DEVELOPMENT OF PLANT BASED PROBIOTIC NUTRITIONAL SUPPLEMENT TO ENHANCE GUT PROBIOTIC MICROFLORA

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By
SUBHASHREE S

Under the guidance and supervision of

Dr. KAVITA M.S.
Assistant Professor (Food and Nutrition),
Department of Home Science, Government Women's College, Trivandrum
Research Guide, Department of Home Science,
St.Teresa's College (Autonomous), Ernakulam

CENTRE FOR RESEARCH IN HOME SCIENCE
ST.TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM

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Declaration

I, Subhashree S, hereby declare that the dissertation entitled **"DEVELOPMENT OF PLANT BASED PROBIOTIC NUTRITIONAL SUPPLEMENT TO ENHANCE GUT PROBIOTIC MICROFLORA"** is a record of original work done by me under the guidance of Dr.Kavita M.S, Assisstant Professor (Food and Nutrition), Department of Home Science, Government College for Women, Thiruvananthapuram and Research Guide, Department of Home Science, St.Teresa's College (Autonomous), Ernakulam. I also declare that the material embodied in this thesis is original and has not formed basis for the award of any Degree/Diploma/ Associateship/Fellowship or similar title to any candidate of any other university.

Ernakulam **Subhashree S**

Certificate

This is to certify that the dissertation entitled **"DEVELOPMENT OF PLANT BASED PROBIOTIC NUTRITIONAL SUPPLEMENT TO ENHANCE GUT PROBIOTIC MICROFLORA"** submitted to Mahatma Gandhi University, Kottayam in fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Home Science-Food and Nutrition (Science) is a record of original work done by Ms.Subhashoursee S during the period of her study in the Centre for Research in Home Science, St.Teresa's College (Autonomous), Ernakulam under my guidance and supervision. This dissertation has not formed basis for the award of any Degree/Diploma/Associateship/ Fellowship or similar title to any candidate of any other university and it represents an independent work on the part of the candidate.

Ernakulam

Dr.Kavita M.S
Supervising Teacher

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CONTENTS

	APPENDICES	

LIST OF TABLES

29	Proportion of ingredients in the food mixture	
30	Acceptability scores of the prepared variations	
31	Viable cell count at different incubation times	
32	Effect of concentration of stabilizer on the product	
33	Effect of heat treatment on physical qualities of the product	
34	Microbial, physicochemical and sensory characteristics of probiotic supplement prepared at varying pH and fermentation time	
35	Regression coefficients and ANOVA of fitted quadratic model for the microbial and physico chemical responses for the developed supplement	
36	Regression coefficients and ANOVA of fitted quadratic model for sensory responses for the developed supplement	
37	Verification of the software predicted value with observed value for the response parameters	
38	Proximate composition of the developed probiotic supplement and controls	
39	SCFA profile and flavour compounds in the probiotic beverage	
40	Physico chemical properties of the developed probiotic supplement	
41	Microbial quality of the probiotic supplement	
42	Changes in the pH and titratable acidity during storage	
43	Changes in the colour during storage	
44	Changes in the viscosity and water holding capacity during storage	
45	Microbial quality during storage	
46	Sensory scores of the probiotic supplement during the period of storage	
47	Shelf life of the probiotic capsule	
48	Functional properties of the RTR probiotic mix	
49	Shelf life of the ready to eat food mix	
50	Cost calculation of the probiotic products	
51	Effect of feeding the probiotic supplement on the gut microflora of rats	

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

XXI	Measurement of viscosity	
XXII	Measurement of pH	
XXIII	Estimation of Titratable acidity	
XXIV	Measurement of Measurement of Colour	
XXV	Measurement of Sedimentation	
XXVI	Measurement of Wheying off %	
XXVII	Water holding capacity	
XVIII	Estimation of Total Viable count	
XVIII	Estimation of Yeast and Mould count	
XVIII	Estimation of Coliform count	

LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
APV	-	Adequate Precision Value
BLIS	-	Bacteriocin like Inhibitory Substance
BSH	-	Bile Salt Hydrolysis
CAGR	-	Compound Annual Growth Rate
CCRD	-	Central Composite Rotatory Design
CLA	-	Conjugated linoleic acid
CMC	-	Carboxy Methyl Cellulose
CSH	-	Cell surface hydrophobicity
DNA	-	Deoxyribo nucleic acid
EE	-	Encapsulation efficiency
EHEC	-	Enterohaemorrhagic *E.coli*
EMB	-	Eosin Methylene Blue
EPS	-	Exo-polysaccharide
FAN	-	Free Amino Nitrogen
FAO	-	Food and Agriculture Organization
FD	-	Freeze Dried
FDA	-	Food and Drug Administration
FOS	-	Fructo-oligosaccharides
FOSHU	-	Foods for Specific Health Use
GC-MS	-	Gas Choursomatography and Mass Spectroscopy
GIT	-	Gastro Intestinal Tract
GOS	-	Galacto-oligosaccharides
GRAS	-	Generally Regarded as Safe
HAART	-	Highly Active Antiretroviral Therapy
HIV	-	Human Immno Virus
HGC	-	High Gene Count
IBD	-	Inflammatory Bowel Disease
IVSD	-	In Vitro Starch Digestibility
LAB	-	Lactic Acid Bacteria
LGC	-	Low Gene Count

MPE	-	Measure of Prebiotic Effect
MRS	-	De Man Rogosa Sharpe
MTCC	-	Microbial Type Culture Collection and Gene Bank
NCDC	-	National Collection of Dairy Cultures
NIST	-	National Institute of Standards and Technology
PAS	-	Prebiotic Activity Score
PC	-	Probiotic Capsule
PSC	-	Probiotic Supplement Capsules
RS	-	Resistant Starch
RSM	-	Response Surface Methodology
RTR	-	Ready-to-Reconstitute
SCFA	-	Short Chain Fatty Acids
SGF	-	Simulated Gastric Fluid
SMP	-	Skim Milk Powder
TA	-	Titratable Acidity
TRS	-	Total Reducing Sugars
TSS	-	Total Soluble Solids
WHO	-	World Health Organization
YMC	-	Yeast and Mold Count

I INTRODUCTION

Consumer interest in attaining wellness thoursough diet has increased the demand for functional foods which, in addition to deliver nutrition, modulate the physiological functions in an advantageous way. Recent focus is on foods that provide an overall state of well being and health benefit beyond providing just nutrients. Japan in the 1980's was the first to name such foods as foods for specific health use (FOSHU). The Food and Drug Administration (FDA) (2004) termed them functional foods and are defined as foods or nutrients whose ingestion leads toimportant physiological changes in the body in addition to deliver nutrients. Food products, with such biologically active ingredients have the potential to be used as non pharmaceutical alternative and possess immense market potential. Of the different category of functional foods, probiotics has received maximum attention.

An abundance of beneficial gut microflora is linked to functional health benefits and for the maintenance of optimal health.The gut microbiota maintains a symbiotic relationship with the gut mucosa and provides metabolic, immunological and gut protective functions to the host. The balance between gut friendly organisms and pathogenic microbes is often altered due to repeated use of antibiotics, unhealthy diet and poor lifestyle.

Probiotic foods are those products that contain viable probiotic cells in an adequate food matrix in sufficient concentration such that the postulated effect is obtained when consumed as a part of normal diet. Organisms coming under the genera Lactobacillus, Bifidobacterium and Saccharomyces are being used successfully in foods as well as in clinical practice as adjuvant. Among the lactic acid bacteria, the genera Lactobacillus and Bifidobacteria are most widely identified for its probiotic characteristics. An ideal probiotic should withstand harsh acid and bile environment prevalent in the gut, show adhesion to epithelial cells and resistant to antibiotics and pathogens. Ease of use in food formulations and maintenance of viability during processing and

storage are other desirable characteristics of probiotics for use in food formulations.

Probiotics and prebiotics as food additives are known to have positive effects on the gut microflora. Few probiotics from genera lactobacillus have shown strong antagonism against many pathogenic microbes. Most promising effects of probiotics and prebiotics have been in management and prevention of colon cancer, inflammatory bowel disease, diarrhoea and hypercholesterolemia. Gut microbial populations have also been strongly linked to obesity. Prebiotics act in synergism with probiotics and is most popularly known to enhance bowel function and producevital nutrients.

Increasing awareness about probiotics, change towards healthy food habits, higher stress and risk of metabolic disorders has made the Indian probiotic market to emerge as one with a highest growth potential. According to "India Probiotic Market Forecast and Opportunities, 2019," India's probiotic market has been projected to grow at a Compound Annual Growth Rate (CAGR) of around 19% (Techsciresearch, 2014). Among the multitude of probiotic products available in the market, most of them are dairy based in the form of fermented milk and yogurt. Lactose intolerance, high fat content and presence of allergenic proteins in milk have ignited the search for non dairy probiotic products.

Prebiotics can evoke a synergistic effect and enhance probiotic growth when ingested along with probiotics. Many of the prebiotics are plant components and hence there is immense scope in exploring the suitability of indigenous plant foods as substrates for probiotic bacteria. Furthermore, these plant foods are a source of indigestible polysaccharides that selectively stimulates healthy bacteria in the large intestine. Food formulations using plant foods as substrates for probiotic bacteria are a novel way for probiotic delivery. Fermentation of plant foods by probiotics makes the food easily digestible and imparts characteristic taste to the product. Several components

produced during fermentation such as short chain fatty acids have highly beneficial functions in the body.

Attaining and maintaining sufficient probiotic counts during processing and storage are still challenges when dealing with plant based probiotic products. Choice of the right plant medium that is indigenous to the local community is essential for assured delivery of probiotics. Processing improves the prebiotic potential of the plant food for probiotic bacteria. Malting, acid hydrolysis, repeated autoclaving and cooling cycles are the proven methods by which plant foods are made more utilizable for probiotic bacteria. Usage of foods indigenous to the region and with right combination of foods can bring about a mutual and synergistic effect on the host.

The recent advances in fermentation technology indicate the use of software such as Response Surface Methodology to enable optimisation of fermentation processes. Optimisation of processing parameters for fermentation conditions such as culture characteristics, substrate characteristics, fermentation time and temperature will be helpful to formulate an acceptable plant based probiotic product.

Novel and innovative technologies are being implemented for the preparation of probiotic products.Encapsulation of sensitive probiotic bacteria within a matrix is an innovative way of protecting the viability of probiotics and extending the shelf life of products. However, susceptibility of the coating matrix to environmental damages, limited choice of wall materials available and the strict post production storage conditions make the process unfeasible for large scale production and preservation.

Freeze drying is the most preferred method of drying the probiotic bacteria. During freeze drying dehydration is attained by sublimation of the frozen food product without affecting the probiotic viability. Freeze dried products have good structural rigidity and rehydration capacity and hence is ideal in the production of ready to reconstitute foods. The process reduces the

bulk and makes transportation less cumbersome. However, high costs associated with freeze drying and the requirement of sophisticated technology hinders its usage in large scale operations.

Therapeutic benefits of probiotic food supplements depend on the population of the probiotic organism in the product. A minimum of 10^5-10^6cfu/ml of probiotic bacteria is necessary to get therapeutic benefit (Shah *et al*., 1995). Higher cell counts of probiotic bacteria have been reported in plant based probiotic beverages and drinks.

Probiotic products in wet form are the most preferred over dry powder form or capsules which evoke the sensation of consuming medicines. Hence majority of commercial probiotic products are in the form of beverage or drink. However, the shelf lives of probiotic beverage are lesser than the freeze dried and encapsulated products. But probiotic beverages are highly acceptable and popular.

Probiotic products are mainly consumed for their beneficial aspect with health restoration and prevention of diseases. These effects are primarily brought about by the re-establishment of good bacteria in gut. Probiotics establishes itself in the gut by competing with pathogens for space and nutrients. Regular consumption of probiotic foods have been shown to improve population of good bacteria and decrease population of harmful bacteria. Apart from providing high probiotic counts, a probiotic product should have acceptable organoleptic and physicochemical properties.

Probiotic products are of immense demand among health and nutrition conscious consumers. However, majority of the existing probiotic products are of dairy based and cannot be provided to lactose intolerant and those who require low residue diets which evoke the need for the development of plant based probiotic products. Hence the present study entitled **"Development of plant based probiotic nutritional supplement to enhance gut probiotic**

microflora” was proposed with the specific objective to develop plant based nutritional supplement to enhance probiotic microflora in the gut.

II REVIEW OF LITERATURE

The review of the literature pertaining to the study titled **"Development of plant based probiotic nutritional supplement to enhance gut probiotic microflora"** is discussed under the following heads:

2.1 Definition of Probiotics, prebiotics and related terms

2.2 Microbial species with applications as probiotics

2.3 Desirable probiotic properties

2.4 Prebiotics

2.5. Plant sources as substrates for probiotics

2.6 Plant based probiotic products

2.7. Optimisation of variables using Response surface methodology

2.8. Acceptability of plant based probiotic products

2.9 Shelf life of probiotic foods

2.10 Mechanisms of probiotic activity

2.11. Health benefits of probiotics

2.1 Definition of Probiotics, Prebiotics and related terms

Gut microflora is the complex community of microorganisms that live in the digestive tracts of humans and animals (Saxena & Sharma, 2016). The human gastrointestinal tract is a reservoir of diverse microbial communities comprising both harmful and beneficial organisms ranging in number of 10^{14} colonies of bacteria, viruses, fungi and protozoa (Whitman *et al*., 1998). Intestinal microflora is a postnatal acquired organ that performs important functions of the host. The gut of an infant is sterile at birth that gets colonized due to acquaintance with the environment and maternal interaction (Clemente *et al*., 2012). The gut of a healthy individual is composed of 80% good and 20% bad bacteria. In an unhealthy gut, it is vice versa where harmful bacteria

stage a revolt leading to multiple problems of the gastro intestinal tract (GIT). Dietary habits (Turnbaugh *et al*., 2009), oxidative stress, environmental toxins (Spor *et al*., 2011) and excessive use of antibiotics (Francino, 2016) greatly affect the fragile good: bad bacterial balance

Probiotics, meaning "for Life" are the good organisms that help in ensuring homeostasis in the gut. Inclusion of large quantity of probiotics helps maintain a balanced colonic flora. Probiotic bacteria are present in fermented foods. As defined by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) in 2001, Probiotics are "Live microorganisms which when administered in adequate amounts, confer a health benefit on the host". Lactobacillus and Bifidobacteria are widely used probiotics (Holzapfel *et al*., 2001).

Diet plays an important role in maintenance and improvement of human health by acting as growth substrates for microbiota. Food after digestion reaches the large intestine where it renders itself for further fermentation by resident microflora before being eliminated from the gut. This undigested food matrix is termed 'Prebiotics' and gains significance thoursough its selective stimulation of beneficial bacteria especially bifidobacteria.

Prebiotics are defined as non digestible component which beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of colonic bacteria, thereby improving the health of the host (FAO/WHO, 2010 & Gibson *et al*., 2010).

2.2 Microbial species with applications as probiotics

Lactic Acid Bacteria (LAB) are gram - positive, usually non-motile, non sporulating bacteria that produce lactic acid as a major product of fermentation. They are anaerobic in nature and produce lactic acid as an end product when correct carbohydrates are provided. Apart from the right energy source, LAB also requires amino acids, vitamins and minerals for their growth and lactic

acid production (Hammes & Hertel, 2006). It is ubiquitous and found in the oral, gastric, intestine and vagina of human beings and hence is an integral part of human gastrointestinal system (Teuber, 1993).

They are both homofermentative and heterofermentative. *Lactococcus* spp, yoghurt strains (*L.delbrueckii* subspecies *bulgaricus, Streptococcus thermophilus*), *Enterococci spp* and *Pediococcus* are some of the homofermentative Lactic acid bacteria (LAB) that produce lactic acid alone. Heterofermentative LAB that produce ethanol/acetic acid and carbon dioxide apart from lactic acid includes *Leuconostoc spp, L.brevis, L.fermentum, L. reuteri, L.plantarum* and *L.casei.*

2.2.1 *L.casei*

L. casei is a facultative anaerobe that ferments galactose, glucose, fructose, mannose, mannitol, N-acetylglucosamine, and tagatose (Cai *et al*., 2007). It is a mesophilic gram positive, rod shaped, nonsporing, nonmotile, anaerobic bacteria (Holzapfel *et al*., 2001). The ideal pH for its growth is 5.5. Mishoursa and Prasad (2005) has reported many *L.casei* strains that have shown good resistance at lower pH of 3 for upto 3 hours. *L.casei* is known to have a varied industrial application. The lactic acid produced by *L.casei* is of use in cheese and yoghurt preparation, enhances immunity, reduce cholesterol, controls diarrhoea and alleviate lactose intolerance. Organic acids produced during fermentation of *L.casei* are known to inhibit pathogens (Mishoursa & Prasad, 2005).

Galdeano and Perdigon (2006) first reported the immune response of *L.casei* strain in vivo on mice. It was concluded that the probiotic strain *L. casei* CRL 431 induced innate immunity response with an influence in the clonal expansion of the IgA β-cell population.

Addition of *L.casei* in foods would have a beneficial effect on human health. Nebesny *et al*. (2007) had successfully supplemented dark chocolate with *L.casei* whose activity lasted for 12 momths when stored in refrigeration. Yakult, a popular commercial probiotic drink contains *L.casei* strain Shirota and is one of the oldest probiotic product in the market. Consumption of the drink has shown to modulate the microbial compositions and metabolic activity of the intestinal flora. An increase in fecal lactobacillus and bifidobacterial counts has been reported (Spanhaak *et al*., 1998).

Koebnick *et al*. (2003) had reported significant improvements in self reported severity of constipation and stool consistency by those who consumed the probiotic drink containing *L.casei* Shirota. A significant reduction in the occurrence of moderate and severe constipation ($P < 0.001$), the degree of constipation ($P = 0.003$) and occurrence of hard stools ($P < 0.001$) and increase in defecation frequency ($P = 0.004$) was seen. However, there was no difference in the occurrence and degree of flatulence or bloating sensation.

2.2.2 *L.acidophilus*

L.acidophilus is a gram positive bacterium in Lactobacillus genus. It means “acid loving milk bacillus” in New Latin. True to its name it can tolerate low pH (<5). Being homofermentative, microaerophilic it produces lactic acid on fermentation of sugars (Bâati *et al*., 2000). Due to its ability to survive in low pH and fermentation it is the most commonly used organism in many products. Yoghurt culture is supplemented with acidophilus for production of acidophilus type yoghurt. Some strains of *L.acidophilus* have exhibited a probiotic potential (Ljungh and Wadstrom, 2006).

The primary commercial strains of *L.acidophilus* include *L. acidophilus* LA-1 and LA-5 (Chours. Hansen, Denmark), NCFM (Dansico, Madison), DDS-1 (Nebraska Cultures, Nebraska) and SBT-2026 (Snow Brand Milk Products, Tokyo, Japan) (Bull *et al*., 2013 & Shah, 2007).

L.acidophilus 5 has been in use as an ingredient in food and dietary supplements since 1979. Hekmat and Mc Mahon (1992) used *L. acidophilus* 5 in the production of probiotic icecream along with Bifidobacterium bifidum. The *L. acidophilus* 5 count after fermenting was 1.5 x 10^8 cfu/ml and had maintained the current minimal standard count of 10^6 cfu/ml even after 17 week storage.

L. acidophilus 5 was also successfully used in probiotic icecream preparation by Senanayake *et al*. (2013). He reported sufficient number of viable cells after a 10 week storage period (1 x 10^7 cfu/g). Additionally, the probiotic icecream had a significantly higher level ($P < 0.05$) of total solids, protein, titratable acidity and lower level of melting than the non probiotic icecream.

Savard *et al*. (2011) studied the impact of Bifidobacterium animalis Subsp. Lactis BB 12 and *L.acidophilus* 5 containing yoghurt on fecal bacteria counts in adults. The fecal count of both probiotics had increased significantly. Viable lactobacillus counts were significantly higher (p=0.05) and enterococci was significantly lower (p=0.04) than the placebo.

L.acidophilus is antagonistic against intestinal and food borne pathogens like *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli* and *Clostridium perfringens* (Gilliland & Speck, 1977) and this activity has been associated partially to the production of hydrogen peroxide.

Bernet *et al*. (1994) reported that *L.acidophilus* exhibited high calcium independent adhesive property and had a good ability to adhere to human enterocyte like caco-2 cells. The organism also showed strong bonding towards mucus secreted by cultured goblet cell line.

2.3 Desirable probiotic properties

For an organism to be called a probiotic, it has to fulfil certain criteria (Plate 1).

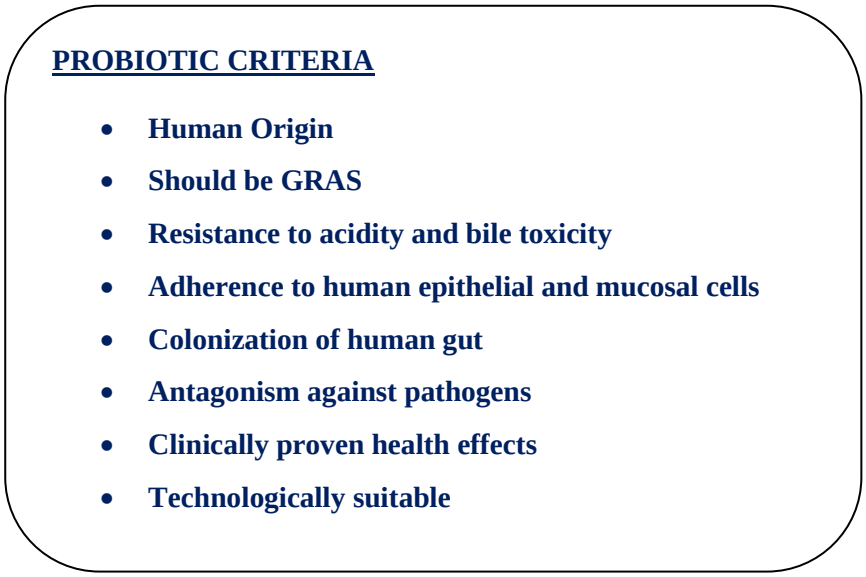

Plate 1: Criteria for selection of probiotic bacteria

- Human origin: It is suggested that microbes perform optimally in the species from which they have been isolated indicating that for probiotics to be beneficial to humans, it has to be from human origin. However, many organisms isolated from foods such as dairy, non dairy, fermented as well as non fermented products have also proven to be effective probiotics (Sornplang & Piyadeatsoontorn, 2016 & Sanders, 2006). Moreover, recent research considers specificity of action more important than the source of microorganism.

- All probiotic strains should have Generally Regarded as Safe (GRAS) status. The organisms should be non-pathogenic, non-toxic and should cause no adverse effects to the recipient
- Resistance to acidity and bile toxicity : For an organism to be considered probiotic, it should be able to show good resistance to gastric and bile conditions so as to reach the bowel in large numbers (Succi *et al*., 2005)

- Adherence to human epithelial and mucosal cells: This is a pre requisite to probiotics as this gives bacteria a competitive advantage in the small bowel where a stable microflora does not exist. Mucosal adherence is needed for prevention of displacement and removal of microbes by the intestinal luminal flow. (Shewale *et al*., 2014)
- Colonization of human gut: Most probiotics are transient colonizers during which the organism remains at high levels to become dominant in the small bowel. Its presence in such high levels is necessary for achievement of probiotic biological activities.
- Antagonism against pathogens: Antagonism to enteropathogens is very important property for a probiotic. (Succi *et al*., 2005). Lactobacillus has demonstrated to inhibit a wide range of enteropathogens.
- Clinically proven health effects: Microbes should demonstrate a specific health effect or claim to be considered probiotic. Health claims should be supported by sound clinical evidence.
- Technologically suitable: Probiotics should be able to resist and withstand processing stresses like pH, salinity, temperature etc so as to grow and survive in high numbers in the end product if considered for designing functional foods (Kechagia *et al*., 2013).

2.4 Prebiotics

Prebiotics is a strategy to manipulate the intestinal microbiota. Rather than supplying an exogenous source of live bacteria, prebiotics are nondigestible food ingredients that selectively stimulate the proliferation and/or activity of desirable bacterial populations already resident in the consumer's intestinal tract. Most prebiotics identified so far are non digestible, fermentable carbohydrates that contain fructose chains with terminal glucose and 10 or fewer sugar molecules.

Mothers' milk is the original source of prebiotics that helps establish beneficial flora in the newborn infant (Kunz & Egge, 2017). Human milk oligosaccharides (HMOs) stimulate development of the intestine, provide protection from pathogens, promote immunity and help establish the gut microbiota (Comstock & Donovan, 2017). Coppa *et al*. (2006) attributed the bifidogenic effect of breast milk to the low levels of protein and phosphate and the presence of lactoferrin, lactose, nucleotides and oligosaccharides.

Fructo-oligosaccharides (FOSs), inulin, galacto-oligosaccharides (GOSs), and soybean oligosaccharides are common prebiotics known. Even dietary fibre that is a complex polysaccharide is considered a prebiotic (Thomas & Greer, 2010). All fibres may be prebiotic but all prebiotic is not necessarily dietary fibre. Resistant Starch (Zaman & Sarbini, 2016), pectin (Gomez *et al*., 2014), beta-glucans (Arena *et al*., 2014), xylooligosaccharides (Linares-Pastén *et al*., 2017) and arabinoxylan (Neyrink *et al*., 2012) are some of the recently identified prebiotics.

For a food ingredient to be classified as a prebiotic it must be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract. According to Roberfroid (2007), a prebiotic need not necessarily be completely indigestible, but a significant amount of the compound should be available in the large intestine where gut microbes can ferment on it.

It should act as a selective substrate for one or a limited number of beneficial bacteria commensal to the colon or are metabolically activated to contribute to health and well being. Roberfroid (2007) suggests that bacterial interactions be taken into account when considering a prebiotic and not growth of organisms in a pure culture.

A prebiotic alters the colonic flora in such a way that it favors a healthier composition. Intestinal populations of bifidobacteria, in particular,

are stimulated to proliferate upon consumption of a range of prebiotics, increasing in numbers by as much as 10–100-fold in faeces. Gibson *et al*. (1995) had reported significant bifidogenic effect of both oligofructose and inulin. While oligofructose supplementation decreased bacteroides, clostridia and fusobacteria counts, inulin supplementation reduced gram-positive cocci counts.

Inulin is the most commercially valuable of all the prebiotics. It occurs naturally in foods like onion, wheat, garlic and leeks that are consumed regularly in Indian diet. Less consumed food sources include asparagus, artichoke and chicory root (Moshfegh *et al.,* 1999). Some preliminary studies also claim honey, oats, some fruits and vegetables to have prebiotic potential. This claim however needs better documentation.

Prebiotics are known to have protective effect against colon cancer (Liong, 2008) by modulation of gene expression. In a rat study by Femia *et al*. (2002), it was found that rats that were fed prebiotics alone or prebiotics in combination with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 had low Glutathione *S*-transferase and pi type GST placental enzyme. Inducible nitric oxide synthase that has an important role in the growth and progression of colon tumor was also depressed in tumors from rats in the prebiotic group. Wollowski *et al*. (2001) highlighted that enzymes induced by butyrate or by the microflora and increased prebiotic activity could be an important mechanism for protection against colon cancers.

Prebiotics also improves sensory qualities of food due to which it has gained huge commercial application. Inulin, a commonly used commercial prebiotic has found wide use as a sugar and fat replacer, and as texture modifier. Akalin and Erisir (2008) tested the use of inulin and oligosachharide at 4% level as a fat replacer in the preparation of low fat icecream. The firmness and melting of icecream had improved with addition of inulin.

Improvements in the viscosity, overrun and melting properties have been reported by the addition of 2% inulin in frozen yoghurt (Rezaei *et al*.,

2012). Furthermore, the sensory scores for flavour, taste and overall acceptability had improved in yoghurt with inulin.

Tárrega and Costell (2006) observed that inulin added fat free dairy dessert was sweeter, thicker and creamier when compared to full fat milk dessert. Yoghurt samples containing inulin have also been reported to have stable colour and water activity, and less syneresis during the storage period (Staffolo *et al*., 2004).

2.5. Plant sources as substrates for probiotics

Fermentation is a metabolic process of converting carbohydrates into organic acids, alcohol and gases by the action of bacteria or yeast or a combination of both. It causes degradation of anti-nutritional factors, increases bio-availability of minerals, improves protein digestibility of tannin-rich cereals and degrades flatulence-causing oligosaccharides (Kohajdová & Karovicova, 2007). Using right starter culture for fermentation inhibits the growth of spoilage organisms and pathogens (Erten, 2000) by production of organic acids and bacteriocin. Fermented products have increased nutritional (Van Boekel *et al*., 2010) and organoleptic properties (Sicard & Legras, 2011).

Fermentation is an age old process of utilizing microorganisms to bring about a change in the nutritional and sensory attributes of foods. Plant foods are the oldest and most commonly fermented food stuffs. Most of the traditional foods are cereal based (Table 1) non alcoholic beverages like Boza (wheat, rye, millet, maize), Bushera (germinated sorghum and millet), mahewu (maize, sorghum, millet malt or wheat flour), pozol (maize) and togwa (maize and finger millet) to name a few (Prado *et al*., 2008). A pan Indian search reveals a wide array of fermented foods where cereals are often combined with vegetables/fruits. A few cereal fermented food preparations like Idli, appam, chole baturae and rice porridge are very popular and regularly consumed in the Indian diet (Kumar *et al*., 2012).

Table 1: Traditional Cereal based probiotic beverages

Name	Plant sources	Strains isolated	Country of origin	Reference
Boza	Wheat Rye Millet Maize	*Lb. plantarum* *Lb. acidophilus* *Lb. fermentum* *Lb. coprophilus*	Turkey	Blandino *et al*. 2003
Bushera	Sorghum, millet flour	*Lactobacillus* *Lactococcus* *Leuconostoc* *Enterococcus and Streptococcus.* *Lb. brevis*	Uganda	Muyanja *et al*. 2003
Mahewu	Maize, sorghum, millet malt, wheat flour	*Lactococcuslactis subsp. lactis*	South Africa	Blandino *et al*. 2003
Togwa	Maize flour finger millet malt	*Lactobacillus* *Streptococcus* *Lb. plantarumA6*	East Africa	Kitabatake *et al*. 2003
Pozol	Maize	Molds, yeasts, bacteria	Mexico	Blandino *et al*. 2003

High ranges of the Himalayan corridor in North East India and Nepal have a rich variety of fermented foods (Table 2) made from locally grown indigenous plant sources. Several strains of lactic acid bacteria have been reportedly isolated from these foods and are a reassurance of the suitability of plant foods in the preparation of probiotic products.

Table 2: Traditional vegetable based fermented foods of India

Name	Plant source	Strain isolated	References
Inziangsang	Mustard leaf (*Brassica juncea)*	*L.plantarum* *L.brevis,* *Pediococcus acidilactici*	Yan *et al*. 2008
Soidon	Bamboo Shoot (*Bambusa vulgaris)*	*L.brevis* *L.fallax* *L.lactis*	Tamang *et al*. 2008
Gundruk	Rayosag (Brassica rapa subsp. campestris var. cuneifolia) mustard leaves (*Brassica juncea)* cauliflowerleaves (Brassica oleracea) cabbages (Brassica oleracea var. capitata)	*L. fermentum* *L. plantarum* *L.casei* *L.casei subsp. pseudoplantarum* *Pediococcus pentosaceus*	Tamang *et al*. 2005
Sinki	radish tap root (Raphanus raphanistrum subsp. Sativus)	*L. plantarum* *L.brevis* *L.casei* *Leuconostoc fallax*	Tamang and Sarkar, 1993
Khalpi	Cucumber (Cucumis sativus)	*L. plantarum* *L.brevis* *Leuconostoc fallax*	Tamang, 2009
Goyang	Maganesaag (Cardamine macrophylla Wild.)	*L. plantarum* *L. brevis* *Lactococcus lactis* *Enterococcus faecium* *Pediococcus pentosaceus* *Yeasts Candida spp.*	Tamang and Tamang, 2007

Natural fermentation is caused by organisms present in the raw material or from a previous batch culture. Many potential lactic acid bacteria (LAB) like *L.plantarum, L. pentosus, L. brevis, L. fermentum, L.casei, Leuconostoc mesenteroides, L. kimchi* and *L. fallax* have been isolated from various traditional naturally fermented foods (Swain *et al*., 2014). Lactic acid bacteria especially have shown good adaptability in cereals and other plant foods like vegetables and fruits that may be a potential prebiotic. Therefore, plant foods

can be used as substrates for probiotics in the preparation of healthy functional foods. Moreover, intolerance to lactose and cholesterol content in milk, two major concerns related to fermented dairy products (Yoon *et al*., 2006) is compelling one to explore potential of plant foods in supporting probiotics that can be crucial for commercial production of more plant probiotic product.

Bourdichon *et al*. (2012) has published an authorized list of microorganisms with documented use in food fermentations. It covers a wide range of food matrices, including vegetables and fruits. Di Cagno *et al*. (2013) suggests that the list be consulted to select starter culture for fermentation of different food matrices.

Charalampopolous *et al*. (2002) recommended the use of potentially probiotic strains as starter culture to produce plant based fermented foods that have a possible health promoting effect. Fermentation of plant foods by potentially probiotic bacteria helps identify ideal plant substrates as a prebiotic source and ideal pro-pre combination.

A number of studies have been carried out to test the ability of cereals in supporting probiotic fermentation Oat bran was found to be utilized by gut flora in an anaerobic fermentation model carried out with human fecal flora (Kedia *et al*., 2009). Many scientists have studied the role of oat flours as a substrate in the formulation of non-dairy probiotic products (Angelov *et al*., 2006; Kedia *et al*., 2009 & Luana *et al*., 2014). A probiotic beverage with 25% oat flakes, enzyme and *L. plantarum* LP09 was successfully developed by Luana *et al*. (2014). The fermentation reportedly increased polyphenol availability and antioxidant activity by 25% and 70% respectively. Another synbiotic functional beverage providing 7.5 x 10^{10} CFU/mL of *L.plantarum* A28 was optimized and formulated from oat mash known for the β-glucan component (Angelov *et al*., 2006). However the content of β-glucan remained constant thoursoughout fermentation and storage indicating that the starter culture did not ferment β-glucan.

On the contrary, barley β-glucan exhibited an increase in bifidobacterial counts in a double blind placebo controlled trial conducted on 52 adults in the age group of 39-70 yrs when ingested at 0.75g/d for 30days (Mitsou *et al*., 2010). This variability could be due to differences in the ability of the bacterial strain in utilizing β-glucan.

Fermentation of sorghum and green gram multimix prepared by Chavan, (2006) markedly increased the crude proteins, free amino acids, soluble proteins and in vitro protein digestibility of the sorghum meal. Among pulses, Soyabean has received a lot of attention due to its protein quality. Suitability of soymilk for lactic acid fermentation has been reported earlier as well (Chou & Hou, 2000). Van Laere *et al*. (2000) studied the fermentation of a number of oligosaccharides like soy arabinogalactan, sugar beet arabinan, wheat flour arabinoxylan, polygalacturonan and rhamnogalacturonan from apples by intestinal bacteria. Recently even unconventional foods like coffee beans and spent coffee have been projected as a potential prebiotic due to the presence of oligosaccharides (mainly hexoses) (Tian *et al*., 2017).

Apart from cereals, several fruits and vegetables also have been used as a culture medium for probiotics with known health benefits. Tomato juice (Yoon, 2004) and red beets (Yoon *et al*., 2005) have been evaluated for its use as substrates by four lactic acid bacteria sp. namely *L.acidophilus* LA39, *L.plantarum* C3, *L.casei* A4 and *L.delbrueckii* 07. Both were found to ably support the growth of the four bacteria and maintained good counts even after 4 weeks storage.

The suitability of celery for probiotic was reported by Moraru *et al*. (2007). The authors found the sugars in celery to be rapidly consumed by probiotics with higher acidity than beetroot. However a profound sourness was noted in the celery product that hindered its commercial value.

Most studies on lactic acid fermentation of vegetables reported a probiotic count in the range of 10^8-10^{10}cfu/ml, except one study by Buruleanu

et al. (2012) that reported very high counts of *L.acidophilus LA-5* on cabbage ($19.25x10^{14}$ CFU/ml), red cabbage($11.9x10^{14}$ CFU/ml), cucumber ($18.6x10^{14}$ CFU/ml) and cucumber with onion juice ($10.25x10^{14}$ CFU/ml). An increase from 10^{5}-10^{14} was reported within just 8 hourss fermentation.

Sreenivas and Lele (2013) demonstrated the production of beneficial short chain fatty acids (SCFA) due to lactic acid fermentation of gourd vegetables. *L.fermentum* on ash gourd fibres had the maximum production of acetic and propionic acid that increased between 24-48 hours fermentation. All other gourd vegetable fibres of bottle, bitter, snake gourds and pumpkin supported the production of acetic acid alone.

The technological challenges in producing non dairy probiotic products are many. Yet, few researches have been conducted in the production of fruit based probiotic product. Fruits contain beneficial nutrients like minerals, vitaminutes, fibre and antioxidants and please the taste profile of all age groups making it ideal for development of a functional product (Luckow & Delahunty, 2004).

Fermentation in the shortest possible time is essential in the development of a probiotic product as rapid decrease in pH causes the lactic acid produced to act as a preservative. Hence the viability, acceptability and commercial feasibility of products with long fermentation as demonstrated in previous studies (Mousavi *et al*., 2011 & Yoon *et al*, 2004 & 2005) is questionable. This is seconded by Sivudu *et al*, (2014) who showed that extension of fermentation time over 24 hours in probiotication of watermelon-tomato, significantly decreased the viable counts of *L.fermentum* and *L.casei*. Both species were reported to survive during cold storage. Addition of sucrose was found to affect the survival of the lactic acid bacteria due to the high acidity.

Nithya Priya and Vasudevan (2016) experimented in formulating probiotic papaya juice and found both *L.plantarum* and *L.acidophilus* to be

capable of utilizing papaya juice. Good viability was reportedly obtained at 48hourss with a 3% inoculum concentration.

2.5.1 Prebiotic potential of plant foods

Consumer interest in non-dairy probiotic products has kindled interest in understanding the prebiotic potential of locally available plant foods. The prebiotic index (Palframan *et al*., 2003), measure of prebiotic effect (MPE) (Vulevic *et al*., 2004) and prebiotic activity score (PAS) (Huebner *et al*., 2007) are a few quantitative approaches to assess the ability of a substrate to support probiotic growth. Several plant foods have exhibited good probiotic potential.

Moongngarm *et al.* (2011) analysed the prebiotic composition and prebiotic activity of 13 foods commonly consumed in Thailand that included 3 species of bulb crops (onion (*Allium cepa .var. cepa* L.), shallot (*Allium cepa var. aggregatum*,), and garlic (*Allium sativum L. var. sativum*)), 7 types of root/tuber crops (sweet potato (*Ipomoea batatas*), white radish (*Raphanus sativus*), yam bean (*Pachyrhizus erosus*), taro (*Colocasia esculenta*), cassava (*Manihot esculenta*), yam (*Dioscorea esculata*), and purple yam (*D. alanta*), and 4 types of rice (*Oryza sativa* (RD6), non waxy rice (KDML105), germinated RD6, and germinated KDML105) .

The highest inulin content was reported in garlic (41.72) followed by shallots (33.22%) and onion (27.17 %). Lactobacillus acidophilus grown on extracts of inulin (2.22), garlic (2.15), shallot (2.09), and onion (1.94) had highest prebiotic activity scores comparable to that of commercial inulin. Least prebiotic potential for *L.acidophilus* was reported in germinated rice.

An analysis of PAS of sweet potato fibre extract taken from Bestak sweet potato revealed good prebiotic potential with *L.plantarum* Mut 7 and *B.longum* JCM1217 (Lestari *et al*., 2013). Other plant foods with proven prebiotic activity are garlic for bifidobacteria (Zhang *et al*., 2013), squash for *L.paracasei* BGP1 (Palacio *et al*, 2014) and Jerusalem artichoke for *L.paracasei* (Rubel *et al*., 2014).

Suitable processing of plant foods improves the prebiotic potential of plant foods. Phosphorylation of corn starch had good prebiotic potential for *B.longum* (3.60) and *L.plantarum* (3.58) (Haryadi *et al*., 2017). Anprung and Sangthawan (2012) compared the PAS of Mangosteen Aril before (A1) and after (A2) hydrolysis with *B.lactis* Bb12 and *L.acidophilus* LA-5. The depolymerised (A2) mangosteen aril showed higher PAS for *B.lactis* Bb12 (0.17) and *L.acidophilus* LA-5 (0.21) than unhydrolysed magaoteen aril (A1) for *B.lactis* Bb12 (0.12) and *L.acidophilus* LA-5 (0.16).

Knowledge of the prebiotic potential of plant foods for specific probiotic bacteria will contribute to selecting plant species as potential sources of prebiotic ingredients for the development of functional foods.

2.6 Plant based probiotic products

Plant probiotic products are gaining popularity as a non dairy medium for delivery of probiotics. Plants foods are widely distributed, low cost and are a storehouse of many essential nutrients. Increasing interest in veganism has caused a spurt in the development of non dairy probiotic products.

Angelov *et al*. (2006) developed an oat based probiotic drink comprising of 5.5% oat flour, 5% *L.plantarum* B28 and 1.5% sucrose. The drink had high viable cell count of 7.5x 10^{10} cfu/ml and shelf life of 21 days. Studies on plant probiotic products using wheat (Sharma *et al*., 2014), barley (Rathore *et al*., 2012) and oats (Gupta *et al*., 2010) gives a promising outlook for commercial marketability.

Products using a blend of different non dairy foods have also been developed. Rodrigues *et al*. (2015) developed a probiotic product using finger millet-soyabean in ratio 70:30 containing *L. casei* (MTCC 1423), *L. plantarum* (MTCC 2621) and *L. fermentum* (MTCC 0903). Probiotic BCGT food mix containing barley flour, milk coprecipitate, sprouted green gram paste and

tomato pulp (2:1:1:1, w/w) fermented with 2 per cent liquid culture (containing 10^6 cells/ml broth) was developed by Sindhu and Khetrapaul (2005).

Yoon *et al*. (2005) has developed a few fruit and vegetable based probiotic beverages. A fermented cabbage juice with *L. plantarum* and *L.delbrueckii* was developed that had a good viable cell count of 10^9 cfu/ml. Probiotic beetroot juice with similar viable cell count was also developed by Yoon *et al*. (2005).

Mousavi *et al*. (2010) produced pomegranate based probiotic drink. Four probiotic strains namely *L*. acidophilus DSMZ 20079, *L. plantarum* DSMZ 20174, L. delbrueckii DSMZ 20006, L. paracasei DSMZ 15996 were used of which all had reached 10^8 cfu/ml after 48hourss fermentation. *L.plantarum* and *L.delbrueckii* consumed the highest amount of substrate as evidenced by citric acid production and metabolism of the sugars and survived for 2 weeks in cold storage. Luckow and Delahunty (2004a) reported to have commercially processed probiotic blackcurrant juice.

Khatoon and Gupta (2015) developed an innovative herbal probiotic sweet lime and sugarcane juice fermented with *L.acidophilus*. The phytochemical content of the juice was enriched due to the presence of ashwgandha, green tea extract, wheat grass juice, whey and oats.

Such studies facilitate the development of innovative and novel fermented, non dairy, nutritionally balanced and sensory acceptable probiotic products for vegetarians and lactose intolerant people.

2.7 Optimisation of variables using Response Surface Methodology

Optimisation of several parameters is important for the development of a probiotic product with good viable count as the outcome is dependent on a multitude of factors ranging from concentration and composition of substrate and inoculum to strain of the culture, pH, and fermentation time.

Most non dairy probiotic product developments have focussed on optimisation of the proportion of food matrix to be used as substrates for probiotic growth. Sharma *et al*. (2014) optimsed the amount of sprouted wheat flour, oat, sprouted wheat bran and stabilizer (guar gum) and found 7.86, 5.42, 1.42 and 0.6 g respectively per 100 mL of water to be used in the development of sprouted wheat based probiotic beverage. A probiotic yoghurt porridge mix with acceptable sensory properties was developed (Fatma *et al*., 2014) after optimisation of different combinations of raw grain oats, pearl millet, sorghum, maize and sugar.

Sharon (2010) optimised the conditions for the development of a raw banana based food mixture along with soyaflour and fruit pulp. Fermentation of 25g substrate at pH 4.5, inoculated with 300µl of *L.acidophilus*, incubated at 37°C for 24 hours gave a product with > 9 log cfu/ml of probiotics.

Process conditions for the development of probiotic beverage using cassava flour and *L.casei* and *L.acidophilus* was optimised by Santos *et al*. (2003). The optimised parameters were 20 percent cassava flour, four percent of each culture, fermented at 35°C for 16 hours. Liong and Shah (2005) optimised cholesterol removal by *L.casei* ASCC 292 in the presence of six prebiotics namely, sorbitol, mannitol, maltodextrin, high amylase maize , inulin and fructooligosaccharide in substrate and found combination of *L.casei* ASCC 292, FOS and maltodextrin to be most effective in removing cholesterol.

Kaur *et al*. (2009) optimised the effect of yoghurt bacteria and probiotic culture on textural characteristics of mango soy fortified probiotic yoghurt (MSFPY). The mean optimum culture addition rate of 0.75 percent *L.acidophilus* yeilded acceptable and good quality MSFPY.

Response surface methodology (RSM) is a statistical technique used for the development and optimization of complex processes, which provides a large amount of information and is more economical approach because a small

number of experiments are performed for monitoring the interaction of the independent variables on the response. It uses quantitative data to simultaneously solve equations and the equation of the model easily clarifies the effects for binary combinations of the independent variables. Central composite, Doehlert, and Box–Behnken designs of RSM are used for optimization (Bezerra *et al*., 2008).

Sharma *et al*. (2014) optimsed the amount of sprouted wheat flour, oats, sprouted wheat bran and stabilizer by using the Box-Behnken design of RSM. A central composite rotatable design of RSM was used by Bunkar *et al*. (2012) to optimize the levels of pearl millet, sugar and dairy whitener in a ready to reconstitute kheer mix. The responses studied for the formulation were consistency, cohesiveness, viscosity and overall acceptability.

RSM was applied by Gupta *et al*. (2010) to investigate the influence of oat, sugar and inoculum concentrations on the growth of *L.plantarum* for the development of a functional oat beverage. They found that X1 (oat concentration), X2 (sugar concentration), (X1)2 (oat×oat) and X1X2 (oat×sugar) to be significant model terms indicating that small variation in their concentrations will cause considerable effects on the growth of L. plantarum.

A central Composite Rotatory Design (CCRD) of response surface methodology was used by Sheba (2015) in the optimization of levels of skim milk powder, sugar and vanilla powder in the probiotic powder. The goal was set to maximize the level of addition of skim milk powder and sensory attributes viz. flavour, mouth feel, appearance and overall acceptability, in range for the level of addition of sugar and minimize the level of addition of vanilla powder. These goals were achieved in the formulation containing 25g skim milk powder, 8.48g sugar and 1.83g vanilla powder.

2.8. Acceptability of plant based probiotic products

Although scientists have been successful in identifying non dairy medium for probiotics, reports on its sensory effects and consumer acceptance has been sparse (Table 3). Luckow and Delahunty (2004b) compared the consumer preference for probiotic orange juice with the conventional orange juice. Majority showed preference to the conventional juice while only a small segment (11%) of people reportedly liked the probiotic orange juice. Luckow and Delahunty (2004a) also conducted a sensory evaluation of commercially processed probiotic blackcurrant juice at a mall. Consumers were instructed about the presence of a special ingredient in one sample added to improve the health. The consumers voted their most preferred juice to be the healthiest sample.

Table 3: Acceptability of plant based probiotic products

S.no.	Probiotic product	Acceptability	Author
1.	Soy protein-passion fruit dessert	Strong liking on 7 pt scale	Granato et al. 2012
2.	Fermented oat flakes beverage	Developed beverage had the features of a yogurt-like beverage Intensity of odor and flavor was enhanced when compared to the non-fermented control.	Luana et al. 2014
3.	Probiotic orange juice	Only 11% preferred the probiotic juice when compared to non fermented orange juice	Luckow and Delahunty, 2004b
4.	Fermented soy beverage with fruit flavouring	Pineapple and guava flavours highly acceptable Strawberry, kiwi and coconut flavoursobtained score close to 6.0 (liked slightly) Hazelnut 43flavor was rejected (acceptance less than 5.0)	Behoursens et al. 2004
5.	Orange and grape juice with probiotic beads	Lower acceptance than yohurt with probiotic beads	Krasaekoopt and Kitsawad, 2010
6.	Barley based fermented food mixture with	Food mix was adjudged as acceptable even after one month storage	Sindhu and Khetarpaul, 2005

	tomato flavour		

Consumer acceptance to orange and grape juices supplemented with probiotic beads was checked by Krasaekoopt and Kitsawad (2010). Overall scores of 6.7 and 6.9 respectively was obtained with more than 80% of the consumers reporting good acceptance. This was lower than the acceptability scores for probiotic bead fortified strawberry yoghurt (Krasaekoopt & Tandhanskul, 2008). The authors reasoned that the beads in a fruit juice were considered more as a foreign particle than as a functional ingredient.

There seemed to be better acceptability for cereal/pulse based probiotic products with fruit flavoring than fruit/ vegetable based products. Sindhu and Khetarpaul (2005) conducted an organoleptic evaluation of barley based probiotic product and concluded that the product was organoleptically acceptable to the human palate.

Behoursens *et al*. (2004) developed a soyabean added probiotic product fermented by S*treptococcus thermophilus, Bifidobacterium lactis*, and *Lactobacillus acidophilus* with added fruit flavours. Among the different fruit flavourings, pine apple and guava flavoured probiotic beverage were significantly better liked than strawberry, kiwi and coconut flavoured ones. Granato et al. (2012) assessed the overall acceptability of soyprotein-passion fruit based dessert on a 7 point hedonic scale. The probiotic nondairy dessert showed great sensory potential as majority indicated a strong liking for the product.

2.9 Shelf life of probiotic foods

Storage and shelf life are essential parameters for commercial viability of probiotic products. Viability of probiotics will depend on its initial count, temperature, time, strain of bacteria and the substrate. All studies reported refrigerated condition as ideal for storage of probiotic products. Table 4 gives a summary of the shelf life of plant based probiotic products reported.

Yoon *et al*. (2006) produced a probiotic cabbage based product with *L.plantarum* C3, *L.casei* A4 and *L.delbrueckii* D7. *L.casei* A4 survived upto 2 weeks in refrigerated conditions while the other two survived till 4weeks. At the end of 4 weeks, a one log decrease was observed in *L.plantarum* C3 while 3 log decrease in *L.delbrueckii* was seen which is more than that observed by most authors. Nature of the strain and its suitability to the substrate may be the reason for such differences. Nematollahi *et al.* (2016) fermented cornelian cherry juice with a few native and industrial strains and found that native strains survived better than industrial strains during a short storage period of 7 days.

Table 4: Shelf life of fruit and vegetable based probiotic products

Sample	**Organism**	**Output**	**Shelf life**	**Authors**
Bitter gourd, Bottle gourd, Carrot	*L.acidophilus* NCDC 11, *L.plantarum* NCDC414 *Pediococcus pantosacous* MTCC 2819	8 log cfu/ml after fermentation for 72 hours and pH dropped to 3.2	Gradual decrease during storage	Sharma and Mishoursa , 2013
Carrot, apple, pear	*L.rhamnosus* IMC 501 *L.paracasei* IMC 502	Good growth on heat treated juices	Decreased to 10^6 during 4 week storage	Coman *et al*., 2010
Cabbage	*L.plantarum* C3 *L.casei* A4 *L.delbrueckii* D7	10^8 cfu/ml after 48hours fermentation	*L.plantarum* C3 - 10^7 cfu/ml *L.delbrueckii* D7 – 10^5 cfu/ml after 4 weeks *L.casei* survived	Yoon *et al*, 2006

			only till 2nd week	
Sweet Lime, Sugar Cane	*L.acidophilus*	10^8 cfu/ml after 24hourss fermentation	Viable cells not detected in sweetlime 10^8 cfu/ml seen after 3 weeks in sugarcane	Khatoon and Gupta, 2015
Pineapple (sweetened and non sweetened)	*L.casei* NRRL B442	8 log cfu/ml after 24hours fermentation, pH dropped to 3.7	6 log cfu/ml in non sweetened and sweetened juice maintained till 42 & 28 days respectively	Costa *et al*., 2013
Cashew apple	*L. casei* B-442	8.8 log cfu/ml at 16hourss fermentation	Cell counts increased from 8.41cful/ml to 8.72cfu/ml at 21st day at 4°C Remained above 8log cfu/ml thoursoughout 42days storage	Pereira *et al*, 2011
Cornelian cherry juice	3 Industrial strains (*L. plantarum* ATCC20174, *L. casei* ATCC 393 and *L. rhamnosus* ATCC 7469) 2 native strains (*L. casei* T4	8 log cfu/ml attained after fermentation in pH adjusted juice	Viability of Native strains (6 log cfu/ml) was better maintained than Industrial strain (4 log cfu/ml) after 7 days	Nematollahi *et al*., 2016

	and TD4)			

Khatoon and Gupta (2005) demonstrated the differences among substrates in supporting probiotics during storage. While sugarcane juice maintained the number of viable cells after 3 weeks of storage, sweet lime did not present any viable cells at 3 weeks. Also, Costa *et al*. (2013) reported longer shelf life for non-sweetened probiotic pineapple juice when compared to sweetened juice. Hence, the nutrient composition of the matrix could be an important contributor for sustainability of the organism and shelf life of the product.

2.9.1 Freeze drying/lyophilisation

Freeze drying is an appealing technique for the preservation of lactic acid bacteria to be used as starter cultures in dairy and food fermentations. This is a low temperature dehydration process where the product is frozen to below the critical temperature of the formulation, at which maximum water is frozen (Jennings, 1999). In the primary drying step, unbound water is removed by sublimation. Finally in the secondary drying stage bound water is removed by desoption (Oetjen, 1999).

Cell viability during freeze drying is affected by the type of strains, the parameters of the lyophilisation process, the physiological cell state, and the use of cryoprotectants (Abadias *et al*., 2001).

Wang *et al*. (2004) reported that milder processing conditions during freeze drying of fermented soy milk resulted in higher survival rate of probiotic organisms when compared to spray drying. They found sufficient viable cells even after four months when stored at $4^{\circ}C$.

Simha *et al*. (2012) compared freeze drying and spray drying of pomegranate juice fermented with *L.acidophilus* MTCC 447. The freeze dried pomegranate powder showed maximum survivability of *L.acdiophilus* MTCC

477 than spray drying. This was seconded by Pandey and Vakil (2017) where the viability loss in freeze dried technique (4-27.5%) was lower than spray drying technique (19-40%). They further rated protective role the of 4 matrixes as skim milk>fructooligosaccharide>maltodextrin>xanthan gum.

The process of dehydration exposes probiotic bacteria to a variety of stresses including extremely high or low temperatures, oxygen and osmotic stresses which lead to the loss of viability during the process and subsequent storage. The main causes leading to loss of viability during freeze drying are mainly by the mechanisms which include the efflux of water from the cell, mechanical stress to cellular components and rupture of cell membranes due to ice crystal formation (Huang *et al*., 2006).

Miao *et al*. (2008) compared the effect of reconstituted skimmed milk (RSM) or either of the cryoprotective disaccharides lactose, trehalose, sucrose, maltose, lactose + maltose and lactose + trehalose on the survival of a freeze dried probiotic culture *L. rhamnosus* GG. The addition of disaccharides to the cells contributed to good survival of *L. rhamnosus* after freeze drying in the order of trehalose> maltose > lactose > sucrose. The disaccharide mixes of lactose + maltose and lactose + trehalose resulted in 98.7% and 97.5% survival, respectively, affording the cells approximately the same level of protection as trehalose alone.

Jalali *et al*. (2012) evaluated the effect of various combination of cryoprotectant namely skim milk powder, trehalose and sodium ascorbate on the stability of freeze-dried Lactobacillus paracasei subsp. tolerance and *L. delbrueckii subsp. Bulgaricus* and during storage at 4°C and 23°C. The combination of 6% skim milk, 8% trehalose and 4% sodium ascorbate had minimal loss of viable cells of Lactobacillus paracasei subsp. Tolerance after freeze drying (12%) and storage at 4°C (24%)and 23°C (63%). The highest survival of *Lactobacillus delbrueckii* subsp. *Bulgaricus* after freeze drying and storage at 4°C and 23°C was also reported at the same concentration and combination of cryoprotectants.

In freeze drying trials of *Lactobacillus rhamnosus* (Saarela et al., 2006), wheat dextrin and polydextrose proved to be promising carriers during freeze-drying and storage at 37 °C and proved to be better carriers than oat flour in chocolate-coated breakfast cereals.

Trachoo *et al*. (2008) developed a low cost freeze dried inoculum of probiotic cultures using prebiotics from cereal crops. They evaluated the shelf life of probiotic *L.acidophilus* culture containing locally grown fruit, legume and cereal powders (banana, soyabean, pearl barley) which have potential prebiotic substances. The shelf life of freeze dried *L.acidophilus* in the banana, pearl barley and soyabean stored at 25°C was 6, 16 and 20 days respectively. Survival of freeze dried *L.acidophilus* on different plant substrates was higher at 4°C when compared to 25°C. It was conluded that inoculum containing soyabean be used as a probiotic freeze dried starter culture.

Freeze dried ready to reconstitute powders have functional use as low bulk powders suitable for combat conditions and in high altitude cold conditions. Tabassum *et al*. (2017) developed a low bulk freeze dried probiotic pineapple lassi powder containing *Bifidobacterium bifidum* (NCDC 235) that had a shelf life of upto 12 months. The freeze dried (FD) probiotic pineapple lassi had initial viable counts of 9.55 log cfu/ml and decreased to 8.87 and 8.95 after storage for 12 months at 25°C and -18°C respectively. The FD probiotic pineapple lassi was reported to have good probiotic count, retained yellow colour of pineapple and had acceptable sensory scores at the end of storage at -18°C.

A single culture fermentation [*L. casei, L. plantarum* (37°C, 24hours.)] and sequential culture fermentation [*S. boulardii* (25°C, 24hours.)+*L. casei* (37°C, 24hours.); *S. boulardii* (25°C, 24hours.)+ *L. plantarum* (37°C, 24hours.)] was conducted on indigenously developed BCGT food mixture containing barley flour, milk coprecipitate, sprouted green gram paste and tomato pulp (2:1:1:1, w/w) by Sindhu and Khetarpaul (2005) to develop ready to reconstitute beverage mix. The food mix was reconstituted with curd to

prepare raita and sensory evaluation was carried out. Single culture fermented raita had significantly (p<0.05) higher acceptability scores as compared to sequential culture fermented raita. Similar trend in sensory quality was reported in the raita with 1 month old freeze dried BCGT food blends.

Sharon (2010) developed a freeze dried raw banana based food mix in combination with defatted soya flour and fruit pulp and reported excellent viability of *L.acidophilus* MTCC 477 until 6 months of storage. Similar freeze dried ready to reconstitute powders using indigenous plant substrates with good probiotic viability has been developed and reported earlier as well (Sindhu & Khetarpaul, 2000).

2.9.2 Microencapsulation

Microencapsulation was developed to protect the sensitive core materials like cells from environmental stresses such as oxygen, high acidity, and gastric conditions. Apart from helping probiotic bacteria survive during processing in the food product, microencapsulation also shields the core material during passage thoursough the stomach.

In the food industry, microencapsulation plays an important role by allowing controlled release of flavours, aroma, drugs and detoxicants (Nag *et al*, 2011). Microencapsulation also makes controlled and targeted release of probiotics possible.

Microencapsulation is a process by which bioactive materials are coated with other protective materials or their mixtures (Huq *et al*., 2013). Alginate, a polymer extracted from seaweed is a widely used encapsulating material. It is nontoxic, biocompatible, inexpensive, and easy to handle. Microcapsules using calcium alginate has been shown to improve survival of *L.acidophilus* and *L.casei* when compared to free cells (Krasaekoopt *et al*., 2004).

Combining plant based substances like prebiotics along with alginate beads has been reported to improve the viability of probiotic bacteria and maintain a longer shelf life. Darjani *et al*. (2016) confirmed that survival of *L.casei* in alginate beads when co-encapsulated with inulin was better than free cells. Moreover, the addition of different chain lengths of inulin (especially inulin with DP≥ 23) together with chitosan coating had significantly affected the survival of the probiotic bacteria during the gastro-intestinal fluid and bile salt tests.

Enterococcus faecalis HZNU P2 when encapsulated in soya protein isolate-alginate matrix showed good resistance in simulated gastric fluid (SGF) at pH 2.5 for 2 hours. This was higher than that of free cells of the culture (Zhang *et al*., 2015).

Anekella and Vale (2014) formulated raspberry juice encapsulated probiotic powder by combining prebiotic fibers (from juice) with maltodextrin and probiotics – *L. acidophilus* and *L. rhamnosus*. Addition of 1% hi-maize and coating with chitosan to calcium alginate capsules improved significantly the survival of *L.casei* in simulated gastric condition and in yoghurt stored at 4°C (Iyer & Kailasapathy, 2005).

Encapsulation has been found to improve storage stability of probiotic cultures. Chen *et al*. (2014) reported better storage stability of free L. bulgaricus cells when encapsulated in alginate-whey protein microspheres. The viability of free *L.bulgaricus* had reduced to 3 log cfu/ml while *L.bulgaricus* in alginate-whey protein microspheres remained at 8.7 log cfu/ml after 4 weeks storage at 4°C.

Shi *et al*. (2013) demonstrated full viability of *L.bulgaricus* during refrigerated storage upto 1 month when encapsulated in an aliginate-milk matrix. The novel combination of encapsulating material offered effective protection against extreme simulated gastrointestinal environment.

2.10 Mechanisms of probiotic activity

The human gastrointestinal tract is a reservatory of diverse microbial communities both harmful and beneficial. A large scale study (Frank *et al*., 2007) estimated gut microbiota to be comprised of more than 35,000 bacterial species. A relatively new concept of high gene count (HGC) and low gene count (LGC) with implications on health and disease has been brought about by researchers in a Danish study (Le Chatelier *et al*., 2013). The HGC microbiome is comprised of butyrate producing beneficial organisms like *Anaerotruncus colihominis, Butyrivibrio crossotus, Akkermansia* sp., *and Fecalibacterium* sp. The LGC microbiome is comprised of higher proportion of pro-inflammatory bacteria such as *Bacteroides* and *Ruminococcus gnavus*, both of which are known to be associated with inflammatory bowel disease (Le Chatelier *et al*., 2013).

The gut microbiota has a symbiotic relationship with the gut mucosa and provides metabolic, immunological and gut protective functions to the host. The abundance of beneficial microflora has been linked to functional health benefits.

A few spp of microorganisms like *Bacteroides, Bifidobacterium, Fecalibacterium,* and *Enterobacteria* spp.produce short chain fatty acids (SCFA) by fermenting the undigested carbohydrates in the colon. The SCFA's like butyrate, propionate and acetate are rich sources of energy for the host. Presence of healthy flora is essential to provide SCFA's for the gut.

Among the SCFA's, only acetate production has been attributed to bacterial group while propionate, butyrate and lactate production seems to be substrate specific and associated with the diet (Morrison & Preston, 2016). A diet rich in complex carbohydrate is known to harbour gut friendly microbial species.

This was reiterated in a comparative study on the fecal microflora of children from the European Union (EU) and an African village of Burkina Faso

(BF) (DeFillipo *et al*., 2010). BF children showed a significant numbers of *Bacteroides* and lower numbers of *Firmicutes* and *Enterobacteriacea* than EU children ($p<0.001$). The presence of short chain fatty acids in faeces of BF children was proof of the saccharolytic type of fermentation happening which can be attributed to polysachharide rich diet of the people of the region.

A diet switch study was done to assess the impact of dietary changes on SCFA production. African Americans were fed a high fibre low fat African style diet and rural Africans were fed a high fat low fibre western style diet (Holmes *et al*., 2012). A profound shift was observed in the butyrate producing organisms *Roseburia intestinalis, Eubacterium rectale* and *Clostridium symbiosum* along with increased butyrogenesis on low-fat, high fiber diet. Whereas, in those on high fat low fibre diet, markers suggestive of increased inflammation was observed in the absence of saccharolytic breakdown of fiber.

Decreased bacterial diversity and loss of butyrate producing organisms such as *F. prausnitzii* have been associated with the genesis of inflammatory bowel disease (IBD) (Wang *et al*., 2014). The bacterium has exhibited anti-inflammatory effects in vitro as well as in vivo in a study by Sokol *et al*. (2008). They had observed low proportion of *F. prausnitzii*, a class of Firmicutes in Chourson's disease patients who exhibited recurrence of the disease within 6 months of surgical resection. The anti-inflammatory effect was due to blocking NF-B activation by some secreted metabolites and IL-8 secretion. The authors further found that supplementing with the bacterium could counterbalance the colitis seen in Chron's disease.

Gut microbiota also imparts a positive impact on lipid metabolism by suppression of the inhibition of lipoprotein lipase activity in adipocytes (Jandhyala *et al*., 2015). Some probiotic bacterial species have shown promising hypocholesterolemic effects in animals as well as humans. The hypocholesterolemic effect is brought about thoursough the bile salt hydrolase enzyme, the main enzyme responsible for bile salt deconjugation in the enterohepatic circulation.

Conjugated linoleic acid (CLA) a known antidiabetic, anti atherogenic, anti obesogenic, hypolipidemic with immunomodulatory properties has been synthesised by members of the Bacteroides spp. (Devillard *et al*., 2007 & Devillard *et al*., 2009).

Some lactic acid bacteria are known to exert strong antagonistic activity against many microorganisms that cause food spoilage and pathogens. This is caused by competitive exclusion, immune modulation, stimulation of host defence systems, production of organic acids or hydrogen peroxide that lower pH and production of antimicrobials such as bacteriocins (Saxelin *et al*., 2005; Millette *et al*., 2007 & Ratsep *et al*., 2014). Lactic acid, acetic acid, formic acid, phenyllactic acid, benzoic acid , organic acids, short chain fatty acids, hydrogen peroxide, carbon dioxide, acetaldehyde, acetoin, diacetyl are some compounds produced by beneficial gut bacteria (Choi & Chang, 2015; Tharmaraj & Shah, 2009 & Ammor *et al*., 2006) that have antimicrobial effect. Feeding of acidophilus-bifidus dahi (AB dahi) was effective than normal dahi in augmentation of lysosomal enzyme activities and exhibited greater phagocytic activity than normal dahi and milk (Rajpal & Kansal, 2009).

Bacteriocins are proteinaceous toxins produced by bacteria that inhibit the growth of undesirable bacterial strain. Some lactic acid bacteria isolated from human milk, infant feces, fermented fruits and caniane gut showed a broad spectrum of inhibitory activities against *S. aureus* (Shokryazdan *et al*., 2014) and *S.typhimurium* (Tzortis *et al*., 2004).

Probiotics have also shown to work against formation of molds like *Fusarium*, *Aspergillus* etc due to which its use in extending the shelf life of breads has been possible. Cizeikiene *et al*. (2013) reported that bacteriocin like inhibitory substance (BLIS) produced by *Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7, *Pediococcus pentosaceus* KTU05-8, KTU05-9 and KTU05-10 strains showed fungicidal and fungistatic activities

against *Fusarium culmorum, Penicillium chourysogenum, Aspergillus fumigatus, Aspergillus versicolor, Penicillium expansum, Aspergillus niger, Debaryomyces hansenii and Candida parapsilosis*. The ropiness in artificially contaminated bread by *Bacillus subtilis* spores was suppressed by the addition of 20% *P. pentosaceus* KTU05-9 sourdough, until 6 days storage at 23 C. The study also reported inhibition of fungal growth on the surface by spraying single cell suspensions of *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8 and KTU05-10 until 8 days of storage.

The gut microbiota has an important role in the development and maturation of the gut immune system, including gut-associated lymphoid tissue, T helper 17 cells, inducible regulatory T cells, IgA-producing B cells and innate lymphoid cells (Kamada *et al*., 2013).

Choursistensen *et al*. (2002) explained the role of Lactobacillus reuteri DSM12246 and *L. casei* CHCC3139in differentially modulating the dendritic cells. The denritic cells play a pivotal role in immune regulation of Th1, Th2 and Th3 cell balance.

SCFAs produced during sacchoursolytic fermentation in the large intestine enhance epithelial barrier function and immune tolerance, production of mucus by intestinal goblet cells, inhibit nuclear factor-κB (NF-κB), activate inflammasomes and subsequent production of interleukin-18 (IL-18); increase secretion of secretory IgA (sIgA) by B cells; reduce expression of T cell-activating molecules on antigen-presenting cells, such as dendritic cells (DCs); and increase number and function of colonic regulatory T (T_{reg}) cells and production of anti-inflammatory cytokines (transforming growth factor-β (TGFβ) and IL-10) (Rooks and Garrette, 2016).

Immune modulating effects have been reported post supplementation of prebiotics (galactooligosaccharides/ fructooligosaccharides/ pectin hydrolysate-derived acidic oligosaccharides) in double blind randomised control trial conducted in highly active antiretroviral therapy (HAART) -naive

Human Immuno Virus (HIV-1) infected patients (Gori *et al*, 2011). Immune markers like reduction of soluble CD14 (sCD14), and activated $CD4^+/CD25^+$ T cells, and significantly increased natural killer (NK) cell activity was seen in the treatment group. This was associated with improvement in bifidobacterial count and decrease in *Clostridium coccoides/ Eubacterium rectal* cluster, *Clostridium lituseburense*/*Clostridium histolyticum.*

2.11. Health benefits of probiotics

Rapid advancement in probiotic research has enabled researchers understand the enormous potential of probiotics in improving human health. Probiotics have been traditionally known to benefit the gastrointestinal tract. Earlier notions of probiotics being beneficial in conditions of the GIT have been quashed by many substantiate research on its benefit beyond the GIT (Lenoir-Wijnkoop *et al*., 2007).

Probiotics have expressed powerful therapeutic and functional properties such as enhancing bowel function, prevention of colon cancer, cholesterol lowering effect, improving immune function, lowering blood pressure and reducing infections and inflammation. These wonder bugs prevent the growth of harmful bacteria, improves mineral absorption and helps in fighting against diseases like candida and eczema.

2.11.1 Maintain gut homeostatsis

One of the earliest effects of probiotics reported was in the modulation of gut microbiome in a way that harbours gut friendly organisms while keeping away harmful pathogens. These gut friendly bacteria prevents colonization of

bacterial pathogens by competing for essential nutrients or attachment sites (Collado *et al*., 2007). Manipulation of gut microflora with probiotic bacteria can regulate gut homeostasis and barrier function partly by production of bacterial metabolites (Bassanganya-Reira *et al*., 2012). This postulate has been proved true time and again by numerous researches.

James (2014) proved that supplementation of 10^8 cells of *Bifidobacterium animalis* subsp. lactic B420/g of feed to mice for 30 days caused a 0.86 log cfu/ml increase in bifidobacterial count and decrease in clostridia (1.11 log cfu/ml) and coliform (0.62 log cfu/ml) counts. The reduction in coliform count was sustained even after cessation of the probiotic supplement.

In a study by Dhiva (2009), probiotics *Weissella confusa* and *Bifidobacterium bifidum* were efficient in removing the pathogenic coliforms from the intestinal mucosa. Moreover the percent reduction in fecal coliform count increased with increasing period of supplementation.

Synbiotics have found to be superior to prebiotics or probiotics in modulating the gut flora (James, 2014). Saulnier *et al*. (2008) compared the gut modulating effect of synbiotic (short-chain fructooligosaccharides/fructooligosaccharides, individually combined with *Lactobacillus fermentum* ME-3, *Lactobacillus plantarum* WCFS1, *Lactobacillus paracasei* 8700:2 or *Bifidobacterium longum* 46), prebiotic and probiotics and found synbiotics to be more efficient. The synbiotic groups had increased *bifidobacteria* and *Eubacterium* rectale–*Clostridium coccoides* count and low *Escherichia coli* count when compared to prebiotic and probiotic group.

2.11.2. Antibiotic associated diarrhoea

Several clinical studies have recorded a reduction in diarrheal frequencies and number of diarrheal days. A placebo controlled trial conducted in Mysore, India (Narayanappa, 2008) showed clinical as well as statistically

significant reduction in number of episodes (frequency) of diarrhea in a day, mean duration of diarrhea (in days), degree of dehydration, duration and volume of oral rehydration salt [ORS] therapy, duration and volume of intravenous fluid [IVF] therapy. Most striking observation with regard to rotaviral shedding was made in the probiotic group than the placebo. Generally, *L.rhamnosus* and *S.boulardi* are more effective than *L.acidophilus* and *L.reuteri* in acute diarrhea.

A number of clinical trials have tested the efficacy of probiotics in the prevention of acute diarrhea, including antibiotic associated diarrhea. Probiotics given along with antibiotic therapy have been shown to decrease the incidence of antibiotic-associated diarrhea in children and in adults. Different strains have been tested including *L. rhamnosus* GG, the yeast *Saccharomyces cerevisiae* (*boulardii*) Lyo, and undefined strains of *Lactobacillus acidophilus* and *L.* delbrueckiisubsp bulgaricus. Meta-analysis of controlled trials concluded that probiotics, *L. rhamnosus* GG and *S. Cerevisiae (boulardii)* Lyo, significantly reduced antibiotic-associated diarrhea (Mc Farland, 2006).

Sindhu *et al*. (2013) developed probiotic fermented food blend using rice flour, milk co precipitate, sprouted green gram paste and tomato pulp in the ratio of 2:1:1:1(w/w) and *S. boulardii* and *L. casei*. Feeding of *S. boulardii* + L. casei *fermented* RCGT food mixtures along with ampicillin successfully prevented associated gastro-intestinal side effects in the mice in the prophylaxis of ampicillin-induced diarrhoea.

2.11.3 Carcenogenesis

There is strong evidence supporting the role of gut microbes in the etiology of cancer. Gut microbiota influence cancer susceptibility by utilising inaccessible nutrients and/or sources of energy from the diet, metabolising beneficial as well as detrimental xenobiotics, regenerating gut epithelial cells, maintaining mucosal integrity; and affecting immune system development and activity (Hullar *et al*., 2013).

Changes in diversity and population of intestinal flora contribute to inflammatory and immunological responses and induce malignant changes to the intestinal mucosal cells (Han *et al*., 2018). *Fusobacterium nucleatum*, *Escherichia* coli, or *Bacteroides fragilis* (Park *et al*., 2018), *Streptococcus bovis* (Tsai *et al*., 2016) and *Streptococcus gallolyticus (*Andres-Franch *et al*., 2017*) have been associated with development of colorectal cancer. In animal models, probiotics have shown to have protective role against cancer development while some strains of probiotic* could diminish the incidence of postoperative inflammation in cancer patients and be used as adjuvant in prevention and treatment of cancer (Yu & Li, 2016).

Modifcation of enteric microflora in IL-10 knockout mice by probiotic lactobacilli was associated with reduced prevalence of colon cancer and mucosal infammatory activity (O' Mahony *et al*., 2001). The authors detected the presence of *L. salivarius* UCC118 in feces, a lower fecal coliform and enterococci counts and nil mortality in the probiotic group when compared to the control.

A spray dried formulation with *L.plantarum* CFR 2194 and fructooligosaccharides (FOS) developed by Madhu and Prapulla (2012) showed significant reduction in the harmful fecal enzymes, -glucuronidase and nitroreductase activities in DMH-induced rats. Feeding of probiotic curd has been found to suppress the elevation of whey proteins-specific IgE and IgG response in whey protein sensitized rats.

2.11.4 Hypocholesterolemic and hypoglycaemic effect

Probiotics have received considerable attention as an alternative to allopathic medicines in the prevention and treatment of many lifestyle diseases due to concerns about the health effects of long term use of those chemical compounds. Incorporating such probiotics in food will make it easy for

consumption and add value as health promoting food. Preliminary in-vivo studies in small animals have been reported in this regard.

Aminlari *et al*. (2018) found a reduction in the total cholesterol, triglycerides, low density lipoprotein, very low density lipoprotein and atherogenic index in wistar rats orally fed with *L.plantarum* and *B. Coagulans*. It was found that inclusion of the two probiotic bacteria along with a high cholesterol diet significantly lowered the total bacterial count.

Kumar *et al*. (2011) reported a reduction of 15-23%, 8-21% and 21-38% in the total cholesterol, triacylglycerol and low density lipoprotein and 7-18% increase in high density lipoprotein of rats whose diet was supplemented with various strains of *L.plantarum*. The excertion of cholic acid and lactobacuillus in feces was also predominant in the probiotic treated rats than in control.

Singroha *et al*. (2014) evaluated the hypocholesterolemic effect of milk fermented with *L.gasseri* strain Lg70 for 90 days and found a 29.71%, 49.54% and 62.52% decrease in total cholesterol, LDL-C and atherogenic index and 36.70% increase e in HDL-C level. The hypo cholesterolemic effect of a probiotic product prepared using a composite mix of Italian millet flour, wheat flour, soya flour, skim milkpowder and roasted Bengal gram powder in proportion of 30:40:11:3:15 was evaluated by Huchchananavar (2013). Significant reduction in total cholesterol levels from 117.5 mg/dl to 66.62 mg/dl was reported in Group II fed with compostite mix + single probiotic culture. Moreover, single culture group showed better reduction in the total cholesterol levels than mixed culture probiotic.

Two types of probiotic dahi developed by Yadav *et al*. (2006a, 2006b and 2007) significantly delayed the progression of streptozotocin and high fructose diet induced diabetes mellitus, suppressed elevation of blood glucose, HbA1c, insulin and blood and hepatic lipids.

2.11.5. Lactose intolerance

Lactose intolerance is a common type of carbohydrate malabsorption. The disability to digest lactose sugar appears due poor activity of lactose digesting enzyme lactase. Supplementation of fermented milk containing beneficial bacteria has been showing promising results in lactose intolerant patients. Some lactic acid bacteria, on reaching the small intestine produce lactase or β-galactosidase that aid in digestion of lactose sugar. Therefore consumption of fermented milk or yoghurt or some probiotics can provide relief for people with lactose intolerance.

This was confirmed in a clinical trial by He *et al.* (2008) where 2 week administration of yoghurt enriched with *Bifidobacterium animalis* and *Bifidobacterium longum* (in capsules) to lactose intolerant adults alleviated the clinical symptoms of lactose intolerance. The authors reported increased bifidobacterial counts and fecal β-galactosidase activity and during the period of supplementation. Changes caused in the colonic bifidobacterial counts by the supplementation led to the alleviation of lactose intolerance.

Secondly, the slowing of transit time due to thicker consistency of fermented milk/ yoghurt exposes the lactose sugars to residual β-galactosidase in the small intestine and reduces osmotic load of lactose (Marteau *et al*., 1990 & De Vrese *et al*., 1995).

2.11.6. Enhance bowel function

Sluggishness in bowel function usually starts during late adulthood and becomes a major daily problem especially for the elderly. Among the many nutrition approaches to tackle constipation, fermented foods or probiotics have gained immense focus. The composition of gut flora is known to affect gut motility. Decrease in bifidobacteria and increase in E.coli, Bacteroides have been observed in patients with constipation (*Gerritsen et al. 2011*). Inclusion of fibre rich food has been the most successful nutritional treatment. The effect

has been attributed to elevated metabolic activity of the colonic flora and a lowering of the pH value in the colon after fibre consumption (Locke, 2000). This same metabolic activity happens during consumption of probiotics. Hence it is considered that probiotics can help enhance bowel function.

Koebnick *et al*. (2003) concluded that consumption of probiotic beverage containing *Lactobacillus casei Shirota* improved gastrointestinal symptoms in patients with choursonic constipation with regard to defecation frequency, strain while defecation and occurrence of hard stools. Significant decrease in use of laxatives has been reported among elderly who were on probiotics (Zaharoni *et al*., 2011).

2.11.7. Production of essential nutrients

Many species of probiotic bacteria have shown ability to synthesis vitaminutes especially water soluble vitaminutes and vitamin K. Some species of Bifidobacteria like *B. bifidum, B. infantis, B. breve, B. adolescentis* and *B. longum* have long been used to produce vitaminutes like thiamine, nicotin, folic acid, pyridoxine and Vitamin B_{12} (Deguchi *et al*., 1985).

Fermented preparations using probiotics have showed to increase the nutrient content of food especially vitamin and protein content (Igbabul *et al*., 2014 & Emire & Buta, 2015). Several strains of probiotic bacteria have been found to synthesise few essential micronutrients. Folate producing cultures *L.helveticus* CD6 *and Bacillus spp*. ST13 have been found to synthesise 5-Methyl-THF involved in synthesis of methionine (Ahire *et al*., 2013) and catechol type siderophores crucial for iron deficiency (Ahire *et al*., 2010) respectively. Therefore fermented foods with naturally enriched micronutrients can be developed using such strains. This can help address a wide range of problems associated with malnutrition.

III MATERIALS AND METHODS

The method followed and materials used for the study titled **"Development of plant based probiotic nutritional supplement to enhance gut probiotic microflora"** was undertaken in four phases and is detailed under the following heads:

3.0 Study design

Phase 1: Assessing the suitability of indigenous plant foods as substrates for selected probiotics

3.1 Selection, isolation and maintenance of purity of cultures

3.2Assessing the probiotic properties of the cultures

3.3 Processing of plant foods and preparation of the substrates

3.4 Assessing the suitability of selected plant foods for the probiotic cultures

3.5 Identification of ideal plant food-probiotic combination

3.6 Processing of the identified plant foods

3.7 Development and standardization of food mix using identified plant foods

Phase 2: Process optimization for the development of a probiotic nutritional supplement

3.8 Process optimization

Phase 3: Development of a probiotic nutritional supplement and its shelf life study

3.9 Development and quality evaluation of the newly developed probiotic supplement

3.10 Shelf life of the newly developed probiotic supplement

3.11 Extending the shelf life of the probiotic supplement

3.12 Feasibility of the developed products

Phase 4: Efficacy of the probiotic supplement in altering gut microflora

3.13 Conduct of a feeding trial on Sprague dawley rats

3.14. Data Management and Analysis

The outline of the study is provided in figure 1.

3.0 Study Design

The present study adopted experimental study design and was conducted in four different phases. During Phase 1, the suitability of indigenous plant foods as substrates for probiotic bacteria was assessed and the ideal plant food-probiotic combination was identified.

Phase 2 of the study focused on process optimization for the development of plant based probiotic product with the ideal combination obtained from the previous phase. In Phase 3, a plant based probiotic product was formulated, standardized and shelf life was studied. Steps were also taken to extend the shelf life of the product.

During Phase 4, the effect of the developed product on gut flora of adult Sprague Dawley rats was studied. Phase 1, 2 and 3 was conducted in the Department of Dairy Microbiology, College of Dairy Science and Technology, Mannuthy and Phase 4 was conducted in the Small Animal Breeding House, Department of Animal Nutrition, Kerala Veterinary and Animal Sciences University, Mannuthy.

Phase 1: Assessing the suitability of indigenous plant foods as substrates for selected probiotics

3.1 Selection, isolation and maintenance of purity of cultures

Two probiotic cultures with long term applications in the probiotic food industry and with proven benefits to the human gastro intestinal tract (Ouwehand, 2002), viz., *Lactobacillus casei* and *Lactobacillus acidophilus* were selected for the study. Lactic acid bacteria especially of *Lactobacillus sp*. are the most common probiotics recommended for gut health (Fuller, 1992). An enteric bacterium,

Escherichia coli MTCC 433 procured from the Institute of Microbial Technology, Chandigarh was used as a control to study the prebiotic potential of substrates.

L. casei, from a commercial probiotic beverage and *L.acidophilus* (LA 5) provided by Chrs Hansen Ltd, Denmark was isolated (Appendix I) and activated before every use. *E.coli* MTCC 433 was opened according to the instructions mentioned in the instruction manual and isolated (Appendix I), without any cross contamination.

The isolated cultures were maintained at 4°C until further use and activated at fortnightly intervals. The purity of all the thoursee fresh cultures was tested by Gram's staining (Gregersen, 1978), Catalase test (Harrigan & McCance, 1976) and Oxidase test (Barrow & Feltham, 1993) (Appendix II).

3.2 Assessing the probiotic properties of the cultures

Probiotic organisms for use in food should be capable of surviving the varied acidic environment in the gastrointestinal tract as indicated by exposure to acids of the stomach and high concentrations of bile in the upper intestine (Soliman *et al*., 2015) which have an influence on the survival of probiotics in the gut. Probiotic properties of the cultures were tested by using a battery of tests as provided in Table 5.

Table 5: Different tests used to assess the probiotic properties of the culture

Test for Probiotic property	Reference	Appendix
Acid tolerance	Gilliland *et al*., 1984	III
Bile tolerance	Gilliland *et al*., 1984	IV
Cell surface hydrophobicity (CSH)	Rosenberg *et al*., 1980	V
Bile salt hydrolysis	Dashkevicz and Feighner,	VI

	1989	
Antibiotic susceptibility test	Bauer *et al*., 1959	VII
Anti-pathogenic effect	Valgas, 2007	VIII
Carbohydrate fermentation test	Barrow and Feltham, 1993	IX

3.3 Processing of plant foods and preparation of the substrates

Cereals, pulses, roots and tubers are important sources of dietary protein and carbohydrates and fermentation is a simple and economic way of improving their nutritive qualities. Thirteen food items from different food groups such as cereals and millets, pulses and roots and tubers were selected as substrates for the growth of probiotics and suitably processed. All commodities were procured from the local market.

Table 6 indicates the various food items selected from different food groups and its pre-processing steps.

Table 6: Food items selected for the study and pre-processing methods

Food Group	**Food Items**	**Pre-processing steps**
Cereals and millets	Rice (*Oryza sativa*) Wheat (*Triticum aestivum*) Oats (*Avena sativa*) Barley (*Hordeum vulgare*) Finger millet (*Eleusine coracana*)	Washing Sun drying Milling
Pulses	Green gram dhal (*Phaseolus aureus*) Bengal gram dhal (*Cicer arietinum*)	Washing Sun drying Milling

	Soyabean (*Glycine max*)	Soaking for 6 hours Blend into a paste Freeze dry in an Operon freeze drier at a condenser temperature of - 70°C at 50-100 mm torr pressure till completely devoid of moisture
Roots and tubers	Arrowroot (Marantha arundinaceae) Powder	NIL
	Tapioca (Manihot esculenta)	Washed till clean of soil particles and skin peeled. Cut into small discsand sundried until brittle. Pulverized into a fine powder and sieved.
	Onion (*Allium sativum*)	Cut into small pieces. Dried in a hot air oven at 60°C till dry and pulverized
	Garlic (*Allium cepa*)	
	Beetroot (*Beta vulgaris*) Powder	NIL

For preparation of plant substrate, individual substrate was mixed with distilled water and made into a paste without lumps. It was then made up to 100 ml with distilled water (Plate 2). The prepared slurry was autoclaved at 15 psi for 15 minutes at 121°C and cooled to room temperature. Each culture was inoculated separately into each plant substrate and incubated at 37°C.

Skim milk powder, common media used in lactic acid fermentation and inulin, a well documented commercial prebiotic supplement was used for comparison with the selected substrates.

3.4 Assessing the suitability of selected plant foods for the probiotic cultures

The suitability of selected plant foods were assessed by observing the viable cell count (Appendix X) of the *L.casei and L.acidophilus LA-5* and *E.coli* cultures on

the selected plant foods before and after fermentation and calculating the relative growth scores.

3.4.1 Viable cell count

Viable cell count measures the number of actively growing/dividing cells in a sample that are capable of growing into distinct colonies. Viable cell count was done (Marth, 1978) (Appendix X) at 0 hours and 24hourss after inoculation. The number of colonies in the sample that was visible to the naked eye on enumeration on MRS agar for *Lactobacillus spp*. and EMB for *E.coli* in a suitable was counted and expressed as colony forming units/ml of the sample. The experiment was carried out thoursice and the mean value was calculated.

3.4.2 Relative growth score

This score was adopted by making slight modification to the prebiotic activity score developed by Heubner *et al*. (2007). The relative growth score was calculated as a measure of the capability of whole food to support probiotic growth relative to the growth of enteric organism on the same food and in comparison to their growth on a pure carbon source like glucose.

3.5 Identification of ideal plant food-probiotic combination

A food is said to be ideal for the probiotic, if it supports the growth of gut friendly probiotic bacteria more than pathogenic enteric organisms (Moongngarm *et al*., 2011). Foods that maximum supported probiotic growth and minimally or nil or negatively supported the growth of E.coli were grouped into clusters based on a dendrogram.

The plant food-probiotic combination that met the criteria was identified using cluster analysis. Three plant foods were identified and selected for the development of a probiotic supplement using the most suitable probiotic bacteria.

3.6 Processing of the identified plant foods

The selected plant foods were processed inorder to improve their nutritional and prebiotic potential. Wheat was malted by soaking (12hours), germination (36 hours) and drying (20hours) to improve its nutritional property. Arrowroot starch was

modified by repeated autoclaving and cooling of hydrolyzed starch to increase the resistant starch content (Jenie *et al*., 2010). For this, 20%w/w of arrowroot starch was suspended in water and autoclaved at 121°C for 30 minutes and stored at 4°C for 24 hourss. This was repeated twice and then freezedried at -70°C at a pressure of 50-100 mmtorr till completely devoid of moisture. The obtained arrowroot RS III flakes was made into a fine powder and stored in airtight container.

In order to assess the prebiotic potential of the processed plant foods, the prebiotic extract was prepared according to Charalampopulous *et al*. (2002). For this, a slurry was prepared with 50g flour in 450ml water, centrifuged at 6000g at 30°C for 30 minutes. The supernatant was immediately sterilized by autoclaving. The extraction and sterilization was repeated twice.

3.6.1 Prebiotic activity Score

Prebiotic activity score reflects the ability of a given substrate to support the growth of an organism relative to other organism (preferably a coliform) and relative to growth on a non-prebiotic substrate such as glucose (Lestari *et al*., 2013).

A 1% (vol/vol) of the overnight probiotic culture was inoculated into separate tubes of De Man Rogosa Sharpe (MRS) broth containing either 1% glucose or 1% previously prepared plant extract and incubated at 37°C for 24 hourss. For E.coli, a 1% (vol/vol) of the overnight culture was inoculated to separate tubes containing M9 minimal media broth (M9 salt+Thiamine+Biotin) containing either 1% glucose or 1% plant extract and incubated at 37°C for 24 hours. Microbial enumeration at 0 hours and 24hourss was carried out by pour plating at suitable dilutions on MRS agar for *L.casei* and EMB agar for *E.coli*.

The prebiotic activity score was determined as per Huebner *et al*. (2007).

3.7 Development and standardization of food mix using identified plant foods

3.7.1 Standardization of proportion of plant food substrates in the food mixture

Suitable variants of food mixtures using malted wheat, arrowroot RS III and beetroot powder was prepared. The proportions of ingredient were chosen based on

preliminary acceptability tests. The food mixture was mixed with 100ml distilled water and autoclaved. *L.casei* was inoculated at 1% concentration and incubated for 24 hourss and presented for sensory analysis. This was evaluated by a panel of judges to choose the most preferred food mix for the development of probiotic supplement.

3.7.2 Acceptability of the standardized food combination

Sensory evaluation of the probiotic supplement was carried out by a panel of six well trained judges on a 9 point hedonic scale ranging from 9 indicating "Like Extremely" to 1 indicating "Dislike Extremely" for attributes like colour/appearance, flavour, mouthfeel, taste and overall acceptability. The evaluation was done individually on pre prepared sensory evaluation score cards (Appendix XI). The judges were selected based on a series of acceptability trials using triangle test conducted according to ISO 4120 (2004).

Phase 2: Process optimization for the development of a probiotic nutritional supplement

3.8 Process optimization

Optimization is a unique set of process conditions that produce best results while satisfying all constraints on the variable. Optimization was carried out for conditions like inoculum, substrate and stabilizer concentrations, heat treatment, pH and fermentation time with the aim of maximizing probiotic count and sensory acceptability of the supplement.

3.8.1 Optimization of inoculum concentration

A 1% overnight active culture of *L.casei* was inoculated into MRS broth and allowed to incubate for 1hour, 3hours and 5hours. The culture was centrifuged at 10000 rpm for 5 minutes, supernatant discarded, washed with sterile water and again centrifuged to collect cell pellet. The cell pellets were dissolved in 1ml of sterile saline and enumerated on MRS agar at two hourly intervals to know the number of L.casei in the pellet.

3.8.2 Optimization of substrate concentration

A slurry was made at 5% and 10% concentrations of the selected food mix prepared with standardized proportions of malted wheat, arrowroot RS III and beetroot powder. It was sterilized, cooled to room temperature and inoculated at optimal concentration of *L.casei* and allowed to ferment for 24 hours. Plating on MRS agar was done by serial dilutions and colony was counted after 48 hours.

3.8.3 Optimization of stabilizer concentration

Two stabilizing agents, guar gum (at 0.4%, 0.6% &1%) and pectin (at 0.1% & 0.4%) were added individually to the slurry prepared with optimized substrate concentration, sterilized and inoculated at optimal concentration of *L.casei* and allowed to ferment for 24 hours. The viscosity, wheying off, consistency score and viable cell counts was measured to decide the ideal stabilizer and its concentration.

3.8.4 Optimization of ideal method of heat treatment

Slurry was prepared at optimal substrate concentration and sterilized by heat treatment in an open pan on direct heat at 90ºC, held for 5minutes or in screw capped bottle in a boiling water bath at 90°C for 5 minutes. It was inoculated at the optimal concentration of *L.casei* and allowed to ferment for 24 hours. The difference in colour and viscosity was measured to decide on the ideal method of heat treatment.

3.8.5 Optimization of pH and fermentation time

In order to optimize initial pH and fermentation time, the response surface methodology (RSM) (Design expert® software version 6.0.8) was used to investigate the influence of varying pH levels and time of fermentation on the responses that included microbial, physicochemical and sensory qualities of the probiotic product.

The level of the two factors pH and fermentation time ranged from pH 4-6 and 9-15 hours respectively. The maximum and minimum level of the factors was chosen based on preliminary trials. The initial pH of the food mix was adjusted using 20% citric acid. The actual values of two factors (pH and fermentation time) at 3 levels

(low, central and high) was obtained by the Central Composite Rotatable Design (CCRD) as shown in Table 7.

Table 7: Level of independent variables, Fermentation time and pH used for Central composite experimental design

Factor	Level of factors		
	Lower limit	Centre coordinate	Higher limit
Fermentation Time (hourss)	9	12	15
pH	4	5	6

The design matrix generated by the software for the two variables: Fermentation time and pH consisted of 13 experiments with the fermentation time ranging from 9 to 15 hours and pH ranging from 4-6 (Table 8). The experiments were conducted in the order specified by the design. The centre point experiment (at pH 5 and 12 hourss fermentation time) was repeated five times to calculate reproducibility of the method. The data of the responses for the experiments was entered into the software for statistical analysis.

Table 8: Central composite experiment design for two variables, Fermentation time and pH

Run order	Factor 1	Factor2
	Fermentation time (hourss)	pH
1	15	6
2	12	4
3	9	4
4	12	5
5	9	5
6	15	4
7	12	5
8	15	5
9	12	5
10	12	5
11	12	6

12	12	5
13	9	6

Experimental results for the response parameters viz., viable cell count, titratable acidity, final pH of the product, wheying off% and sensory characters like colour/appearance, mouthfeel, taste, flavour and overall acceptability were fitted to a full quadratic polynomial equation by applying multiple regression analysis. In order to determine the significance of the quadratic model, ANOVA analysis was conducted. The P-values were used as a tool to check the significance of each co-efficient, which also indicated the interaction strength of each parameter. The smaller the P-values are, the bigger the significance of the corresponding co-efficient (Murthy *et al.*, 2000). The goodness of fit of the model was examined by F-test and the determination co-efficient R^2. The greater the F-value is from unity, the more certain it is that the factors explain adequately the variation in the data around its mean, and the estimated factor effects are real.

Optimization of the responses was carried out using the numerical optimization technique. The pH and fermentation time that results in maximum probiotic count and sensory scores with minimal wheying off was desired for the optimization of the supplement.

Viability of probiotic bacteria is affected during storage and on exposure to gastric and bile juices after consumption. Hence for sufficient numbers of such bacteria to reach the body, a product with a high probiotic count is desirable. Consumer acceptance is of prime importance for a food product and generally measured by the sensory scores. A product with high scores for sensory parameters is considered highly acceptable. Wheying-off negatively affects perception of a product as consumers consider such a product as spoilt. Hence, the goal was to maximize probiotic count and sensory attributes viz. flavour, taste, mouth feel, appearance and overall acceptability while minimizing wheying off.

Based on the goals, after response surface analysis, one solution was obtained with pH of 4.93, fermentation time 13.6hourss and desirability of 0.749. The values for the different response parameters of the probiotic supplement prepared from the

suggested optimized pH and fermentation time was predicted by the software and is given in Table 9.

Table 9: Predicted values for the response parameters of probiotic supplement by the design expert RSM software for the suggested optimized pH anf fermentation time

Parameters	Predicted values
Titratable acidity	0.25
Final pH	4.4
Probiotic count (log_{10}cfu/ml)	8.25
Wheying off (%)	3.1
Colour/appearance	7.05
Mouthfeel	6.85
Taste	7.1
Flavor	6.69
Overall acceptability	7.29

The results were verified by preparing the probiotic supplement in optimized pH and fermentation time and subjecting it to microbial, sensory, and physico chemical evaluation. All tests were conducted in triplicates. The results thus obtained were compared statistically with the predicted values.

The regression co-efficients obtained was used to predict polynomial model for the responses when substituted with the response values. The predicted values were then compared with the actual experimental values to assess the efficiency of the model in optimization of fermentation time and pH. Thoursee-dimensional response surfaces were generated to study the interaction among the two factors tested and to visualize the combined effects of factors on the responses.

Phase 3: Development of a probiotic nutritional supplement and its shelf life study

3.9 Development and quality evaluation of the newly developed probiotic supplement

After optimizing the variables for fermentation, the probiotic supplement was prepared under optimized conditions. A 10% slurry of the food mix using wheat malt, arrowroot RS III and beetroot powder was prepared, pH adjusted to 4.9, heated to 90°C for 5minutes and cooled to room temperature in sterile covered bottles. *L.casei* culture was added to the slurry (approx4 log cfu/ml), mixed well and allowed to ferment for 13.5hourss at 37°C. This served as the sample for the study.

In order to compare the effect of fermentation and processing of plant substrates on the physic-chemical, microbial and sensory characteristics of the supplement, unfermented supplement and unprocessed substrates was used in combination as controls (Table 10).

Table 10: Treatments provided for the sample and controls

Code	Treatments	Test/control
S 1	Fermented supplement using processed substrates	Test sample
S 2	Unfermented supplement using processed substrates	Control sample
S 3	Fermented supplement using unprocessed substrates	Control sample
S 4	Unfermented supplement using unprocessed substrates	Control sample

The quality of the newly developed probiotic supplement was evaluated with respect to its nutrient composition, physicochemical parameters and microbial count.

3.9.1 Nutrient composition of the developed product

The nutrient content of the developed supplement was carried out using standard procedures as shown in Table 11.

Table 11: Method for evaluation of nutrient content

S.No	Test	Method	Reference	Appendix

1.	Moisture and Total solids	Thermogravimetric	IS 11623: 1997	XII
2.	Ash	Thermogravimetric	Raghuramulu *et al*, 2003	XIII
3.	Starch	Anthoursone method	Sadasivam and Manickam, 2008	XIV
4.	Total Protein	Micro-Kjeldahl	Sadasivam and Manickam, 2008	XV
5.	Crude fat	Rose-Gottlieb	FSSAI, 2015	XVI
6.	Crude Fibre	Maynard, 1970	Sadasivam and Manickam, 2008	XVII
7.	Reducing sugars	Miller, 1972	Sadasivam and Manickam, 2008	XVIII
8.	Free Amino Nitrogen	European Brewery Convention Method 8.8.1	European Brewery Convention, 1987	XIX
9.	Invitro starch digestion	Dinitrosalicylic acid method	Satterlee *et al*, 1979	XX

3.9.2 Composition of organic acids

The sample for analysis of organic acids was prepared according to Sreenivas and Lele (2013) with slight modification. Four ml of the sample was mixed with 1.25 ml of 20% metaphosphoric acid, precipitated at room temperature for 20 minutes and centrifuged at 13,845 g for 30 minutes at 10°C. The supernatant was collected and filtered thoursough 0.25µm filters and used for gas choursomatographic analysis. The analysis was outsourced to Sophisticated Analytics and Instrumentation Facility, Indian Institute of Technology, Chennai.

For the analysis, the gas choursomatograph (Jeol GC Mate 11) was equipped with HP 5 Ms coloumn. High purity helium was used as carrier gas at a flow rate of 1ml/min. The temperature of the injector, coloumn and detector were 22°C, 250°C and 250°C respectively. The analysis was done in a quadruple double focusing mass analyzer. A photon multiplier tube detector was used to detect organic acids.

3.9.3 Physicochemical parameters of the probiotic supplement

The physicochemical parameters measured and the method followed is highlighted in Table 12

Table 12: Method for evaluation of physico-chemical parameters

S.No	Parameters	Instrument/Reference	Appendix
1.	Viscosity	Brookefields Viscometer	XXI
2.	pH	Systronics pH meter 361	XXII
3.	Titratable Acidity	IS 11765: 1986	XXIII
4.	Colour	Hunter Lab colour meter	XXIV
5.	Sedimentation	Modha and Pal, 2011	XXV
6.	Wheying Off	Modha and Pal, 2011	XXVI
7.	Water Holding Capacity	Harte *et al*, 2003	XXVIII

3.9.4 Microbial quality

Microbial enumeration of the probiotic supplement was carried out as described in Table 13

Table 13: Method for evaluation of microbial count

S.No	Test	Reference	Appendix
1.	Total Viable count	Marth, 1978	XXVIII
2.	Yeast and mould count	Marth, 1978	XXVIII
3.	Coliform count	Marth, 1978	XXVIII

3.10 Shelf life of the newly developed probiotic supplement

The developed probiotic supplement was stored in airtight glass bottle at refrigeration temperature (5-7°C) to assess the physico-chemical (Table 5) and microbial (Table 6) quality. A fermented supplement prepared using unprocessed substrates was used as a control so as to maintain uniformity with regard to the microbial counts. In order to find the shelf life and "best before consume date" of the newly developed probiotic supplement, sensory evaluation was conducted. For this, the products were served chilled to the panel of judges in the morning time in a well lit and ventilated room and evaluated on a 9 point hedonic scale.

3.11 Extending the shelf life of the probiotic supplement

Encapsulation and freeze drying of the developed product was carried out to make probiotic supplement capsules and ready to reconstitute probiotic mix to extend the shelf life of the supplement.

3.11.1 Probiotic supplement capsules

Microencapsulation is the process of retaining the cell within an encapsulating material (Krasaekoopt *et al*., 2004). The probiotic supplement was encapsulated within an alginate matrix. The extrusion technique for microencapsulation given by Zhou *et al.* (1998) was followed with slight modification. A 20% vol/vol of the probiotic supplement was incorporated into 20 ml of 2% sterile sodium alginate and homogenized. The homogenized mixture was injected into sterile 0.05M calcium chloride solution with 1% beetroot powder and allowed to gel for 30 minutes, rinsed with sterile 0.1% peptone water and stored in 0.1% sterile peptone water at 4°C.

Properties of the probiotic supplement capsule with respect to physical characteristics, encapsulation efficiency, viability of L.casei, shelf life and feasibility were studied.

The yield, size and shape of the capsule were measured by light microscope. The encapsulation efficiency was evaluated by suspending 1g of capsules into Phosphate Buffered Saline (PBS) (pH 7.2) and incubating for 2 hours. The colony count was done by plating at ideal dilution and encapsulation efficiency was calculated as below (Ayama *et al.*, 2014).

$$EE\ (\%) = (Xt / Xi) \times 100$$

where Xt is the total amount of probiotic loaded in alginate beads and Xi represents the initial amount of probiotic added in the preparation process.

The viability of *L.casei* in the capsules was studied by dispersing 1g of the beads in 9ml PBS and vortexed to homogenize. It was then enumerated at ideal dilution by pour plating and incubated for 48 hours at 37°C. The viability of *L.casei* in the probiotic supplement capsules stored in refrigerated condition was enumerated every week until week 4 and fortnightly then after to assess the shelf life.

3.11.2 Ready to reconstitute probiotic mix

The formulated supplement was poured into sterile jars; cold trapped and immediately freeze dried in an Operon lyophilizer (Plate 3) at -70°C till it was completely devoid of moisture. The flakes were powdered in sterile conditions and stored in airtight capped bottles at refrigeration temperature. Physical characteristics of the mix were evaluated by measuring the loose and packed bulk density, wettability and insolubility index. Viability of *L.casei* and shelf life and feasibiliy of the ready to eat food mix was also evaluated.

The viability of the culture was assessed by serially diluting 1g of the ready to reconstitute probiotic mix in normal saline, pour plated on MRS agar and counted after 48 hours incubation at 37°C.

Loose bulk density was measured by filling the food mix upto the 10ml mark into preweighed (W0) 10ml graduated cylinder and weighed again (W1). Loose bulk density was calculated as mentioned by Onwuka (2005).

Weight of 10 ml of sample (W2) =W1-W0

Loose bulk density(g/ml) = W2/10

Packed bulk density was determined by tapping the bottom of the cylinder till no further diminution of sample level after filling to the 10ml mark occurs and calculated according to Onwuka (2005).

Packed bulk density = Weight of sample (g) / Vol. of sample (ml)

Wettability of the powder was measured by releasing 1g of the ready to reconstitute probiotic mix from an inverted 25ml graduated cylinder over 500 ml of distilled water taken in a beaker below. The time taken for the powder to become completely wet was noted (Onwuka, 2005).

Insolubility index of the ready to reconstitute probiotic mixwas determined by ADMI (1971) method. 14g of the powder was mixed in 100ml water and allowed to stand for 10 secs. 50ml of the liquid was centrifuged for 5minutes. Supernatant was decanted, the residue washed with water and again centrifuged for 5 minutes. The volume of the sediment at the bottom of the tube was noted as insolubility index.

The freeze dried probiotic product was stored in sterile airtight containers at refrigeration temperature. The viability of the culture in the probiotic food mix was assessed thoursoughout the storage period.

3.12 Feasibility of the developed products

The cost involved in the production of the thoursee products namely, probiotic supplement, probiotic supplement capsule and read-to-reconstitute mix was calculated. The costs involved in the procurement of raw materials, processing,

equipment utility charges and electricity charges were taken into account while calculating the cost. The most feasible product was chosen for the biological study.

Phase 4: Efficacy of the probiotic supplement in altering gut microflora

3.13. Conduct of feeding trial on Sprague dawley rats

A dietary intervention study was conducted on Sprague dawley rats to assess the impact of the developed probiotic supplement. The intervention study was conducted in 3 phases that lasted 15 days: a 1 day adaptation period, a 7 day supplementation period and a 7 day follow up period to assess the persistent effect of the intervention post withdrawal of the probiotic supplement. Approval of the Institutional Review Board was obtained prior to the study (Appendix XXIX).

3.13.1 Selection of animal

Tweleve adult Sprague Dawley rats from both genders weighing between 120-150 g was obtained from the Small Animal Breeding House, Kerala Veterinary and Animal Sciences University, Mannuthy. They were housed in individual cages and kept in a well ventilated room with 12 hours light and dark cycles. Guidelines for the care, maintenance and handling were strictly adhered to during the conduct of the study.

3.13.2 Feeding of the developed probiotic supplement

The rats were randomly divided into 2 groups i.e experimental (Plate 4) and control (Plate 5) group ensuring both groups had equal number of male and female rats . A basal diet consisting of wheat, gingelly oil cake, wheat bran, soyabean cake, mineral and vitamin supplements formulated institutionally was fed to the animals in both groups. The control group were maintained and fed according to the standard protocol followed at the Small Animal Breeding House. The experimental group was additionally fed 1 ml of the newly developed probiotic supplement, twice a day for a period of one week. The actual food intake of the animal was recorded every day. The rats had free access to water thoursoughout the period of intervention.

3.13.3 Analysis of the effect on the gut flora

The effect of feeding of the developed probiotic supplement was done by observing the change in the fecal micro flora before and after the intervention and during the follow up period. Fresh feces were collected under sterile conditions and suspended in 1% peptone broth until evaluation. The *Lactobacillus* and *E.coli* count was enumerated by plating after serially diluting the samples. The plates were incubated at 37°C for 48hours and 24 hours to enumerate *Lactobacillus* and *E.coli* respectively.

Plate 2 - Slurry of individual plant foods prior to autoclaving

Plate 3 - Operon freeze drier in operation

Plate 4: Representative rats from experimental group

Plate 5: Representative rats from control group

3.14 Data management and analysis

3.14.1. Scores and Indices and regression equations used /developed

3.14.1.1. Relative growth score

The relative growth score of the selected plant foods were calculated using the following equation

{(probiotic logcfu/mL on plant food at 24h - probiotic logcfu/mL on plant food at 0 h) (prebiotic log cfu/mL on glucose at 24 h - probiotic log cfu/mL on the glucose at 0 h)}

-{(enteric log cfu/mL on plant food at 24 h - enteric log cfu/mL on plant food at 0 h) (enteric log cfu/mL on glucose at 24 h - enteric logcfu/mL on the glucose at 0 h)}

3.14.1.2 Prebiotic activity score

The prebiotic activity score was determined using the equation described by Huebner *et al*. (2007).

Prebiotic activity score =

{(probiotic logcfu/mL on the prebiotic at 24h -probiotic logcfu/mL on the prebiotic at 0 h) (prebiotic log cfu/mL on glucose at 24 h - probiotic log cfu/mL on the glucose at 0 h)}

- {(enteric log cfu/mL on the prebiotic at 24 h - enteric log cfu/mL on the prebiotic at 0 h) (enteric log cfu/mL on glucose at 24 h - enteric log cfu/mL on the glucose at 0 h)}

3.14.1.3. Regression equations developed during the study

The various regression equations developed by Response Surface Methodology (RSM) during the study is given in Table 14

Table 14: Regression equations for the response parameters developed by RSM

Response parameters	Type of equation	Equation
Viable cell count (VCC)	Linear	VCC = +7.76 +0.35 X Fermentation time +0.67 X pH
Titratable Acidity(TA)	Quadratic	TA== +0.25 +0.023 X Fermentation time -6.667E-003 X pH +0.026 X Fermentation time^2 -0.084 X pH^2 -2.500E-003 X Fermentation time X pH
End pH of probiotic supplement	Quadratic	End pH = +4.40 -0.26 X Fermentation time +0.28 X pH -0.089 X Fermentation time^2 +0.44 X pH^2 -0.18 X Fermentation time X pH
Wheying off	Quadratic	Wheying off = +24.54598 -0.42002 X Fermentation time -8.80632 X pH +0.018774 X Fermentation time^2 +1.01897 X pH^2 -8.33333E-003 X Fermentation time X pH
Mouthfeel	Quadratic	Mouthfeel =+6.86 +0.18 X Fermentation time -0.10 X pH -5.172E-003 X Fermentation time ^{2} -0.46 X pH ^{2} +0.25 X Fermentation time X pH
Taste	Quadratic	Taste = +7.10 -0.18 X Fermentation time -0.38 X pH -0.25 X Fermentation time ^{2}-0.85 X pH ^{2}+0.28 X Fermentation time X pH
Flavour	Quadratic	Flavour = +6.69 -0.033 * Fermentation time -0.10 X pH +0.27 X Fermentation time ^{2}-0.63 X pH ^{2}+0.20 X Fermentation time X pH
Over All Acceptability Score(OAAS)	Quadratic	OAAS = +7.29-0.083 X Fermentation time -0.33 X pH-0.33 X Fermentation time ^{2}-0.98 X pH^2+0.18 X Fermentation time X pH

3.14.1.4 Data Analysis

The data was entered in excel format and was exported and analyzed using SPSS (Version 22).Various analytical measures used to interpret the data included cluster analysis and tests for significance such as student's t test .

Analysis of variance (ANOVA) and multiple regression analysis were conducted using Design Expert Software version 11 (Stat-ease Inc., Minneapolis, USA). Response Surface Methodology software version 6 was used to examine statistical significance of responses with respect to changes in physico chemical, microbial and sensory characters.

The adequacy of developed models were determined using F values, lack-of fit test, coefficient of determination(R^2), Coefficient of variation (CV), Predicted sum of squares (PRESS) and adequate precision ratio (APR). Thoursee dimensional response surfaces were generated and numerical and graphical optimization was performed by Design Expert Software version 11 (Stat-ease Inc., Minneapolis, USA).

IV RESULTS AND DISCUSSION

The results of the study **"Development of plant based probiotic nutritional supplement to enhance gut probiotic microflora"** is presented under the following headings

Phase 1: Suitability of indigenous plant foods as substrates for selected probiotics

4.1 Selection, isolation and maintenance of purity of cultures

4.2 Probiotic properties of the cultures

4.3 Suitability of selected plant foods for the probiotic cultures

4.4 Identification of ideal plant food-probiotic combination

4.5 Probiotic growth potency of processed plant substrates

4.6 Development and standardization of probiotic nutritional supplement

Phase 2: Process optimization for the development of a probiotic nutritional supplement

4.7 Process optimization

Phase 3: Development of the probiotic nutritional supplement and its shelf life study

4.8 Development and Quality evaluation of probiotic supplement

4.9 Shelf life of the newly developed probiotic supplement

4.10 Extending the shelf life of the probiotic product

4.11 Feasibility of the developed products

Phase 4: Efficacy of the probiotic supplement in altering gut microflora

4.12 Effect of the probiotic supplement on the gut floraof rats

Phase 1: Suitability of indigenous plant foods as substrates for selected probiotics

4.1 Selection, isolation and maintenance of purity of cultures

Lactic acid bacteria (LAB) are lactic acid producing gram positive organisms that are commonly used as starter cultures in the preparation of fermented products in the dairy industry and are considered as GRAS (Generally Regarded As Safe) by the Food and Drug Administration (FDA) (Ammor *et al*., 2007). There has been an influx of probiotic products with cultures from this genus in the market. For the study, *L.casei* was isolated from the commercial probiotic product, by suspending in De Man Rogosa Sharpe (MRS) broth which was incubated and plated on MRS agar plates to isolate individual colonies. The white spindle shaped colonies of *L.casei* obtained after plating were isolated for the study. Isolations of *L casei* from dairy products have been reported by Bao *et al*. (2010). Pure cultures of *L.acidophilus LA-5* and *E.coli* were purchased and enumerated on MRS and Eosin Methylene Blue (EMB) agar plates respectively. The probiotic and enteric cultures were stored in MRS and nutrient broth correspondingly at 4^{o}C. The cultures were also preserved in 70 per cent sterile glycerol and stored at -20^{o}C for further study and were revived every fortnight.

A pure culture is one that contains only single kind of organism and is free from other microbial contaminants. Pure culture is essential to know the cultural, morphological and physiological characteristics, and functional properties of the individual culture (Pelczar *et al*., 1993). The results of the experiments conducted to test the purity of the isolated cultures are detailed in Table 15.

Table 15: Purity of the isolated cultures

Name of culture	Gram's staining Test		Catalase Test (+/-)	Oxidase Test (+/-)	Colony Characteristics
	Reaction	Morphology			
L.casei	+	violet short	-	-	White, spindle

		rods			shaped and opaque
L.acidophilus LA-5	+	violet rods	-	-	White , spindle shaped and opaque
E.coli	–	pink short rods	+	-	Pinpoint colonies with green metallic sheen

Lactobacillus casei is a gram positive, catalase negative, oxidase negative, endospore forming, rod shaped bacterium (Corsetti *et al*., 2003). *L. acidophilus* is obligate and homofermentative while *L.casei* is facultative and heterofermentative (Kandler & Weiss, 1986). From Table 15, it is clear that the probiotic cultures used in this study were both catalase and oxidase negative and violet colour rods were seen on gram's staining (Plate 6). White spindle shape colonies were noticed in the subsurface of the plate (Plate 7) that is characteristic of *Lactobacillus*. Hence the purity of the probiotic cultures was confirmed.

E.coli is a gram negative rod shaped facultative anaerobe and is commonly present in the intestine of mammals (Tenaillon *et al*., 2010). *E.coli* used in the study was catalase positive (Table 15), oxidase negative and dark pinpoint colonies appeared on the surface of the plate and had a green metallic sheen (Plate 8) which confirmed the purity of the coliform.

4.2 Probiotic properties of the cultures

A probiotic is an organism which can withstand low pH, bile salts, enzymes, and antibiotics in the human gut and should be non-pathogenic. In

the study, the cultures were subjected to acid, bile, bile salt, and antibiotic treatment and were exposed to pathogen to study the probiotic properties. The results of these experiments are presented below.

4.2.1 Acid tolerance

Probiotics in food are exposed to multiple stress factors that influence their survival during transit in the Gastro-intestinal tract (GIT) (Marteau *et al*., 1993). Upper GI is extremely acidic with a pH range of 1.5 to 3.5 (Marieb and Hoehn, 2010), where food stays for a short period of time (i.e. 3 hours) (Soliman *et al*., 2015 & Wang *et al*., 2009). A potential probiotic should survive this environment and reach the site of action i.e. the colon in large numbers. Therefore, the ability of the *Lactobacillus* cultures to tolerate and survive acidic condition for upto 3 hours was assessed by suspending the cultures in a medium with pH adjusted at different ranges. Turbidity was qualitatively observed at different time intervals to assess the cultures' survival in different pH ranges and is presented in Table 16.

Table 16: Acid tolerance of the probiotic cultures at varying time and pH levels

pH of the medium	Acid tolerance of probiotic cultures at varying time intervals							
	L.casei				*L.acidophilus* LA-5			
	0hour	1 hour	2 hour	3 hour	0	1	2	3
2.5	++**	++	++	+*	++	++	++	++

3	+++***	++	++	++	+++	++	++	++
3.5	++	++	+++	+++	+++	++	++	+++
4	+++	+++	+++	+++	+++	++	+++	+++

+ less growth **++moderate growth *+++ high growth*

L.casei exhibited moderate resistance at pH 3.5 at 0 hour that recovered to luxurious growth after 2 hours and remained the same 3 hours after incubation (Table 16). This could be due to sudden initial shock and adaptation thereafter of the culture to the acidic condition. The growth on medium with pH 4 was luxurious from the beginning and remained constant thouroughout incubation indicating high resistance. A similar trend of growth was reported by Mishra and Prasad (2005) where *L.casei* strains showed resistance to pH 3 for upto 3 hours. A gradual decline resistance was noted with increasing acidity as evident by poor growth at pH 2.5 and moderate growth at pH 3 after 3 hours indicative of less acid tolerance. Zavaglia *et al*. (2002) opined that hydrochloric acid secreted in the stomach oxidizes the biological components of microbial cell resulting in reduced growth and viability.

L.acidophilus LA-5 at all levels of pH showed only moderate resistance at 1hour that later increased with longer incubation time at pH 3.5 and 4.0 (Table 16).This could be due to adaptation of the culture to the acidic conditions in the media. Such adaptation was reported in a study by Both *et al*. (2010) where an increase in cell counts of *L.acidophilus* at 90 mins was observed after a sharp decline at 30 mins at pH 2, 3 and 4. This increase was noted only at pH 4 but not at pH 2 and 3 even after 90 mins in the study by Both *et al*. (2010). The same was exhibited by *L.acidophilus* LA-5 in this study. No change was seen at pH 2.5 and 3.0 even during the extended incubation period where the growth remained moderate. Jin *et al*. (1998) had also reported moderate survival of *Lactobacillus* at pH 3 and good survival at pH 4 when compared to pH 2 or lesser where nil or poor survival was

observed. Balasingham *et al*. (2017) found that *L.acidophilus* could tolerate a wide range of pH (3-9) and is resistant than *Bifidobacterium* spp. due to tolerance to high acidity (Boylston *et al*., 2004). Soliman *et al*. (2015) had also reported on the good acid tolerance of *L. acidophilus* and *L.casei*.

Since high acidity of stomach reach a pH of 1.5 during periods of fasting (Sahadeva *et al*., 2011) and under normal dietary conditions the stomach acidity is lower at around pH 4.5 (Wang *et al*., 2009), Liong and Shah (2005) and Hassanzadazar *et al*. (2012) set pH of 3 as a thoureshold point for acid tolerance for probiotic cultures as it simulates bacterial growth conditions in the stomach.

In the present study, both cultures, *L.casei* and *L.acidophilus* observed to be endured at pH 2.5, even after 3 hours of exposure and were acid tolerant. However the cell concentration decreased gradually from 0 hour to 3hour exposing its vulnerability at lower pH. Resistance to even lower pH of 2.0 has been observed in NCDC 17, C1 and Y strains of *L.casei* (Mishra & Prasad, 2005).

4.2.2 Bile Tolerance

Exposure to bile is one among the many stresses that probiotics have to counter in order to reach the colon in a viable form. Table 17 indicates in-vitro tolerance to bile exhibited by selected cultures.

Table 17: Tolerance of the probiotic cultures at varying time and bile salt concentrations

Bile salt-concentration	Bile Tolerance of probiotic cultures at varying time intervals							
	L.casei				*L.acidophilus LA-5*			
	0 hour	1 hour	2 hour	3 hour	0 hour	1 hour	2 hour	3 hour
0.30%	+++	+++	++	++	+++	+	NS	++
0.60%	+++	+ +	<++	NS	++	+*	NS	++

+ less growth, **++moderate growth, *+++ luxurious growth, ****NS Not significant*

Probiotics are exposed to bile of concentration ranging from 0.05% to 2% and bile is responsible for shaping the microbial diversity in the gut (Islam *et al*., 2011). Bile possesses strong antimicrobial activity as it disorganizes the cell membrane structure and triggers DNA damage (Ruiz *et al*., 2013). Hence it is one of the most crucial factors to be overcome by probiotic bacteria in order to reach the colon in large numbers.

L.casei showed good tolerance with bile and healthy growth at 0.3% bile until incubation for 1 hour (Table 17). Moderate cell growth was observed till about 2 hours exposure at 0.3% bile salt level after which it remained constant. Moderate resistance was exhibited by *L.casei* at 0.6% bile concentration. However the cell concentration showed a gradual decline with increasing duration of incubation at higher bile concentrations. Disruptions in cellular homeostasis occurs on prolonged exposure to bile salts leading to dissociation of the lipid bilayer and cell membrane protein resulting in cell leakage and cell death eventually (Mandal *et al*., 2006).

In a study by Hassanzadazar *et al*. (2012), *L.casei* and *L.plantarum* showed resistance to bile at 0.3% concentration until 4 hours. In vitro tests conducted on a novel strain of potential probiotic *L.casei Zhang* isolated from Koumiss, a fermented alcoholic beverage indicated high tolerance to bile salts similar to commercial probiotic strains *like Lactobacillus acidophilus NCFM, Lactobacillus rhamnosus GG, L. casei Shirota* and *Bifidobacterium animalis Bb12* (Guo *et al*., 2009).

Healthy growth of *L.acidophilus LA-5* was observed at 0.3% level initially (Table 17). It further decreased gradually until 2 hours and later increased at 3hours. A similar trend was observed at 0.6% bile concentration. Moderate growth was seen at the end of 3 hours in both cases. Innate adaptability to the presence of bile could be a reason for increase in growth at 3 hours. This property of *L.acidophilus* has been demonstrated earlier (Vinderola & Reinheimer, 2003). *L.acidophilus* NIT has been shown to have good bile tolerance at 1-3% concentrations by Pan *et al*. (2009).

Both *L.casei* and *L.acidophilus* LA-5 cultures exhibited resistance to bile and met one of the preconditions for probiotic cultures. This must be due to some specific defense mechanism that *Lactobacillus* must have evolved to fight against these detrimental factors.

4.2.3 Cell surface hydrophobicity (CSH)

Adhesion to hydrocarbon is an important attribute for probiotic bacteria. It determines the ability of the organism to adhere to intestinal epithelial cells (Dhewa *et al*., 2009). Higher CSH indicates greater adhesive forces and hence a higher level of adhesion (Marin *et al*., 1997). The level of adhesion to organic solvents of the two probiotic cultures was done and is shown in Table 18

Table 18: Cell surface hydrophobicity towards organic solvents

Solvent	*L.casei*	*L.acidophilus LA-5*
n-Hexadecane	27.30%	19.70%
Chloroform	70%	72.71%
Ethyl acetate	25.09%	16.76%
Xylene	33.75%	17.21%

Lactic acid bacteria occupy sites on the epithelium thus denying space for competing enteric pathogens. In this study, *L. casei* revealed 70% hydrophobicity to chloroform (Table 18). This was higher than that shown with non-polar solvents (n-hexadecane, xylene) and an acidic polar solvent, ethyl acetate (~25%). Greater affinity of *L.casei Shirota* towards chloroform, a polar solvent than non-polar solvents has been reported in a recent study (Melgar-Lalanne & Hermandez, 2015). High affinity towards chloroform and low affinity towards non polar solvents has been primarily related to the presence of exopolysaccharide in the cell wall. *L.casei* Shirota showed Exo-polysaccharide (EPS) production and presence of capsular EPS in a study that affirmed the reason for higher hydrophobicity towards chloroform (Melgar-Lalanne & Hermandez, 2015).

The basic nature of the culture, presence of carboxyl group (Bellon-Fontaine *et al*., 1996) and capsular exopolysaccharide (Melgar-Lalanne & Hermandez, 2015) is the reason attributed for its affinity with chloroform. The CSH value reported by Pelletier *et al*. (1997) for *L.casei* towards chloroform was reported to be 76.8 per cent.That is similar to the level of affinity of *L.casei* towards chloroform that has been observed in the present study.

The CSH of *L.acidophilus LA-5* (Table 18) was also highest with chloroform (73%). Affinity of *L.casei* to other solvents (16-19%) in this study was higher than that of *L.acidophilus* NCDC 15 (6-12%) reported by Dhewa *et al*. (2009). Vinderola and Reinheimer (2003) had observed higher hydrophobicity values for *L.acidophilus* (38.1 to 67.8%). Differences in the strain and experimental conditions could explain such differences.

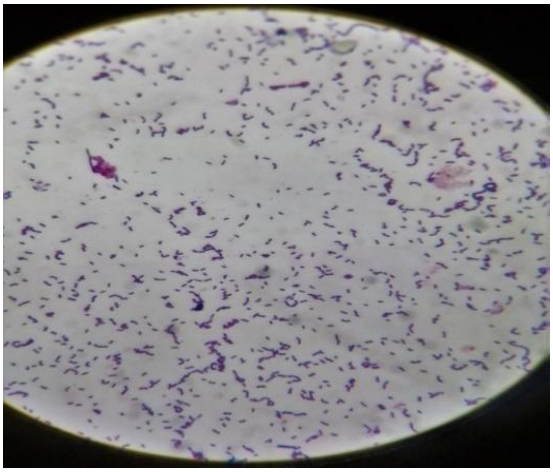

Plate 6: Microscopic image of L.casei under oil emulsion

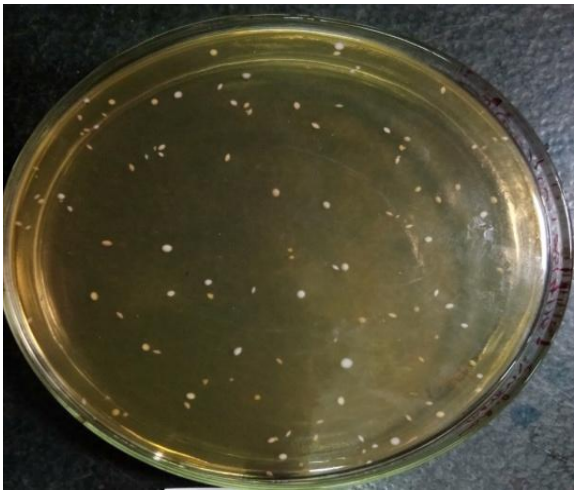

Plate 7: Subsurface white coloured spindle shaped colonies of L.casei on MRS agar plate

Plate 8: Dark pink coloured pinpoint colonies of E.coli with green metallic sheen on surface of EMB agar plate

4.2.4 Bile salt hydrolysis (BSH)

The property of probiotic strains to hydrolyze bile salts is one of the criteria in their selection. It is an indirect method of decreasing cholesterol levels in humans. (Sedláčková *et al*., 2015).

Bile salt hydrolase activity of the selected probiotic cultures was carried out and observed that *L.casei* was found to have BSH activity that was confirmed by the appearance of white opaque colonies (Plate 9).

Bile salt hydrolase activity has an influence on the cholesterol mechanism. It hydrolyses the amide bonds and liberates the deconjugated bile acids (glycine/taurine) from the steroid core (Bortolini *et al*., 1997). Appearance of opaque white colonies is indicative of BSH activity.This activity benefits the bacteria by enhancing resistance to conjugated bile salts which in turn increase their survival rate and colonization (Jones *et al*., 2008).

Even though *L. acidophilus* exhibited moderate tolerance to bile salts, no BSH activity was observed. Some studies have reported BSH negative strains of Lactobacilli capable of growing in media containing bile. This is suggestive of the fact that bile tolerance is not necessarily the outcome of only BSH production. The presence or absence of precipitate and level of deconjugation of bile salts has been reported to vary with the strain of the organism (Ramasamy *et al*., 2010).

4.2.5 Anti bio gram profile

Antibiotics affect the probiotic population in the human gut. Hence, for probiotics to thourive, they must have resistance to antibiotics. The antibiogram profile of the selected cultures to a few common antibiotics is presented in Table 19 and Figure 2 and 3

Table 19: Reaction of the probiotic cultures towards common antibiotics

Mechanism of action	Antibiotics/dosage (mcg)	*L.casei*		*L.acidophilus* LA-5	
		Size of zone (mm)	Resistant/Sensitive	Size of zone (mm)	Resistant/Sensitive
Cell wall inhibitors	Amoxyclav AMC30	17	Intermediate	22	Sensitive
	Amoxycillin AMX 10	11	Resistant	11	Resistant
	Ceftazidine CAZ30	0	Resistant	0	Resistant
	Cefpodoxime CPD 10	0	Resistant	0	Resistant
	Erythouromycin E15	24	Sensitive	39	Sensitive
	Oxacilin Ox 5	0	Resistant	13	Resistant
	Penicillin P10	0	Resistant	28	Sensitive
	Vancomycin VA30	0	Resistant	0	Resistant
Protein synthesis inhibitor	Azithromycin AzM15	19	Intermediate	30	Sensitive
	Chloramphenical C30	20	Intermediate	0	Resistant
	Gentamycin G50	22	Sensitive	22	Sensitive
	Streptomycin S10	16	Intermediate	20	Intermediate
DNA gyrase inhibitor	Rifampicin RIF 5	15	Resistant	23	Sensitive

Zone of clearance Key- Resistant (≤ 15 mm); Intermediate (16-20 mm); Sensitive (≥ 21 mm)

The cultures may be resistant, susceptible or intermediate in response to antibiotics. In general, antibiotics act by inhibiting synthesis of bacterial cell wall, protein, and folate or tDNA gyrase.

L.casei was resistant to 7 of the 13 antibiotics; namely amoxycyclin, ceftazidine, cefpodoxomine, oxacilin, penicillin, vancomycin, and rifampicin. All except for rifampicin are inhibitors of cell wall synthesis. *L.casei* displayed sensitivity to gentamycin and erythouromycin. Intermediate resistance was observed for 4 of the 13 antibiotics tested. Out of the 4, azithromycin, chloramphenical and streptomycin are protein synthesis inhibitors. *L.acidophilus LA-5* showed resistance to amoxycyllin, oxacilin,

cefpodoxomine, ceftazidine chloramphenicol, and vancomycin. It was sensitive to 6 of the 13 antibiotics; namely rifampicin, penicillin, erythromycin, azithromycin and amoxyclav. *L.acidophilus LA-5* displayed sensitivity (azithromycin, gentamycin), resistance (chloramphenicol) and intermediate resistance (streptomycin) to protein synthesis inhibitors. It has been suggested that susceptibility of *L.acidophilus* to vancomycin be used to identify *Lactobacillus* sp. (Hamilton-Miller & Shah, 1998). However, both cultures used in this study were resistant to vancomycin. Similar resistance to vancomycin in *L.acidophilus* has been reported by Klein *et al*. (1998). In a study by Ocana *et al*. (2006) 4 of 6 lactobacilli tested were able to grow at concentrations lower than 1µg/mL of vancomycin.

The results of this study are in contrast to earlier studies that reported sensitivity of *Lactobacillus* to cell wall inhibitory antibiotics (Ammor *et al*., 2007). However, *L.casei* and *L. acidophilus* LA-5 showed resistance to 6 and 4 of the 8 cell wall inhibiting antibiotics respectively. Similar result has been reported where four isolates of *Lactobacillus* from carrot, idli batter, curd, and duck faeces showed resistance to cell wall inhibiting antibiotics namely ampicillin, cefalexin and cefixime (James *et al*., 2016). Resistance exhibited by both cultures against cephalosporin antibiotics used in this study (ceftazidine and cefpodoxime) is in agreement with the earlier reports of resistance of lactobacilli to cephalosporins (Ammor *et al*., 2007).

Variation among the two cultures with regard to cell wall inhibiting antibiotics was found with penicillin. While *L.casei* displayed resistance, *L.acidophilus LA-5* was sensitive to penicillin. Figures 2 and 3 shows the reaction of *L.casei* and *L.acidophilus LA-5* towards common antibiotics

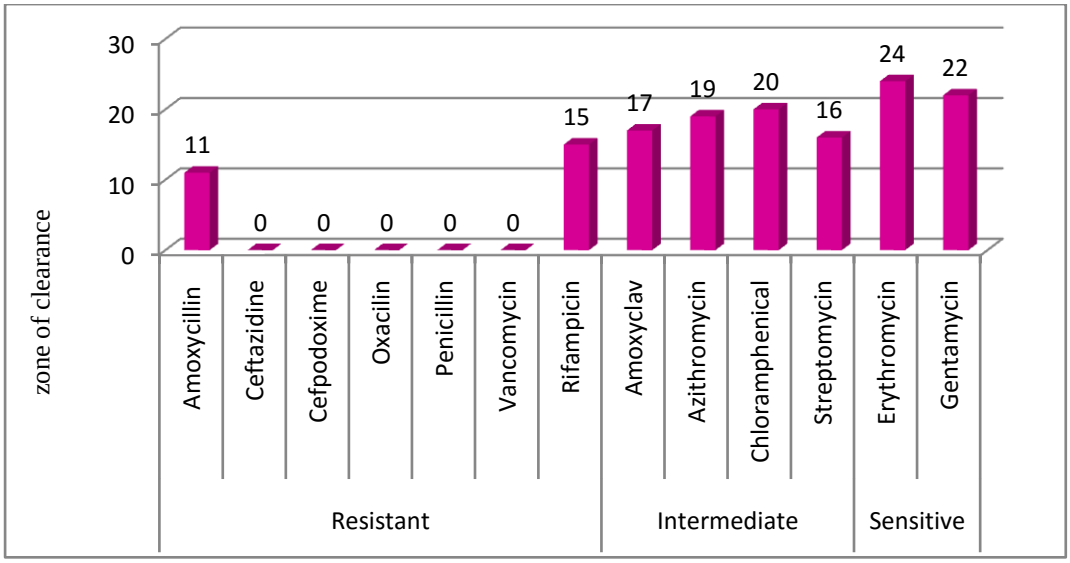

Fig 2: Reaction of *L.casei* towards common antibiotics

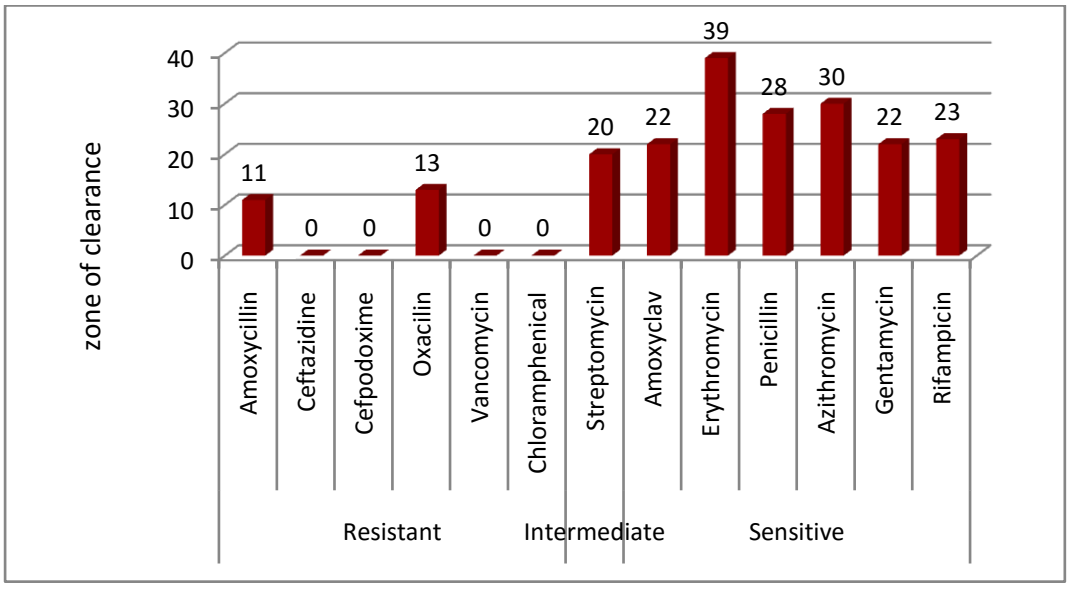

Fig 3: Reaction of *L.acidophilus LA-5* towards common antibiotics

4.2.6 Anti-pathogenic effect

The most important virtue of probiotics is to displace or destroy pathogens present in unhealthy gut and transiently establish itself in the colon. The ability of the probiotic strains to act against selected pathogens was measured.

Antimicrobial/antagonistic ability is one of the functional and beneficial effects that probiotics have on enteric pathogens. Competitive exclusion, immune modulation, low pH due to organic acids, hydrogen peroxide production, and production of antimicrobial components like bacteriocin are the different mechanisms by which probiotics exert an anti-pathogenic effect (Fijan, 2016). A cell free supernatant of the culture was suspended in wells bored on the lawn culture of the pathogen and the zone of clearance was measured after incubation

In this study, *L.casei* was antagonistic against *S.aureus* (Plate 10) and *E.coli*. The zone of clearance of *L.casei* against *S.aureus* and *E.coli* was 19mm and 14mm respectively indicating intermediate inhibition. Similar antagonism of 3 strains of *L.casei* (BF1, BF2 and BF3) against *E.coli* and *S.aureus* has been reported by Shokryazdan *et al*. (2014). In the 2014 study, the zone of clearance of *L.casei* BF1, BF2 and BF3 against *E.coli* was 14.8mm, 14mm and 13 mm respectively and against *S.aureus* was 20mm, 19.2 and 18mm respectively. Further, the thouree *L.casei* strains isolated from infant faeces displayed strong antagonistic effect to H.Pylori.

The strain of *L.acidophilus* HM1 used by Shokryazdan *et al*. (2014) displayed intermediate inhibition against *E.coli* (11mm) and low inhibition against *S.aureus* (6.7mm). Fredua-Agyeman *et al*. (2017) has also reported that the commercial *L.acidophilus LA-5* strain inhibited *Clostridium difficile*. This is in contrast to this study where *L.acidophilus LA-5* showed intermediate inhibition to both S.auerus (19 mm) (Plate 11) and *E.coli* (15mm). Differences in the strain of *L.acidophilus* could be a reason for this.

Some molecules contained in probiotic La-5 cell free spent medium fraction have been found to block/interfere with the virulence of enterohaemorrhagic *E.coli* (EHEC) (Medellin-Peña *et al.*, 2007). Tzortis *et al*. (2004) also recorded the antipathogenic activity of *L.acidophilus* against two *E.coli* strains and *Salmonella enterica* serotype *typhimurium* and attributed it to the production of antimicrobial compounds.

Both probiotic cultures showed better antagonism towards staphylococcus sp (zone 19-20mm) than *E.coli* (14-15mm).These findings are significant especially with regard to *S.aureus*. *S.aureus* has acquired resistance to many antibiotics in the past (Chambers & De Leo, 2009). Hence probiotics that were used in this study could be used to tackle *S.aureus* infections.

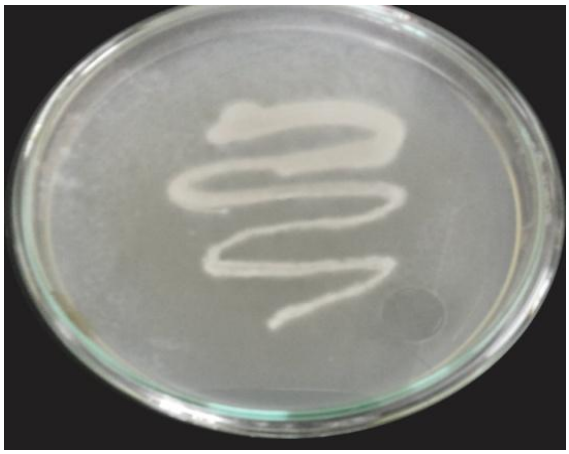

Plate 9: Positive bile salt hydrolysis by L.casei

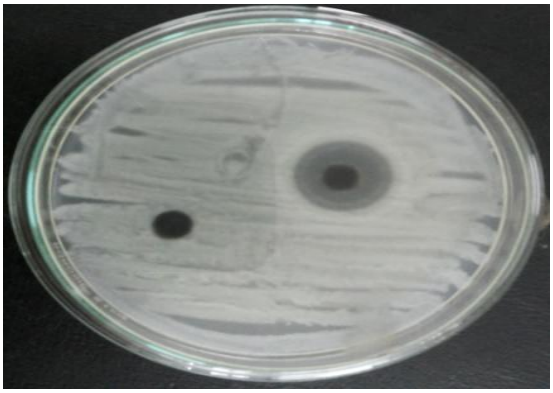

Plate 10: Zone of clearance exhibited by L.casei against S.aureus

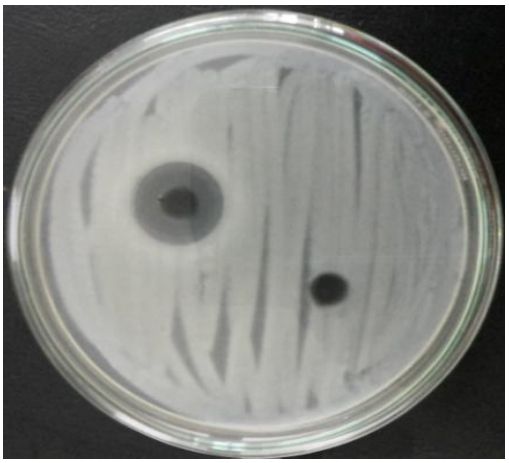

Plate 11: Zone of clearance exhibited by L.acidophilus LA-5 against S.aureus

4.2.7 Carbohydrate fermentation test

Carbohydrate source utilized by probiotics is helpful in finding an ideal medium for probiotic growth. Hence the ability of the selected strains to utilize some common sugars was measured. The observations are given in Table 20

Table 20: Carbohydrate utilization by *L.casei* and *L.acidophilus* LA-5

Carbo Hydrate*	*L.casei*							*L.acidophilus* LA-5						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	+	++	++	++	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	S.P	S.P	S.P	S.P	S.P	S.P	S.P
4	++	++	++	++	++	++	++	++	++	++	++	++	++	++
5	V.S.P	S.P	+	++	++	++	++	++	++	++	++	++	++	++
6	-	-	-	-	-	-	-	S.P	S.P	S.P	S.P	+	+	+
7	S.P	+	+	+	++	++	++	S.P	S.P	+	+	+	+	++
8	-	-	-	-	-	S.P	S.P	V.S.P	V.S.P	V.S.P	V.S.P	-	-	-
9	-	V.S.P	+	+	+	++	++	S.P	++	++	++	++	++	++
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	V.S.P	V.S.P	V.S.P	S.P	S.P	S.P	S.P	S.P	S.P	S.P	S.P	-	-	-

*1. Lactose, 2. Mannitol, 3. Arabinose, 4. Fructose, 5. Cellobiose, 6. Raffinose, 7. Sucrose, 8.Inulin, 9.Maltose, 10.Xylose, 11.Rhamnose

*V.S.P-Very slight pink, S.P-Slight pink, (-) no change, (+) moderate change, (++) dark pink

Table 20 clearly shows that, *L.casei* was able to utilize disaccharides (lactose, sucrose, cellobiose, and maltose) and monosaccharide (fructose) and sugar alcohol (mannitol). There was poor or nil utilization of rhamnose, raffinose, arabinose, xylose and inulin. A strain of *L.casei* isolated from idli batter also showed similar results in a study conducted by Sheba (2015). Gas production was seen during fermentation of lactose. This confirmed the heterofermentative nature of the organism. *L.casei* is a facultative heterofermentative organism (Kandler &Weiss, 1986).

From Table 20, it was observed that, lactose, fructose, sucrose, cellobiose and maltose were well utilized by *L.acidophilus* LA-5. *L.acidophilus* is an obligate homofermenter that produces lactic acid from fermentation of carbohydrates (Bull *et al*., 2013). Inulin, rhamnose, and mannitol were not utilized efficiently. Ozer *et al*. (2005) also reported poor utilization of inulin by *L.acidophilus* LA-5. Raffinose sugar was moderately utilized by the culture in this study. *Lactobacillus* Strain NRRL B-1910 has been found to utilize raffinose and stachyose well as measured by change in pH by Stern *et al*. (1977).Wheater (1955) tested the utilization of sugars by 29 *L.acidophilus* cultures and found that all utilized glucose, galactose, lactose, fructose, mannose, amygdalin, cellobiose, salicin and sucrose. 89% and 62% of the strains utilized maltose and raffinose. According to Barrow and Feltham (1993), *L.acidophilus* is arabinose negative. However, in the present study, the isolate was found to moderately use arabinose. Strain variation could be a reason for this.

4.3 Suitability of selected plant foods for the probiotic cultures

The suitability of plant foods in supporting growth of the probiotic cultures were assessed and the results are discussed below

4.3.1 Viable cell count

Viable cell count before and after fermentation revealed the change in the number of viable cells which is indicative of the ability of the plant food to support probiotic cultures during fermentation. The cell counts of all the cultures on the selected plant foods and the percent change after fermentation is given in Tables 21-24.

Table 21: *L.casei* count on selected plant food

Plant foods	*L.casei* (log_{10} cfu/ml)		Difference (log_{10} cfu/ml)
	0 hour	24 hour	
Eleusine coracana	7.78±0.212	8.95±0.07	1.17ns
Triticum aestivum	7.44±0.275	9.31±0.169	1.86*
Oryza sativa	8.04±0.289	7.58±0.155	-0.46ns
Avena sativa	7.72±0.169	8.20±0.134	0.48*
Hordeum vulgare	7.66±0.148	9.17±0.183	1.50*
Phaseolus aureus	7.8±0.282	8.76±0.657	0.96ns
Cicer arietinum	7.99±0.014	8.79±0.353	0.8ns
Glycine max	7.38±0.544	9.15±0.212	1.76ns
Manihot esculenta	7.8±0.282	7.68±0.304	-0.11ns
Marantha arundinaceae	5.33±0.36	7.70±0.063	2.37ns
Beta vulgaris	7.4±0.791	8.68±0.777	1.28**
Allium sativum	7.95±0.304	8.82±0.247	0.87ns
Allium cepa	7.31±0.537	8.48±0.162	1.17*
Glucose	7.57±0.601	8.07±0.098	0.49ns
Inulin	7.15±0.494	7.63±0.19	0.48ns
Skim Milk Powder	7.69±0	9.285±0.841	1.59ns

** Significant at 0.01 level; * significant at 0.05 level; ns non significant

From Table 21, it is clear that among the selected plant foods, *Marantha arundinaceae, Triticum aestivum, Glycine max, Hordeum vulgare*, and *Beta vulgaris* were found to support growth of *L.casei*. A 2 log increase was seen with *Marantha arundinaceae* and >1- <2 log increase was observed in the other above mentioned substrates. *Marantha arundinaceae* is a source of resistant starch and has a good prebiotic index of >2 (Faridah *et al*., 2017). Charalampopoulos *et al*. (2002) saw a 3

log increase in LAB count with *Triticum aestivum* substrate. Arabinoxylan oligosaccharide has been considered as the prebiotic nutrient in *Triticum aestivum* and has shown to increase *bifidobacterial* counts (Neyrinck *et al*., 2012).

High concentration of fructan tri and tetra saccharides and raffinose has been reported in *Hordeum vulgare* (Henry & Saini, 1989). *L.casei* was found to utilize *Hordeum vulgare* well with a 1.5 $\log_{10}$ cfu/ml increase in counts. Successful experiments of single and mixed cereal substrate with *Hordeum vulgare* and malt in increasing cell counts of *Lactobacillus* has been reported earlier (Rathore *et al*., 2012).

Inulin is considered a prebiotic as the lower degree of polymerization allows easy availability of carbon for probiotics to use (Van De Wiele *et al*., 2006). However, Inulin did not seem to support *L.casei* in this study as there was only <0.5 $\log_{10}$ cfu/ml increase after fermentation (Table 21). This may be due to lack of growth nutrients such as protein, vitamins, and minerals. Hence, it is ideal to use inulin as a non-nutritional additive along with a nutritional medium to reveal its prebiotic effect. Faridah *et al*. (2017) reported high prebiotic index (3.88) for inulin when added at 2.5% concentration along with MRS agar.

Skim milk powder (SMP) due to the availability of lactose sugar is the most commonly used growth medium for LAB. There was a 1.5 log increase in *L.casei* which was lower than that seen in *Hordeum vulgare* and *Glycine max* substrates. *Oryza sativa* has been used in fermented food preparations across the world. However it was not an ideal substrate for *L.casei* as a decline in the number of colonies was observed. A similar decline was observed on *Manihot esculenta* as well. A very small increase of 0.7 $\log_{10}$ cfu/ml has been reported by Moongngarm *et al*. (2011) for *Oryza sativa*. The absence of inulin and fructose oligosaccharides in *Oryza sativa* could be a reason for this. Moongngarm *et al*. (2011) also reported nil inulin and FOS in germinated and ungerminated *Oryza sativa*. Moreover, the amount of glucose, maltose, sucrose, maltotriose, and iso maltotriose in *Oryza sativa* is also less.

The ability of *L.acidophilus* LA-5 to use plant based substrate was carried out in a similar manner as *L.casei. L.acidophilus* LA-5 (Table 22) was found to use

Glycine max, Allium sativum, Beta vulgaris, Triticum aestivum, Hordeum vulgare, Phaseolus aureus and *Eleusine coracana* better than the other substrates. A difference of >1 log was seen on all the above said substrates. A similar effect of *Glycine max* oligosaccharide on *bifidobacteria* counts has been reported. Also, addition of just 0.1% soybean oligosaccharide to the medium brought about a 3-fold increase in the proportion of *bifidobacteria* to total bacteria (Saito *et al*., 1992). Soy oligosaccharides contain stachyose, raffinose and other saccharides. The higher difference in viable counts of *L.acidophilus LA- 5* on *Glycine max* may be due to its ability to utilise raffinose sugar as confirmed in the carbohydrate fermentation test (Table 20).

Table 22: *L.acidophilus* LA-5 count on selected plant foods

Plant foods	*L.acidophilus* LA-5 (log_{10}cfu/ml)		Difference (log_{10}cfu/ml)
	0 hour	24 hour	
Eleusine coracana	7.78±0.021	9.08±0.12	1.3*
Triticum aestivum	7.97±0.141	9.54±0.098	1.57*
Oryza sativa	7.95±0.007	7.66±0.933	-0.29ns
Avena sativa	7.85±0.169	8.39±0	0.54ns
Hordeum vulgare	8.03±0.049	9.19±0.275	1.16ns
Phaseolus aureus	8.14±0.424	9.39±0.07	1.25ns
Cicer arietinum	7.80±0.473	8.45±0.643	0.64ns
Glycine max	8.44±0.084	10.06±0.233	1.62ns
Manihot esculenta	8.08±0.438	8.35±0.169	0.27ns
Marantha arundinaceae	8.06±0.113	7.45±0.636	-0.61ns
Beta vulgaris	7.52±0.268	8.85±0.261	1.33**
Allium sativum	7.29±0.247	9.11±0.261	1.82ns
Allium cepa	6.98±1.209	7.4±0.848	0.41*
Glucose	8.06±0.289	8.45±0.438	0.38ns
Inulin	6.28±1.52	7.19±0.707	0.91ns
Skim Milk	7.38±0.431	8.59±0.13	1.21ns

Powder			

** Significant at 0.01 level; * significant at 0.05 level; ns non significant

There was 1.82 log increase of *L.acidophilus LA-5* on Allium *sativum* (Table 22). Moongngarm *et al*. (2011) reported a slightly higher increase of 2.5 $\log_{10}$ cfu/ml of *L.acidophilus* on *Allium sativum*. The inulin content in *Allium sativum* (41%) was attributed as the reason for its prebiotic effect.

Beta vulgaris contains high concentration of minerals (Fajkowska & Korzeniowe, 1982) and when fermented, provides high nutritive value and taste. *L.acidophilus LA-5* displayed good utilization *of Beta vulgaris* with 8.8 $\log_{10}$ cfu/ml after fermentation for 24 hours. Tomczak and Zielińska (2006) also reported > 8 $\log_{10}$ cfu/ml of *L.plantarum* when allowed to ferment *Beta vulgaris*. Yoon *et al*. (2005) observed that fermentation of *Beta vulgaris* by *L.acidophilus* produced lactic acid that brought about a rapid decline in pH.

Eleusine coracana is called poor man's milk due to its high calcium content. *L.acidophilus LA-5* count after fermentation of *Eleusine coracana* was 9 $\log_{10}$ cfu/ml (Table 22). The effectiveness of finger millet soybean combination as a medium for enhanced *L.casei* growth has been demonstrated in a study by Rodrigues *et al*. (2013). Xylo oligosaccharide and arabinose have been identified as the prebiotics in *Eleusine coracana*. *L.acidophilus LA-5* strain used in this study was found to moderately utilize arabinose (Table 20). In vitro studies have been carried out with *Lactobacillus* sp. have proved the prebiotic nature of xylooligosaccharides extracted from *Eleusine coracana* (Manisseri & Gudipati, 2012). The above results show that *Glycine max, Allium sativum, Beta vulgaris, Triticum aestivum* and *Eleusine coracana* had good prebiotic effect on *L.acidophilus*.

Table 23: *E.coli* count on selected plant substrates

Plant foods	*E.coli* (log_{10}cfu/ml)		Difference (log_{10}cfu/ml)
	0hour	24hour	
Eleusine coracana	6.83±0.332	7.82±0.247	0.99ns
Triticum aestivum	6.38±0.12	6.84±0.643	0.46ns
Oryza sativa	6.49±0.275	7.35±0.636	0.85ns
Avena sativa	6.4±0.41	8.60±0.19	2.20*
Hordeum vulgare	6.5±0.282	8.63±0.19	2.13ns
Phaseolus aureus	7.23±0.332	8.80±0.431	1.57*
Cicer arietinum	6.12±0.247	8.15±0.212	2.02ns
Glycine max	5.25±0.353	8.99±0.134	3.74ns
Manihot esculenta	5.89±0.289	7.98±0.12	2.09ns
Marantha arundinaceae	6.25±0.494	7.66±0.763	1.41ns
Beta vulgaris	6.29±0.975	6.96±0.268	0.57ns
Allium sativum	6.10±0.275	7.74±0.226	1.63**
Allium cepa	6.27±0.381	6.73±0.374	0.46*
Glucose	6.67±0.466	8.57±0.806	1.9ns
Inulin	6.13±1.173	6.97±0.707	0.84ns
Skim Milk Powder	5.88±0.586	8.42±0.601	2.54ns

** Significant at 0.01 level; * significant at 0.05 level; ns non significant

Enumeration of *Escherichia coli* on the substrate was carried out to identify food sources that least supported *E.coli*. The cell densities of *E.coli* grown on food substrates should be low relative to the growth on glucose. From Table 23, it was observed that among the substrates studied, *Triticum aestivum, Allium cepa, Allium sativum, Beta vulgaris, Phaseolus aureus, Oryza sativa, Marantha arundinaceae* and inulin had lower cell densities than glucose (1.9). *Glycine max*, skim milk powder, *Avena sativa, Hordeum vulgare, Cicer arietinum* and *Manihot esculenta* had >2 log increase in *E.coli* at the end of 24 hours. Most of the above foods are rich sources of protein that is needed for multiplication. *E.coli* flourished on those substrates due to the high protein content.

A substrate is said to be beneficial if it supports the growth of gut friendly probiotics more than harmful disease causing enteric organisms. The percent increase in the growth of the two *Lactobacillus* strains and *E.coli* is presented in Table 24 and Fig 4.

Table 24: Percent change in viable cell counts during fermentation

Plant foods	*L.casei*	*L.acidophilus LA-5*	*E.coli*
Eleusine coracana	15%	16%	14%
Triticum aestivum	25%	19%	7.20%
Oryza sativa	-5%	-3%	13%
Avena sativa	6.20%	6%	34%
Hordeum vulgare	19%	14%	32%
Phaseolus aureus	12%	15%	21.70%
Cicer arietinum	10%	8.30%	33%
Glycine max	23%	19%	71%
Manihot esculenta	-1.47%	3%	35.40%
Marantha arundinaceae	44%	-7.50%	22.50%
Beta vulgaris	17%	17.70%	10%
Allium sativum	10.90%	24.90%	26.70%
Allium cepa	16%	5.90%	7.40%
Glucose	6.50%	4.70%	28%
Inulin	6.70%	14.40%	13%
Skim Milk Powder	20%	16%	43.10%

Table 24 shows that *Triticum aestivum, Marantha arundinaceae, Beta vulgaris, Eleusine coracana* and *Allium cepa* showed a higher increase in *L.casei* than that of *E.coli*. Among them, *Triticum aestivum and Allium cepa* showed the least growth of *E.coli* at 7.2% and 7.4% respectively. With regard to *L.acidophilus LA-5*, only *Triticum aestivum* and *Beta vulgaris* supported *L.acidophilus LA-5* more than that of *E.coli*. The percent increase in *E.coli* was greater than that of *L.acidophilus LA-5* in

rest of the substrates. A 44% increase of *L.casei* on *Marantha arundinaceae* was noted which is lower than 58-63% reported by Faridah *et al*. (2017).

4.3.2 Relative growth score

The relative growth score of probiotic bacteria on selected plant foods was measured by slightly modifying the prebiotic activity analysis detailed by Huebner *et al*. (2007). Relative growth reflects the ability of a given plant food to support the growth of an organism relative to other organisms and relative to the growth on a purified substrate such as glucose. Foods that show a higher growth rate for probiotic bacteria than that of *E.coli* reported higher positive score and are considered suitable for the probiotic bacteria.

Table 25: Relative growth score of the plant substrates

	E.coli	*L.casei*		*L. acidophilus LA-5*	
Plant foods	Growth rate	Growth rate	Relative growth score	Growth rate	Relative growth score
Eleusine coracana	0.52	2.36	1.84	3.37	2.85
Triticum aestivum	0.24	3.75	3.51	4.07	3.83
Oryza sativa	0.45	-0.93	-1.38	-0.76	-1.21
Avena sativa	1.16	0.97	-0.18	1.4	0.24
Hordeum vulgare	1.12	3.04	1.91	3.01	1.88
Phaseolus aureus	0.82	1.94	1.12	3.24	2.42
Cicer arietinum	1.06	1.61	0.55	1.67	0.6
Glycine max	1.97	3.56	1.59	4.22	2.24
Manihot esculenta	1.1	-0.23	-1.33	0.7	-0.39
Marantha arundinaceae	0.74	4.78	4.04	-1.58	-2.32
Beta vulgaris	0.3	2.58	2.28	3.46	3.16
Allium sativum	0.86	1.75	0.89	4.72	3.86
Allium cepa	0.24	2.37	2.12	1.07	0.83
Inulin	0.44	0.97	0.53	2.36	1.92
Skim Milk Powder	1.33	3.22	1.88	3.14	1.8

From Table 25, it was observed that the highest relative growth score for *L.casei* was on *Marantha arundinaceae* (4.04), followed by *Triticum aestivum* (3.51), *Beta vulgaris* (2.28) and *Allium cepa* (2.12). *L.casei* had negative scores or scores near zero on *Oryza sativa* (-1.38), *Avena sativa* (-0.18), *Manihot esculenta* (-1.33), *Inulin* (0.53) and *Cicer arietinum* (0.55). Although high growth rate of *L.casei* was also seen on *Eleusine coracana, Hordeum vulgare, Glycine max,* and skim milk powder, they had a low relative growth score when compared to the foods specified earlier due to the high growth rate of *E. coli*. This had lowered their relative growth score.

Relating to *L.acidophilus LA-5*, *Allium sativum* (3.86), *Triticum aestivum* (3.83), *Beta vulgaris* (3.16), *Eleusine coracana* (2.85) and *Phaseolus aureus* (2.42) had high relative growth scores, while *Oryza sativa, Manihot esculenta and Marantha arundinaceae* had negative relative growth scores (Table 25). *Manihot esculenta* was found to inhibit both probiotic cultures and supported the growth of gram –ve pathogen *E.coli*. Similar inhibition towards gram positive organisms of tapioca starch/decolorized hsian-tsao leaf gum (dHG) matrix with green tea extracts (GTEs) has been reported in earlier studies (Chiu & Lai, 2010). Such an inhibition was not reported on gram negative *E.coli* and *salmonella*.

Oryza sativa had a negative effect on the growth of both probiotic cultures while supporting *E.coli* growth resulting in negative relative growth scores. This result is contrary to that of Giri *et al*. (2018) who isolated *L.plantarum* from rice based fermented beverage and used it as a starter culture for the production of a fermented beverage. Rapid acidification, generation of organic acids, increased nutritional and digestibility properties were proof of its suitability on the rice media. This could be due to differences within the strains of *Lactobacillus* spp. in utilization of substrates.

Both cultures showed poor growth on inulin with relative growth scores 0.53 and 1.91 for *L.casei* and *L.acidophilus LA-5* respectively. This result is in concurrence with earlier reports where inulin exhibited high growth rates with *Bifidobacterium* (Huebner *et al*., 2007 & Gopal *et al*., 2001) than with *Lactobacillus* strains (Tsuda & Miyamoto, 2010).

The reason for good relative growth scores of the above substrates can be attributed to two factors. The growth rate of probiotic bacteria on *Triticum aestivum*, *Marantha arundinaceae*, *Beta vulgaris*, and *Allium sativum* was greater than that of *E.coli*. Additionally the probiotic cultures were found to metabolize these plant foods better than glucose.

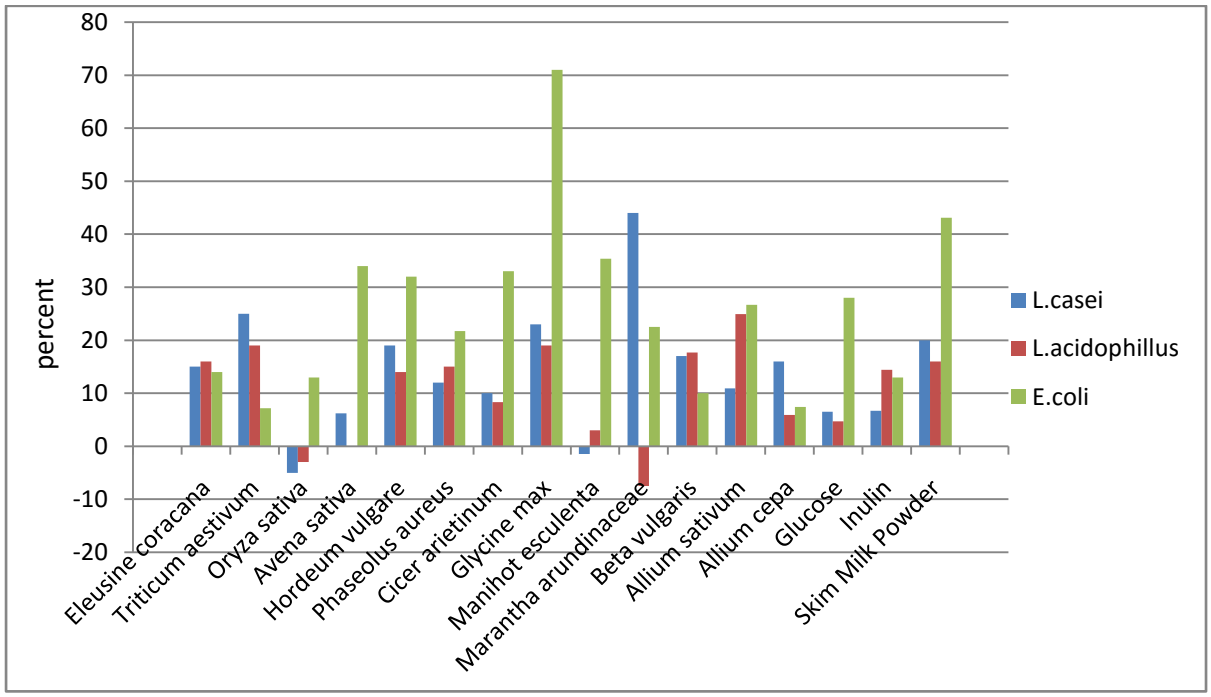

Fig 4: Percent change in viable cell counts during fermentation

4.4 Identification of ideal plant food-probiotic combination

The mean difference in the viable cell counts of the thouree cultures, for each of the selected plant food was calculated and is presented in Table 26

Table 26: Mean difference of viable cell counts on selected plant substrates

Plant foods	*L.casei*	*L.acidophilus* LA-5	*E.coli*
Eleusine coracana	1.17	1.3	0.99
Triticum aestivum	1.86	1.57	0.46
Oryza sativa	-0.465	-0.295	0.855
Avena sativa	0.485	0.54	2.205
Hordeum vulgare	1.505	1.16	2.135
Phaseolus aureus	0.965	1.25	1.57
Cicer arietinum	0.8	0.645	2.025
Glycine max	1.765	1.625	3.745
Manihot esculenta	-0.115	0.27	2.09
Marantha arundinaceae	2.37	-0.61	1.41
Beta vulgaris	1.28	1.335	0.57
Allium sativum	0.87	1.82	1.635
Allium cepa	1.175	0.415	0.465
Glucose	0.495	0.385	1.9
Inulin	0.485	0.91	0.84
Skim Milk Powder	1.595	1.21	2.54

Among the selected plant foods, *Marantha arundinaceae, Triticum aestivum, Glycine max, Hordeum vulgare*, and *Beta vulgaris* were found to support growth of *L.casei* as evident from Table 26. Among them, a 2 log increase was seen with Marantha arundinaceae and >1-<2 log increase was observed in the rest.

L.acidophilu s LA-5 was found to use *Glycine max, Allium sativum, Beta vulgaris, Triticum aestivum, and Eleusine coracana* better than the other substrates. A difference of >1 log was seen on all the above said substrates.

Enumeration of *Escherichia coli* on the substrates was carried out to identify food sources that least supported *E.coli*. Among the substrates studied, *Triticum aestivum, Allium cepa, Beta vulgaris, Phaseolus aureus, Oryza sativa, Marantha arundinaceae* and inulin had lower cell densities than glucose (1.9). *Glycine max,*

skim milk powder, *Avena sativa, Hordeum vulgare, Cicer arietinum and Manihot esculenta* had >2 log increase in *E.coli* at the end of 24 hours.

Inorder to identify ideal plant substrate - probiotic combination, plant foods that maximum supported growth of probiotic and minimum/nil/negative supported growth of E.coli were grouped into clusters based on a dendrogram (Fig 5) is presented in Table 27.

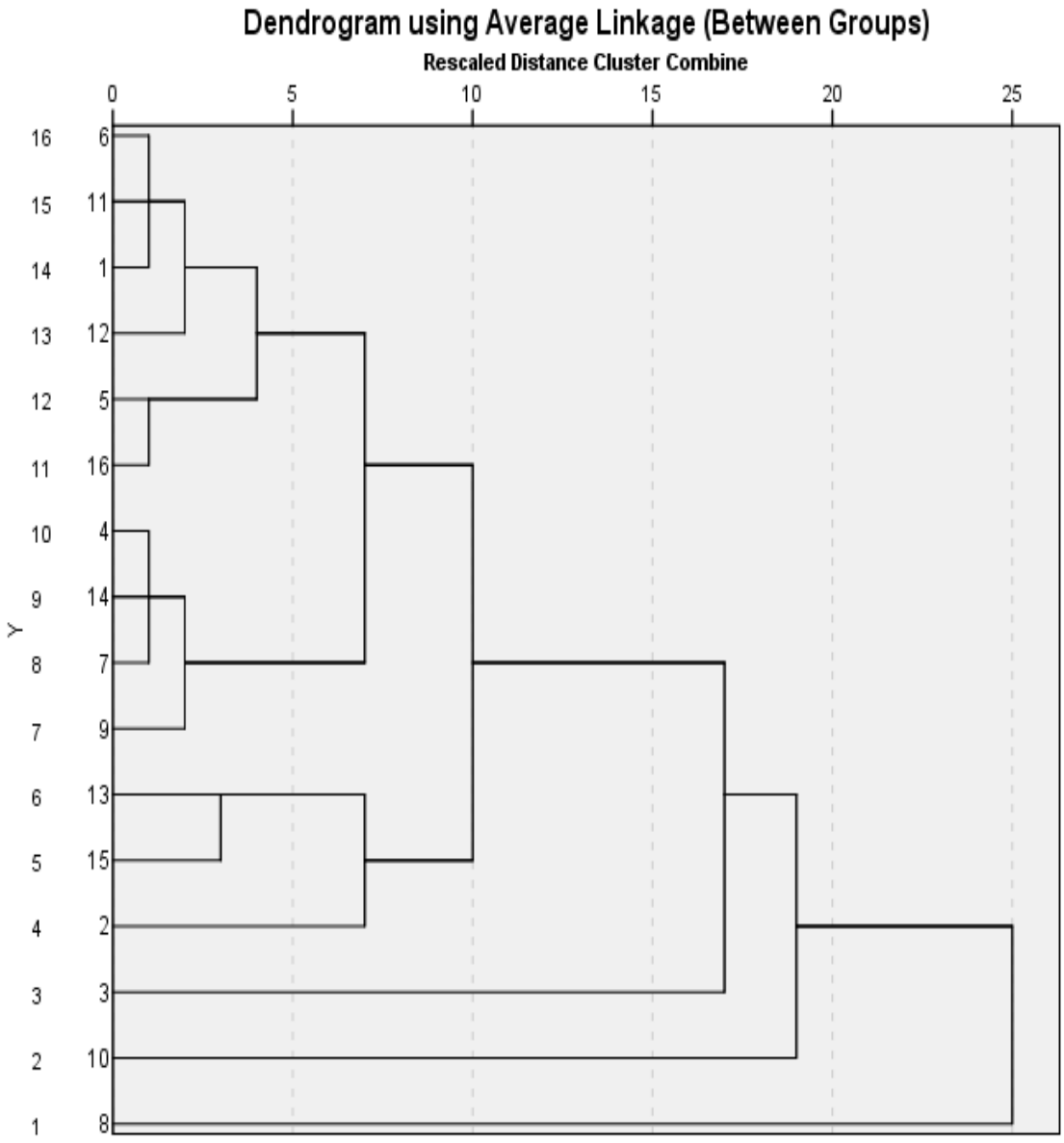

Fig 5 - Dendrogram showing dissimilarities between clusters

Table 27 – Cluster analysis of the different treatments

Cluster No.	Plant foods	Mean Difference			Cluster Mean		
		L.casei	*L.acidophilus* LA-5	*E.coli*	*L.casei*	*L.acidophilus* LA-5	*E.coli*
1	*Eleusine coracana*	1.17	1.3	0.99	1.23	1.34	1.72
1	*Avena sativa*	1.50	1.16	2.13			
1	*Phaseolus aureus*	0.96	1.25	1.57			
1	*Beta vulgaris*	1.28	1.33	0.57			
1	*Allium sativum*	0.87	1.82	1.63			
1	Skim milk powder	1.59	1.21	2.54			
2	*Triticum aestivum*	1.86	1.57	0.46	1.86	1.57	0.46
3	*Oryza sativa*	-0.46	-0.29	0.85	-0.46	-0.29	0.85
4	*Avena sativa*	0.48	0.54	2.20	0.41	0.46	2.05
4	*Manihot esculenta*	-0.11	0.27	2.09			
4	Glucose	0.49	0.38	1.9			
4	*Cicer arietinum*	0.8	0.64	2.02			
5	*Glycine max*	1.76	1.62	3.74	1.76	1.62	3.74
6	*Marantha arundinaceae*	2.37	-0.61	1.41	2.37	-0.61	1.41
7	*Allium cepa*	1.17	0.41	0.46	0.83	0.66	0.65
7	Inulin	0.48	0.91	0.84			

Table 27 shows that maximum growth (2.37 log colonies) of *L.casei* was seen on *Marantha arundinaceae* followed by *Triticum aestivum*. *L.acidophilus LA*5 had the highest growth (1.63 log colonies) on *Glycine max*. A negative count of both the probiotic cultures was observed on *Oryza sativa* (cluster 3). All plant foods in cluster 1 had shown an increase of 1 log colonies of both probiotic cultures and *E.coli*. Plant foods in cluster number 4 and 5 had a higher increase in *E.coli* count than probiotic count. A marginal and similar increase in probiotic as well as *E.coli* counts was seen in plant foods in cluster 7. Hence plant foods in cluster numbers 1, 3, 4, 5, and 7 are not ideal for product formulation.

From table 27, *Triticum aestivum* (cluster 2) seems to be an ideal substrate as it supports the growth of probiotic organisms while causing only a minimum increase in *E.coli* count. Although *Marantha arundinaceae* also supports the growth of *L.casei*, its *E.coli* count is higher than that of *Triticum aestivum* (0.46). Hence *Triticum aestivum* can be considered an ideal substrate for *L.casei*. *Triticum aestivum* supports the growth of *L.casei* more than *L.acidophilus LA*5. Hence *Triticum aestivum-L.casei* was considered as an ideal substrate-probiotic combination and was chosen as the major component for formulation of a probiotic supplement.

This finding is a reassurance that plant foods could be used as a good medium for the growth of health beneficial probiotic bacteria.

4.5 Probiotic growth potency of processed plant substrates

Triticum aestivum (Wheat) and *Marantha arundinaceae* (Arrowroot) were chosen for use in the development of the probiotic supplement due to its ability in supporting growth of *L.casei*. Both substrates were processed to improve their nutritional as well as functional properties. Wheat was malted while arrowroot starch was modified into resistant starch (RS III)

4.5.1 Prebiotic Activity Score (PAS)

The prebiotic activity score indicates the ability of a potential prebiotic substrate to support the growth of a probiotic organism relative to an enteric organism and relative to growth on a non-prebiotic substrate like glucose (Huebner *et al*., 2007). Substrate with higher score is considered to have more prebiotic activity. The prebiotic activity scores of the identified plant foods are given in Table 28.

Table 28: Prebiotic activity score of selected plant food extracts

Plant food extract		Culture	Growth percent	Growth rate	Prebiotic activity score
Wheat malt	Sample	*L.casei*	54%	1.2	0.712
		E.coli	33%	0.488	
Arrowroot RS III	Sample	*L.casei*	47%	1.25	0.89
		E.coli	20%	0.36	
Inulin	+ve control	*L.casei*	18%	1.15	0.95
		E.coli	7%	0.197	
MRSB	-ve control	*L.casei*	86%	2.91	NA
		E.coli	NA	NA	

The prebiotic activity scores (PAS) of wheat malt and arrowroot RS III were positive indicating that they are potential prebiotics. Although the growth rates of *L.casei* on both wheat malt and arrowroot RS III was similar, the PAS of arrowroot RSIII was higher than that of wheat malt.The differences exhibited in the growth rates of *E.coli* on the two substrates had produced a variation in the PAS.

The growth rates of *L.casei* on both wheat malt and arrowroot RS III extracts were lower than that exhibited on whole wheat flour and unmodified arrowroot flour as mentioned in Table 25. The process of extraction involving centrifugation and sterilization resulted in the loss of protein as sediments, leading to the decrease in growth rates. An increase in the growth rate of *E.coli* was observed on malted wheat

extract when compared to whole wheat. This could be due to the presence of easily assimilated sugars formed during malting.

The growth of *L.casei* on malted wheat was 54% when compared to 47% growth on arrowroot RS III. This difference could be due to the specific preference for one or more sugars during fermentation by the culture. Such differences due to sugars have been demonstrated by Charalampopulous *et al*. (2002), where each of the four *Lactobacillus* strains studied exhibited differences in the amount and type of sugars utilized. The growth rate of *E.coli* on arrowroot RS III (0.36) was less when compared to that on arrowroot powder (0.74) (Table 25).

Commercial prebiotic Inulin brought about an 18% growth of *L.casei* which is significantly lower than 71% reported earlier (Faridah *et al*., 2017). However, this is in accordance with earlier reports that inulin is bifidogenic in nature and is not suitable for *Lactobacillus* (Tsuda & Miyamoto, 2010). Inulin seemed to least support the growth of *E.coli* and hence had a PAS of 0.95.

The highest growth percent (86%) of *L.casei* was seen on the selective media, MRS Broth. The media contains yeast and meat extracts as well as peptone that are sources of carbon, nitrogen and vitamins, which are conducive for general bacterial growth. Yeast extract also contains vitamins and acids specifically required by *Lactobacilli* (De Man *et al*., 1960). These results prove that plant foods are potential sources of prebiotic for probiotic bacteria. Wheat and Arrowroot RS III have a great prebiotic potential especially for *L.casei*. Among the plant foods assessed, *Beta Vulgaris* is a natural source of pigment betalin and had a good relative growth score for L.casei. Hence, *Beta Vulgaris (*beetroot) was chosen to impart colour to the probiotic product.

4.6 Development and standardization of probiotic nutritional supplement

A probiotic supplement incorporating a food mix of malted wheat, arrowroot RS III and beetroot powders was intended to be developed. The standardization of the proportion of the thouree plant foods in the food mix to be used for as a substrate for

fermentation by *L.casei* in the formulation of the probiotic supplement is discussed below

4.6.1 Standardization of proportion of plant food substrates in the food mixture

The proportion of ingredients in the preparation of food mixtures is provided in Table 29 below

Table 29: Proportion of ingredients in the food mixture

Variation number	Proportion (Per cent)		
	Malted Wheat	Arrowroot RS III	Beetroot powder
V1	90	9	1
V2	60	39	1
V3	70	26	4
V4	51	47	2

Four food mixtures (Table 29) using malted wheat powder, arrowroot RS III powder and beetroot powder was prepared by dry mixing of the ingredients. The proportions of ingredients were decided based on preliminary sensory acceptability tests. The proportion of malted wheat powder and arrowroot powder ranged from 51 to 90 % and 9 to 47% respectively. Beetroot powder was incorporated in small quantities (1-4%) to enhance the appearance of the product.

4.6.2 Acceptability of the standardized food combination

Probiotic supplement was prepared using the four food mixes and evaluated on a 9-point scale by a panel of judges. Mean scores for the sensory parameters are presented below.

Table 30: Acceptability scores of the prepared variations

Variations	Sensory parameters (Mean ± SD)				
	Appearance	**Mouthfeel**	**Taste**	**Flavor**	**Overall acceptability**
Variation 1	4.25±0.28[b]	6.25±0.28[c]	4.12±0.25[c]	5.25±0.28[c]	5±0[c]
Variation 2	5.75±0.64[a]	7.12±0.25[a]	7.37±0.25[a]	7.37±0.62[a]	7.37±0.25[a]
Variation 3	5.37±0.25[a]	7.12±0.47[a]	6.87±0.47[b]	6.12±0.25[b]	6.37±0.25[b]
Variation 4	5.25±0.28[a]	6.5±0[b]	6.75±0.28[b]	6±0[b]	6.37±0.25[b]
Chi-square	10.118	10.881	11.517	13.772	13.714
p-value	0.018[*]	0.012[*]	0.009[**]	0.003[**]	0.003[**]

[a-c] *Means having different superscript differ significantly within a column,*
* *Significant at 1% level, * Significant at 5% level

Table 30 shows that with respect to appearance, all variations were rated average or below average with scores ranging from 4.25 indicating 'dislike slightly' to 5.75 indicating 'neither like nor dislike'. Variation 2 had the highest score for appearance ($p<0.05$). It did not differ significantly from the scores for variations 3 and 4. This shows that beetroot powder did not have any effect on the colour/appearance of the supplement as there was no significant difference between Variation 2, 3 and 4 that had beetroot powder in varying concentrations of 1%, 4% and 2% respectively. Variation 1 had the least score for appearance when compared to all other variations.

Variations 2 and 3 had the highest score for mouthfeel and were rated as 'liked moderately' ($p<0.05$). Variation 4 had a score of 6.5 that was significantly lesser than variations 2 and 3 but significantly higher than variation 1. Arrowroot RS III when used in moderate proportions contributed to a good mouthfeel as evident in variation 2 (39%) and 3 (26%). However, too high or too less of arrowroot resulted in poor scores for mouthfeel in variation 4 (47%) and 1 (9%).

With respect to taste, Variation 2 had a significantly higher score than all other variations and was rated as 'liked moderately' ($p<0.001$). The taste score for variation 3 was significantly lower than variation 2, but significantly higher than variation 1. Maximum flavour score of 7.37 was obtained for variation 2 that was significantly higher than variations 1, 3 and 4 ($p<0.001$). Variation 1 had significantly low scores for flavour when compared to all other variations. The higher concentration of wheat malt in variation 1 and 3made the supplement bitter and hence was low on taste and flavour scores. Although variation 2 had higher concentration of wheat malt than variation 4, it had better scores for taste and flavour. The addition of arrowroot RS III would have masked the bitterness of the wheat malt in this case.

The overall acceptability scores ranged from 5 to 7.37. Variation that had the highest amount of wheat malt (90%), i.e. variation 1 had the least overall acceptability score indicating neither like nor dislike. Variations 3 and 4 were liked slightly by the judges and did not differ significantly. The overall acceptability score of Variation 2 was significantly higher than the rest with a score of 7.37 indicating 'Liked moderately'.

Among the four variations, Variation 2 comprising 60% wheat malt, 39% arrowroot RS III, and 1% beetroot powder was adjudged the best combination for preparation of a probiotic supplement by the panel members and was chosen for the optimization process.

Phase 2: Process optimization for the development of a probiotic nutritional supplement

4.7 Process optimization

Parameters like inoculum, substrate and stabilizer concentration, heat treatment, pH and fermentation time were optimized for the development of the probiotic beverage. Response surface methodology was used to optimize pH and fermentation time.

4.7.1 Optimization of inoculum concentration

Optimization of the inoculum concentration was done by plating of cell pellets at different times of incubation (Table 31).

Table 31: Viable cell count at different incubation times

Time	Absorbance	Viable cell count (log_{10}cfu/ml)
1hour	0.071	6.6
3hour	0.096	7
5hour	0.173	7.9

A gradual increase in the cell count was observed between 1 and 5 hours. A high colony count of ~8 log cfu/ml was obtained within 5 hours of incubation. The required count of ~7 log cfu/ml was attained after 3 hours of incubation and hence was taken as ideal time for preparation of starter culture.

4.7.2 Optimization of substrate concentration

Substrates provide the required nutrients and act as a medium for the growth of probiotic bacteria. Concentration of the substrate determines the microbial and nutritional quality of the product. The probiotic supplement with a 5% and 10% concentration of the chosen food mix was prepared. The viable count was done before and after fermentation. A greater increase in lactobacillus count (~ 4 log_{10} cfu/ml) was seen on the supplement with 10% substrate concentration when compared to 5% substrate levels. Only 2 log increase was noted in the supplement prepared with 5%

substrate concentration. Hence substrate concentration at 10% was taken as the ideal level for the formulation of the probiotic supplement.

4.7.3 Optimization of stabilizer concentration

The effect of stabilizer concentration on the microbial and physicochemical parameters of the product is presented below.

Table 32: Effect of concentration of stabilizer on the product

Level of stabilizer	Sediment(ml)	Wheying off (%)	Viscosity (cP)	Mean Consistency score	Viable count (log10 cfu/ml)
Control	0.23±0.05	4.24 ± 0.25^{a}	366.6 ± 57.7^{f}	$4.83\pm0.28^{c,d}$	7.65 ± 0.05^{d}
Pectin					
0.40%	0	0^{e}	3966 ± 57.7^{a}	4.3 ± 0.28^{d}	8.71 ± 0.11^{a}
0.10%	0	3.03 ± 0.05^{c}	1433 ± 115^{c}	7.6 ± 0.28^{a}	8.37 ± 0.06^{b}
Guar gum					
1.00%	0	0^{e}	3433 ± 57.7^{b}	4.3 ± 0.28^{d}	8.03 ± 0.06^{c}
0.60%	0	2.26 ± 0.25^{d}	1166 ± 57.7^{d}	$5\pm0^{b,c}$	$7.81\pm0.10^{c,d}$
0.40%	0	3.9 ± 0.11^{b}	566.6 ± 57.7^{e}	6.16 ± 0.28^{b}	7.63 ± 0.23^{d}
F-value	-	439.68	1376.44	$15.67^{\#}$	37.32
p-value	-	<0.001	<0.001	0.008	<0.001

#Chi square value Means having different superscript differ significantly within a column

Sedimentation affects the appearance and acceptability of the food product. This can be minimized or avoided by increasing the viscosity of the product by addition of stabilizers. Stabilizers are hydrophilic particles that bind free water by its swelling action to concentrate the solids in the solution.

In this study, as evident from Table 32, both pectin and guar gum when added did not leave any sediment when compared to the control without stabilizer where slight sedimentation (0.23ml) was seen. Girish (2006) assessed the effect of incorporation of Carboxy Methyl Cellulose (CMC), pectin and combination of these

two on the scores for sedimentation of a rabadi like wheat based fermented milk beverage. The addition of stabilizers reduced the tendency of sediment formation in the rabadi-like wheat based fermented milk beverage. The effect of stabilizers was highly significant. It was noted that CMC incorporation in beverage formulation improved the sediment scores of the beverage significantly while pectin had no significant effect.

In the present study, no wheying off was observed at 0.4% pectin and 1% guargum while mild wheying off was seen at 0.1% (Table 32). This was significantly lesser than the wheying off% observed in the control and 0.4% guargum levels. A similar trend of increasing wheying off with decreasing pectin level has been reported in a rabadi-like fermented beverage with pearl millet (Modha & Pal, 2011). An inverse relation was seen between guar gum concentration and percent wheying off. An increase in wheying off was seen with decrease in guar gum concentration. Highest wheying off was observed in the control that was significantly different from all other treatment groups.

A direct relation with regard to stabilizer concentration and viscosity was also noted. The supplement became thicker with increasing levels of stabilizer. A highly viscous supplement was obtained at 0.4% pectin (3966 cP) and 1% guar gum (3433 cP) that was significantly higher than at 0.1% pectin, 0.6% and 0.4% guargum level. The control had the least viscosity of 366cP. An ideal level of viscosity was obtained at 0.1% level pectin that was similar to the viscosity of cereal based fermented beverages reported earlier (Gupta *et al*., 2010).

Consistency indicates the physical nature of the substance with respect to thick or thin, smooth or coarse, or easy or difficulty to pour. Stabilizers play a very important role as far as consistency of beverage is concerned. Data from Table 32 revealed that the scores for consistency ranged from 4.3 (dislike slightly) to 7.6 (like moderately). Maximum scores for consistency were obtained for supplement with 0.1% pectin which was significantly higher than consistency scores of all other treatments. There was lower acceptability for supplements with 0.4% guargum (Like

slightly), 0.6% guargum (neither like nor dislike), 1% guargum (dislike slightly) and 0.4% pectin (dislike slightly).

Girish (2006) compared the average consistency scores for a rabadi-like wheat based fermented beverage made with incorporation of CMC, pectin and combination of these two and reported 6.89, 7.08 and 7.17 scores respectively. The beverages prepared with combined stabilizers were significantly superior to those made with CMC alone. The incorporation of stabilizers and by increasing their levels in general increased the consistency scores significantly. Among the two stabilizers, CMC was reported to improve the consistency score significantly while no significant effect of pectin levels on consistency scores was reported.

The viable cell count of the supplement for all treatments ranged from 7.63 to 8.71cfu/ml (Table 32). The highest cell counts were observed with 0.4% pectin level. This was significantly higher than at 0.1% pectin levels. With regard to the effect of guargum, there was no significant difference in the viable cell counts between 1% and 0.6% levels and 0.6% and 0.4%. The addition, type and concentration of stabilizers did not affect the viable cell count of the supplement. This is in conjunction with the results of Ghasempour *et al*. (2011) who used zedo gum in the production of probiotic yoghurt and found no significant effect on the viability of probiotics.

In the present study, addition of pectin at 0.1% level gave a product with no sedimentation, minimal wheying off, acceptable viscosity, desired viable cell counts and good score for consistency and hence was considered as the optimal level for the development of the probiotic supplement.

4.7.4 Optimization of ideal method of heat treatment

The kind of heat treatment affects the physical as well as microbiological qualities of the product. Colour and viscosity has a great influence on the appearance, processing and acceptance of food materials (Woolfe, 1979). Betacyanin, being a

natural pigment is unstable, heat labile and degrades easily (Priatni & Praditha, 2015) in its natural form which thereby affects the appearance of the product. Hence the effect of two kinds of heat treatments i.e open pan direct heat and closed container heat treatments on the colour and viscosity was tested and is presented below.

Table 33: Effect of heat treatment on physical qualities of the product

Parameter		Open pan	Closed
Hunter's colour scale	Lightness 'L'	54.58±0.02	58.76±0.52
	Redness/ Greenness 'a'	14.43±0.04	6.04±0.28
	Yellowness/ Blueness 'b'	13.43±0.06	20.3±0.85
Viscosity (cP)		1533	1400

The open pan direct heat treatment had a better retention of red colour as evident from the higher 'a' scale value when compared to the closed container heat treatment (Table 33). However, the supplement that was heat treated in a closed container had higher 'L' and 'b' scale value. Heat treatment affects the viscosity of the product. In this study, the supplement treated in the open pan under direct heat had a higher viscosity than closed container treatment method. The higher loss of water as vapors during evaporation in open pan cooking could be a reason for higher viscosity. Since open pan treatment retained red colour compared to closed pan method, it was chosen as the ideal method of heat treatment for probiotic supplement.

Chandran *et al*. (2014) studied the degradation of beet pigment in 3 methods of cooking viz. open pan, pressure cooking and slow cooking using an Eco-cooker. The retention of colour in beetroot by open pan was the highest, followed by pressure cooking and Eco cooker. The results in the present study were also in concurrence with the above study.

4.7.5 Optimization of pH and fermentation time

Effect of varying levels of fermentation time and pH on the viable cell count, physico chemical characteristics like titratable acidity, end pH, wheying off, and sensory characteristics such as colour/ appearance, mouth feel, taste, flavour, and overall acceptability is presented in Table 34

Table 34: Microbial, physicochemical and sensory characteristics of probiotic supplement prepared at varying pH and fermentation time

Run Order	Factors		Responses								
	Fermentation Time (Hours)	pH	Cell count (log_{10}cfu/ml)	Sensory characteristics					Titratable Acidity (g/L)	End pH	Wheying off (%)
				Colour	Mouthfeel	Taste	Flavour	Overall Acceptability			
1	15	6	8.39	6.66±1.0	6.83±0.7	5.58±0.4	6.41±0.91	5.83±0.25	0.2	4.49	5.5
2	12	4	6.47	7±1.09	6.66±1.0	6.66±0.6	6.25±0.88	6.5±0.89	0.12	4.55	3
3	9	4	6	7±1.09	6.5±1.26	7±1.04	6.66±1.08	6.68±1.4	0.2	4.63	3
4	12	5	7.6	7±0.81	7.37±0.9	7.37±0.7	6.25±1.25	7.25±0.95	0.27	4.43	3.1
5	9	5	7.91	6.87±1.0	6.75±0.6	6.87±0.6	7.12±0.85	7.12±0.25	0.25	4.5	3.5
6	15	4	7.85	6.45±0.8	6.45±1.1	5.83±0.5	6.29±0.87	6.2±1.22	0.261	4.33	3
7	12	5	8.36	7.25±0.5	7±0.57	7.25±0.6	6.87±0.85	7.25±0.64	0.27	4.3	3.1
8	15	5	7.77	7.3±0.47	7.05±.82	7.05±1.4	6.92±0.86	6.72±1.21	0.28	4.27	3.4
9	12	5	8.44	7.6±0.22	6.7±0.27	7.1±0.41	6.5±0.86	7.6±0.41	0.28	4.51	3.1
10	12	5	8	7±0.35	6.7±0.27	6.8±0.27	6.8±0.75	7.2±0.5	0.28	4.3	3.1
11	12	6	8	7.3±0.57	6.2±0.27	6±0.35	6±0.35	6±0.5	0.19	5.2	5.6
12	12	5	8.34	6.8±0.27	6.6±0.22	7±0.5	7±0	7.3±0.75	0.2	4.4	3.1
13	9	6	7.9	7.6±0.41	5.9±0.54	5.6±0.41	6±0.35	5.5±0.35	0.15	5.5	5.6

Table 35: Regression coefficients and ANOVA of fitted quadratic model for the microbial and physico chemical responses for the developed supplement

Partial Coefficients	Cell count (log_{10}cfu/ml)		Titratable Acidity		end pH		Wheying off (%)	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Intercept	7.76		0.25		4.4		3.15	
A- Fermentation Time	0.35	0.15	0.02	0.19	-0.26	0.0006**	-0.03	0.513
B- pH	0.67	0.01*	0.006	0.69	0.28	0.0003**	1.28	<0.0001**
AB	-	-	0.002	0.90	-0.18	0.01*	-0.02	0.68
A^2	-	-	0.02	0.32	-0.08	0.21	0.17	0.04*
B^2	-	-	-0.08	0.01*	0.44	0.0003**	1.02	<0.0001*
Lack of fit	3.64	0.11ns	1.93	0.26ns	2.3	0.219ns	-	-
Model F value	5.68	0.02*	2.91	0.09	27.26	0.0002**	195.3	<0.0001*
R^2	0.53	-	0.67	-	0.951	-	0.99	-
Press	5.55	-	0.07	-	0.54	-	0.68	-
Adequate Precision Value	7.72	-	5.13	-	19.55	-	34.64	-

* *Significant at 1% level, * Significant at 5% level

The optimization of the level of factors i.e. pH and fermentation time was carried out based on viable cell counts, physico chemical characters like titratable acidity, end pH, wheying off and sensory characteristics such as colour/ appearance, mouth feel, taste, flavour and overall acceptability (Table 34). The quadratic models for the response variables were obtained thourough multiple linear regression analysis. The regression coefficients and ANOVA of the fitted quadratic model for microbial and physico chemical parameters (Table 35) revealed that the model F-value for all attributes except titratable acidity was significant ($p<0.01$). This indicated that the

model was a good fit. The coefficient of determination (R^2) was more than 0.80 for end pH and wheying off and adequate precision value (APV) which measures the signal to noise ratio, varied from 5.138 to 34.643 which was higher than the minimum desirable value (4.00). The final models fit well for all parameters except titratable acidity. These results suggested that the model can be used to validate the design.

4.7.5.1.1 Effect of fermentation time and pH on the viable cell count

The viable cell count for the probiotic supplement in the experimental design ranged from 6 $\log_{10}$cfu/ml to 8.44 $\log_{10}$cfu/ml (Table 34). Least count was obtained in the supplement prepared at pH 4 with 9 hours incubation time indicating that the conditions are not conducive for growth of *L.casei*. Initial pH had a significant impact on the viable cell counts in the supplement ($p<0.05$). Hydrogen ion concentration of the medium is known to have maximum influence on microbial growth. pH affects the functioning of enzymes and the transport of nutrients into the microbial cell (Panesar *et al*., 2010). Though *L.casei* can grow at pH as low as 3.5, the optimal pH reported is 6.5 (JHeimbach, 2012). However, not much difference was seen in *L.casei* growth at pH 6 (mean 8.09 $\log_{10}$ cfu/ml) and pH 5 (mean 8.05 $\log_{10}$ cfu/ml) in the study. The highest cell count was obtained in the experimental conditions of pH of 5 and 12 hour incubation period. This could be due to differences in the nature of the strain.

The regression coefficients and ANOVA of the fitted quadratic model (Table 35) revealed that the model F- value of the viable cell count (5.68) was significant at 5% level and lack of fit was found to be non-significant. Both factors, pH and fermentation time affected the viable cell counts. However, interaction effect of the two factors was non-significant. The fitted quadratic model accounted for about 53 per cent of the variation in the data as the coefficient of determination (R^2) was 0.53. The adequate precision value was 7.72 which was higher than the minimum desirable value of 4. These results suggested that the model can be used to validate the design. Figure 6 shows the response surface plots in 3-D graphs obtained for viable cell count.

4.7.5.1.2 Effect of fermentation time and pH on the titratable acidity

The titratable acidity (TA) for the probiotic supplement was in the range from 0.15 to 0.28 g/L. Supplement prepared at pH 6 with 9 hour incubation had the least TA while that prepared at pH 5 and incubated for 12 hours had the highest TA. The acidity of food increases with decrease in pH. Fermentation results in the production of lactic and other short chain fatty acids resulting in an increase in the titratable acidity (Wilkowske, 1954), thus providing an acidic taste. The model F- value (2.91) for TA was found to be non-significant, which was an indication that the final model was not good enough to explain the variation in titratable acidity. The p value corresponding to all the factors except the quadratic term pH reveal the same. Figure 7 shows the response surface plots in 3-D graphs obtained for titratable acidity

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4.7.5.1.3 Effect of fermentation time and pH on the final pH

The final pH for the probiotic supplement in the experimental design ranged from 4.3 to 5.5 (Table 34). Low final pH was obtained in the supplement prepared at a pH 5 with 12 hours incubation time while high final pH was obtained for the supplement prepared at a pH of 6 with 9 hour incubation.

The regression coefficients and ANOVA of the fitted quadratic model (Table 35) revealed that the model F- value (27.26) of pH was significant and lack of fit was found to be non-significant. Both factors, pH and fermentation time affected the end pH significantly. Soukoulis *et al*. (2007) found a linear correlation between incubation time and pH in the final product quality in the industrial manufacture of yoghurt. The interaction effect of the two factors was also found to be significant.

The fitted quadratic model accounted for about 95 per cent of the variation in the data as the coefficient of determination (R^2) was 0.95. The adequate precision value was 19.55, which was higher than the minimum desirable value of 4. These results suggested that the model can be used to explain the design. Figure 8 shows the response surface plots in 3-D graphs obtained for final pH.

4.7.5.1.4 Effect of fermentation time and pH on percent wheying off

The percent wheying off for the probiotic supplement in the experimental design ranged from 3 to 5.6 (Table 34). Minimal wheying was obtained for the supplement prepared at pH 4 with 15 hours incubation time while the maximum wheying was obtained at 6 pH and 9 hours incubation. Wheying off for the supplement prepared at pH 5 with 12 hour incubation was also on the lower side (3.1). The linear factor pH and quadratic term fermentation time and pH significantly affected the wheying off. This is contrary to that reported by Akgun *et al*. (2017). They identified starter culture to be the reason for higher syneresis in yoghurt than fermentation time and pH.

The model F- value for wheying off (195.3) was significant and lack of fit was negligible (Table 35). The coefficient of determination (R^2) was 0.99, which indicated that the fitted quadratic model accounted for more than 99 per cent of the variation in the data. The adequate precision value was 34.64 which was higher than the minimum desirable value of 4. These results suggested that the model can be used to explain the design. Figure 9 shows the response surface plots in 3-D graphs obtained for wheying off.

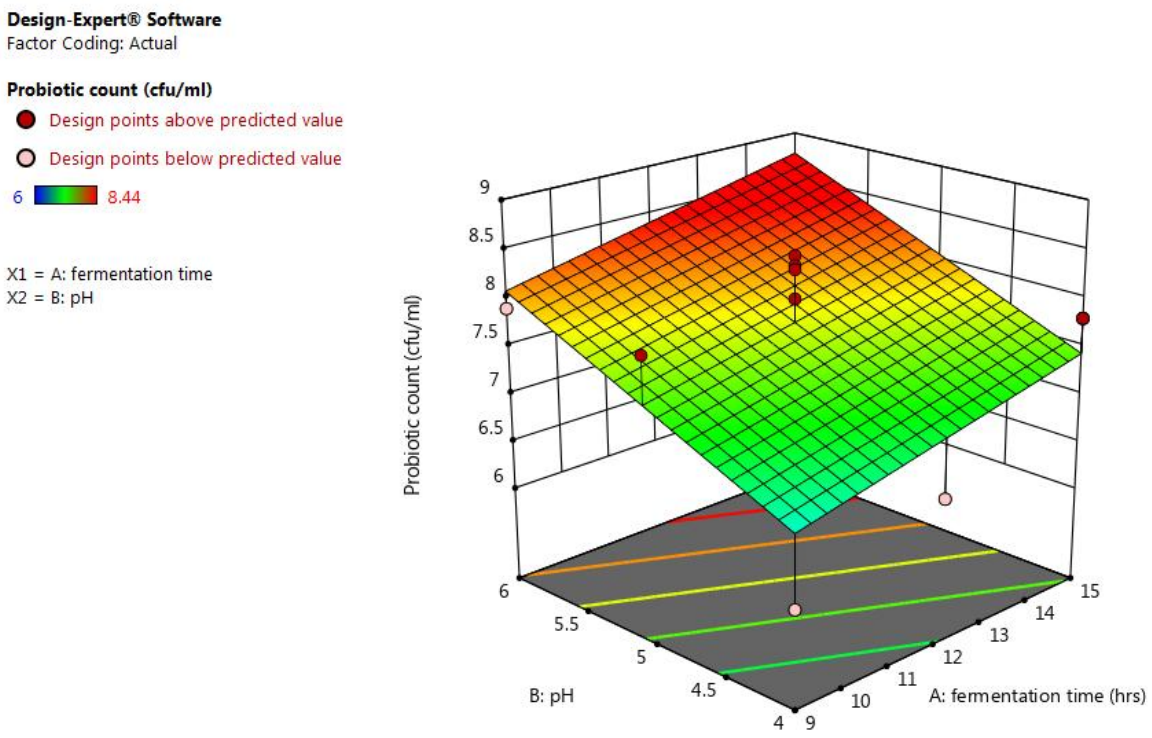

Figure 6: Effect of pH and fermentation time on viable cell count

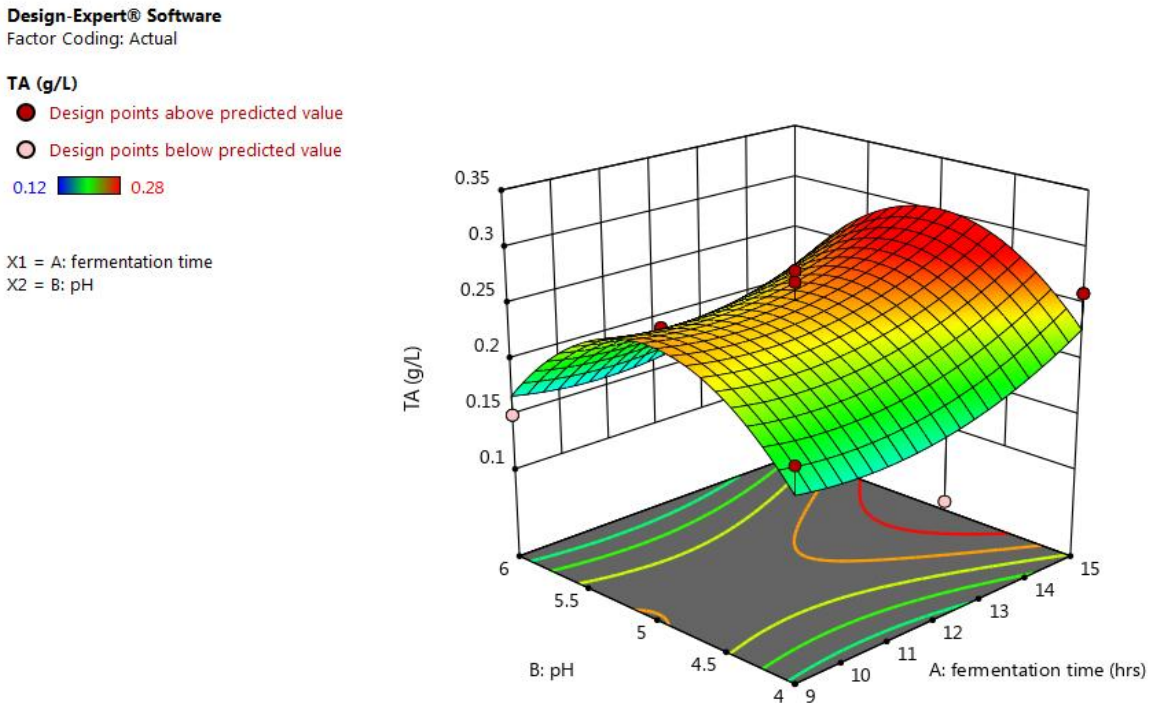

Figure 7: Effect of pH and fermentation time on titratable acidity

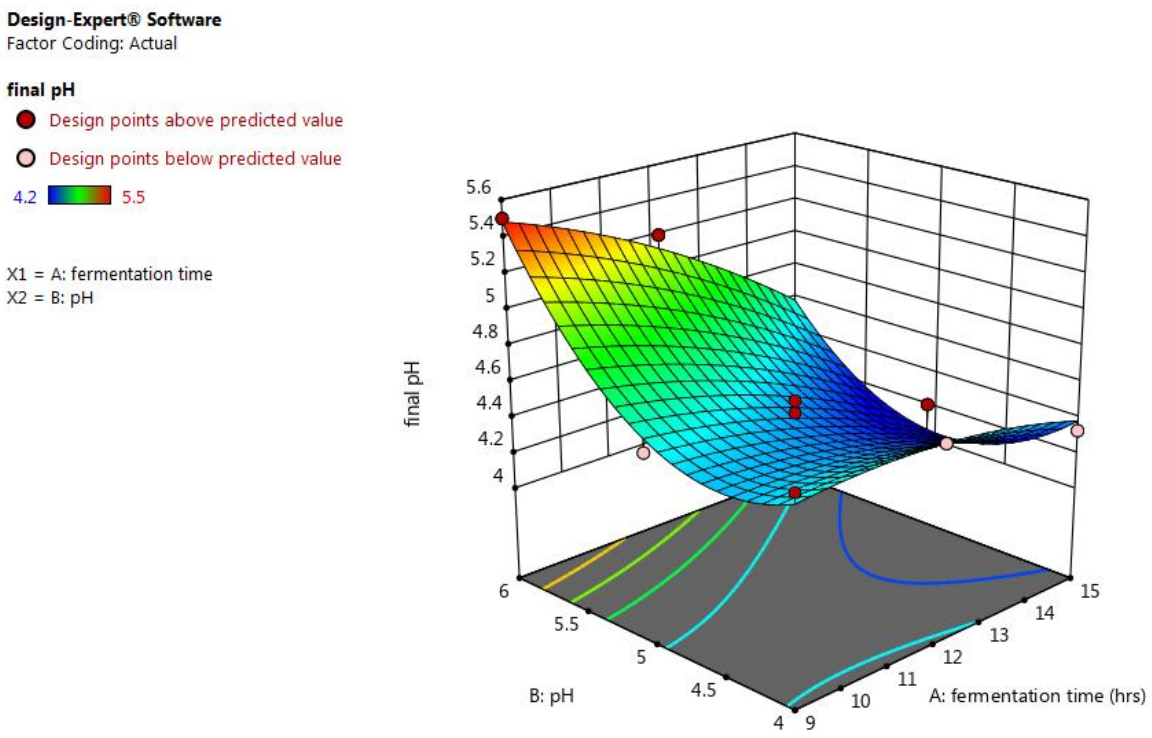

Figure 8: Effect of pH and fermentation time on final pH

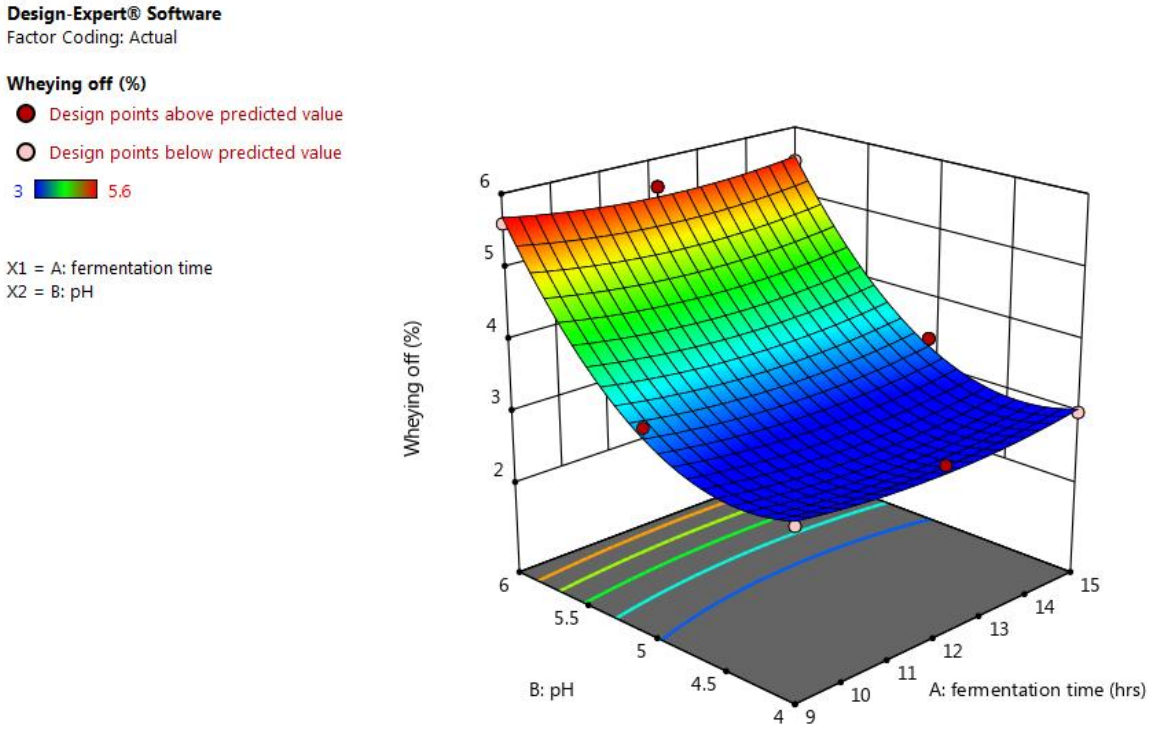

Figure 9: Effect of pH and fermentation time on percent wheying off

Table 36: Regression coefficients and ANOVA of fitted quadratic model for sensory responses for the developed supplement

Partial Coefficients	Colour		Mouthfeel		Taste		Flavour		Overall Acceptability	
	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Intercept	7.06	-	6.86	-	7.1	-	6.69	-	7.29	-
A- Fermentation Time	-	-	0.18	0.09	-0.18	0.11	-0.03	0.75	-0.08	0.25
B- pH	-	-	-0.1	0.31	-0.38	0.006**	-0.1	0.36	-0.33	0.001**
AB	-	-	0.25	0.06	0.28	0.06	0.2	0.15	0.18	0.07
A^2	-	-	0.005**	0.97	-0.25	0.13	0.27	0.12	-0.33	0.01**
B^2	-	-	-0.46	0.01**	-0.85	0.007**	-0.63	0.004	-0.98	<0.001**
Lack of fit	*1.48*	0.37^{ns}	*0.13*	*0.93^{ns}*	*2.49*	*0.19^{ns}*	*0.078*	*0.96*	*1.14*	*0.43^{ns}*
Model F value	-	-	4.55	0.03*	14.67	0.001**	4.27	0.04*	36.91	<0.001**
R^2	-	-	0.76	-	0.91	-	0.75	-	0.96	-
Press	1.76	-	0.82	-	2.98	-	0.82	-	0.96	-

* *Significant at 1% level, * Significant at 5% level

The regression coefficients and ANOVA of the fitted quadratic model for sensory characteristics (Table 36) reveals that the model F-value for all attributes except colour/ appearance was significant ($p<0.05$),which indicated that the model fitted the data well. The lack of fit was found to be non-significant for all sensory attributes. The coefficient of determination (R^2) was more than 0.80 for taste and overall acceptability and the adequate precision value (APV) which measures the signal to noise ratio, varied from 7.55 to 15.39 which was higher than the minimum desirable value (4.00). These results suggested that the model can be used to navigate the design.

4.7.5.1.2 Effect of fermentation time and pH on the Colour and Appearance

The sensory scores of appearance for different experimental design are shown in Table 34. The response values of colour/appearance for the probiotic supplement ranged from 6.45 to 7.6. Minimum score was obtained for the supplement prepared at a pH 4 with 15 hours incubation time while the maximum value was obtained at 5 pH and 12 hours incubation. The regression coefficients and ANOVA of the fitted model (Table 36) reveals that the factors did not have any significant effect on the colour/appearance score of the probiotic supplement.

4.7.5.1.3 Effect of fermentation time and pH on the Mouthfeel

The response values of mouthfeel for the probiotic supplement were in the range of 6.00 to 7.12 (Table 34). Supplement prepared at pH 6 with 9 hour incubation had the least scores while that prepared at pH 5 and incubated for 12hours had the maximum scores for mouthfeel. The regression coefficients and ANOVA of the fitted quadratic model (Table 36) reveals that the model F- value for mouthfeel (4.55) was significant and lack of fit was found to be non-significant. At quadratic level, pH alone had a significant effect on the mouthfeel score of the supplement. The coefficient of determination (R^2) was 0.76, which indicated that the fitted quadratic model accounted for more than 76 per cent of the variation in the data. The adequate precision value was found to be 7.55 which was higher than the minimum desirable value of 4. These results suggested that the model can be used to explain the design. Figure 10 shows the response surface plots in 3-D graphs obtained for mouth feel.

4.7.5.1.4 Effect of fermentation time and pH on the Taste

The sensory scores of taste for the probiotic supplement were in the range from 5.58 to 7.37 (Table 34). Supplement prepared at pH 6 with 15 hour incubation had the least scores while that prepared at pH 5 and incubated for 12hours had the

maximum scores for taste. The model F- value for taste (4.55) was significant and lack of fit was found to be non-significant (Table 36). The factor pH was found to significantly affect ($p<0.001$) the taste score of the supplement both in linear as well as quadratic terms. The coefficient of determination (R^2) was 0.91, which indicated that the fitted quadratic model accounted for more than 91 per cent of the variation in the data. The adequate precision value was 9.41 which were higher than the minimum desirable value of 4. These results suggested that the model can be used to explain the design. Figure 11 shows the response surface plots in 3-D graphs obtained for overall acceptability.

4.7.5.1.5 Effect of fermentation time and pH on the flavour

The response values of flavour for the probiotic supplement were observed in range from 6.00 to 7.12 (Table 34). The minimum value was obtained for the supplement prepared at a pH of 6 with both 9 and 12 hours incubation time while the maximum value was obtained at pH 5 and 9 hours incubation. The regression coefficients and ANOVA of the fitted quadratic model (Table 36) reveal that the model F- value for flavour (4.27) was significant and lack of fit was found to be non-significant. The quadratic term pH had a significant effect ($p<0.01$) on the flavour profile. The coefficient of determination (R^2) was 0.75, which indicated that the fitted quadratic model accounted for more than 75 per cent of the variation in the data. The adequate precision value was found to be 6.06 which was higher than the minimum desirable value of 4. These results suggested that the model can be used to explain the design. Figure 12 shows the response surface plots in 3-D graphs obtained for flavour.

4.7.5.1.6 Effect of fermentation time and pH on the Overall Acceptability

The response values of overall acceptability for the probiotic supplement ranged from 5.55 to 7.6 (Table 34). Minimum score was obtained for the supplement

prepared at pH 6 with 9 hours incubation time while the maximum value was obtained at 5 pH and 12 hours incubation. The regression coefficients and ANOVA of the fitted quadratic model (Table 36) reveals that the model F- value for overall acceptability (36.91) was significant and lack of fit was found to be non-significant. pH had a significant effect on the overall acceptability of the supplement. The effect of fermentation time on overall acceptability in linear terms and the interaction of the two factors was found to be non-significant ($p<0.05$). However at the quadratic level, both fermentation time and pH had a significant effect on the overall acceptability. The fitted quadratic model accounted for more than 96 per cent of the variation in the data as the coefficient of determination (R^2) was 0.96. The adequate precision value was 15.39 which was higher than the minimum desirable value of 4. These results suggested that the model can be used to explain the design.

Results from Table 35 and 36 clearly shows that the factor initial pH had a highly significant effect on the viable cell count, final pH, wheying off, taste score and overall acceptability scores of the supplement indicating that a slight variation in pH can have a huge effect on these responses. This is contrary to the results reported by Akgun *et al.* (2017) that incubation pH did not significantly affect the flavour scores of buffalo milk yogurt samples ($p >0 .05$). Differences in the nature of the substrate where one is dairy based and the other non-dairy could be the reason for differences in the effect of pH.

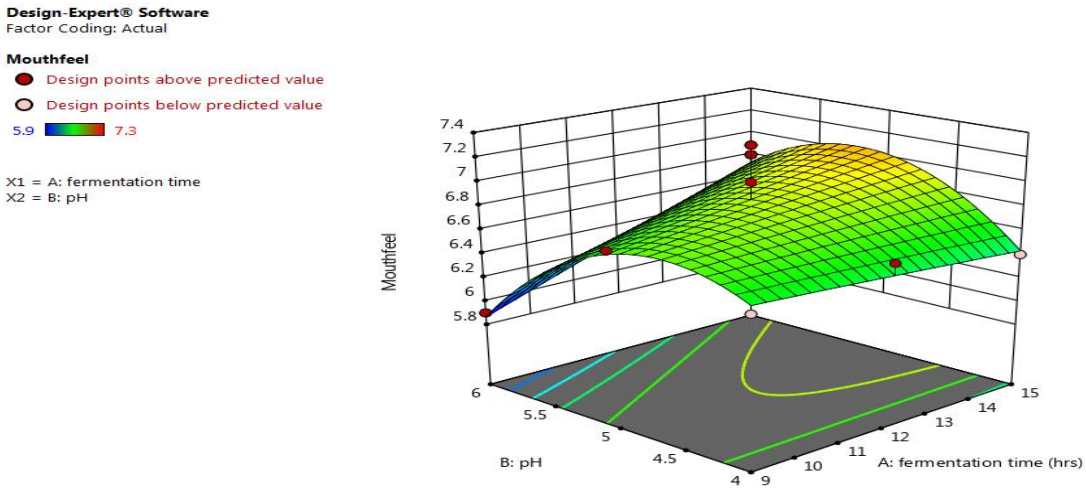

Figure 10: Effect of pH and fermentation time on mouthfeel

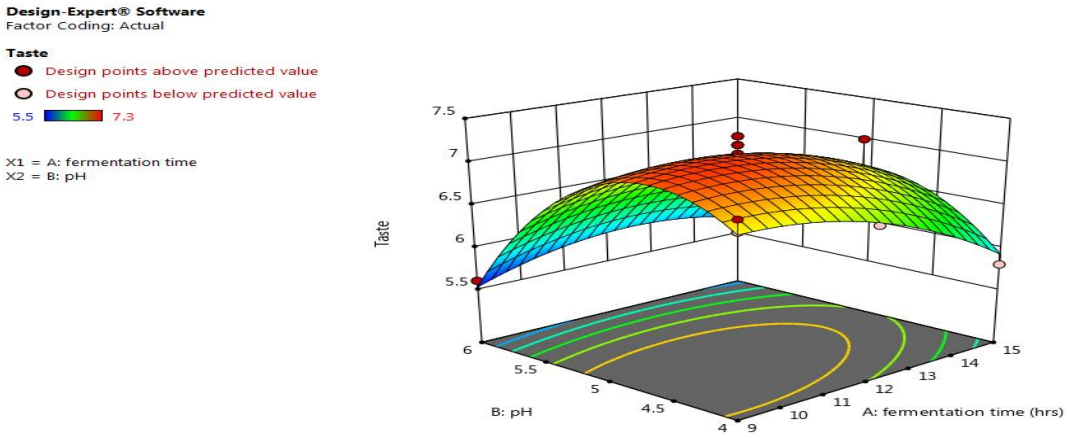

Figure 11: Effect of pH and fermentation time on taste

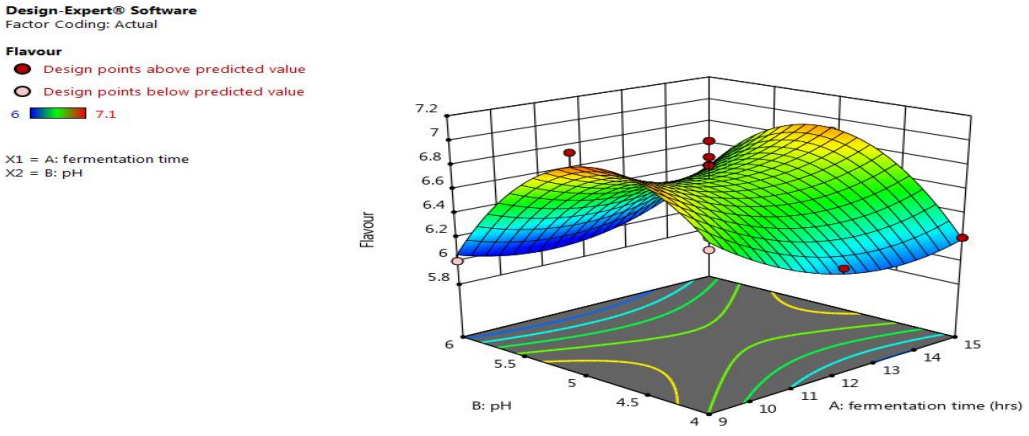

Figure 12: Effect of pH and fermentation time on flavour

4.7.5.2 Optimization and Verification of software predicted results vs actual results

Optimization of the responses was carried out using the numerical optimization technique. The pH and fermentation time that results in maximum probiotic count and sensory scores and minimal wheying off was desired for the optimization of the supplement. The validity of the predicted model for microbial, physico chemical and sensory qualities of the probiotic supplement was assessed by comparing the predicted values with the experimental values for all the parameters and is presented in the below table

Table 37: Verification of the software predicted value with observed value for the response parameters

Parameters	Predicted values	Observed values
Titratable acidity	0.25	0.26 ± 0^{ns}
Final pH	4.4	4.41 ± 0.12^{ns}
Viable cell count	8.25	8.35 ± 0.38^{ns}
Wheying off	3.1	3.05 ± 0.07^{ns}
Colour/appearance	7.05	7±0
Mouthfeel	6.85	6.75 ± 0.35^{ns}
Taste	7.1	7.25 ± 0.35^{ns}
Flavour	6.69	7±0
Overall acceptability	7.29	7.25 ± 0.35^{ns}

The results (Table 37) showed that the difference between the predicted and observed values were statistically non-significant ($p>0.05$). This showed that the predicted model for the effect of pH and fermentation time on microbial, physicochemical and sensory qualities was validated. Hence the probiotic product

with pH adjusted to 4.93 and fermented for 13.6 hours was selected for preparation of the product.

Phase 3: Development of the probiotic nutritional supplement and its shelf life study

4.8 Development and quality evaluation of probiotic supplement

The probiotic supplement was prepared under optimised condition as follows:

10% slurry of the plant substrates (6g malted wheat flour, 3.9g arrowroot RS III, 0.1g beetroot powder) was prepared with distilled water and pH was adjusted to ~ 4.93 using 20% citric acid. To it 0.1g pectin was added and sterilized by direct heat treatment at 90°C for 5 mins. The slurry was inoculated with ~ 4 log cfu/ml of *L.casei* culture and allowed to ferment for ~ 13.6 hours at 37°C (Plate 12). The nutrient content, organic acid profile, physio chemical and microbial quality of the probiotic supplement was assessed.

4.8.1 Nutrient composition of the developed product

The chemical composition of different variations of the formulated probiotic nutritional supplement was analyzed and the results are presented in Table 30.

Table 38: Proximate composition of the developed probiotic supplement and controls

Nutrient	S1 (Test Sample)	S2 (Control)	S3 (Control)	S4 (Control)
Moisture (%)	90.5 ±0.5	92.9 ±0.5	90.3±0.3	90.7 ±0.55
Ash (%)	0.61 ±0.01	0.6± 0.05	0.67 ±0.01	0.66 ±0.01
Protein (g)	1.7 ±0.011	0.7± 0.1	5.6 ±0.05	2.8 ±0.05
Fat (%)	0.03 ±0.005	0.029± 0.001	0.032 ±0.002	0.027 ±0
Crude fibre (%)	5 ±0.1	11.5 ±0.16	Nil	16 ±0.4
Starch (g)	9.43 ±0.01	13.16 ±0.01	16.4 ±0.1	17.4 ±0.08
Reducing sugars (%)	1.97 ±0.005	5.2± 0.25	Nil	2.7 ±0
Total Soluble Solids (°brix)	6.57 ±0.01	7.44 ±0.11	6.73 ±0.13	7.2± 0.09
Free Amino Nitrogen (mg/L)	98.79±5.23	177.22±1.27	82.06±3.54	154.19±1.23
In Vitro Starch Digestibility (%)	76 ±1.52	50 ±1.5	56 ±1.52	35 ±2

S1- Fermented supplement using processed foods S2- Unfermented supplement using processed foods, S3- Fermented supplement using unprocessed foods, S4- unfermented supplement using un processed foods

The moisture and ash content of all the samples (Table 30) ranged from 90.33 to 92.95% and 0.6 to 0.67% respectively. Both processing as well as fermentation did not affect moisture and ash content. It was observed that fermentation increased the protein content of the product irrespective of incident of processing. Increased protein content due to fermentation has been reported in pearl millet-soya blends (Ojokoh & Bello, 2014) and ragi (Basappa *et al*, 1997).

Emire and Buta (2015) prepared a weaning food from fermented maize and soyabean. They also reported a significant increase in crude protein content after fermentation. Moreover, the protein content of the fermented mix increased with increased duration of fermentation.

Wang (2007) found an increase in protein content and degree of protein hydrolysis in peanut flour when fermented by *L.plantarum* P9. Ojokoh and Bello, (2014) studied the effect of fermentation on nutrient composition of a millet soyabean blend and observed a huge increase in protein content in most blends after fermentation.

The increase in protein content is often a reflection of the decrease of other constituents like carbohydrates which the microorganism might have consumed for its growth (Onyango *et al*., 2005). Pranoto (2013) attributed the increase in protein content to the production of peptides and amino acids due to proteolysis of protein during fermentation.

Processing of plant foods had brought about a considerable decrease in the protein content. Wheat is the primary protein source in this supplement. Proteases produced during malting process would have broken down the protein present in the grain. Similar results have been reported by Banusha and Vasantharuba (2013) who demonstrated a significant decrease in protein content during different periods of malting of finger millet and mungbeans. Contrasting to the above results, Okporo *et al*. (2016) observed an increase in protein content of thouree varieties of sorghum after malting.

Generally, cereals and legumes are low in fat content. There was not much difference between fermented and unfermented supplements prepared using processed or unprocessed plant foods (Table 38). The findings of the present study are in agreement with those of previous workers (Sindhu & Khetarpaul, 2005 & Goyal & Khetarpaul, 1994) who observed no change due to fermentation in the fat content of cereal-legume food blends.

The crude fibre content of the fermented supplement was lower than the unfermented supplement (Table 38). This decrease may be due to solubilisation of fibre by microbial enzymes. During fermentation, the lactic acid bacteria utilize fibre as carbon source thereby reducing the fibre content (Raimbault, 1998).

A decrease in the starch content was noted after fermentation (Table 38). This suggests that *L.casei* had utilized the starch as an energy source. Elkhalifa *et al*. (2004) reported a decrease in the starch content of sorghum flour from 74.45% to 61.93% after fermentation for 36 hours.The decrease in starch content is due to the breakdown of starch to fermentable sugars by the microbial amylase.

Table 38 shows that the reducing sugars in fermented supplements were lesser than the unfermented supplements. The decrease in the reducing sugar content could be attributed to the consumption of sugars by microorganisms during fermentation. Moreover, it was noted that the reducing sugar content in supplements that were prepared using processed plant substrates was higher than supplements prepared using unprocessed ones. Malting lead to breakdown of complex carbohydrates to simpler forms thereby increasing the reducing sugar concentration. Rathore *et al* (2012) studied the total reducing sugars (TRS) in barley, malt and barley-malt combination and found media with malt and barley-malt combination to have higher initial TRS concentration when compared to raw barley. A decrease in reducing sugar content in all thouree media was reported after fermentation by *L.acidophilus.*

Sindhu *et al*. (2000) conducted a sequential fermentation of an indigenous food mixture containing rice flour, whey, sprouted green gram paste and tomato pulp (2:1:1:1, w/w) with S. boulardii + *L. casei* and S. boulardii + *L. plantarum* and observed a 58% reduction in the reducing sugar content in both fermentations.

Total soluble solids (TSS) in fermented supplements were lower than that of unfermented supplements (Table 38). Microorganisms utilize sugars from the substrates for their metabolism that could have resulted in the decrease in the TSS. Similar decrease in TSS in fermented tomato, red chilli, bottle gourd and carrot juice from 2.9, 1.1, 4.2 and 6.90Bx to 2.1, 0.4, 3.1 and 0.200Bx has been reported earlier (Thorat *et al*, 2017).

The Free Amino Nitrogen (FAN) content for unfermented supplements ranged from 154- 177 mg/L (Table 38). This decreased to 82-98mg/L after fermentation. Fermentation brought about a decrease while processing had brought about an

increase in FAN content. This could be due to partial break down of proteins into amino acids during malting. Similar to the findings of this study, Rathore *et al.* (2012) also observed a higher FAN in malt and barley-malt when compared to barley flour. Irrespective of the initial content of FAN, there was a reduction in all the thouree medias.

The digestibility of starch ranged between 35-50% in unfermented supplements (Table 38). This increased to 56-76% after fermentation. Processing affected the In vitro starch digestibility (IVSD). Supplements prepared using processed plant foods had higher IVSD than that prepared using unprocessed plant foods. These results are in concordance with that reported by Alka *et al.* (2012). Fermentation had caused a significant ($p \leq 0.05$) increase in in vitro starch digestibility of cereal flours. Increase in starch digestibility was highest for fermented sorghum (70 %), followed by pearl millet (49 %) and maize flour (41 %) respectively. Restrictions in the accessibility of starch caused by endosperm proteins (Waniska *et al.*, 1990) were reversed during fermentation making starch more accessible (Hassan & Tinay, 1995).

Fermentation had caused an increase in crude protein content and improved starch digestibility. Decrease in crude fibre, starch, reducing sugars and FAN content affirmed that the substrate has been utilized by *L.casei* for its metabolic activity. Results of the proximate analysis confirmed that the plant substrate was ideal in supporting the growth of probiotic *L.casei*. The use of right processing technique further enhanced the suitability of plant foods for probiotic bacteria.

4.8.2 Composition of short chain fatty acids

Bacterial fermentation produces several byproducts of which short chain fatty acids (SFCA) are of prime nutritional importance. The SCFA's and flavour compounds present in the probiotic supplement were identified from the National Institute of Standards and Technology (NIST) 17 mass spectral library based on the retention time of peaks (Fig 13). The details are presented in the below table

Table 39: SCFA profile and flavour compounds in the probiotic supplement

Retention Time (Mins)	Compound	Molecular formula	Peak area	Area %	Biological activity
1.6	Ethanol	CH_3CH_2OH	117255392	5.9	Energy source
4.82	Propionic acid	$CH_3CH_2CO_2H$	214567368	10.97	Hypocholesterolemic
4.15	Acetic acid	CH_3COOH	241630552	12.3	Preservative
7.35	Butyric acid	$CH_3CH_2CH_2$-COOH	137863960	7.05	Energy for colonocytes, Anti cancer property
10.8	Lactic acid	CH_3CHCO_2H	179590224	9.18	Antimicrobial
9.18	Acetoin	$C_4H_8O_2$	175360952	8.98	Flavour agent, Secondary Energy source
17.37	Cyclohexane carboxylic acid	$C_7H_{12}O_2$	243255064	12.44	Flavour agent
19.03	Ethyl 2(2 oxycyclopentyl propionate	$C_7H_{12}O_2$	254642176	13.02	-

The results of gas chouromatography- mass spectroscopy (GC-MS) analysis (Table 39) show the presence of essential organic acids butyric (7.05%), acetic (12.3%), propionic (10.97%) and lactic (9.18%) acids. The compounds identified possessed biological activities helpful in the food and pharmaceutical industry.

Propionic acid was found at 10.97% level in the probiotic supplement (Table 39). Propionate is a gluconeogenerator and has been shown to inhibit gluconeogeneis from lactate and indirectly contribute to lowering of cholesterol (Wong *et al*., 2006). Due to

this property propionate could have a possible role in prevention of carbiovascular disease.

Among the organic acids in the supplement (Table 39), acetic acid was found at highest concentration (12.3%). This is true in the case of the human gut also where acetate is the principal SCFA (Salminen *et al*., 1998). Acetate has been found to be a key player in the ability of bifidobacteria to inhibit enteropathogens (Fukuda *et al*., 2011). Acetate and propionate reach systemic circulation and is known to affect metabolism and function of peripheral organs (e.g. liver, pancreas, brain, muscle) (Peoker, 2018).

The presence of lactic acid (9.18%) confirmed fermentation by lactic acid bacteria (Table 39). Though many studies say lactic acid should be the dominant metabolite of lactic acid fermentation, it is also to be noted that lactic acids are converted to other SCFA's by microbial action during fermentation (Hernandez-Hernandez *et al*., 2011). This could be a reason for low lactic acid level in the supplement. Lactic acid has displayed good antimicrobial properties especially due to the production of bacteriocins (De Vuyst a& Leroy, 2007).

Among the SCFA's, butyric acid is considered as the major health supporting factor (Belenguer *et al*., 2008) as they are key contributors of energy to the colonocytes (Rios-Covian *et al*, 2016). Butyrate is known to prevent cancer by promoting cell differentiation, apoptosis of malignant colonocytes and inhibiting histone deacetylase enzyme (Wong *et al*., 2006). The presence of butyric acid in the newly developed beverage (Table 39) confirmed its prebiotic potential as reported by Sreenivas and Lele (2013). SCFA's in general is known to lower the luminal pH which inhibits pathogenic microorganisms and increases absorption of some nutrients (Macfarlane & Macfarlane, 2012).

Several flavour compounds are produced due to microbial, enzymatic, or chemical transformations of lactose, lipids, citric acid, and proteins/amino acids during fermentation (Boelrijk *et al*., 2003). Acetaldehyde, diacetyl and acetoin are the most common flavour compounds. In this study, acetoin (8.98%) and cyclohexane

carboxylic acid (12.44%) were the flavour compounds present (Table 39) in the newly developed probiotic supplement. Acetoin is mainly produced from citrate and lactose by the activity of lactic acid bacteria (Xaio & Lu, 2014). It is also naturally present in some fruits (Aurore *et al*., 2011) and vegetables (Sharma *et al*., 2010) and nuts (Rochat *et al*., 2000) and is responsible for natural distinct flavour. Commercially synthesised acetoin is used as a flavouring agent in bakery, alcoholic and non alcoholic beverages, gelatins and puddings (Xaio & Lu, 2014). Moreover, the accumulation of 2,3 butanediol (acetoin) during fermentation can avoid cellular acidification. In the absence of primary fermentable carbohydrates, acetoin is utilized as an alternative carbon source (Xaio *et al*., 2010).

Cyclohexane carboxylic acid is a flavouring agent and imparts an acidic, cheese and fruity taste to foods. Its flavouring effect has been well documented (Human metabalome database# HMBD0031342 & Joint FAO/WHO expert committee on food additive #961) and GRAS limits in baked goods, frozen dairy, galatins, puddings and beverages has been fixed (Oser & Ford, 1978). This compound has been used as antifibrinolytic agent after Andersson *et al*. (1965) recommended it as a useful clinical fibrinolytic inhibitor.

The relative proportion of organic acids produced during fermentation varies with the kind of substrate. However, in most fermented beverages, acetate is the major anion (Henningsson *et al*., 2001). In the present study also, acetic acid was the predominant one (Table 39). This could be due to the presence of pectin, a known source of acetic acid (Casterline *et al*., 1997) or due to secondary fermentation of lactic acid. Similar dominance of acetic acid has been reported earlier as well (Oliveira *et al*., 2012).

Fermentation of arabinogalactan and starches are known to produce propionic acid (McBurney & Thompson, 1987) and butyric acid (Bradburn *et al*., 1993) respectively. The presence of wheat and arrowroot RS III, a source of arabinogalactan and starch respectively in the probiotic supplement could have contributed to the production of propionic and butyric acids.

Plate 12: Newly developed probiotic supplement

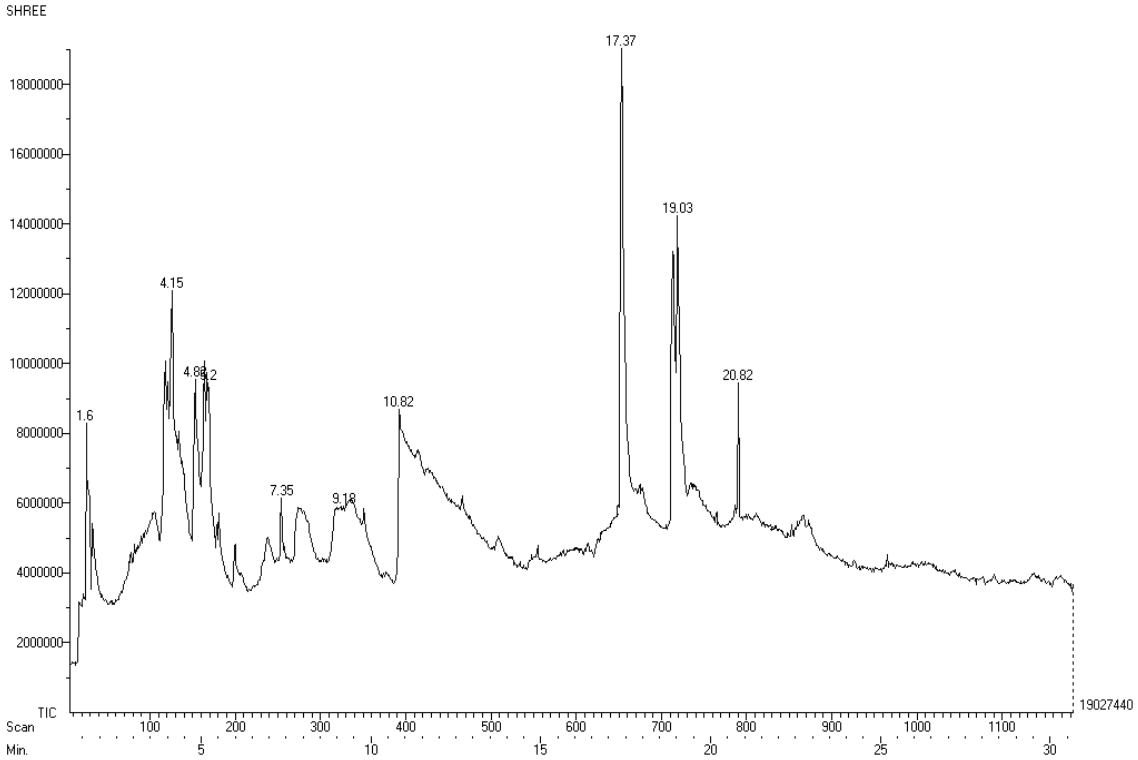

Fig 13: Chromatogram of the probiotic supplement showing peaks and retention time

4.8.3 Physico-chemical parameters of the developed product

The physical and chemical properties of the developed probiotic supplement and the controls are presented in Table 40.

Table 40: Physico chemical properties of the developed probiotic supplement

Physico chemical parameter	S1 (Test Sample)	S2 (Control)	S3 (Control)	S4 (Control)	F-value	p-value
pH	4.4	4.9	4.7	4.9		
Colour						
Lightness 'L'	54.58 ± 0.02^{b}	49.66 ± 0.07^{d}	60.11 ± 0.09^{a}	51.61 ± 0.13^{c}	3722	<0.001
Redness/ Greyness 'a'	14.43 ± 0.04^{c}	18.69 ± 0.07^{a}	5.58 ± 0.05^{d}	17.73 ± 0.06^{b}	2966	<0.001
Yellowness/ Blueness'b'	13.43 ± 0.06^{b}	11.01 ± 0.02^{d}	15.3 ± 0.07^{a}	11.52 ± 0.10^{c}	2223	<0.001
Viscosity	1533 ± 57.73^{d}	2800 ± 200^{c}	3933 ± 115.4^{b}	7333.3 ± 577.3^{a}	190.7	<0.001
Sedimentation (ml)	0	0.1	0	0		
Wheying off (%)	2.9	1.9	0	0		

S1- Fermented supplement using processed foods, S2- Unfermented supplement using processed foods,

S3- Fermented supplement using unprocessed foods, S4- unfermented supplement using un processed food

$^{a-d}$ *Means having different superscript differ significantly within a column*

From Table 40 it is clear that, a decrease in pH was observed in the probiotic supplement after fermentation. A higher decline was noted in the newly developed probiotic supplement S1 (0.5). The decrease in pH is due to the production of acids (primarily lactic acid) due to fermentation by lactic acid bacteria. This has been earlier confirmed by Nurhartadi *et al*., (2017) who observed an increase in lactic acid content corresponding to a decrease in pH during fermentation of yoghurt. Hydrogen ion concentration increases during the fermentation due to a decreasing pH level (Misrianti, 2013).

Table 40 shows that, with respect to colour, all samples had a positive score for redness (a scale) of the beet pigment. Anthocyanin is said to be stable in acidic medium (Markakis, 1982 & Bae & Suh, 2007). The medium of all the samples and control (S1) being acidic retained the redness effectively. The unfermented samples had higher retention of red colour. In case of fermented samples, a slight reduction in redness was observed. Among the fermented samples, the test sample (S1) prepared using processed substrates had significantly higher redness scale value than its unprocessed substrate (S3) ($p<0.001$). A corresponding increase in yellowness was also seen during fermentation. The Hunter's 'L' scale indicates lightness/darkness of sample. Fermented samples were found to have a higher number on L scale indicating lightness. The reduction in 'a' scale number and corresponding increase in 'b' scale number made the fermented supplement light in colour. Fermented/unfermented upplements prepared using processed substrates had lower 'L' scale number and were found to be darker when compared to fermented/unfermented supplements with unprocessed substrates. The process of malting of wheat contributed to the mild brownness in the supplements. The 'L', 'a' and 'b' scores of the test sample and three controls were significantly different from each other. The dominant colour in the probiotic supplement was red that was represented by positive value for hunter's colour 'a' (redness).

Viscosity is one of the most important determinants of food acceptability. Viscosity of the test sample (S1) (Table 40) was significantly lesser than the control

samples ($p<0.001$). Fermentation is known to cause a reduction in the viscosity of foods and has been reported earlier (Wanink *et al*, 1994 & Saalia *et al*, 2012).Wanink *et al.* (1994) observed that natural fermentations of maize, sorghum, and soyabean blend resulted in low viscous porridge and minimum viscosity of porridge fermented with pure culture of *Lactobacillus plantarum* and *Candida famata* was obtained at adjusted pH range 5.0-5.5. This is in conjunction with the present study where pH of the supplement was adjusted to 4.9. Saalia *et al*. (2012) formulated millet based porridge (Koko) as weaning food for babies. The viscosity of Koko porridge made using dough obtained by co-fermenting with malt was lower than koko made using dough that was unfermented and had no malt in it.

Table 40 indicates that the viscosity of the controls was significantly higher and thicker than the probiotic supplement with viscosity in the range of 2800cP to 7333cP ($p<0.001$). The viscosity of the supplements prepared using unprocessed substrates (S3 and S4) was significantly higher than that of supplements using processed substrates (S1 and S2). Hence in the present study, both processing as well as fermentation were found to significantly impact the viscosity of the supplement. Low viscosity in the newly developed probiotic supplement (S1) is an indication of the change in properties of cooked starch of processed arrowroot. Moreover, denaturation of protein in wheat and partial hydrolysis of starch that take place during malting of wheat could have contributed to the low viscosity. Addis *et al*. (2013) attributed this to the high presence of α-amylase in malted flours. During heat treatment, the 1, 4 α-D-glucosidic linkages of polysaccharides are hydrolyzed into lower molecular weight dextrins, thus contributing to the low viscosity of the heated paste (Dogan, 2002). Moreover, the enzyme being moderately stable to heat, gets activated and liquefies starch during cooking and found that even 5% addition of malted finger millet flour decreased the viscosity from 23733 to 450 mPas (Dogan, 2002). Similar results have been reported by Amankwah *et al*. (2009) who concluded that both malting and fermentation of maize-soya bean blend caused a reduction in the viscoamylograph indices.

Mshelisa *et al*. (2018) assessed the impact of fermentation and roasting, fermentation alone, and roasting alone of sorghum-soyabean blend on the viscosity of the porridge. The fermented and roasted sample had the least viscosity than fermentation alone, and roasted alone blends. The untreated samples had the highest viscosity.

Sedimentation is a major hurdle for beverages with high solid content. Low pH in fermented products causes sedimentation of proteins leading to wheying off during long periods of storage (Amice- Quemeneur *et al*., 1995). In the present study, no sedimentation and wheying off (Table 40) was seen in fermented (S3) and unfermented supplements (S4) prepared using unprocessed substrates. This could be due to the very high viscosity of the supplement. From Table 40 it was found that there was no sedimentation seen in the newly developed probiotic supplement (S1). The wheying off% in the probiotic supplement was 2.9% which is higher than the controls. Microorganisms' breakdown starch to smaller molecules that is soluble and unable to hold water (Cronk *et al*., 1977). The free water thus released decrease the viscosity and causes wheying off. However, the percent wheying off observed was minimal and did not affect the acceptability of the product in any way as it became homogenized with minimal agitation before consumption. The results of the present study indicated that neither fermentation nor processing of substrate seemed to affect the sedimentation rate and/ or wheying off %.

Modha and Pal (2011), successfully developed pearl millet based rabbadi like fermented milk beverage without any sediment and wheying off. Sudha *et al*. (2016) studied the effect of milk of fermented fingermillet (FM), sorghum (SM) and pearl millet (PM) on the sedimentation and wheying off in beverages and was observed that FM milk and SM milk contributed to increase sedimentation of beverages. Wheying off was observed to increase in FM milk and PM milk and decreased in SM milk (Sudha *et al*., 2016).

The probiotic supplement displayed a reduction in pH, zero sediment and minimal wheying off and had an acceptable appearance. The physico-chemical

properties of the probiotic supplement were superior to the controls in terms of all parameters.

4.8.4 Microbial count

Microbial quality is an indication of the safety of a food product. The *Lactobacillus* (LAB) count, coliform count, yeast, and mold count (YMC) of the newly developed supplement is presented in table 33.

Table 41: Microbial quality of the probiotic supplement

Parameter	S1 (Test Sample)	S2 (Control)	S3 (Control)	S4 (Control)	F-value	p
Lactobacillus count	8.88 ± 0.12^{a}	4.56 ± 0.15^{c}	7.46 ± 0.35^{b}	4.63 ± 0.15^{c}	296.60	<0.001
Coliform count	Nil	Nil	Nil	Nil	-	-
Yeast and mold count	Nil	Nil	Nil	Nil	-	-

S1- Fermented supplement using processed foods, S2- Unfermented supplement using processed foods, S3- Fermented supplement using unprocessed foods, S4- unfermented supplement using un processed food

$^{a-c}$ *Means having different superscript differ significantly within a row*

From Table 41, it is observed that the *Lactobacillus* count of the probiotic supplement (8.88 $\log_{10}$ cfu/ml) was higher than the minimum expected viable count of $>10^5$ cfu/g for a probiotic product (Shah, 1995). A ~4 $\log_{10}$cfu/ml increase was seen in the lactobacillus count of fermented supplements (S1 and S3). Zero or only marginal change in lactobacillus counts was seen in unfermented supplements ($p<0.001$). During the process of fermentation, *L.casei* utilized the substrates for their growth and increased in number. Processing of substrates had a significant effect ($p<0.001$) on the probiotic count of fermented supplements. Hence it can be concluded that both processing of substrates and the fermentation process contributed significantly to the increase in probiotic count.

No coliforms, yeast and moldwere present in any of the samples. This is indicative of the hygienic quality of the product. Inorder to exert a beneficial effect, probiotic products are expected to retain a minimum viable count of 6 $\log_{10}$ cfu/ml (Dave *et al.*, 1998). The viable cell count in the developed probiotic supplement (S1) was higher than the minimum expected. Therefore the developed supplement has the potential to offer therapeutic benefits.

4.9 Shelf life of the newly developed probiotic supplement

The shelf life of the probiotic supplement along with one control (fermented supplement prepared using unprocessed substrates) was assessed by storing at refrigeration temperature (5-7°C) and observing the changes with respect to physico-chemical, microbial and sensory parameters at weekly intervals.

4.9.1 Physio-chemical characteristics

Changes in the pH and titratable acidity during storage at refrigerated condition are depicted in Table 42

Table 42: Changes in the pH and titratable acidity during storage

Parameter	Samples	Initial	Week 1	Week 2	Week 3	p-value between
pH	Control	4.7 ± 0.1^{Ac}	5.14 ± 0.1^{Ab}	5.5 ± 0.1^{Aa}	4.7 ± 0.1^{Ac}	week=<0.001 sample=<0.001 interaction=<0.001
	Probiotic supplement	4.4 ± 0.1^{Ba}	4.4 ± 0.1^{Ba}	4.4 ± 0.1^{Ba}	3.8 ± 0.3^{Bb}	
Titratable acidity	Control	0.17 ± 0^{Bab}	0.23 ± 0.04^{Ba}	0.11 ± 0.05^{Bb}	0.11 ± 0.05^{Bb}	week =NS sample=<0.001 interaction=<0.005
	Probiotic supplement	0.37 ± 0.05^{Ab}	0.46 ± 0.05^{Aab}	0.43 ± 0.09^{Aab}	0.49 ± 0.05^{Aa}	

Means with superscript $^{A\text{-}C}$ depict difference between samples Means with superscript $^{a\text{-}c}$ depict difference between weeks

From the Table 42, it was observed that the pH of the probiotic supplement did not change significantly until week 2 ($p<0.001$). A gradual decrease to pH 3.8 was seen at week 3 ($p<0.001$). A pH level above 4.0 is generally required for a fermented beverage thouroughout storage (Gupta *et al*., 2010). The pH of the probiotic supplement remained above 4 until week 2.

In the control, there was a significant increase in the pH of the supplement at weeks 1 and 2. However by third week, the pH had become similar to that of the pH observed before storage. It was found that the pH of the probiotic supplement was significantly lower than that of the control thouroughout the storage period ($p<0.001$).

The accumulation of organic acids due to continuous growth and metabolic activity of lactic acid bacteria causes reduction in pH of fermented milks (Ruggeri *et al*., 2008). The presence of prebiotic polysaccharides and other growth promoting substances of the plant substrate might have sustained the metabolic activity of *L.casei* leading to a decrease in pH of the probiotic supplement.

In an oat based fermented beverage prepared by Gupta *et al*. (2010), there was no significant change in pH and it remained above 4 at the end of the storage period. Hussain *et al*., (2014) studied the changes in the physico chemical characteristics of Aloe barbadensis Miller supplemented probiotic lassi stored at 5±1°C and found that the pH had reduced from 3.95 to 3.45 by the 12^{th} day and the storage days significantly affected the pH of the lassi.

With regard to the titratable acidity, there was a significant increase during the course of the storage period from 0.37 to 0.49 in the probiotic supplement ($p<0.001$). This increase is expected as the pH of the supplement had also become low during storage due to metabolic activity of *L.casei* and production of organic acids. The titratable acidity of the probiotic supplement was significantly higher than the control and remained the same thouroughout the storage period ($p<0.001$).

The developed probiotic supplement had acceptable pH and titratable acidity until 2 week of storage.

Table 43: Changes in the colour during storage

Parameter	Samples	Initial	Week 1	Week 2	Week 3	p-value between
Lightness/ Darkness 'L'	Control	64.07 ± 0.03^{a}	64.78 ± 0.21^{b}	65.87 ± 0.15^{c}	67.54 ± 0.03^{d}	week=<0.001 sample=<0.001 interaction=NS
	Probiotic supplement	57.16 ± 0.02^{a}	57.58 ± 0.01^{b}	58.82 ± 0.15^{c}	60.62 ± 0.15^{d}	
Redness/ Greenness 'a'	Control	4.88 ± 0.01^{Ba}	3.98 ± 0.05^{Bb}	3.55 ± 0.11^{Bc}	3.04 ± 0.02^{Bd}	week =<0.001 sample=<0.001 interaction=<0.001
	Probiotic supplement	12.85 ± 0.02^{Aa}	12.48 ± 0.0^{Ab}	11.65 ± 0.1^{Ac}	10.03 ± 0.01^{Ad}	
Yellowness/Blueness 'b'	Control	14.74 ± 0.04^{Ans}	14.98 ± 0.1^{Ans}	14.86 ± 0.04^{Bns}	14.73 ± 0.01^{Bns}	week =<0.001 sample=<0.005 interaction=<0.001
	Probiotic supplement	13.90 ± 0.27^{Bc}	14.03 ± 0.2^{Bc}	15.05 ± 0.1^{Ab}	15.66 ± 0.08^{Aa}	

Means with superscript $^{A\text{-}C}$ depict difference between samples

Means with superscript $^{a\text{-}c}$ depict difference between weeks

. The table 43 shows that a significant increase in the ‘L’ scale value was observed in both the probiotic supplement as well as the control thouroughout the period of storage ($p<0.001$). The interaction between the two was non-significant. The pattern of variation between the weeks was the same for both the probiotic supplement as well as the control. Also the pattern of variation between the probiotic supplement and control was the same for all the weeks.

The probiotic supplement had a significantly higher ‘a’ scale value (indicating redness) than the control thouroughout the period of storage ($p<0.001$). A gradual yet significant decrease in the redness indicated by ‘a’ scale value was observed every week in both the probiotic supplement and control. With regard to the yellowness indicated by ‘b’ scale value, a significant increase (($p<0.001$) was seen in the probiotic supplement every week. This could be due to the reduction in betalin pigment of beetroot. Jagannath *et al*. (2015) have reported 50% reduction in betalin pigment during refrigerated storage. A significant decrease in ‘a’ values with a corresponding increase in the ‘b’ values has been reported during longer exposure of beetroot to heat (Chandran *et al*., 2014). Considering the fact that the red pigment, betacyanin in beetroot is unstable when compared to the yellow pigment, betaxanthins (Gokhale & Lele 2011) the change observed in this study is justified. The scale values remained positive indicating that the colour of the supplement remained red thouroughout the period of storage.

Akgun *et al*. (2017) investigated the combined effect of starter culture type and incubation final pH on the colour of buffalo milk yoghurt stored at refrigerated temperature for 20 days. No significant change was reported in the L, a, and b scale values in most yoghurt samples during the storage period. Also, the pH did not significantly affect the colour scale values.

The newly developed probiotic supplement had acceptable colour and retained mild redness thouroughout storage period

Table 44: Changes in the viscosity and water holding capacity during storage

Parameter	Samples	Initial	Week 1	Week 2	Week 3	p-value between
Viscosity(cP)	Control	15833 ± 208^{Aa}	15500 ± 100^{Ab}	15066 ± 115.4^{Ac}	13600 ± 200^{Ad}	week=<0.001 sample=<0.001 interaction=<0.001
	Probiotic supplement	1700.00 ± 00^{Ba}	1633.33 ± 57.7^{Bab}	1433.33 ± 57.7^{Bb}	1200 ± 20^{Bc}	
Water holding capacity	Control	$85.8\pm.55^{Aa}$	$75.35\pm.08^{Ab}$	$75.19\pm.38^{Ab}$	75.56 ± 39^{Ab}	week=<0.001 sample=<0.001 interaction=<0.001
	Probiotic supplement	$52.07\pm.23^{Ba}$	$47.09\pm.18^{Bb}$	$47.05\pm.26^{Bb}$	$40.63\pm.24^{Bc}$	

Means with superscript $^{A-C}$ depict difference between samples

Means with superscript $^{a-c}$ depict difference between weeks

Table 44 indicates that the viscosity of the probiotic supplement was significantly lesser than the control thouroughout the period of storage ($p<0.001$). There was no significant change in the viscosity of the probiotic supplement when compared to initial values and values at week 1 and weeks 1 and 2. However, by the end of the third week, there was a significant decrease in the viscosity. Degradation of starch by microorganisms during storage could be a possible reason for decrease in viscosity. The breakdown products of starch namely mono and di sacharides are water soluble and unable to hold water anymore. The free water thus released decreases the viscosity of the products during storage (Moda & Pal, 2011 & Cronk *et al.*, 1977).

The water holding capacity of the probiotic supplement during the storage period was significantly lesser than the control ($p<0.001$). A significant decrease in the water holding capacity of the probiotic supplement was observed by third week and with prominent wheying off. According to Shirai *et al*. (1992) wheying off is common during cold storage. Akgun *et al*. (2017) also attributed syneresis, as a reason for fluctuations in' a' scale value in refrigerated probiotic buffalo milk yogurt sample.

The developed probiotic supplement displayed superior physico-chemical properties. Though changes had taken place during storage, the pH, titratable acidity, viscosity and water holding capacity of the probiotic supplement remained acceptable until two weeks storage.

4.9.2 Microbial quality

Deterioration in the quality of a probiotic product is brought about by metabolic action of microorganisms during the period of storage. These changes dramatically compromise the therapeutic benefits and acceptability of the product. Hence the microbial quality of the developed probiotic supplement was assessed during storage at refrigeration temperature and is presented in Table 45.

Table 45: Microbial quality of the probiotic supplement during storage

Microbial parameter	Samples	Initial	Week 1	Week 2	Week 3	p-value between
Lactobacillus count	Control	7.71 ± 0.35^{Ba}	7.77 ± 0.17^{Ba}	7.1 ± 0.09^{Bb}	6.45 ± 0.15^{Bc}	week=<0.001 sample=<0.001 interaction =<0.001
	Probiotic supplement	8.65 ± 0.1^{Aa}	8.11 ± 0.06^{Ab}	8.59 ± 0.03^{Aa}	8.55 ± 0.04^{Aa}	
Coliform count	Control	Nil	Nil	Nil	Nil	-
	Probiotic supplement	Nil	Nil	Nil	Nil	-
Yeast and Mold Count	Control	Nil	Nil	Nil	Nil	-
	Probiotic supplement	Nil	Nil	Nil	Nil	-

Means with superscript $^{A-C}$ depict difference between samples

Means with superscript $^{a-c}$ depict difference between weeks

The initial lactobacillus count in the probiotic supplement was 8.65 $\log_{10}$cfu/ml (Table 45) which was within the recommended level of probiotics needed to exert a therapeutic benefit (Dave & Shah, 1998). The viability of lactobacillus in the probiotic supplement decreased significantly during the 1st week of storage ($p<0.001$). However, it gradually increased during the extended storage period and reached levels similar to that of the initial count. There was no significant difference in the lactobacillus count at weeks 2 and 3 when compared to the initial count. In the control, there was no significant change in the lactobacillus count until the 1st week. A gradual yet significant decrease was seen on further storage ($p<0.001$).

On comparing the lactobacillus count of the probiotic supplement and control during the period of storage, it was clear that the probiotic supplement (Plate 13) had a significantly higher ($p<0.001$) count when compared to the control (Plate 14) from the beginning till the full duration of storage. A significantly higher (8.55 $\log_{10}$cfu/ml) count was found in the probiotic supplement when compared to the control (6.45 $\log_{10}$cfu/ml) at week 3.

In order to provide the intended health benefit, Shah (1995) had suggested that viable probiotic bacteria in the levels of 10^5cfu/g be available in a product. The *L.casei* count in the probiotic supplement at the end of the storage period was higher (8.55 $\log_{10}$cfu/ml) than the minimum suggested.

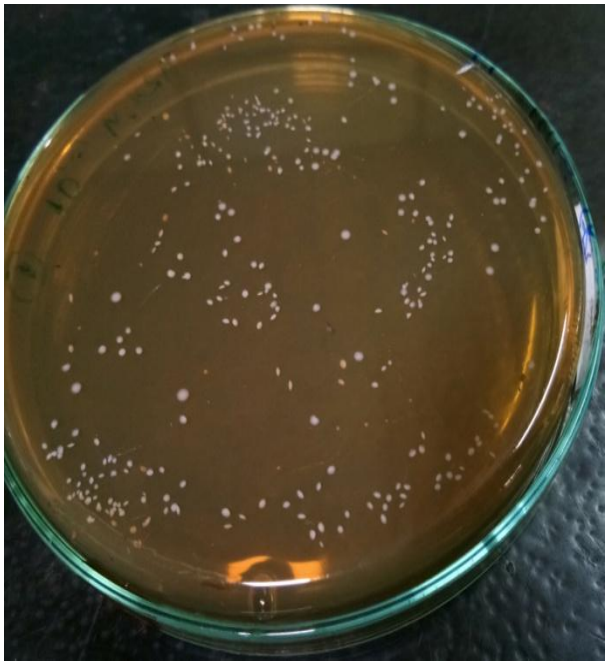

Plate 13: Lactobacillus growth observed in the probiotic supplement after 3 week storage (x 10^6cfu/ml)

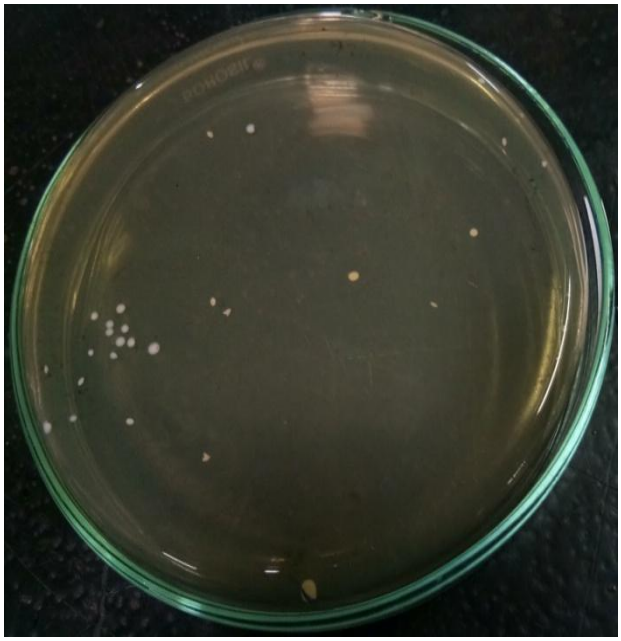

Plate 14: Lactobacillus growth observed in control after 3 week storage (x10^6cfu/ml)

4.9.3 Sensory evaluation

The sensory properties of the newly developed probiotic supplement stored at refrigerated conditions (7± 1°C) was carried out and compared with that of the control supplement. Table 46 shows the change in scores for all sensory parameters during the storage period.

Table 46: Sensory scores of the probiotic supplement during the period of storage

Parameter	Samples	Initial	Week 1	Week 2	Week 3	Chi-square	p-value
Appearance	Control	4.62 ± 0.47^{a}	4.37 ± 0.25^{a}	4 ± 0^{b}	2.75 ± 0.95^{c}	10.53	0.015^{*}
	Probiotic supplement	8.12 ± 0.25^{a}	7.75 ± 0.28^{ab}	7.25 ± 0.28^{b}	5.5 ± 0.57^{c}	13.14	0.004^{**}
p-value		0.017*	0.017*	0.013*	0.019*		
Mouthfeel	Control	4.62 ± 0.47^{a}	4.5 ± 0^{a}	4.12 ± 0.25^{a}	2.37 ± 0.47^{b}	11.3	0.010^{*}
	Probiotic supplement	8.12 ± 0.25^{a}	7.62 ± 0.2^{b}	7.37 ± 0.25^{b}	4.75 ± 1.5^{c}	13.24	0.004^{**}
p-value		0.017*	0.011*	0.015*	0.027*		
Taste	Control	4.87 ± 0.25^{a}	4.37 ± 0.47^{ab}	4.25 ± 0.28^{b}	2.37 ± 0.47^{c}	11.46	0.009^{**}
	Probiotic supplement	8.37 ± 0.25^{a}	7.87 ± 0.25^{b}	7.25 ± 0.28^{c}	3.5 ± 0.57^{d}	13.69	0.003^{**}
p-value		0.015*	0.017*	0.018*	0.036*		
Flavour	Control	4.87 ± 0.25^{a}	4.37 ± 0.47^{ab}	4.25 ± 0.28^{b}	2.37 ± 0.47^{c}	11.46	0.009^{**}
	Probiotic supplement	8.37 ± 0.25^{a}	7.87 ± 0.25^{b}	7.25 ± 0.28^{c}	3.5 ± 0.57^{d}	13.69	0.003^{**}
p-value		0.015*	0.017*	0.018*	0.036*		
Overall acceptability	Control	4.87 ± 0.25^{a}	4.5 ± 0.4^{ab}	4.25 ± 0.28^{b}	2.37 ± 0.47^{c}	11.65	0.009^{**}
	Probiotic supplement	8.25 ± 0.28^{a}	7.87 ± 0.25^{a}	7.25 ± 0.28^{b}	3.5 ± 0.57^{c}	13.41	0.004^{**}
p-value		0.017*	0.017*	0.018*	0.036*		

[a-d]Means with different superscript vary significantly within a row * *Significant at 1% level, * Significant at 5% level

Fermented foods undergo numerous chemical changes during storage due to persistent microbial activity, which affects the palatability and acceptability. The table 46 shows that the score for appearance of the probiotic supplement was 8.12 indicating 'Like very much'. This was higher than the score of the control (4.62). There was no significant decrease in the appearance scores of the probiotic supplement during the 1st week, after which it decreased significantly ($p<0.001$). The supplement had decreased WHC at 2 weeks that led to mild syneresis (Table 44). This could have affected the colour and apprarance scores of the supplement after the 2nd week of storage. A similar pattern of decrease was seen in the appearance scores of the control supplement as well ($p<0.05$). The appearance score of the probiotic supplement remained significantly ($p<0.05$) higher than that of the control thouroughout the period of storage.

In case of mouthfeel, the probiotic supplement (8.12) had a significantly ($p<0.05$) higher score than the control (4.62) (Table 46). Arrowroot RS III provided a grainy texture which was liked by the panel members (8.12) than the control that uses arrowroot starch which gave it paste/porridge like mouthfeel. The scores for the probiotic supplement decreased significantly in the first week. However, not much decrease was noted in the second week. By the 3rd week, the mouthfeel scores had significantly decreased to 4.75 indicating 'dislike slightly'. In the control, there was no significant change in mouthfeel scores until week 2, after which it decreased significantly. The score for mouthfeel of the probiotic supplement was significantly ($p<0.05$) higher than that of the control thouroughout the period of storage.

It was observed from the table 46 that the probiotic supplement (8.37) had a significantly higher ($p<0.001$) taste score indicating 'Like very much' than the control (4.87). The taste score of the probiotic supplement decreased significantly ($p<0.001$) every week and was rated 3.5 by the panel of judges, indicating 'dislike moderately' at week 3. The low score at week 3 indicates poor acceptability and hence was considered unfit for consumption. The scores for taste of the control also had decreased significantly by the 3rd week. Throughout the storage period, the probiotic supplement had significantly ($p<0.05$) higher scores for taste than the control.

The flavour score of the probiotic supplement was significantly ($p<0.05$) higher than that of the control (Table 46). Malted wheat imparted a good taste and flavour to the supplement unlike the control where unprocessed wheat flour gave a raw taste. It remained so thouroughout the storage period.

The scores for flavour of the probiotic supplement was 8.37 initially and significantly ($p<0.001$) decreased to 3.5 by the third week. This could be due to increased perception of acidity in the supplement brought about by the metabolic activity of the probiotic bacteria on the prebiotic substrate. Donkor *et al.* (2007) also reported that presence of prebiotic substances in probiotic yoghurts altered the organic acid production, proteolysis patterns and flavour profile of probiotic yoghurts during storage.

The probiotic supplement was found to be more acceptable than the control with regard to overall acceptability (Table 46). No significant change in overall acceptability was seen until week 1 after which a significant decrease ($p<0.001$) was noted every week. A similar decrease ($p<0.001$) was also seen in the control during the 3 week storage. The probiotic supplement had significantly ($p<0.05$) higher acceptability scores thouroughout the period of storage when compared to the control.

Hussain *et al.* (2015), set a minimum score of 7 to test the acceptability of Aloe barbadensis Miller supplemented probiotic lassi (APL) stored at 5±1°C. Scores of all sensory attributes decreased to below 7 in just 9 days of refrigerated storage and was rejected owing to its unacceptable sensory quality thereafter. The sensory scores of the newly developed probiotic supplement were above 7 for all parameters until 2 weeks in refrigerated storage.

On assessing the sensory properties, it was found that the probiotic supplement was rated as ‘like very much’ by the panel of judges. This was higher than the control for all sensory parameters. The probiotic supplement was found to have poor acceptability by the 3rd week of storage as evident by the low scores for all sensory attributes. Though a significant decrease in the scores of all attributes was seen by the second week, it was still rated as ‘like moderately’ with score above 7. Hence it can be

concluded that the newly developed probiotic supplement is fit for consumption until 2 weeks from the date of manufacture.

4.10 Extending the shelf life of the probiotic product

An effort was made to extend the shelf life of the probiotic supplement by subjecting it to two kinds of treatment - microencapsulation and lyophilization. Microencapsulation is the process of applying a shell to sensitive probiotic bacteria to protect them from their external environment (Capela *et al*., 2007) thereby improving their stability and extending their shelf life (Kailasapathy, 2015). Freeze drying is a conventional dehydration technique used for probiotic bacteria as it helps maintain viability of cells (Goderska, 2012).

4.10.1 Probiotic supplement capsules

Probiotic supplement capsules (PSC) were produced by encapsulating the developed supplement within alginate matrix. The probiotic capsule (PC) was produced by encapsulating *L.casei* cell pellets alone and was devoid of plant substrate. The physical characteristics of the capsules and viable cell count during the period of storage are presented in the below table.

Table 47: Shelf life of the probiotic capsule

Characteristics of the capsule	Probiotic capsule	Probiotic supplement capsule
Encapsulation efficiency (%)	85	84
Yield (g)	95.1	103.65
Size of bead (mm)	0.7	0.7
Viable count (log_{10}cfu/g)		
Initial	8.69	8.77
Week 1	8.47	9.26
Week 2	8.3	8.97
Week 3	8.2	8.9
Week 4	8.01	8.8
Week 6	7.98	8.76
Week 8	7.85	8.6
Week 10	7.69	8.4
Week 12	7.46	8.3

From Table 47, it is clear that, the encapsulation technique followed in the study yielded 95.1g and 103.65g of PC and PSC respectively with approximate diameter of 0.7mm/bead (Plate 15). The encapsulation efficiency (EE) of the probiotic capsule (PC) and the probiotic supplement capsule (PSC) were85% and 84% respectively. This was higher than that reported for *L.plantarum* (82%) (Ayama *et al*., 2014). Zajani *et al*. (2014) compared the EE% of microcapsules using different matrices by emulsion technique and found no significant difference between chitosan coated and uncoated alginate gelatinized starch beads. The average EE% was higher (97.4%) than the findings of the present study. This contrast could be due to differences in the technique used for encapsulation. Apart from the technique, the kind of matrix, its interaction with the entrapped microorganism, concentration, initial microbial biomass, and processing conditions also affect the EE (Mortazavian *et al*., 2007).

An EE above 80% indicates that no profound damages have been caused during the encapsulation process, thus, proving it to be a good technique for encapsulation (Plate 16).

The shelf life of the PC and PSC stored at 5°C was evaluated. There was an expected gradual decline thouroughout storage period. 90% survival was seen at 8 weeks storage period for PC. 85% of the *L.casei* in the PC had survived at the end of 12 weeks. These results are similar to that of Dianawati (2011) who reported 82% survival of freezedried encapsulated B.animalis Bb12 after 10 weeks of storage.

The viability of *L.casei* in PC stored at 5°C was above 7 log cfu/ml until 12 weeks showing a reduction of only 1.23 log cfu/ml (Table 47). In contrast, De Prisco *et al*. (2015) who encapsulated *L.reuterii* with alginate and chitosan matrix by vibration technology in an encapsulator obtained a reduction of 1 log cfu/ml within just 28 days of storage at 4°C.

In yet another study, Holkem *et al.* (2016) obtained a reduction close to 1.67 log cfu/ml in viability of freeze dried and encapsulated *B.animalis* Bb12 capsules after 120 days of storage at 7°C. The viable cell count of 7.32 log cfu/ml at 120 days was similar to the viable cell count (7.46 $\log_{10}$ cfu/ml) after 96 days in the present study. The capsules in the former study had a lower water activity as they were freeze dried. In the current study, capsules were not subjected to any heat preservation treatments.

In contrast to PC, there was a significant increase in the viable cell counts of PSC at 1 week of storage with viable count increasing by 0.49 $\log_{10}$ cfu/ml and reaching 9.26 $\log_{10}$ cfu/ml. Such increase in counts during storage of microcapsules have been reported by Pedroso *et al.* (2012) who encapsulated *B.lactis* and *L.acidophilus* by spray chilling due to a probable rearrangement of the chain of microorganism that interfered with the cell viability which required additional validation.

Holkem *et al.* (2016) observed such an increase after 60 days of storage of freeze dried encapsulated *L.reuterii* at room, freezer, and refrigeration temperatures after an initial decrease.

From week two onwards, there was a slow decline in the viable cell counts in PSC (Table 47). The decrease of 0.47 $\log_{10}$ cfu/ml by the end of storage was lesser

than the PC. The presence of prebiotic oligosaccharides in the supplement developed could have provided protection for the *L.casei* in the capsule.

Encapsulating the probiotic supplement in an alginate matrix prevented oxidative damage, provided protection for the probiotic *L.casei* in maintaining good viability and was successful in extending the shelf life. These results of the study suggested that encapsulating the probiotic supplement could be a possible alternative to consider tomaintain the viability of the culture for longer periods at refrigeration temperature.

4.10.2 Ready-to-reconstitute (RTR) probiotic food mix

Freeze drying method is the most common technique to dehydrate probiotic bacteria within coating materials (Meng *et al*., 2008). This helps to maintain acid and bile tolerance, surface hydrophobicity and metabolic functions upon rehydration (Archacka *et al.*, 2018) and makes transportation and storage easy (Dianawati *et al*., 2016). The newly developed probiotic supplement was freeze dried to study its effect on extending the shelf life of the supplement. The functional properties of the ready-to-reconstitute probiotic mix are presented in Table 48

Table 48: Functional properties of the RTR probiotic mix

Attribute	Observed value
Yield of powder (g)	15.72
Loose bulk density (g/ml)	0.24
Packed bulk density(g/ml)	0.25
Insolubility index (ml)	0.5
Wettability (sec)	8.2

Freeze drying of 100ml of the probiotic supplement yielded about 15g of the ready-to-reconstitute (RTR) probiotic mix (Plate 17). Bulk density of the powder is an important property which determines the structure and flow characteristics of the powder. It is directly proportional to the moisture content that in turn affects the shelf life of the product. The loose and packed density of the RTR probiotic mix was 0.24

and 0.25 g/ml respectively. Simha *et al*. (2012) reported a bulk density of 0.372 g/cm^3 for freeze dried pomegranate powder that increased to 0.709 g/cm^{3}during four week storage.

The RTR probiotic mix got completely wet at 8.2 sec. Freeze dried powders are known to have better wettability than spray dried powders (Rasekh, 1974). The insolubility index of the probiotic powder was found to be 0.5 ml. This could be due to the presence of lactic acid in the probiotic supplement produced during fermentation. Lactic acid is known to contribute to low solubility in previous studies (Sharma *et al*., 2012 & Kubantseva & Hartel, 2002).

Table 49: Shelf life of the ready to eat food mix

Duration	Viable count
Initial	9.1
Week 1	8.84
Week 2	9
Week 3	8.97
Week 4	8.72
Week 6	8.69
Week 8	8.52
Week 10	8.48
Week 12	8.4

The changes in viable cell count of freeze dried RTR probiotic mix stored at refrigeration temperature was monitored periodically during storage (Table 49). The initial count was 9.1 $\log_{10}$cfu/ml. A slight decrease was noted in the initial week after which the counts had increased to initial levels by week 2. Post the second week, a gradual decline in the *L.casei* count was observed. A 92% survival was observed after 12 weeks of storage at 5°C i.e a decrease of 0.7 $\log_{10}$cfu/ml. Adequate storage conditions and the presence of prebiotic oligosaccharides in the food mix has contributed to maintaining the viability of the culture. Tracoo *et al.* (2008) reported a similar decline in freeze dried *L.acidophilus* in pearl barley powders stored at 4°Cfor

30days. However, storing at -18°C extended the shelf life of a FD probiotic pineapple lassi powder till one year (decrease of 0.6 $\log_{10}$cfu/ml).

Freeze drying of the probiotic supplement preserved the viability of *L.casei* to a good extent. The viable cell count remained above 8 $\log_{10}$cfu/ml until 12 week of refrigerated storage which is on par with the recommended level of probiotics needed during ingestion (Nazzaro *et al*., 2009). Freeze drying of the probiotic supplement was successful in preserving and maintaining the viability and extending the shelf life of the supplement.

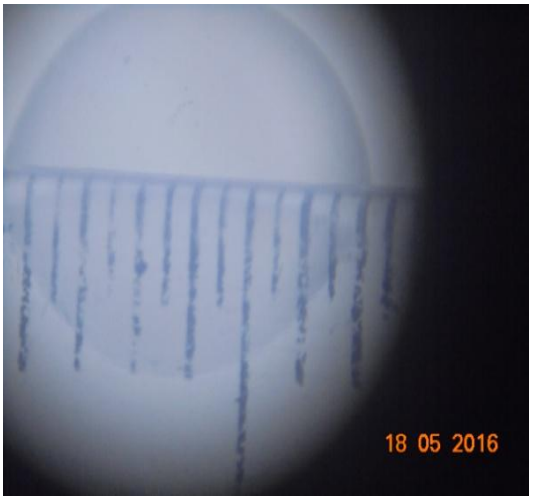

Plate 15: Size of a probiotic capsule viewed under light microscope

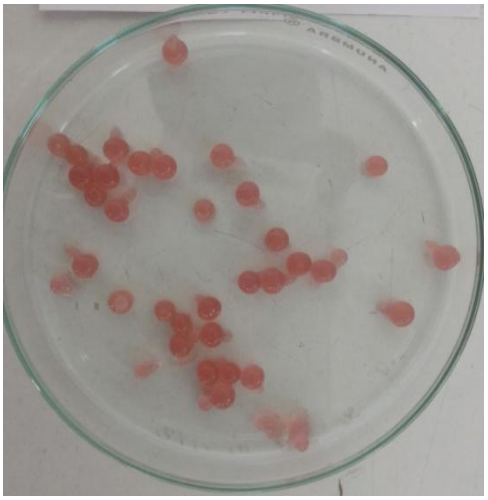

Plate 16: Microencapsulated probiotic supplement capsules

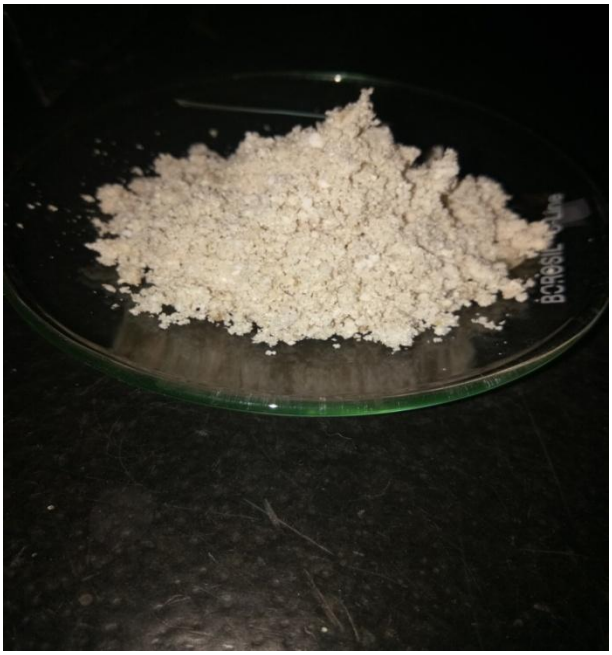

Plate 17: Ready-to-Reconstitute probiotic mix

4.11 Feasibility of the developed products

A cost analysis of the probiotic supplement, the probiotic supplement capsules and probiotic ready-to-reconstitute (RTR) mix was calculated to decide on the most feasible product.

Table 50: Cost calculation of the probiotic products

Cost components	Probiotic supplement (100 ml)	Probiotic supplement capsule (100 g)	Probiotic ready-to-reconstitute mix (100 g)
Raw material (Rs)	6.37	13.37	69.75
Processing/Storage (Rs)	2	2	2082.5
Total (Rs)	8.37	15.37	2152.2

A cost analysis of the thouree products (Table 50) revealed that the cost of production and storage of the probiotic supplement was the least and cost

only Rs.8.37. The cost of probiotic supplement capsule (Rs.15.37) was higher than that of the probiotic RTR mix. Freeze drying/lyophilisation incur a high operation cost and hence is not widely used in the food industry.

Among the thouree probiotic products developed, the probiotic supplement was ideal in terms of ease of production, need of less sophisticated equipments and technology and had low operational cost. Therefore, the probiotic supplement was chosen for the conduct of the biological study.

Phase 4: Efficacy of the probiotic supplement in altering gut microflora

4.12 Effect of the probiotic supplement on the gut floraof rats

The effect of the feeding trial on the fecal lactobacillus and E.coli count of the rats was analysed. The observations are presented in Table 51

Table 51 : Effect of feeding the probiotic supplement on the gut microflora of rats

Period	Parameter	Experimental group	Control group	p-value
Baseline period	Lactobacillus count (log_{10} cfu/ml)	6.70 ± 0.55^{b}	6.53 ± 0.56	0.616^{ns}
Post Intervention period		7.46 ± 0.37^{a}	6.81 ± 0.18	0.003^{**}
Follow up period		6.88 ± 0.30^{c}	6.7 ± 0.28	0.302^{ns}
p-value		0.012^{*}	0.409^{ns}	
Baseline period	Coliform count (log_{10} cfu/ml)	3.53 ± 0.88^{a}	4.15 ± 0.41	0.153^{ns}
Post Intervention period		2.1 ± 1.21^{b}	4.13 ± 0.69	0.005^{**}
Follow up period		3.02 ± 1.66^{a}	4.05 ± 0.61	0.187^{ns}
p-value				

		0.003**	0.852 ^{ns}	

** significant at 0.01 level; * significant at 0.05 level; ns non significant
Means having different letter as superscript differ significantly within a column

From Table 51 it is clear that the baseline lactobacillus count (LAB) in the experimental group was 6.7 $\log_{10}$ cfu/ml and had increased significantly ($p<0.0.5$) to 7.46 $\log_{10}$ cfu/ml at the end of the seven days intervention period. On withdrawal of the supplementation, the LAB count had reduced to 6.88 $\log_{10}$ cfu/ml which was significantly different from the LAB counts at baseline as well as post intervention period ($p<0.0.5$). With regard to the E.coli count, there was a significant ($p<0.01$) decrease after one week intervention period in the experimental group. However, the E.coli count increased to 3.02 $\log_{10}$ cfu/ml after cessation of the probiotic supplement which was not significantly different from the baseline E.coli counts (Fig 14).

On the contrary, no significant changes were seen in the lactobacillus count of rats in the control group. Similar trend was noticed with regard to the E.coli counts also which remained almost the same thouroughout the period of study (Table 51)

A comparison among the control and experimental groups revealed (Table 51) a highly significant ($P<0.01$) increase in the post intervention lactobacillus count in the experimental group that was fed the probiotic supplement. No significant difference exhibited in the LAB count between the two groups at the baseline and follow up periods. An opposite trend was noted with regard to the E.coli count which was lower in the experimental group in post intervention period when compared to the control group. There was no significant difference in the E.coli count at the baseline and follow up periods among the two groups.

Such increase in fecal lactobacillus and decrease in fecal coliform count is a common trend and has been reported for dairy based probiotic/synbiotic

products (James, 2014). Such trend has also been reported in dairy-cereal based composite substrates (Ganguly *et al*., 2014). However, very few studies have reported such alterations in gut flora upon feeding a completely plant based probiotic product.

Thourough this study it has been proven that non-dairy probiotic products can bring about a similar effect as dairy products with regard to change in gut microflora. The fact that count returned to the baseline values after one week of stopping the intervention signifies that for the beneficial effect to be persistant, regular consumption of the probiotic beverage is essential.

Moreover, most studies on plant foods have been performed on substrates tested as single sources of indigestible carbohydrates, which is not representative of a human diet that contains a complex mixture of carbohydrates. In this study a combination of different indigestible carbohydrates have been used that may be representative of a normal diet and influence the fermentation pattern in a different way.

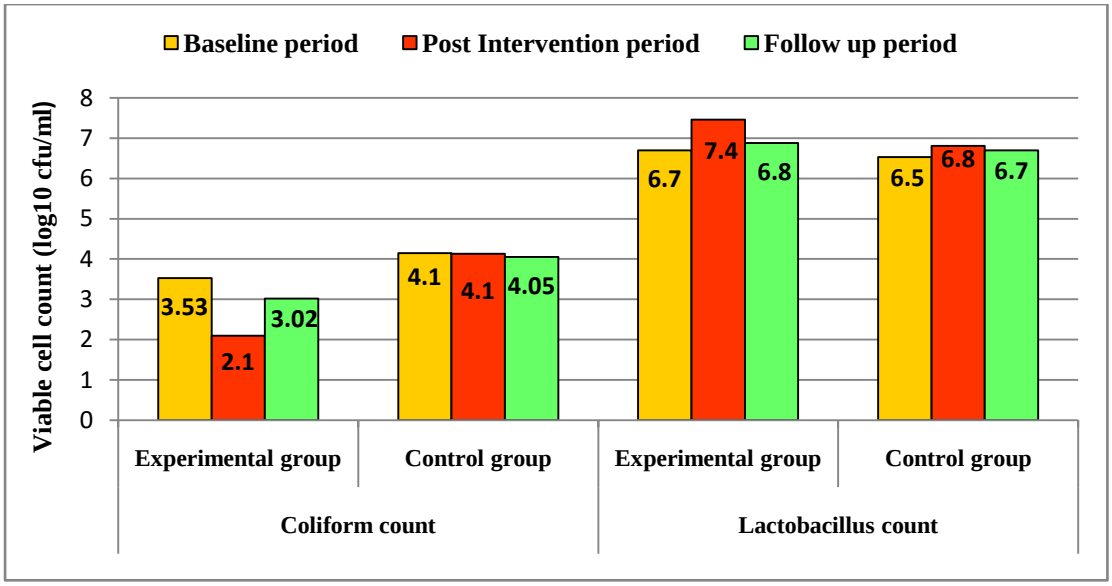

Fig 14: Change in fecal coliform and lactobacillus count in the experimental and control group on day 0, 8 and 15

V SUMMARY AND CONCLUSION

Functional foods are those foods that provide health benefits beyond basic nutrition**.** Among different category of functional foods, probiotic foods have received maximum attention due to their beneficial influence on gastrointestinal physiology and function. Increased adaption of veganism has kindled a pressing demand for the development of plant based probiotic products. Many plant foods are a source of prebiotic that acts in selectively stimulating the growth of health benefiting organisms. Therefore, utilising indigenous plant foods in the development of probiotic product seems promising by way of the synergistic effect that probiotics have when ingested along with a prebiotic.

The present study was carried out to explore the prebiotic potential of commonly consumed plant foods in supporting the growth of probiotic bacteria. A food mix was developed using foods that exhibited good prebiotic potential. Parameters for the preparation of the probiotic product like inoculum, substrate and stabiliser concentration, initial pH of the substrate and fermentation time was optimised. A plant based probiotic supplement with desirable microbial, physicochemical and sensory qualities was developed. Lyophilisation and freeze drying was done to extend the shelf life of the supplement. The efficacy of the developed probiotic supplement in altering the gut microflora was tested by a feeding trial on Sprague dawley rats.

The results and major findings of the study are summarized below.

- Both probiotic cultures *Lactobacillus casei*, isolated from a probiotic drink and *Lactobacillus acidophilus* LA-5, from Chrs Hansen Ltd. displayed good probiotic properties.
- *Marantha arundinaceae, Triticum aestivum, Glycine max, Hordeum vulgare,* and *Beta vulgaris* supported the growth of *L.casei.* The highest relative growth score for *L.casei* was on *Marantha arundinaceae* (4.04), followed by *Triticum aestivum* (3.51) and *Beta vulgaris* (2.28).
- *L.acidophilus* LA-5 had shown >1 log cfu/ml increase on *Glycine max, Allium sativum, Beta vulgaris, Triticum aestivum, Hordeum vulgare, Phaseolus aureus* and *Eleusine coracana*.

- *Allium sativum* (3.86), *Triticum aestivum* (3.83), *Beta vulgaris* (3.16), *Eleusine coracana* (2.85) and *Phaseolus aureus* (2.42) had high relative growth scores with *L.acidophilus* LA-5, while *Oryza sativa, Manihot esculenta* and *Marantha arundinaceae* had negative relative growth scores.
- *Triticum aestivum* with *L.casei* was found to be ideal combination and was taken as the major substrate for the development of probiotic supplement. *Marantha arundinaceae* and *Beta vulgaris* (Beetroot) were incorporated in minor proportions to enhance the prebiotic property and sensory appeal of the product.
- *Triticum aestivum* (Wheat) malt (0.71) and *Marantha arundinaceae* (arrowroot) RS III (0.89) had positive prebiotic activity score that indicated that they are potential prebiotics.
- Four food mixes using malted wheat powder, arrowroot RS III powder and beetroot powder was developed to choose the most acceptable food mix.
- Malted wheat powder, arrowroot RS III powder and beetroot powder in the ratio of 6:3.9:0.1 was the most acceptable food mix and was used as substrate in the probiotic product.
- The parameters for the production of the probiotic supplement were optimized.
- Starter culture with an inoculum concentration required to develop count of ~7 log cfu/ml after 3 hours of incubation was prepared.
- Substrate concentration at 10% level brought about a greater increase in lactobacillus count (~ 4 log cfu/ml) when compared to 5% substrate levels and hence was chosen as the ideal substrate concentration
- Pectin when added at 0.1% level gave a product without sedimentation, minimal wheying off, acceptable viscosity, desired viable cell counts and good score for consistency. Therefore this was chosen as the ideal stabilizer concentration to clarify the product.
- Open pan heat treatment at 90°C for 5 mins showed better retention of innate red colour of beet pigment in the probiotic supplement and was chosen as the ideal method of heat treatment.
- Effect of varying the initial pH and fermentation time on the microbial, physico chemical and sensory characteristics was optimized using response surface methodology Design expert version 6.

- Initial pH ($p<0.05$) had a significant effect on the viable cell count, end pH, wheying off, mouthfeel, taste and overall acceptability scores of the supplement.
- Adjusting the initial pH to 4.93 and allowing a fermentation time of 13.6 hours resulted in a probiotic product with high viable cell counts and sensory acceptability scores, and minimum wheying off and hence was optimised for preparation of the probiotic supplement.
- The probiotic supplement was prepared by making a10% slurry using the food mix and the intial pH was adjusted to 4.93 using 20% citric acid. 0.1g pectin was added to the prepared slurry and sterilized by direct heat treatment at 90°C for 5 mins. The slurry was inoculated with ~ 4 log cfu/ml of *L.casei* culture and allowed to ferment for ~ 13.6 hrs at 37°C.
- The probiotic supplement prepared under optimised processing conditions, had probiotic counts of 8.88±0.12 log cfu/ml which is higher than the minimum viable count required in a probiotic product to provide a therapeutic effect.
- The supplement was had a pleasing colour (redness scale 14.43±0.04), as measured by Hunters Lab Colour meter, ideal viscosity (1533±57.73 cP), as measured by Brookesfield Viscometer, no sedimentation and moderate wheying off.
- Fermentation of the indigenous food mix with *L.casei* increased the crude protein (142%) and starch digestibility (52%) and decreased crude fibre (56%), starch (28%), reducing sugars (62%) and free amino nitrogen (FAN) (44%) content.
- Butyric (7.05%), acetic (12.3%), propionic (10.97%) and lactic (9.18%) acids were detected in the fermented product on GC-MS. The presence of butyric acid in the newly developed beverage confirmed its prebiotic potential as reported by Sreenivas and Lele (2013).
- Acetoin (8.98%) and cyclohexane carboxylic acid (12.44%), flavour compounds was formed during fermentation gave a characteristic taste.
- The developed probiotic supplement had acceptable pH and titratable acidity until 2 weeks of storage at refrigerated temperature.

- Significant reductions in the Hunters ‘a’ scale value and significant increase in the ‘L’ and ‘b’ scale values was seen during 3 week storage. Even after significant reductions ($p<0.01$), the red scale value remained positive.
- Significant reductions ($p<0.01$) in the viscosity and wheying off% was seen during storage. However, the viscosity and wheying off% remained under desirable levels throughout storage
- The viable cell count of the supplement remained above the minimum desirable levels throughout the storage period.
- The probiotic supplement had good acceptability scores until 2 weeks after manufacture when stored at refrigerated temperature.
- Encapsulation of the supplement into capsules and lyophilisation into ready to reconstitute probiotic mix maintained the viability of the *L.casei* until 12 weeks at refrigerated storage.
- Among the three products developed, the probiotic supplement was found to be feasible in terms of cost and availability of technological expertise and hence was chosen for the biological study.
- Feeding of the probiotic supplement for seven days to adult Sprague dawley rats increased the fecal lactobacillus count by 1 log cfu/ml but the count reduced to the baseline value after withdrawal of the feed.
- Coliform count of the faeces of the experimental group decreased by 1.43 log cfu/ml during feeding but reverted back to original value on cessation of the feed.
- The probiotic supplement was found to be effective in altering the gut microflora in Sprague Dawley rats.

CONCLUSIONS

The results of the study show that substrates based on indigenous plants are suitable for the growth and development of probiotic bacteria. Optimisation of the process parameters had resulted in a product with good populations of viable probiotic bacteria. For vegan diets such probiotic formulations are of immense potential. Among the multitude of commercial dairy probiotic products, a plant based probiotic product like the one formulated could offer variety to the health conscious and lactose

intolerant consumers. Microencapsulation and lyophilisation of the probiotic bacteria on a plant based substrate provided additional protection for the organism and thereby extended the shelf life. The gut altering ability displayed in this study is a promising evidence of the crucial role that probiotics can have on human healthcare especially in the prevention and treatment of diseases of the GI tract.

Probiotication of plant foods done in this study has the potential to add value to indigenous and staple foods. This study has showed the possibility of utilising indigenous foods for the development of probiotic product. Although the overall acceptability of the developed product was moderate, good probiotic viability, beneficial effects on the gut microflora and presence of beneficial organic acids makes the product worthy of extrapolation.

Recommendations for future line of work

- The efficacy of the product on human beings should be validated by conducting clinical trials.
- Commercialisation and promotion of plant based probiotic products should be carried out targeting specific health benefits
- Further research needs to be carried out to explore other indigenous and staple plant foods for its prebiotic potential.

BIBLIOGRAPHY

Abadias, M., Benabarre, A., Teixidó, N., Usall, J., & Viñas, I. (2001).Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake.*International Journal of Food Microbiology*, 65(3), 173-182. doi:10.1016/s0168-1605(00)00513-4

Abed, M. S., Abdelmoneim, H. A., Anwar, N., Sobia, N., Al-Farga, A., & Amr, M. B. (2016). Inulin as Prebiotics and its Applications in Food Industry and Human Health; A Review. *International Journal of Agriculture Innovations and Research*, 5(1), 2319-1473.

Addis, G., Singh, V., Pratape, V., Srivastav, S., Gowda, L., Asha, M., & Bhattachary, S. (2013).Development and functional properties of low-cost complementary food.*African Journal of Food Science*, 7(9), 274-284. doi:10.5897/ajfs12.143

Ahire, J. J., Mokashe, N. U., Patil, H. J., & Chaudhari, B. L. (2013). Antioxidative potential of folate producing probiotic Lactobacillus helveticus CD6.*Journal of Food Science and Technology*, 50(1), 26-34. doi:10.1007/s13197-011-0244-0

Ahire, J. J., Patil, K. P., Chaudhari, B. L., & Chincholkar, S. B. (2010). Bacillus spp. of Human Origin: A Potential Siderophoregenic Probiotic Bacteria. *Applied Biochemistry and Biotechnology*, 164(3), 386-400. doi:10.1007/s12010-010-9142-6

Akalın, A., & Erişir, D. (2008).Effects of Inulin and Oligofructose on the Rheological Characteristics and Probiotic Culture Survival in Low-Fat Probiotic Ice Cream.*Journal of Food Science*, 73(4), M184-M188. doi:10.1111/j.1750-3841.2008.00728.x

Akgun, A., Yazici, F., & Gulec, H. A. (2017).The combined effect of probiotic cultures and incubation final pH on the quality of buffalo milk yogurt

during cold storage.*Food Science & Nutrition*, 6(2), 492-502. doi:10.1002/fsn3.580

Alka, S., Yadav, N., & Sharma, S. (2012). Effect of fermentation on physicochemical properties & in vitro starch and protein digestibility of selected cereals. *International Journal of Agriculture and Food Science*, 2(3), 66-70. Retrieved from https://pdfs.semanticscholar.org/1719/755d1b864cd9fde16b0c2402925bd915a7b3.pdf

Amankwah, E., Barimah, J., Acheampong, R., Addai, L., & Nnaji, C. (2009).Effect of Fermentation and Malting on the Viscosity of Maize-Soyabean Weaning Blends.*Pakistan Journal of Nutrition*, 8(10), 1671-1675. doi:10.3923/pjn.2009.1671.1675

Amice-Quemeneur, N., Haluk, J., Hardy, J., & Kravtchenko, T. (1995). Influence of the Acidification Process on the Colloidal Stability of Acidic Milk Drinks Prepared from Reconstituted Nonfat Dry Milk. *Journal of Dairy Science*, 78(12), 2683-2690. doi:10.3168/jds.s0022-0302(95)76899-0

Aminlari, L., Shekarforoush, S. S., Hosseinzadeh, S., Nazifi, S., Sajedianfard, J., & Eskandari, M. H. (2018). Effect of Probiotics Bacillus coagulans and Lactobacillus plantarum on Lipid Profile and Feces Bacteria of Rats Fed Cholesterol-Enriched Diet.*Probiotics and Antimicrobial Proteins*.doi:10.1007/s12602-018-9480-1

Ammor, M. S., Belén Flórez, A., & Mayo, B. (2007).Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria.*Food Microbiology*, 24(6), 559-570. doi:10.1016/j.fm.2006.11.001

Ammor, S., Tauveron, G., Dufour, E., & Chevallier, I. (2006). Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria

isolated from the same meat small-scale facility. *Food Control*, 17(6), 454-461. doi:10.1016/j.foodcont.2005.02.006

Andersson, L., Nilsson, I. M., Niléhn, J., Hedner, U., Granstrand, B., & Melander, B. (1965). Experimental and Clinical Studies on AMCA, the Antifibrinolytically Active Isomer of p-Aminomethyl Cyclohexane Carboxylic Acid. *Scandinavian Journal of Haematology*, *2*(3), 230-247. doi:10.1111/j.1600-0609.1965.tb01300.x

Andres-Franch, M., Galiana, A., Sanchez-Hellin, V., Ochoa, E., Hernandez-Illan, E., Lopez-Garcia, P., … Soto, J. L. (2017). Streptococcus gallolyticus infection in colorectal cancer and association with biological and clinical factors.*PLOS ONE*, 12(3), e0174305. doi:10.1371/journal.pone.0174305

Anekella, K., & Orsat, V. (2014). Shelf life stability of lactobacilli encapsulated in raspberry powder: Insights into non-dairy probiotics. *International Journal of Food Sciences and Nutrition*, 65(4), 411-418. doi:10.3109/09637486.2013.869793

Angelov, A., Gotcheva, V., Kuncheva, R., & Hristozova, T. (2006). Development of a new oat-based probiotic drink. *International Journal of Food Microbiology*, *112*(1), 75-80. doi:10.1016/j.ijfoodmicro.2006.05.015

Anprung, P., & Sangthawan, S. (2012). Prebiotic activity and bioactive compounds of the enzymatically depolymerized Thailand-grown mangosteen aril. *Journal of Food Research*, *1*(1), 268-276. doi:10.5539/jfr.v1n1p268

Archacka, M., Białas, W., Dembczyński, R., Olejnik, A., Sip, A., Szymanowska, D., … Rogodzińska, M. (2018). Method of preservation and type of protective agent strongly influence probiotic properties of Lactococcus lactis: A complete process of probiotic preparation manufacture and use. *Food Chemistry*, *274*, 733-742. doi:10.1016/j.foodchem.2018.09.033

Arena, M., Caggianiello, G., Fiocco, D., Russo, P., Torelli, M., Spano, G., & Capozzi, V. (2014). Barley β-Glucans-Containing Food Enhances Probiotic Performances of Beneficial Bacteria. *International Journal of Molecular Sciences*, *15*(2), 3025-3039. doi:10.3390/ijms15023025

Aurore, G., Ginies, C., Ganou-parfait, B., Renard, C. M., & Fahrasmane, L. (2011). Comparative study of free and glycoconjugated volatile compounds of three banana cultivars from French West Indies: Cavendish, Frayssinette and Plantain. *Food Chemistry*, *129*(1), 28-34. doi:10.1016/j.foodchem.2011.01.104

Ayama, H., Punnanee, S., & Suphitchaya, C. (2014). Effect of encapsulation of selected probiotic cell on survival in simulated gastrointestinal tract condition. *Journal of Science and Technology*, 36(3), 291-299. Retrieved from rdo.psu.ac.th/sjstweb/journal/36-3/36-3-6.pdf

Bâati, L., Fabre-Gea, C., Auriol, D., & Blanc, P. J. (2000). Study of the cryotolerance of Lactobacillus acidophilus: effect of culture and freezing conditions on the viability and cellular protein levels. *International Journal of Food Microbiology*, 59(3), 241-247. doi:10.1016/s0168-1605(00)00361-5

Baddini Feitoza, A., Fernandes Pereira, A., Ferreira da Costa, N., &Gonçalves Ribeiro, B. (2009). Conjugated linoleic acid (CLA): effect modulation of body composition and lipid profile. *Nutr Hosp*, 24, 422–428.

Bae, S., & Suh, H. (2007).Antioxidant activities of five different mulberry cultivars in Korea.*LWT - Food Science and Technology*, 40(6), 955-962. doi:10.1016/j.lwt.2006.06.007

Balasingham, K., Valli, C., Radhakrishnan, L., & Balasuramanyam, D. (2017). Probiotic characterization of lactic acid bacteria isolated from swine intestine. *Veterinary World*, 10(7), 825-829. doi:10.14202/vetworld.2017.825-829

Banusha, S., & Vasantharuba, S. (2013).Effect of Malting on Nutritional Contents of Fingermillet and Mungbean.*American-Eurasian Journal of Agriculture & Environmental Science*, 13(12), 1642-1646.

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., … Zhang, H. (2010). Screening of potential probiotic properties of Lactobacillus fermentum isolated from traditional dairy products. *Food Control*, 21(5), 695-701. doi:10.1016/j.foodcont.2009.10.010

Barrow, G.I., and Feltham, R.K.A. (1993). Cowan and Steel's manual for the identification of medical bacteria. 3rd Edition, Cambridge University Press, Cambridge, 331.

Basappa, S. C., Somashekar, D., Agrawal, R., Suma, K., & Bharathi, K. (1997). Nutritional composition of fermentedragi(chhang) byphaband defined starter cultures as compared to unfermentedragi(Eleucine coracanaG.). *International Journal of Food Sciences and Nutrition*, 48(5), 313-319. doi:10.3109/09637489709028577

Bassaganya-Riera, J., Hontecillas, R., Horne, W. T., Sandridge, M., Herfarth, H. H., Bloomfeld, R., & Isaacs, K. L. (2012). Conjugated linoleic acid modulates immune responses in patients with mild to moderately active Crohn's disease. *Clinical Nutrition*, 31(5), 721-727. doi:10.1016/j.clnu.2012.03.002

Bauer, A. W., Perry, D. M., & Kirby, W. M. M. (1959).Single-Disk Antibiotic-Sensitivity Testing of Staphylococci.*A.M.A. Archives of Internal Medicine*, 104(2), 208. doi:10.1001/archinte.1959.00270080034004

Behrens, J., Roig, S., & Da Silva, M. (2004). Fermentation of soymilk by commercial lactic cultures: Development of a product with market potential. *Acta Alimentaria*, 33(2), 101-109. doi:10.1556/aalim.33.2004.2.2

Belenguer, A., Duncan, S., Holtrop, G., Flint, H., & Lobley, G. (2008). Quantitative Analysis of Microbial Metabolism in the Human Large Intestine. *Current Nutrition & Food Science, 4*(2), 109-126. doi:10.2174/157340108784245957

Bellon-Fontaine, M., Rault, J., & Van Oss, C. (1996). Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids and Surfaces B: Biointerfaces*, 7(1-2), 47-53. doi:10.1016/0927-7765(96)01272-6

Bernet, M. F., Brassart, D., Neeser, J. R., & Servin, A. L. (1994). Lactobacillus acidophilus LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut*, 35(4), 483-489. doi:10.1136/gut.35.4.483

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965-977. doi:10.1016/j.talanta.2008.05.019

Blandino, A., Al-Aseeri, M., Pandiella, S., Cantero, D., & Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Research International*, 36(6), 527-543. doi:10.1016/s0963-9969(03)00009-7

Boelrijk, A. E., De Jong, C., & Smit, G. (2003). Flavour generation in dairy products. In *Dairy Processing - Improving Quality* (pp. 130-154). Boca Raton, FL: CRC Press.

Bortolini, O., Medici, A., & Poli, S. (1997). Biotransformations on steroid nucleus of bile acids.*Steroids*, 62(8-9), 564-577. doi:10.1016/s0039-128x(97)00043-3

Both, E., Gyorgy, E., Kibédi-Szabó, C. Z., Tamás, E., Ábrahám, B., Miklóssy, I., & Lányi, S. (2010). Acid and Bile Tolerance, Adhesion to Epithelial Cells Of Probiotic Microorganisms. *UPB Scientific Bulletin,* Series B,

72(2), 1454-2331. Retrieved from https://www.scientificbulletin.upb.ro/rev_docs_arhiva/full2746.pdf

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., ... Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. International *Journal of Food Microbiology*, 154(3), 87-97. DOI: 10.1016/j.ijfoodmicro.2011.12.030

Boylston, T. D., Vinderola, C. G., Ghoddusi, H. B., & Reinheimer, J. A. (2004). Incorporation of bifidobacteria into cheeses: challenges and rewards. *International Dairy Journal*, 14(5), 375-387. doi:10.1016/j.idairyj.2003.08.008

Bradburn, D. M., Mathers, J. C., Gunn, A., Burn, J., Chapman, P. D., & Johnston, I. D. (1993). Colonic fermentation of complex carbohydrates in patients with familial adenomatous polyposis. *Gut, 34*(5), 630-636. doi:10.1136/gut.34.5.630

Bull, M., Plummer, S., Marchesi, J., & Mahenthiralingam, E. (2013). The life history ofLactobacillus acidophilusas a probiotic: a tale of revisionary taxonomy, misidentification and commercial success. *FEMS Microbiology Letters*, 349(2), 77-87. doi:10.1111/1574-6968.12293

Bull, M., Plummer, S., Marchesi, J., & Mahenthiralingam, E. (2013). The life history ofLactobacillus acidophilusas a probiotic: a tale of revisionary taxonomy, misidentification and commercial success. *FEMS Microbiology Letters*, 349(2), 77-87. doi:10.1111/1574-6968.12293

Bunkar, D. S., Jha, A., & Mahajan, A. (2012). Optimization of the formulation and technology of pearl millet based 'ready-to-reconstitute' kheer mix powder. *Journal of Food Science and Technology, 51*(10), 2404-14.

Buruleanu, L., Manea, I., Bratu, M.G., Avram,D. and Nicolescu, C.L. 2012. Evaluation of the cabbage and cucumber juices as substrate for

Lactobacillus acidophilus LA-5. Romanian Biotechnological Letters, 17(4), 2012 7418

Cai, H., Rodriguez, B. T., Zhang, W., Broadbent, J. R., & Steele, J. L. (2007). Genotypic and phenotypic characterization of Lactobacillus casei strains isolated from different ecological niches suggests frequent recombination and niche specificity. Microbiology, 153(8), 2655-2665. doi:10.1099/mic.0.2007/006452-0

Capela, P., Hay, T., & Shah, N. (2007). Effect of homogenisation on bead size and survival of encapsulated probiotic bacteria. *Food Research International*, 40(10), 1261-1269. doi:10.1016/j.foodres.2007.08.006

Casterline, J. L., Oles, C. J., & Ku, Y. (1997). In Vitro fermentation of various food fiber fractions. *Journal of Agricultural and Food Chemistry*, *45*(7), 2463-2467. doi:10.1021/jf960846f

Chambers, H. F., & De Leo, F. R. (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology*, 7(9), 629-641. doi:10.1038/nrmicro2200

Chandran, J., Nisha, P., Singhal, R. S., & Pandit, A. B. (2014). Degradation of colour in beetroot (Beta vulgaris L.): a kinetics study. *Journal of Food Science and Technology*, 51(10), 2678-2684. doi:10.1007/s13197-012-0741-9

Charalampopoulos, D., Pandiella, S., & Webb, C. (2002). Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. *Journal of Applied Microbiology*, 92(5), 851-859. doi:10.1046/j.1365-2672.2002.01592.x

Chavan, U., Chavan, J., & Kadam, S. (1988). Effect of Fermentation on Soluble Proteins and In Vitro Protein Digestibility of Sorghum, Green Gram and Sorghum-Green Gram Blends. *Journal of Food Science*, *53*(5), 1574-1575. doi:10.1111/j.1365-2621.1988.tb09329.x

Chen, M., Zheng, W., Dong, Q., Li, Z., Shi, L., & Tang, Z. (2014).Activity of encapsulated Lactobacillus bulgaricus in alginate-whey protein microspheres.*Brazilian Archives of Biology and Technology*, 57(5), 736-741. doi:10.1590/s1516-8913201402377

Chiu, P., & Lai, L. (2010). Antimicrobial activities of tapioca starch/decolorized hsian-tsao leaf gum coatings containing green tea extracts in fruit-based salads, romaine hearts and pork slices. *International Journal of Food Microbiology*, 139(1-2), 23-30. doi:10.1016/j.ijfoodmicro.2010.01.010

Choi, E. A., & Chang, H. C. (2015). Cholesterol-lowering effects of a putative probiotic strain Lactobacillus plantarum EM isolated from kimchi. *LWT - Food Science and Technology*, 62(1), 210-217. doi:10.1016/j.lwt.2015.01.019

Chou, C., & Hou, J. (2000). Growth of bifidobacteria in soymilk and their survival in the fermented soymilk drink during storage. International Journal of Food Microbiology, 56(2-3), 113-121. doi:10.1016/s0168-1605(99)00201-9

Christensen, H. R., Frokiaer, H., & Pestka, J. J. (2002). Lactobacilli Differentially Modulate Expression of Cytokines and Maturation Surface Markers in Murine Dendritic Cells. *The Journal of Immunology*, 168(1), 171-178. doi:10.4049/jimmunol.168.1.171

Cizeikiene, D., Juodeikiene, G., Paskevicius, A., & Bartkiene, E. (2013). Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. Food Control, 31(2), 539-545. doi:10.1016/j.foodcont.2012.12.004

Clemente, J., Ursell, L., Parfrey, L., & Knight, R. (2012). The Impact of the Gut Microbiota on Human Health: An Integrative View. Cell, 148(6), 1258-1270. doi:10.1016/j.cell.2012.01.035

Collado, M., Meriluoto, J., & Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Letters in Applied Microbiology, 45(4), 454-460. doi:10.1111/j.1472-765x.2007.02212.x

Coman MM, Silvi S, Verdenelli MC, Cecchini C, Orpianesi C and Cresci A (2010) Fruit and vegetable juices tested as possible probiotic beverage .Agro Food Industry Hi-Tech, 21 (2):28-31

Comstock, S. S., & Donovan, S. M. (2017).Human Milk Oligosaccharides as Modulators of Intestinal and Systemic Immunity.*Prebiotics and Probiotics in Human Milk*, 223-248. doi:10.1016/b978-0-12-802725-7.00008-7

Coppa, G., Zampini, L., Galeazzi, T., & Gabrielli, O. (2006). Prebiotics in human milk: a review. *Digestive and Liver Disease*, 38, S291-S294.doi:10.1016/s1590-8658(07)60013-9

Corsetti, A., De Angelis, M., Dellaglio, F., Paparella, A., Fox, P., Settanni, L., & Gobbetti, M. (2003). Characterization of sourdough lactic acid bacteria based on genotypic and cell-wall protein analyses. *Journal of Applied Microbiology*, 94(4), 641-654. doi:10.1046/j.1365-2672.2003.01874.x

Costa, M. G., Fonteles, T. V., De Jesus, A. L., & Rodrigues, S. (2013). Sonicated pineapple juice as substrate for L. casei cultivation for probiotic beverage development: Process optimisation and product stability. *Food Chemistry*, 139(1-4), 261-266. doi:10.1016/j.foodchem.2013.01.059

Cronk, T. C., Steinkraus, K. H., Hackler, L. R., & Mattick, L. R. (1977).Indonesian tape ketan fermentation.*Applied and Environmental Microbiology*, 33, 1067–1073. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC170828/

Darjani, P., Hosseini Nezhad, M., Kadkhodaee, R., & Milani, E. (2016). Influence of prebiotic and coating materials on morphology and survival of a probiotic strain of Lactobacillus casei exposed to simulated gastrointestinal conditions. *LWT*, 73, 162-167. doi:10.1016/j.lwt.2016.05.032

Dashkeviez, M. P., & Feighner, S. D. (1989).Development of a differential medium for bile salt hydrolase active lactobacillus species.*Applied and Environmental Microbiology*, 55, 11- 16. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC184046/

Dave, R., & Shah, N. (1998). Ingredient Supplementation Effects on Viability of Probiotic Bacteria in Yogurt. *Journal of Dairy Science*, 81(11), 2804-2816. doi:10.3168/jds.s0022-0302(98)75839-4

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., … Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.*Proceedings of the National Academy of Sciences*, 107(33), 14691-14696. doi:10.1073/pnas.1005963107

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., … Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.*Proceedings of the National Academy of Sciences*, 107(33), 14691-14696. doi:10.1073/pnas.1005963107

De Man, J. C., Rogosa, M., & Sharpe, M. E. (1960).A medium for the cultivation of lactobacilli.*Journal of Applied Bacteriology*, 23(1), 130-135. doi:10.1111/j.1365-2672.1960.tb00188.x

De Prisco, A., Maresca, D., Ongeng, D., & Mauriello, G. (2015). Microencapsulation by vibrating technology of the probiotic strain Lactobacillus reuteri DSM 17938 to enhance its survival in foods and in gastrointestinal environment. *LWT - Food Science and Technology*, 61(2), 452-462. doi:10.1016/j.lwt.2014.12.011

De Vrese, M., Suhr, M, & Barth, C. A. (1995). Differentiation of Intestinal From Bacterial B-Galactosidase In The Small Intestine Of Rats By Affinity Chromatography. *ZeitschriftFürErnährungswissenschaft*, 34(40).

De Vuyst, L., & Leroy, F. (2007). Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications. *Journal of Molecular Microbiology and Biotechnology*, *13*(4), 194-199. doi:10.1159/000104752

Deguchi, Y., Morishita, T., & Mutai, M. (1985).Comparative Studies on Synthesis of Water-soluble Vitamins among Human Species of Bifidobacteria.*Agricultural and Biological Chemistry*, 49(1), 13-19. doi:10.1080/00021369.1985.10866683

Devillard, E., McIntosh, F. M., Duncan, S. H., & Wallace, R. J. (2007). Metabolism of Linoleic Acid by Human Gut Bacteria: Different Routes for Biosynthesis of Conjugated Linoleic Acid. *Journal of Bacteriology*, 189(6), 2566-2570. doi:10.1128/jb.01359-06

Devillard, E., McIntosh, F. M., Paillard, D., Thomas, N. A., Shingfield, K. J., & Wallace, R. J. (2009). Differences between human subjects in the composition of the faecal bacterial community and faecal metabolism of linoleic acid. Microbiology, 155(2), 513-520. doi:10.1099/mic.0.023416-0

Dhewa, T., Pant, S., Goyal, N., & Mishra, V. (2009). Adhesive properties of food and faecal potential probiotic lactobacilli.*Journal of Applied and Natural Science*, 1(2), 138-140. doi:10.31018/jans.v1i2.52

Dhiva, S. (2009). Studies on isolation characterization and evaluation of microorganisms for the formulation of probiotic product (http://hdl.handle.net/10603/115002) (Doctoral dissertation). Retrieved from Shodhganga.(http://hdl.handle.net/10603/115002)

Di Cagno, R., Coda, R., De Angelis, M., & Gobbetti, M. (2013).Exploitation of vegetables and fruits through lactic acid fermentation.*Food Microbiology*, 33(1), 1-10. doi:10.1016/j.fm.2012.09.003

Dianawati, D., & Shah, N. P. (2011).Survival, Acid and Bile Tolerance, and Surface Hydrophobicity of Microencapsulated B. animalis ssp. lactis Bb12 during Storage at Room Temperature.*Journal of Food Science*, 76(9), M592-M599. doi:10.1111/j.1750-3841.2011.02422.x

Dianawati, D., Mishra, V., & Shah, N. P. (2015). Survival of Microencapsulated Probiotic Bacteria after Processing and during Storage: A Review. *Critical Reviews in Food Science and Nutrition*, *56*(10), 1685-1716. doi:10.1080/10408398.2013.798779

Dogan, I. S. (2002).Dynamic rheological properties of dough as affected by amylases from various sources.*Nahrung/Food*, 46(6), 399-403. doi:10.1002/1521-3803(20021101)46:6<_x0033_99:_x003a_aid-food399>3.0.co;2-u

Donkor, O., Nilmini, S., Stolic, P., Vasiljevic, T., & Shah, N. (2007).Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage.*International Dairy Journal*, 17(6), 657-665. doi:10.1016/j.idairyj.2006.08.006

Elkhalifa, A. E., Schiffler, B., & Bernhard, R. (2004).Effect of fermentation on the starch digestibility, resistant starch and some physicochemical

properties of sorghum flour.*Nahrung/Food*, 48(2), 91-94. doi:10.1002/food.200300322

Emire, S. A., & Buta, M. B. (2015).Effects of Fermentation on the Nutritional Quality of QPM and Soybean Blends for the Production of Weaning Food.*Journal of Food Processing & Technology*, 6(11). doi:10.4172/2157-7110.1000507

Erten, H. (2000). Fermentation of Glucose and Fructose by Leuconostocmesenteroides.*Turkish Journal of Agriculture and Forestry*, 24(4), 527-532.

European Brewery Convention. 1987. Free amino nitrogen, p. E141-E142. Analytica-EBC, 4th ed.. (1987).

Fajkowska, H., & Korzeniowe, W. (1982).Szczegółowa uprawa warzyw (ed. Z. Borna). PWRiL, Warszawa(in Polish), 129-136.

FAO/WHO. (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina: Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report.

FAO/WHO. (2010). Guidelines for the evaluation of probiotics in food: joint FAO/WHO Working Group report on drafting guidelines for the evaluation of probiotics in food. Food and Agriculture Organization of the United Nations and World Health Organization.

Faridah, D. N., H Riana, R. Z., & Kusumaningrum, H. D. (2017). Potential Prebiotic Properties by In vitro in Arrowroot Starch (Marantha arundinaceae L.) a Combination of Acidic, Enzymatic, and Physical Modification. *Der Pharma Chemica*, 9(1), 15-21. Retrieved fromhttps://www.derpharmachemica.com/pharma-chemica/potential-prebiotic-properties-b...

Fatma, S., Singh, S. P., & Sharma, N. (2014).Optimization of a process for the development ofprobiotic yoghurt porridge mix. In 2nd Annual Conference on Probiotics and Microbiome: Gut and Beyond. New Delhi, India.

FDA [Food and Drug Administration]. 2004. *Probiotics*. Available: http://www.webdietitians.org/Public/GovernmentAffairs/92_adap1099.cfm. [13 Aug. 2014]

Femia, A. P., Luceri, C., Dolara, P., Giannini, A., Biggeri, A., Salvadori, M., …Caderni, G. (2002). Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics Lactobacillus rhamnosus and Bifidobacteriumlactis on azoxymethane-induced colon carcinogenesis in rats. *Carcinogenesis*, 23(11), 1953-1960. doi:10.1093/carcin/23.11.1953

Fijan, S. (2016).Chapter 10.Antimicrobial effect of probiotics against common pathogens.In *Probiotics and prebiotics in human nutrition and health*. Retrieved from DOI: 10.5772/63141

Francino, M. P. (2016). Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Frontiers in Microbiology*, 6. doi:10.3389/fmicb.2015.01543

Frank, D. N., St. Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases.*Proceedings of the National Academy of Sciences*, 104(34), 13780-13785. doi:10.1073/pnas.0706625104

Fredua-Agyeman, M., Stapleton, P., Basit, A. W., Beezer, A. E., & Gaisford, S. (2017). In vitro inhibition of Clostridium difficile by commercial probiotics: A microcalorimetric study. *International Journal of Pharmaceutics*, 517(1-2), 96-103. doi:10.1016/j.ijpharm.2016.12.005

FSSAI (2012). Ministry of Health and Family Welfare, Government of IndiaManual of methods of analysis of foods- Milk and Milk products. Retrieved from Food Safety and Standards Authority Of India website :https://old.fssai.gov.in/Portals/0/Pdf/Draft.../MILK_AND_MILK_PRODUCTS.pdf

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., … Ohno, H. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature*, *469*(7331), 543-547. doi:10.1038/nature09646

Fuller, R. (1992). History and development of probiotics.In Probiotics (pp. 1-8). Dordrecht: Springer.

Galdeano, C. M., & Perdigon, G. (2006).The Probiotic Bacterium Lactobacillus casei Induces Activation of the Gut Mucosal Immune System through Innate Immunity.*Clinical and Vaccine Immunology*, 13(2), 219-226. doi:10.1128/cvi.13.2.219-226.2006

Ganguly, S., & Sabikhi, L. (2012). Fermentation dynamics of probiotic Lactobacillus acidophilus NCDC-13 in a composite dairy-cereal substrate. *International Journal of Fermented Foods*, *1*, 33-46. Retrieved from file:///C:/Users/Dr%20Sujatha/Downloads/003_Fewwmented_Food1.pdf

Gerritsen, J., Smidt, H., Rijkers, G. T., & De Vos, W. M. (2011). Intestinal microbiota in human health and disease: the impact of probiotics. *Genes & Nutrition*, 6(3), 209-240. doi:10.1007/s12263-011-0229-7

Ghasempour, Z., Alizadeh, M., & Bari, M. R. (2011).Optimisation of probiotic yoghurt production containing Zedo gum.*International Journal of Dairy Technology*, 65(1), 118-125. doi:10.1111/j.1471-0307.2011.00740.x

Gibson, G. R., Beatty, E. R., Wang, X., & Cummings, J. H. (1995).Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin.*Gastroenterology*, 108(4), 975-982. doi:10.1016/0016-5085(95)90192-2

Gibson, G. R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., … Buddington, R. (2010). Dietary prebiotics: current status and new definition. *Food Science & Technology Bulletin: Functional Foods*, 7(1), 1-19. doi:10.1616/1476-2137.15880

Gilliland, S. E., & Speck, M. L. (1977). Antagonistic Action of Lactobacillus acidophilus Toward Intestinal and Foodborne Pathogens in Associative Cultures. *Journal of Food Protection*, 40(12), 820-823. doi:10.4315/0362-028x-40.12.820

Gilliland, S., Staley, T., & Bush, L. (1984).Importance of Bile Tolerance of Lactobacillus acidophilus Used as a Dietary Adjunct.*Journal of Dairy Science*, 67(12), 3045-3051. doi:10.3168/jds.s0022-0302(84)81670-7

Giongo, A., Gano, K. A., Crabb, D. B., Mukherjee, N., Novelo, L. L., Casella, G., … Triplett, E. W. (2010). Toward defining the autoimmune microbiome for type 1 diabetes. *The ISME Journal,* 5(1), 82-91. doi:10.1038/ismej.2010.92

Giri, S. S., Sen, S. S., Saha, S., Sukumaran, V., & Park, S. C. (2018). Use of a Potential Probiotic, Lactobacillus plantarum L7, for the Preparation of a Rice-Based Fermented Beverage.*Frontiers in Microbiology*, 9. doi:10.3389/fmicb.2018.00473

Girish, L. (2006). Development Of A Rabadi-Like Wheat Based Fermented Milk Beverage, (Unpublished master's thesis). National Dairy Research Institute, Karnal, India.

Goderska, K. (2012). Chapter 24-Different Methods of Probiotics Stabilization. In Probiotics, Edited by E.C Rigobelo.Rejeka, Croatia,. Retrieved from DOI: 10.5772/50313

Gokhale, S. V., & Lele, S. S. (2011). Dehydration of red beet root (Beta vulgaris) by hot air drying: Process optimization and mathematical modeling. *Food Science and Biotechnology*, 20(4), 955-964. doi:10.1007/s10068-011-0132-4

Gómez, B., Gullón, B., Remoroza, C., Schols, H. A., Parajó, J. C., & Alonso, J. L. (2014). Purification, Characterization, and Prebiotic Properties of Pectic Oligosaccharides from Orange Peel Wastes.*Journal of Agricultural and Food Chemistry*, 62(40), 9769-9782. doi:10.1021/jf503475b

Gopal, P. K., Sullivan, P. A., & Smart, J. B. (2001).Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including Bifidobacterium lactis DR10 and Lactobacillus rhamnosus DR20.*International Dairy Journal*, 11(1-2), 19-25. doi:10.1016/s0958-6946(01)00026-7

Gori, A., Rizzardini, G., Van't Land, B., Amor, K. B., Van Schaik, J., Torti, C., … Clerici, M. (2011). Specific prebiotics modulate gut microbiota and immune activation in HAART-naive HIV-infected adults: results of the ―COPA‖ pilot randomized trial. *Mucosal Immunology*, 4(5), 554-563. doi:10.1038/mi.2011.15

Goyal, R., & Khetarpaul, N. (1994). Preparation and fermentation of rice-defatted soy flour blends: Effect on protein, fat and ash content. *Plant Foods for Human Nutrition*, 45(1), 81-89. doi:10.1007/bf01091232

Granato, D., Masson, M. L., & Ribeiro, J. C. (2012). Sensory acceptability and physical stability evaluation of a prebiotic soy-based dessert developed

with passion fruit juice. *Ciênciae Tecnologia de Alimentos*, 32(1), 119-126. doi:10.1590/s0101-20612012005000004

Gregersen, T. (1978).Rapid method for distinction of gram-negative from gram-positive bacteria.*European Journal of Applied Microbiology and Biotechnology*, 5(2), 123-127. doi:10.1007/bf00498806

Guo, Z., Wang, J., Yan, L., Chen, W., Liu, X., & Zhang, H. (2009).In vitro comparison of probiotic properties of Lactobacillus casei Zhang, a potential new probiotic, with selected probiotic strains.*LWT - Food Science and Technology*, 42(10), 1640-1646. doi:10.1016/j.lwt.2009.05.025

Gupta, S., Cox, S., & Abu-Ghannam, N. (2010). Process optimization for the development of a functional beverage based on lactic acid fermentation of oats.*Biochemical Engineering Journal*, 52(2-3), 199-204. doi:10.1016/j.bej.2010.08.008

Hamilton-Miller,& Shah. (1998). Vancomycin susceptibility as an aid to the identification of lactobacilli.*Letters in Applied Microbiology*, 26(2), 153-154. doi:10.1046/j.1472-765x.1998.00297.x

Hammes W.P., Hertel C. (2006) The Genera Lactobacillus and Carnobacterium. In: *The Prokaryotes.* Springer, New York, NY 320-403. doi:10.1007/0-387-30744-310

Han, S., Gao, J., Zhou, Q., Liu, S., Wen, C., & Yang, X. (2018). Role of intestinal flora in colorectal cancer from the metabolite perspective: a systematic review. *Cancer management and research*, 10, 199-206. doi:10.2147/CMAR.S153482

Harrigan, W. F., & McCance, M. E. (1976).Laboratory methods in food and dairy microbiology. London: Academic Press Inc. Ltd.

Harte, F., Luedecke, L., Swanson, B., & Barbosa-Cánovas, G. (2003). Low-Fat Set Yogurt Made from Milk Subjected to Combinations of High Hydrostatic Pressure and Thermal Processing. *Journal of Dairy Science*, 86(4), 1074-1082. doi:10.3168/jds.s0022-0302(03)73690-x

Haryadi, Putri, A. W., & Cahyanto, M. N. (2017). Prebiotic activity of phosphorylated resistant corn starch. *Faculty of agricultural technology Universitas Gadjah Mada*. Retrieved from https://tp.ugm.ac.id/wp-content/uploads/2017/11/15082013145416-haryadi-prebioticalactivityofphosphorylated-.pdf

Hassan, I. A., & Tinay, A. H. (1995).Effect of fermentation on tannin content and in-vitro protein and starch digestibilities of two sorghum cultivars.*Food Chemistry*, 53(2), 149-151. doi:10.1016/0308-8146(95)90780-b

Hassanzadazar, H., Ehsan, A., Mardani, K., & Hesari,, J. (2012). Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. *Veterinary Research Forum*, 3(3), 181-185. Retrieved from https://europepmc.org/abstract/med/25610566

He, T., Priebe, M., Zhong, Y., Huang, C., Harmsen, H., Raangs, G., … Vonk, R. (2008). Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects.*Journal of Applied Microbiology*, doi:10.1111/j.1365-2672.2007.03579.x

Hekmat, S., & McMahon, D. J. (1992).Survival of Lactobacillus acidophilus and Bifidobacterium bifidum in Ice Cream for Use as a Probiotic Food.*Journal of Dairy Science*, 75(6), 1415-1422. doi:10.3168/jds.s0022-0302(92)77895-3

Henningsson, A., Björck, I., & Nyman, M. (2001). Short-chain fatty acid formation at fermentation of indigestible

carbohydrates. *Näringsforskning*, *45*(1), 165-168. doi:10.3402/fnr.v45i0.1801

Henry, R. J., & Saini, H. S. (1989).Characterisation of cereal sugars and oligosaccharides. , 66(5):.*Cereal chemistry*, 66(5), 362-365. Retrieved from https://espace.library.uq.edu.au/view/UQ:232842

Hernandez-Hernandez, O., Côté, G. L., Kolida, S., Rastall, R. A., & Sanz, M. L. (2011). In Vitro Fermentation of Alternansucrase Raffinose-Derived Oligosaccharides by Human Gut Bacteria. *Journal of Agricultural and Food Chemistry*, *59*(20), 10901-10906. doi:10.1021/jf202466s

Holkem, A. T., Raddatz, G. C., Nunes, G. L., Cichoski, A. J., Jacob-Lopes, E., Ferreira Grosso, C. R., & De Menezes, C. R. (2016). Development and characterization of alginate microcapsules containing Bifidobacterium BB-12 produced by emulsification/internal gelation followed by freeze drying.*LWT - Food Science and Technology*, 71, 302-308. doi:10.1016/j.lwt.2016.04.012

Holmes, E., Li, J., Marchesi, J., & Nicholson, J. (2012). Gut Microbiota Composition and Activity in Relation to Host Metabolic Phenotype and Disease Risk. *Cell Metabolism*, 16(5), 559-564. doi:10.1016/j.cmet.2012.10.007

Holzapfel, W. H., Haberer, P., Geisen, R., Björkroth, J., & Schillinger, U. (2001).Taxonomy and important features of probiotic microorganisms in food and nutrition.*The American Journal of Clinical Nutrition*, 73(2), 365s-373s. doi:10.1093/ajcn/73.2.365s

Huang, L., Zhang, M., Mujumdar, A. S., Sun, D., Tan, G., & Tang, S. (2009). Studies on Decreasing Energy Consumption for a Freeze-Drying Process of Apple Slices. *Drying Technology*, *27*(9), 938-946.doi:10.1080/07373930902901844

Huchchananavar, S. (2013). Development Of Probioticated Functional Food Mixes (Doctoral dissertation, University Of Agricultural Sciences , BENGALURU, India). Retrieved from krishikosh.egranth.ac.in/bitstream/1/82451/1/thesis.pdf.

Huebner, J., Wehling, R., & Hutkins, R. (2007). Functional activity of commercial prebiotics. *International Dairy Journal*, *17*(7), 770-775. doi:10.1016/j.idairyj.2006.10.006

Hullar, M. A., Burnett-Hartman, A. N., & Lampe, J. W. (2013). Gut Microbes, Diet, and Cancer. *Advances in Nutrition and Cancer*, 377-399. doi:10.1007/978-3-642-38007-5_22

Huq, T., Khan, A., Khan, R. A., Riedl, B., & Lacroix, M. (2013).Encapsulation of Probiotic Bacteria in Biopolymeric System.*Critical Reviews in Food Science and Nutrition*, 53(9), 909-916. doi:10.1080/10408398.2011.573152

Hussain, A. S., Patil, G. R., Yadav, V., & B Singh, R. R. (2015). Effect of storage on sensory quality, pH, wheying-off and probiotic count of lassi supplemented with Aloe barbadensis Miller juice. *Indian Journal of Dairy Science*, 68(2).

Igbabul, B., Hiikyaa, O., & Amove, J. (2014).Effect of Fermentation on the Proximate Composition and Functional Properties of Mahogany Bean (Afzelia africana) Flour.*Current Research in Nutrition and Food Science Journal*, 2(1), 01-07. doi:10.12944/crnfsj.2.1.01

IS 11623. (2008). *Dried milk - Determination of moisture content*. Retrieved from Bureau of Indian Standards (BIS) website: https://archive.org/details/gov.in.is.11623.2008/page/n5

IS 11766. (1987). *Method for Determination of Titratable Acidity in Milk Powder and Similar Products (Reference Method)*. Retrieved from Bureau Of Indian

Standards; New Delhi website:

https://archive.org/details/gov.in.is.11766.1986

Islam, K. S., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., … Yokota, A. (2011). Bile Acid Is a Host Factor That Regulates the Composition of the Cecal Microbiota in Rats. *Gastroenterology*, 141(5), 1773-1781. doi:10.1053/j.gastro.2011.07.046

ISO 4120:2004. (2004).Sensory analysis. Methodology. Triangle test. doi:10.3403/03067804u

Iyer,C., & Kailasapathy, K. (2005). Effect of Co-encapsulation of Probiotics with Prebiotics on Increasing the Viability of Encapsulated Bacteria under In Vitro Acidic and Bile Salt Conditions and in Yogurt.*Journal of Food Science*, 70(1), M18-M23. doi:10.1111/j.1365-2621.2005.tb09041.x

Jagannath, A., Kumar, M., & Raju, P. (2015).Fermentative Stabilization of Betanin Content in Beetroot and Its Loss during Processing and Refrigerated Storage. *Journal of Food Processing and Preservation*, 39(6), 606-613. doi:10.1111/jfpp.12267

Jalali, M., Abedi, D., Varshosaz, J., Najjarzadeh, M., Mirlohi, M., &Tavakoli, N. (2012).Stability evaluation of freeze-dried Lactobacillus paracasei subsp. Tolerance and Lactobacillus delbrueckii subsp. bulgaricus in oral capsules.*Research in Pharmaceutical Sciences*, 7(1), 31-36.

James, L. (2014). Effect of Inulin on the Growth and Antimicrobial Activity of Bifidobacteriumanimalis Subsp. Lactis 420- an Assessment. *World Applied Sciences Journal*, 32(4), 553-559.

James, L., Beena, A. K., Anupa, A., & Sreeshma, N. (2016). Antibiogram of Lactobacilli Isolated from Four Different Niches. *Journal of Microbiology & Microbial Technology*, 1(1), 4. doi:10.13188/2474-4530.1000002

Jandhyala, S. M.,Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Nageshwar Reddy, D. (2015). Role of the normal gut microbiota.*World Journal of Gastroenterology*, 21(29), 8787. doi:10.3748/wjg.v21.i29.8787

Jenie, B. S., Nurjanah, S., Firlieyanti, A. S., & Firlieyanti, C. C. (2010). Properties of modified starch of arrowroot, cassava, and kimpul starch by autocla ving-freeze-drying and chemical treatments,. In International Seminar Emerging Issues and Technology Developments in Foods and Ingridients. (pp. 6-8).

Jennings, T. A. (1999). Lyophilisation - introduction and basic principles (1st ed.). Boca Raton: CRC Press.

JHeimbach LLC. (2012). Generally Recognized as Safe (GRAS) Determination for the Use of Lactobacillus casei Strain Shirota As a Food Ingredient. Retrieved from httphttps://pdfsecret.com/.../lactobacillus-casei-strain-shirota_59f70ccdd64ab20a7512da59.

Jin, L. Z., Ho, Y. W., Abdullah, N., & Jalaludin, S. (1998). Acid and bile tolerance of Lactobacillus isolated from chicken intestine. *Letters in Applied Microbiology*, 27(3), 183-185. doi:10.1046/j.1472-765x.1998.00405.x

Joint FAO/WHO expert committee on food additives (JEFCA)-59th session. (2002). *Compendium of food additive specifications*. Geneva, Switzerland.

Jones, B. V., Begley, M., Hill, C., Gahan, C. G., & Marchesi, J. R. (2008).Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome.*Proceedings of the National Academy of Sciences*, 105(36), 13580-13585. doi:10.1073/pnas.0804437105

Kailasapathy, K. (2015). Chap 11: Biopolymers for administration and gastrointestinal delivery of functional food ingredients and probiotic bacteria. In In Functional Polymers in Food Science: From Technology to Biology (2nd ed), pp. 231-260

Kamada, N., Seo, S., Chen, G. Y., & Núñez, G. (2013).Role of the gut microbiota in immunity and inflammatory disease.*Nature Reviews Immunology*, 13(5), 321-335. doi:10.1038/nri3430

Kandler, O., & Weiss, N. (1986). Bergey's manual of systematic bacteriology. (9th ed.). Baltimore (MD): Williams and Wilkins.

Kaur, H., Mishra, H. N., & Kumar, P. (2009). Textural properties of mango soy fortified probiotic yoghurt: optimisation of inoculum level of yoghurt and probiotic culture. International Journal of Food Science & Technology, 44(2), 415-424. doi:10.1111/j.1365-2621.2008.01789.x

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., & Fakiri, E. M. (2013). Health Benefits of Probiotics: A Review. *ISRN Nutrition*, 2013, 1-7. doi:10.5402/2013/481651

Kedia, G., Vázquez, J. A., Charalampopoulos, D., & Pandiella, S. S. (2008).In Vitro Fermentation of Oat Bran Obtained by Debranning with a Mixed Culture of Human Fecal Bacteria.*Current Microbiology*, 58(4), 338-342. doi:10.1007/s00284-008-9335-1

Kedia, G., Vázquez, J. A., Charalampopoulos, D., & Pandiella, S. S. (2009). In Vitro Fermentation of Oat Bran Obtained by Debranning with a Mixed Culture of Human Fecal Bacteria. Current Microbiology, 58(4), 338-342. doi:10.1007/s00284-008-9335-1

Khatoon, N. and Gupta, R.K. (2015). Probiotic beverages of sweet lime and sugarcane juices and its physiochemical, microbiological & shelflife Studies. *Journal of Pharmacognosy and Phytochemistry*. 4(3): 25-34

Kitabatake, N., Gimbi, D. M., & Oi, Y. (2003). Traditional non-alcoholic beverage, Togwa, in East Africa, produced from maize flour and germinated finger millet. International *Journal of Food Sciences and Nutrition*, 54(6), 447-455. doi:10.1080/09637480120092053

Klein, G., Zill, E., Schindler, R., & Louwers, J. (1998). Peritonitis associated with vancomycin-resistant Lactobacillus rhamnosus in a continuous ambulatory peritoneal dialysis patient: organism identification, antibiotic therapy, and case report. *Journal of ClinicalMicrobiology*, 36(6), 1781–1783.Retrieved fromhttps://europepmc.org/abstract/med/9620421

Koebnick, C., Wagner, I., Leitzmann, P., Stern, U., & Zunft, H. F. (2003).Probiotic Beverage ContainingLactobacillus caseiShirota Improves Gastrointestinal Symptoms in Patients with Chronic Constipation.*Canadian Journal of Gastroenterology*, 17(11), 655-659. doi:10.1155/2003/654907

Kohajdová, Z., &Karovičová, J. (2007).Fermentation of cereals for specific purpose.*Journal of Food and Nutrition Research*, 46(2), 51-57.

Krasaekoopt, K., &Tandhanskul, A. (2008).Sensory and acceptance assessment of yogurt containing probiotic beads in Thailand.*Kasetsart Journal: Natural Science*, 42, 99-106.

Krasaekoopt, W., & Kitsawad, K. (2010).Sensory Characteristics and Consumer Acceptance of Fruit Juice Containing Probioitcs Beads in Thailand.*AU Journal of Technology*, 14(1), 33-38.

Krasaekoopt, W., Bhandari, B., & Deeth, H. (2004). The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria.*International Dairy Journal*, 14, 737–743.

Kruse, H., Kleessen, B., & Blaut, M. (1999).Effects of inulin on faecal bifidobacteria in human subjects.*British Journal of Nutrition*, 82(05), 375-382. doi:10.1017/s0007114599001622

Kubantseva, N., & Hartel, R. W. (2002). Solubility of calcium lactate in aqueous solution. *Food Reviews International*, *18*(2-3), 135-149. doi:10.1081/fri-120014355

Kumar, R., Grover, S., & Batish, V. K. (2011). Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing Lactobacillus plantarum strains in Sprague–Dawley rats.*British Journal of Nutrition*, 105(04), 561-573. doi:10.1017/s0007114510003740

Kumar, S.R., Kanmani, P., Yuvaraj, N., Paari, K. A., Pattukumar, V., & Arul, V. (2012). Traditional Indian fermented foods: a rich source of lactic acid bacteria. *International Journal of Food Sciences and Nutrition*, 64(4), 415-428. doi:10.3109/09637486.2012.746288

Kunz, C., & Egge, H. (2017). From Bifidus Factor to Human Milk Oligosaccharides: A Historical Perspective on Complex Sugars in Milk. In Prebiotics and Probiotics in Human Milk-Origins and Functions of Milk-Borne Oligosaccharides and Bacteria.(1st ed., p. 223–248). Academic Press.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., & Pedersen, O. (2013). Richness of human gut microbiome correlates with metabolic markers. *Nature*, 500(7464), 541-546. doi:10.1038/nature12506

Lenoir-Wijnkoop, I., Sanders, M. E., Cabana, M. D., Caglar, E., Corthier, G., Rayes, N., …Wolvers, Danielle A. W. (2007). Probiotic And Prebiotic Influence Beyond The Intestinal Tract. *Nutrition Reviews*, 65(11), 469–489.

Lestari, L. A., N. E. Soesatyo, M. H., Iravati, S., &Harmayani, E. (2013). Characterization of Bestak sweet potato (Ipomoea batatas) variety from

Indonesian origin as prebiotic.*International Food Research Journal*, 20(5), 2241-2245.

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America*, 102(31), 11070–11075.

Linares-Pasten, J. A., Aronsson, A., & Karlsson, E. N. (2017).Structural Considerations on the Use of Endo-Xylanases for the Production of prebiotic Xylooligosaccharides from Biomass.*Current Protein & Peptide Science*, 19(1).doi:10.2174/1389203717666160923155209

Liong, M. (2008). Roles of Probiotics and Prebiotics in Colon Cancer Prevention: Postulated Mechanisms and In-vivo Evidence. *International Journal of Molecular Sciences*, 9(5), 854-863. doi:10.3390/ijms9050854

Liong, M., & Shah, N. (2005).Acid and Bile Tolerance and Cholesterol Removal Ability of Lactobacilli Strains.*Journal of Dairy Science*, 88(1), 55-66. doi:10.3168/jds.s0022-0302(05)72662-x

Ljungh, A., &Wadström, T. (2006).Lactic acid bacteria as probiotics.*Current Issues in Intestinal Microbiology*, 7(2), 73-89.

Locke, G. R., Pemberton, J. H., & Phillips, S. F. (2000). AGA Technical Review On Constipation. American Gastroenterological Association, *Gastroenterology*.119, 1766-1778.

Luana, N., Rossana, C., Curiel, J. A., Kaisa, P., Marco, G., & Rizzello, C. G. (2014). Manufacture and characterization of a yogurt-like beverage made with oat flakes fermented by selected lactic acid bacteria. *International Journal of Food Microbiology*, 185, 17-26. doi:10.1016/j.ijfoodmicro.2014.05.004

Luckow, T., & Delahunty, C. (2004). Which juice is ‗healthier'? A consumer study of probiotic non-dairy juice drinks. *Food Quality and Preference*, 15(7-8), 751-759. doi:10.1016/j.foodqual.2003.12.007

Luckow, T., & Delahunty, C. (2004).Consumer acceptance of orange juice containing functional ingredients.*Food Research International*, 37(8), 805-814. doi:10.1016/j.foodres.2004.04.003

Macfarlane, G. T., & Macfarlane, S. (2012). Bacteria, colonic fermentation, and gastrointestinal health. *Journal of AOAC International*, *95*(1), 50-60. doi:10.5740/jaoacint.sge_macfarlane

Madhu, A. N., &Prapulla, S. G. (2012). In vitro fermentation of prebiotics by Lactobacillus plantarum CFR 2194: selectiveity, viability and effect of metabolites on β- glucuronidase activity. *World Journal of Microbiology and Biotechnology*, 28(3), 901-908.

Mandal, S., Puniya, A., & Singh, K. (2006). Effect of alginate concentrations on survival of microencapsulated Lactobacillus casei NCDC-298. *International Dairy Journal*, 16(10), 1190-1195. doi:10.1016/j.idairyj.2005.10.005

Manisseri, C., & Gudipati, M. (2012). Prebiotic Activity of Purified Xylobiose Obtained from Ragi (Eleusine coracana, Indaf-15) Bran. *Indian Journal of Microbiology*, 52(2), 251-257. doi:10.1007/s12088-011-0176-4

Marieb, E. N., & Hoehn, K. (2010). Human anatomy & physiology (8th ed.). San Francisco: Benjamin Cummings.

Marin, M. L., Benito, Y., Pin, C., Fernandez, M. F., Garcia, M. L., Selgas, M. D., & Casas, C. (1997). Lactic acid bacteria: hydrophobicity and strength of attachment to meat surfaces. *Letters in Applied Microbiology*, 24(1), 14-18. doi:10.1046/j.1472-765x.1997.00334.x

Markakis. (1982). Chap 6: stability of anthocyanin in foods. In Anthocyanin as food colours (1st ed., p. 192).Academic press.

Marteau, P., Flourie, B., Pochart, P., Chastang, C., Desjeuxj, F., &Rambaudj, C. (1990). Effect Of The Microbial Lactase (EC 3.2.1.23) Activity In Yoghurt On The Intestinal Absorption Of Lactose: An In Vivo Study On Lactase-Deficient Humans. *British Journal of Nutrition*, 64, 71-79.

Marteau, P., Pochart, P., Bouhnik, Y., & Rambaud, J. (1993). The Fate and Effects of Transiting, Nonpathogenic Microorganisms in the Human Intestine. *World Rev. Nutr.Diet*, 74(1). Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8212727

Marth, E. H. (1978). Standard methods for the examination of dairy products (14th ed.). Washington D C: American Public Health Association.

McBurney, M. I., & Thompson, L. U. (1987). Effect of human faecal inoculum on in vitro fermentation variables. *British Journal of Nutrition*, *58*(02), 233. doi:10.1079/bjn19870091

McFarland, L. V. (2006). Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of Clostridium difficile disease. *The American Journal of Gastroenterology*, 101, 812–822.

Medellin-Pena, M. J., Wang, H., Johnson, R., Anand, S., & Griffiths, M. W. (2007). Probiotics Affect Virulence-Related Gene Expression in Escherichia coli O157:H7. *Applied and Environmental Microbiology*, 73(13), 4259-4267. doi:10.1128/aem.00159-07

Melgar-Lalanne, G., & Hermandez, H. (2015).Cell surface properties of halotelorent probiotic lactobacilli. .4(3),.*J.of advances in biotechnology*, 4(3), 405-413.

Meng, X., Stanton, C., Fitzgerald, G., Daly, C., & Ross, R. (2008). Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry*, *106*(4), 1406-1416. doi:10.1016/j.foodchem.2007.04.076

Miao, S., Mills, S., Stanton, C., Fitzgerald, G. F., Roos, Y., & Ross, R. P. (2008). Effect of disaccharides on survival during storage of freeze dried

probiotics. *Dairy Science and Technology*, 88(1), 19-30. doi:10.1051/dst:2007003

Millette, M., Luquet, F., & Lacroix, M. (2007).In vitro growth control of selected pathogens by Lactobacillus acidophilus- and Lactobacillus casei-fermented milk.*Letters in Applied Microbiology*, 44(3), 314-319. doi:10.1111/j.1472-765x.2006.02060.x

Mishra, V., & Prasad, D. (2005).Application of in vitro methods for selection of strains as potential probiotics.*International Journal of Food Microbiology*, 103(1), 109-115. doi:10.1016/j.ijfoodmicro.2004.10.047

Misrianti, B. (2013). Effects of Sucrose on Making Additions Buffalo Whey Fermentation against Inhibition of Bacterial Pathogens (Thesis) ((Doctoral dissertation, Makassar: Faculty of Animal Science, University of Hasanuddin Makassar).

Mitsou, E. K., Panopoulou, N., Turunen, K., Spiliotis, V., & Kyriacou, A. (2010). Prebiotic potential of barley derived β-glucan at low intake levels: A randomised, double-blinded, placebo-controlled clinical study. Food Research International, 43(4), 1086-1092. doi:10.1016/j.foodres.2010.01.020

Modha, H., & Pal, D. (2010).Optimization of Rabadi-like fermented milk beverage using pearl millet.*Journal of Food Science and Technology*, 48(2), 190-196. doi:10.1007/s13197-010-0146-6

Modha, H., & Pal, D. (2010). Optimization of Rabadi-like fermented milk beverage using pearl millet. *Journal of Food Science and Technology*, *48*(2), 190-196. doi:10.1007/s13197-010-0146-6

Moongngarm, A., Trachoo, N., &Sirigungwan, N. (2011). Low molecular weight carbohydrates, prebiotic content, and prebiotic activity of selected food plants in Thailand. *Advance Journal of Food Science and Technology*, 3(4), 269-274.

Moraru, D., Bleoancă, I., & Segal, R. (2007).Probiotic vegetable juices.The Annals of the University Dunarea de Jos of Galati – 2007 Fascicle IV – Food Technology at International Symposium Euro - aliment, 87.

Morrison, D. J., & Preston, T. (2016).Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism.*Gut Microbes*, 7(3), 189-200. doi:10.1080/19490976.2015.1134082

Morrison, D. J., & Preston, T. (2016).Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism.*Gut Microbes*, 7(3), 189-200.

Mortazavian, A., Razavi, S. H., Ehsani, M. R., & Sohrabvandi, S. (2007). Principles and methods of microencapsulation of probiotic microorganisms.*Iranian Journal of Biotechnology*, 5(1), 1-18. Retrieved from http://www.ijbiotech.com/article_7032.html

Moshfegh, A. J., Friday, J. E., Goldman, J. P., & Ahuja, J. K. (1999). Presence of Inulin and Oligofructose in the diets of Americans. *The Journal of Nutrition*, *129*(7), 1407S-1411S. doi:10.1093/jn/129.7.1407s

Mousavi, Z. E., Mousavi, S. M., Razavi, S. H., Emam-Djomeh, Z., & Kiani, H. (2011). Fermentation of pomegranate juice by probiotic lactic acid bacteria. World Journal of Microbiology and Biotechnology, 27(1), 123-128. doi:10.1007/s11274-010-0436-1

Msheliza, E. A., Hussein, J. B., JOY I., & Nkama, I. (2018). Effect of Fermentation and Roasting on the Physicochemical Properties of Weaning Food Produced from Blends of Sorghum and Soybean. *Journal of Nutrition & Food Sciences*, 08(02). doi:10.4172/2155-9600.1000681

Murthy, M. S., Swaminathan, T., Rakshit, S. K., & Kosugi, Y. (2000). Statistical optimization of lipase catalyzed hydrolysis of methyloleate by response surface methodology. *Bioprocess Engineering*, 22(1), 35-39. doi:10.1007/pl00009097

Muyanja, C., Narvhus, J., Treimo, J., & Langsrud, T. (2003). Isolation, characterisation and identification of lactic acid bacteria from bushera: a Ugandan traditional fermented beverage. International Journal of Food Microbiology, 80(3), 201-210. doi:10.1016/s0168-1605(02)00148-4

Nag, A., Han, K., & Singh, H. (2011). Microencapsulation of probiotic bacteria using pH-induced gelation of sodium caseinate and gellan gum. International Dairy Journal, 21(4), 247-253. doi:10.1016/j.idairyj.2010.11.002

Narayanappa, D. (2008). Randomized double blinded controlled trial to evaluate the efficacy and safety of Bifilac in patients with acute viral diarrhea. The Indian Journal of Pediatrics, 75(7), 709-713. doi:10.1007/s12098-008-0134-2

Nazzaro, F., Fratianni, F., Coppola, R., Sada, A., & Orlando, P. (2009). Fermentative ability of alginate-prebiotic encapsulated Lactobacillus acidophilus and survival under simulated gastrointestinal conditions. *Journal of Functional Foods*, *1*(3), 319-323. doi:10.1016/j.jff.2009.02.001

Nebesny, E., DorotaŻyżelewicz, D., Motyl, I., &Libudzisz, Z. (2007). Dark chocolates supplemented with Lactobacillus strains. *European Food Research and Technology*, 22(1), 33-42.

Nematollahi, A., Sohrabvandi, S., Mortazavian, A. M., & Jazaeri, S. (2016). Viability of probiotic bacteria and some chemical and sensory characteristics in cornelian cherry juice during cold storage. Electronic Journal of Biotechnology, 21, 49-53. doi:10.1016/j.ejbt.2016.03.001

Neyrinck, A. M., Van Hée, V. F., Piront, N., De Backer, F., Toussaint, O., Cani, P. D., & Delzenne, N. M. (2012). Wheat-derived arabinoxylan oligosaccharides with prebiotic effect increase satietogenic gut peptides and reduce metabolic endotoxemia in diet-induced obese mice. *Nutrition & Diabetes*, *2*(1), e28-e28. doi:10.1038/nutd.2011.24

Nithyapriya, S., &Vasudevan, A. (2016). Effect Of Lactic Acid Bacteria In Development Of Papaya Juice Using Response Surface Methodology. *International Journal of Biotechnology and Biochemistry*, 12(1), 27-32.

Nurhartadi, E., Utami, R., Nursiwi, A., Sari, A. M., Widowati, E., Sanjaya, A. P., & Esnadewi, E. A. (2017).Effect of Incubation Time and Sucrose Addition on the Characteristics of Cheese Whey Yoghurt. In International Conference On Food Science and Engineering IOP Conf. Series: *Materials Science and Engineering* (p. 193). IOP Publishing.

Ocaña, V., Silva, C., & Nader-Macías, M. E. (2006).Antibiotic Susceptibility of Potentially Probiotic Vaginal Lactobacilli.*Infectious Diseases in Obstetrics and Gynecology*, 2006, 1-6. doi:10.1155/idog/2006/18182

Oetjen, G. W. (1999). Freeze-drying. Weinheim: Wiley-VCH.

Ojokoh, A., & Bello, B. (2014).Effect of Fermentation and Extrusion on Nutrient and Anti-nutrient Composition of Millet (Pennisetum glaucum) and Soyabean (Glycine max) Blend Flours.*Journal of Life Sciences*, 8(8), 668-675. Retrieved from https://www.researchgate.net/publication/316605503_Effect_of_Fermentation_on_Nutrient_and_Anti-nutrient_Composition_of_Millet_Pennisetum_glaucum_and_Soyabean_Glycine_max_Blend_Flours

Okporo, V. E., Ameh, D. A., Sallau, A. B., & Aba, D. A. (2016).Effect of Malting on Chemical Composition and Protein Invitro Digestibility of Three New Varieties of Sorghum Grains.*International Journal of Science and Research (IJSR*), 5(7), 2107-2110. doi:10.21275/v5i7.27071601

Oliveira, D. L., Costabile, A., Wilbey, R. A., Grandison, A. S., Duarte, L. C., & Roseiro, L. B. (2012). In vitroevaluation of the fermentation properties and potential prebiotic activity of caprine cheese whey

oligosaccharides in batch culture systems. *BioFactors*, *38*(6), 440-449. doi:10.1002/biof.1043

O'Mahony, L., Feeney, M., O'Halloran, S., Murphy, L., Kiely, B., Fitzgibbon, J., … Collins, J. K. (2001). Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. *Alimentary Pharmacology and Therapeutics*, *15*(8), 1219-1225. doi:10.1046/j.1365-2036.2001.01027.x

Onwuka, G. I. (2005). Food analysis and instrumentation theory and practice . Lagos, portugal: Napthali prints.

Onyango, C., Noetzold, H., Ziems, A., Hofmann, T., Bley, T., & Henle, T. (2005). Digestibility and antinutrient properties of acidified and extruded maize–finger millet blend in the production of uji. *LWT - Food Science and Technology*, 38(7), 697-707. doi:10.1016/j.lwt.2004.09.010

Oser, B. L., & Ford, R. A. (1978). Recent Progress in the Consideration of Flavoring Ingredients Under the Food Additives Amendment 11. GRAS Substances. *Food TEchnology*, 61-70.

Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: an overview of beneficial effects. *Lactic Acid Bacteria: Genetics, Metabolism and Applications*, 279-289. doi:10.1007/978-94-017-2029-8_18

Özer, D., Akin, S., & Özer, B. (2005).Effect of Inulin and Lactulose on Survival of Lactobacillus AcidophilusLA-5 and Bifidobacterium Bifidum BB-02 in Acidophilus-Bifidus Yoghurt.*Food Science and Technology International*, 11(1), 19-24. doi:10.1177/1082013205051275

Palacio, M. I., Etcheverría, A. I., & Manrique, G. D. (2014). Fermentation by Lactobacillus paracasei of galactooligosaccharides and low-molecularweight carbohydrates extracted from squash (Curcubita maxima) and lupin (Lupinusalbus) seeds. *Journal of Microbiology, Biotechnology and Food Sciences*, 3(4), 329-332.

Palframan, R., Gibson, G., & Rastall, R. (2003). Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. Letters in Applied Microbiology, 37(4), 281-284. doi:10.1046/j.1472-765x.2003.01398.x

Pan, X., Chen, F., Wu, T., Tang, H., & Zhao, Z. (2009). The acid, bile tolerance and antimicrobial property of Lactobacillus acidophilus NIT.*Food Control*, 20(6), 598-602. doi:10.1016/j.foodcont.2008.08.019

Pandey, K.R., & Vakil, B.V.(2017). Encapsulation of Probiotic Bacillus coagulans for Enhanced Shelf Life. . Journal of Applied Biology & Biotechnology. doi:10.7324/jabb.2017.50409

Panesar, P. S., Kennedy, J. F., Knill, C. J., & Kosseva, M. (2010). Production of L(+) lactic acid using Lactobacillus casei from whey. *Brazilian Archives of Biology and Technology*, 53(1), 219-226. doi:10.1590/s1516-89132010000100027

Pedroso, D. D., Thomazini, M., Heinemann, R. J., & Favaro-Trindade, C. S. (2012).Protection of Bifidobacterium lactis and Lactobacillus acidophilus by microencapsulation using spray-chilling.*International Dairy Journal*, 26(2), 127-132. doi:10.1016/j.idairyj.2012.04.008

Pelczar, M. J., Chan, E. C., & Krieg, N. R. (1998). pure cultures and cultural characteristics. In *Microbiology: Concepts and Applications*. New York, NY: McGraw-Hill College.

Pelletier, C., Bouley, C., Cayuela, C., Bouttier, S., Bourlioux, P., & Bellon-Fontaine, M. N. (1997).Cell surface characteristics of Lactobacillus casei subsp. casei, Lactobacillus paracasei subsp. paracasei, and Lactobacillus rhamnosus strains.Applied and environmental microbiology, 63(5), 1725-31.(n.d.). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC168469/

Pereira, A. L., Maciel, T. C., & Rodrigues, S. (2011). Probiotic beverage from cashew apple juice fermented with Lactobacillus casei. Food Research International, 44(5), 1276-1283. doi:10.1016/j.foodres.2010.11.035

Poeker, S. A., Geirnaert, A., Berchtold, L., Greppi, A., Krych, L., Steinert, R. E., … Lacroix, C. (2018). Understanding the prebiotic potential of different dietary fibers using an in vitro continuous adult fermentation model (PolyFermS). *Scientific Reports*, *8*(1). doi:10.1038/s41598-018-22438-y

Prado, F. C., Parada, J. L., Pandey, A., & Soccol, C. R. (2008). Trends in non-dairy probiotic beverages. Food Research International, 41(2), 111-123. doi:10.1016/j.foodres.2007.10.010

Pranoto, Y., Anggrahini, S., & Efendi, Z. (2013). Effect of natural and Lactobacillus plantarum fermentation on in-vitro protein and starch digestibilities of sorghum flour.*Food Bioscience*, 2, 46-52. doi:10.1016/j.fbio.2013.04.001

Priatni, S., & Pradita, A. (2015).Stability Study of Betacyanin Extract from Red Dragon Fruit (Hylocereus Polyrhizus) Peels.*Procedia Chemistry*, 16, 438-444. doi:10.1016/j.proche.2015.12.076

Raghuramalu, N., Nair, K. M., & Kalyanasundaram, S. (2003). A manual of laboratory techniques. Hyderabad, India: National Institute for nutrition.

Raimbault, M. (1998).General and microbiological aspects of solid substrate fermentation.*Electronic Journal of Biotechnology*, 1(2), 174-188. doi:10.2225/vol1-issue3-fulltext-9

Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. *Electronic Journal of Biotechnology*, *1*(2), 174-188. doi:10.2225/vol1-issue3-fulltext-9

Rajpal, S., &Kansal, V. K. (2009). Probiotic dahi containing Lactobacillus acidophilus and Bifidobacteriumbifidum stimulates immune system in mice. *Milchwissenschaft*, 64, 147-150.

Ramasamy, K., Abdullah, N., Wong, M. C., Karuthan, C., & Ho, Y. W. (2010). Bile salt deconjugation and cholesterol removal from media byLactobacillusstrains used as probiotics in chickens. *Journal of the Science of Food and Agriculture*, 90(1), 65-69. doi:10.1002/jsfa.3780

Rasekh, J. (1974). Effect of freeze drying, spray drying, and stabilizers on functional characteristics of fish protein concentrate. *Journal of Milk and Food Technology*, *37*(2), 78-82. doi:10.4315/0022-2747-37.2.78

Rathore, S., Salmerón, I., & Pandiella, S. S. (2012). Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. Food Microbiology, 30(1), 239-244. doi:10.1016/j.fm.2011.09.001

Rathore, S., Salmerón, I., & Pandiella, S. S. (2012). Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. *Food Microbiology*, *30*(1), 239-244. doi:10.1016/j.fm.2011.09.001

Ratsep, M., Naaber, P., Koljalg, S., Smidt, I.,Shkut, E., & Sepp, E. (2014). Effect of Lactobacillus plantarum Strains on Clinical Isolates of Clostridium difficile in vitro. Journal of Probiotics & Health, 02(01). doi:10.4172/2329-8901.1000119

Rezaei, R., Khomeiri, M., Aalami, M., & Kashaninejad, M. (2012). Effect of inulin on the physicochemical properties, flow behavior and probiotic survival of frozen yogurt. Journal of Food Science and Technology, 51(10), 2809-2814. doi:10.1007/s13197-012-0751-7

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavilán, C. G., & Salazar, N. (2016). Intestinal Short Chain

Fatty Acids and their Link with Diet and Human Health. *Frontiers in microbiology*, *7*, 185. doi:10.3389/fmicb.2016.00185

Roberfroid, M. (2007). Prebiotics: The Concept Revisited. *The Journal of Nutrition*, *137*(3), 830S-837S. doi:10.1093/jn/137.3.830s

Rochat, D., Meillour, P. N., Esteban-Duran, J. R., Malosse, C., Perthuis, B., Morin, J., & Descoins, C. (2000). Identification of pheromone synergists in American palm weevil, Rhynchophorus palmarum, and attraction of related Dynamis borassi. *Journal of Chemical Ecology*, *26*(1), 155-187. doi:10.1023/a:1005497613214

Rodrigues, M., Mandalika, S. and Jamdar, N.S. (2015). Influence of finger millet soyabean matrix on te viability of probiotic lactic acid bacteria exposed to high temperature. *International Journal Of Researches In Biosciences, Agricultureand Technology*, 6, ISSN 2347-517

Rodrigues, M., Mandalika, S., Jamdar, S., & Sharma, A. (2013, October). Influence of a ragi-soybean combination on the viability of probiotic organisms during storage and under simulated gastrointestinal conditions. Paper presented at *BARC Newsletter*, Special Issue,, Mumbai.

Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. Nature Reviews Immunology, 16(6), 341-352. doi:10.1038/nri.2016.42

Rosenberg, S. A., Schwarz, S., Spiess, P. J., & Brown, J. M. (1980). In vitro growth of murine T cells. III. Method for separation of T cell growth factor (TCGF) from concanavalin A and biological activity of the resulting TCGF. *Journal of Immunological Methods*, *33*(4), 337-350. doi:10.1016/0022-1759(80)90004-6

Rubel, I. A., Pérez, E. E., Genovese, D. B., & Manrique, G. D. (2014). In vitro prebiotic activity of inulin-rich carbohydrates extracted from Jerusalem artichoke (Helianthus tuberosus L.) tubers at different storage times by

Lactobacillus paracasei. Food Research International, 62, 59-65. doi:10.1016/j.foodres.2014.02.024

Ruggeri, S., Straniero, R., Pacifico, S., Aguzzi, A., & Virgili, F. (2008). French Marine Bark Extract Pycnogenol as a Possible Enrichment Ingredient for Yogurt. *Journal of Dairy Science, 91*(12), 4484-4491. doi:10.3168/jds.2008-1250

Ruiz, L., Margolles, A., & Sánchez, B. (2013).Bile resistance mechanisms in Lactobacillus and Bifidobacterium.*Frontiers in Microbiology*, 4. doi:10.3389/fmicb.2013.00396

Saalia, N. K., Ankamah, S., Otubuah, B., Barfi, N. K., & Tano-Debrah, K. (2012). Development and Characterization of Low Viscosity Porridge (Koko) Flour by CoFermenting with Millet Malt.*Open Access Scientific Reports* , 1, 551. doi:10.4172/scientificreports.551

Saarela, M., Virkajärvi, I., Nohynek, L., Vaari, A., & Mättö, J. (2006). Fibres as carriers for Lactobacillus rhamnosus during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals. International Journal of Food Microbiology, 112(2), 171-178. doi:10.1016/j.ijfoodmicro.2006.05.019

Sadasivam, S., & Manickam, A. (2008). Biochemical Methods (3rd ed.). New Delhi, India: New Age International Publishers.

Sahadeva r, P. K., Leong, S. F., Chua, K. H., Tan, C. H., Chan, H. Y., Tong, E. V., … Chan, H. K. (2011). Survival of commercial probiotic strains to pH and bile. *International Food Research Journal*, 18(4), 1515-1522. Retrieved from www.ifrj.upm.edu.my/18%20(04)%202011/(44)IFRJ-2011-285.pdf

Saito, Y., Takano, T., & Rowland, I. (1992).Effects of Soybean Oligosaccharides on the Human Gut Microflora in In vitro Culture.

Microbial Ecology in Health and Disease, 5(2), 105-110. doi:10.3109/08910609209141296

Sanders, M. E. (2006). Summary of Probiotic Activities of Bifidobacterium lactis HN019. Journal of Clinical Gastroenterology, 40(9), 776-783. doi:10.1097/01.mcg.0000225576.73385.f0

Santos M, C. R., Soccol, M., Waszczynsky, J. N., & Raimbault, M. (2003).Development of a New Beverage with Probiotic Activity.In New Horizons in Biotechnology. (pp. 353-364). Dordrecht: Springer.

Satterlee, L. D., Marshall, H. F., & Tennyson, J. M. (1979).Measuring protein quality.*Journal of the American Oil Chemists' Society*, 56(3Part1), 103.doi:10.1007/bf02671431

Saulnier, D. M., Gibson, G. R., & Kolida, S. (2008). In vitro effects of selected synbiotics on the human faecal microbiota composition. FEMS Microbiology Ecology, 66(3), 516-527. doi:10.1111/j.1574-6941.2008.00561.x

Savard, P., Lamarche, B., Paradis, M., Thiboutot, H., Laurin, É., & Roy, D. (2011). Impact of Bifidobacterium animalis subsp. lactis BB-12 and, Lactobacillus acidophilus LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. International Journal of Food Microbiology, 149(1), 50-57. doi:10.1016/j.ijfoodmicro.2010.12.026

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., & De Vos, W. M. (2005). Probiotic and other functional microbes: from markets to mechanisms. Current Opinion in Biotechnology, 16(2), 204-211. doi:10.1016/j.copbio.2005.02.003

Saxena, R., & Sharma, V. (2016). A Metagenomic Insight Into the Human Microbiome. Medical and Health Genomics, 107-119. doi:10.1016/b978-0-12-420196-5.00009-5

Sedláčková, P., Horáčková, Š., Shi, T., Kosová, M., & Plocková, M. (2016). Two different methods for screening of bile salt hydrolase activity in Lactobacillus strains. *Czech Journal of Food Sciences*, 33(No. 1), 13-18. doi:10.17221/299/2014-cjfs

Sedláčková, P., Horáčková, Š., Shi, T., Kosová, M., & Plocková, M. (2016). Two different methods for screening of bile salt hydrolase activity in Lactobacillus strains. *Czech Journal of Food Sciences*, *33*(No. 1), 13-18. doi:10.17221/299/2014-cjfs

Shah, N. P. (2007). Functional cultures and health benefits. International Dairy Journal, 17(11), 1262-1277. doi:10.1016/j.idairyj.2007.01.014

Shah, N. P., Lankaputhra, W. E., Britz, M. L., & Kyle, W. S. (1995).Survival of Lactobacillus acidophilus and Bifidobacterium bifidum in commercial yoghurt during refrigerated storage.*International Dairy Journal*, 5(5), 515-521. doi:10.1016/0958-6946(95)00028-2

Sharma, M., Mridula, D., & Gupta, R. K. (2014). Development of sprouted wheat based probiotic beverage. *Journal of Food Science and Technology*, 51(12), 3926–3933. doi:10.1007/s13197-013-0959-1

Sharma, V., & Mishra, H. N. (2013). Fermentation of vegetable juice mixture by probiotic lactic acid bacteria. Nutrafoods, 12(1), 17-22. doi:10.1007/s13749-012-0050-y

Sharma, A., Jana, A. H., & Chavan, R. S. (2012). Functionality of Milk Powders and Milk-Based Powders for End Use Applications-A Review. *Comprehensive Reviews in Food Science and Food Safety*, *11*(5), 518-528. doi:10.1111/j.1541-4337.2012.00199.x

Sharon, C. L. (2010). Standardisation and Quality evaluation of banan based probiotic fermented food mixtures, 2010, Kerala Agriculture University (Unpublished doctoral dissertation). Kerala Agriculture University, Thrissur, India.

Sheba, S. (2015).Development of a ready to reconstitute probiotic drink using indigenous lactic acid bacteria, (Unpublished master's thesis). Kerala Veterinary and Animal Sciences University, Thrissur, India.

Shewale, R. N., Sawale, P. D., Khedkar, C. D., & Singh, A. (2014). Selection criteria for probiotics: a review. *International Journal of Probiotics and Prebiotics*, *9*(1), 17-22. Retrieved from http://www.nchpjournals.com/manuscript/uploads/article_649.pdf

Shi, L., Li, Z., Li, D., Xu, M., Chen, H., Zhang, Z., & Tang, Z. (2013). Encapsulation of probiotic Lactobacillus bulgaricus in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. Journal of Food Engineering, 117(1), 99-104. doi:10.1016/j.jfoodeng.2013.02.012

Shirai, K., Pedraza, G., Gutierrez-Durán, M., Marshall, V. M., Revah-Moiseev, S., & García-Garibay, M. (1992). Production of a yogurt-like product from plant foodstuffs and whey.Substrate preparation and fermentation.*Journal of the Science of Food and Agriculture*, 59(2), 199-204. doi:10.1002/jsfa.2740590210

Shokryazdan, P., Sieo, C. C., Kalavathy, R., Liang, J. B., Alitheen, N. B., Faseleh Jahromi, M., & Ho, Y. W. (2014). Probiotic Potential ofLactobacillusStrains with Antimicrobial Activity against Some Human Pathogenic Strains. BioMed Research International, 2014, 1-16. doi:10.1155/2014/927268

Shokryazdan, P., Sieo, C. C., Kalavathy, R., Liang, J. B., Alitheen, N. B., Faseleh Jahromi, M., & Ho, Y. W. (2014). Probiotic Potential ofLactobacillusStrains with Antimicrobial Activity against Some Human Pathogenic Strains. *BioMed Research International*, *2014*, 1-16. doi:10.1155/2014/927268

Sicard, D., & Legras, J. (2011). Bread, beer and wine: Yeast domestication in the Saccharomyces sensu stricto complex. Comptes Rendus Biologies, 334(3), 229-236. doi:10.1016/j.crvi.2010.12.016

Simha, H. V., Sharanakumar, H., Nidoni, U., Ramachandra, C., Tamil Vendan, K., & Prakash, K. (2012).Studies on spray-drying and freeze-drying of pomegranate (punicagranatum l.) Juice fermented with l.acidophilus. *International Journal of Food and Nutritional Sciences,*1(1), 118-127.

Simwaka, J. E., Chamba, M. V., Huiming, Z., Masamba,, K. G., & Luo, Y. (2017). Effect of fermentation on physicochemical and antinutritional factors of complementary foods from millet, sorghum, pumpkin and amaranth seed flours.*International Food Research Journal*, 24(5), 1869-1879. Retrieved from https://docplayer.net/62902913-Effect-of-fermentation-on-physicochemical-and-antinutritional-factors-of-complementary-foods-from-millet-sorghum-pumpkin-and-amaranth-seed-flours.html

Sindhu, S. C., & Khetarpaul, N. (2000). Development, acceptability and nutritional evaluation of probiotic fermented indigenous food blends. *Haryana Agricultural University Journal of Research*, 3(4), 141-151.

Sindhu, S. C., & Khetarpaul, N. (2005). Development, acceptability and nutritional evaluation of an indigenous food blend fermented with probiotic organisms. *Nutrition and Food Science,* 35(1), 20-27.

Sindhu, S. C., Khetarpaul, N., & Sindhu, A. (2013). Antidiarrhoeal effect of feeding probiotic fermented indigenous food blend in ampicillin treated mice. *International Journal of Microbial Resource Technology*, 2(1), 2278 – 3822.

Sindhu, S., Khetarpaul, N., & Sindhu, A. (2005).Effect of probiotic fermentation on carbohydrate and mineral profile of an indigenously

developed food blend.*Acta Alimentaria*, 34(1), 41-47. doi:10.1556/aalim.34.2005.1.7

Singoha, G., Pandey, N., Malik, R. K., & Mishra, S. K. (2014). Hypocholeterolemic effect of Lactobacillus gasseristarin Lg70 isolated from breast fed human infant faeces. *Journal of Innovative Biology*, 1(2), 105-111.

Sivudu, S. N., Umamahesh, K., & S Reddy, O. V. (n.d.). A Comparative study on Probiotication of mixed Watermelon and Tomato juice by using Probiotic strains of Lactobacilli. *International Journal of Current Microbiology and Applied Sciences*, 3(11), 977-984.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G., Gratadoux, J., … Langella, P. (2008). Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proceedings of the National Academy of Sciences, 105(43), 16731-16736. doi:10.1073/pnas.0804812105

Soliman, A. H., Sharoba, A. M., Bahlol, H. E., Soliman, A. S., & Radi, O. M. (201). Evaluation of Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus plantarum for probiotic characteristic.*Middle East J. Agric. Res*, 5(1), 10-18. Retrieved from https://www.researchgate.net/publication/272564816_Evaluation_of_Lactobacillus_acidophilus_Lactobacillus_casei_and_Lactobacillus_plantarum_for_probiotic_characteristics

Soliman, A. H., Sharoba, A. M., Bahlo h, E. M., Soliman, A. S., & Radi, O. M. (2015). Evaluation of Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus plantarum for probiotic characteristics. *Middle East Journal of Applied Sciences*, *5*(1), 10-18. Retrieved from www.curresweb.com/mejas/mejas/2015/94-101.pdf

Sornplang, P., & Piyadeatsoontorn, S. (2016). Probiotic isolates from unconventional sources: a review. Journal of Animal Science and Technology, 58(1). doi:10.1186/s40781-016-0108-2

Soukoulis, C., Panagiotidis, P., Koureli, R., & Tzia, C. (2007). Industrial Yogurt Manufacture: Monitoring of Fermentation Process and Improvement of Final Product Quality. *Journal of Dairy Science*, 90(6), 2641-2654. doi:10.3168/jds.2006-802

Spanhaak, S., Havenaar, R., & Schaafsma, G. (1998). The effect of consumption of milk fermented by Lactobacillus casei strain Shirota on the intestinal microflora and immune parameters in humans. European Journal of Clinical Nutrition, 52(12), 899-907. doi:10.1038/sj.ejcn.1600663

Spor, A., Koren, O., & Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. Nature Reviews Microbiology, 9(4), 279-290. doi:10.1038/nrmicro2540

Sreenivas, K., & Lele, S. (2013). Prebiotic activity of gourd family vegetable fibres using in vitro fermentation. Food Bioscience, 1, 26-30. doi:10.1016/j.fbio.2013.01.002

Sreenivas, K., & Lele, S. (2013). Prebiotic activity of gourd family vegetable fibres using in vitro fermentation. Food Bioscience, 1, 26-30. doi:10.1016/j.fbio.2013.01.002

Staffolo, M., Bertola, N., Martino, M., & Bevilacqua, Y. A. (2004). Influence of dietary fiber addition on sensory and rheological properties of yogurt. International Dairy Journal, 14(3), 263-268. doi:10.1016/j.idairyj.2003.08.004

Stern, N., Konishi, F., Hesseltine, C., & Wang, H. (1977).Lactobacillus acidophilus Utilization of Sugars and Production of a Fermented

Soybean Product.*Canadian Institute of Food Science and Technology Journal*, 10(3), 197-200. doi:10.1016/s0315-5463(77)73502-3

Succi, M., Tremonte, P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S., & Coppola, R. (2005). Bile salt and acid tolerance ofLactobacillus rhamnosusstrains isolated from Parmigiano Reggiano cheese. FEMS Microbiology Letters, 244(1), 129-137. doi:10.1016/j.femsle.2005.01.037

Sudha, A., Priyenka devi, K. S., & Sangeetha, V. (2016). Development of millet sprout milk beverage based on physicochemical property studies and consumer acceptability data. *Journal Of Scientific And Industrial Research*, 75, 239-243. Retrieved from http://nopr.niscair.res.in/handle/123456789/34056

Sudha, M. R., Chauhan, P., Dixit, K., Babu, S., & Jami, K. (2009).Probiotics as complementary therapy for hypercholesterolemia.*BiolMed*, 1(4), 1-13.

Swain, M. R., Anandharaj, M., Ray, R. C., & Parveen Rani, R. (2014). Fermented Fruits and Vegetables of Asia: A Potential Source of Probiotics. Biotechnology Research International, 2014, 1-19. doi:10.1155/2014/250424

Tabassum, A., Shiby, V. K., & Pandey, M. C. (2017). Quality Aspects and Storage Evaluation of Freeze Dried Probiotic Pineapple Lassi Powder. Defence Life Science Journal, 2(2), 186. doi:10.14429/dlsj.2.11364

Tamang, B., & Tamang, J. P. (2007).Role of lactic acid bacteria and their functional properties in goyang, a fermented leafy vegetable product of the Sherpas.*Journal of Hill Research*, 20(20), 53-61.

Tamang, B., Tamang, J. P., Schillinger, U., Franz, C. M., Gores, M., & Holzapfel, W. H. (2008). Phenotypic and genotypic identification of lactic acid bacteria isolated from ethnic fermented bamboo tender shoots

of North East India. International Journal of Food Microbiology, 121(1), 35-40. doi:10.1016/j.ijfoodmicro.2007.10.009

Tamang, J. P. (2009). Himalayan Fermented Foods: Microbiology, Nutrition and Ethnic Values(1st ed.). NewDelhi, India: CRCPress.

Tamang, J. P., & Sarkar, P. K. (1993). Sinki: A traditional lactic acid fermented radish tap root product. The Journal of General and Applied Microbiology, 39(4), 395-408. doi:10.2323/jgam.39.395

Tamang, J. P., Tamang, B., Schillinger, U., Franz, C. M., Gores, M., & Holzapfel, W. H. (2005). Identification of predominant lactic acid bacteria isolated from traditionally fermented vegetable products of the Eastern Himalayas. International Journal of Food Microbiology, 105(3), 347-356. doi:10.1016/j.ijfoodmicro.2005.04.024

Tárrega, A., & Costell, E. (2006). Effect of inulin addition on rheological and sensory properties of fat-free starch-based dairy desserts. International Dairy Journal, 16(9), 1104-1112. doi:10.1016/j.idairyj.2005.09.002

Tenaillon, O., Skurnik, D., Picard, B., & Denamur, E. (2010).The population genetics of commensal Escherichia coli.*Nature Reviews Microbiology*, 8(3), 207-217. doi:10.1038/nrmicro2298

Teuber, M. (1993).Lactic acid bacteria.In Biotechnology. (2nd ed.). Weinheim: Verlagsgesellschatt mbH.

Tharmaraj, N., & Shah, N. P. (2009). Antimicrobial effects of probiotic bacteria against selected species of yeasts and moulds in cheese-based dips. *International Journal of Food Science & Technology*, *44*(10), 1916-1926. doi:10.1111/j.1365-2621.2009.01986.x

Thomas, D. W., & Greer, F. R. (2010). Probiotics and Prebiotics in Pediatrics. PEDIATRICS, 126(6), 1217-1231. doi:10.1542/peds.2010-2548

Thorat, P., Sadawarte, S., Sawate, A., & Machewad, G. (2017).Studies on Effect of Fermentation on Physicochemical Properties of Vegetables and Preparation of Sauce.*International Journal of Current Microbiology and Applied Sciences*, 6(8), 3537-3545. doi:10.20546/ijcmas.2017.608.423

Tian, T., Freeman, S., Corey, M., German, J. B., & Barile, D. (2017). Chemical Characterization of Potentially Prebiotic Oligosaccharides in Brewed Coffee and Spent Coffee Grounds. Journal of Agricultural and Food Chemistry, 65(13), 2784-2792. doi:10.1021/acs.jafc.6b04716

Tomczak, D. W., & Zielińska, A. (2006).Effect of fermentation conditions on red-beet leaven quality.*Pol. J. Food Nutr*. Sci., 15/56(4), 437–444. Retrieved from journal.pan.olsztyn.pl/fd.php?f=813

Trachoo, N., Wechakama, P., Moongngarm, A. & Sutlajit, M. (2008). Stability of Freeze-Dried Lactobacillus acidophilus in Banana, Soybean and Pearl Barley Powders. Journal of Biological Sciences, 8(1), 119-124. doi:10.3923/jbs.2008.119.124

Trachoo, N., Wechakama, P., Moongngarm, A., & Suttajit, M. (2008). Stability of freeze dried Lactobacillus acidophilus in Banana, Soyabean and Peral Barley flours. *Journal of Biological Sciences*, *8*(1), 119-124. Retrieved from docsdrive.com/pdfs/ansinet/jbs/2008/119-124.pdf

Tsai, C., Chiu, C., Rayner, C. K., Wu, K., Chiu, Y., Hu, M., … Wang, H. (2016). Associated factors in Streptococcus bovis bacteremia and colorectal cancer. The Kaohsiung Journal of Medical Sciences, 32(4), 196-200. doi:10.1016/j.kjms.2016.03.003

Tsuda, H., & Miyamoto, T. (2010).Production of Exopolysaccharide by Lactobacillus plantarum and the Prebiotic Activity of the Exopolysaccharide.*Food Science and Technology Research*, 16(1), 87-92. doi:10.3136/fstr.16.87

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., … Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature*, 457(7228), 480-484.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R., and Gordon, J.I. 2009. A core gut microbiome in obese and lean twins. Nature. 457(7228):480-4

Tzortzis, G., Baillon, M., Gibson, G., & Rastall, R. (2004).Modulation of anti-pathogenic activity in canine-derived Lactobacillus species by carbohydrate growth substrate.*Journal of Applied Microbiology*, 96(3), 552-559. doi:10.1111/j.1365-2672.2004.02172.x

Valgas, C., Souza, S. M., Smânia, E. F., & Smânia Jr., A. (2007).Screening methods to determine antibacterial activity of natural products.*Brazilian Journal of Microbiology*, 38(2), 369-380. doi:10.1590/s1517-83822007000200034

Van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., … Eisenbrand, G. (2010). A review on the beneficial aspects of food processing. Molecular Nutrition & Food Research, 54(9), 1215-1247. doi:10.1002/mnfr.200900608

Van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H., & Verstraete, W. (2007). Inulin-type fructans of longer degree of polymerization exert more pronounced in vitro prebiotic effects. *Journal of Applied Microbiology*, *102*(2). doi:10.1111/j.1365-2672.2006.03084.x

Van Laere, K. M., Hartemink, R., Bosveld, M., Schols, H. A., & Voragen, A. G. (2000). Fermentation of Plant Cell Wall Derived Polysaccharides and Their Corresponding Oligosaccharides by Intestinal Bacteria. Journal of Agricultural and Food Chemistry, 48(5), 1644-1652. doi:10.1021/jf990519i

Vinderola, C., & Reinheimer, J. (2003). Lactic acid starter and probiotic bacteria: a comparative “in vitro” study of probiotic characteristics and biological barrier resistance. *Food Research International*, 36(9-10), 895-904. doi:10.1016/s0963-9969(03)00098-x

Vlková, E., Rada, V., Popelářová, P., Trojanová, I., & Killer, J. (2006). Antimicrobial susceptibility of bifidobacteria isolated from gastrointestinal tract of calves. *Livestock Scienc*e, 105(1-3), 253-259. doi:10.1016/j.livsci.2006.04.011

Vulevic, J., Rastall, R. A., & Gibson, G. R. (2004). Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides. FEMS Microbiology Letters, 236(1), 153-159. doi:10.1111/j.1574-6968.2004.tb09641.x

Wang, J., Guo, Z., Zhang, Q., Yan, L., Chen, W., Liu, X., & Zhang, H. (2009).Fermentation characteristics and transit tolerance of probiotic Lactobacillus casei Zhang in soymilk and bovine milk during storage.*Journal of Dairy Science*, 92(6), 2468-2476. doi:10.3168/jds.2008-1849

Wang, N., Shi, Y., Sun, J., & Le, G. (2007). Evaluation of Peanut Flour Fermented with Lactic Acid Bacteria as a Probiotic Food. *Food Science and Technology International*, 13(6), 469-475. doi:10.1177/1082013208088370

Wang, W., Chen, L., Zhou, R., Wang, X., Song, L., Huang, S., … Xia, B. (2014). Increased proportions of Bifidobacterium and the Lactobacillus group and loss of butyrate-producing bacteria in inflammatory bowel disease.*Journal of Clinical Microbiology*, 52, 398-406.

Wang, Y., Yu, R., & Chou, C. (2004). Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration

and storage. International Journal of Food Microbiology, 93(2), 209-217. doi:10.1016/j.ijfoodmicro.2003.12.001

Wanink, J. F., Van Vliet, T., & Nout, M. J. (1994). Effect of roasting and fermentation on viscosity of cereal-legume based food formulas. *Plant Foods for Human Nutrition*, 46(2), 117-126. doi:10.1007/bf01088763

Waniska, R. A., Kotarski, S., & Thurn, S. K. (1990). Proceedings of the International Conference on Sorghum Nutritional Quality.Purdue University, West Lafayette.

Wheater, D. M. (1955).The Characteristics of Lactobacillus acidophilus and Lactobacillus bulgaricus.*Journal of General Microbiology*, 12(1), 123-132. doi:10.1099/00221287-12-1-123

Whitman, W. B., Coleman, D. C., & Wiebe, W. J. (1998). Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences, 95(12), 6578-6583. doi:10.1073/pnas.95.12.6578

Wilkowske, H. (1954). Relationship between Titratable Acidity and pH during Lactic Acid Fermentation in Reconstituted Nonfat Milk.*Journal of Dairy Science*, 37(1), 22-29. doi:10.3168/jds.s0022-0302(54)91227-8

Wollowski, I., Rechkemmer, G., & Pool-Zobel, B. L. (2001). Protective role of probiotics and prebiotics in colon cancer. The American Journal of Clinical Nutrition, 73(2), 451s-455s. doi:10.1093/ajcn/73.2.451s

Wong, J. M., De Souza, R., Kendall, C. W., Emam, A., & Jenkins, D. J. (2006). Colonic Health: Fermentation and Short Chain Fatty Acids. *Journal of Clinical Gastroenterology*, *40*(3), 235-243. doi:10.1097/00004836-200603000-00015

Woolfe, M. L. (1979). Effect of heat processing on food stuffs.In Pigments (p. 76–120). Priestley RJ, London: Applied Science Publishers Ltd.

Xiao, Z., & Lu, J. R. (2014). Generation of Acetoin and Its Derivatives in Foods. *Journal of Agricultural and Food Chemistry*, *62*(28), 6487-6497. doi:10.1021/jf5013902

Xiao, Z., Qiao, S., Ma, C., & Xu, P. (2010). Acetoin production associated with the increase of cell biomass in Bacillus pumilus ATCC 14884. *Afr. J. Microbiol. Res*, *4*, 1997−2003.

Yadav, H., Jain, S., & Sinha, P. (2006a). Effect of Skim Milk and Dahi (Yogurt) on Blood Glucose, Insulin, and Lipid Profile in Rats Fed with High Fructose Diet. Journal of Medicinal Food, 9(3), 328-335. doi:10.1089/jmf.2006.9.328

Yadav, H., Jain, S., & Sinha, P. (2006b). Effect of Skim Milk and Dahi (Yogurt) on Blood Glucose, Insulin, and Lipid Profile in Rats Fed with High Fructose Diet. Journal of Medicinal Food, 9(3), 328-335. doi:10.1089/jmf.2006.9.328

Yadav, H., Jain, S., & Sinha, P. (2007). Antidiabetic effect of probiotic dahi containing Lactobacillus acidophilus and Lactobacillus casei in high fructose fed rats. Nutrition, 23(1), 62-68. doi:10.1016/j.nut.2006.09.002

Yan, P., Xue, W., Tan, S., Zhang, H., & Chang, X. (2008). Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. *Food Control*, *19*(1), 50-55. doi:10.1016/j.foodcont.2007.02.008

Yoon, K. Y., Woodams, E. E., & Hang, Y. D. (2004).Probiotication of tomato juice by lactic acid bacteria.*Journal of Microbiology*, 42(4), 315-318.

Yoon, K. Y., Woodams, E. E., & Hang, Y. D. (2005). Fermentation of beet juice by beneficial lactic acid bacteria. LWT - Food Science and Technology, 38(1), 73-75. doi:10.1016/j.lwt.2004.04.008

Yoon, K. Y., Woodams, E. E., & Hang, Y. D. (2006). Production of probiotic cabbage juice by lactic acid bacteria. Bioresource Technology, 97(12), 1427-1430. doi:10.1016/j.biortech.2005.06.018

Yu, A., & Li, L. (2016). The Potential Role of Probiotics in Cancer Prevention and Treatment. Nutrition and Cancer, 68(4), 535-544. doi:10.1080/01635581.2016.1158300

Zaharoni, H., Rimon, E., Vardi, H., Friger, M., Bolotin, A., & Shahar, D. R. (2011). Probiotics improve bowel movements in hospitalized elderly patients — The proage study. The journal of nutrition, health & aging, 15(3), 215-220. doi:10.1007/s12603-010-0323-3

Zaman, S. A., & Sarbini, S. R. (2016). The potential of resistant starch as a prebiotic. *Crit Rev Biotechnol.*, *36*(3), 578-584. doi: 10.3109/07388551.2014.993590

Zanjani, M. A., Tarzi, B. G., Sharifan, P. A., & Mohammadi, N. (2014).Microencapsulation of Probiotics by Calcium Alginate-gelatinized Starch with Chitosan Coating and Evaluation of Survival in Simulated Human Gastro-intestinal Condition.*Iranian Journal of Pharmaceutical Research,* 13(3), 843-852. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4177644/

Zavaglia, A. G., Kociubinski, G., Perez, P., Disalvo, E., & De Antoni, G. (2002).Effect of bile on the lipid composition and surface properties of bifidobacteria.*Journal of Applied Microbiology*, 93(5), 794-799. doi:10.1046/j.1365-2672.2002.01747.x

Zhang, N., Huang, X., Zeng, Y., Wu, X., & Peng, X. (2013). Study on prebiotic effectiveness of neutral garlic fructan in vitro. Food Science and Human Wellness, 2(3-4), 119-123. doi:10.1016/j.fshw.2013.07.001

Zhang, Y., Zheng, W., Gu, J., Ni, J., Wang, L., Tang, Z., & Shi, L. (2015). Soy Protein Isolate-Alginate Microspheres for Encapsulation ofEnterococcus faecalis HZNU P2. Brazilian Archives of Biology and Technology, 58(5), 805-811. doi:10.1590/s1516-89132015050260

Zhou, Y., Martins, E., Groboillot, A., Champagne, C. P., & Neufeld, R. J. (1998). Spectrophotometric quantification of lactic bacteria in alginate and control of cell release with chitosan coating. *Journal of Applied Microbiology*, *84*(3), 342-348. doi:10.1046/j.1365-2672.1998.00348.x

http://www.hmdb.ca/metabolites/HMDB0031342

https://www.techsciresearch.com/report/india-probiotic-market-forecast-and-opportunities-2019/375.html

APPENDIX I

ISOLATION OF CULTURES

Isolation of *L.casei*

1. 1 ml of the commercial probiotic beverage containing *L.casei* was suspended in 10 ml of skim milk and incubated for 24hrs at 37°C.
2. A loopful of the one day old culture was sub cultured twice into MRS broth and incubated at 37°C for 24 hrs.
3. One ml of the activated broth culture was serially diluted and plated by pour plating at 10^{-5}, 10^{-6} and 10^{-7} dilutions.
4. White spindle shaped colonies typical of LAB was selected from each plate and inoculated into MRS broth and incubated at 37°C for 48 hrs.
5. The culture was then streaked to isolate individual colonies and maintained on MRS agar slant.
6. Single colony from the plate was then sub cultured on MRS broth and used for further study.

Isolation of *L.acidophilus* LA-5

L.Acidophillus 5 (LA-5) culture, provided by Chrs Hansen Ltd, Denmark was activated as follows

1. A pinch of freeze dried culture was suspended in 10 ml of skim milk and incubated for 24hrs at 37°C.
2. A loopful of the one day old culture was sub cultured twice into MRS broth and incubated at 37°C for 24 hrs.
3. One ml of the broth culture was serially diluted and plated by pour plating at 10^{-5}, 10^{-6} and 10^{-7} dilutions.
4. White spindle shaped colonies typical of LAB was selected from each plate and inoculated into MRS broth and incubated at 37°C for 48 hrs.

5. The culture was then streaked to isolate individual colonies and maintained on MRS agar slant.
6. Single colony from the plate was then sub cultured on MRS broth and used for further study.

Isolation of *Escherichia Coli* MTCC 433

1. The ampoule was disinfected with 70% ethanol and opened according to the instructions mentioned in the instruction manual.
2. The complete contents were transferred to a test tube with 10 ml of Nutrient broth and mixed well.
3. 0.5 ml of this culture was suspended into N.broth for activation and incubated at 37°C overnight.
4. The culture was streaked on EMB agar to isolate individual colonies.
5. Single colony of the culture was then sub cultured on Nutrient broth before use.

APPENDIX II

Gram's Staining (Gregersen, 1978)

1. A small amount of the culture from agar plate was taken and placed over a glass slide with a drop of sterile distilled water and spread well in small circular motion.
2. After air drying of the smear, the culture was heat fixed
3. The smear was flooded with Crystal violet and allowed to stand for 1 min after which it was washed with distilled water until clear
4. Flooded the smear with Gram's iodine and allowed to stand for 1 min and washed until clear with distilled water
5. The glass slide was titled and added 95% ethyl alcohol for 5 sec and washed immediately with distilled water
6. The smear was flooded with Safranin and allowed to stand for 45 sec and washed until clear of stains with distilled water.
7. Allowed to dry
8. The smear was viewed under light microscope in oil emulsion

Catalase Test (Harrigan and Mc Cance, 1976)

1. A small amount of the culture was placed in the centre of a glass slide
2. Placed a drop of 3% hydrogen peroxide over the culture
3. Observe the presence or absence of bubbles

Oxidase Test (Barrow and Feltham, 1993)

1. Place a small amount of the culture on tetramethyl-p-phenylenediamine dihydrochloride disc
2. Observe the colour change, if any

APPENDIX III

Estimation of Acid Tolerance (Gilliland *et al*, 1984)

1. 10ml of MRS broth was taken in each conical flask.
2. The medium was adjusted to pH 2.5, 3.0, 3.5 and 4.0 using concentrated HCl.
3. 1 ml of the overnight culture was inoculated into the flask and allowed to incubate.
4. Survival of the isolate was qualitatively evaluated by streaking on MRS agar plates at 0, 1, 2 and 3 hourly intervals.

APPENDIX IV

Estimation of Bile Tolerance (Gilliland *et al*, 1984)

1. 10ml of MRS broth was taken in each conical flask.
2. Bile salt was added at 0.3% and 0.6% levels and mixed well.
3. 1 ml of the overnight culture was inoculated into the flask and allowed to incubate.
4. The tolerance to bile was assessed qualitatively by streaking on MRS agar plates at 0, 1, 2 and 3 hourly intervals.

APPENDIX V

Estimation of Cell Surface Hydrophobicity (Rosenberg *et al* , 1980)

1. Fresh culture of the isolate was prepared.
2. To harvest cells, the fresh culture was centrifuged at 10,000 rpm for 15mins at 4°C.
3. The pellet obtained was washed twice, resuspended in PUM buffer (Phosphate Urea magnesium sulphate) and absorbance adjusted between 0.8 and 0.9 at 610nm using the buffer with the help of a spectrophotometer. Noted the initial O.D reading
4. 4.8 ml of the prepared cell suspension was taken in test tubes
5. 0.8 ml of the solvent (n-hexadecane/ethyl alcohol/chloroform /xylene) was added to the cell suspension
6. The aqueous phase was collected in glass cuvette and the absorbance was determined at 610nm. Final O.D was noted
7. CSH% was calculated using the formula

$$\text{CSH\%} = \frac{\text{Initial OD- Final OD}}{\text{Initial OD}} \times 100$$

APPENDIX VI

Bile Salt Hydrolysis Activity (Dashkevicz and Feighner, 1989)

1. Active culture of the isolate was streaked on sterile oxgall agar plates
2. The plates were incubated in an anaerobic incubator maintained at 5% CO_2, 37°C for 24hrs.
3. Formation of precipitate halos around colonies or formation of opaque granular white colonies was considered as positive.

APPENDIX VII

Antibiotic susceptibility test (Bauer *et al*., 1959)

1. The culture was smeared on the surface of a sterile agar plate using a sterile swab and allowed to dry
2. Antibiotic discs were placed at a distant from each other on the surface of the smear plate
4. The plates were incubated at 37°C for 24hrs.
3. The zone of clearance around each antibiotic disc was measured using a metric ruler
4. The culture was classified as sensitive, S (≥ 21 mm); intermediate, I (16-20 mm) or resistant, R (≤ 15 mm) according to Vlková *et al*, (2006).

APPENDIX VIII

Anti-pathogenic effect (Valgas *et al*., 2007)

1. The pathogen was smeared on the surface of a sterile agar plate using a sterile swab and allowed to dry
2. Wells were drilled on the agar plate using a sterile borer.
3. 100 µl of the probiotic culture was placed inside the well and incubated at 37°C for 24hrs.
4. The zone of clearance around the well was measured using a metric ruler
5. The culture was classified as strong (>20mm), intermediate (10-20mm) and low (<10mm) inhibition according to Shokryazdan *et al.*(2014).

APPENDIX IX

Carbohydrate fermentation test (Barrow and Feltham, 1993)

1. 10 ml of Andrade peptone broth was filled into each test tube.
2. An inverted durham's tube was inserted into the media carefully without any air bubbles, corked and the tubes were autoclaved.
3. One carbohydrate disc was added to each test tube
4. Each test tube was inoculated with a loopfull of fresh culture
5. The tubes were incubated at 37°C for 7 days
6. Colour change and production of gas was noted everyday

APPENDIX X

Estimation of viable cell count

1. 1% of an active culture of either Lactobacillus casei or Lactobacillus acidophilus or *Escherichia Coli* was inoculated separately into individual slurries of each plant food and mixed well in a dancing shaker for 20 mins.
2. 1 ml of the inocluated slurry was suspended into 9ml of 0.95% N.Saline and serially diluted until suitable dilution was obtained.
3. Pour plating was carried out at ideal dilutions on MRS agar for LAB cultures.
4. Spread plating was done at ideal dilutions on EMB agar for E.coli culture.
5. Number of white spindle shaped opaque colonies was counted after 48 hrs for LAB cultures.
6. Number of dark pink pinpoint colonies with a green metallic sheen characteristic of E.coli was counted after 24hrs incubation.
7. The number of colonies was expressed as cfu/ml
8. Steps 2-5 were repeated after 24 hr fermentation of the slurries incubated at 37°C

APPENDIX XI

Score card for sensory evaluation of the probiotic supplement

SCORE CARD

DATE: SET NO:

NAME:

ATTRIBUTES	Sample No 1	Sample No 2	Sample No 3	Sample No 4
COLOUR/APPEARANCE				
MOUTHFEEL				
FLAVOUR				
TASTE				
OVERALL ACCEPTABILITY				

Score the products for its attributes on a scale of 1-9 using the key given below

9 POINT HEDONIC SCALE	
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike moderately
2	Dislike very much
1	Dislike extremely

APPENDIX XII

Estimation of Moisture and Total solids (IS 11623: 1997)

1. Five gram of the sample was taken in a petridish (W1).
2. The sample was allowed to dry at 60-70°C in a hot air oven and cooled in a dessicator
3. The dish was weighed (W2).
4. The process of heating and cooling was repeated until concordant weight was obtained.
5. The loss in weight during drying was calculated as the moisture content.
6. Total solids was calculated by the formula

$$TS\% = W2/W1 \times 100$$

APPENDIX XIII

Estimation of Ash (Raghuramulu *et al*., 2003)

1. Ten grams of the sample (W1) was taken in a porcelain crucible.
2. The crucible was heated over a low flame until the sample was completely charred.
3. The sample was heated in a muffle furnace at 600 °C for 5 hours and allowed to cool in a dessicator.
4. The weight of the crucible with the sample was measured.
5. The sample was heated in the muffle furnace at 600°C for 1 hour and weight noted. This was repeated until concordant weight was achieved (W2).

$$\text{Ash content (g/100g)} = \frac{W2}{W1} \times 100$$

APPENDIX XIV

Estimation of Starch by Anthrone reagent (Sadasivam and Manickam, 2008)

1. 0.5g of the sample was homogenised with hot 80% ethanol to remove sugars.
2. The solution was centrifuged and residue was collected.
3. The residue was washed using hot 80% ethanol until the washings did not give colour with anthrone reagent
4. The residue was dried over water bath
5. Five ml water and 6.5 ml of 52% perchloric acid was added to the residue and extracted at 0°C for 20 mins.
6. The extract was then centrifuged and supernatant collected.
7. The extraction and centrifugation was repeated and the supernatants pooled and made upto 100ml
8. 0.2ml of the supernatant was pipetted and made upto 1 ml with water
9. The standards were prepared by taking 0.2,0.4 0.6, 0.8 and 1 ml of the working standard and made upto 1 ml with water in each tube
10. Four millilitre of anthrone reagent was added to each tube and heated for 8 mins ina boiling water bath
11. It was then radily cooled and the intensity of the green colour was read at 630 nm
12. The glucose content of the sample was found by using a standard graph
13. The starch content was calculated s follows

Starch content = glucose content x 0.9

APPENDIX XV

Estimation of Total Protein by Micro-Kjeldahl method (Sadasivam and Manickam, 2008)

1. 100mg of the sample (containing 1-3mg nitrogen) was transferred to a digestion flask
2. To it was added,1.9 g potassium sulphate, 80mg mercuric oxide, 2 ml sulphuric acid and boiling chips and allowed to digest till the solution became clear
3. After cooling, the digest was diluted with ammonia free water and transferred to the distillation apparatus.
4. A 100 ml conical flask containing 5 ml boric acid solution and indicator was placed such that the tip of the condenser was dipping below the surface of the solution
5. 10 ml of sodium hydroxide-sodium thiosulphate solution was added to the test solution in the apparatus.
6. The solution was distilled and the ammonia was collected on boric acid
7. The tip if the condenser was rinsed well.
8. The solution was titrated against standard acid until the appearance of violet colour.
9. A reagent blank with equal volume of distilled water was run .
10. The titration volume was subtracted from the sample volume
11. The nitrogen content was calculated as follows

$$\text{Ng/Kg} = \frac{(\text{ml HCL} - \text{ml Blank}) \times \text{Normality} \times 14.01 \times \text{Final volume}}{\text{Weight(g)} \times \text{volume of aliquote}}$$

APPENDIX XVI

Estimation of fat (FSSAI, 2015)

1. 10 grams (W0) of the sample was weighed into a majonnier extraction tube and added 1 ml of ammonia solution and mixed thoroughly
2. 10 ml of ethyl alcohol was added and corked before shaking vigourously for 30 sec.
3. To it, 25 ml of solvent ether was added and mixed well for 1 min.
4. To it 25 ml of petroleum ether was added and shaken vigorously for 1 min
5. The tube was allowed to stand for 30 mins
6. Decanted ether layer into previously weighed (W1) conical flask
7. The extraction was repeated using 15 ml petroleum ether and 15 ml diethyl ether and allowed to stand for 30 mins and the ether solution was poured into the conical flask
8. Step 7 was repeated once again
9. The ether was evaporated in a boiling water bath
10. The conical flask was allowed to dry in a oven at 100°C for 1 hour and cooled in a desiccator. Weigh the flask
11. The heating and cooling was repeated until constant weight was obtained (W2)

$$\text{Fat content (\%)} = 100 (W2-W1) / W0$$

APPENDIX XVII

Estimation of Crude Fibre (Sadasivam and Manickam, 2008)

1. Two grams of the sample was boiled with 200ml sulphuric acid for 30 mins with bumping chips
2. The sample was then filtered using muslin cloth and washed with boiling water until the washings become non-acidic
3. 200 ml of sodium hydroxide was added to the solution and boiled for 30mins
4. The solution was filtered using muslin cloth and washed with 25ml of boiling 1.25% H_2SO_4,three 50 ml portions of water and 25ml alcohol
5. The residues were transferred to a preweighed (W1) ashing dish and dried for 2 hours at 130 C.
6. The dish was allowed to cool in a dessicator and weight taken (W2)
7. The ash was ignited for 30 mins at 600C, cooled in a dessicator and weight taken (W3)
8. Crude fibre content was calculated as follows

$$\% \text{ crude fibre} = \frac{(W2-W1) - (W3-W1)}{\text{Weight of sample}} \times 100$$

APPENDIX XVIII

Estimation of Reducing sugars by Miller (1972) (Sadasivam and Manickam, 2008)

1. 100 mg of the sample was homogenised with hot 80% ethanol to extract the sugars.
2. The supernatant was collected and and evaporated on a water bath at 80C
3. To it, 10 ml of water was added and was mixed until sugar dissolved completely
4. 0.5 to 3 ml of the extract was taken in test tubes and the volume equalized to 3ml with water in all tubes
5. To it 3 ml of dinitrosalysilic reagent was added
6. One ml of 40% Rochelle salt solution was added to the warm tubes
7. The intensity of the dark red colour at 510nm was measure after cooling
8. The steps 5-7 was also conducted on a series of standards using glucose in the range of 0-500µg
9. The reducing sugar content was calculated using the standard graph

APPENDIX XIX

Estimation of Free Amino Nitrogen (European Brewery Convention, 1987)

1. One ml of the test solution was diluted with 99 ml distilled water.
2. Two ml each of the diluted sample, glycine standard and water (as blank) was taken in separated tubes
3. To the tubes, 1ml of ninhydrin colour reagentwas added and heated at 100C for 15mins
4. This was then cooled at 20°C for 20mins.
5. Five ml of dilution solution was added and absorbance was noted at 570nm against blank within 30 mins.
6. The concentration of FAN was estimated using the nin-hydrin colorimetric method prescribed by the European Brewery Convention Method 8.8.1 (European Brewery Convention, 1987)
7. Free amino nitrogen [mg/L] = $\frac{A_S - A_B}{A_G - A_B} \times 2 \times F$

   Where A_S = average absorbance of the sample

   A_G = average absorbance of the glycine standard solution

   A_B = average absorbance of the blank value (H2O)

   F = dilution factor of the sample

   2 = concentration of the glycine standard solution in mg/L

APPENDIX XX

Estimation of Invitro starch digestion (Saterlee *et al*., 1979)

1. One gram of the sample mixed in 100 ml water was gelatinised and allowed to boil for one hour and filtered. .
2. To one ml of the gelatinized solution , one ml of enzyme solution (saliva diluted with equal quantity water) was added and mixed well.
3. The mixture was incubated at 37°C for 1-2 hours.
4. The reaction was stopped by the addition of one ml of sodium hydroxide.
5. Glucose content was estimated by the Somoygi method (1952).

APPENDIX XXI

Measurement of Viscosity (using Brookefields Viscometer)

1. About 100 ml of the sample at 20 ± 0.1°C was placed in the apparatus.
2. Spindle 3 was slowly inserted into the sample till mark without air bubbles and allowed to rotate at 10rpm.
3. The reading was noted after allowing 5 rotations.
4. The viscosity was calculated as follows

   Visocsity (cP) = Reading x Brookfield factor

APPENDIX XXII

Measurement of pH (Systronics pH meter 361)

1. A clean and calibrated pH meter was slowly inserted into the sample such that the complete tip of the probe is immersed in the sample.
2. It was held till the reading appeared static in the pH meter.
3. The reading on the pH meter was noted as the pH of the sample.

APPENDIX XXIII

Estimation of Titratable acidity (IS 11765: 1986)

1. One ml of the sample was taken in a 25ml conical flask.
2. Phenolphthalein indictor was added and mixed well.
3. 0.1 N sodium hydroxide was taken in the burette
4. The sample was titrated against 0.1N NaOH until pale pink colour that stayed for 10 seconds appeared.
5. The titration was repeated until concordant volume occurred.

   Titratable acidity of the sample expressed as g lactic acid/100g was calculated as follows

$$\% \text{ lactic acid} = \frac{\text{Vol. of NaOH} \times 0.009}{\text{Vol of sample} \times \text{specific gravity}} \times 100$$

APPENDIX XXIV

Measurement of Colour (Hunter's colour lab)

1. The sample was taken in a suitable glass jar
2. The apparatus was calibrated using black and white tiles
3. The sample was placed in the apparatus
4. The 'L', 'a' and 'b' scale values were noted.

APPENDIX XXV

Measurement of Sedimentation (Modha and Pal, 2011)

1. Ten ml of the sample was taken in a 15ml graduated capped tube
2. The tube was left undisturbed in refrigeration temperature for 24hrs.
3. The amount of sediment at the bottom that did not dislodge on inverting the tube was noted as sediment in ml and amount of whey separated above was noted and expressed as % wheying off

APPENDIX XXVI

Measurement of Wheying off (Modha and Pal, 2011)

1. Ten ml of the sample was taken in a 15ml graduated capped tube
2. The tube was left undisturbed in refrigeration temperature for 24hrs.
3. The amount of whey separated above was noted and expressed as % wheying off

APPENDIX XXVII

Measurement of Water holding capacity (Harte *et al*., 2003)

1. Twenty gram of the sample (DE) was taken in a 50ml centrifuge tube
2. The sample was centrifuged at 5000 rpm for 40mins at 5°C.
3. The amount of whey expelled (WE) was removed and weighed
4. WHC calculated as follows

$$WHC = 100[(DE-WE)/DE].$$

APPENDIX XXVIII

Estimation of Total Viable count (Marth, 1978)

1. One ml of the sample was drawn using a sterile 2 ml syringe and suspended in 9 ml of 0.95% Normal Saline to obtain the first dilution
2. Transfered one ml from first dilution using a sterile micropipette to 9ml of 0.95 % N.saline to obtain second dilution
3. This was repeated until suitable dilution was obtained.
4. Pour plating was carried out at ideal dilutions on MRS agar
5. The plates were incubated at 37°C for 48 hours
6. Number of white spindle shaped opaque colonies was counted and expressed as cfu/ml

Estimation of Yeast and Mould count (Marth, 1978)

1. One ml of the sample was drawn using a sterile 2 ml syringe and suspended in 9 ml of 0.95% Normal Saline to obtain the first dilution
2. Transferred one ml from first dilution using a sterile micropipette to 9ml of 0.95 % N.saline to obtain second dilution
3. This was repeated until suitable dilution was obtained.
4. Spread plating was done on Yeast and mould agar plates at ideal dilution and spread evenly using a sterile spreader
5. It was incubated at 25°C for 7 days
6. Number of colonies was counted and noted

Estimation of Coliform count (Marth, 1978)

1. One ml of the sample was drawn using a sterile 2 ml syringe and suspended in 9 ml of 0.95% Normal Saline to obtain the first dilution
2. Transferred one ml from first dilution using a sterile micropipette to 9ml of 0.95 % N.saline to obtain second dilution
3. This was repeated until suitable dilution was obtained.
4. Spread plating was done on EMB agar plates at ideal dilution and spread evenly using a sterile spreader and incubated at 37°C for 24 hours
5. Number of dark pink pinpoint colonies with a green metallic sheen was counted as expressed as cfu/ml

APPENDIX XXIX