Isolation and Characterization of Probiotic Strain from Traditional Fermented Food

Dissertation submitted in partial fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

by

Anwesha Chowdhury

(Roll No: 151824**)**

Vidushi Sharma

(Roll No:151832**)**

Under the Guidance of

Dr. Garlapati Vijay Kumar

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

DEPT. OF BIOTECHNOLOGY AND BIOINFORMATICS

HP-173234, INDIA

MAY 2019

Isolation and Characterization of Probiotic Strain from Traditional Fermented Food

Dissertation submitted in partial fulfillment of the requirement for the degreeof

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

by

Anwesha Chowdhury

(Roll No: 151824)

Vidushi Sharma

(Roll No: 151832)

Under the Guidance of

Dr. Garlapati Vijay Kumar

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

DEPT. OF BIOTECHNOLOGY AND BIOINFORMATICS

HP-173234, INDIA

MAY 2019

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the B. Tech. thesis entitled **"*Isolation and Characterization of Probiotic Strain from Traditional Fermented Food*"**, submitted by Anwesha Chowdhury (151824) andVidushi Sharma (151832) at Jaypee University of Information Technology, Waknaghat, India, is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

(**Dr. Garlapati Vijay Kumar**)

Dept. of Biotechnology and Bioinformatics,

Jaypee University of Information Technology (JUIT), Waknaghat, Solan.

Himachal Pradesh, India – 173234

Date:

DECLARATION

We hereby declare that the work reported in the B. Tech. thesis entitled **"*Isolation and Characterization of Probiotic Strain from Traditional Fermented Food*"** submitted at Jaypee University of Information Technology, Waknaghat, India, is an authentic record of our work carried out under the supervision of Dr. Garlapati Vijay Kumar, Dept. of Biotechnology and Bioinformatics, JUIT, Waknaghat, HP-173234, India. We have not submitted this work elsewhere for any other degree or diploma.

(**Anwesha Chowdhury, 151824**)

(**Vidushi Sharma, 151832**)

Department of Biotechnology and Bioinformatics,

Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India.

Date:

ACKNOWLEDGEMENTS

It is indeed a great pleasure to thank all those individuals who have, directly or indirectly, contributed and extended their valuable assistance in the progress and completion of this project.

We would like to acknowledge **"Dr. Garlapati Vijay Kumar"** for granting us the opportunity to work on **"Isolation and Characterization of Probiotic Strain from Traditional Fermented Food"**and trusting us with the work and supervising us with finite patience. We are also thankful to Lab Staff of Biotechnology department for their continuous support and encouragement. Also, we would like to thank the officials of Jaypee University of Information Technology (JUIT), Waknaghat, Solan, Himachal Pradesh for their help and cooperation.

We thank **Dr. JitendraVashist**, Dept. of Biotechnology and Bioinformatics for his valuable suggestions and experimental help and **Dr. Sudhir Kumar**, Head, Department of Biotechnology and Bioinformatics, for providing me the opportunity and facilities to carry out the project and for guiding and motivating me whenever required.

We would also like to thank PhD scholars**Ms. Monika Choudhary**for helping in implementation of microbiological testsand**Ms. Swati Sharma** and for providing us all the necessary guidelines, helping me in all the experiments and to clarify all the doubts without any hesitation.

It is our privilege to thank our parents and friends for their constant motivation and encouragement. They were always there to provide an affectionate shoulder at odd times or when unsatisfactory results led to frustrations. I am and will always be indebted by their support and care.

(Anwesha Chowdhury, 151824) **(Vidushi Sharma, 151832)**

TABLE OF CONTENTS

LIST OF SYMBOLS AND ACRONYMS

°C	Degree Celsius
µg/ml	Microgram per milliliter
CFC	Cell free supernatant
GIT	Gastrointestinal Tract
LAB	Lactic acid Bacteria
LB	Luria BertaniAgar
mg/ml	Milligram per milliliter
MHA	Mueller Hinton Agar
Mm	Millimeter
MRS	De Man, Rogosa and Sharpe Agar
PBS	Phosphate Buffer Saline
RPM	Revolutions per minute
µl	Micro-liter
S	Susceptible
I	Inhibitory
R	Resistant

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

There was a time when our food source was used to be whole but nowadays our daily requirement for fruits and veggies has doubled in the past fifteen years. This study mainly focuses on providing improved health benefits using traditional food as probiotic source. Probiotics are live organisms when consumed in sufficient amount improve gut performance and traditional food is persistent in our daily life since forever. These two aspects together can deliver healthy life style without spending loads of cash! Fermented foods are one major part of traditional food chain. From Japan's tofu to Korean kimchee, the list of fermented food items is quite long and it's a fact that in ancient times they were consumed because of their health improving qualities. Here, we are trying to prepare a fermented probiotic item which will not only improve health but also prevent and treat gut related conditions, that too at affordable price.

CHAPTER 1

INTRODUCTION

==========

1.1.Brief insight

The reason for the fast growth of the probiotics in the international market is mainly credited to the increased interest in functional foods along with the rising incidences of digestive and gastrointestinal disorders occurring these days. Functional foods are those food which when consumed provide indispensable benefits to our human body. Fermented food's application in order to provide nutritional value to the food, increase pliability, use them for their medicinal values all of these are the practices that are being performed since ancient times. Fermentation of the food articles as well as beverages is continuously helping to make a notable contribution to the overall patterns of the traditional dietary practices.

'*The processes required for fermented foods were present on earth when man appeared on the scene… When we study these foods, we are in fact studying the most intimate relationships between man, microbe and foods.*' (Selhub *et al*., 2014)

As said in the above quotation, our Paleolithic ancestors always used plenty of opportunities for the consumption of food products (for example- honey, fruits, berries and their juices etc.) which were unknowingly subjected to the process of today what we call natural microbial fermentation. Without having knowledge of different types of the microbes, our ancestors recognized, over time, the palatability, the preserving nature, the analgesic properties, and mentally stimulating or sedating qualities of the fermented foods and beverages (Steinkraus, 1983)

In this way, the stage was somehow formed so that the application of the fermentation could be done with a meaningful purpose so as to provide value in the areas of human nutrition, traditional medicines and culture (ceremonies, and so on) .It is difficult to comment with certainty that when was intentional fermentation began in the earnest ; however, the sophisticated measurements of the chemical contents within the ancient Neolithic vessels indicate that the intentional fermentation of different fruit, rice, or honey beverages has been present in common practice for close to 10,000 years .When across the world agricultural

practices evolved and expanded, the intentional fermentation techniques did too. Apart from the clear references to the alcohol production, it is now quite obvious that the household and artisanal fermentation of cereals, dairy, vegetables, seafood, fish and meat were always a notable part of the ancestral dietary practices.

As the years passed by there were much advancement in the chemical preservation techniques, the ways in which refrigeration was done and also in the transportation methods but still it has not resulted in the abandonment of the fermented food. At least when it comes to the traditional dietary practices that are followed, fermented foods and beverages still remain widespread, currently accounting for approximately one-third of the human diet globally across the world. Scientists are still trying to uncover and unfold the health-promoting properties of these ancestral dietary patterns (e.g.: Mediterranean diet, traditional Japanese diet and hunter-gatherer diets), by extension there is a renewed examination of the fermented foods that are so often a part of such ancient diets. The consumption of fermented food has been providing health benefits in various forms as prepared and consumed in several parts of the world and across distinct cultures.

1.2 Probiotics

Probiotics are the live microorganism which after intake provides health benefits. People usually believe that the microorganisms are always harmful for our bodies but it's not like that at all. Some of these microorganisms have many health benefits and they help our body to function properly.

For example : bacteria that are usually present in our intestines help to digest the food more efficiently , they destroy the disease-causing bad microorganisms and also produce some vitamins .There is a huge number of bacteria's living on and inside our bodies for that matter. Most of the times microorganisms present in these probiotics contain bacteria's that live inside our body naturally. The concept behind the probiotics came into picture in the early 20th century. Nobel laureate Elie Metchnikoff who is also known as the "father of probiotics" proposed the idea that consumption of the beneficial microorganisms could improve people's health. After this Researchers continued to investigate this idea and with the ever evolving researches the term "probiotics" eventually came into existence as we know it today.

The definition used at present was given by the Food and Agriculture Organization of the United Nations World Health Organization, according to which probiotics are redefined as

"live microorganisms which when administered in adequate amounts confer a health benefit on the host."

Prebiotics basically acts as the food for the probiotic bacteria. Prebiotics essentially help in the growth of the beneficial bacteria's over harmful bacteria. Gibson and Roberfroid coined this term in 1995 to describe the food supplements that can't be digestible by the host but are surely capable to provide the beneficial effects by selectively stimulating the growth or the activity of the microorganisms that are located in the intestine.

Synbiotics are the products which contain both the probiotics and the prebiotics together.

All probiotics are not same; they all don't work the same. Suppose, if one type of lactobacillus is helping to treat one condition then it does not necessarily means that another type of lactobacillus will also help to treat the same condition.

Table 1. Microorganisms considered as probiotics **(**Adapted from Holzapfel *et al.*, 2001).

***Lactobacillus* species**	***Bifidiobacterium*species**	**Other lactic acid bacterias**	**Non lactic acid bacteria**
L.acidophillus	*B.adoloscentis*	*Enterococuus faecalis*[1]	*Bacillus cereus var toyoi*[1]
L.crispatus	*B.animalis*	*E.faecium*	*Escherichia coli strain nissle*
L.gallinarium	*B.bifidium*	*Lactococcus lactis*[3]	*Propionibacteriumfreudenreichii*
L.gasseri	*B.breve*	*Leuconostoc*	*Saccharomyces cerevisiae*
L.johnsonii	*B.infantis*	*Mesenteroides*	*S.baulardii*
L.paracasei	*B.lactis*[2]	*Pediococcus acidilactici3*	
L.plantarum	*B.longum*	*Sporolactobacillus inulinus*[1]	
L.reuteri		*Streptococcus thermophilus*[3]	
L.rhamnasus			

[1]Mainly used for animals; [2]Recently reclassified as *B. animalis* subsp. *lactis;* [3]Little is known about probiotic properties.

Probiotics can work inside the body in various ways:

Probiotics might –

- Be useful to support the growth of one type of microorganism.
- Can make intestinal linings resistant towards the harmful microorganisms or produce substances that can prevent the growth of such harmful microorganisms inside the body.
- Maintain the healthy number of good microorganisms and also help them to get stabilize after they were disturbed maybe because of some antibiotic course etc.
- Competitively eliminate the harmful microorganisms from the body.
- Stimulate the immune responses.

There are plenty of strains of the probiotics out there and the healthiest diets will or can incorporate several of these, because they all are capable to provide different benefits. During the recent times the concept of food containing all sorts of medicinal values has been reborn as the functional foods. List of health benefits because of functional foods continue to increase. The gut is an obvious target for the development of these functional foods because it acts as an interface between the diet and all functions of the body. One of the most promising areas for the functional food component's development lies in the usage of prebiotics and probiotics. The two most popular types of the probiotic bacteria are Lactobacillus sp. and Bifidobacteria sp. There are several strains of each of these bacteria, and most of the probiotic supplements usually contain one or more of these bacteria. Apart from the nutritional value, ingestion of these LAB and their fermented food, has indicated to provide a enormous range of benefits such as increased resistance towards malignancy, immune system modulation etc. LAB was first derived from the milk. They are diverse in their presence and can be found variety of the fermented products like milk products, meat, beverages, vegetables and bakery products etc.

CHAPTER 2

REVIEW OF LITERATURE

==========

2.1. Probiotics

Probiotics are live microorganisms mostly bacteria and yeasts that have numerous health benefits. These yeasts and bacteria are especially useful for the digestive system. They are used to keep the gut healthy. Probiotics help to keep the balance between good and bad bacteria so that the body works in a systematic way (Fuller, 1991). Probiotics have numerous health benefits. Some of them are:

- Treating disorders of digestion such as diarrhea; irritable bowel syndrome; and inflammatory bowel disease.
- Treating liver diseases.
- Treatment of common cold.
- Allergic disorders such as hay fever and eczema.
- Tooth decay and gum problems.
- Colic in infants.

Probiotics are known to affect the body. Different probiotics act in its own unique ways. Probiotic help in maintaining a favorable community of microorganisms and also it stabilizes the digestive tract to protect against undesirable microorganisms. Probiotics also help to inhibit the growth of harmful microorganisms. Probiotics also help to restore the natural health of the digestive system after any illness or disease. It helps in restoring the immune system and outcompetes undesirable microorganisms.

The following types of bacteria are classified as probiotics:

Lactobacillus. This is the probiotic strain which is most common. It is found in fermented foods like yoghurt. Lactobacillus also helps with diarrhea and also helps people who cannot digest lactose.

Bifidobacterium. This is present in many dairy products. It supports to cure the irritable bowel syndrome symptoms and some other stomach conditions.

Saccharomyces boulardii is a probiotic and is a type of yeast. It helps in fighting diarrhea and other digestive and intestinal problems.

2.2. General properties of probiotics

For many years microbial cultures were used to ferment foods and for preparing alcoholic beverages. In the past century, different microorganisms were tested for preventing and curing diseases in both humans and animals. Microorganisms have also been added to domestic animal feed as it helps in enhancing growth. The word probiotic was then used based on these observations to describe beneficial micro-organisms by Lilley & Stillwell in 1965. Füller in 1992 defined a probiotic as 'a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance'.

A probiotic isolated from one animal can be less effective in another animal. However, inter-species use of probiotics is common. Probiotics include the ability by adhering to the intestinal epithelial cell lining, survive transit through the gastrointestinal tract, stabilizing the intestinal micro flora, producing antimicrobial substances towards pathogens, a short generation time , antigen toxic properties, a good shelf life in food or powdered preparations and nonpathogenic properties (Goldin, 1998).

Table 2. List of microorganisms those are used as probiotics by humans as well as animals.

***Lactobacillus* species**	***Bifidobacterium*species**	**Others**
L.acidophilus	*B.bifidum*	*Streptococcus thermophiles*
L.rhamnosus	*B.longum*	*Escherichia coli*
L.gasseri	*B.breve*	*Bacillus cereus*
L.rueteri	*B.infantis*	*Clostridium butyricum*
L.bulgaricus	*B.lactis*	*Enterococcus faecalis*
L.plantarum	*B.adolescentis*	*Enterococcus faecium*
L.johnsonii		Yeast
L.casei		*Saccharomyces boulardii*
L.salivaricus		*Saccharomyces cerevisiae*
L.lactis		VSL #3

Probiotics must have the ability to survive in the gastrointestinal tract. It must have the potential ability to be able to survive in the low pH environment and also to be able to survive in the presence of bile- this has been used as a screening technique. Many probiotics have a past history that do not contain any medical illness are now currently used. But there can be differences in species in pathogenicity which is an important condition in the selection of probiotics. In recent times, most probiotics used meet the criteria's listed above. Moreover, some probiotic microorganisms those are in use for many years in the food industry and are used as health supplements do not meet the selection criteria for experiments and hence do not survive in the GIT.

The mechanism of action of probiotics is as follows:

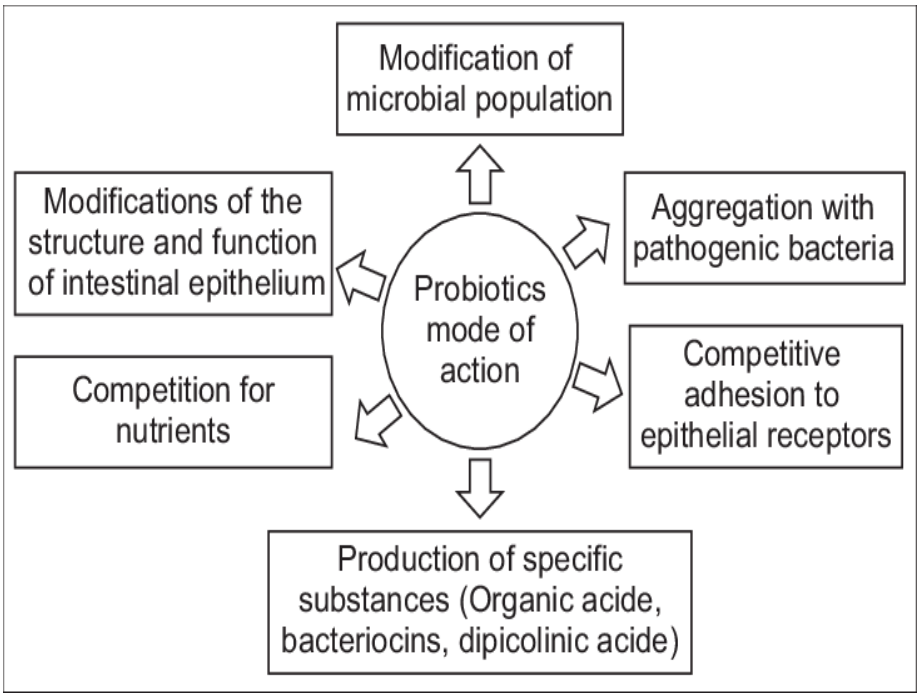

Fig 2.1. Mechanism of action of probiotics (Tiwari *et al*., 2012)

Mechanism of probiotics in pharmaceutical industry:

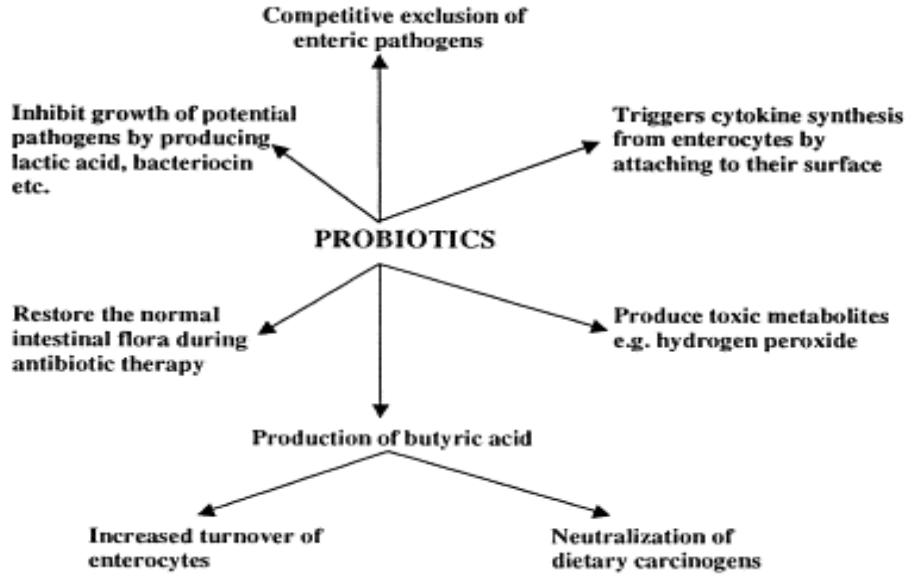

Fig. 2.2. Mechanism of action of probiotics in the pharmaceutical industry (Kaur *et al.*, 2002)

2.3. Pharmacokinetics of probiotics

Probiotics influences physiology through both direct and indirect effects in the gastrointestinal tract. Pharmacokinetics is a very important concept to understand how much probiotics should be consumed, for how long and how often. Pharmacokinetics also helps to correlate the effects of probiotics at the target site. Pharmacokinetics also help to anticipate the effect of other probiotics and it also establishes the concentration at which the probiotics must be present for commercial use. Pharmacokinetics also determines the safety of probiotics at the commercial level (Marteau and Vesa, 1987). Most pharmacokinetic studies are used to determine the fate of probiotics. They are mostly measured in *in vitro*. Some *in vitro* techniques can be used to predict and determine the survivability of probiotics *in vivo* and determines the adhering property to the intestinal epithelium. One of the best techniques to ensure pharmacokinetic of a probiotic in GIT is to measure it *in vivo.*

2.4. Gut health and probiotics

Maintenance of gut health is very important as many physical health problems are found closely associated to improper functionality of the gut. Probiotic helps to modify or alter the composition of micro floras in the gut by introducing beneficial microorganisms. The prebiotic strategy is an alternative way to improve the wellbeing of the gut. It has impact on

the metabolism of intestinal microorganisms. The potential synergy between prebiotic and probiotic, combination of foods containing prebiotic and probiotic are known as synbiotic foods (Manap*et al*., 1997). The prebiotic serves as preliminary growth substance and also introduces special functionality of probiotic into the gut. It acts as an important way to determine the weakness of probiotic surviving in the GIT to reach an allocated site. The correct pair of probiotic and prebiotic should be designed to ensure a successful establishment of probiotic in the gut among large microbial flora.

2.5. Survival of probiotics in gastrointestinal tract

The survival of probiotics depends on many factors such as host factors, intrinsic resistance of probiotics and the vehicle of the probiotic (Marteau *et al*., 1997).

2.5.1 Host factors

The gastric acid secretions constitute a major defense mechanism against the invading microbes. Bile salts also act defense mechanisms. Other than this, mucous, pancreatic secretions and enteric secretions are limited. The gastrointestinal motility also acts a major defense mechanism of the gut. The fate of the probiotics and its equilibrium also depend on microbial interactions which include competition for adhesion sites and substrates and modifications of the environment (by bacteriocins).The immune system can also be involved in the control of the flora of microbes.

2.5.2 Intrinsic resistance of probiotics

For the intrinsic resistance of probiotics to gastric acid and bile, the effect of the age of probiotic culture on the growth conditions and on intrinsic resistance is important.

2.5.3 Survival of ingested probiotics

Various genus and strains of probiotics determine the survivability of probiotics that have been ingested in different sites of the gastrointestinal tract. Some microorganisms are easily destroyed in the stomach whereas some have high rates of survival till feces.

Lactobacillus bulgaricus and *Streptococcus thermophilus* have very poor resistance to acid and are destroyed within few minutes at pH 1. (Pochart *et al*., 1989) observed that in human's concentrations of viable bacteria from yoghurt human were around 10^5Cfu/ml after ingestion of 430 gm. of yoghurt. This survival represented about 1% of ingested bacteria. Using an in

vitro model, it was observed that 26% of ingested *L.bulgaricus* survived in the stomach passage when the pH and gastric emptying of yoghurt was simulated.

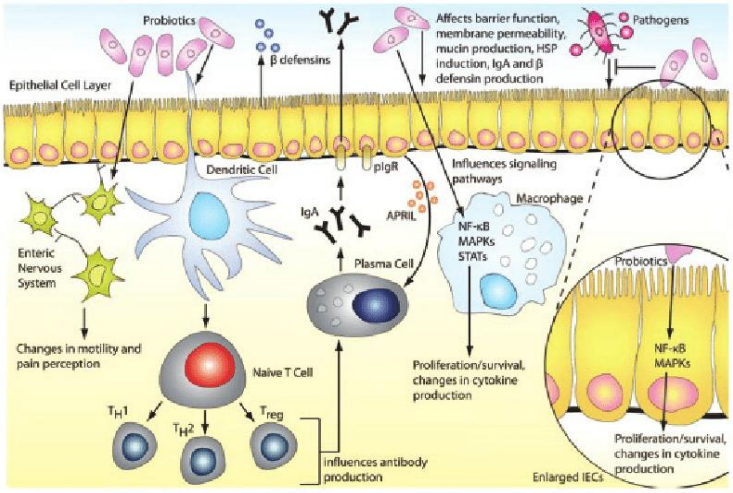

Fig.2.3. Effect of probiotics in the gut microbiota (Hemarajata *et al.*, 2012)

2.6. Benefits of various probiotic microorganisms

The benefits of various probiotic microorganisms are given in the table below.

Table 3. Benefits of variousprobiotic microorganisms

Lactic acid bacteria	**Improve nutritional value of food, control intestinal infections, improve lactose digestion, control cancer and cholesterol levels.**
***Bifidobacterium* sp.**	Help digest dietary fibers, produce vitamins and other essential chemicals, help in treatment of certain diseases and infections.
Streptococcus thermophiles	Stimulates immune system, reduces lactose intolerance, help in treating diarrhea, improves oral health, prevent gastritis.
E.coli	Reduces infectious diarrhea in children, treat constipation and provides normal bowel function.
Yeast	Boost immune system, growth of hair, skin and nails, improve glucose sensitivity and boosts energy.
Saccharomyces boulardii	Treats gastrointestinal problems, treats acne, urinary tract infections and also yeast infections.
Enterococcus faecalis	Boosts cell immune function, improve cell proliferation, elevated fat burning capacity.
Bacillus sp.	Preventing oxidating DNA damage, production of pectinase, amylase, protease and cellulose, prevents cellular damage.

2.7. Colonization of gastrointestinal tract by probiotics

It had been observed that the persistence of some probiotic strains in the feces of subjects for longer periods was different from normal persistence with normal intestinal transit. Colonization is indicated from this phenomenon. Probiotics are mainly excreted within few days after ingestion in the feces. Sometimes this rate can be quicker or at the same speed than a transit marker. Johansson et al. observed colonization occurs of the jejunal and rectal mucosa with lactobacilli.

A soup which contained 19 different strains of lactobacilli was given to healthy volunteers. Then the colonization was searched in the jejunam and rectum on biopsies using probes on days one and eleven after the ingestion of the lactobacillus soup. Two strains of *lactobacillus plantarum* were detected on the 11^{th} day among which one was of human origin and the other was from sour dough. It appeared of important issue of fundamental research in intestinal; ecology and also safety aspects of probiotics.

Thus knowledge on pharmacokinetics is very important to determine the safety of probiotics and it is increasing due to the progresses of in vitro models. There are tools which help in reliable identification of probiotics in complex ecosystems and sampling techniques in the human gut. Strains are different in their survival capacity in different levels in the GIT and also adhesion properties to epithelial cells in vitro.

2.8. Survival of probiotic strain in gastrointestinal tract

Fredua-Agyeman *et al*. (2014) observed that there is no global agreement that describes the minimum number of bacteria required for functionality but probiotics must have a minimum concentration of $>10^6$cfu/ml and total of 10^8 to 10^9 probiotic microorganisms.

The potential effectiveness of probiotics depends on the survival and health of bacteria in probiotic products through processes like storage, manufacture and transit time through stomach and its arrival at small intestine. The transit through the gut induces the most losses of viable microorganisms. There are many barriers of the GI tract which includes pH, composition, volume and buffer capacity of gastric juice; transit time and presence of foods. To mitigate the effects of these factors, the dose and formulation must be optimized.

Gastric juice is one of the most important barriers for pathogens. Under fasting conditions, the pH of healthy human stomach ranges from 1 and 3 which is acidic (Dressman *et al*., 1990; Kalantzi *et al*., 2006). After food intake, the stomach environment varies over the

gastric residence of the meal (Kalantzi *et al*., 2006) and the pH may climb to 6.7 before it declines back to the fasting condition within two hours or less (Dressman *et al*., 1990). The stomach also determines a large variation in emptying times between fed and fasted state which shows that the emptying times in the fasted states are much quicker than the emptying times of the fed state. This shows that liquids which are water based traverse faster than non-water based liquids in the fasting state.

Studies said that half time for emptying of saline from the human stomach is approximately 12 minutes (Granger *et al*,. 1985) and half time for emptying of water is in region of 15-16 minutes (Steingoetter *et al*., 2006). Variability in complexity also occurs such as and proteolytic enzymes and bile salts in the volume of gastric components.

Gastric tolerance assays for new probiotic formulations (Chandramouli *et al.*, 2004; Cook *et al.*, 2011; Cui *et al.*, 2000; Ding *et al.*, 2009; Mokarram *et al.*, 2009) and commercial probiotic formulations (Sahadeva *et al.*, 2011)use buffer solutions in the pH range of 1.2 and 4 or growth media in the same pH for simulating gastric juice.

Mostly plate count technique is used in the gastric tolerance assays to observe the survival of bacteria after exposure. This may have many advantages but lots of inherent problems also arises such as it is time consuming and labor intensive and can result in underestimation or overestimation of viable organisms. Stress occurring during plating also leads to underestimation of organisms. Organisms may be unevenly distributed and may also occur in chains and clumps. Some organisms are also difficult to grow on agar medium rather than liquid medium. Plate counting is not a suitable model for assessment of in vivo growth because plates enable visible counting by designing in such a way to provide a nutrient rich environment. But it can allow any bacteria to survive and recover and allows environment that do not mimic the harsh in vivo condition. In case of probiotics, any surviving bacteria will go on to face transit through small intestine.

2.9. Probiotics and fermented foods

Foods rich in probiotic bacteria are fermented foods such as kimchi and kombucha, tofu, bone broth, pickles, cheese, dark chocolate, miso, soy sauce, yoghurt etc. These foods contain large amounts of lactobacilli bacteria which are beneficial for the digestion of vegetables and grains and also have important probiotic properties. Pickles prepared by lactic acid bacteria (LAB) fermentation have unique flavor and beneficial health effects (Choi *et al*.2013). Lactobacillus helps to enhancing human nutrition by providing nutrients such as

carbohydrates, vitamins and minerals. LAB produces aroma components. In addition, LAB also produces bacteriocins and exopolysaccharides. These help in imparting taste, texture and longer shelf life to products (Leroy and de Vuyst, 2004).

Lactobacillus also helps in detoxifying toxic compounds and degrading mycotoxins which helps to reduce harmful health risks. Lactic acid bacteria are also known as safe additives and Generally Recognized as Safe (GRAS). Lactic acid bacteria help control development of pathogens and spoilage microorganisms in both food and feed (Namasivayam *et al.*, 2014).

Fermented foods containing LAB are used every day in many cultures. One of the food items is curd. Curd is prepared by fermenting milk with a previously made curd which is used as an inoculum. Foods containing probiotic properties fall under functional food. These have a positive effect on health and vitality of human beings. Traditional fermented foods have a very rich source of microorganisms. Among fermented foods, dairy products are considered to contain a very large amount of healthy bacteria (Pavli *et al*., 2016).

Performing in vitro tests on probiotic foods help in focusing on the technological properties of isolated strains with improved characteristics in fermented meat and dairy products and food industry in general.

The consumption of fermented foods influences the nutritional availability, quality and safety of the final products. So, to improve the quality of fermented foods consisting of quality and beneficial health effects, isolating wild type strains from traditional fermented products are used as starter cultures in food fermentation (Owusu-Kwarteng *et al*., 2015). The functional starter cultures should contribute to food quality and safety and also should have organoleptic, nutritional, technological and health advantages. Thus, the strains must be carefully selected as starter cultures help achieving in situ expression of the desired property. This helps to maintain a perfect natural product and still functions as probiotics wherever applicable.

2.10. Probiotic potential and safety

The probiotic strains must have survivability in the passage of the gastrointestinal tract, antimicrobial properties, hemolytic properties and susceptibility to several antibiotics.

Lactobacillus and Bifidobacteria are the most commonly used bacteria in human foods and animal feeds. Several species such as *L.plantarum* and *L.fermentum* have received a

Qualified Presumption of Safety (QPS) status given by European Food Safety Authority (EFSA). For the evaluation of probiotics, probiotic strains must be screened for essential functional properties such as production of antimicrobial compounds, resistance to bile salts and gastric acidity, adherence to gut tissues ability to modulate immune responses, antibiotic resistance, ß-galactosidase activity and production of biogenic amines in in vitro tests. Hemolytic activity and antibiotic resistance should also be absent where safety must be proven in animal models (Belicová *et al*., 2013).

*L.plantarum*isolateshave shown the ability by surviving in the gastric transit and by colonizing the intestinal tract of humans and other mammals. To assess the probiotic potential of strains, the food is a good source of various suitable isolates in finding new probiotic strains for different functional food products. It is traditionally recommendation that probiotic strains must come from humans which is now being mitigated. At present, several probiotic products include nonstarter LAB such as *L.paracasei* and *L.plantarum*. The food and health products which contain probiotic strains are now commercially available.

2.11. Probiotic bacteria from pickles

Monica *et al*., 2017 observed isolated a total of 15 lactic acid bacteria from traditional pickles from Himachal Pradesh. The lactic acid bacteria that were isolated were *Enterococcus faecalis, Lactobacillus plantarum, Pediococcus pentosaceus, Leuconostoc mesenteroides, Lactococcus lactis* and *Enterococcus* sp.

Pickling has been around in India for thousands of years. It is one of the oldest methods of food preservation with the help of fermentation. In pickling, sugar is converted to acids by microorganisms. Pickles help in digestion of grains and vegetables due to the high content of lactobacillus. All pickles have beneficial effects on the body and also unique flavor. Lactobacillus also imparts taste, longer shelf life and texture to the products. Lactobacillus can also reduce health risks by detoxifying toxic compounds.

All the lactic acid bacteria that were isolated had antibiotic susceptibility, ß-galactosidase activity, ß- glucosidase activity, amylase, protease and produced exopolysaccharide activity. Heamolytic activity was not shown in any of the isolates of lactic acid bacteria.

The lactic acid bacteria also showed antimicrobial activity against pathogenic bacteria such as *Escherichia coli, Bacillus cereus, Staphylococcus aureas* etc. This determined that the lactic acid bacteria can be used as probiotics.

2.12. Probiotics and cancer

It was observed that people who took high amounts of fermented products had very high life expectancy. Consuming fermented milk products is associated with human health benefits. Many experimental observations were made where a protective function was indicated of LAB against various types of tumors. (Wollowski *et al*., 2001) stated various factors that within the gut microflora consists of $>10^{11}$ living bacteria/g colon content. LAB belongs to the class of bacteria with many beneficial effects. Lactobacillus plays an important role in helping to retard carcinogenesis by help in influencing metabolic, protective and immunogenic functions in the body. Concentration of lactobacillus might beincreasedin the body after consuming foods rich in probiotics. Prebiotic ingestion is also responsible for the increasing the metabolic activity of Lactobacillus in humans and animals. In animals, Lactobacillus ingestion help in preventing carcinogen induced preneoplastic tumors and lesions. Some studies also observed a much lowered rate of colon cancer in persons who consume fermented milk products.

Raftar (2003) observed that the anticancer activity of probiotics mainly deal with studies related to colon cancer and helps to fight against it. There is no direct expression of cancer inhibition and suppression in humans by taking probiotic cultures in fermented and non-fermented products but there are indirect evidences largely based on experimental and laboratory studies. The anticancer activity of LAB falls into the categories of epidemiological studies, in vitro studies, animal studies and human dietary intervention studies.

Probiotics can be used as a potential complement for treatment because they are inexpensive and associated with very less or no major adverse effects. Some evidences also suggest that probiotics may have a significant clinical impact. In one study of 168 patients evaluated after colorectal surgery for cancer, those who received probiotics had a significantly decreased rate of all postoperative major complications when compared to placebo arm (Hendler *et al*., 2018). But effect of probiotics in cancer patients are currently very less in number and more evidences are needed to determine the situation in which probiotics will be beneficial.

2.13Probiotics and pregnancy

Jarde *et al.,* 2018 observed that about 1.3 – 3.6% use probiotics in the United States and Canada and 13.7% use probiotics in Netherlands. It has been observed that probiotics might help in preventing preterm birth. Intrauterine infection is an important factor and is frequent

during preterm birth. Probiotics may act as etiologic agent during pregnancy. Probiotics displace and kill pathogens by enhancing anti-inflammatory cytokines and also it reduces pH which makes the vaginal environment friendly to the beneficial bacteria. Prebiotics also help the probiotics by help to stimulate their growth and activity.

One study described a data from a Norwegian cohort that women who take a lot of probiotic products have a significant protective effect of preterm delivery. In contrast, two British Charities (Cochrane) reviewed randomized trials which were controlled- the first one being on gestational diabetes and the second on association of probiotics with preterm labor. These tests obtained relative risks between 3 and 4. In both, the results came from the identical trial and it was difficult to examine data from high risk group such as gestational diabetes but these data says that probiotics can produce adverse outcomes which should be addressed in a review which is updated and systematic. Another review showed a study where prebiotics were compared with placebo and not much significant differences were found in the gestational age at birth. So the risks of preterm birth in women taking prebiotics or synbiotics should be analyzed further.

Elias *et al.,* 2011 suggested that the risk of probiotic rich bacteria and virus is low so probiotics should not reach the systemic circulation of fetus and it will not cause any harm. A review was published of more than 1500 pregnant women in 8 randomized control trials of probiotic use where most pregnant women began their probiotic treatment between thirty two and thirty six weeks gestation and they persisted till delivery. This study estimated *Lactobacillus* sp alone and also in combination with *Bifidobacterium* sp. with placebo. The study confirmed that there was no increasingmalformationsor miscarriages; it was expected because use of probiotics mostly occurred from the third trimester and therefore should be unlikely in affecting organogenesis. Moreover, no significant difference in gestational age, birth weight, or the incidence of cesarean section.

As probiotics are systemically and rarely absorbed, they do not transfer into breast milk. One control trial was made and it examined for *Lactobacillus reuteri* levels in 174 colostrum samples after oral supplementation of maternal and infant. Although it was higher than in the placebo group, the occurrence of *L. reuteri* in colostrum was low which was not clinically important. (Abrahamsson *et al.,* 2009) said that the most likely origin of *L reuteri* in colostrum was contamination from external sources in the GIT. There were also no adverse

effects in breastfed infants. In several studies, infants who received probiotic therapy after delivery also showed no adverse reactions and effects.

Studies also suggest that probiotics may also help in postpartum depression as it reduces inflammation and the risk of a variety of diseases. Additional studies are needed, but the antioxidant properties of various probiotics and its ability to increase GABA (gamma-aminobutyric acid) help in improving the mood and alleviating depressive symptoms especially after pregnancy. Probiotics also have positive effect on post-partum weight loss in mothers as well by helping pregnant women lose weight more quickly once they have given birth.

Constipation causes one of the most common health conditions and discomfort in pregnant women. Probiotics act as an additional good nutrition therapy for constipation especially in pregnant mothers. Several studies have showed that yogurt can help in treating constipation but very few studies have been conducted in a pregnant population. In one random trial of 60 women, it was found that three hundred gram of probiotic enriched yogurt *Bifidobacterium* and *Lactobacillus* 4.8 × 1010 [CFU] per day helped in alleviating constipation better than normal yogurt in pregnant women.

Studies using *B. lactis* BB12 and *L. rhamnosus* GG and have shown that atopic dermatitis which is a skin condition where severe skin rashes in up to 15 % of babies can be prevented in 50% of cases if pregnant women intake probiotics during pregnancy and newborn's ingest probiotics during the first six months of birth.

More research is needed to completely understand the action of probiotic supplements on the health of mothers. But much current research prevents clinicians from recommending specific doses for supplementation of probiotics in pregnancy, a review of studies which evaluated the safety of *Lactobacillus* and *Bifidobacterium* and this said that no risk to expectant mothers or newborns occurred.

Probiotics are a protective way to introduce in pregnant women to consume healthy microbes during pregnancy which will provide positive health benefits. These include fermented foods such as yogurt and kefir (providing calcium and vitamin D) and sauerkraut (a source of fiber). Pregnant clients should also stop taking unpasteurized milk and juice products as there can be a greater risk of food borne illness in unpasteurized food products. Prescott said to obtain a healthy life, whole foods and fermented foods are the best. However, "there may be a role for

supplements in some situations as long as they're seen as supplements and not replacements for healthful nutrition," she said.

Dietitians should tailor made recommendations to the individual patients and make recommendations according to their preferences. Pregnant women who fall into the high risk categories are good candidates for probiotic supplements. In fact, consuming fermented and probiotic foods regularly may benefit everybody.

2.14. Probiotics and depression

Depression is a very serious mental disorder which can be recurrent or long lasting. People living with depression may feel anxious, empty, sad, guilty, worthless, irritable, restless or ashamed. There may also be loss of appetite, or overeating, problems with concentrating, or making decisions and in very serious condition can also lead to suicide. Bases on certain studies, about 20% of the population will suffer from some kind of depression in their lifetime. Currently about three fifty million people suffer from depression and the number is gradually increasing.

In 2001, the World Health Organization said that consuming probiotics lead to beneficial effect on the host's body. Probiotics reduce gastrointestinal discomfort, bloating and improve bowel irregularity. Probiotics also enhance immune system, improve skin elasticity,decrease body pathogens, reduce pollen allergy, and protect DNA, proteins, lipids from oxidative damage. They also help in maintaining the intestinal microbiota. Scientists have claimed that the vast assembly of microbiota in the intestines has a great impact on our condition of our minds. The gut microbiota affects the immune system, development of the brain, and also behavior of individuals. Gut microbiota also activates the immune and the central nervous system. Gut microorganisms can produce and deliver neuro-active substances such as serotonin and gamma-amino butyric acid (Huang *et al*., 2016)

Bacteria in the intestines have an important role in the neuropsychiatric conditions such as anxiety or depression. The nervous system of the intestines is a separate one which generates many neurotransmitters including acetylcholine and serotonin. These acetylcholine and serotonin are mainly generated by the brain. The neurotransmitters are promoting motility of the gut, and an imbalance may result in constipation or diarrhea. It is also believed that the brain and the gut are connected with each other. So it is has been stated that anxiety and depression may trigger stomach problems such as abdominal pain or other GI symptoms. It is

also believed that abdominal pain, constipation or other gastrointestinal symptoms may also result in anxiety or depression.

Naseribafrouei *et al*., 2014 performed an analysis of fecal samples from 55 individuals (37 patients and 18 non-depressed controls).It was found that potential correlations between depression and human fecal microbiota. Jiang *et al*., 2015 also performed analysis of fecal samples from 46 patients who suffer from depression and 30 healthy patients and observed that the healthy patients have increased fecal bacteria.

In a double blind placebo controlled and random group study conducted by Messaoudi *et al*., 2011, healthy volunteers took *Lactobacillus helveticus* R0052 and *Bifidobacteriumlongum* or a placebo for 30 days and it was observed that subjects who took the probiotics regularly, their depression scores have decreased and also psychological stress levels . Another study by Mohammadi *et al*., 2015 showed that in petrochemical workers by consuming probiotic yogurt or a probiotic capsule containing multispecies for 6 weeks helped in improving the mental health biomarkers.

The effects of probiotics on human health which includes psychological disorders have recently emerged as an area of interest in neuroscience. Recent studies have suggested that beneficial effects on mood increases with the consumption of probiotics. A poor diet is acts as a risk factor for depression whereas a healthy diet acts as a positive effect on depression. Probiotics may be regulated through diet which may have beneficial effect on preventing and treating depression.

2.15 Probiotics and side effects

Many health benefits are related to probiotics but there can also be some possible side effects. Most of these affect only a small percentage of population. While most people do not suffer from any side effects but there can be temporary intestinal discomfort like gas and bloating. Yeast based probiotics may cause constipation and increased thirst (Ann Pharmacother, 2007). But these side effects decrease after a few weeks of continued usage. So one should always start with a low dosage of probiotics and gradually increase its amount so that the body adjusts to them.

Some probiotics such as kimchi and yoghurt contain biogenic amines. Biogenic amines are substances that form when protein containing foods are fermented by bacteria. The most common amines found are histamine, tryptamine, tyramine and phenylethylamine (Alvarez

etal., 2014).. Amines can cause problems such as exciting the central nervous system, cause headaches, increase or decrease blood flow in people sensitive to amines. It was also observed that low histamine diets reduced headaches up to 75%. A probiotic supplement is a better choice if probiotic foods trigger these symptoms (Liang *et al.*, 2003).

Histamine can be produced by probiotic supplements inside the digestive tract of humans (Pessione, 2012). Due to the rise in histamine levels, Blood vessels may be dilated to bring more blood to the affected area. It can cause redness and swelling and can cause allergic reactions such as itching, runny nose, watery eyes and breathing difficulty. People with histamine intolerance may have problem in breaking down of the histamine in their bodies (Maintz *et al*., 2007). The histamine which is absorbed through the intestinal tract lining into the bloodstream can produce allergic reactions. People with histamine intolerance should select probiotic supplements that do not include any bacteria which produces histamine. Some histamine-producing probiotic strains include *Lactobacillus helveticus*, *Lactobacillus buchneri, Streptococcus thermophiles* and *Lactobacillus hilgardii* (Rossi*et al*., 2011 ;Gezginc *et al*., 2013 ; Joosten *et al*., 1989)

People who have allergies or intolerances should read and understand the labels of probiotic supplements carefully, since they can contain ingredients they may have allergic reactions to. For example, some allergens such as dairy, egg or soy are present in many probiotic supplements. These ingredients must be avoided by people who are allergic as they may produce undesirable effects on the body. Some studies have shown that people who suffer from lactose intolerance can take up to maximum of 400 mg of lactose in medications or supplements (Monatalto *et al*., 2008; Petrini *et al*., 1997). Some supplements may also contain *pre*biotics which are generally plant fibers that cannot be digested by humans but it can be consumed by bacteria as food. The most common types of prebiotics are inulin, lactulose and other oligosaccharides. When a supplement contains both probiotic microbes and prebiotic fibers, it is called a synbiotic supplement. It has been reported that some people experience stomach problems such as gas and bloating when consuming synbiotics (Cummings *et al*., 2002). People who experience these side effects of gas and bloating should select supplements that do not contain prebiotics.

In some rare cases, the probiotics which contain specific bacteria and yeasts can cause infections in some susceptible individuals. People who are at higher risk for infection from

probiotics and prebiotics include people who have suppressed immune systems, venous catheters, hospitalization since a prolonged time or people who may have undergone any recent surgeries. But the risk the infection being developed to a serious state is very low and in clinical studies of the general population, no serious infections have been reported. It is also observed that only one in one million people who intake probiotics containing *Lactobacilli* bacteria develops an infection and it can be treated with traditional antibiotics or antifungals (Borriello *et al*., 2003; Karpa, 2007). Researchers also observed that people who suffer from severe acute pancreatitis should not consume probiotics as it might increase the death risk (Bessilink *et al*., 2008).

Table 4.PROBIOTICS AVAILABLE IN THE INDIAN MARKET.

1) **Yakult – fermented milk drink.**
2) **DanActive from Dannon.**
3) **NOW Foods probiotic-10.**
4) **HealthAid Acidophilus plus.**
5) **Health it Pre- Probiotic Daily Health.**
6) **Vista Nutrition Probiotic Blend.**
7) **Healthy Organ Probiotic.**
8) **Nature Made.**

OBJECTIVES:

- ✓ Isolation of probiotics strain from traditional fermented food
- ✓ Screening for Probiotics activity.
- ✓ Testing of Probiotics strain for health benefits

CHAPTER 3

MATERIALS AND METHODS

==========

3.1. Materials and Media

The following materials were required for performing various tests:

1. **MRS Agar (1 ltr):**
   - Peptone : 10 g
   - Beef extract : 10 g
   - Yeast extract : 5 g
   - Dextrose : 20 g
   - Polysorbate 80 : 1 g
   - Ammonium citrate : 2 g
   - Sodium citrate : 5 g
   - Magnesium sulphate : 0.1 g
   - Manganese sulphate :0.05 g
   - Dipotassium phosphate: 2 g
   - Agar : 12 g
   - Final pH : 6.5±0.2
2. **LB Agar (1 ltr):**
   - Peptone : 10 g
   - Yeast extract :5 g
   - Sodium chloride : 5 g
   - Agar : 12 g
   - Final pH : 6.5±0.2
3. **Mueller Hinton Agar (1 ltr):**
   - Beef extract : 2 g
   - Hydrolysate of casein : 17.5 g
   - Starch : 1.5 g
   - Agar : 17 g

4. **MRS Agar without glucose (500 ml):**
   - Tryptone : 5 g
   - Beef extract : 5 g
   - Yeast extract : 2.5 g
   - Starch : 10 g
   - $K_2HPO_4.3H_20$: 1 g
   - Sodium acetate : 2.5 g
   - Triammonium citrate : 1 g
   - $MgSO_4.7H_2O$: 0.1 g
   - Manganese(II) Sulphate monohydrate : 0.025 g
   - Tween 80 : 0.05%
5. **Gram Staining reagents:**
   - Gram crystal violet
   - Gram iodine
   - Ethanol
   - Gram safranin
6. **PBS Buffer composition (1 ltr):**
   - NaCl : 8 g
   - KCl : 200 mg
   - Na_2HPO_4 :1.44 g
   - KH_2PO_4 : 240 mg
   - pH : 7.4
7. **Antibiotics:**
   - Imepenum
   - Streptomycin
   - Vancomycin
   - Kanamycin
   - Tetracycline
   - Gentamycin
   - Rifampicin
8. **Lugol's Iodine**
   - 0.33% (w/v) iodine
   - 0.66% (w/v) potassium iodine

9. **Blood Agar (Infusion Agar) (1 ltr)**
   - Beef Heart, infusion :500 g
   - Tryptose : 10 g
   - Sodium chloride : 5 g
   - Agar : 15 g
   - Final pH : 7.3±0.2

10. **Food grain**

11. **Plant leaves.**

3.2. Protocols and Techniques

The following protocols and techniques were implemented for carrying out the isolation of probiotic strains.

3.3. Preparation of the fermented food for isolation studies:

- Some amount of food grain was taken and it was boiled at low heat.
- At low heat only, it was let to sit for 15-20 minutes.
- The boiled broth was collected in a container and $3/4^{th}$ water of the broth's amount was added when the broth becomes lukewarm.
- The plant leaves were added to the broth and the water mixture.
- It was kept for fermentation for 3-4 days.
- The microbial growth of the broth was checked on the petri plates as sample.
- If no results were obtained, the previous broth was decanted and fresh broth of the same traditional food was added and $3/4^{th}$ quantity of water was added to the fresh broth's amount.
- It was kept for fermentation for 3-4 days.
- The microbial growth was again checked in the petri plates.
- The strain was subculture into MRS broth for future use.

3.4. Tests performed

3.4.1. Gram Staining

The bacterial culture was taken on a slide and it was heat fixed. A primary stain ,i.e, crystal violet stain was applied on the heat fixed smear of bacterial culture. Crystal violet stains all cells blue or purple. The slide was gently washed in a gentle stream of tap water for few seconds. Grams iodine was applied next and left for 1 minute. Then the slide was gently washed for a few seconds under tap water. Ethanol was then used to wash the slide for 10 seconds until the slide ran clear. Counter-stain safranin was then applied and left for 30 seconds. The slide was then washed for a few seconds under tap water. The results of the staining were observed under light microscope.

3.4.2. Determination of curdling activity

Curdling potential of the sample was checked at two different volumes to see at what volume curdling activity is better.

- In flask 1, 15 ml sample was added to 60 ml lukewarm milk.
- In flask 2, 15 ml buttermilk was added to 60 ml lukewarm milk.
- In flask 3, 30 ml sample was added to 45 ml lukewarm milk.
- In flask 4, 30 ml buttermilk was added to 45 ml lukewarm milk.
- The curdling activity was checked after 48 hours.

3.4.3. Resistance to low pH

The resistance of the strains to low pH was examined. The bacterial culture was first harvested overnight in MRS broth. The bacterial cells from the overnight culture were then harvested at 10000 rpm for 5 minutes at 4°C. The cells were then washed with PBS buffer at pH 7.2 twice by centrifugation. The cells were then resuspended in PBS buffer and the pH was adjusted to pH 3, pH 4, pH 6 and pH 7. It was then enumerated on MRS agar. The plates were then incubated at 37°C for 4 hours.

3.4.4. Antibiotic susceptibility

The antibiotic susceptibility was performed by using antibiotic discs of Imepenum, Gentamycin, Rifampicin, Streptomycin, Vancomycin, Kanamycin and Tetracycline. First, inoculation was made with the broth culture diluted to match 0.5 McFarland Turbidity Standard. The media used for this experiment was Mueller-Hinton Agar. Under aseptic techniques, using a sterile swab the broth culture of the specific organism was taken. Then

using the swab, the Mueller-Hinton agar plate was streaked to obtain uniform growth. The plate was rotated 5 times and it was then streaked in that direction. Then the antibiotics were dispensed into the plate using flame sterilized forceps. Plates were incubated overnight at 37°C.

3.4.5. Amylase activity

The bacterial culture was harvested overnight. It was then point inoculated on modified MRS agar without glucose. Starch was used instead of glucose. Inoculated plates were then incubated anaerobically for 48 hours at 37°C. The culture plates were then covered by spraying Lugol's Iodine to detect starch hydrolysis. The production of clear halo zones around the colony was taken as indication of starch hydrolysis, i.e, production of alpha-hydrolysis.

3.4.6. Antimicrobial activity

Antimicrobial activity was determined against 2 strains (ATCC 19606 and ATCC 25922) - *Escherichia coli* strain (ATCC 25922) and *Acinetobacter baumannii* strain (ATCC 19606). Fresh overnight bacterial culture was taken. The culture was harvested by centrifugation at 10000 rpm for 15 minutes at 4°C. The cell free supernatants (CFC) of the bacterial strain were tested for antimicrobial activity using well diffusion assay. Initial inoculum was incorporated into the MHA and CFC was also transferred into holes drilled into the agar. The plates were incubated at 37°C and the growth free zone was determined. 20 µl of the culture and supernatant was transferred onto paper and 100 µl of the culture and the supernatant was transferred onto the wells.

3.4.7. Haemolytic Activity

Overnight grown culture was used to perform the haemolytic activity. Blood agar was prepared and the overnight culture was streaked into the blood agar plates. The plates were incubated at 30°C for 24 hours. The reaction can be observed by partial hydrolysis of RBC and α-haemolysis or greening zone, ß-haemolysis or clear zone and ɤ-haemolysis or no reaction.

CHAPTER 4

RESULTS AND DISCUSSION

==========

4.1. Gram staining result.

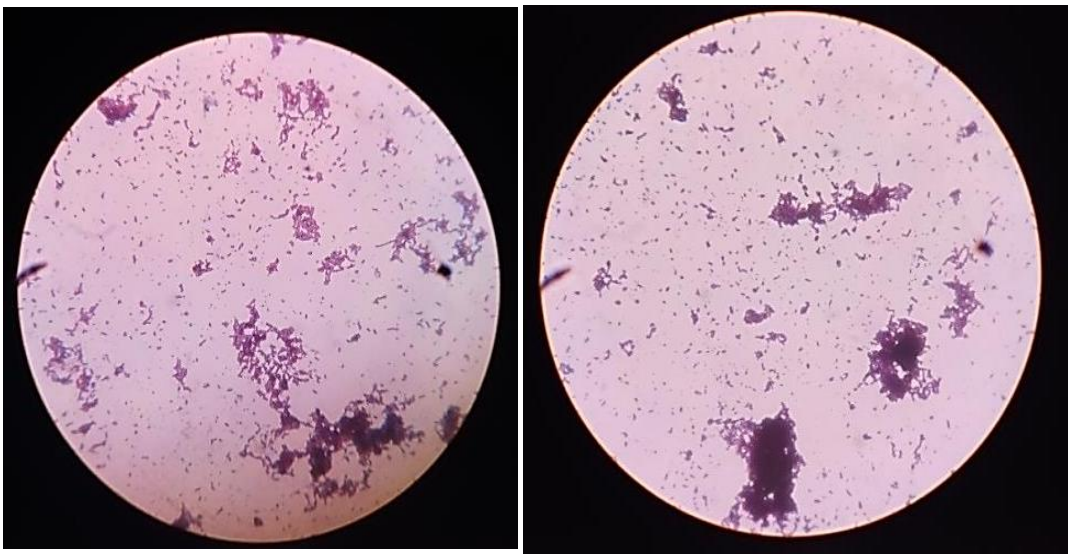

Fig.4.1. Gram staining results showing gram positive, rod shaped bacteria.

Individual colonies were identified and selected for gram staining. The gram staining showed that the bacteria are gram positive in nature and it is rod shaped. The bacteria are identified to be of *lactobacillus* sp. The bacteria is stained violet because of thick layer of peptidoglycan in their cell walls which help in retaining the crystal violet stain

4.2.Curdling activity result.

Fig.4.2. The curdling activity was best observed in 30 ml of sample after 48 h

The curdling activity in 15 ml sample and 60 ml milk was not found to be very prominent even after 48 h. The control containing buttermilk and milk showed curdling activity after 48 h.

The curdling activity in 30 ml sample and 45 ml milk was found to be prominent after 48 h. The control containing 30 ml buttermilk and 45 ml milk also showed curdling activity.

The curdling activity thus found to be best in 30 ml of sample after 48 h

4.3. Resistance to low pH result.

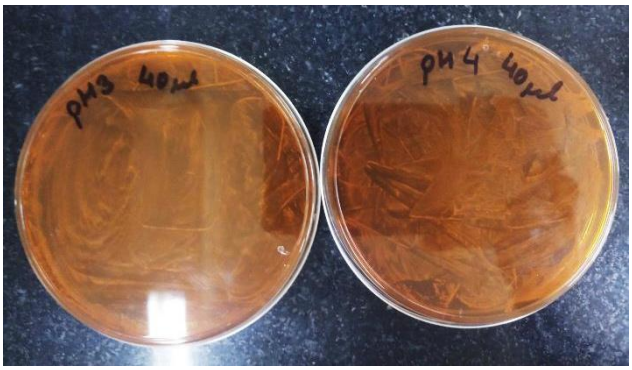

Fig.4.3. pH test result after 4 hours at pH 3 and pH 4.

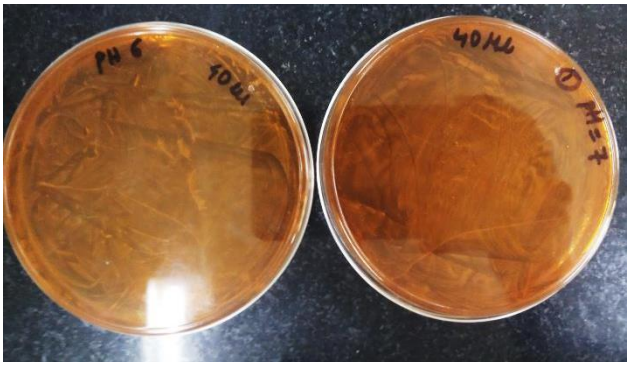

Fig.4.4. pH test result after 4 hours at pH 6 and pH 7.

After adjusting the pH at pH 3, pH 4, pH 6 and pH 7, the plates were incubated at 37°C for 4 hours. Bacterial growth was seen in all the pH tested for. Lactobacillus strains were able to maintain their viability when exposed to low pH. The pH of the stomach is mainly between 1.5 to 3.5. So, as the bacteria could grow at pH 3 and pH 4, it can survive the acidic conditions of the stomach. Very low pH guaranties the isolation of the acid tolerant strains. In vitro studies tell to select the strains exposed to pH adjusted PBS. Foods containing high level of fat and also containing certain proteins help in providing additional protection to the bacteria from gastric acid. This increases the survival to gastric transit.

4.4. Antibiotic susceptibility Result.

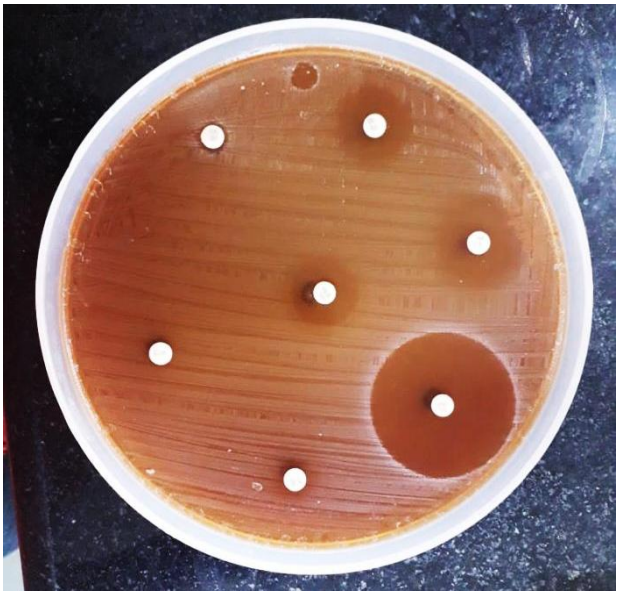

Fig.4.5. The antibiotic susceptibility test result of the culture.

Table 5. List of antibiotics and the zone diameter imperative criteria for the culture.

Antibiotics	**Diameter of zone of inhibition(mm)**	**S/I/R**
Imepenum	42	S
Gentamycin	20	S
Rifampicin	25	S
Streptomycin	0	R
Vancomycin	0	R
Kanamycin	0	R
Tetracycline	18	S

The culture was found to be susceptible to all the antibiotics tested except Streptomycin and Vancomycin.According to Zone diameter Interpretative criteria given in ICMR SOP 2015 it was found that Streptomycin, Vancomycin, Kanamycin were resistant towards the bacterial culture and Imepenum, Gentamycin , Rifampicin, Tetracycline was susceptible towards the bacterial culture. None of the antibiotics showed inhibitory action towards the bacterial culture.

The antibiotic susceptibility result of ATCC 19606 (*A.baumnnii*) and ATCC 25922 (*E.coli*):

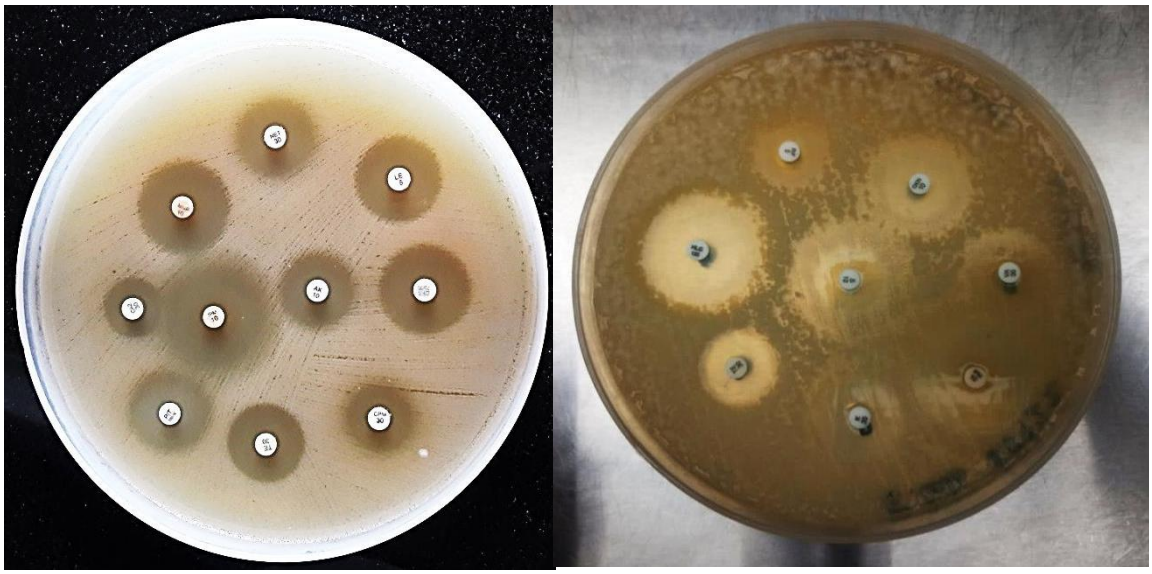

Fig. 4.6. The antibiotic susceptibility result of ATCC 19606 (*A.baumannii*) and ATCC 25922 (*E.coli*).respectively.

Table6. List of antibiotics and zone diameter imperative criteria antibiotic susceptibility test of ATCC 19606 (*A.baumannii*).

Antibiotics	**ATCC 19606(*A.baumannii*)**	
	Diameter(mm)	**S/I/R**
Ceftazidime (CAZ)	16	I
Levofloxacin (LEV)	23	S
Amikacin (AMK)	18	S
Piperacillin-tazobactam (PTZ)	19	I
Tetracycline (TET)	20	S
Cefepime (FEP)	17	I
Netilmicin (NET)	20	-
Imipenem (IPM)	26	S
Meropenem (MEM)	24	S
Cefoperazone-sulbactam (CSL)	24	S

Table7.List of antibiotics and zone diameter imperative criteria antibiotic susceptibility test of ATCC 25922 (*E.coli*).

Antibiotics	**ATCC 25922 (*E.coli*)**	
	Diameter(mm)	**S/I/R**
HLG	22	S
Rifampicin	16	S
Gentamycin	20	S
Vancomycin	17	S
Kanamycin	16	S
Tetracyclin	14	I
Streptomycin	23	S
Imepenum	22	S

4.5. Amylase test result.

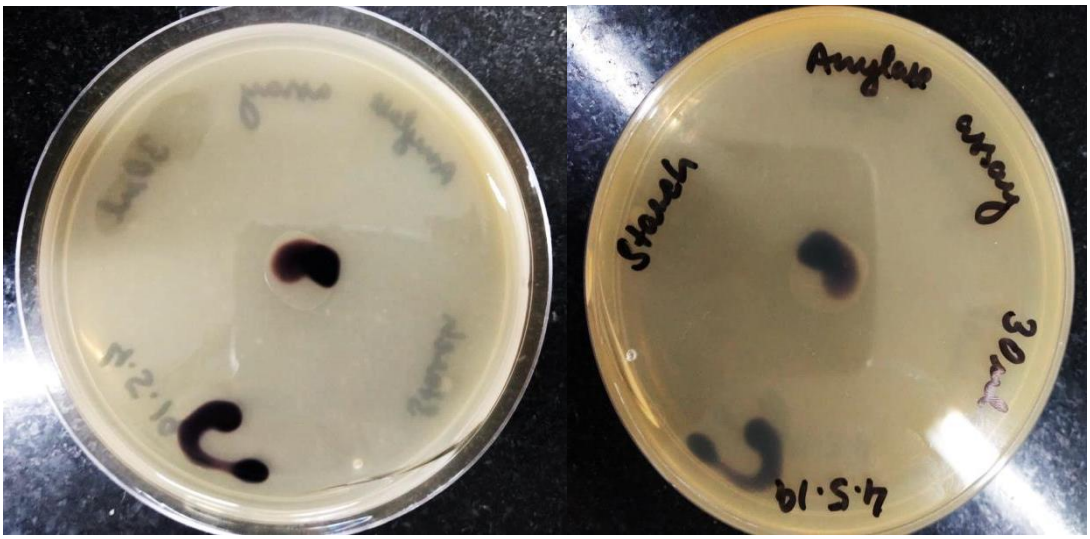

Fig.4.7. Amylase test result.

After spraying Lugol's iodine over the sample, starch stained into blue-black colour which means starch was undegraded. If we would have obtained clear halo zone around the tested colony, it would have indicated that starch was degraded and α-amylase was produced.

4.6. Antimicrobial activity result.

Fig.4.8. Antimicrobial activity result.

20 µl of the cell culture and the supernatant showed prominent anti-microbial action. But when the concentration was increased to 100 µl, the culture and the supernantantshowed much better anti-microbial action and inhibited the growth of the pathogenic strains tested by well-diffusion assay. This showed that no bacteriocin like action exists. Thus the culture and the supernatant were able to efficiently kill the pathogenic strains.

4.7. Haemolytic activity result.

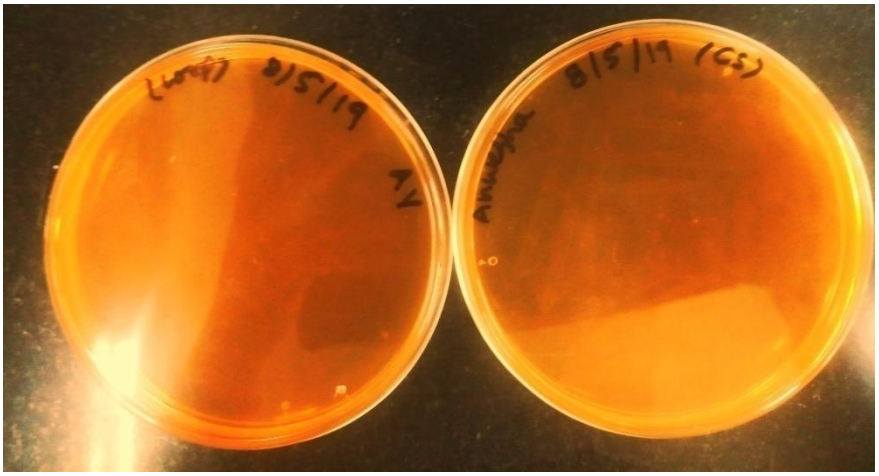

Fig. 4.9.Haemolytic test result.

Haemolytic activity must be absent for safety prerequisite for selecting a probiotic strain. The isolated neither showed α-haemolysic and ß-haemolysis when grown in blood agar. The isolates only showed ɤ-haemolysis which is no haemolysis after incubation at 30°C for 24 hours under anaerobic conditions.

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTS

==========

CONCLUSION

- The screened the lactobacilli strain showed
  - positive result in gram staining,
  - resistant to low pH,
  - showed curdling activity,
  - could kill the pathogenic bacteria,
  - showed antibiotic susceptibility and did not show any haemolytic activity.
- Thus, the culture can be used as a probiotic product as it can be easily prepared in home and is a cost effective option. These qualities give the culture good probiotic power.
- Thus, the culture is a viable option for a formulation of a probiotic product.

FUTURE PROSPECTS

- Identification and characterization study of the isolated Lactobacillus sp.
- Formulation and pharmacokinetic study of the probiotic compound
- Probiotic enrichment of health drinks, curd and its sensory analysis

REFERENCES:

Abrahamsson TR, Sinkiewicz G, Jakobsson T, Fredrikson M, Björkstén B (2009) Probiotic Lactobacilli in Breast Milk and Infant Stool in Relation to Oral Intake During the First Year of Life. Journal of Pediatric Gastroenterology and Nutrition 49: 349-354.

Alvarez-Olmos MI ,OberhelmanRA (2001) Probiotic Agents and Infectious Diseases: A Modern Perspective on a Traditional Therapy. Clinical IinfectiousDiseases 32: 1567-1576.

BelicováA, Mikulasova M, Dušinský R (2013) Probiotic Potential and Safety Properties of Lactobacillus plantarum from Slovak Bryndza Cheese.BioMedResearch International. 2013: 1-8.

Besselink MG, van Santvoort HC, Buskens E, Boermeester MA et al., (2008) Probiotic prophylaxis in patients with predicted severe acute pancreatitis: A randomised, double-blind, placebo-controlled trial. Lancet 371(9613): 685-696.

Borriello PS, Hammes PW , Holzapfel W ,Marteau P ,Schrezenmeir J, Vaara M, ValtonenV. (2003) Safety of Probiotics That Contain Lactobacilli or Bifidobacteria. Clinical Infectious Diseases36: 775-780.

Chandramouli V, Kailasapathy K, PeirisP , Jones M. (2004). An improved method of microencapsulation and its evaluation to protect Lactobacillus sp. in simulated gastric condition.Journal of Microbiological Methods56: 27-35.

Choi JY , Kim JS , Ingale SL , Kim KH , Shinde PL, Kwon IK,Chae BJ (2011a) Effect of potential multimicrobe probiotic product processed by high drying temperature and antibiotic on performance of weanling pigs. Journal of Animal Science 89: 1795–1804.

Choi JY, Shinde PL, Ingale SL , Kim JS , Kim YW, Kim KH , Kwon IK,Chae BJ (2011b) Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. Livestock Science 138: 144–151.

Cook MT ,Tzortzis G , Charalampopoulos D, Khutoryanskiy VV (2012) Microencapsulation of probiotics for gastrointestinal delivery. Journal of Control Release 162: 56–67.

Cui JH, Goh JS, Dim PH, Choi SH, Lee BJ (2000) Survival and stability of bifidobacteria loaded in alginate poly-l-lysine microparticles. International Journal of Pharmaceutics 210: 51-59

Cummings JH, Macfarlane GT (2002) Probiotics, infection and immunity. Current Opinion Infectious Diseases 15:501–506.

Ding J, Zhou ZM, Ren LP, Meng QX (2008) Effect of Monensin and live yeast supplementation on growth performance, Nutrient digestibility, carcass characteristics and ruminal fermentation parameters in lambs fed steam-flaked corn-based diets. Asian-Australasian Journal of Animal Science 21: 547- 554.

Dressman JB, Berardi RR, Dermentzoglou LC et al., (1990) Upper gastrointestinal (GI) pH in young, healthy men and women. PharmaeuticalResearch 7: 756-761.

EliasJ, BozzoP, EinarsonA(2011)Are probiotics safe for use during pregnancy and lactation?.CanadianFamily Physician 57: 299-301.

Fredua-Agyeman M, Gaisford S (2015) Comparative survival of commercial probiotic formulations: tests in biorelevant gastric fluids and real-time measurements using microcalorimetry. Beneficial Microbes 6 (1): 141 - 151.

Gezginc Y, Akyol I, Kuley E, Ozogul F (2013)Biogenic amines formation in Streptococcus thermophilus isolated from home-made natural yogurt.Food chemistry138: 655-662.

Goldin BR(1998) Health Benefits of Probiotics. The British Journal of Nutrition80: 203-207.

Hemarajata P, Versalovic J (2013)Effects of probiotics on gut microbiota: Mechanisms of intestinal immunomodulation and neuromodulation. Therapeutic Advances in Gastroenterology6: 39-51.

Hendler R, Zhang Y (2018) Probiotics in the Treatment of Colorectal Cancer.Medicines (Basel) 5:101.

http://www.renewlife.ca/blog/10-reasons-supplements-daily/

https://wellnessmama.com/8487/traditional-foods/

https://www.health.harvard.edu/staying-healthy/the-benefits-of-probiotics

https://www.intechopen.com/books/probiotics/encapsulation-technology-to-protect-probiotic-bacteria

https://www.mcgill.ca/cine/research/food/benefits

Huang R, Wang K, Ho J (2016) Effect of Probiotics on Depression: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Nutrients8(8): 483

Ito M , Kim Y , Tsuji H, Kiwaki M, Nomoto K, Tanaka R, Okada N ,Danbara H. (2010) A practical random mutagenesis system for probiotic *Lactobacillus casei* using Tn 5 transposition complexes.Journal of Applied Microbiology109(2):657-66.

Jarde A, Mikhael AM, Moayyedi P, Stearns J, CollinsMS, Beyene J, Mcdonald S (2018) Pregnancy outcomes in women taking probiotics or prebiotics: A systematic review and meta-analysis. BMC Pregnancy and Childbirth18(1):14.

Jiang M, Zhang F, Wan C, Xiong Y, ShahPN, Wei H, Tao X (2016) Evaluation of probiotic properties of *Lactobacillus plantarum* WLPL04 isolated from human breast milk .Journal of Dairy Science 99(3):1736-1746.

JoostenMLJH , Northolt DM (1989) Detection, Growth, and Amine-Producing Capacity of Lactobacilli in Cheese.Applied and Environmental microbiology 55:2356-2359.

Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman BJ, Reppas C (2006) Characterization of the Human Upper Gastrointestinal Contents Under Conditions Simulating Bioavailability/Bioequivalence Studies.Pharmaceutical research23: 165-176.

Karpa K (2007)Probiotics for Clostridium difficile Diarrhea: Putting It into Perspective. The Annals of pharmacotherapy 41: 1284-1287.

Kaur IP, Chopra K, Saini A (2002) Probiotics: Potential pharmaceutical applications. European Journal of Pharmaceutical Sciences 15: 1-9.

Leroy F, Vuyst DL (2004) Functional lactic acid bacteria starter cultures for the food fermentation industry.Trends in Food Science and Technology15: 67-78.

Liang SC,Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, Sharpe AH (2003)Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses.European Journal of Immunology 33(10):2706-2716.

Maintz L, BenfadalS,Allam JP, HagemannT,Fimmers R, Novak N (2006) Evidence for a reduced histamine degradation capacity in a subgroup of patients with atopic eczema. J Allergy and Clinical Immunology 117: 1106-1112.

Marteau P, Ruault BMC (2002)Nutritional advantages of probiotics and prebiotics. British Journal of Nutrition 87 (S2): S153-S157.

Marteau P, Tuure T. (1998). Pharmacokinetics of Probiotics and Biotherapeutic Agents in Humans.Bioscience and Microflora17:1-6.

Messaoudi M, Violle N ,Bisson JF ,Desor D ,JavelotHervé, Rougeot C (2011) Beneficial psychological effects of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacteriumlongum* R0175) in healthy human volunteers. Gut microbes2: 256-261.

Mohammadi AA , Jazayeri S , Khosravi-Darani K, Solati Z , Mohammadpour N , Asemi Z , Adab Z , Djalali M, Tehrani-Doost M , Hosseini M , Eghtesadi S (2015) The effects of probiotics on mental health and hypothalamic–pituitary–adrenal axis: A randomized, double-blind, placebo-controlled trial in petrochemical workers. Nutritional Neuroscience19(9):387-395.

Mokarram RR, Mortazavi SA, Najafi MBH, Shahidi F (The influence of multistage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. Food Research International 42(8):1040-1045.

Monika,Savitri, Kumari A, Angmo K, Bhalla TC (2016) Traditional pickles of Himachal Pradesh. Indian Journal of Traditional Knowledge 15:330–336.

Montalto M, Vastola M, Marigo L, Covino M, Graziosetto R , Curigliano V (2004). Probiotic treatment increases salivary counts of lactobacilli: A double-blind, randomized, controlled study. Digestion 69:53-56.

Namasivayam SKR, Angel JCR, Bharani RSA, Karthik MY (2014) Effect of media on bacteriocin production by *Lactobacillus brevis* and evaluation of anti-bacterial activity. Research Journal of Pharmaceutical, Biological and Chemical Sciences 5(5):1129–1136.

Naseribafrouei A, Hestad K, Avershina E , Sekelja M , Linlokken A , Wilson Robert , Rudi K (2014)Correlation between the human fecal microbiota and depression.Neurogastroenterology and Motility26(8):1155-1162.

Owusu-Kwarten J, Debrah TK, Akabanda F, Jespersen L (2015)Technological properties and probiotic potential of *Lactobacillus fermentum* strains isolated from West African fermented millet dough.BMC microbiology15: 1-10.

Pavli F, Argyri A, Papadopoulou O , George NJ,Chorianopoulos N , Tassou C (2016) Probiotic Potential of Lactic Acid Bacteria from Traditional Fermented Dairy and Meat Products: Assessment by In Vitro Tests and Molecular Characterization. Journal of Probiotics & Health 4:157.

Pessione E (2012) Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Frontiers in Cellular and Infection Microbiology 2:86.

Pessione E (2014)The double face of bacteria and the ambiguous role of some probiotics. In: Interactive probiotics (Ed.Pessione E), Taylor and Francis,USA, pp. 196-214.

Petrini L, Usai P, Caradonna A, Cabula R, Mariotti S (1997)Lactose intolerance following antithyroid drug medications.J Endocrinology Investment 20(9):569-570.

Pochart P, Dewit O, DesjeuxJ ,Bourlioux P (1989) Viable starter culture, betagalactosidase activity, and lactose in duodenum after yogurt ingestion in lactase-deficient humans. American Journal of Clinical Nutrition 49: 828–831.

Rafter J (2005)The effect of probiotics on colon cancer development. Nutrition Research Reviews 17: 277-284.

Rossi M, AmarettiA, Raimondi S (2011) Folate Production by Probiotic Bacteria.Nutrients. 3: 118-134.

Sahadeva R, Leong S, Chua K, Tan C, Chan H, Tong E, Wong S, Chan H (2011) Survival of commercial probiotic strains to pH and bile. International Food Research Journal 18(4):1515-1522.

Selhub EM, Logan AC, Bested AC (2014) Fermented foods, microbiota, and mental health: Ancient practice meets nutritional psychiatry. Journal of Physiological Anthropology33 (1): 2.

Steingoetter A, Fox M, Treier R, Weishaupt D, Marincek B, Boesiger P, FriedM,Schwizer W (2006) Effects of posture on the physiology of gastric emptying: A magnetic resonance imaging study, Scandinavian Journal of Gastroenterology41(10): 1155-1164.

Steinkraus KH (2002) Fermentations in world food processing. Comprehensive Reviews in Food Science and Food Safety 1(1):23–32.

Tiwari G, Tiwari R, Pandey S, Pandey P (2012) Promising future of probiotics for human health: Current scenario. Chronicles of Young Scientists.3(1): 17-28.

Wollowski I, Rechkemme G, Zobel LPB (2001) Protective role of probiotics and prebiotics in colon cancer.The American Journal of Clinical Nutrition73: 451S-455S.