causing a drop in pH, the methyl red will turn the solution red. This color change indicates a positive result for mixed acid fermentation, which is often associated with certain types of bacteria, such as *Escherichia coli*. The methyl red test is commonly used in microbiology laboratories to distinguish between different bacterial metabolic pathways based on their acid production capabilities.

No color change was observed after the addition of methyl red indicator to the glucose broth culture of Y1, Y2, D3, V3 and C1, so, they are methyl red negative. While the addition of methyl red indicator to the glucose broth culture of O2 strains resulted in the immediate development of a stable red color so O2 was methyl red positive as shown in figure 4.7.

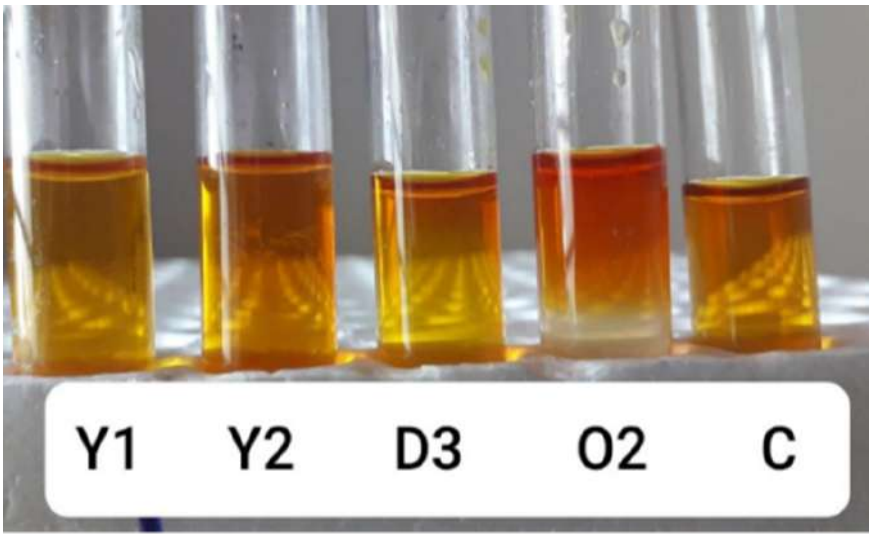

FIGURE 4.7: Results showing Methyl Red negative bacteria except O2 that was Methyl Red Positive

4.4.5 Voges Proskeur Test

The Voges-Proskauer Test is a biochemical test used to determine whether an organism produces acetoin as a metabolic product during glucose fermentation. It is often performed as part of the IMViC (Indole, Methyl Red, Voges-Proskauer, Citrate) test series to identify and differentiate members of the Enterobacteriaceae family, especially Escherichia coli and Enterobacter aerogenes. A reagent containing alpha-naphthol and potassium hydroxide (KOH) is added to the bacterial culture and if acetoin is present, a red color develops due to the formation of a complex. This color change indicates a positive Voges-Proskauer reaction, providing valuable information about the organism's metabolic pathways. The test helps in distinguishing between bacteria that produce a significant amount of acetoin during glucose fermentation from those that do not. There was no detectable change in color following the introduction of reagents A and B to bacterial strains found in commercial yogurt (Y1, Y2), Dahi (D3), olives (O2), and cocoa powder(C1). This VP-negative result suggests the absence of acetoin production, as illustrated in Figure 4.8. Conversely, only vinegar (V3) exhibited a VP-positive reaction, indicating acetoin production upon the addition of reagents A and B, as depicted in the figure 4.8.

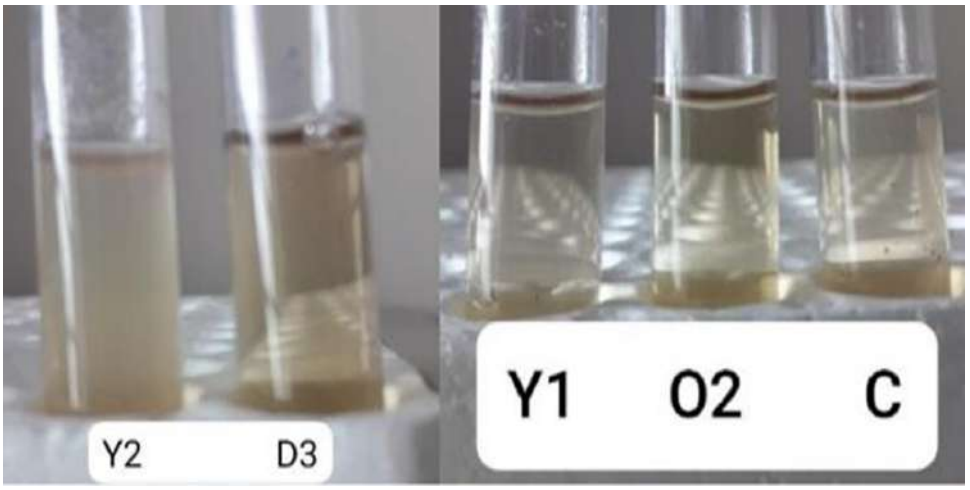

FIGURE 4.8: Results showing Voges Proskeur negative bacteria except V3 that was Voges Proskeur Positive

4.4.6 Simmon Citrate Agar

The Simmon's Citrate Test is a biochemical test used to determine the ability of bacteria to utilize citrate as a sole carbon source for growth. This test is often employed to differentiate between members of the *Enterobacteriaceae* family, particularly *Escherichia coli* and *Enterobacter aerogenes*. The medium contains citrate as the only source of carbon, along with other essential nutrients. If the bacteria possess the enzyme citrate-permease, they can transport citrate into the cell and utilize it for growth.

A positive result in the test is indicated by the alkalization of the agar, usually observed as a color change from green to blue. This change in color is due to the utilization of citrate and the subsequent production of alkaline byproducts.

The Simmon's Citrate Test is part of the battery of tests used for the biochemical characterization of bacteria and aids in the identification of different bacterial species based on their metabolic capabilities.

Robust growth was evident on the Simmon Citrate Agar plate, accompanied by a distinct transition in medium color from green to deep blue in the bacterial cultures derived from olives (O2), vinegar (V3), and cocoa powder(C1). This marked citrate- positive response indicated their ability to utilize citrate, as illustrated in

Figure 4.9. Conversely, no discernible growth or alteration in color was observed on the Simmon Citrate Agar plate for bacterial cultures originating from commercial yogurt (Y1, Y2) and Dahi (D3). These findings establish them as citrate-negative, underscoring their in ability to utilize citrate, as depicted in the figure.

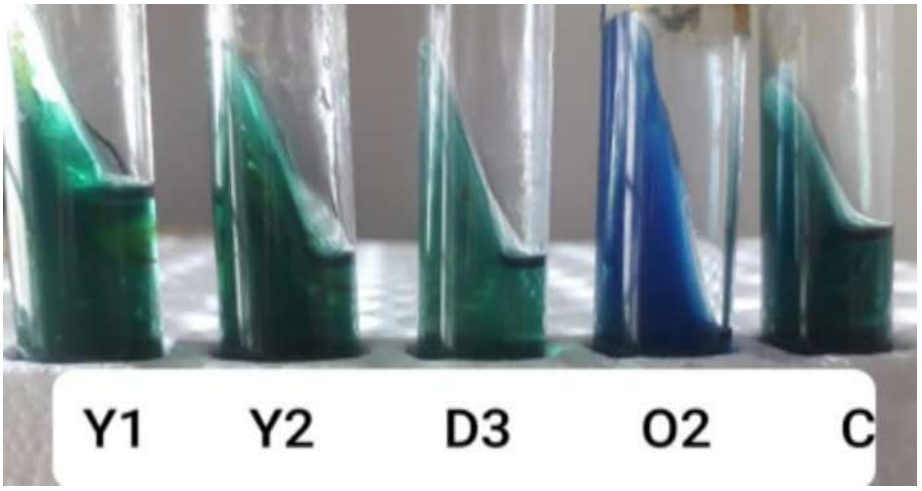

FIGURE 4.9: Results showing Simmon Citrate Agar negative bacteria except O2, V3 and C1 that were Simmon Citrate Agar Positive

4.4.7 Motility-Ornithine Test

Commercial yogurt (Y1), Dahi (D3), and cocoa powder(C1) were identified as motile and ornithine positive. This means that these bacterial isolates exhibited growth away from the point of inoculation on the Motility-Ornithine agar, indicating their ability to move. Furthermore, they were able to utilize ornithine, resulting in the production of alkaline byproducts that caused a color change in the medium, typically turning it dark purple or black.

Vinegar (V3) and Olives (O2), on the other hand, was characterized as non-motile but ornithine positive. This suggests that the bacterial isolate did not display movement away from the point of inoculation in the agar medium. However, it did demonstrate the ability to metabolize ornithine, as indicated by the color change in the medium. Commercial yogurt (Y2) exhibited contradictory characteristics. It was found to be motile, similar to Commercial yogurt (Y1) and Dahi (D3), but it was ornithine negative. This means that Commercial yogurt (Y2) displayed movement away from the point of inoculation on the agar, suggesting motility.

However, it did not utilize ornithine, as evidenced by the lack of color change in the medium (Fig: 4.10).

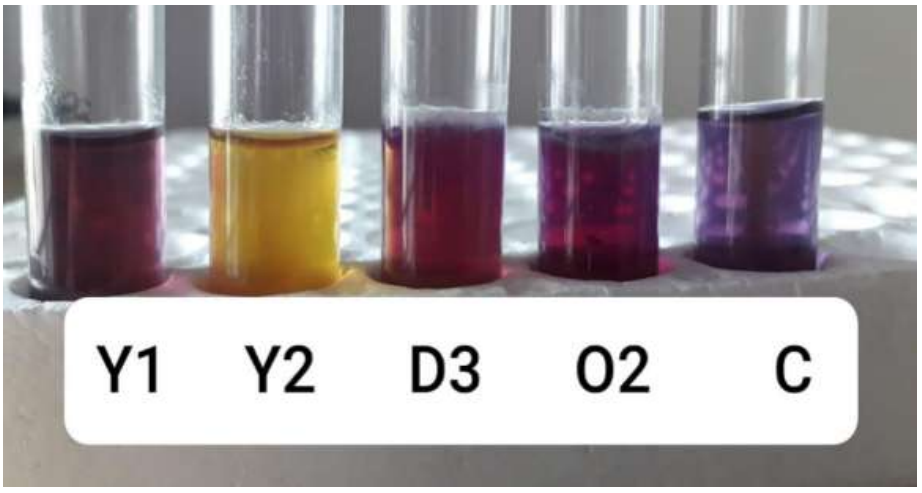

FIGURE 4.10: Y1, D3 and C1 were motile and ornithine positive. O2 was non-motile and ornithine positive. Whereas Y2 was motile and ornithine negative

4.5 Probiotic Activity Test

4.5.1 Lactic Acid Test

Commercial yogurt (Y1, Y2) and vinegar (V3) were lactic acid producing bacteria. It showed that the bacterial strains were capable of producing lactic acid indicating a positive result for lactic acid production on MRS with 1% CaCO3. Dahi (D3), Cocoa powder(C1) and Olives (O2) was found to be negative for lactic acid production. This indicates that the bacterial isolate D3 does not possess the ability to metabolize glucose into lactic acid as shown in (Figure 4.11).

FIGURE 4.11: Y1, Y2 and V3 were lactic acid producing while D2, C1 and O2 were not lactic acid producing strains

4.5.2 Hemolysis Test using Blood Agar

The Hemolysis Test using Blood Agar is a laboratory procedure employed to assess the ability of bacteria to lyse or break down red blood cells. This test is particularly useful for distinguishing different types of bacteria based on their hemolytic activities. Blood agar, typically containing sheep's blood, serves as the medium for this test. All bacterial strains i.e. Commercial Yogurt (Y1, Y2), Dahi (D3), Olives (O2), Coca powder(C1) and Vinegar (V3) were found to be non-hemolytic showed Gamma hemolysis. The bacterial isolates indicates the absence of hemolysis, suggesting that the microorganism being tested does not produce hemolysins and there is no change in the appearance of the blood agar as shown in (Figure 4.12).

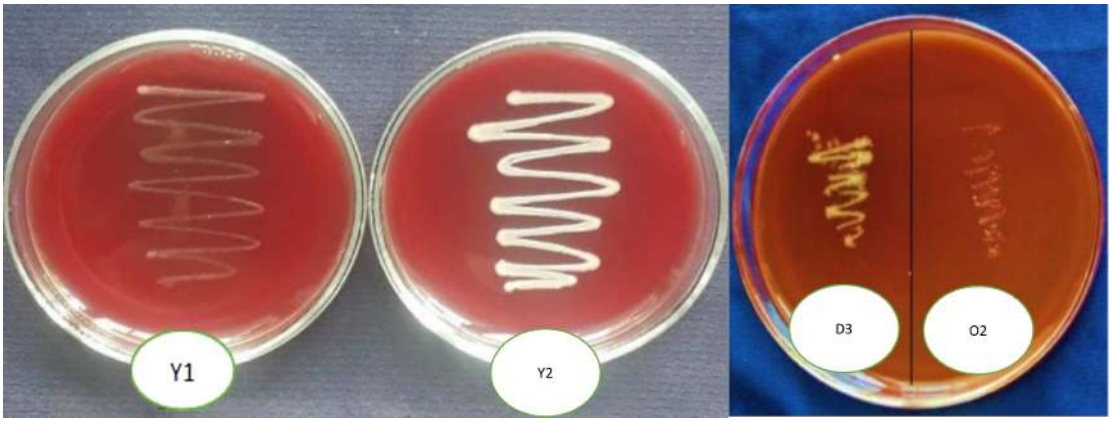

FIGURE 4.12: All bacterial strains i.e. Y1, Y2, D3, O2, C1 and V3 were found to be non-hemolytic

4.6 Antibiotic Sensitivity Test

The antibiotic sensitivity test was conducted to determine the impact of antibiotics against a specific bacterial strain. Probiotics are sometimes recommended alongside antibiotic treatments to mitigate the negative effects of antibiotic therapy on the gut microbiota. Checking antibiotic susceptibility ensures that the prescribed probiotics will not be adversely affected by co-administration with antibiotics.

After incubation for 24 hours at 370°C observation revealed varying antimicrobial activity against antibiotics (Table: 4.4). The strains isolated from the commercial

yogurt (Y1 and Y2) were highly susceptible to all the antibiotics tested whereas strains isolated from olives (O2) were highly susceptible to Trimethoprim/Sulfamethoxazole (30 mm) and no susceptibility to Cefotaxime (0mm). Strain isolated from Dahi (D3) less susceptibility against Trimethoprim/ Sulfamethoxazole.

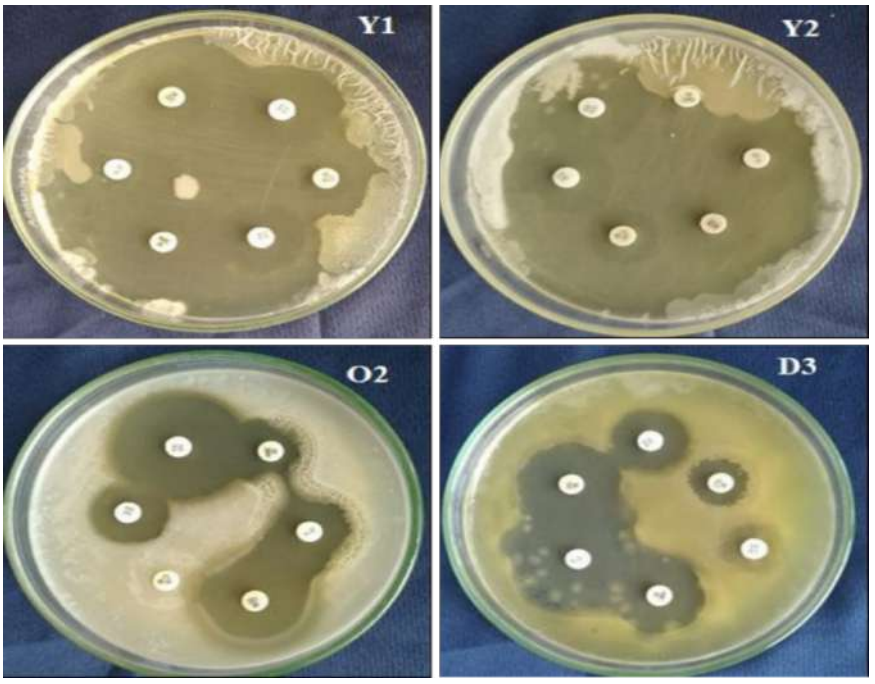

FIGURE 4.13: Antimicrobial Activity against Antibiotics

TABLE 4.4: Antimicrobial Activity against Antibiotics.

Antibiotic	**Y1**	**Y2**	**D3**	**O2**
Amplicine AMC (30 μg)	18mm	17mm	16mm	18mm
Amikacin AK (30 μg)	36mm	20mm	19mm	18mm
Trimethoprim/ Sulfamethoxazole TS (10 μg)	30mm	34mm	10mm	32mm
Doxycycline DO (30 μg)	27mm	20mm	26mm	20mm
Penciline P (30 μg)	30mm	30mm	30mm	18mm
Cefotaxime CTX (30 μg)	15mm	18mm	15mm	0mm

4.7 Molecular Characterization using 16S rRNA

According to samples, identified five bacterial species from different sources such as yogurt, brine cured olives, vinegar and cocoa powder based on their 16S rRNA

gene sequences. These were *Lactiplantibacillus plantarum*, *Lactiplantibacillus plantarum*, *Kurthia gibsonii*, *Bacillus pumilus*, *Acinetobacter baumannii*, *Bacillus subtilis* as shown in table 4.5.The sequencing results of isolated strains received from sequencing company give us the following results as shown 4.5:

TABLE 4.5: molecular characterization using 16sRNA

Sr. No	**Sample ID**	**Scientific Name**	**Accession No.**	**Query Cover**	**% Identity**
1	Y1	*Lactiplantibacillus plantarum*	OR484908	98%	99.5%
2	Y2	*Lactiplantibacillus plantarum*	OR484910	99%	99.70%
3	D3	*Kurthia gibsonii*	OR484904	97%	99%
4	O2	*Bacillus pumilus*	OR484905	100%	100%
5	C1	*Acinetobacter baumannii*	PP098451	99%	99.78%
6	V3	*Bacillus subtilis*	PP098412	100%	100%

Chapter 5

Discussion

Probiotics are live microorganisms that confer health benefits to the host when ingested in adequate amounts. They can modulate the gut microbiota, enhance the intestinal barrier function, produce antimicrobial substances, modulate the immune system and influence the metabolism. The most common probiotics belong to the genera i and *Bifidobacterium*, but other bacteria, such as *Bacillus* and *Enterococcus*, and yeasts, such as *Saccharomyces*, have also been used as probiotics.

Probiotics have been proven to be beneficial promoters of improved aquaculture productivity, and their use as an alternative technique to revitalize the therapeutic channel has gained traction recently [165]. Probiotics are live, non-carcinogenic microorganisms that can lessen pathogen adherence, strengthen the immune system of the host, break down indigestible substances, and enhance the synthesis of vitamins and enzymes [166]. Probiotics are foods or medications that, when consumed, contain living bacteria that enhance an animal's physiological function.

Probiotics have shown promise in both disease prevention and the treatment of host GIT inflammation [167]. The mechanisms that are thought to be responsible for this could include nutrient competition [168], adhesion to the mucosal epithelium of the gastrointestinal tract [169], competitive exclusion of probiotics with intestinal epithelium and mucus to prevent pathogen colonization [170], improved digesting enzymes [171], production of fatty acids, organic acids, and vitamin B12 [172], and elevated feed digestibility. Probiotics have been shown to be an effective

alternative to antibiotics and chemotherapeutants [173] because they compete with adhesion receptors to decrease pathogen colonization through antagonistic activity [174]. Probiotics compete with infections for nutrients, which has antagonistic effects [175]. Probiotics have also been shown to enhance innate immunity in Nile tilapia and olive flounder by inducing blood respiratory burst activities by *Lactobacillus lactis* against Streptococcus iniae and Pseudomonas fluorescens, as well as increasing serum peroxidase and lysozyme.

Kurthia gibsonii, a spore-forming bacterium, exhibits notable resilience in harsh environments, reminiscent of Bacillus subtilis. Known for its robust survival in elevated temperatures and acidic conditions, this strain shows promise as a probiotic. With a focus on digestive health, *Kurthia gibsonii* has been studied for its antimicrobial properties, producing substances that effectively inhibit pathogens like *Escherichia coli* [156]. Notably, this bacterium has demonstrated immune-modulating effects by influencing cytokine production. Although ongoing research continues to unveil specific characteristics, the spore-forming ability, antimicrobial production, and immune modulation of *Kurthia gibsonii* hint at its potential benefits, particularly in gastrointestinal health applications [158].

Bacillus pumilus is a robust spore-forming bacteria that is related to hardy bacteria such as *Bacillus subtilis*. It is a robust organism that can survive in harsh settings, including high temperatures and acids. *Bacillus pumilus* is interesting because it produces antimicrobial compounds that are efficient against diseases like *Escherichia coli* and may find use as a probiotic. Furthermore, the bacteria exhibits immune-modulating properties that impact the generation of cytokines. Although further research is needed to identify specific characteristics, *Bacillus pumilus* may be important for maintaining gastrointestinal health based on its ca- pacity to create spores, produce antimicrobials, and modulate the immune system. Bacillus species (*Bacillus pumilus*) carried in five commercial probiotic products consisting of bacterial spores were characterized for potential attributes (colonization, immune stimulation, and antimicrobial activity) that could account for their claimed probiotic properties [116].

Antimicrobial peptides, bacteriocin, siderophores, lysozyme, proteases, and the generation of organic acids to lower pH could be the mechanism of action of probiotics to perform antibacterial phenomena [176]. Low pH levels and high bile concentrations were found to have an impact on the bactericidal activity of *Bacillus licheniformis* and *B. pumilus* [177]. Extracellular products (ECPs), the primary pathogenic component, inhibited the proliferation and cell density of the pathogen.

Lactiplantibacillus plantarum, a resilient lactic acid bacterium, distinguishes itself with its versatile characteristics. Widely acknowledged for its probiotic potential, this strain excels in promoting digestive health in both humans and animals. *L. plantarum* is renowned for its robust survival in acidic environments, a trait attributed to its acid-tolerant nature. Furthermore, it exhibits antimicrobial activity, producing substances that inhibit the growth of various pathogens, including harmful bacteria like *Escherichia coli*. Its probiotic efficacy extends to immune modulation, influencing the production of cytokines. With ongoing research, *Lactiplantibacillus plantarum* stands out for its acid tolerance, antimicrobial production, and immune modulating capabilities, making it a promising candidate for gastrointestinal well-being [31].

Bacillus subtilis is a sporeforming bacterium that can survive harsh environmental conditions, such as high temperature, low pH and bile salts. It has been used as a probiotic for humans and animals, especially for poultry and pigs. It can produce antimicrobial substances, such as bacitracin, subtilin and subtilosin that can inhibit the growth of pathogens, such as *Escherichia coli*, *Salmonella* and *Clostridium*. It can also modulate the immune system by stimulating the production of cytokines and immunoglobulins. Moreover, it can produce enzymes, such as amylase, protease and lipase that can improve the digestion and absorption of nutrients. *Bacillus subtilis* has been used for the prevention and treatment of diarrhea, constipation, inflammatory bowel disease, irritable bowel syndrome and hepatic encephalopathy [69].

Acinetobacter baumannii is a gram-negative bacterium that is usually considered as an opportunistic pathogen that can cause infections in immunocompromised patients. It is resistant to many antibiotics and disinfectants, making it difficult to treat. However, some strains of Acinetobacter baumannii have been isolated from healthy human feces and have shown probiotic properties in vitro and in vivo. These strains can produce antimicrobial substances, such as acinetobactin and baumannoferrin that can inhibit the growth of pathogens, such as *E. coli*, *Staphylococcus aureus* and *Candida albicans*. They can also adhere to the intestinal epithelial cells and modulate the gut microbiota by increasing the abundance of beneficial bacteria, such as *Bifidobacterium* and Lactobacillus. Moreover, they can enhance the intestinal barrier function by increasing the expression of tight junction proteins and mucins. *Acinetobacter baumannii* has been used for the prevention and treatment of diarrhea, colitis and sepsis in animal models [106].

Chapter 6

Conclusion and Recommendations

Tiny living things in different foods were studied by us using various methods like growing them, checking how they look, and doing tests. A diverse range of bacteria in common foods was revealed by our research. Each type of bacteria had its own unique features, showing that there's a lot of diversity in the tiny world of our daily meals. How they grow, their appearance under a microscope, and their response to antibiotics was examined. Differences in the way these bacteria grow and look on different types of agar were also highlighted by the tests, providing clear evidence of the lively microbial world present in the foods we eat every day.

Methods, including culturing, looking at their shapes and sizes, testing their reactions to antibiotics, and studying their genes, were employed by us. Specific types of bacteria like *Bacillus subtilis*, *Acinetobacter baumannii*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, and *Streptococcus thermophilus* were identified by doing this. Our discoveries can be checked and confirmed by others when this information is shared with a big science database. Moving forward, a solid foundation for future research into the tiny living things in different foods is formed by our study, helping us understand more about what we eat and ensuring our food remains safe.

A variety of techniques were used by us to evaluate the microscopic organisms in our food, and their capabilities were better grasped through these experiments. More about the metabolic capacities of these microscopic creatures was discovered by us through a variety of assays, ranging from simple ones like Catalase and Oxidase to more intricate ones like Indole, Methyl Red, Voges-Proskauer, and Simmon's citrate. Furthermore, the antibiotic resistance of these organisms was investigated by us, and it was found that, in contrast to the strains found in olives, those found in commercial yogurt are readily impacted by specific antibiotics. This emphasizes how crucial it is to understand how these microscopic organisms—which are mostly found in food—can interact with the bacteria in our digestive system while we are taking antibiotics. Put concisely, the diverse antibiotic-responses of these microscopic organisms were demonstrated by our experiments in addition to revealing their capabilities. This emphasizes the need of understanding how, particularly while receiving antibiotic therapy, probiotics in our food may affect our gut flora. Promoting a balanced and healthy gut microbiota requires an understanding of these relationships.

The genes of the microscopic organisms in our food were studied by us through 16S RNA sequencing. Our ability to distinguish between different kinds of bacteria, such as *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Acinetobacter baumannii*, and *Bacillus subtilis*, was significantly aided by this. Our findings can be verified and validated by others if this data is posted on a large scientific database. It also lays the groundwork for more in-depth investigations comparing their genetic data. It not only demonstrates the effectiveness of this genomic technology for recognizing the diversity of these microscopic organisms. We make our results easy to duplicate and access for other researchers by providing reference numbers when our work is submitted to the National Center for Biotechnology Information (NCBI).

A few recommendations for more investigation in the future are made by us. In order to determine whether the found small creatures have any health benefits particularly in terms of improving the nutritional value of our food we need first to delve deeper into their capabilities. This might make a big difference in the

expanding field of functional foods, which are meant to enhance human health. Furthermore, further research into the microorganisms found in particular foods is advised by us to determine potential health effects. The intricate interaction between the microorganisms in our bodies and the food we eat may be clarified by this. A good starting point for further research into the microscopic organisms found in many meals is provided by our work, with its solid techniques and unique findings. The methods we use to assess its quality can eventually be enhanced by this, helping us make our food safer.

In conclusion, investigating the possible health advantages of the discovered microorganisms and examining the ways in which particular foods may affect human health due to the microorganisms they contain is suggested by us. The sound methodology and insightful discoveries of our study are expanded upon by this, opening the door to additional research into the tiny world of our food and assisting in the improvement of safety protocols

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Appendix A

Following are the NCBI submitted strains with their Accession numbers and Fasta format.

D3: Kurthia gibsonii strain TBTS1 16S ribosomal RNA gene, partial sequence

GenBank: OR484904.1

>OR484904.1 Kurthia gibsonii strain TBTS1 16S ribosomal RNA gene, partial sequence.

GGACGTGTACGAGTCTGATGGAGCACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGTTAGGAAATGA
ACGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGT
AAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCA
ACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGATA
GTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACAC
CAGTGGCGAAGGCGACTGTCTGGTCTGTAACTGACACTGAGGCGCGAAA
GCGTGGGGAGCAAACAGGATTAGATAGCGGGGGGGAAAGTCGAC

O2: Bacillus pumilus strain TBTS2 16S ribosomal RNA gene,partial sequence

GenBank: OR484905.1

>OR484905.1 Bacillus pumilus strain TBTS2 16S ribosomal RNA gene, partial sequence

GCCCGGTGGACGAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGTTT
TCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACT
GCTTGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGG
GCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG
GCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGA
GGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGG
AACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAG
CGAAAGCGTGGGGAGCGAACAGGATTAGATACCCCCGGTAGTCCGGA

Y1: Lactiplantibacillus plantarum strain TBTS3 16S ribosomal RNA gene, partial sequence

GenBank: OR484908.1

¿OR484908.1 Lactiplantibacillus plantarum strain TBTS3 16S ribosomal RNA gene, partial sequence.

GTCGTTGGTACGCGTCTGATGGAGCACGCCGCGTGAGTGAAGAAGGGTTT
CGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGT
TCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGT
AAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCA
ACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACA
GTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACC
AGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGT
ATGGGTAGCAAACAGGATTAGATACCGGGTTATTCCGA

Y2: Lactiplantibacillus plantarum strain TBTS4 16S ribosomal RNA gene, partial sequence.

GenBank: OR484910.1

>OR484910.1 Lactiplantibacillus plantarum strain TBTS4 16S ribosomal RNA gene, partial sequence.

GACGTGGGACGGAAGGTCTGATGGAGCACGCCGCGTGAGTGAAGAAGGG
TTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACT
GTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGC
GTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTC
AACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGAC
AGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACAC
CAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAG
TATGGGTAGCAAACAGGATTAGATACCCGGGTTAGTCCGAA