DEVELOPMENT OF BARLEY-MILK BASED FERMENTED PROBIOTIC DRINK

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRY TECHNOLOGY

BY

KUNAL KUMAR AHUJA
M. Tech. (Dairy Technology)

DAIRY TEHNOLOGY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
KARNAL-132 001 (HARYANA), INDIA

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Approved by

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Certificate

This is to certify that the thesis entitled **"DEVELOPMENT OF BARLEY-MILK BASED FERMENTED PROBIOTIC DRINK"** submitted by **Mr. KUNAL KUMAR AHUJA** towards the partial fulfillment of the award of the degree of **DOCTOR OF PHILOSOPHY in DAIRY TECHNOLOGY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY),** Karnal (Haryana), India, is a bonafide piece of research work carried out by him under my supervision and no part of the thesis has been submitted for any other degree or diploma.

(Ashish Kumar Singh)
Major Advisor and Chairman
(Guide)

DEDICATED TO MY BELOVED PARENTS

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ABSTRACT

Barley being a rich source of soluble and insoluble dietary fiber also provides good substrate for the growth of probiotic lactic acid bacteria (LAB). However, presence of certain anti-nutrients such as phytate and tannins limit its suitability for human consumption. In the present study, *L. plantarum* 344 was selected as suitable culture for the development of barley-milk based fermented probiotic drink amongst five randomly selected strains of probiotic LAB (*L. acidophilus* 13, *L. casei* 297 & 299, *L. plantarum* 344 and *L. rhamnosus* RSI3) based on its antinutrient reduction potential, compatibility with co-culture *S. thermophilus* 20, and sensory acceptability of barley-milk based fermented probiotic curd. Based on preliminary trials type of milk and flour were selected for the preparation of probiotic drink. A central composite rotatable design of response surface methodology consisting of 3 variables namely barley flour, probiotic culture and co-culture concentration at 5 levels was adopted to optimize the level of variables. Overall acceptability score, probiotic count and β-glucan content of fermented probiotic drink were selected as responses. The optimized drink rated 7.8 (in between like very much and like moderately) for overall acceptability on 9 point hedonic scale and contained 8.59 log cfu/ml of probiotic strain, and 0.14 g/100 g β-glucan. Barley flour concentration was the most critical variable amongst the three, which significantly ($\rho<0.01$) affected the overall acceptability, probiotic count and β-glucan content of probiotic drink. When analysed for antioxidant potential, the optimized probiotic drink exhibited 0.40 and 0.79 mg/ml trolox equivalent ABTS and DPPH radical scavenging activity, respectively. Antimicrobial activity of the supernatant obtained from optimized product was high against tested pathogens, namely, *E. coli* (22 mm) and *S. typhii* (20 mm), whereas, moderate activity was observed against *S. dysenteriae* (16 mm) and *S. aureus* (12 mm). Angiotensin-converting enzyme (ACE) inhibitory activity of developed product was less than 15%. The optimized drink remained best acceptable up to a period of 9 days when packed in PET bottles and stored at 5±1°C. However, product packed in LDPE pouches, when compared with the PET bottles had significantly ($\rho<0.01$) higher non-LAB count and yeast and mould count. Changes in sensory quality of stored product in two packages exhibited almost similar trend. Responses from 100 respondents representing potential consumers belonging to mixed age, location and economic group indicated, 89% of the respondents liked the optimized product with score closer to 'liked extremely' side on 9-point hedonic scale. Cost of production per 200 ml product in PET bottle was estimated to be around ₹12.09.

विषय : जौ - दूध आधारित किण्वित प्रोबायोटिक पेय का विकासिकरण

छात्र का नाम : कुणाल कुमार आहूजा **प्रमुख सलाहकार :** डॉ० आशीष कुमार सिंह

सारांश

जौ, घुलनशील एवं अघुलनशील आहार रेशाओं के समृद्ध स्रोत होने के साथ-साथ प्रोबायोटिक जीवाणुओं के विकास के लिए अच्छा अधः स्तर प्रदान करता है । लेकिन, कुछ पोषक विरोधी तत्व, जैसे कि फाइटेट एवं टैनिन की उपस्थिति जौ के मानवीय उपभोग को सीमित करती है। वर्तमान अध्ययन में, एल० प्लांटेरम एनसीडीसी344 को पांच लैक्टिक अम्ल जीवाणुओं (एल० एसिडोफिलस एनसीडीसी13; एल० केजाइ एनसीडीसी297; एल० केजाइ एनसीडीसी 299; एल० प्लांटेरम एनसीडीसी344 एवं एल० रहेमनोसस आरएसआई3 में से पोषक विरोधी तत्वों के पराभव; सह-जामन (एस० थर्मोफिलस 20) के साथ संगतता; एवं जौ - दूध सयुंक्त आधारित किण्वित प्रोबिओटिक के संवेदी मूल्यांकन के आधार पर, जौ दूध आधारित प्रोबायोटिक पेय के विकासिकरण के लिए चयनित किया गया । प्रारंभिक परीक्षणों के आधार पर प्रोबायोटिक पेय के अनुकूलन के लिए जौ के आटे, एवं दूध के प्रकार का चयन किया गया । तीन चर जैसे कि, जौ का आटा; प्रोबायोटिक संरोपण; एवं सह-जामन के संरोपण का अनुकूलन पांच स्तर वाले प्रतिक्रिया-सह-कार्यप्रणाली के केंद्रीय-समग्र-घूर्णन-योग्य-परिकल्पना द्वारा किया गया । अनुकूलन के लिए, स्वीकार्यतांक (कुल मिलाकर); प्रोबायोटिक की गिनती; एवं प्रोबायोटिक पेय में बीटा-ग्लूकन का स्तर को प्रतिकिर्यात्मक चर के रूप में चुना गया। अनुकूलित पेय को 9-बिन्दुओं वाले हेडोनिक पैमाने पर 7.8 मूल्यांकित किया गया, जिसमे प्रोबायोटिक की गिनती 8.59 प्रति मिली०, एवं बीटा-ग्लूकन 0.14 ग्रा० प्रति 100 ग्रा० निहित थे । तीनो चरों में जौ के आटे की सांद्रता सबसे महत्वपूर्ण थी जिसने सार्थक ($\rho<0.01$) रूप से स्वीकार्यतांक (कुल मिलाकर); प्रोबिओटिक की गिनती, एवं बीटा-ग्लूकन के स्तर को प्रभावित किया । ऑक्सीकरण-रोधी गतिविधि की क्षमता का विश्लेषण करने पर, अनुकूलित प्रोबायोटिक पेय ने 0.40 एवं 0.79 मिग्रा० प्रति ग्रा० ट्रोलॉक्स के बराबर क्रमशः डी० पी० पी० एच० एवं ए० बी० टी० एस० के मुक्तकणों को समार्जित किया । अनुकूलित प्रोबायोटिक पेय के सतह-तरल की रोगाणुरोधी क्षमता का विश्लेषण करने पर इ० कोलाई (22 मिमी०) एवं सेल्मोनेला टाइफी (20 मिमी०) के विरूद्ध अधिक गतिविधि एवं शिजेला डिसेन्ट्री (16 मिमी०) एवं स्टेफ़० ओरीअस (12 मिमी०) के विरुद्ध मध्य्यम गतिविधि पायी गयी । विकसित उत्पाद की ACE निरोधी क्षमता 15 प्रतिशत से कम थी । अनुकूलित पेय पी० ई० टी० बोतलों में 9 दिनों तक सबसे अधिक स्वीकार्य बना रहा, जबकि एल० डी० पी० ई० थैली में पैक किये गए पेय में गैर- लैक्टिक अम्ल जीवाणुओं, एवं खमीर की संख्या अधिक ($\rho<0.01$) पायी गयी। भण्डारण के दौरान दोनों प्रकार के पैकेज में संवेदी गुणवत्ता में परिवर्तन लगभग सामान प्रविृति में पाये गए । विभिन्न आयु, स्थान, आर्थिक समूह वाले 100 संभावित उपभोक्ताओं की प्रतिक्रियाओं का विश्लेषण करने पर 89 प्रतिशत उपभोक्ताओं को अनुकूलित पेय 9-बिन्दुओं वाले हेडोनिक पैमाने पर "अत्यंत पसंद आया" के करीब लगा । पी० ई० टी० बोतलों में पैक किये गए 200 मिली० उत्पाद की लागत लगभग 12.09 ₹ आंकी गयी ।

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PHOTOPLATES

LIST OF APPENDICES

LIST OF ANNEXURES

ABBREVIATIONS

%	:	Percent
% LA	:	Percent lactic acid
@	:	at the rate of
₹	:	Indian rupee
° C	:	Degree Celsius
µg	:	Microgram
µl	:	Microlitre
µm	:	Micro meter
ANOVA	:	Analysis of variance
ANOVA	:	analysis of variance
AOAC	:	Association Of Analytical Chemists
AR	:	Analytical reagents
BMFPD	:	Barley-milk based fermented probiotic drink
cfu	:	Colony forming units
cm	:	Centimeter
cP	:	Centipoise
et al.	:	and co-workers
FAO	:	Food and Agricultural Organization
FFA	:	Free fatty acid
g	:	gram
h	:	hour
i.e.	:	That is
kg	:	Kilogram
l	:	litre
LAB	:	Lactic acid bacteria
LDPE	:	Low density polyethylene
M Pa	:	Mega Pascal
mg	:	milligram
min	:	minute
ml	:	millilitre
mm	:	millimeter
MRS	:	deman Rogasa sharpe medium

MT	:	Million tons
N	:	Normality
NCDC	:	National Collection of Dairy Cultures
nm	:	nanometer
Pa.S.	:	Pascal Second
PCA	:	Plate Count Agar
PET	:	Polyethylene terephthalate
ppb	:	Parts per billion
ppm	:	parts per million
psi	:	Pounds per square inch
rpm	:	Rotations per minute
s	:	Second
s^{-1}	:	Per second
SNF	:	Solids not fat
SPC	:	Standard Plate Count
spp.	:	Species
ssp.	:	Subspecies
TCA	:	Trichloro acetic acid
v	:	Volume
viz	:	Namely
VRBA	:	Violet Red Bile Agar
w	:	Weight

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

According to the US Census Bureau's World Population Clock (2014), the current world population is 7.1 billion that will reach 9 billion by 2044. Ensuring food as well as nutritional security to growing population is daunting task. Food choices and preferences among people is a dynamic process that depends on several factors including the agricultural production scenario, climatic conditions, culture, availability, socio-economic status, education and awareness regarding the nutritional and health promoting virtues of foods available. Our ancestors received their daily dietary requirements on annual basis from at least five hundred plants and that too in minimally processed form. In this form most of the native beneficial microflora also used to reach our gut and impart health benefits (Bengmark, 2012). The diversity of our food basket has narrowed down over the years and many raw materials especially in their un-processed form have diminished drastically. Present day nutrient supply is restricted to only 17 plant species and 50% calorie contribution from 8 grains. In our country, trend is towards the consumption of wheat-rice based food stuffs that too in refined form resulting in deprivation of essential micronutrients. Moreover, consumption of processed, specially thermally processed products further reduce the availability of vital nutritional components and in certain cases may even dispose the consumers for harmful substances such as peroxides and maillard reaction products (MRP). It has fuelled the "functional food" movement towards improving the variety of foods in our daily diet by incorporating food raw materials from wide range of sources with minimal processing and including either healthy bacteria or components, which shift the balance towards beneficial gut microflora.

In this regard, coarse cereal grains including oat, barley and millets should be given more emphasis to efficiently utilize them for human consumption because of their ability to grow in diverse climatic conditions with little inputs besides being nutritionally superior compared to other staples. Barley is fourth important cereal crop of India ranking next only to the rice, wheat and maize both in terms of acreage and production. India produced 1.75 MT constituting only about 2% of global barley production (FAO, 2014). It is a versatile cereal crop produced over a wide range of environmental conditions as compared with other

cereal grains (Jadhav *et al.,* 1998). In India major proportion of barley is used either as animal feed or for the production of malt which is utilized for the manufacture of malted milk foods, alcoholic beverages and other specialty foods. A very small proportion of it is consumed at household level.

On average, barley contains about 64% starch, 11% protein and 5% β-glucan, a complex carbohydrate having cardio-protective role (MacGregor and Fincher, 1993). Barley**,** an excellent source of B-complex vitamins, minerals and wide array of complex carbohydrates (Kalra and Jood, 2000) is now gaining renewed interest as a dietary component primarily because of its soluble dietary fiber (*i.e.* β-glucan) content and suitability for malting. Moreover, natural antioxidants present in barley such as benzoic acid and cinnamic acid derivatives, pro-anthocyanidins, flavonols, chalcones, flavones, flavanones and amino-phenolic complexes (Goupy *et al*., 1999; Hernanz *et al*., 2001; Gamel and Abdel-Aal, 2012) exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, anti allergic, and antithrombotic effects, which may also be involved in vasodilatory actions (Cook and Sammon, 1996). Barley contains high levels of phenolic components mainly p-coumaric acid, ferulic acid and its derivatives (Bonoli, 2004; Gamel and Abdel-Aal, 2012) which are the major group of compounds contributing to the antioxidant activity of cereal grains. However, certain phenolic compounds may adversely interfere with the colour (Axtell and Baik, 2006) and flavour (Baik and Ullrich, 2008) of the product during processing and storage.

Consumption of barley based products lower blood glucose and insulin responses, induce greater satiety (Granfeldt *et al.,* 1994), minimizes the risk for type 2 diabetes (McKeown, 2004; Hinata *et al*., 2007, Lattimer and Haub, 2010) and cardiovascular diseases (Truswell, 2002; Behall *et al.,* 2004a; Behall *et al.,* 2004b). On the other side, presence of certain anti-nutritional factors (ANFs) like phytate (Eklund-Jonsson *et al.,* 2006) and tannins (El-Emery and Amin, 2010) limits its popularity as “functional” dietary component. Lack of awareness regarding health benefits of barley; and lack of primary processing equipments further hinder its consumption as staple. Phytic acid content of barley adversely affects the bioavailability of essential minerals like iron by forming insoluble mineral-phytate complexes and thereby inhibits their effective absorption in gut (Sandström and Sandberg, 1992; Bohn *et al.,* 2004), whereas, tannins form

complexes with proteins and may decreases their biological value (Hagerman *et al.* 1998).

Barley contains certain essential amino acids such as threonine, lysine, and tryptophan in minor quantity, rendering them less nutritious. Nutritional value of barley can be enhanced by combining barley with milk, which is a good source of vitamin A, vitamin B_2, vitamin B_{12} and essential amino acids including lysine, tryptophan and threonine to develop a composite dairy food. Milk barley based composite foods complement and supplement to each other, enhance the organoleptic quality of finished product and assist in minimization of anti-nutrients. It also seems to be an approach for diversification of existing dairy product profile economically and logically.

Fermentation is one of the most economical and effective method of food preparation and preservation (Buckenhüskes, 2001), which has also been proven to degrade ANFs like phytate, tannins and digestive enzyme inhibitors present in various cereals and legumes. Wide range of lactic acid bacteria (LAB) belonging to the species of *L. plantarum*, *L. casei*, *L. acidophilus*, *L. fermentum*, *L. gasseri*, *L salivarius*, *L. pentosus*, *L. sanfranciscensis etc*. have been reported to liberate phytase (De Angelis *et al*., 2003; Palacios *et al*., 2008; Haros *et al*., 2008). Degradation of phytate in the small intestine of humans was attributed to the activity of phytogenic phytase, or microbial phytase produced by gut microbiota (Sandberg and Andlid 2002). Thus, fermentation can be effectively utilized as a processing intervention to degrade ANFs, and fermented mass can be utilized for the development of health foods. The functionality of such health food can further be enhanced when fermenting microbes possess probiotics potential.

Probiotics, as live microorganisms when administered in adequate amounts confer health benefit to the host (FAO/WHO, 2002).The market size of probiotic market in the country is estimated to be around $12 million in 2011, and expected to have a compound annual growth rate of 11 percent till 2016 (Sharma *et al*., 2013). Probiotics favourably alter the intestinal microflora balance, promote intestinal mucosal integrity and mobility, inhibit harmful bacteria and increase resistance to infection. Probiotic foods have already captured the Indian market with presence of strong players like Yakult, Amul, Nestle and Mother Dairy.

Cereals serves as suitable substrates for the growth of probiotic lactic acid bacteria (LAB) and improved functionality of colonic strains due to the presence

of specific non-digestible components of the cereal matrix that act as prebiotics (Charalampopoulos *et al.,* 2002a). Barley β-glucan has been proven to extend the viability of *L. acidophilus* LA-5 and *Bifidobacterium lactis* Bb-12 (Elsanhoty *et al.,* 2009). The growth of probiotic LAB in barley-milk mix alone or in consortium of strains, offer a newer opportunity for food formulators to utilize the mix for the development of health promoting food products. However, there is limited or no information on fermentation characteristics of probiotic LAB in barley-milk composite and utilization of fermented mass for the development of heath drinks are available. Moreover, knowledge regarding various biochemical changes during fermentation and their impact on product quality needs to be elucidated. Therefore, the present research project is being proposed with the following objectives:

1. Selection of suitable probiotic culture for the manufacture of Barley-Milk based Fermented Probiotic Drink.
2. Optimization of processing parameters for Barley-Milk based Fermented Probiotic Drink.
3. Evaluation of physico-chemical, microbiological, sensory, nutritional, therapeutic attributes and shelf life of the optimized drink.
4. Techno-economic feasibility and consumer acceptance of Barley-Milk based Fermented Probiotic Drink.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Barley

Barley (*Hordeum vulgare* L.) is one of the major cereal crop, cultivated throughout the world since ancient time (Zohary and Hopf, 1988). Barley is ranked as the world's fourth food crop in terms of acreage and production (Saidi *et al.,* 2005). The largest use of barley for food is found in regions with low rainfall or soil salinity stresses and where cultivation of other food crops is difficult. Of the total production about two-thirds of the barley is being used for feed, one-third for malting and remaining only about 2% for food (Baik and Ullrich, 2008). Barley is used for food as pearled barley flour, whole and cracked grain preparations and malted barley for the manufacture of alcoholic beverages, malted milk food or used as flavoring, sweetener and colouring agent. The various traditional food uses of barley are included the preparation of fermented pancake, bread, porridge, roasted whole grains, flour of roasted whole grains, beer etc. Barley-malt products are used in preparation of breakfast cereals, confectionery products and other specialty foods (Ismail, 2005). Barley, which is an excellent and rich source of therapeutically important nutrients such as dietary fiber, β-glucan, anthocyanins and antioxidants, has been used as a staple food in several areas of the world for centuries (Ames *et al.,* 2006; Mahdi *et al.,* 2008).

In recent years, there has been a growing interest in utilizing barley into the human diet, since it is naturally healthy, readily available, and relatively inexpensive (Newman and Newman, 2006). Barley-based foods may succeed as an alternative to wheat-based foods, and barley does not need to be considered only as a minor ingredient in food formulations. The wide range of sensory and functional properties offered by diverse barley sources provides food manufacturers with unlimited product opportunities that span multiple market sectors including breakfast cereals, snack foods, pasta, beverages, bakery goods, and more (Ames and Rhymer, 2008).

2.1.1 Classification of Barley

Barley may be grouped as hulled, thin-hulled, or hulless; two rowed or six-rowed; normal or high lysine and amylose varieties; and, low or high β-glucan barley (Jadhav *et al.,* 1998). Barley is classified as six-row or two-row, describing

the physical arrangement of kernels on the plant. Barley grain that is clean, bright yellow-white, plump, thin hulled, medium hard and uniform in size, is generally suitable for food uses and preferred for pearling (Pomeranz, 1974).

2.1.2 Nutritional and other health benefits of barley

Cereals contribute as major staple globally and contribute significant amounts of energy, protein, selected micronutrients to the human diet. They also contain a large variety of biologically active substances that may influence human health positively. The composition of major cereal grains have been presented in **Table 2.1**. Barley and oats, two major coarse cereals contain appreciable amounts of crude fiber and are referred to as coarse cereal grains. In recent years, the demand for natural, functional, and healthy foods has increased.

Table 2.1 Proximate composition and nutritive value of major cereal grains

Parameters ($100g^{-1}$)	Maize	Oat	Rice	Barley	Wheat
Moisture (g)	14.9	10.7	13.6	12.5	13.3
Protein (g)	11.1	13.6	6.4	11.5	11.0
Fat (g)	3.6	7.6	0.4	1.3	0.9
Carbohydrate (g)	66.2	62.8	78.9	69.4	74.1
Fibre (g)	2.7	3.5	0.2	3.9	0.3
Calcium (g)	0.01	0.05	0.01	0.03	0.02
Phosphorus (g)	0.33	0.38	0.15	0.23	0.09
Iron (g)	2.1	3.8	2.2	3.7	1.0
Carotene (mg)	90	0	0	10	29
Thiamine (mg)	0.42	0.54	0.21	0.47	0.12
Niacin (mg)	1.4	1.1	3.8	4.7	1.2
Riboflavin (mg)	0.10	0.16	0.05	0.20	0.07
Calories (kcal)	342	374	345	335	349

Source: Chavan and Kadam (1989); Swaminathan (1995) and Haard (1999)

Most of the bioactives in cereal grains have demonstrated the antioxidant effect, namely free radical scavenging activity, reducing agents, potential of making complexes with prooxidant metals, and quenching the formation of singlet oxygen species (Zielinski, 2002). DPPH radical scavenging activity of waxy type whole, milled barely, and barley by-product extracts were reported in the range of 30.8 to 65.4 % with minimum scavenging capacity for milled barley and highest

scavenging capacity for whole waxy type barley (Lee *et al.*, 2010). Cereals also provide B-group vitamins and minerals (Miller 1996).

Barley has great potential to reclaim its importance as a food grain, due to its high nutritional value. Barley provides low fat; higher amounts of carbohydrates for energy; relatively well-balanced profile of amino acids to meet the requirements of essential amino acids; minerals; vitamins, especially vitamin E; antioxidants and dietary fibers with general and specific health benefits. The composition and properties of barley grain vary with cultivar and environmental factors. Barley provides 50 mg Ca/100 g, 6 mg of iron per 100 g (Haard and Chism, 1996).

Nutritive value of barley is comparable to wheat, maize and rice. β-glucan is a predominant water soluble fiber found in barley and oats. β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic linkage, and are diverse group of molecules that can vary with respect to their molecular mass, solubility, viscosity, and three-dimensional configuration. Barley β-glucans contain a large amount of (1→3) and (1→4) side chains. Barley β-glucan is known to have various physiological effects such as cholesterol lowering effect (Newman *et al.,* 1989; McIntosh *et al.,* 1991), regulating blood glucose level, insulin response among diabetics (Cavallero *et al.,* 2002) and reducing risk of cancer (Jacobs *et al.,* 1998). Besides being its richness in β-glucans, barley contains rich in a wide range of physiologically active components such as phenolic acid derivatives, proanthocyanidins, quinones and flavonoids (Bonoli *et al.,* 2004; Kim *et al.,* 2007). Barley is a rich source of tocols, including tocopherols and tocotrienols, which are known to reduce serum LDL cholesterol (also known as bad cholesterol) through their radical scavenging activities (Qureshi *et al.,* 1991).

Hodzic *et al.,* (2009) investigated the antioxidant activity of water extract of different cereals including barley, oat, buckwheat, corn, wheat, white rice and brown rice; and found significant correlation between total phenolic content and their antioxidant potential. Zhao *et al.,* (2008) analysed 14 malting barley varieties for their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonate (ABTS) radical cation and superoxide anion radical scavenging activities, reducing power, metal chelating activities, and total phenolic contents. All barley samples were reported to have significant

antioxidant activities and contained significant levels of phenolic compounds. Total phenolic content were reported to have strong positive correlations with DPPH radical scavenging activity, ABTS radical cation scavenging activity and (Ragee *et al.,* 2006; Zhao *et al.,* 2008), whereas its correlations with superoxide anion radical scavenging activity and metal chelating activity were poor (Zhao *et al.,* 2008). Various health benefits of barley consumptions are briefly reviewed under following subheadings.

2.1.2.1 Barley and cardio vascular disease

Modifying risk factors through dietary intervention offers great potential for reducing the incidence of cardio vascular disease (CVD). The consumption of dietary fiber, especially water-soluble fiber is inversely associated with the incidence of CVD (Anderson, 1995; Pereira *et al.,* 2004). The risk factors associated with the occurrence of CVD include higher total cholesterol and low density lipoprotein (LDL) cholesterol levels in blood. Barley intake exhibits beneficial effects on lipid metabolism (Li *et al.,* 2003) by lowering plasma total and low-density lipoprotein cholesterol concentrations, and reduced plasma triacylglycerol concentration. Incorporating barley in diet has been found effective in lowering plasma total cholesterol and LDL cholesterol levels (McIntosh *et al.,* 1991; Behall *et al.,* 2004a).

Canadian Heart and Stroke Foundation recommend consumption of 21 to 38 g/d of fiber and suggest inclusion of soluble dietary fiber for lowering the bad cholesterol and blood sugar levels (Heart and Stroke Foundation, 2008). The FDA concluded that daily consumption of 3 g of soluble β-glucan from barley products would lower plasma total cholesterol by 5–8%. Various mechanisms attributed to cholesterol reduction after soluble fiber consumption in higher amounts include delayed intestinal uptake of glucose and lipids, and inhibition of absorption and reabsorption of cholesterol through gut epithelium and bile acids accompanied by increased excretion of bile acids (Anderson and Bridges, 1993; Wilson *et al.,* 2004; Pins *et al.,* 2007). The reduced absorption may be caused by the β-glucan, which increases the viscosity of the intestinal contents (Wursch and Pi-Sunyer, 1997; Lairon, 2001; Jenkins *et al.,* 2002; Wood, 2002). Wilson *et al.*, (2004) suggested that cholesterol biosynthesis is also affected by the production of short chain fatty acids (SCFAs) due to fermentation of β-glucan in colon region.

Pins *et al.* (2005) and Keenan *et al.* (2007) conducted randomized, controlled human studies to determine the cholesterol-lowering effects of different doses of barley β-glucan extracts added to food products. These studies showed a greater reduction in cholesterol was observed with 5 g/d than 3 g/d of β-glucan consumption, but both doses reduced LDL cholesterol by 14% and 9%, respectively. They also noticed that difference in molecular weight of β-glucan, whether low (50–400 kDa) or high (1000 kDa) molecular weight, didn't significantly affected the cholesterol lowering properties. Shimizu *et al.,* (2008) investigated the effect of consuming diet rich in barley β-glucan on the visceral fat, serum LDL-cholesterol and total cholesterol in hypercholesterolemic Japanese men and reported that, it significantly reduced serum concentrations of LDL cholesterol and total cholesterol as well as visceral fat.

In another study LDL-cholesterol lowering effect of a concentrated barley β-glucan extract was investigated and suggested food products containing concentrated barley β-glucan is an effective option for improving blood lipid profile (Keenan *et al.,* 2007). AbuMweis *et al.,* (2010) quantified the effect of barley β-glucan on blood lipid profile in humans and concluded that increased consumption of barely products should be considered as a dietary approach to reduce LDL cholesterol concentrations. Behall *et al.,* (2006a) compared the effects of insoluble and soluble dietary fiber in a whole-grain diet on blood pressure (BP) and concluded that whole grain diet, irrespective of richness in soluble and insoluble fibers is effective in reducing BP and in controlling the weight.

Feeding of Wistar rats with fiber-rich barley products on metabolic reactions in intestinal tract was carried out by Dongowski *et al.* (2002), and they observed that diets containing higher soluble macromolecular dietary fibers such as β-glucans affected the excretion of bile acids and neutral sterols the most. Moreover, the fermentation of dietary fiber, including resistant starch, influenced the level of steroids in feces. It clearly reflected that dietary fiber rich barley-based foods have beneficial physiological effects.

2.1.2.2 Barley and diabetes melitus

Barely bread as substitute for wheat bread has been traditionally used by diabetics in Iraq, especially in rural areas (Naismith *et al.,* 1991). Glycemic response of barley bread has been reported to be significantly lower than that of

wheat bread both in healthy volunteers and in type-II diabetics (Shukla *et al.,* 1991). Health effects of barley consumption on the diabetes–hyperglycemia, polyphagia and polydypsia were related to its chromium constituent (Mahdi and Naismith, 1991). A linear decrease in glycemic index has been reported with increasing β-glucan content in diet. Ajgaonker (1972) described the dietary approach practicised by ancient Indian physicians for treatment of type-II diabetics.

Kochar *et al.,* (2007) analyzed the physiological data of 21,152 male respondents participated in Physicians' Health Study carried out to examine the association between breakfast cereal consumption and associated risk of type 2 diabetes and concluded that intake of breakfast cereals can confer lower risk of type-II diabetes. Behall *et al.,* (2006b) conducted a study to determine, whether barley β-glucan and pre-formed resistant starch reduces glucose and insulin responses in mildly insulin resistant men independently or in a synergetic matter and concluded that consumption of barley β-glucan, rather than resistant starch was effective in reducing glucose and insulin responses. Behall *et al.,* (2005) reported both oat and barley can reduce glycemic responses, and the high soluble fiber content of oat and barley appeared as major factor. Feeding of purified β-glucan to the db/db diabetic mice (a model for diabetic dyslipidemia) increased their liver superoxide dismutase activities (detoxifying enzyme) and reduced their malondialdehyde levels. Zhao *et al*., (2014) investigated the effect of physicochemical properties such as molecular weight, viscosity and rheological properties of β-glucans extracted from oat, wheat and barley on regulation of cholesterol metabolism and antioxidant property db/db diabetic mice. With the molecular weight or viscosity of β-glucan increased, the alleviating effect on db/db diabetic mice became better.

2.1.2.3 Barley and obesity

Barley, in spite of having a rich nutty flavor and an appealing consistency has also found to protect against obesity. Beck *et al.,* (2009) recorded acute biochemical and subjective measures of satiety, followed by energy intake from a subsequent meal, after varying doses of β-glucan in extruded breakfast cereals and concluded β-glucan that mediated satiety and release of cholecystokinin, likely to be major causative factor.

Kim *et al.,* (2005) reported acute reduction in glycemic response and hunger ratings among overweight women requires consumption of at least 2g and 1g of β-glucan per meal, respectively. However, in overweight men greater amounts of β-glucan per meal is likely to be required for reducing glucose and improve satiety rating.

2.1.3 Issues related to human consumption of barley

2.1.3.1 Anti-nutrients in Barley

Presence of wide array of anti-nutrients in barley could be considered as major limiting factor for its use wide-spread in human consumption. Anti-nutrients can be defined as dietary components, which affect digestion and absorption of vital nutrient from the diet. Anti-nutrients in barley vary greatly with the cultivars and package of practice followed for their cultivation. Various anti-nutrients identified in barley include phytate, polyphenolics, and digestive enzyme inhibitors; which, are briefly reviewed under following subheadings.

2.1.3.1.1 Phytate

Phytate represents a complex class of naturally occurring phosphorus compounds that can significantly influence the functional and nutritional properties of foods. Phytic acid, myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate), is the main phosphorus store in mature seeds. Phytic acid has a strong binding capacity, resulting in rapid formation of complex with multivalent cations and proteins. Most of the phytate-metal complexes are insoluble at physiological pH, hence phytate binding renders several essential minerals biologically unavailable to human beings. Barley though contain appreciable amount of minerals, but their availability is low, because of the formation of insoluble complexes with phytate, a salt of phytic acid (Sandberg *et al*. 1999; Eklund-Jonsson *et al.* 2006).

However, few studies reported beneficial effect of phytic acid as well, such as chelation of dietary Pb^{2+} (Wise and Gilburt, 1981), protection against a fatty liver resulting from elevated hepatic lipogenesis (Onomi *et al.,* 2004), lowering of serum cholesterol and triglyceride levels due to decrease in serum zinc level (Jariwalla *et al.*, 1990), protection against breast and prostate cancer (Vucenik and Shamsuddin, 2003) and therapy for uranium removal (Cebrian *et al.,* 2007). Onomi *et al.,* (2004) reported, phytic acid content should be lowered to a level of

25 mg per 100 g or to about 0.035%, to obtain health benefits. Mohammed *et al.,* (2010) estimated phytic acid content in five germplasms of barley and reported the values in the range of 0.99 to 6.78 mg g^{-1} (db), whereas, according to El-Emery and Amin, (2010) phytate content in three genotypes of barley varied in between 1.2–1.41%. A decrease in the phytic acid content was reported to improve the absorption of minerals (Hurrell *et al.*, 2003; Anastasio *et al*, 2010; Sandberg, 2002). Reduction in phytate level can render more available phosphorus than phytate in the grain (Coulibaly *et al.*, 2011).

2.1.3.1.2 Total Phenols

Role of total phenols as an anti-oxidant or anti-nutrient is quite thin and debatable (Beta, 2003). Phenolic compounds including phenolic acids, flavonoids, flavanols and proanthocyanidins (polymeric flavanols, also referred to as condensed tannins) are secondary plant metabolites naturally present in cereals and other plants and could be considered as non-nutritive components (Beta *et al.,* 1999; Nyamambi *et al.,* 2000). Total phenolic content of barley vary in the range 18.9-35mg/g gallic acid equivalent (El-Emery *et al.,* 2010). According to Bendelow and LaBerge, (1979) phenolic content of barley ranged between 0.2 to 0.4%, both in the free and bound forms including benzoic and cinnamic acid derivatives, proanthocyanidins, quinines, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds which are generally concentrated in outer layers of barley grain (Goupy *et al.,* 1999). Proanthocyanidins found in barley exhibits antioxidant activity (Tamagawa *et al.,* 1999). Dordevic *et al.,* (2010) reported total phenolic content in a barley variety was 16.4mg gallic acid equivalent/g. Phenolic components of cereals may interact with protein and thereby alter their nutritional significance (Beta, 2003).

Tannins, generally defined as naturally occurring polyphenolic compounds of higher molecular weight (500-3000 Da), which may form insoluble complexes with the proteins (Hagerman *et al*., 1998). Tannins are polymers resulting from condensation of flavan-3-ols, which adversely affect the grain's nutritional quality (Salunkhe *et al.,* 1982; Salunkhe *et al.,* 1990; Butler *et al.,* 1984, 1986) and functionality of the complexed constituents by binding with proteins, carbohydrates and minerals (Parawira and Muchuweti, 2008). Tannins impart an astringent taste which adversely affects palatability, thus mat reduce food intake

(Butler *et al.,* 1984). Tannins bind to both exogenous and endogenous proteins including digestive enzymes, thereby affecting the digestion and biological value of proteins (Eggum and Christensen, 1975; Griffiths, 1985; Asquith and Butler 1986). Ford and Hewitt (1979) observed higher protein digestibility in the cereals with lower tannin content. El-Emery and Amin (2010) reported tannin content of three barley genotypes in the range of 14.23 to 18mg/g, whereas Mohamed *et al.,* (2010) reported tannin content of five barley germplasm in the range of 0.64 to 1.98 mg/g. The slightly lower variations in the tannin content may be attributed to varietal differences, differences in cultivation practices, soil nutrient status, as well as composition of extraction solvent or solvent mixture.

2.1.3.1.3 Digestive enzyme inhibitors

Certain legumes, millets, and coarse cereals have been reported to posses enzyme blockers including amylase inhibitor, trypsin inhibitor *etc*. Many plant seeds contain relatively large amounts of proteinase inhibitors, and their major biological role appears to be a natural defense component of plants against various pests and parasites. Many of these compounds inhibit digestive enzymes of animals and human beings, thereby act as an anti-nutrient (Ryan, 1973). Odani *et al.,* (1983) determined the complete amino acid sequence of barley trypsin inhibitor and observed a significant sequence homology between barley trypsin inhibitor and castor-bean seed glutamine-rich storage protein. Barley contains a variety of serine proteinase inhibitors collectively referred to as barley protein Z, which are found in varying concentrations in different parts of the plant at various stages of development. For instance, BSZ4 and BSZ7 serpins are found in the highest concentrations in mature barley seed, while BSZx was present in the developing grain and foliage (Roberts *et al.,* 2003).

2.1.3.2 Other issues limiting human consumption of barley

There is a lack of awareness among people regarding the nutritional and disease preventing role of coarse cereals including barley, oats and millets. Majority of barley produced in the country is utilized as cattle feed, for malting, in brewing and for the preparation of ethnic foods. Non-availability of value added health products based on barley in the market too limits its popularity among mass. Presence of tightly cemented husk too pose difficulty in thick pericarp results difficulty in its primary processing such as dehulling and milling and also

cause poor colour and mouthfeel in resultant products. There is a need for strategies promotion through involvement of producer, technologists, health professionals, food manufacturers, nutritionists industry and government to enhance the utilization of barley and barley based products for human consumption.

2.1.4 Processing treatments to enhance the nutritional quality of barley

Cereal grains are subjected to various processing treatments like pearling, milling, refining, soaking, germination, fermentation, roasting, puffing, extrusion and hydrothermal treatments either in combination or individually before their consumption to improve convenience, nutritional quality and palatability. Pearling, milling and refining of cereal grains are primary processes for removal of hulls and preparation of products such as grits, brokens, semolina and flour to be used in wide range of products such as bread, biscuits, cakes, pasta, noodles, breakfast cereals *etc.* The degree of pearling and refining significantly influences the composition of intermediate products mentioned above, whereas milling parameters influences the size of starch granules, degree of starch damage, proportion of cell wall constituents, minerals, vitamins and level of other constituents. Processing treatments like fermentation, soaking and germination have been reported to enhance relative nutritional value of barley by lowering the anti-nutritional factors, improving protein digestibility and enhancing amino acid profile. Degradation of phytic acid content using different processes such as autoclaving, moistening and pearling in combinations or as individual parameter in whole grain cereal tempeh was studied by Eklund-Jonsson *et al.*, (2006). All the treatments were reported to improve hydrolysis of phytate during preparation of tempeh. Effect of few of the above processes important in context to preparation of suitable barley preparations that could be utilized for value addition is discussed hereunder.

2.1.4.1 Pearling, milling and refining

The outer bran layer of barley is fibrous, off coloured and contains bitter or astringent compounds. The outer layer is tightly adhered to endosperm and many interventions have been attempted to remove it. Pearling and milling of barley is therefore required to enhance consumer acceptability of the resulting products. A partial refining of the grains by removing the outermost coarse layers

of the bran would increase the overall acceptability of the products (Rao and Desikachar, 1964). Removal of husk through normal milling process is practically impossible as observed in other food grains such as rice and wheat. Barley contains approximately 10 per cent coarse fibrous seed coat, where majority of anti-nutritional factors such as phytate, tannins and polyphenols are concentrated. For grains possessing hard endosperm are generally conditioned with 2-3 per cent moisture followed by abrasive milling before removal the outer bran (Desikachar, 1984). An equilibration time of 5-10 min after spraying and mixing with water allows higher diffusion of water in the endosperm and may leads to higher breakage during milling (Rao and Desikachar, 1964). Pearling efficiently remove husk to maximum extent.

Nutrient level of milled grains is relatively poor due to significant nutrient losses particularly of B-vitamins and minerals (Deosthale, 1984). However, the bioavailability of certain nutrients such as iron has been reported to increase by milling of wheat (Rao and Belavady, 1978; Prabhavathi and Rao, 1979). This improved bioavailability of iron is due to the reduction in polyphenols, tannins and phytate levels (Disler *et al.,* 1975). Moreover, milled grains hydrate faster and cook to soft texture in a short time.

2.1.4.2 Soaking

Steeping or soaking of cereals sometime referred as “conditioning” before further processing is a common practice employed under household as well on industrial conditions. Overnight soaking of pulses resulted in 25-50 per cent reduction in tannin through leaching in wash water (Deosthale, 1984). Soaking of food grains increases activity of various enzymes such as of α-glucosidases, α-amylases, phytases and proteases present in cereals. Hydrolysis of complex carbohydrates and proteins to a certain extent results in reduced cooking time and improved digestibility of foods (Platel and Srinivasan, 2004). Starch is major storage polysaccharide of cereal (MacGregor *et al.,* 1971; Granules, 1980; MacGregor and Fincher, 1993), which undergoes partial hydrolysis into simple sugars during soaking process by the action of hydrolytic enzymes α-amylases and α-glucosidases. Soaking bring about several physico-chemical changes in food grains besides cleaning and washing. It includes softening of grains, easier removal of husk, efficient separation of anatomical parts, reduction in the levels of

anti-nutrients located in outer layers, slight modification of endosperm and decreases energy requirement during milling.

2.1.4.3 Fermentation

Fermentation is a most economic and effective processing method in which foods are subjected to the action of microorganisms to bring desirable biochemical changes resulting in a significant modification of the food leading to enhanced nutritional value and acceptability of raw materials (Campbell-Platt, 1987; Motarjemi and Nout, 1996). Fermentation is one of the most economic approaches for reducing the anti-nutritional factors, in wide range of food products.

Studies have shown that both spontaneous fermentations as well as fermentations with starter cultures significantly reduced the content of phytic acid in millets (Sharma and Kapoor, 1996; Elyas *et al.,* 2002; Murali and Kapoor, 2003; El Hag *et al*., 2002). Lactic acid fermentation could enhance the protein digestibility (Antony and Chandra, 1998; Taylor and Taylor, 2002; Onyango *et al.,* 2004) and reduces tannin content, thereby increasing bioavailability of iron (Khetarpaul and Chauhan, 1989, 1990; Motarjemi and Nout, 1996; Antony and Chandra, 1998; Elyas *et al*, 2002; Onyango *et al.,* 2005). Increase in the proteins, free amino acids, soluble proteins and *in-vitro* protein digestibility has been reported within 24 h fermentation of the meals of sorghum, green gram and sorghum-green gram blend (Chavan *et al*., 1988). Dordevic *et al.,* (2010) investigated the effect of fermentation by *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* on antioxidant activities and total phenolics in buckwheat, wheat, barley and rye, and reported its positive influence on antioxidant activity and total phenolic content. Sequential fermentation by yeasts at 30°C for 72 h followed by 72 h lactobacilli fermentation have been reported to cause a significant reduction in phytic acid and polyphenol content of pearl millet flour (Khetarpaul and Chauhan, 1991).

Gupta and Nagar, (2010) studied the effect of dehulling, cooking and fermentation on proximate composition and flavonoids in pearl millet *rabadi,* a buttermilk-millet based fermented product. They concluded that cooking increased ash and flavonoids content, and reduced crude fibre while fermentation enhanced the crude protein and reduced fat and crude fibre content. Other major nutrients were unaffected after dehulling except crude fibre.

Anastasio *et al.,* (2010) investigated the phytate-degradation activity of few LAB isolates to improve the mineral solubilization during dough fermentation. The highest phytase activity was reported for *Enterococcus faecium* A86 *and Lactobacillus plantarum* H5. Other strains namely, *L. plantarum* L3, *L. paracasei* ssp *paracasei* EL7 and *Leuconostoc gelidum* A16 were reported to degrade phytates to a lower extent (de Angelis *et al.,* 2003; Anastasio *et al.,* 2010). Hosseinkhani *et al.,* (2009) isolated phytase producing bacteria from soil and poultry faeces. Among different isolated strains, *Pseudomonas* sp. had higher phytase activities. Palacios *et al*., (2008) selected two lactobacilli *Lactobacillus reuteri* M15 and *Lactobacillus salivarius* ID15 exhibiting high phytate degrading activity to be used in whole wheat bread making. Certain strains of *L. plantarum* have ability to degrade raw starch (Giraud, 1994).

Twenty-six strains of LAB isolated from French and Bulgarian sourdoughs have been reported for their phytase and phosphatase activity (Valcheva *et al.*, 2009). *Leuconostoc mesenteroides* KC51 isolated from *Kimchi*, one of the Korean traditional fermented foods can also produce phytase (Oh and In, 2009). In a different study, *Lactobacillus* sp. KV1 isolated from fermented Chinese cabbage was found to produce phytase (Luechai and Dharmsthiti, 2010). Potential probiotic *L. johnsonii* LT171 for chicken nutrition showed high clear zone in culture medium containing calcium phytate (Taheri *et al.,* 2009). Effect of pH on the phytate degrading activity of different lactobacilli was studied by Haros *et al.*, (2008) and reported optimal pH for most of the lactobacilli in the range of 6.0 to 6.5.

Phytase from different cereal sources such as malted barley flour, malted oat flour and rye sour dough were studied by Marklinder *et al.* (1995) to degrade phytic acid content in an oat based nutrient solution. Depending upon the sources of phytase, degradation was varied from 72 to 81% of initial phytate content in oat based nutrient solution, which was prepared by mixing of oat-meal with malted barley flour or other phytase sources, followed by heat processing and fermentation with *L. plantarum* 299v. Addition of malted barley flour containing enzymes like proteases provides rich source of free amino acids thus supports the growth of LAB (Marklinder and Lonner, 1992).

Khetarpaul and Chauhan (1989) improved HCl-extractability of minerals from pearl millet when fermented with yeast (*Saccharomyces diastaticus* or *Saccharomyces cerevisiae*) and lactobacilli (*Lactobacillus brevis* or *Lactobacillus fermentum*). Gupta and Khetarpaul (1993) reported that fermentation of freshly ground wheat flour-buttermilk mixture brought about a significant increase in HCl-extractability of vital micronutrients calcium, iron, copper, zinc, manganese and phosphorus when fermented at 30, 35 and 40°C for the period ranging from 6, 12, 18, 24 and 48 h. The longer the period and the higher the temperature of fermentation the greater was the increase in HCl-extractability of minerals.

There was significant decrease in the level of phytic acid and starch, as well as improved protein digestibility (*in-vitro*) with an increase in temperature and period of fermentation during wheat-*raabadi* manufacture (Gupta *et al.,* 1992). Similar results were also obtained for barley-*raabadi* at similar time-temperature combinations (Gupta *et al.,* 1992). Grewal and Chauhan (1993) reported that soy-*raabadi* fermentation was associated with increased *in-vivo* and *in-vitro* calcium availability and decreased phytic acid and saponin levels. Fermentation by the probiotic strain *L. acidophilus* NCDC 13 reduced phytic acid and polyphenols contents in composite pearl millet-barley-dairy based substrate with concomitant increase in *in-vitro* protein and starch digestibility, and Ca and Fe bioavailability (Ganguly *et al*., 2014).

2.1.4.4 Germination

Germination leads to considerable changes in the nutritive quality of a cereal grain by modifying the grain constituents such as partial degradation of macromolecules, synthesis of vitamins (Malleshi and Desikachar, 1986; Pedersen *et al.*, 1989; Kaukovirta-Norja *et al*., 2004), minerals (Hemalatha *et al*., 2007 and Hubner *et al*., 2010) and enhanced antioxidants (Bicka *et al.*, 2011 and Donkor *et al.*, 2012). Germination also decreased anti-nutritional components such as phytic acid (Egli *et al*., 2002; Coulibaly *et al,* 2011) and tannins present in cereals and millets (Rao and Prabhavathi, 1982; Hemalatha *et al.*, 2007; Osuntogun *et al.,* 1989; El Khalifa, and El Tinay, 1994). Hubner *et al*., (2010) reported a 26 percent decrease in the phytic acid content of barley, when the grains were germinated for 96 hours at 18°C. During germination, activity of native phytase increases, which catalyzes the hydrolysis of phytate thereby resulting in breakdown of phytic acid (Bartnik and Szafranska, 1987; Laboure *et*

al., 1993). A decrease in the phytic acid content improves the absorption of minerals (Hurrell *et al*., 2003; Anastasio *et al*, 2010; Sandberg, 2002). Level of phytic acid lowers significantly by germination; and, germinated flours contained 50 percent less phytic acid compared to the non-germinated semi-refined flour. However, level of phytic acid was similar whether the grains were germinated for three, five, or seven days (Pedersen *et al.*, 1989).

Germination has been reported to increase the simple sugar content by hydrolyzing starch molecules and decrease dietary fibre content (Pedersen *et al.,* 1989). There was no appreciable change in the levels of other nutrients including minerals reported. Germination caused only minor changes in amino acid composition; the content of glutamic acid decreased, whereas the concentration of proline increased. Germination has been reported to enhane the lysine content and improve the amino acid profile of cereal grains (Robbins and Pomeranz, 1979; Malleshi and Desikachar, 1985). Germinated flours contained higher levels of reducing sugars that results in the formation of Maillard products during heat processing, thus lowering available lysine from processed foods (Oste, 1984). Wang and Fields (1978) found that germination of sorghum increased the relative nutritive value from 54.6 to 63 percent and the protein efficiency ratio (PER) from 1.5 to 1.7. There were substantial increase in lysine, methionine and tryptophan content. Germination was also reported to lower tannins in sorghum (Osuntogun *et al.,* 1989), which may otherwise interfere with the absorption of protein. Arora *et al.,* (2010) formulated food mixtures using non-germinated and germinated barley flour, whey powder and tomato pulp (2:1:1w/w); fermented using 5% *Lactobacillus acidophilus,* and reported combination of germination followed by fermentation with probiotic is a potential process for developing products of improved nutritional quality .

Germination period of 48 h has been previously reported for a considerable loss of 67 % β-glucan which has been caused by the activity of β-glucanases in barley grains (Hubner *et al.*, 2010). Imbibition of cereal grains is another type of treatment, in which cereal grains are subjected to multiple cycle of immersion and non-immersion of grains in water. Kihara *et al* (2007) studied the effect of 48 h imbibition (four cycles of 5 h immersion plus 7 h non-immersion at 15°C over 95% humidity) and subsequent germination up to 144 h on the changes in β-glucan and γ-aminobutyric acid content of barley, and found 48 h

imbibition process is optimal for accumulation of γ-aminobutyric with a 15.7 % loss in β-glucan. Increase in germination time has been reported to increase γ-aminobutyric acid content in barley (Chung *et al.*, 2009; Donkor *et al.*, 2012). During malting process activation of β-glucanase led to hydrolysis of β-glucan resulting in substantial lowering in its level in malted grains.

2.1.5 Effects of processing on barley β-glucan

The physiochemical properties of β-glucan such as viscosity and solubility, as well as their molecular weight and concentration, affect its functionality of in food systems (Knuckles *et al.,* 1997; Anderson *et al.,* 2007). The higher viscosity of β-glucan soluble fiber is considered as a contributing factor to health benefits of oat and barley (Pins and Kaur, 2006; Kim *et al.,* 2006). Processing barley into food involves a wide range of unit operations including milling, hydrothermal treatments, mixing, blending, extrusion, germination, fermentation, boiling, roasting and baking all of which may affect the functionality of barley β-glucan. It has been reported that processing methods can alter molecular structure and viscosity of β-glucan without affecting its concentration (Wood, 2002; Lazaridou and Biliaderis, 2007, Andersson *et al.,* 2007; Tosh, 2007). The major challenges lies ahead are to develop processed food with emphasis on to retain the physiological effects associated with β-glucan.

2.2 Milk nutrients for well being

Since ancient time milk has been quoted as “complete food” that possess all the vital macronutrients, micronutrients and bioactive molecules required for the growth and development of all age groups. The consumption pattern of milk and milk product usually vary from region to region. Highly imbalance consumption pattern due to difference in purchasing power and availability of milk significantly contributes towards the malnutrition in India. India harbour largest numbers of persons suffering from or prone to diabetes, cardiovascular diseases and cancer. Moreover, incidences of infectious diseases are also on rise. One of the common reasons for these diseases could be attributed to weak immune system. The healing power of milk nutrients is known for centuries and recent scientific investigations have proved the disease preventing or alleviating role of milk nutrients. Milk is a rich source of vitamins like A, B_2, B_{12}, Niacin, *etc.* and contains various physiological functional components such as immunoglobulins,

galactooligosaccharides, lactoferrin, β-lactoglobulin, α-lactalbumin and conjugated linoleic acid (Shah, 2000a). Milk proteins have high biological value and provide essential amino acids (Vijayalakshmi and Anbiah, 2010). Milk protein also serve as precursor for the generation of wide array of bioactive peptides that may modulate the various metabolic reactions positively and thus play important role for healthy life. Role of milk nutrients specially the minor milk proteins such as β-lactoglobulin, α-Lactalbumin and lactoferrin, in modulating the immune system has been well documented. However, in spite of being superior food, milk is deficient in certain essential micronutrients such as iron and ascorbic acid. Better availability of added nutrients in milk and milk nutrients offers newer opportunities for the fortification of bioactive components such as essential fatty acids, micronutrients and amino-acids; addition of dietary fibres of physiological health significance; probiotics and prebiotics. New product development focused on addition of healthy ingredients to enhance the nutritional image of dairy products is required.

2.3 Fermented milks

International Dairy Federation (IDF, 1988) defined fermented milk as “a milk product prepared from milk, skimmed or not, with specific cultures; the microflora is kept alive until sale to the consumer and may not contain any pathogenic germ”. According to the revised definition by IDF (Standard No. 163 & 164) fermented milks are prepared from milk and/or milk products (e.g. any one or combinations of whole, partially or fully skimmed, concentrated or powdered milk, buttermilk powder, concentrated or powdered whey, milk protein (such as whey proteins, whey protein concentrates, soluble milk proteins, edible casein and caseinates), cream, butter or milk fat- all of which have been manufactured from raw materials that have been at least pasteurized), fermented by the action of specific microorganisms, which results in a reduction of the pH and coagulation. Fermented milk products have been classified on the basis of metabolite produced by the principal microflora of the product, as; (i) lactic fermented milks *e.g.* dahi, cultured butter milk, zabadi, acidophilus milk and yakult etc., (ii) yeast-lactic fermented milks *e.g.* kefir, koumiss, *etc*. and (iii) mold-lactic fermented milks *e.g.* villi *etc*. (Robinson and Tamime, 1995). In pursuit of functional dairy products, fermented milk appears to be most preferable and logical choice, owing to its well documented health benefits.

2.4 Cereal-milk based fermented foods

2.4.1 Rabadi

Raabadi, a cereal based fermented milk product, is popular in rural parts of Haryana, Rajasthan and Punjab. *Raabadi* serves as a refreshing and thirst quenching beverage during hot summers in these regions. In the preparation of *raabadi,* cereal flour is used as an essential ingredient which offers opportunities for utilizing the minor crops in an effective manner. The cereals used for rabadi manufacture used are barley, wheat, maize, pearl millet and sorghum. It is prepared by mixing and fermenting flour of wheat / pearl millet / barley / sorghum or maize with surplus buttermilk in summer months and allowed to ferment at around 40-45°C for 4-6 h. The fermented mass is boiled, mixed with water, spiced and salted to taste, cooled and consumed. It is a lactic acid fermented food in which lactose undergoes acid fermentation naturally and readily (Economidou and Steinkrauss, 1983; Gupta*,* 1989).

In the traditional method *raabadi* is prepared by adding the cooked and cooled cereal flour to buttermilk and allowing the mixture to ferment overnight. The resulted *raabadi* is consumed as such (Ramakrishnan, 1977). Traditional process of *raabadi* making yields a product with limited shelf life (one to two days) with unpredictable sensory quality.

In recent years attempts have been made to develop the technological package for the preparation of *raabadi* using three different cereals, *viz*., wheat (Girish, 2006), pearl millet (Modha, 2006) and sorghum (Pintu, 2006). Modha and Pal (2011) optimized *rabadi* like fermented milk beverage based on pearl millet. Briefly process consists of addition of germinated cereal flour in milk, heating to pasteurization temperature and the mix is cooled and inoculated with suitable starter culture and incubated for sufficient time to obtain 0.9 to 1% lactic acidity. The curd obtained is broken down and mixed thoroughly with pasteurized water, spices and salt. The product has keeping quality of 7 days when kept under refrigeration (7-8°C) conditions.

Grewal and Chauhan (1993) prepared soy *rabadi* by blending autoclaved dehulled soybean slurry and the curd mass (obtained from reconstituted skim milk). The blend was added with water and fermented at 30°C for 12 h. Soy *raabadi* appeared to be a low cost wholesome food which was free from beany flavour and rich in protein, fat and dietary essential minerals. Gupta *et al.,* (2007)

prepared *raabadi* from buttermilk and mothbean by two methods. Buttermilk was mixed with mothbean flour to get homogeneous paste and this was diluted with plain water and boiled for 7 min with constant stirring while salt was added during stirring. In the first method the mix was fermented before cooking and in the second method the mix was fermented after cooking. They reported that 4 h fermented and cooked and cooked and 12 h fermented *raabadi* had better acceptability.

2.4.2 Tarhana

Fermented cereal-yoghurt mixtures play an important role in the diets of many people in the Middle East, Asia, Africa and some parts of Europe (Ibanoglu and Ibanoglu, 1999; Altay *et. al*, 2013). Tarhana is a traditional fermented food product of Turkey. Tarhana is prepared by mixing yoghurt, wheat flour, yeast and a variety of vegetables and spices followed by fermentation for 1-7 days. After fermentation the mixture is sun dried and grounded. Tarhana has an acidic and sour taste with a yeasty flavour and is used for soup making (Ibanoglu and Ibanoglu, 1997).

In all cases, the milk or buttermilk undergoes a typical lactic acid fermentation in which the pH ranges from 3.5-3.8 and titratable acidity from 1.3 to 1.8 percent of as lactic acid. Tarhana though a traditional fermented product, has been continuously researched by various workers with major focus on improving the nutritional and organoleptic attributes. Georgala (2013) studied the nutritional value of tarhana and reported it as good source of B vitamins, minerals, organic acids and free amino acids. Use of barley flour in preparation of tarhana yields product with high β-glucan content, while use of wheat bran in preparation gives increased fibre content and antioxidant properties (Erkan *et al.*, 2006; Georgala, 2013).

2.4.3 Kishk

The name “kishk” refers to a group of popular fermented milk-cereal mix products and their variants. A wide range of traditional fermented and dried foods are produced in many countries. One such product, which is widely produced in the rural regions located between the Middle East and the Indian subcontinent, is known as Kishk, Kushuk, Keshkeh or Kichk. This product is normally made from low-fat yoghurt or the buttermilk of full fat churned yoghurt (Tamime *et al*., 2000).

The yoghurt is mixed with cereal (commonly known as Burghol, made from parboiled cracked wheat), sundried and ground to a powder.

According to Tamime and O'Connor (1995) there are three main kinds of food referred to as kishk. They include foods based on curdled milk products like yoghurt or fresh cheese; foods based on barley broth, bread, or flour; and foods based on cereals combined with curdled milk. Tamime *et al*. (1997) found that the mouthfeel attributes (grainy, sticky and slimy character) of kishk are associated with the type of bulgur used (*e.g.* made from wheat, barley or oats).

Milk from different species of mammals (cow, goat, sheep or buffalo) or a mixture of these has been used for the production of kishk. Traditionally, skimmed milk or buttermilk from churned fermented milk is normally used. The ratio of cereal to fermented milk ranges between 1:2 and 1:4. The main cereal additive (*i.e.* parboiled cracked wheat) is known by different names such as burghol, bourghoul, burghul or bulgur (El-Tinay *et al.,* 1979). Kishk (as a dish) is prepared by reconstituting the dried product with water and then simmering the mix gently over the fire. The consistency of this product is rather similar to porridge and it is normally consumed with bread. In some instances, flavoring agents such as chopped onions, tomatoes and/or coriander are added to the gruel mix (Tamime and Robinson, 1999). In Egypt principle microorganism reported in Kishk are the heterofermentative lactobacilli i.e. *Lactobacillus brevis* and the homofermentative *Lactobacillus casei* and *Lactobacillus plantarum* (Gaden *et al.*, 1992). Dried *Kishk* and *Tarhanas* are not hygroscopic, therefore, can be stored in a container for several years (Steinkraus, 1992).

2.4.4 Curd rice

Curd rice (yoghurt rice) is most popular in the Southern Indian states, namely, Karnataka, Kerala, Andhra Pradesh and Tamil Nadu (Plunkett *et al.,* 2001) usually served chilled. It is prepared by simply mixing cooked rice grains and curd. In more elaborated method rice is boiled so that it breaks down properly, giving almost a paste like consistency and cooled to room temperature. It is then seasoned with a finely fried mix of chopped green chillies, ginger, and curry leaves. To enhance the aroma and flavour it is tempered with urad dal, mustard seeds, cumin seeds, and asafetida followed by addition of yoghurt and salt. Curd rice is often eaten along with meal and served with pickles. Seeds of fresh pomegranate can be added to decorate the curd rice (Chandra, 1991).

2.4.5 Cereal based low fat yoghurt

Cereal flours, *viz*., rice, wheat, corn, oats and barley can be used in the preparation of yoghurt using skim milk. Among the different cereal flours used, corn flour was reported to be most acceptable (Vijayalakshmi, 2005). The nutritive value of such product can be enhanced by addition of fruit pulps and probiotic organisms during fermentation. Cereal flour or starch incorporation in preparation of low fat yoghurt improves textural and rheological characteristics.

2.4.6 Medida

Medida, the Sudanese thin cereal porridge is prepared for fasting, sick or convalescent people, nursing mothers and weaned infants. Kabeir *et al.,* (2004) improved the nutritional quality of *medida* by malting the brown rice grains for two days followed by addition of skim milk. The prepared mass was fermented with *Bifidobacterium longum* BB 536. The essential amino acid contents including lysine and methionine increased during fermentation.

2.4.7 Akpan

Akpan is a yoghurt-like product prepared from a partially fermented cooked maize gruel, named ogi. It is usually mixed with condensed milk, ice and sugar just before consumption. It is the most commonly consumed beverage in Benin (Pallet, 2011).

2.4.8 Bircher Muesli

The term is derived from German word “Mus”, means porridge. Bircher Muesli a homemade dietetic yoghurt, is the precursor of the new commercially made fruit yoghurt preparation. It was developed by Dr. Bircher-Benner, physician and nutritional physiologist (Kurmann *et al*., 1992). Bircher muesli is the preferred breakfast food for many people it contains milk, grated fruits such as apple, strawberry, raspberry, *etc*.; nuts and rolled breakfast cereals such as oat flakes, barley flakes, wheat flakes or any other precooked cereal grains. A number of stirred yoghurts are available commercially which contains all these ingredients (Kurmann *et al*., 1992).

2.4.9 Crowdies

Crowdie is a traditional product prepared in Scotland and generally prepared by pouring buttermilk to finely ground oatmeal with stirring till

consistency similar to pancake batter is obtained (Fitzgibbon, 1976). Crowdies is also the name of soft cheese from soured milk curd or butter milk.

2.4.10 Other composite fermented milk

Mugocha *et al*. (2000) prepared composite beverage containing finger millet gruel and skimmed milk powder; and fermented with yoghurt culture. In another study yoghurt extended with chickpea (*Cicer arietinum*), inoculated with *S. thermophilus* and *L. bulgaricus* was prepared and results indicated that yoghurt made with 80:20 mixture and modified starch removed syneresis and yielded pleasant flavor and textural characteristics (Morales *et al*., 2000). A yoghurt-like oat based functional food product formulated by Bekers *et al.* (2001) “oat bio lacto” supports oat beta-glucan, oat lipid unsaturated fatty acids and living probiotic lactic acid bacteria. The number of lactic acid bacteria in ‘‘oat bio lacto’’ was 10^9 per ml. The commercial product ‘‘oat bio lacto’’ contains a fat-free milk additive which improves the texture of yoghurt and lowering the caloric value. Cadena and Robinson (1979) studied the acceptability of yoghurt-cereal mixtures to a rural community in Mexico and observed that the production of yoghurt-cereal flours could be a valuable approach of preventing losses of liquid milk in rural areas. The material could form a useful dietary adjunct, especially for pre-school children.

2.5 Barley based fermented foods

2.5.1 Injera

Injera (Enjera) could be considered as the undisputed national food of Ethiopians (Chavan and Kadam, 1989; Oda *et al.,* 1983). It can be made from different cereals including barley, sorghum, tef, corn and finger millet. For the preparation of injera, the grains are dehulled and milled into flour. Flour is mixed with water to form a dough, the starter is also added, and the dough is fermented for 2 or 3 days. After fermentation, the dough is mixed with water to a thick batter and poured onto a lightly oiled pan, which is then covered with a tightly fitting lid to retain the steam (Parker *et al*., 1989). Within 2-3 h, it is ready to be removed from the pan and placed on a basket. A normal and typical injera is round, soft, spongy and resilient, about 6mm thick, 60 cm in diameter with uniformly spaced honeycomb like “eyes” on the top (Gebrekidan and Gebrettiwat, 1982). The major

quality attribute of a good quality injera is its slightly sour flavor. Injera has a very high nutritional value, as it is rich in calcium and iron (Zegeye, 1997).

2.5.2 Aara

Aara is a fermented alchololic beverage based on barley and popular in Himachal Pradesh of India. For its preparation, barley grains are washed and ground into a paste with water (1:1). The barley slurry is cooked for 2-3 hours in an open vessel. The cooked slurry is cooled and sprinkled with powdered *phab* (one granule for 1-2 kg barley grains), fermented for 2-3 days at 25-30°C in container containing water (30-35 litres of water for 50 kg of barley). '*Phab*' a traditional inoculum used for fermentation of alcoholic products. It is a starter culture or a traditional inoculum used for fermentation of alcoholic products. It is commercially available as dried white cakes or granules having millet or rice powder as basic ingredients. Approximate weight of each granule (*phab*) is 3-5 g. Predominating yeast flora identified in Aaara and phab granules were *Saccharomyces cerevisiae and Saccharomyces fermentati,* respectively, whereas, bacterial flora identified in Aara were *L. plantarum*, *B. subtilis* and *Acetobacter aceti.* Fermented product obtained is locally called as "Aaara". Distillation is done by using a copper container and product after evaporation is collected through an outlet (Kanwar *et al.,* 2011). Alcohol content in undistilled and distilled Aaara were reported to be around 11.5 and 18.8 percent, respectively.

2.5.3 Barley tempeh

Tempeh is immensely popular in far-east countries and usually made from rice or soy bean. Tempeh is defined as a compact and sliceable mass of cooked particles of raw materials covered, penetrated and held together by dense non-sporulated mycelium of *Rhizopus* spp. (Nout and Rombouts, 1990). A patented barley tempeh procedure has been developed by fermentation of whole pearled barley kernels with selected strains of *Rhizopus oligosporus* (Berg *et al.,* 2001). Although different substrates can be used to produce tempeh, the basic fermentation process is similar for all substrates. It includes soaking, dehulling, boiling and fermenting. The main differences between different substrates used in tempeh fermentation are the selection of optimal pre-treatments (e.g. when cereals are used it is sometimes necessary to modify the surface of the grain by

cutting, cracking or pearling to obtain good growth of the mold), optimal soaking, boiling and incubating time, and optimal fermentation strain and inoculation level of *Rhizopus oligosporus*.

2.6 Shelf-life of fermented milks

The acid environment created by metabolic reactions of LAB in the fermented milks is responsible for the enhanced shelf life of fermented milks. Several strains of LAB used as starter have been reported to produce number of anti-microbial metabolites such as hydrogen peroxides, bacteriocin, organic acids, and production of antimicrobial peptides by degrading native milk proteins by microbial peptidases. Normally shelf-life of fermented milks is 2-3 days at ambient temperatures and 1-3 weeks at refrigeration temperatures (Prajapati, 1995; Iniguez and Paz, 1998; Driessen, 1998; Tamime and Robinson, 1999). Kumar (1992) found 7 and 14 days of shelf-life of untreated *lassi* stored at 37±2°C and 7±1°C, respectively. Fruit yoghurt can be stored up to 10 days at refrigeration temperature (5°C), but spoiled within 24 hours when kept at ambient temperature (Shukla, 1982). Sanyal *et al.,* (1990) found shelf-life of *dahi* as 2 and 7 days at 30±2°C and 4±2°C, respectively. *Dahi* and yoghurt were stable up to 5 days, whereas, their shelf life was only 3 days at room temperature. Continuous metabolic activity exhibited by viable starter often led to over-acidification resulting in poor sensory characteristics. Moreover, under highly acidic conditions milk protein lost their water holding capacity.

Yoghurt stored at room temperature (23-32°C) had been reported to be stable up to only for 12 hours, whereas storage at refrigeration temperature (0-7°C) enhanced its stability up to 14 days with good body and texture, and acceptable flavour and aroma (Barraquio *et al*., 1981). Hassan *et al.,* (1994) reported that acceptability of acidophilus milk was up to 8 days at 7°C. Dahi prepared with slow acidifying culture was found stable up to 30 days and 36 h at refrigeration temperature and room temperature, respectively (Baxi, 1996).

2.7 Shelf-life of cereal-milk based fermented milk

Modha (2006) reported that the shelf life of pearl millet based *raabadi* was 7 days at refrigeration temperature (6-8°C). Similar results were obtained by Pintu (2006) and Girish (2006) for sorghum based *raabadi* and wheat based *raabadi,* respectively. Hussain (2008) and Kakde (2010) attempted to extend the shelf life of sorghum based and pearl millet based lassi like beverage using bio-

preservatives (bacteriocin), chemical preservatives and their combination with thermization treatment. Hussain (2008) reported that sorghum-milk based fermented beverages added with potassium sorbate, nisin, and MicroGARD had shelf life of 35, 28, and 21 days, respectively under refrigeration storage. Kakde (2010) reported salted pearl millet *lassi* samples added with potassium sorbate, nisin and pediocin as separate treatment remained acceptable up to 20 days in comparison to control (only 12 days).

Oat based fermented probiotic drink was reported to be stable up to 21 days with adequate cell viability (Angelov *et al*., 2006; Gupta *et al.*, 2010). However, a reduction of less than only one log cfu/ml was reported during the refrigeration storage. Use of LAB strains having potential to produce antifungal metabolite, could be utilized to improve the shelf life of milk-cereal based fermented drinks (Basu *et al*., 2011a; Basu *et al*., 2011b).

2.8 Probiotics, prebiotics and synbiotics

The word probiotics is derived from the Greek meaning 'for life'. Even though probiotics products *per se* have always existed, it was Metchnikoff at the beginning of the 20^{th} century who first identified the health benefits related to the regular consumption of fermented milks (Metchnikoff, 1907a; Fuller, 1989). According to Metchenikoff, the regular consumption of live beneficial bacteria such as lactic acid bacteria (LAB) through fermented dairy products are needed to maintain a good equilibrium of the intestinal microflora that minimize putrefactive microbial fermentations.

Probiotics could be defined as live microorganisms when administered in adequate amounts confer health benefits to the host (FAO/WHO, 2002). Ross *et al.,* (2002), defined probiotic as living microorganisms, which upon ingestion in certain numbers exert health benefits beyond their inherent basic nutrition. A number of genera of bacteria are considered as probiotics such as *Lactobacillus, Leuconostoc, Pediococcus, Bifidobacterium* and *Enterococcus*, but the strains of genera *Lactobacillus* and *Bifidobacterium* have the most credible and safe history as probiotic for human consumption (Shah, 2007).

It has been suggested that probiotic organisms should be present in a food at a minimum concentration of 10^6 cfu/g (Kurmann and Rasic, 1991; Ishibashi and Shimamura, 1993; Gomes and Malcata, 1999), or the daily intake should be

about 10^8 cfu/g. Such high numbers have been recommended to compensate for possible losses in the numbers of the probiotic organisms during passage through the upper part of gut *i.e.* stomach and intestine. Despite the importance of viability of probiotic microorganisms, surveys have shown poor viability in yoghurts (Shah *et al*., 1995; Dave and Shah, 1997; Shah, 2000b). Therefore, in order to provide the therapeutic benefits, attempts to increase viability in dairy products have drawn the attention of researchers in recent years. On the other hand, there is much evidence to show that *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* survive at high concentration (>10^7 cfu/g) in yoghurts after manufacture until the time of consumption (Rohm *et al*., 1990) in comparison to probiotic microorganisms. Several factors had been reported to affect the viability of probiotic cultures in fermented milk products.

These factors include the strains, interactions among mixed strains, acidity, pH and hydrogen peroxide produced due to bacterial metabolism, have been identified to affect the viability of probiotic microorganisms during manufacture and storage of yoghurt (Dave and Shah, 1997). Other factors such as storage temperature, oxygen content, concentrations of acetic and lactic acids, nutrients limitations in milk, prebiotics, inhibitors, inoculation level, fermentation time and post-acidification have also been reported to affect viability of probiotic organisms in yoghurt (Shah and Ravula, 2000; Dave and Shah, 1998; Shah, 2000b; Tamime, 2005).

In addition to probiotics, the terms ‘prebiotics’ and ‘synbiotics’ are often used. A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers health benefits to host wellbeing (Gibson *et al*., 2004). Prebiotics are non-digestible carbohydrates that pass undigested through the upper part of the gastrointestinal tract and stimulate the growth and/or activity of beneficial bacteria that colonize in the colon by acting as substrate for them. Most commercial prebiotics are carbohydrates, predominantly oligosaccharides and some polysaccharides. The nondigestible character of prebiotics is a feature shared with dietary fibre, but their physiological functions are often different (Macfarlane *et al.,* 2006). A synbiotic refers to a food product, which contains both a probiotic and a prebiotic (Roberfroid, 1998). This combination has shown

to confer benefits, which are beyond the benefits of the probiotic and prebiotic on their own. If the prebiotic carbohydrate is utilised by a probiotic strain, its growth and proliferation in the gut will be selectively promoted (Holzapfel and Schillinger, 2002).

2.8.1 Type of probiotics

Probiotic microorganisms are delivered in food or dairy products via supplementation or fermentation. A wide range of microorganisms have been incorporated into dairy products for their probiotic potential (Fuller, 1997; Gibson and Fuller, 1998). Examples of probiotic organism include Lactobacilli, such as *L. acidophilus*, *L. casei*, *L. delbrueckii* ssp. *bulgaricus*, *L. reuteri*, *L. brevis*, *L. cellobiosus*, *L. carvatus*, *L. fermentum* and *L. plantarum*; Gram-positive cocci, such as *Lactococcus lactis* ssp. *cremoris*, *Streptococcus thermophilus*, *Enterococcus faecium*, *S. diacetylactis* and *S. intermedius* and Bifidobacteria, such as *B. bifidum*, *B. adolescentis*, *B. animalis*, *B. infantis*, *B. longum* and *B. thermophilum etc*. Nonpathogenic microorganisms that occupy important niches in the host gut or tissues, such as yeasts, enterococci and *Enterobacteriaceae,* are also used as probiotics. *Lactobacillus* and *Bifidobacterium* are the most common species of bacteria used as probiotics for the production of fermented milks and other dairy products (Fuller, 1992). A number of food products including yoghurt (Kailasapathy and Rybka, 1997), frozen fermented dairy desserts (Ravula and Shah, 1998), spray dried milk powder (Stanton *et al.,* 2001), cheeses (Ross *et al.,* 2002), ice cream (Haynes and Playne, 2002), freeze-dried yoghurt (Capela *et al.,* 2006) and fruit juices (Saarela *et al.,* 2006) have been successfully employed as delivery vehicles for probiotics to consumer.

2.8.2 Health benefits of probiotics

Probiotics have been reported to exert numerous health benefits such as balancing of intestinal microflora, stimulation of the immune system, prevention of diarrhoea and anticarcinogenic activity (Sanders, 1999; Salminen *et al*., 1998). Naidu *et al.,* (1999) extensively reviewed the probiotic spectra of LAB and concluded that LAB could prevent adherence, establishment, and replication of several enteric mucosal pathogens through several antimicrobial mechanisms. A number of health benefits for product containing live probiotic bacteria have been claimed including alleviation of symptoms of lactose intolerance, treatment of diarrhoea, anticarcinogenic properties, reduction of blood cholesterol and

enhancement in immunity (Shah, 2000a). Consumption of LAB fermented dairy products with LAB may exert antitumor effects. Health benefits imparted by probiotic bacteria are strain specific and It is important to note that no strain will provide all proposed benefits, not even strains of the same species will be effective against defined health conditions. Few of the health benefits of probiotic organisms are reviewed briefly hereunder.

2.8.2.1 Antimicrobial activity and control of gastrointestinal infection

Probiotic bacteria produce organic acids, hydrogen peroxide and bacteriocins as antimicrobial substances that suppress the multiplication of pathogenic and putrefying bacteria. Lowering of pH due to lactic acid or acetic acid produced by these bacteria in the gut has a bactericidal or bacteriostatic effect (Sandine, 1979). *Bifidobacterium* and *L. acidophilus* show antagonistic effects towards enteropathogenic *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Cl. Perfringens* (Shah, 2007).

2.8.2.2 Effectiveness against diarrhoea

The ability of probiotics to decrease the incidence or duration of certain diarrhoeal illnesses is probably the most accepted of probiotic health effects. Investigations suggest that treatment of diarrhoea with probiotics is most effective towards diarrhoea caused by rotavirus, followed by bacterial and parasitic diarrhoea (Shornikova *et al.,* 1997; Heyman and Ménard, 2002). LAB are capable of producing antimicrobial substances which inhibit many of the diarrhoea causing pathogens. It is believed that by production of these substances by LAB assist in the maintenance of existing intestinal microflora and inhibit proliferation of pathogenic microorganism and thereby reduce or prevent diarrhoea (Holzapfel and Schillinger, 2002). Many *in-vitro* studies suggests that the anti-infective properties of lactobacilli are due to their ability to adhere intestinal mucosal surfaces thereby replacing the adhesion of pathogens; deplete nutrients for pathogens; and modulate the host immune response and microenvironment and reducing the risk of infection (Dunne and Shanahan, 2002, Gionchetti *et al*. 2002, Heyman and Ménard, 2002, Penner *et al.,* 2005 and Santosa *et al.,* 2006).

The antibiotics are invariably used to kill harmful microorganism. Unfortunately, these antibiotics are often active towards a broad spectrum of bacteria in GIT and may lead to destruction of beneficial microorganisms as well

as disruption of microbial balance, thus may induce diarrhea. Supplementation of probiotics through diets during and after antibiotic therapy has been shown to minimize disruptive effects of antibiotics (Thomas *et al.,* 2001; Johnston *et al.,* 2006). Rotavirus is one of the most widely prevalent causative organisms of acute diarrhoea among children worldwide. Probiotics have been proved to minimize the incidences of rotaviral diarrhoea in children (Saavedra, *et al.,* 1994). The strongest evidence of a beneficial effect of defined strains of probiotics has been established using *L. rhamnosus* GG and *B. lactis* Bb-12 for prevention and treatment of diarrhoea and acute diarrhoea in children mainly caused by rotavirus. There is also strong evidence that probiotic strains can prevent traveller's diarrhoea (Hilton, *et al.,* 1997), which is mainly caused by enterotoxigenic strains of *E. coli*.

2.8.2.3 Improvement in lactose metabolism

Lactose intolerant individuals have insufficient levels of lactase, an enzyme that catalyzes the hydrolysis of lactose into glucose and galactose, in their digestive system. In most cases, this causes symptoms which may include abdominal bloating and cramps, flatulence, diarrhea, nausea, borborygmi (rumbling stomach), or vomiting. Most mammals normally cease to produce lactase, becoming lactose intolerant, after weaning (Swallow, 2003). A study of 21 African American girls, showed 75% had some decrease in lactase activity during adolescence (Pribila *et al*., 2000). The frequency of lactose intolerance ranges from 5% in Northern European to more than 90% in some African and Asian countries (Bulhões et al., 2007). Relief of the symptoms of lactose intolerance is another widely accepted health benefit of probiotics. Lactose malabsorption results from a deficiency of the enzyme β-D-galactosidase. β-D-galactosidase hydrolyses the lactose into its constituents monosaccharides, glucose and galactose. Lactose intolerant people often complain of gastric distress after consuming milk or non-fermented milk products due to the formation of hydrogen gas by microbial action on undigested lactose in the gut (Shah *et al.,* 1992; Shah,1993). Probiotic of LAB group contains substantial quantity of β-D-galactosidase and help in improvement in lactose metabolism (Shah *et al.,* 1992).

2.8.2.4 Antimutagenic properties

It is well accepted fact that majority of carcinogenic or mutagenic substances are present in diet. Probiotic organisms are reported to bind mutagens to the cell surface, neutralize them, and assist in their excretion (Orrhage, *et al.,* 1994). Certain probiotics have been reported to reduce or check faecal enzymes including β-glucuronidase, azoreductase and nitroreductase, which are implicated in the activation of mutagens (Goldin and Gorbach, 1984). Lankaputhra and Shah (1998) studied the antimutagenic activity of organic acids produced by probiotic bacteria against several mutagens and promutagens and reported butyric acid for exhibiting broad spectrum antimutagenic activity for all tested mutagens or promutagens.

2.8.2.5 Anticarcinogenic properties

Few bacterial metabolites such as genotoxic compounds (nitrosamine, heterocyclic amines, phenolic compounds, and ammonia) are responsible for colorectal cancer. The colonic microflora are also reported to induce carcinogenesis mediated by microbial enzymes such as β-glucuronidase, azoreductase, and nitroreductase, which convert procarcinogens into carcinogens. The anticarcinogenic effect of probiotic microbes could be attributed to inhibition of the conversion of procarcinogens into carcinogenic forms or minimizing the enzymes that lead to their formation, normalized intestinal permeability, strengthening of intestinal barrier mechanisms, and activation of non-specific cellular components such as macrophages *via* regulation of γ-interferon production. Certain strains of *L. acidophilus* and *Bifidobacterium* spp. Have been observed to reduce the levels of β-glucuronidase, azoreductase and nitroreductase; and consequently decrease the risk of tumour development (Yoon *et al.,* 2000). Short chain fatty acids (SCFA) produced by *L. acidophilus* and *Bifidobacterium*, *L. plantarum* and *L. rhamnosus* are reported to inhibit the generation of carcinogenic products by reducing enzyme activities (Cenci, *et al.,* 2002). SCFA mainly butyrate induces apoptosis, promote cell differentiation and activate cellular immune components.

2.8.2.6 Reduction in serum cholesterol

The level of serum cholesterol particularly LDL-cholesterol is a major factor associated with coronary heart diseases (Liong and Shah, 2006). Probiotic

bacteria are reported to de-conjugate bile salts. Deconjugated bile acid does not absorb lipid as readily as its conjugated counterpart, leading to a reduction in cholesterol level. Bile salt deconjuagation activity is due to liberation of bile salt hydrolase (BSH) enzyme by certain microbes. *L. acidophilus* is reported to take up cholesterol during growth and this makes it unavailable for absorption into the blood stream (Klaver and Meer, 1993; Fukushima and Nakano, 1996).

2.8.2.7 Helicobacter pylori infection reduction

Helicobacter pylori is a pathogen that causes peptic ulcers, type B gastritis and chronic gastritis, and is normally present in the stomach as an opportunistic pathogen without causing pathogenicity (Armuzzi *et al.,* 2001; Sakamoto *et al.,* 2001). Antibiotic treatments can successfully eradicate *H. pylori*. However, long term antibiotics usage often may lead to several side effects and could render the bacteria antibiotic resistant. Probiotic organisms do not appear to eradicate *H. pylori*, but they are able to reduce the bacterial load in patients infected with *H. pylori* (Felley *et al.,* 2001). Similarly, *L. casei* Shirota, *L. reuteri* and *L. acidophilus* are reported to inhibit the growth of *H. pylori* (Cats *et al.,* 2003).

2.8.2.8 Improvement in inflammatory bowel disease (IBD)

Occurrence of inflammatory bowel disease (IBD) is related to the type of intestinal microflora. Symptoms of IBD include a disturbance in bowel habits and mucosal inflammation. Probiotics do not alleviate the disease, but once patients are in remission through treatment with corticosteroids, certain probiotics can reduce the incidence of relapse. *L. acidophilus, L. plantarum, L. reuteri* etc are reported for their role against inflammatory bowel disease (Marteau *et al.,* 2001; Schultz and Sartor 2000; Vanderhoof, 2000). Probiotic mechanisms may include competitive metabolic interactions, the production of antimicrobials, and inhibition of adherence or translocation of pathogens. Stabilization of the gut barrier by probiotics may be important for therapeutic efficacy in IBD (Sheil *et al*., 2007).

2.8.2.9 Immune system stimulation

The GI tract contains 70 percent of the immune system and the diversity of intestinal microflora and the collective metabolic activity is equivalent to virtual organ within an organ (O'hara and Shanahan *et al*., 2006). Probiotics may directly or, by changing the composition or activity of the intestinal microflora, influence the body's immune function (Marteau *et al.,* 1997). Probiotic cultures

produce γ-interferon by T-cells and stimulate cytokines as represented by tumour necrosis factor α and interleukins 6 or 10. Probiotics can enhance nonspecific cellular immune response by activation of macrophages, natural killer cells, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines (Ashraf and Shah, 2014). Various studies has shown that *L. acidophilus* NCFM, *L. plantarum*, *L. rhamnosus*, *L. helveticus*, *L.casei* Shirota etc. play role in immune system stimulation (Kato *et al*., 1999; Martar *et al.,* 2001; Sanders and Klaenhammer, 2001; Leblanc *et al*., 2002; Morita *et al.,* 2002; Borruel *et al.,* 2003; Walker, 2000).

2.8.3 Use of lactobacilli as probiotics

Beyond the fermentative abilities of the lactobacilli, an additional role has been attributed to those species that reside in the intestinal tracts of humans. Lactobacilli are the major component of the commensal microflora of both small and large intestine. **Table 2.2** represents various bacterial species primarily used as probiotic cultures in formulation of various food or pharma foods. However, only few probiotics related to present investigation are being discussed briefly in the review under the following sub-headings.

2.8.3.1 *Lactobacillus acidophilus*

L. acidophilus constitutes a major part of the natural human intestinal microflora (Gilliland and Speck, 1977; Gilliland, 1979). It has long been suggested that *L. acidophilus* may enhance resistance to common intestinal disorders through stabilization of normal intestinal microflora (Metchnikoff, 1907b; Fuller, 1991; Sandine, 1979). *L. acidophilus* shows antagonistic activity towards enteropathogenic *E. coli, S. typhimurium, Staph. aureus* and *Cl. perfringens*. *L. acidophilus* produces various bacteriocins and antibacterial substances such as lactocidin, acidolin, acidophilin, lactacium-B and inhibitory proteins (Shah, 2007). *L. acidophilus* are reported for their antimutagenic properties due to their ability to bind mutagens, produce organic acids and reduce faecal enzymatic activities (Ayebo *et al.,* 1982; Goldin and Gorbach, 1984; Hosoda *et al.,* 1992; Orrhage *et al.,* 1994). *L. acidophilus (*NCDC13) isolated from milk products (Laxminarayan, 1952) have been studied for its probiotic attributes (Kushal *et al.,* 2006a, Kushal *et al.,* 2006b, Kushal *et al.,* 2005; Kapila *et al.,* 2006; Saran *et al*., 2012; Arora *et al*., 2012).

Table 2.2 Bacterial species primarily used as probiotic cultures

Species	Strains
Lactobacillus acidophilus	La2, La5, Lj1, NCFM, DDS-1, SBT-2062
L. bulgaricus	Lb12
L. lactis	La1
L. plantarum	299v, Lp01
L. rhamnosus	GG, GR-1, 271, LB21
L. reuteri	SD2112 (also known as MM2)
L. casei	Shirota, Immunitass, 744, 01
L. paracasei	CRL 431
L. fermentum	RC-14
L. helveticus	B02
Bifidobacterium adolescentis	-
B. longum	B536, SBT-2928
B. breve	Yakult
B. bifidus	Bb-11
B. essensis	Danone, (Bio Activia)
B. lactis	Bb-02
B. infantis	Shirota, Immunitass, 744, 01
B. laterosporus	CRL 431

Source: Krishnakumar and Gordon (2001)

Arora *et al*., (2012) studied anti-obesity potential of *L. acidophilus* NCDC 13 by administering in the form of *dahi* containing native starter in diet-induced obese mice up to 8 weeks. No significant changes in body fat composition, liver and muscle adiposity were reported. However, *L. acidophilus* NCDC 13 supplementation was reported to be beneficial in shifting the gut microbiota positively. A fermented indigenous food mixture based on barley flour, whey powder and tomato pulp and having adequate cell viability (8.88 cfu/ml) of probiotic *Lactobacillus acidophilus* NCDC-16 was reported to be beneficial in controlling *E. coli* induced diarrhoea in mice (Jood *et al*., 2012a).

2.8.3.2 *Lactobacillus casei*

L. casei is a group of heterogeneous mesophilic lactic acid bacteria widely distributed in environment. The strains are characterized as Gram-positive, non-

sporulating, catalase-negative, short or long rods with rounded ends (Ferrero *et al.,* 1996). A number of fermented milk products containing *L. casei* have been used since long. *L. casei* has been found to reduce the incidence and duration of certain types of diarrhoea. Gorbach *et al*. (1987) observed that *L. casei* aided in prevention and treatment of antibiotic associated diarrhoea. Travelers diarrhoea was also reduced by ingestion of freeze dried viable cells of *L. casei* (Oksanen *et al.,* 1990). The severity of diarrhoea in children in day care centers in France was controlled by consumption of milk fermented with *L. casei* (Pedone *et al.,* 1999). Saxelin *et al*. (1991) reported that feeding of *L. casei* @ 10^{11} to 10^{12} cfu/d, decreased the carcinogenic enzyme activity, including β-glucuronidase, nitroreductase, etc. Various studies in mice model, demonstrated that *L. casei* can modulate both specific and non-specific immune system (Perdigon *et al.,* 1991; Moineau and Goulet, 1991). *L. casei* strain Shirota enhanced the production of T helper-1 cell associated cytokines like IFN-γ and IL-2 by spleen cells. *L. casei* is also reported to play role in reducing serum cholesterol (Liong and Shah, 2006; Kawase *et al.,* 1999). Studies showed that *L. casei* Shirota and *L. acidophilus* inhibits the growth of *H. pylori* (Cats *et al.,* 2003). Mishra and Prasad (2005) isolated three strains (C1, C2 and Y) of *L. casei* from milk products and investigated them extensively for the probiotic attributes. *L. casei* NCDC-297 and 298, both isolates from milk products, indicated promising results for acid tolerance and adherence properties. *L. casei* ATCC 393 (other collection codes: NCDC299, *L. casei* VTT E/85225, DSM 20011, NCIMB 11970) (NCDC catalogue, 2002; www.straininfo.net/strains/ 287934; Mishra and Prasad 2005) contained in fermented milk survived GI transit and modulated intestinal microbiota (Sidira *et al.,* 2010)

2.8.3.3 *Lactobacillus plantarum*

L. plantarum is a gram positive bacterium that is found in wide range of fermented products such as cheese, fermented sausages, fermented vegetables namely sauerkraut, pickeles, brined olives, ogi, sourdough, besides harbouring in human GIT. It is a facultative heterofermentative LAB that utilizes an extensive range of fermentatable carbon sources. *L. plantarum* also produces anti-microbial peptides and exopolysaccharides. It has the ability to maintain a pH gradient between the inside and outside of the cell in the presence of large amounts of

acetate or lactate. Ceratin strains of *L. plantarum* have been identified as probiotics (Niedzielin *et al.,* 2001). Few companies currently sell products containing *L. plantarum* as a probiotic to help in intestinal problems including IBS and IBD, stating that these bacteria assist in balancing the Intestinal ecosystem (Bronze *et al.,* 2008). Frias *et al*. (2008) utilized *L. plantarum* to reduce the allergenicity of soy flour and observed the highest reduction in IgE immunoreactivity (96–99%), depended upon the sensitivity of the plasma. The 299v strain of the *L plantarum* family originated from the human intestine has been studied much more extensively than other strains. In human trials, Lp299v have shown to adhere the cells lining the colon forming a defensive barrier (Adlerberth *et al.,* 1996), improved antibiotic associated diarrhoea (Wullt *et al.,* 2007), decreased abdominal bloating in patients with IBD (Nobaek, 2000), improved mucosal status (Johansson *et al.,* 1993; Molin, 2001; Mao *et al.,* 1996b), increased the concentration of carboxylic acids in feces, decreased fibrinogen (a protein that helps with blood clotting) concentrations in the blood (Molin, 2001), decreased translocation (Mao *et al.,* 1996a) and improvd liver status (Kasravi *et al.,* 1997).

Kaushik *et al.,* (2009) considered effectiveness of probiotic organism is population-specific due to variation in gut microflora, food habits and specific host-microbial interactions, and hence extensively studied an indigenous isolate of *Lactobacillus plantarum* lp9 for its functional and probiotic attributes. The isolate exhibited high resistance against low pH and bile and possessed antibacterial, antioxidative and cholesterol lowering properties with a potential for exploitation in the development of functional foods containing indigenous probiotic strain. Kumar *et al.*, (2011) evaluated the anti-hypercholesterolaemic effects of putative probiotic bile salt hydrolase (Bsh)-producing indigenous isolate of *Lactobacillus plantarum* lp91 in rats and observed higher excretion cholic acid and lactobacilli counts in faeces, suggesting strain has the potential to be explored as a probiotic in the management of hypercholesterolaemia.

2.8.3.4 *Lactobacillus rhamnosus*

Lactobacillus rhamnosus is a bacterium that was originally considered to be a subspecies of *L. casei*, but later genetic research found it to be a species of its own (Prajapati *et al*., 2012). Some strains of *L. rhamnosus* are being used as probiotics. The species is sometimes used in yoghurt and other dairy products.

The clinical health effects of *Lactobacillus rhamnosus* GG have been widely studied. Gorbach and Goldin have published studies of *Lactobacillus rhamnosus* GG demonstrating the tolerance to acidic conditions of the stomach, and the bile acids of the small intestine (Conway *et al.,* 1987). As a probiotic, *Lactobacillus rhamnosus* GG is claimed to colonize in the digestive tract, and to balance intestinal microflora, however, *Lactobacillus rhamnosus* is more likely a transient inhabitant, not autochthonous (Walter, 2008). In 2005, *Lactobacillus rhamnosus* GG was first successfully used to treat gastrointestinal carriage of vancomycin-resistant enterococcus in renal patients (Manley *et al*., 2007). Tannock *et al.,* (2000) analysed the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20 and concluded that consumption of the DR20 containing milk product transiently altered the *Lactobacillus* and enterococcal contents of the feces of the majority of consumers without markedly affecting biochemical or other bacteriological factors.

2.8.4Therapeutic dose requirements

Adequate numbers of viable cells, namely the “therapeutic minimum dose” needs to be consumed regularly for transfer of the probiotic associated health benefit. The IDF (1997) proposed that in probiotic foods, “the specific microorganisms shall be viable, active and abundant at the level of at least 10^7 cfu/g in the product to the date of minimum durability (Ouwehand and Salminen, 1998; Samona and Robinson, 1991). It has been suggested that approximately 10^9 cfu/d of probiotics is necessary to elicit health effects. Based on daily consumption of 100 gram of a probiotic food, it has been suggested that a product should contain at least 10^7 cfu/g, a level paralleling current Japanese recommendations (Ishibashi and Shimamura, 1993). The ingestion of 10^6 to 10^9 viable cells per day is necessary for humans in order to develop beneficial effects from probioyic organisms (Lee and Salminen, 1995). Regular consumption of 400-500 g/week of bio-yoghurt, containing 10^6 viable cells/ml would provide these numbers (Tamime *et al.,* 1995). Assuming a daily consumption of fermented milk equal to 100 g, a minimum level for probiotic bacteria of 10^6 cfu/g can be suggested in these products, reported by Vanderhoof and Young (1998) and this level is now recommended by a French Standard (norm AFNOR NF V04-600) (Lucas *et al.,* 2004).

2.9. Cereal-milk based probiotic products

Cereals serves as suitable substrates for the growth of probiotic LAB and improved functionality of colonic strains because of the presence of specific non-digestible components of the cereal matrix that act as prebiotics (Charalampopoulos *et al.,* 2002a). Cereals may contain water soluble fibre such as β-glucan and arabinoxylan, oligosaccharides such as galacto- and fructo-oligosaccahrides and resistant starch, all of which have been suggested to fulfil the prebiotic concept. Non-digestible carbohydrates (NDC) such as β-glucan content in barley besides providing beneficial physiological effects can stimulate the growth of LAB present in the colon (Charalampopoulos *et al.,* 2002b). Barley β-glucan has known to extend the viability of *L. acidophilus* LA-5 and *Bifidobacterium lactis* bb-12 (Elsanhoty *et al.,* 2009). Incorporation of fructooligosaccharide (FOS) in reduced fat yoghurt has been reported to improve the viability of starter culture and probiotic organism (Akalin *et al*., 2007). Higher *in vivo* effectiveness of *Bifidobacterium* probiotic strains in synbiotics has been reported by Bielecka *et al.* (2002). Single culture fermentation or sequential culture fermentation (by lactobacilli and yeast) of an indigenously developed mixture containing barley flour, milk coprecipitate, sprouted green gram paste and tomato pulp have been reported to drastically reduce the levels of certain anti-nutrients such as phytic acid, polyphenols and trypsin inhibitor activity, while improved the *in vitro* digestibilities of starch and protein (Sindhu and Khetarpaul, 2001). Similar results were also reported for food mixture containing rice flour, whey, sprouted green gram paste and tomato pulp (Sindhu and Khetarpaul, 2002), when either singly fermented with *L. casei* or *L. plantarum* at 37°C for 24 h, or sequential culture fermentation using *S. boulardii* for 25°C for 24 h followed by fermentation with *L. casei* or *L. plantarum* at 37°C for 24 h.

Incorporation of certain dietary polysaccharides could be effective in increasing the beneficial bacteria in gut. However, such polysaccharides must escape digestion in upper gastrointestinal tract, and must be soluble, hydrolysable, and fermentable by the gut flora mainly in colon region. These polysaccharides may be soluble dietary fibre, resistant starch, and unabsorbed sugars and oligo- and poly-saccharides (Charalampopoulos *et al.,* 2002a). In cereal grains the indigestible polysaccharides and dietary fibre components are arabinoxylans or pentosans present predominantly in the cell walls of wheat and

rye, and mixed linkage β-glucan found in the cell wall of oats and barley (Salovaara, 1998).

Human derived strains of *L. reuteri*, *L. plantarum*, *L. acidophilus* and *L. fermentum* isolated from cereals, when cultured in malt, barley, and wheat extracts exhibited better cell growth in malt medium than in barley and wheat extracts due to the higher proportion of maltose, sucrose, glucose, and fructose (Charalampopoulos *et al.,* 2002b; Charalampopoulos and Pandiella, 2010). *L. plantarum* exhibited the highest cell population owing to its unique ability to tolerate low pH by maintaining a proton and charge gradient between the inside and the outside of the cells even in the presence of high amounts of lactate and protons (Giraud *et al.,* 1998). Helland *et al.,* (2004) studied the growth and metabolism of selected strains of probiotic bacteria namely *L. acidophilus* La5 and 1748, *B. animalis* Bb12, and *L. rhamnosus* GG in milk- and water-based cereal puddings, and reported good growth and survival in milk-cereal based puddings, with the highest viable cell count of *L. rhamnosus* GG in both milk- and water-based puddings. Ouwehanda *et al.,* (2004) tried inclusion of a probiotic strain *Bifidobacterium lactis* Bb-12 in an oat-based cereal bar, to eliminate dairy based probiotic because it needs refrigeration storage during distribution and storage. *In vivo* study indicated presence of *Bifidobacterium lactis* Bb-12 in the faces of healthy human volunteers after one week cessation or probiotic oat-based cereal bar. Benefical health effects of a probiotic strain and oat prebiotic β-glucan were combined by Angelov *et al.,* (2006) and a new oat based probiotic drink was developed with a shelf life of 21 days under refrigeration with adequate cell viability. Probiotic fermentation was carried out using *L. plantarum* B28, isolated from a cereal based product.

Survival of a potentially probiotic *L. plantarum* strain found much better in the malt extracts compared to barley and wheat extracts during refrigerated storage (Charalampopoulos and Pandiella, 2010). Kedia *et al.,* (2007) developed a new cereal-based probiotic food with suitable aroma, flavor and pH using mixed culture fermentation by yeast and LAB, and observed that growth of LAB was enhanced by the introduction of the yeast. In another study conducted by Kalui *et al.* (2009), different strains of *L. plantarum* and *L. rhamnosus* isolated from *ikii*, a traditional fermented maize porridge, were studied for their probiotic attributes, utilisation of fructooligosaccharide, production of exopolysaccharides and safety

attributes and found that *L. plantarum* involved in production of *ikii* do have potential probiotic attributes.

Arora *et al.,* (2010) formulated food mixtures from non-germinated and germinated barley flour, whey powder and tomato pulp and fermented it with *L. acidophilus* reported that, combined processes of germination and fermentation significantly increased thiamine, niacin, lysine and soluble dietary fibre contents indicating, combination of germination and fermentation is a potential process to enhance the nutritional value of food mixtures based on coarse cereals. Gupta *et al.,* (2010) developed an oat based functional beverage based on lactic acid fermentation of oats by *Lactobacillus plantarum* by optimizing three different levels of oat, sucrose and starter culture with a response of final viable cell population using Box–Behnken optimization design. The developed beverage was found acceptable up to 21 days without any change in β-Glucan content during fermentation and entire storage period.

Ganguly *et al.*, (2014) developed a composite dairy-cereal substrate consisting of whey-skim milk, germinated pearl millet flour, and liquid barley malt extract which was heat processed, and fermented using probiotic *L. acidophilus* NCDC 13. A high count of 13.22 log cfu/ml was reported in the substrate with at 4% inoculum level with 8 h incubation at 37°C. Phytic acid, polyphenol contents and phytate phosphorous were reported to be reduced by 80.0, 47.2 and 76.5%, respectively with concomitant increase by 69.0 and 64.0% in the bio-availability of Ca and Fe. The protein and starch digestibility of the mixture were reported to increase from 45.4 and 43.4% to 62.4 and 57.8%, respectively.

2.10 Commercial potential of milk-cereal based fermented foods

Majority of health foods attract little effective demand in Indian market and face competitions from established brands. The recent growth of health foods in Indian market offers new opportunities for the development of health foods by judicious blending of milk or milk constituents and cereals, into convenient form with proven health benefits to consumers. Rapid advances in science and technology, increasing healthcare costs, changes in food laws affecting label and product claims, an aging population, and rising interest in attaining wellness through diet are among the factors fuelling interest in functional foods. Credible scientific research indicates many potential health benefits from food

components. Milk nutrients are considered essential components of diets among all age groups. Likewise, many agricultural commodities including cereals, millets, and legumes are important constituents of the diets of poor across the globe. Although certain milk-cereal based products are already in the marketplace but they are not affordable to all segment of society. Lack in availability of such products and consumer awareness towards health beneficial aspect of milk-cereal based product also limits their consumption. Hence, still there is requirement of milk-cereal based composite and fermented foods that can assist us in alleviating the problems of malnutrition and hunger. The capacity of India to penetrate world markets depends on its ability to meet various emerging challenges both at production and processing levels. It could be possible through research initiatives for storage and processing of raw materials, novel food product development, development of indigenous processing equipments, appropriate packaging materials and techniques and rapid and reliable quality control methods.

2.11 Effective Packaging

Wider range of packaging materials namely glass bottles, earthenware vessels, metal cans, laminated pouches, flexible plastic containers, laminated paperboard cartons, polyethylene pouches are available for packaging of cultured milk products (Tamime and Robinson, 1999). *Lassi* (stirred yoghurt) is generally available in tetra packs as well as in polyethlene pouches whereas *dahi* is available earthen pots, polyethylene pouches and high impact polystyrene cups. According to an study by Patidar and Prajapati (1998), *lassi* packed in bottles can be better preserved than pouches and found acceptable up to 21 days. Shukla (1988) studied the effect of packaging material on the quality of fruit yoghurt and found that type of packaging material did not show a marked effect on the quality of the fruit yoghurt. However, the consumer acceptance was found to be more in favour of polystyrene cups rather than wax coated paper cups. Kefir filled in aluminium-lined cartons had shelf-life of more than 13 days (Castberg *et al.,* 1986). Modha (2006) found that the shelf life of pearl millet-*rabadi* packed in glass bottles is 7 days under refrigeration (5-7°C) conditions; there after the product became unacceptable because of increased acidity and wheying-off. Kakde (2010) packed pearl millet based lassi in LDPE pouches and extended shelf life of the product up to 20 days with the use of certain preservatives,

whereas the control samples without any treatment remained acceptable up to 13 days in LDPE pouches.

2.12 Sensory evaluation

Sensory evaluation is defined as the science of judging and evaluating the quality of a food by the use of the senses, i.e. taste, smell, sight, touch and hearing. Sensory testing has been developed into a precise, formal, structured methodology that is continually being updated to refine existing techniques. The developed methods serve economic interests and can establish the worth or acceptance of a commodity. Before a product reaches the market, it has gone through many tests to accurately judge how well the public will accept it. This is especially true in the food industry (Meilgaard *et al*., 1999). Sensory evaluation is used as a practical application in product development by aiding in product matching, improvements, and grading (Weinreich, 1999). Research is another area where sensory evaluation is frequently used. Evaluation of a product may be needed to determine the effects of an experiment. Quality control and marketing is another application of sensory testing (Stone *et al*., 1974; Stone and Sidel, 1993; Meilgaard *et al*, 1999). Sensory evaluation is divided into two methods, subjective and objective testing. Subjective tests involve panelists, while objective testing employs the use of laboratory instruments with no involvement of the senses (Meilgaard *et al*., 1991).

2.13 Hedonic scale

Hedonic scale method is a type of subjective test in sensory evaluation. This rating scale method measures the level of the liking of foods, or any other product. This test relies on people's ability to communicate their feelings of like or dislike. Hedonic testing is popular because it may be used with untrained people as well as with experienced panel members. A minimum amount of verbal ability is necessary for reliable results (Poste *et al*., 1991). In hedonic testing, samples are presented in succession and the subject is told to decide how much he likes or dislikes the product and to mark the scales accordingly. The nature of this test is its relative simplicity (O'Mahony, 1986). The hedonic scale is anchored verbally with nine different categories ranging from like extremely to dislike extremely. These phrases may be placed on a linegraphic scale either

horizontally or vertically. Hedonic ratings are converted to scores and treated by rank analysis or analysis of variance (ASTM, 1968).

2.14 Central composite rotary experiment

A central composite design (CCD) is an experimental design, useful in response surface methodology (Myers, 1971), for building a quadratic model for the response variable without needing to use a complete three-level factorial experiment. CCD contains factorial or fractional factorial design with center points that is augmented with a group of star points that allow estimation of curvature. A CCD always contains twice as many star points as there are factors in the design. The star points represent new extreme values for each factor in the design. If the distance from the center of the design space to a factorial point is ±1 unit for each factor, the distance from the center of the design space to a star point is ± α with | α | > 1. The precise value of α depends on certain properties desired for the design and on the number of factors involved. A design is rotatable if the variance of the predicted response at any point x depends only on the distance of x from the design center point. To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the central composite design. If the factorial is a full factorial, then $\alpha = [2^k]^{1/4}$ (Montgomery, 1997). In rotatable and near-rotatable CCDs, each experiment variable is represented at five levels, whereas, face-centered CCD requires only three levels of each experiment variable. However, compared to face-centered CCDs, rotatable or near-rotatable CCDs offer reduced prediction error for, and improved estimation of quadratic effects (Verseput, 2011). Experimental design and application of response surface methodology for process modeling and optimization has been extensively reviewed by Myers *et al*., (2009). Singh *et al*., (2008) optimized levels of ingredients for the manufacture of malted milk beverage using rotatable CCD response surface methodology. Cultivation conditions and medium components for the production of folate by *S. thermophilus* using rotatable CCD of response surface methodology were optimized by Iyer *et al*., (2010). Central composite rotatable design of response surface methodology has been successfully applied in the optimization of ingredients in fermented milks (Kumar and Mishra, 2003; Modha and Pal, 2011; Shilpi and Kumar, 2013).

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The materials used and the experimental procedures adopted to study various technological, physico-chemical, sensory, textural, microbiological and statistical aspects are described in this chapter. According to the objectives, the study has been divided into different phases. In phase-I, suitable probiotic lactic acid bacteria was selected for the manufacture of barley-milk based fermented probiotic drink (BMFPD) following a series of experiments. Phase-II, dealt with the optimization of different processing parameters for production of BMFPD. In phase-III, developed probiotic drink was evaluated for physico-chemical, nutritional, sensory, therapeutic properties and shelf life of developed probiotic drink. Techno-economic feasibility and consumers responses were analysed in phase-IV.

3.1 MATERIALS / INGREDIENTS

3.1.1 Milk

Fresh cow milk was collected from the Experimental Dairy of National Dairy Research Institute, Karnal.

3.1.2 Skim milk powder

The skim milk powder (SMP) was procured from Modern Dairies Limited, Karnal.

3.1.3 Barley

Wholesome, sound, healthy and insect free grains of barley (var. DWR-28) were procured from the Farm section of National Dairy Research Institute, Karnal.

3.1.4 Salt and spices

Commercial grade iodized salt (Tata Chemicals Ltd., Mumbai), cumin seeds (Catch, Dharampal Satyapal Ltd, Noida, Uttar Pradesh) and black pepper powder (Mahashian Di Hatti Ltd., Delhi) were procured from the local market of Karnal. The cumin seeds were roasted and ground in to fine powder and sieved through 52 mesh sieve before use.

3.1.5 Chemicals

All the chemicals and microbiological media used in the study were procured from standard laboratory chemical suppliers and have been detailed in **Appendix-I**.

3.1.6 Bacterial cultures

Bacterial cultures used in the study are listed in **Table 3.1** and were obtained from National Collection of Dairy Cultures (NCDC) and Ultrastructure Laboratory (UL) of Dairy Microbiology Division of National Dairy Research Institute, Karnal. Functional attributes of probiotic lactic acid bacteria used in the study have been summarized in **Table 3.2.**

Table 3.1 Standard cultures used in the study

Bacterial species	Origin /Source	Type
Lactobacillus acidophilus NCDC13	Indigenous (Milk), NCDC	Probiotic
Lactobacillus casei NCDC297	Indigenous (Cheese), NCDC	Probiotic
Lactobacillus casei NCDC299	European (Cheese), NCDC	Probiotic
Lactobacillus plantarum NCDC344	Indigenous (Milk), NCDC	Probiotic
Lactobacillus rhamnosus RSI3	Indigenous (*Rabari*), UL	Probiotic
Streptococcus thermophilus 20	Indigenous (*Dahi*), UL	Co-culture
Staphylococcus aureus NCDC109	NCDC	Pathogen
Shigella dysentriae NCDC107	NCDC	Pathogen
Escherichia coli NCDC135	NCDC	Pathogen
Salmonella typhii NCDC113	NCDC	Pathogen

3.1.7 Packaging material

LDPE film (co-extruded food grade milky white pigmented in two colour for lassi packaging; thickness: 60µm; width: 323-325 mm) manufactured by M/s Swastik Industries, Parwanoo, Himachal Pradesh, was used for the packaging of BMFPD using pouch filling machine (200ml; 50 pouhes/min) at the Experimental Dairy of the National Dairy Research Institute, Karnal. Polyethylene terephthalate (PET) bottles (colour: transparent; weight~16 g; diameter of bottle~52mm; length of bottle~124 mm; capacity~180 ml; opening mouth diameter~25mm; colour of cap: white; weight of cap~3.25 g) manufactured by Alpha packaging, Surat (Gujarat) were procured from local supplier in Karnal.

3.2 METHODS

3.2.1 Analysis of Milk

Acidity, fat content and solid not fat content of milk were estimated by following the procedure of Bureau of Indian Standards as described in IS:SP:18, part XI (1981).

Table 3.2 Functional attributes of probiotic lactic acid bacteria used in the study

Bacterial species	Previous studies
Lactobacillus acidophilus 13	Probiotic attributes (Kushal *et al.,* 2005; Kushal *et al.,* 2006a; Saran *et al*., 2012); enhanced immune response (Kushal *et al.,* 2006b); positive shift in gut microbiota (Arora *et al*., 2012)
Lactobacillus casei 297	Probiotic attributes (Mishra, 2001; Mishra and Prasad, 2005); β-glucosidase activity and isoflavone bioconversion (Hati *et al*., 2014); Antioxidant potential (Subrota *et al*., 2013)
Lactobacillus casei 299	Probiotic attributes (Mishra, 2001; Mishra and Prasad 2005), modulation of intestinal microbiota (Sidira *et al.,* 2010; Saxami *et al*., 2012)
Lactobacillus plantarum 344	Potential probiotic attributes (Kaushik *et al.,* 2009); expression of *atpD* gene under *in vitro* acidic (Duary *et al.*, 2010); its relative gene expression of bile salt hydrolase activity *in vitro* gut conditions (Duary *et al.*, 2012); adhesion to human colonic epithelial cells (Duary *et al.*, 2011); antioxidative potential (Achuthan *et al*., 2012)
Lactobacillus rhamnosus RSI3	Anti-fungal properties (Basu *et al*., 2011a); probiotic potential (Minj and Tomar, 2011; Minj and Tomar, 2012); increased level glutathione peroxidase and improvement in lipid profiles (Minj and Tomar, 2013)

3.2.2 Preparation of barley flour

The grains were cleaned to remove foreign materials and broken seeds. Three different types of barley flour were prepared from non-germinated, germinated and imbibed barley grains. Barley grains were processed in manner as detailed in **Figure 3.1**. Photographs of barley grains before and after processing are represented in **Photoplates I to III.**

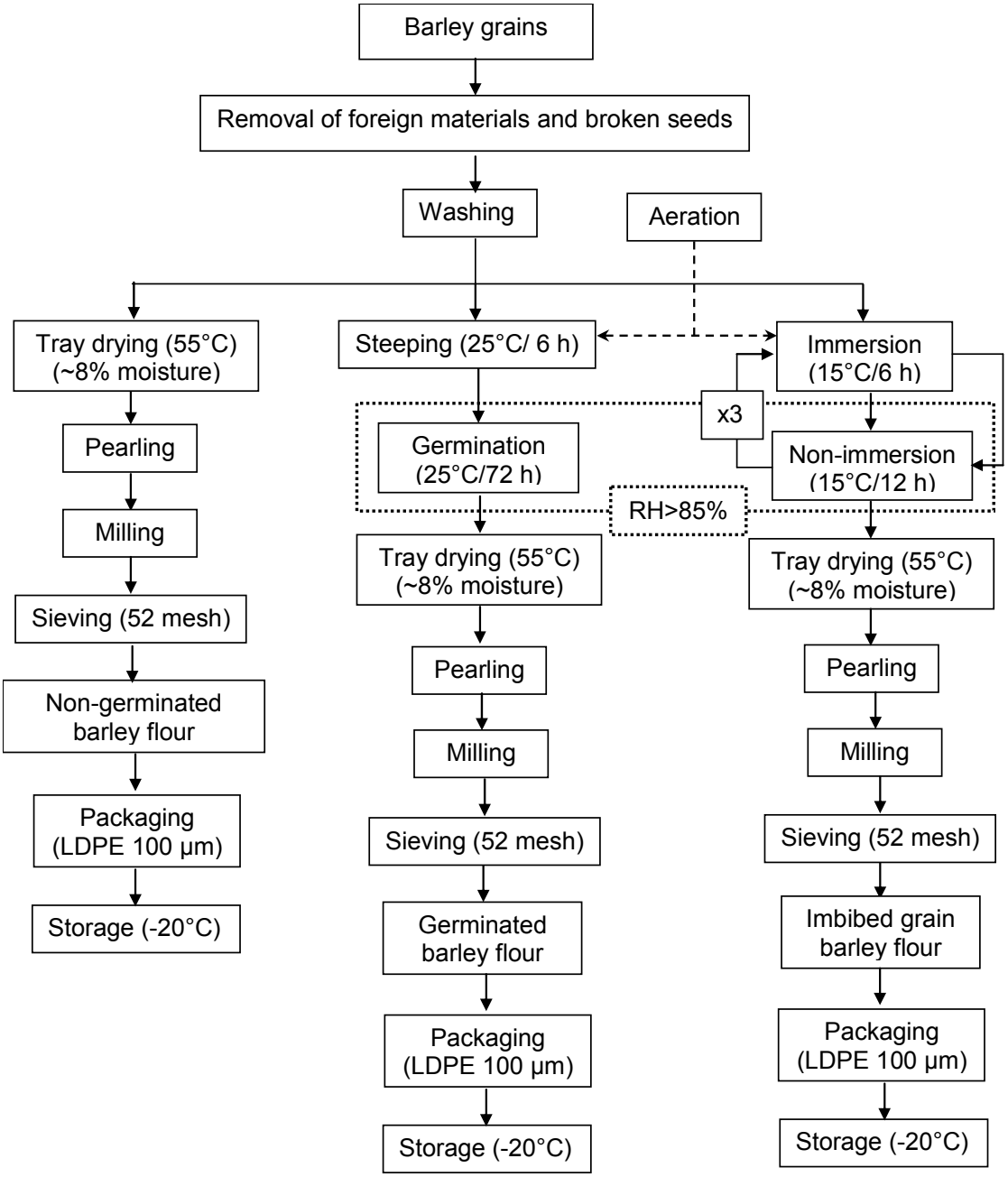


Figure 3.1 Preparation of flour from non-germinated, germinated and imbibed barley grains

Pearling of grains was done by using a prototype electro mechanical thresher (Vivek-thresher-cum-pearler, Punjab Agricultural Implement Pvt. Ltd., Saharanpur) and milled in a domestic flour mill (Natraj Aata Maker, Ahmedabad) and sieved through 52 mesh sieve (Precision Scientific Equipment Corp., New Delhi).

Plate I: (a) Non-germinated barley grains (b) Non-germinated barley grains after pearling

Plate II: (a) Imbibed barley grains (b) Imbibed barley grains after drying (c) Imbibed barley grains after pearling

Plate III: (a) Germinated barley grains (b) Germinated barley grains after drying (c) Germinated barley grains after pearling

3.2.3 Analysis of barley flour

Analyses of barley flour samples were performed in triplicate for moisture, ash, protein, crude fat, crude fibre, phytic acid, β-glucan, total dietary fibrer, reducing sugar, glucose and free amino nitrogen content. Crude fat and crude fibre content of barley flour were estimated using SOCS PLUS and FIBRA PLUS instruments, Pelican Equipments, Chennai, India.

3.2.3.1 Moisture content

The moisture content of barley flour was estimated by the method of Association of Official Analytical Chemists (AOAC, 1995). Empty dishes were dried in the oven at 130°C for 1 h and transferred to desiccator to cool. Two gram of well mixed sample was weighed in to the dish and uniformly distributed. Dish with sample were dried in the oven at 130°C for 1 h or to achieve constant weight. Dish was covered when still in oven and transferred in desiccator to cool and weighed.

$$\text{Moisture \%} = \frac{\text{Initial weight of sample} - \text{Weight of sample after drying}}{\text{Initial weight of sample}} \times 100$$

3.2.3.2 Ash content

Ash content of barley flour samples was estimated by the method of AOAC (1995). Three gram of flour sample was weighed and transferred into a pre-weighed porcelain crucible, dried at 100°C for 10 h or overnight and weighed. The weighed samples were ignited till smoke ceased. The crucibles were then transferred to muffle furnace maintained at 550±5°C and incinerated until light grey ash was obtained. The crucible was then cooled in desiccator and weighed.

$$\text{Ash \%} = \frac{\text{Weight of crucible after ashing} - \text{Weight of empty crucible}}{\text{Weight of sample}} \times 100$$

3.2.3.3 Protein content

The protein content of barley flour was determined by micro Kjeldahl method (AOAC, 1984). Accurately weighed 0.5 g sample was transferred to the Kjeldahl digestion tube followed by addition of 2 g of digestion mixture (K_2SO_4: $CuSO_4$:SeO_2~100:10:1) and 20 ml concentrated H_2SO_4. The contents were then digested to a blue greenish transparent fluid. The digested material was transferred to a 100 ml volumetric flask and distilled water was added to make the volume up to the mark. An aliquot (10 ml) of digest was distilled with 50% sodium hydroxide and the liberated ammonia was collected in 10 ml saturated boric acid containing 2-3 drops of mixed indicator (prepared by dissolving 100 mg

methyl red and 30 mg methylene blue in 60 ml of 95% ethyl alcohol and then making up the volume to 100 ml with distilled water).

Approximately 75 ml of distillate was collected in a 100 ml conical flask. The contents of the flask were titrated against 0.02N HCl. A blank determination using distilled water in place of sample was also carried out. The total nitrogen and percent protein were calculated as follows:

$$\text{Per cent Nitrogen} = \frac{14.007 \times (V\text{-}B) \times N \times \text{dilution factor} \times 100}{1000 \times W}$$

where,

V = ml of HCl required for sample

B= ml of HCl required for blank

N= Normality of HCl used, and

W= Weight of the sample in mg.

Percent total protein = Percent total nitrogen x 5.83 (Merrill and Watt, 1973)

3.2.3.4 Crude fat

Crude fat content of barley flour samples was estimated using method of AOAC (2000) using SOCS PLUS (Pelican Equipments, India). Fat extraction beakers were rinsed and placed in oven at 102°C for 1 h and cooled in desiccator for about 10 min. Weight of empty beaker was taken. Thimbles were inserted in thimble holder and placed in beaker. Five g of each sample was weighed accurately in thimbles. Approximately 100 ml solvent (petroleum ether, boiling point 40-60°C) was poured in beaker. Beakers were loaded to SOCS PLUS and boiling point was adjusted to 20°C higher than the boiling point of solvent (80°C). The crude fat was extracted for about 60 minutes. After the process time recovery temperature was increased to 120°C (2 times of boiling point of solvent). Thimbles were rinsed for about 2-3 times to collect the remaining fat present in the sample. The beakers with extracted fat were taken out from SOCS PLUS and thimbles were removed. The beakers containing extracted fat were placed in an oven at 102°C and the contents were dried until a constant weight achieved (1-2 h). The beaker was cooled in a desiccator and weighed. The content left in the beaker after the extraction was estimated as crude fat in the sample and expressed as per cent crude fat in the sample.

$$\text{Crude Fat (\%)} = \frac{\text{Weight of beaker with fat} - \text{Weight of empty beaker}}{\text{Weight of sample}} \times 100$$

3.2.3.5 Crude fibre

The crude fibre content of barley flour was estimated using Fibra Plus (Pelican Equipments, India) following the method of AOAC (2000). One gram of defatted flour sample was accurately weighed in the dried extraction crucibles. Weight of empty crucible and crucible with sample was recorded nearest to 0.1 mg. Crucibles were placed into the metal adapters of Fibra Plus hot extraction unit and proper sealing of crucible against adapter rubber was ensured. Approximately 150 ml of 1.25% sulfuric acid was poured into the extraction tubes from the top. Instrument was switched on and initial temperature was adjusted to 500°C. After boiling started, temperature was reduced to at 400°C. Samples were digested for about 45 minutes in 1.25% sulfuric acid. Acid was drained and samples were washed thrice with the help of vacuum pump keeping the knob in vacuum mode. If draining was not effective due to clogging of samples then knob was changed to pressure mode, and pressure button was pressed twice or thrice and immediately turned to vacuum mode. Subsequently, 150 ml sodium hydroxide (1.25%) solution was poured into the extractors from the top. Samples were boiled with alakali solution, drained and washed in manner similar as in case of acid. After washing crucibles were taken out of Fibra Plus and kept in an oven at 102°C for 2-3 h to remove moisture. Crucibles were cooled down in desiccators for 10 minutes and weight was recorded. All crucibles were then transferred to the muffle furnance maintained at 400°C for 3-4 h. Weight of crucibles after ashing was recorded.

$$\text{Crude fibre\%} = \frac{\text{Weight of crucible with fibre – Weight of crucible after ashing}}{\text{Weight of sample}} \times 100$$

3.2.3.6 Total starch content

Total starch content in barley flour samples were estimated by using Megazyme kit assay (2011a). The process of estimation employs treatment of samples with thermostable α-amylase during following the starch gelatinisation step (Batey, 1982). Samples were pre-treated with dimethyl sulphoxide to aid the gelatinization of resistant starch, if present (Englyst and cummings, 1988 and McCleary *et al.*, 1994). Thermostable α-amylase hydrolyses starch into soluble branched and unbranched maltodextrins (Anon, 1987). Samples were then treated with amyloglucosidase at pH 5.0 at 50°C, which hydrolyses maltodextrins into D-glucose units. D-Glucose is oxidised to D-gluconate with the release of one mole of hydrogen peroxide (H_2O_2) which is quantitatively measured in a

colourimetric reaction employing peroxidase and the production of a quinoneimine dye. Flour samples obtained from germinated and imbibed grains containing high levels of D-glucose and maltodextrins were washed with aqueous ethanol (80 % v/v) before analysis.

$$\text{Starch} + H_2O \xrightarrow{\text{thermostable } \alpha\text{–amylase, pH 5.0, 100°C}} \text{Maltodextrins} \quad \text{---(i)}$$

$$\text{Maltodextrins} \xrightarrow{\text{amyloglucosidase, pH 5.0, 50°C}} \text{D-glucose} \quad \text{---(ii)}$$

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconate} + H_2O_2 \quad \text{---(iii)}$$

$$2H_2O_2 + \textit{p}\text{-hydroxybenzoic acid} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + 4\ H_2O \quad \text{---(iv)}$$

Reagents provided in kit were thermostable α-amylase (3,000 U/ml on Ceralpha at pH 6.5 and 40°C), amyloglucosidase (3300 U/ml on soluble starch at pH 4.5 and 40°C), GOPOD reagent buffer, GOPOD reagent enzymes (glucose oxidase plus peroxidase and 4-aminoantipyrine), D-Glucose standard solution (1.0 mg/ml in 0.2 % benzoic acid) and maize starch control.

3.2.3.6.1 Preparation of reagents for estimation of total starch content

GOPOD reagent buffer concentrate was diluted to 1 litre with distilled water. GOPOD reagent enzymes were mixed with 20 ml of GOPOD reagent buffer and mixed with remainder of the solution to make 1 l. Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM) was prepared by mixing 5.8 ml of glacial acetic acid (1.05 g/ml) to 900 ml of distilled water and pH was adjusted to 5.0 by the addition of 1 M (4 g/100 ml) sodium hydroxide solution followed by addition of 0.74 g of calcium chloride. One ml of thermostable α-amylase was diluted to 30 ml using sodium acetate buffer (100 mM, pH 5.0, 5mM calcium chloride).

3.2.3.6.2 Estimation of total starch

Accurately, 100 mg of flour sample was weighed in polypropylene centrifuge tube. Samples were added with 5.0 ml of aqueous ethanol (80 % v/v), mixed by votexing and incubated at 80-85°C for 5 min. Contents were again mixed by vortexing and added with another 5 ml of 80% v/v aqueous ethanol. Tubes were then centrifuged for 10 min at 3,000 rpm and supernatant was discarded carefully. Pellet was resuspended 10 ml of 80 % v/v aqueous ethanol and stirred on a vortex mixer. Tubes were again centrifuged as above and supernatant was carefully poured off. Samples were added with 0.2 ml of

aqueous ethanol (80 % v/v) to aid dispersion, and tubes were stirred using a vortex mixer. Immediately 1 ml of dimethyl sulphoxide was and contents were mixed on vortex mixer. Tubes were placed in a vigorously boiling water bath and removed after 5 min. Immediately, 3 ml of thermostable α-amylase (diluted 1:30 in sodium acetate buffer, pH 5.0 with 5mM calcium chloride) was added into the tubes and incubated in a boiling water bath for 12 min, with intermittent stirring at 4, 8 and 12 min. Tubes were then transferred to a water bath at 50°C, allowed for 5 minutes to cool and added with 0.1 ml of the contents of amyloglucosidase. Followed by stirring on a vortex mixer, tubes were incubated at 50°C for 30 min. Entire contents of the tubes were transferred to a 100 ml volumetric flask and volume was adjusted deionized with distilled water. Contents were mixed thoroughly, and an aliquot of the solution was centrifuged at 1,500 rpm for 10 min. Clear supernatant was used for the assay. In duplicate, 0.1 ml of clear supernatant was transferred to bottom of glass test tubes and added with 3.0 ml of GOPOD reagent to each tube. D-glucose standards, controls and reagent blanks were also included in the assay. D-Glucose controls consisted of 0.1 ml of D-glucose standard solution (1 mg/ml) and 3.0 ml of GOPOD Reagent. Reagent blank solutions consisted of 0.1 ml of water and 3.0 ml of GOPOD Reagent. Tubes were incubated at 50°C for 20 min and absorbance was read at 510 nm against blank.

Calculations

$$\text{Starch (\%)} = \frac{\Delta A_{\text{sample}} \times F \times FV \times 1 \times 100 \times 162}{0.1 \times 1000 \times W \times 180}$$

where,

ΔA = Absorbance (reaction) read against the reagent blank.

$F = \frac{100\ (\mu g \text{ of D-glucose})}{\Delta A_{\text{Standard}}}$ (Conversion from absorbance to µg)

FV = final volume (ml)

0.1 = volume of sample analysed

$\frac{1}{1000}$ = conversion from µg to mg

$\frac{100}{W}$ = Factor to express 'starch' as percentage of flour weight

W = weight of sample (mg)

$\frac{162}{180}$ = A factor to convert from free D-glucose, as determined, to anhydro-D-glucose, as occurs in starch

3.2.3.7 Total Carbohydrate

Total Carbohydrate content in barley flour samples were estimated by the method of Hedge and Hofreiter (1962). The principle of analysis involved hydrolysis of complex carbohydrates into simple sugars by boiling in diluted hydrochloric acid. Simplest sugar glucose in hot acidic medium is dehydrated to hydroxymethyl furfural, which forms a green coloured product with anthrone reagent.

3.2.3.7.1 Preparation of reagents for total carbohydrate estimation

Anthrone reagent was prepared by dissolving anthrone in 100 ml of ice-cold 95% H_2SO_4. The reagent was prepared fresh before use. Standard glucose solution was prepared by dissolving 100 mg of glucose in 100 ml water.

3.2.3.7.2 Estimation of total carbohydrates

Accurately, 400 mg of sample was weighed in a boiling test tube. Samples were hydrolysed for 3 hours by boiling in 20 ml of 2.5 N HCl. Tubes were cooled down to room temperature and neutralized with sodium carbonate till effervescence ceased. Contents of tubes were transferred to measuring flask and final volume was made up to 100 ml. An aliquot of sample was centrifuged at 3000 rpm for 10 min. Working solution of sample was prepared by diluting 100 µl of supernatant to 2000 µl with distilled water. One ml of ice cold working sample solution was mixed with 4 ml of anthrone reagent and heated for eight minutes in a boiling water bath. Samples were cooled rapidly to develop green to dark green colour and read at 630 nm. Calibration curve was plotted using glucose as standard (10-120 µg/ml) on the X-axis *vs.* absorbance on the Y-axis (**Appendix-II**). The equation for the glucose calibration curve was $\mathrm{Y} = 0.008\mathrm{X} + 0.0507$ (where, $\mathrm{X} =$ concentration of glucose equivalent expressed as µg/ml; Y = absorbance), and correlation coefficient was R^2= 0.99). Results were expressed as % total carbohydrates on dry basis.

3.2.3.8 Reducing sugar

Extent of starch hydrolysis during imbibition and germination process was assessed by estimating reducing sugar in flour samples following the method of Miller (1972). Dinitrosalicylic acid (DNSA) reagent was prepared by dissolving 1 g of 3, 5-dinitrosalicylic acid in 50 ml of water, followed by slow addition and mixing of 30 g sodium potassium tartrate tetrahydrate. Twenty ml of 2 N NaOH was added and final volume was made up to 100 ml with distilled water.

For extraction of sugars, 100 mg of sample was accurately weighed in polypropylene tubes and mixed with 10 ml 80% ethanol by vortexing (Serna-Saldivar, 2012). The contents were kept in a water bath maintained at 80°C for 5 min followed by centrifugation at 2500 × g for 10 min. Supernatant was decanted carefully to glass test tubes and evaporated to dryness in a water bath maintained at 80^{o}C. Contents in the tubes were re-suspended in 10 ml distilled water. In a test tube, 3 ml sample extract was mixed with 3 ml DNSA reagent, and kept in a boiling water bath for 5 min. Tubes were then cooled to room temperature under tap water. Absorbance was read at 525 nm. Calibration curve was prepared using maltose as standard within range of 30 to 300 µg/ml and results were expressed in mg maltose per g sample. Equation for the maltose calibration curve (**Appendix-III**) was $Y = 0.0007X + 0.4061$ (where, $X =$ concentration of maltose expressed as µg/3ml; Y = absorbance at 525 nm), and correlation coefficient was R^2 = 0.99). Results were reported in terms of mg maltose/g flour.

3.2.3.9 Glucose content

Glucose content in flour samples were estimated by employing high purity glucose oxidase and peroxidase (GOPOD) reagent using Megazyme kit assay (Megazyme International Ireland Ltd. 2011b) which is specific for the measurement of D-glucose in extracts of plant materials or foods. Test principle for the measurement of glucose employs a modification of the glucose oxidase / peroxidase method described by Trinder (1969).

Reagents provided in kit were, GOPOD reagent buffer (pH 7.4, with *p*-hydroxybenzoic acid and sodium azide), GOPOD reagent enzymes (Glucose oxidase >12,000 U/l plus peroxidase > 650 U/l and 4-aminoantipyrine 0.4mM) and D-glucose standard (1 mg/ml in 0.2% benzoic acid).

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconate} + H_2O_2 \qquad \text{---(i)}$$

$$2\ H_2O_2 + p\text{-hydroxybenzoic acid} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine dye} + 4\ H_2O \qquad \text{---(ii)}$$

For estimation of glucose content, 100 mg of flour samples was weighed nearest to 0.1 mg in polypropylene tubes. Two hundred microlitre of 80 % ethanol was added to aid the dispersion of flour and final volume was made up to 10 ml by adding 0.8 ml of deionized distilled water. Samples were mixed thoroughly by

vortex mixing, followed by incubating in a water bath for 5 min at 80°C. Tubes were then centrifuged at 1000× g for 10 min. Accurately, 100 µl of supernatant was transferred in to the bottom of test tubes and added with 3 ml of GOPOD reagent. Contents were mixed by vortexing and incubated in a water bath at 50°C for at least 20 min, and absorbance taken against blank at 510 nm. Blank was prepared by mixing 100 µl of distilled water with 3 ml of GOPOD reagent. D-glucose standard in quadripulate was prepared by mixing 100 µl of glucose standard (1 µg/µl) with 3 ml of GOPOD reagent.

$$\text{D-glucose (mg/g)} = \frac{\Delta A_{\text{sample}} \times F \times FV}{0.1 \times 1000 \times W}$$

where,

ΔA = Absorbance (reaction) read against the reagent blank.

$F = \frac{100\ (\mu g \text{ of D-glucose})}{\Delta A_{\text{Standard}}}$ (Conversion from absorbance to µg)

FV = final volume (ml)

0.1 = volume of sample analysed

$\frac{1}{1000}$ = conversion from µg to mg

W = weight of sample (g)

3.2.3.10 β-glucan

The β-glucan content in barley flour samples were estimated by following the method **3.11.1** of European Brewery Convention (EBC) using the Megazyme mixed-linkage-β-glucan kit assay (Megazyme International Ireland Ltd., 2011e). The principle of estimation was based on suspending and hydrating the samples in a buffer solution of pH 6.5 followed by incubation with purified lichenase enzyme and filtration. An aliquot of the filtrate is hydrolysed with purified β-glucosidase, and the D-glucose liberated was measured by using glucose oxidase / peroxidase (GOPOD) reagent (McCleary and Glennie-Holmes, 1985; McCleary and Codd, 1991).

The reagents provided with kit were lichenase [specific, *endo*-(1-3)(1-4)-β-D-glucan 4-glucanohydrolase] suspension (1 ml, 1,000 U/ml); β-glucosidase (1 ml, 40 U/ml) suspension; GOPOD reagent buffer [Potassium phosphate buffer (1 M, pH 7.4), *p*-hydroxybenzoic acid (0.22 M) and sodium azide (0.4 % w/w)]; GOPOD reagent enzymes [Glucose oxidase (>12,000 U) plus peroxidase (> 650

U) and 4-aminoantipyrine (80 mg)]; D-Glucose standard solution [(1.0 mg/ml) in 0.2 % (w/v) benzoic acid] and standardized barley flour control.

3.2.3.10.1 Preparation of reagents and working solution

Lichenase (1 ml) suspension was diluted to 20.0 ml with sodium phosphate buffer [20 mM, pH 6.5; 3.12 g of sodium dihydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_2O$) dissolved in 900 ml of distilled water and pH was adjusted with 100 mM sodium hydroxide; final volume was made up to 1 l with distilled water]. β-glucosidase (1 ml) suspension was diluted to 20.0 ml with sodium acetate buffer [50 mM, pH 4.0; 2.9 ml of glacial acetic acid added to 900 ml of water and pH 4.0 was adjusted with1 M sodium hydroxide solution; final volume was made up to 1 l with distilled water]. GOPOD reagent buffer supplied with the kit was diluted to1 l with distilled water before use. GOPOD reagent enzymes was dissolved in 20 ml of diluted GOPOD reagent buffer and quantitatively transferred to remainder 880 ml. The bottle was covered with aluminum foil to protect the GOPOD reagent from light. When the GOPOD reagent is freshly prepared it may have light yellow or light pink in colour. The absorbance of this solution should be less than 0.05 when read against distilled water. All the reagents prepared were divided into appropriate sized aliquots and stored at -20°C. Sodium acetate buffer (200 mM, pH 4.0) was prepared by adding 11.6 ml of glacial acetic acid to 900 ml of distilled water and adjusting the pH 4.0 by 1 M sodium hydroxide solution. Final volume was adjusted to 1 litre.

3.2.3.10.2 Standard assay procedure for β-glucan

Barley flour samples of known moisture content along with one standard barley flour sample (of known β-glucan content) were accurately weighed (0.5 g) into polypropylene tubes. An aliquot (1.0 ml) of aqueous ethanol (50 % v/v) was added in each tube to aid in the subsequent dispersion of samples followed by addition of 5.0 ml sodium phosphate buffer (20 mM, pH 6.5). The contents in the tubes were mixed thoroughly using vortex mixer and incubated in boiling water bath for 2 min. After that, tubes were removed and again vigorously stirred on a vortex mixer to prevent the formation of gel material. The tubes were further heated for 3 min in the boiling water bath and cooled to 40°C. Each tube was added with 0.2 ml of lichenase (10 U) enzyme followed by capping, stirring and incubation at 40°C for 1 h. After completion of incubation period the volume in each tube was made up to 30.0 ml with distilled water. The contents of the tubes

were mixed thoroughly, centrifuged (1,000 × g; 10 min) and filtered through Whatman No. 42 filter circle. Aliquot (0.1 ml) from each filtrate was carefully and accurately transferred to the bottom of three test tubes. One of the tubes (the reaction blank) was added with an aliquot (0.1 ml) of sodium acetate buffer (50 mM, pH 4.0), while the other two (the reaction) tubes were added with 0.1 ml of β-glucosidase (0.2 U) and incubate the tubes at 40°C for 15 min. In each tube 3.0 ml of GOPOD reagent was added and incubated at 40°C for 20 min. The absorbance was measured at 510 nm.

With each set of determination reagent blanks and one D-glucose standard (100 µg / 0.1 ml) were also included in duplicate. The reagent blank comprised of 0.1 ml distilled water, 0.1 ml sodium acetate buffer and 3.0 ml of GOPOD reagent. Whereas, the glucose standards were comprised 0.1 ml sodium acetate buffer, 0.1 ml D-glucose standard (100 µg/0.1 ml) and 3.0 ml GOPOD reagent.

The β-glucan content of flour samples were calculated by using the following equation:

$$\beta\text{-glucan} = \frac{\Delta A \times F \times 300 \times 1 \times 100 \times 162}{1000 \times W \times 180} = \frac{\Delta A \times F \times 27}{W}$$

where,

ΔA = Absorbance after β-glucosidase treatment (reaction) minus reaction blank absorbance.

F = A factor for the conversion of absorbance values to µg of glucose.
= 100 (µg of D-glucose) / absorbance of 100 µg of D-glucose.

300 = Volume correction (i.e. 0.1 ml taken from 30.0 ml).

$\frac{1}{1000}$ = Conversion from µg to mg

$\frac{162}{180}$ = A factor to convert from free D-glucose, as determined, to anhydro-D-glucose, as occurs in β-glucan

W = Calculated dry weight of the sample analysed, in mg

3.2.3.11 Total dietary fiber

The total dietary fiber was estimated by following AOAC (1995) method (992.16) using total dietary fibre kit assay (Megazyme International Ireland Ltd., 2011c). This assay determines the total dietary fiber content of flour using a combination of enzymatic and gravimetric methods, in which the fat free samples are gelatinized with heat stable α-amylase and then enzymatically digested with

protease and amyloglucosidase to remove the protein and starch present in the sample. Ethanol is added to precipitate the soluble dietary fiber and which is filtered. Residue obtained is washed with ethanol and acetone, followed by drying. Two replicates of a sample need to be run simultaneously, out of which one is analyzed for protein content and the other for ash. Total dietary fiber represents the weight of the residue before ashing minus the weight of protein and ash.

Reagents provided in kit were heat stable α-amylase (3000 U/ml); protease (50 mg/ml, 350 tyrosine U/ml) and amyloglucosidase (3300 U/ml on soluble starch). Acid washed celite™ (C8656) was procured from Sigma (St. Louis, Missouri).

3.2.3.11.1 Standard assay procedure for total dietary fibre

One gram of each flour samples (in triplicate) was accurately weighed in tall form beakers and weights were recorded nearest to 0.1 mg. Blank samples (in duplicate) were also ran simultaneously throughout the procedure to measure any contribution from reagent to residue. Fifty ml of phosphate buffer [0.08 M, pH 6.0; 1.4 g of Na_2HPO_4 and 8.4 g of NaH_2PO_4 mixed in 900 ml of water and pH was adjusted with either NaOH or H_3PO_4; final volume was made up to 1 l with distilled water] was added to each beaker. Each beaker were then added with 50 µl of α-Amylase and mixed well. The beakers were covered with aluminium foil and placed in a boiling water bath. The beaker were agitated gently in every 5 min intervals and incubated for 15 min after internal temperature of contents achieved 95°C. All the solutions were cooled to room temperature and pH of solutions was adjusted to 7.5±0.1 by the adding 10 ml of 0.275 N NaOH. Contents of each beaker was checked for the pH, and if necessary, adjusted with either NaOH or HCl solution. Subsequently, 100 µl (5 mg protease) of protease solution was added in to each beaker. Covered beakers were then incubated in 60°C water bath with continuous agitation, for 30 minutes after the internal temperature of beakers reached 60°C. Contents of beakers were cooled to room temperature and pH of the solutions was adjusted to in between 4.0 and 4.6 by adding 10 ml of 0.325 M HCl. If necessary, pH of the solutions was adjusted with NaOH or HCl. To each beaker, 200 µl of amyloglucosidase was added, covered with aluminium foil and with continuous agitation kept in a water bath at 60°C. After, internal temperature of contents in beaker reached 60°C, were further

incubated for 30 minutes. Four volumes of 95% ethanol were added to each beaker. Beakers were covered with Parafilm® and allowed to complete precipitation for overnight at room temperature.

Fritted crucible (coarse 40-60 microns) were thoroughly washed and kept in muffle furnace at 550°C for 1 h. Cooled crucibles were soaked and rinsed using distilled water and dried in air. In each crucible 0.5 g of acid washed Celite was added and dried in air oven set at 130 °C to constant weight. Crucibles were cooled in desiccator and weighed nearest to 0.1 mg (W_1). Using 78% ethanol Celite in each crucible was made wet and the bed of Celite was redistributed on frit to an even mat. Precipitate and suspension from each beaker was quantitatively transferred to crucible and gentle suction was maintained to assist filtration process. Residue was washed with three 20 ml portions of 78% ethanol, two 10 ml portions of 95% ethanol, and two 10 ml portions of acetone. Surface film if formed due to gum formation was broken with a spatula. The crucibles containing residues were dried overnight at 105 °C in air oven and cooled in desiccator. The weight (W_2) of each crucible was recorded.

One out of the two residues was analysed for protein content by micro-Kjeldahl nitrogen analysis as specified in the previous sub-section (**3.2.3.3**) using 6.25 as conversion factor. Second residue sample was incinerated in muffle furnace at 525°C for 5 h and cooled in desiccator. Weight (W_3) of crucible containing Celite and ash was recorded nearest to 0.1 mg. Total dietary fibre (TDF) was calculated by using the following equations.

Residue weight = W_2-W_1

Ash weight = W_3-W_1

B = R_{BLANK} - P_{BLANK} - A_{BLANK}

$$\%TDF = \frac{R_{SAMPLE} - P_{SAMPLE} - A_{SAMPLE} - B}{\text{Average Sample weight (mg)}} \times 100$$

where,

R = Average residue weight (mg)

P = Average protein weight (mg)

A = Average ash weight (mg)

3.2.3.12 Phytic acid

Different barley flour samples were analysed for phytic acid content using the phytic acid kit assay (Megazyme International Ireland Limited, 2011d). The principle involved acid extraction of inositol phosphates followed by treatment

with a phytase which is specific for phytic acid (IP_6) and the lower *myo*-inositol phosphate forms (i.e. IP_2, IP_3, IP_4, IP_5). Subsequent treatment with alkaline phosphatase ensures the release of the final phosphate from *myo*-inositol phosphate (IP_1) which is relatively resistant to the action of phytase. The total phosphate released is measured by using a modified colorimetric method and expressed as g of phosphorus per 100 g of sample (Fiske and Subbarow, 1925; Lowry and Lopez, 1946).

Reagents provided with kit were sodium acetate buffer (200 mM, pH 5.5; 0.02% sodium azide), phytase suspension (12,000 U/ml), glycine buffer (400 mM, pH 10.4; $MgCl_2$ 4 mM; $ZnSO_4$ 0.4 mM; sodium azide 0.02 % w/v), alkaline phosphatase suspension (80 U/ml) and phosphorus standard solution (50 µg/ml; sodium azide 0.02 % w/v). Colour reagent was freshly prepared by mixing 1 part of solution I (ammonium molybdate 5%) with 5 parts of solution II (ascorbic acid 10%, sulfuric acid 1M).

3.2.3.12.1 Standard assay procedure for phytate

One gram of flour sample was accurately weighed in 75 ml glass beaker. Twenty ml of hydrochloric acid (0.66 M) was added and the beaker was covered with Parafilm®, stirred for overnight using magnetic stirrer at room temperature. One ml of extract was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 10,500 × g for 10 min. Resulting extract supernatant (0.5 ml) was immediately transferred to a fresh 1.5 ml microfuge tube and neutralized by addition of 0.5 ml of sodium hydroxide solution (0.75 M). This neutralized sample extract was used in the enzymatic dephosphorylation reaction procedure.

Enzymatic dephosphorylation reaction for total phosphorus was initiated by mixing of 0.60 ml distilled water, 0.20 ml sodium acetate buffer, 0.05 ml sample extract and 0.02 ml phytase in 2 ml microcentrifuge tube and mixing the contents by vortexing followed by incubation at 40°C for 10 min. The next reaction was initiated by adding 0.02 ml distilled water, 0.20 ml glycine buffer and 0.02 ml of alkaline phosphatase. Contents were mixed by vortexing and incubated at 40°C for 15 min. The reaction was terminated by addition of 0.30 ml of 50% (w/v) trichloroacetic acid. For estimating the free phosphorus in samples, reactions were initiated without addition of enzymes and making up similar volume with distilled water. After terminating the reaction, contents were

centrifuged at 10,500 × g for 10 min. The supernatant was carefully pipetted out, for colorimetric determination of phosphorus.

For colorimetric determination of phosphorus, 1 ml of supernatant (or standard phosphorus solution) and 0.5 ml of colour reagent were mixed in 1.5 ml micro-centrifuge tube. The contents were mixed by vortexing and incubated in a water bath set at 40°C for 1 hour. After incubation the contents were mixed again and absorbance was taken at 655 nm against water.

For calibration curve, phosphorus solutions of different concentrations (STD0: 0.0 µg, STD1: 0.5 µg, STD2: 2.5 µg, STD3: 5.0 µg and STD4: 7.5 µg) were prepared by using standard phosphorus solution (50 µg/ml) and treated as samples for the colorimetric determination of phosphorus. The absorbance (A_{655}) of each phosphorus standard (STD 0 - 4) was determined and $\Delta A_{phosphorus}$ was obtained by subtracting the absorbance of STD0 from the absorbance of the other standards (STD1 to STD4). M was calculated according to the following equation, for each standard (STD 1 - 4)

$$M = \frac{P\ (\mu g)}{\Delta A_{phosphorus}} \qquad [\mu g/\Delta A_{phosphorus}]$$

The mean M was calculated as follows:

$$\text{mean } M = \frac{(MSTD1 + MSTD2 + MSTD3 + MSTD4)}{4} \qquad [\mu g/\Delta A_{phosphorus}]$$

Mean M was used to calculate the phosphorus content of the test samples.

3.2.3.12.2 Phytic acid content estimation

The absorbance (A_{655}) for both the 'free phosphorus' sample and the 'total phosphorus' sample was obtained. The absorbance of the 'free phosphorus' sample was subtracted from the absorbance of the 'total phosphorus' sample, thereby obtained $\Delta A_{phosphorus}$.

The concentration of phosphorus was calculated by using following equation:

$$M = \frac{\text{mean M x 20 x F}}{\text{10,000 x 1.0 x V}} \times \Delta A_{phosphorus} \quad [g/100g\ ]$$

where,

mean M = mean value of phosphorus standards [$\mu g/\Delta A_{phosphorus}$]

20 = original sample extract volume [ml]

F = dilution factor

ΔA = absorbance change of sample

10,000 = conversion from µg/g to g/100 g

1.0 = weight of original sample material [g]

V = sample volume (used in the colorimetric determination step)

Phosphorus content was calculated by using following equation:

$$c = \frac{\text{mean M x 20 x 55.6}}{\text{10,000 x 1.0 x 1.0}} \times \Delta A_{\text{phosphorus}} \quad [\text{g}/100\text{g}]$$

$$= \text{mean M} \times 0.112 \times \Delta A_{\text{phosphorus}} \quad [\text{g}/100\text{g}]$$

The calculation of phytic acid content assumes that the amount of phosphorus measured is exclusively released from phytic acid and that this comprises 28.2 % of phytic acid. Therefore, phytic acid content was calculated by using the following equation:

$$p = \frac{\text{phosphorus}}{0.282} \quad [\text{g}/100\text{g}]$$

3.2.3.13 Alcoholic acidity of flour

Five 5 g of sample was weighed into a conical flask and added with 50 ml of neutral ethyl alcohol (90%). Flask was stoppered, shaken and allowed to stand for 24 hours, with occasional stirring. Alcoholic extract was filtered through a dry filter paper (Whatman Filter Paper No. 1). Ten ml of alcoholic extract was titrated against 0·05 N sodium hydroxide solution using phenolphthalein as indicator. Percentage of alcoholic acidity (% sulfuric acid) was calculated using the following formula (IS:1155:1968).

$$\text{Alcoholic acidity (as \% } H_2SO_4) = \frac{0.00245 \times 50 \times \text{Titre volume} \times 100 \times 100}{10 \times 5 \times (100 - \text{Moisture content of sample})}$$

3.2.3.14 Free amino nitrogen

Free amino nitrogen (FAN) content in barley flour was estimated using ninhydrin assay described by Lie (1973). One g of barley flour sample was mixed by vortexing with 10 ml of deionized water (Yan *et al*., 2010). Vortexing for 60 s was done five times in 10 min. Tubes were centrifuged at 2500 × g for 10 min. One ml of supernatant was diluted to 50 ml using deionized water and used for estimation of FAN.

Colour reagent was prepared by mixing 4 g anhydrous di-sodium hydrogen phosphate (Na_2HPO_4), 6.0 g potassium dihydrogen phosphate (KH_2PO_4), 0.5 g ninhydrin, and 0.3 g fructose in 80 ml of water, and pH of the

solution was adjusted (if necessary) in between 6.6 and 6.8 with hydrochloric acid or sodium hydroxide solution. Final volume was made to 100 ml with water (stable for 2 weeks when stored at 4°C in dark bottles). Dilution solution was prepared by dissolving 2 g of potassium iodate in a mixture of 600 ml of water with 400 ml of 96 % ethanol (stable for 1 week at 4 °C in dark bottles). Glycine stock solution 200 mg/l amino nitrogen was prepared by dissolving 107.2 mg of glycine in 100 ml water.

In a glass test tube, 2 ml of diluted sample extract was mixed with 1 ml of colour reagent and mixed by vortexing, followed by incubation in a boiling water bath for exactly 16 min. Test tubes were loosely closed to prevent evaporation. Test tubes were allowed to cool in a water bath for 10 min at 20°C. Five ml of dilution solution was added to each test tube and mixed by vortexing. Blank determination was also carried out simultaneously using 2 ml deionized water instead of sample. Absorbance after 3 min at 575 nm was taken against blank. A calibration curve using glycine as standard was prepared within range of 0.4 to 6 mg/l. Equation for the glycine calibration curve (**Appendix-IV**) was $\mathrm{Y} = 0.1843\mathrm{X} + 0.0336$ (where, $\mathrm{X} =$ concentration of glycine expressed as mg/l; Y = absorbance at 575 nm), and correlation coefficient was R^2 = 0.99). Results were reported in terms of mg glycine/g flour.

3.2.4 Preliminary examination of procured bacterial cultures

3.2.4.1 Morphological examination of procured cultures

Lactic acid bacterial cultures were streaked on their respective media and incubated at their respective temperatures for 24-48 h. *L. rhamnosus* RSI3, *L. acidophilus* 13, *L. casei* 299, *L. casei* 297 and *L. plantarum* 344 were grown on MRS Agar, whereas, *S. thermophilus* 20 was grown on M17 agar. The morphology of the appeared colonies was inspected visually for their size, shape, color and growth patterns. Microscopic characterization and purity *i.e.* shape, and arrangement of individual organisms was determined by standard Gram staining of heat fixed smears of activated standard cultures.

3.2.4.2 Catalase Test

The catalase test was performed by using slide method. Using an inoculating needle, culture from a well-isolated colony was placed on a clean glass slide. A drop of 3% H_2O_2 solution was added on to this culture and closely observed for the effervescence, indicating positive test.

3.2.5 Maintenance, propagation and preservation of cultures

Stock cultures (10^{11} cfu/ml) were maintained in 40% glycerol stock (0.5 ml of 40% glycerol + 0.5 ml grown cells in respective medium) at −20°C. Before, their use, *Lactobacillus* was propagated on 3 successive days by 2 % inoculation at 37°C for 12-18 h, in MRS (de Man, Rogosa and Sharpe) broth (de Man *et al.*, 1960), *Streptococcus thermophilus* in M17 (Terzaghi and Sandine, 1975) broth at 40°C. Non-lactic cultures were maintained in Brain Heart Infusion (BHI) broth and stored in BHI agar slants between transfers. Grown cells of LAB were harvested (6000 × g, 20 min, 5°C), washed with buffered peptone water (Himedia) and re-suspended in sterilized skim milk to its original volume and incubated at 37°C for 12-18 h. The probiotic and co-culture inoculum were prepared by 2% propagation of the activated cells in sterilized skim milk followed by incubation at 37°C (12-18 h) for probiotic LAB and 40°C (4-6 h) for *S. thermophilus*. The developed inoculum gave probiotic count of approximately 7 log cfu/ml.

3.2.6 Selection of suitable probiotic lactic acid bacteria for the manufacture of barley milk based fermented probiotic drink (Phase-I)

Lactic acid bacteria are the most important group of bacteria used in the preparation of fermented food products. Apart from their general requirements such as safety, economy and technological effectiveness, numerous specific aspects should have to be considered while selecting a strain for a targeted food substrate. These selection criteria of a single strain or mix of two or more will depend on the type and desired characteristics for the final product, desired metabolic activities, characteristic of the raw materials and the applied technology. A series of experiments including phytase plate assay, phytase assay in liquid medium, anti-nutrient reduction potential of probiotic culture in a barley-milk composite model system, co-culture compatibility test and sensory evaluation of a barley-milk based fermented probiotic curd were conducted to screen out a suitable probiotic culture with desired attributes amongst five probiotic LAB.

3.2.6.1 Phytase Plate Assay

Method described by Anastasio *et al.* (2010) with some modifications was followed to determine the phytase activity of procured lactic cultures. Briefly, activated cultures of Lactobacilli in MRS broth and *Streptococcus thermophilus* in M17 broth were preliminary grown for 24 h at 37^{o}C and 40^{o}C respectively in

modified Chalmers broth (**Appendix-V**) supplemented with 1% sodium phytate and 0.1% ammonium sulfate (Merck). Five microlitres of microbial suspension was spotted on modified Chalmers agar plate supplemented with 1% of hexacalcium phytate (Himedia). Addition of hexacalcium phytate makes the medium opaque. The plates were incubated at 37^{o}C and examined after 2-3 days of incubation for clearing zones around the spots. Microbes expressing phytase activity produce zone of clearance on agar medium containing sodium or calcium phytate (Shieh and Ware, 1968; Howson and Davis, 1983). Petri plates were flooded twice with 2% (w/v) aqueous cobalt chloride solution. After 20 min of incubation at room temperature, the cobalt chloride solution was decanted and phytase activity was evaluated by measuring the diameter (mm) of clear halo zones. Comparison of phytase activity was done by comparing the diameter of clear zone around spot.

3.2.6.2 Phytase Assay

To determine the phytase activity of lactic cultures, methods of Anastasio *et al.* (2010) and Engelen *et al.* (2001) were applied with few modifications. Phytase activity was measured in terms of inorganic phosphate released from phytate complex. One Phytase (fytase) unit (FTU/ml) was defined as the amount of enzyme required to liberate 1 µmol of inorganic phosphate per minute under the test conditions (Cheryan, 1980). Lactic acid bacteria having highest activity in phytase assay in solid medium were subjected to phytase assay in liquid medium. Cells were grown in phytase specific medium (modified Chalmers broth supplemented with 0.5% sodium phytate and 0.1% ammonium sulfate) at 37°C for 24 h. Five ml of cell suspension was mixed with 15 ml extraction buffer (250mM sodium acetate buffer, pH 5.5 containing 68.4 mM calcium chloride dihydrate and 0.01 % Tween 20) and stirred using a magnetic stirrer for 60 min. The suspension was then centrifuged at 3000 × g for 10 min at 5°C and supernatant was kept under ice till analysed.

The color stop solution was prepared by mixing 25 ml of ammonium heptamolybdate solution [10 g ammonium molybdate.4H_2O in 90 ml distilled water, 1 ml ammonia (25%), final volume adjusted to 100 ml with distilled water] and 25 ml ammonium vanadate solution [0.235 g ammonium vanadate added to 40 ml distilled water at 60^{0}C followed by slow addition of 2 ml nitric acid and final volume adjusted to 100 ml with distilled water] followed by adding 16.5 ml nitric

acid (65%) with slow stirring. After cooling to room temperature, the volume was adjusted to 100 ml with distilled water (prepared fresh).

Two milliliter of phytic acid substrate (9.09 mM phytic acid doedcasodium salt, 250 mM sodium acetate buffer, pH 5.5, 0.1 mM calcium chloride dihydrate and 0.01 % Tween 20) was pre-incubated for 5 minutes at 37°C into six glass test tubes. One ml of sample aliquot was added in to three test tubes to start the enzymatic reactions. All the test tubes were incubated at 37°C for exactly 60 min. The enzymatic reaction was stopped by adding 2.0 ml of freshly color stop solution. After the blank reaction tubes have been quenched with colour stop solution 1 ml of sample aliquot was added. Samples were kept for 5 min at room temperature. Contents were then centrifuged at 3000 × g for 10 minutes to clarify the reaction mixture. The released inorganic phosphate was calculated by measuring the corrected absorbance at 415 nm and comparing with standard curve prepared with inorganic phosphate (K_2HPO_4).

3.2.6.2.1 Plotting phosphate calibration curve

Calibration curve was prepared by using 7.2mM potassium dihydrogen phosphate (KH_2PO_4: 0.980 g/1000ml) solution, as a standard. Different dilutions were prepared by diluting 0.00, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 ml of 7.2mM standard to 1 ml with dilution buffer, which liberated 0.144, 0.216, 0.288, 0.360, 0.432, 0.504 and 0.576 (7.20x0.40/5=0.576) µmol/5ml phosphate, when added with 4 ml of colour stop solution.

Corrected absorbance at 415 nm (ΔOD_{415nm} the absorbance measurement of each phosphate standard calibration solution after subtracting the absorbance measured for the 0 µmol phosphate solution) was plotted as a function of phosphate concentration (in µmol) on the X-axis (**Appendix-VI**). Next, using linear regression program the 'best fit' line and its corresponding equation was generated.

$$y = mx + b$$

where,

y = ΔOD_{415nm} (Y-axis of the plot)

m = slope of the standard phosphate calibration curve

x = µmol of phosphate (X-axis of the plot)

b = intercept of the standard phosphate calibration curve

3.2.6.2.2 Estimating the concentration of released inorganic phosphate due to phytase catalyzed enzymatic reaction

Concentration (μM) of phosphate ion was calculated using the following equation:

$$C = \frac{(d - b)}{m}$$

where,

C = concentration (μmol) of inorganic phosphate (P_i)

d = ΔOD_{415nm}=OD_{415nm} for enzyme reaction sample - OD_{415nm} for the enzyme background sample

b = intercept of the calibration standard curve

m = slope of the calibration standard curve.

3.2.6.2.3 Determining the phytase activity of bacterial cell culture

Phytase activity of bacterial cell culture was determined using the equations provided below:

$$A = \frac{\left(C \times \frac{V_r}{V_a}\right) \times V_e}{t \times V_s} \times D_f$$

where,

A = phytase activity expressed in μmol of P_i released per minute per ml of sample

C = concentration of released P_i, expressed in μM

V_r = total volume (ml) of the reaction mixture after addition of the color stop solution (5 ml as per the present assay)

V_a = volume (ml) of diluted enzyme extract used for each assay (1 ml as per the present assay)

V_e = total volume (ml) of extraction mixture after addition of sample (20 ml as per the present assay)

D_f = working solution dilution factor $\left[D_f = \frac{V_t}{V_d}\right]$ where, V_t=final volume of diluted enzyme extract & V_d=volume of extracted enzyme used for working dilution

t = time-period (in minute) of incubation during phytase assay (60 minute as per the present assay)

V_s = initial volume (ml) of sample used for each extraction

3.2.6.3 Evaluation of lactic acid bacterial cultures for anti-nutrient reduction potential in barley-milk composite model system

The potential of lactic cultures was assessed to degrade anti-nutritional factors including phytate and tannins present in barley grains using standard protocols. A barley-milk composite model system was prepared as discussed below and individually fermented with different lactic cultures. Fermented barley-milk composite model system was evaluated for various chemical parameters as discussed hereunder.

3.2.6.3.1 Preparation and fermentation of barley-milk mix model system

Barley-milk composite model system was prepared by systematic mixing of 400 g non-germinated barley flour, 120 g skim milk powder and 600 ml distilled water for about 10 min in planetary mixer (Hobart Corporation, Troy). The mix was individually inoculated with 1% of activated LAB, mixed thoroughly and allowed to ferment for 12 h at 37°C. Samples without cultures (0 h and 12 h fermented) were served as control. Samples were stored at -20°C till analyzed.

3.2.6.3.2 Analysis of barley-milk composite model system

3.2.6.3.2.1 Titratable acidity and pH

Titratable acidity of each sample was determined by mixing 5 g of sample with 15 ml of distilled water (25°C) and titrating against 0.1N NaOH, using phenolphthalein as an indicator, till pink colour developed which should persist for at least 30 sec Results were expressed in % lactic acid.

$$\text{TA (\%lactic acid)} = \frac{9 \times \text{Normality of NaOH} \times \text{ Titre Value}}{\text{Weight of sample}}$$

For determination of pH, 5 g of sample was mixed with 15 ml distilled water. An Orion 3 star (Thermo Scientific) pH meter was used for the measurement of pH at 20°C. All estimations were carried out in triplicate.

3.2.6.3.2.2 Phytic acid

Phytic acid content of control and LAB fermented barley-milk composite model system was estimated by using the method previously mentioned in sub-section **3.2.3.12**.

3.2.6.3.2.3 Tannin content

Tannin content of the each sample (along with non-germinated flour sample) was determined according to the modified Vanillin-HCl methanol method as described by Price, *et al.* (1978). The Vanillin-HCl reagent was freshly prepared by mixing

equal volumes of 8% concentrated HCl in methanol and 1 % vanillin in methanol. For determination of tannin content, extracts were obtained by mixing 2 g of samples (1 g in case of barley flour) in 20 ml acidified methanol (1% concentrated HCl). The suspension was vigorously stirred for 90 minutes using magnetic stirrer and centrifuged at 3000 × g for 10 min. About 1.0 ml of the supernatant was pipetted into a test tube containing 5ml of Vanillin-HCl reagent. Absorbance at 450 nm recorded using spectrophotometer (DU® 720, Beckman Coulter, Fullerton, CA) after 30 minutes incubation at 30°C. A sample blank was prepared by using 1.0 ml of acidified methanol in place of sample extract and processed subsequently in similar manner. A set of catechin hydrate standard solutions ranging from 0 to 120 ml/l was prepared (**Appendix-VII**) using methanol as a solvent to draw calibration curve and results were expressed in terms of catechin equivalent (mg/g).

3.2.6.3.2.4 Extraction of samples for total phenolics and antioxidant activity

Extracts for the determination of total phenols and antioxidant activity were prepared by mixing 1 g of sample with 20 ml of 60% methanol (acidified with 0.1% conc. HCl). The extracts were transferred carefully into culture tubes and kept at -20 °C until analyzed. Acid addition in the extraction solvent increased the stability of the phenolic compounds, antioxidants and favors their dissolution (Campos et al. 2008). Similar methanolic extraction has been previously (Oki et al., 2002; Sreeramulu *et al*., 2009) used to determine total pehnolics and antioxidant activity of various cereals.

3.2.6.3.2.5 Total phenolics content

The total phenolic content (TPC) in extracts was determined by modified Folin-Ciocalteu method (Singleton and Rossi, 1956). Briefly, 100 µl of each extract were shaken for 60 s with 500 µl Folin-Ciocalteu reagent and 5 ml of distilled water. After the mixture was shaken, 2 ml of 15% Na_2CO_3 was added and the mixture was shaken once again for 45 s. Finally, the volume was made up to 10 ml by addition of distilled water. After 2 h, the absorbance was read on the UV/Vis spectrophotometer (Beckman Coulter, DU® 720, Fullerton, CA) at 734 nm. The, TPC was assessed by plotting the gallic acid calibration curve (from 5 to 150 µg/ml, **Appendix-VIII**) and expressed as mg of gallic acid equivalent (GAE) per g sample. The equation for the gallic acid calibration curve was $Y =$

$0.0015\mathrm{X} - 0.0044$ (where, $\mathrm{X} =$ concentration of gallic acid equivalent expressed as µg/ml; Y = absorbance), and correlation coefficient was R^2 = 0.99).

3.2.6.3.2.6 DPPH free radical scavenging activity

Antioxidant activity of extract was measured on the basis of scavenging activities of the stable 1,1-diphenyl-2-pcrylhydrazyl (DPPH) radical (Cuendet *et al.*, 1997). In a test tube, 50 µl extracts of test sample was mixed with 3.95 ml of methanol followed by adding 1 ml of 0.2mM DPPH solution in methanol. After 30 min of incubation in dark at room temperature, the absorbance was read against a blank (methanol) at 517 nm using UV/Vis spectrophotometer (Model: DU® 720, Beckman Coulter, CA). Inhibition DPPH radical was calculated as percentage (%) using the formula:

$$\text{Percentage inhibition } (\%) = \frac{\mathrm{A_{control}} - \mathrm{A_{sample}}}{\mathrm{A_{control}}} \times 100$$

Results were expressed in terms of trolox equivalent antioxidant capacity (mg/g) sample. Calibration curve was plottied (from 20 to 350 µg/ml) using Trolox as standard (**Appendix-IX**). The equation for the assessment of DPPH radical scavenging activity was $\mathrm{Y} = 0.247\mathrm{X} - 2.9739$ (where, $\mathrm{X} =$ concentration of trolox equivalent expressed as µg/ml; Y = % inhibition), and correlation coefficient was R^2 = 0.99).

3.2.6.3.2.7 ABTS free radical scavenging activity

The radical scavenging activity of the extract against ABTS radical cation was measured using the method of Awika *et al.* (2003). Equal volumes of 8 mM 2,2-azinobis [3-ethyl-benzothiazoline-6-sulphonic acid] (ABTS) and 3 mM potassium per sulphate were mixed and allowed to react for 12 h in dark to obtain a radical cation ($ABTS^{\bullet+}$) solution. The ABTS solution was diluted with a pH 7.4 phosphate buffer solution containing 150 mM NaCl to obtain an initial absorbance of about 1.5 at 734 nm. Sample extracts / standards (100 µl) were reacted with 2900 µl ABTS solution for 30 min in dark and absorbance at 734 nm was then read. Inhibition of ABTS radical was calculated as percentage (%) using the formula mentioned in sub-section **3.2.6.3.2.6**. Calibration curve was plotted (from 40 to 350 µg/ml) using Trolox as standard (**Appendix-X**) and the result were expressed in mg/g sample trolox equivalent antioxidant capacity (TEAC). The equation for the assessment of ABTS radical scavenging capacity was $\mathrm{Y} =$

$0.2422X - 2.9574$ (where, X =concentration of trolox expressed as µg/ml; Y = % inhibition), and correlation coefficient was R^2 = 0.99).

3.2.6.4 Co-culture compatibility assay

The probiotic cultures with highest anti-nutrient reduction potential were tested for compatibility against co-culture. Well diffusion assay as described by Schillinder and Lucke (1989) was employed to assess the compatibility of different strains. MRS agar plates were overlaid with 7 ml soft agar inoculated containing 20 µl of washed cells from overnight grown indicator strain (probiotic culture). Wells (6 mm diameter) were bored into these agar plates, and sealed with 10 µl of soft agar. Co-culture to be tested for compatibility with probiotic bacteria was grown overnight in M17 broth. Cell free culture supernatant was obtained by centrifugation of broth at 10,000 × g for 10 min at 4ºC (Sigma Laborzentrifugen, Germany). Fifty µl of cell free culture supernatant was added into each well. Plates were kept at refrigeration temperature for 2-4 h for diffusion. The plates were incubated under anaerobic (Anaerogas Pack, Himedia, Mumbai) conditions at 37°C for 24 h and examined for zones of inhibition (if any).

3.2.6.5 Preparation of barley-milk based fermented probiotic composite

After examining the compatibility of co-culture with highest anti-nutritional factors reducing probiotic strains, they were utilized for the manufacture of barley-milk based fermented probiotic composite. To prepare the barley-milk based probiotic composite, 4 % barley flour was added in skimmed cow milk (total solids adjusted to 12% using skim milk powder) at 70°C (**Figure 3.2**). Barley-milk composite was mixed thoroughly using a blender for 2 min (Orpat® Model 107E; Ajanta Ltd., Gujarat). The temperature of barley-milk composite was raised to 90°C in a waterbath with continuous stirring, maintained for 30 min, and finally cooled to 37°C in a refrigrated waterbath (Lauda, Germany) maintained at 5°C. The barley-milk composite samples were inoculated with 1.5 % probiotic culture along with 0.5 % co-culture. The cultured barley-milk composite (100 ml) was filled in to sterile container (Himedia) and incubated at 37°C till acidity reaches 0.65% (% lactic acid). The fermented samples were transferred to refrigerator (5±1°C) till analyzed.

3.2.6.6 Sensory evaluation of barley-milk based curd

Barley-milk based probiotic curd samples were evaluated organoleptically for different sensory attributes such as colour and appearance, body and texture, flavour and overall acceptability by a semi trained panel of judges.

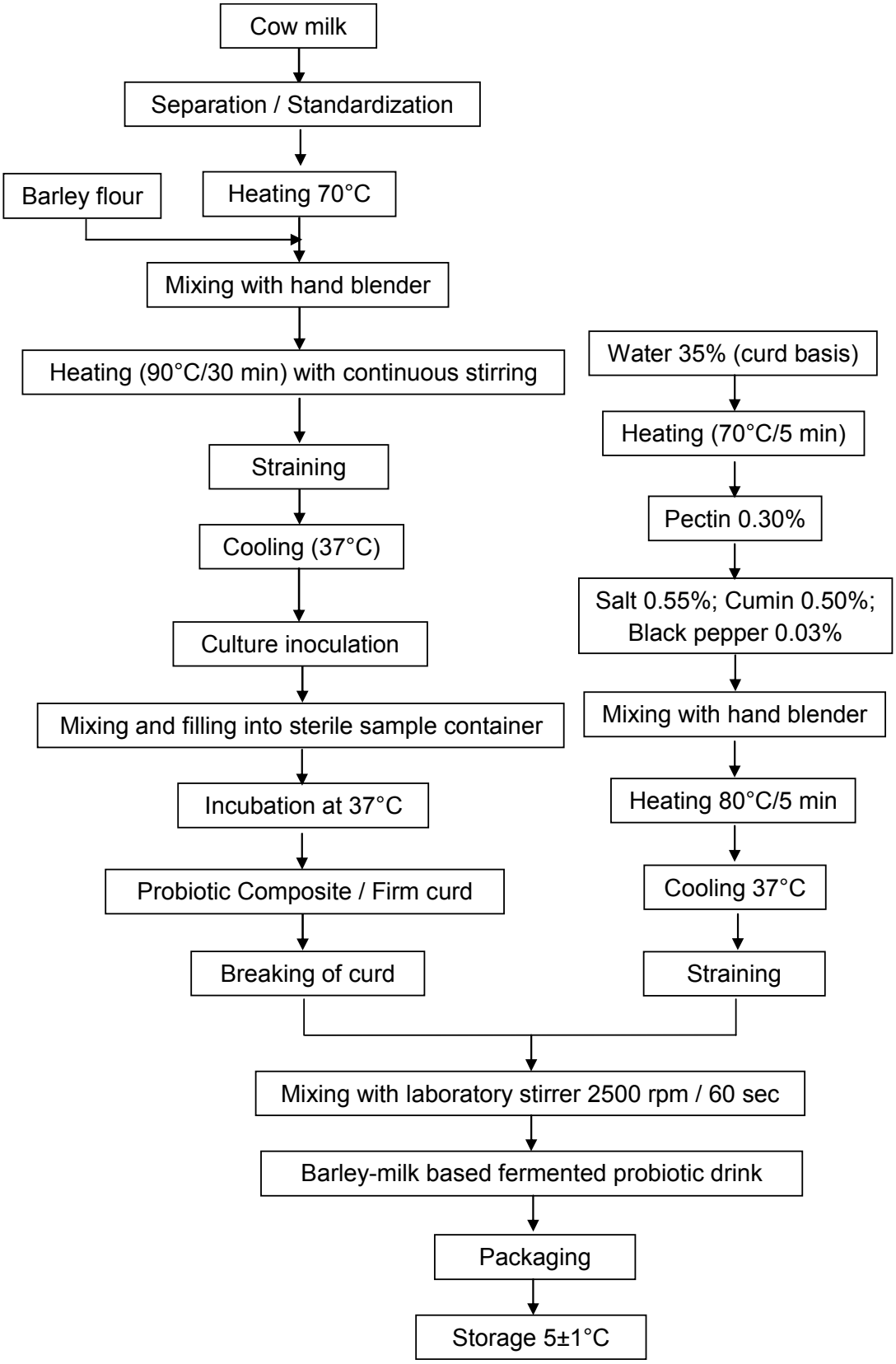


Figure 3.2 Flow diagram for the preparation of barley-milk based fermented probiotic composite / drink

Samples were taken out from the refrigerator just before presenting to the panel of sensory judges and evaluated using 9-point Hedonic scale. A score of 9 represented 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 and 1 dislike extremely (**Appendix-XI**).

3.2.6.7 Selection of suitable culture with desired attributes

After examining the co-culture compatibility of phytase producing probiotic cultures, the probiotic strain with maximum potential to degrade the anti-nutritional factors, and yielding composite of maxium sensory acceptability when co-cultured with *S. thermophilus* 20 was selected as suitable probiotic culture for the development of barley-milk based fermented probiotic drink.

3.2.7 Evaluation of *in vitro* low pH, bile salt tolerance and cell surface hydrophobicity of selected probiotic lactic acid bacteria (confirmatory test)

3.2.7.1 *In vitro* tolerance to Low pH

Acid tolerance of the culture was studied in different pH solutions. Hundred ml MRS broth solutions were prepared and pH was adjusted to 1.5 and 2.0 by HCl. The adjusted pH broths were divided into aliquots of 10 ml and sterilized (121°C, 15 min, 15 psi). Previously activated culture (~10^{11} cfu/ml) was inoculated (1%) in adjusted pH broth and incubated at 37°C. Sample for 0 h was withdrawn immediately after inoculation and appropriate serial dilutions were prepared using buffered peptone water (Himedia, Mumbai). Broth of pH 1.5 and 2.0 were taken out exactly after 1 and 2 h. Appropriate dilutions were pour plated on MRS agar and incubated at 37°C for 24-48 h. Viable cells were expressed in colony forming units per ml (cfu/ml).

3.2.7.2 *In vitro* tolerance to high bile concentrations

MRS broth was supplemented with 1.5% and 2.0% (w/v) bile salts (Merck). Broth without bile salt was served as control. Freshly activated cells of probiotic bacterial culture (~10^{11} cfu/ml) in MRS was inoculated in each broth followed by incubation at 37°C. Samples from each broth was taken at 0, 1 and 2 h. Appropriate dilutions were pour plated on MRS agar and incubated at 37°C for 24-48 h. Bile tolerance was assessed in terms of colony forming units per ml.

3.2.7.3 Cell surface hydrophobicity

Ability of the organisms to adhere to hydrocarbons is a measure of their adherence to the epithelial cells in the gut *i.e.* cell surface hydrophobicity. Cell

surface hydrophobicity was determined according to the method described by Rosenberg *et al*., (1980) with slight modification using n-hexadecane. Previously activated probiotic culture were grown in MRS broth at 37°C for 18 h. Grown bacterial cells were centrifuged (6000 × g for 30 min at 5°C) and the cell pellet was washed twice with phosphate urea magnesium (PUM) buffer. The washed cell pellet was resuspended in PUM buffer and the absorbance (A_o) was adjusted in between 0.7 and 0.9 OD at 600 nm. Lactobacilli cell suspension (3.0 ml) and n-hexadecane (1.0 ml) were mixed by vortexing and incubated at 37°C for 10 min for temperature equilibration. The mixture was again briefly vortexed and incubated at 37°C for 1 h for phase separations and the hydrocarbon layer was allowed to rise completely. The aqueous phase was gently siphoned out and absorbance (A_o) was taken at 600 nm. The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (%H) calculated as:

$$\%\mathrm{H} = \frac{\mathrm{A_o} - \mathrm{A}}{\mathrm{A_o}} \times 100$$

where, A_o = initial absorbance and A = final absorbance hydrocarbons at 610nm.

3.2.8 Optimization of processing parameters for barley-milk based fermented probiotic drink (Phase-II)

3.2.8.1 Selection of type of milk

Aimed to minimize the cost of production and decrease calorific value contributed by fat, three different types of milk namely skimmed cow milk, double toned cow milk and toned cow milk were subjected to preliminary trials in the preparation of barley-milk based probiotic curd and drink. For the preparation of barley-milk based fermented probiotic composite 4% non-germinated barely flour was added to skimmed cow milk (total solids adjusted to 12% using SMP), double toned cow milk (1.5% fat and 9.0% SNF) and toned cow milk (3% fat and 8.5% SNF) at 70°C and mixed thoroughly using blender. The temperature of milk-flour mix was raised to 90°C, maintained for 30 minutes and finally cooled to 37°C. The mix was then inoculated with 1.5% probiotic inoculum (selected from **3.2.6.7**) and 0.5% co-culture inoculum, transferred to sterile containers (100 ml), incubated at 37°C till acidity reaches 0.65% (% lactic acid) and stored under refrigeration at 5±1°C. The barley-milk based curd samples was analysed for colour and appearance, body and texture, flavour and overall acceptability (**Appendix-XI**).

For the preparation of barley-milk based fermented probiotic drink, barley-milk fermented composite (curd) was broken down manually and added with water-spice mix. Water-spice mix was prepared by addition of salt, pectin, cumin and black pepper at the rate of 0.55, 0.30, 0.50 and 0.03 % (on curd basis), respectively to the water (35% of the curd w/v) at 70°C. The temperature of water-spice mix was raised to 85°C, maintained for 5 min and cooled to 5°C. Cumin and black pepper were chosen as flavouring agents, which impart appealing flavour, improve the sensory perception, and mask the native flavour of barley flour. The cooled water spice mix was added to the broken curd and mixed using a laboratory stirrer (2500 rpm) for 60 s.

Prepared drink was filled in pre-sterilized PET bottles. The bottles and caps were sterilized by dipping in to 20 % hydrogen peroxide (H_2O_2) solution followed by drying at 55°C for the complete evaporation of H_2O_2. Before filling, bottles and caps were exposed to Ultra Violet (UV) rays for 15 min. After filling of BMFPD bottles were capped tightly and stored under refrigeration at 5±1°C. Barley-milk based fermented probiotic drink was analysed for colour and appearance, sedimentation, consistency, flavour and overall acceptability (**Appendix-XII**) by a panel of sensory judges using 9-point hedonic scale.

3.2.8.2 Selection of type of flour

Three different types of flour obtained from non-germinated, germinated and imbibed grains barley flour (**Figure 3.1**) were subjected to preliminary trials for selection of one to develop barley milk based fermented probiotic drink. Germination of barely grains has been reported to improve the nutritional quality (Pedersen *et al.*, 1989), and enhances the lactobacilli count approximately by 1 log cycle in a food mixture comprised of barley flour, whey powder and tomato pulp when compared with non germinated barley flour based food mixture (Arora *et al.*, 2010). However, germination of barley grains has also been reported to significantly decrease the functional component *i.e.* β-glucan content in barley grain (Kihara *et al*., 2007; Hubner *et al*., 2010). Germination hydrolyses starch content in to more simple carbohydrate forms, thereby interferes with the gelatinization properties of barley starch.

Imbibtion of barley grains is other type of treatment for barley grains, which comprises of multiple cycle of immersion and non-immersion process. Imbibition of 48 h, which comprised 4 cycles of 5 h immersion and 7 h non-immersion

process over 95% relative humidity (15°C) has been reported for accumulation of a functional component γ-aminobutyric acid (GABA) with minimum degradation of barley β-glucan content (Kihara *et al*., 2007). Germination and imbibition are time taking process as well as, adding to the cost of production. However, hydrolysis of starch during germination and imbibition will decreases the water holding capacity of starch and reduce the viscosity of composite, thereby provide an opportunity to increase the flour incorporation rate in the milk.

Considering the above, the three types of flour namely non-germinated, germinated and imbibed barley grain flour were used at 2 different levels i.e. 3.5 % and 4.5 % in milk (selected from **3.2.8.1**) to prepare barley milk based probiotic composite and drink (**Figure 3.2**). The fermented probiotic composite (**Appendix-XI**) and corresponding drink (**Appendix-XII**) thus prepared were subjected to sensory evaluation for colour and appearance, sedimentation, consistency, flavour and overall acceptability (based on 9 point hedonic scale). Effect of type of flour on the probiotic count in drink was investigated. Based on the sensory evaluation and effect of flour type on probiotic count, one out of the three type of flour was selected and used in the subsequent experiments.

3.2.8.3 Effect of type of flour on the pasting properties of barley-milk composite

Native starch in cereals exists as microscopic granules which are arranged radially, and form a series of concentric layers. The size and shape of such granules are very dependent on their sources (Atwell *et al*., 1988; McArthur and D' Appolonia, 1979). Each starch molecule is a large polymer made up of anhydrous glucose units. There are basically two distinct polymer types of starch, amylase and amylopectin. Amylose is linear in structure, whereas, amylopectin exhibits substantial branching in its structure (Blanshard, 1987; Zobel, 1988; Zobel and Stephen, 1995). Unmodified starch is generally insoluble in water below 50°C. When starch is heated in water beyond a critical temperature, it absorbs a large amount of water and swells to many times of their original size. Over a critical temperature range, the starch granules undergo an irreversible process known as gelatinization, which is marked by crystalline melting, loss of birefringence and starch solubilisation (Autio, 1990; Leach *et al*., 1959). In the pasting test the temperature of starch slurry is kept below the gelatinization temperature of the starch, and viscosity of solution remains low. With the rise in temperature above the gelatinization temperature, the starch granules begins to

swell, and viscosity of starch slurry increases on shearing when these swollen starch granules have to squeeze past each other.

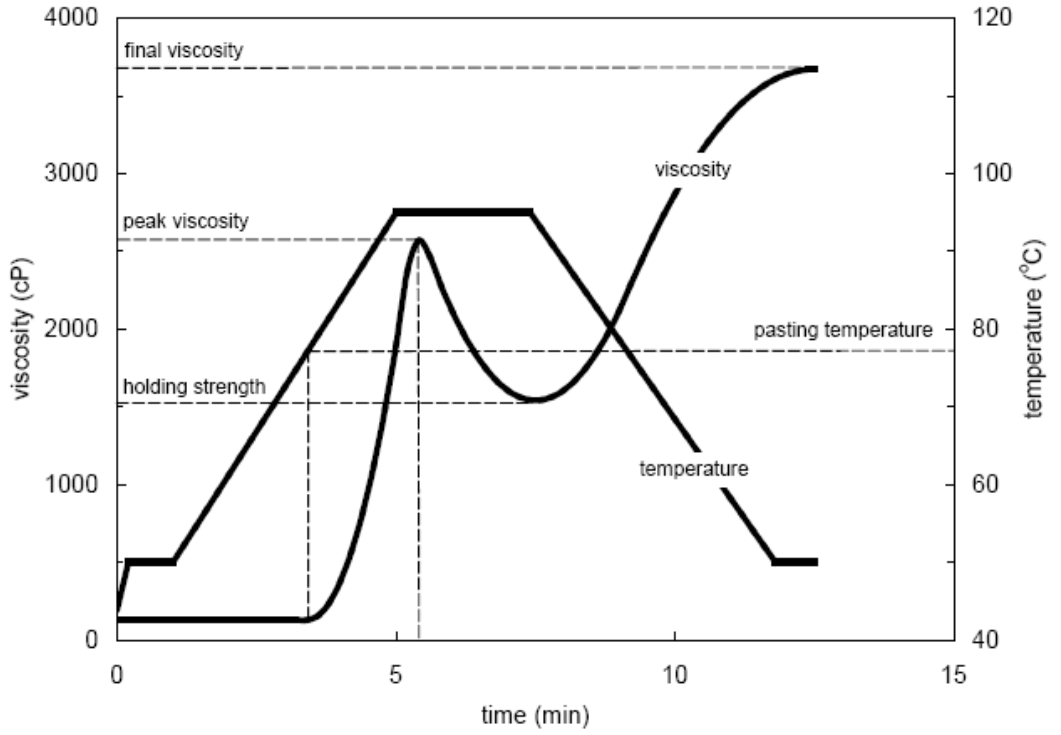


Figure 3.3 A typical temperature profile and pasting curve.

The temperature at the onset of this rise in viscosity is known as the pasting temperature, and provides an indication of the minimum temperature required to cook a given sample (Newport Scientific, 1998). When a sufficient number of granules become swollen, a rapid increase in viscosity occurs, given sufficient concentration of starch. Granules swell over a range of temperatures, indicating their heterogeneity of behaviour. This range is reflects the sleepness of the initial rise in viscosity in the pasting curve. As the temperature increases further, the starch granules rupture and become the more soluble, due to polymer realignment by shearing action. These combined processes that follow gelalinisation are known as pasting. A typical temperature profile and pasting curve (Bason *et al*., 1993) is represented in **Figure 3.3**. Peak viscosity occurs at the equilibrium point between swelling and polymer leaching which cause an increase in viscosity, and rupture and polymer realignment which cause it to decrease. Peak viscosity indicates the water-binding capacity of the starch (Newport Scientific, 1998).

During the hold period of the test, the sample is subjected to a period of constant high temperature (usually 95°C) and shear stress, resulting further

disruption of the granules and amylose molecules will generally leach out into solution. This period is commonly accompanied by a breakdown in viscosity to a holding strength, hot paste viscosity or trough. The rate of reduction depends on the temperature and degree of mixing, or shear stress, applied to the mixture, and the nature of the material itself. As the mixture is subsequently cooled, re-association between starch molecules, especially amylose, occurs to a greater or lesser degree. In sufficient concentration this usually causes the formation of a gel, and viscosity will normally increase to a final viscosity. This phase of the pasting curve is commonly referred to as the setback region, and involves retrogradation, or re-ordering, of the starch molecules. Final viscosity is the most commonly used parameter to define a particular sample's quality, as it indicates the ability of the material to form a viscous paste or gel after cooking and cooling. Different factors affecting the pasting curve are starch source, test conditions, modifications such as hydrolysis, annealing, pre-gelatinisation in starch, and, interactions with other ingredients.

Table 3.3 Instrument profile for RVA

Time (h:min:s)	RVA Parameters	Value
00:00:00	Temperature (°C)	50
00:00:00	Rotational speed (rpm)	960
00:00:10	Rotational speed (rpm)	160
00:00:30	Temperature (°C)	50
00:03:00	Temperature (°C)	90
00:23:00	Temperature (°C)	90
00:26:00	Temperature (°C)	50
00:35:00	Temperature (°C)	50
00:35:00	End of test	-
Idle temperature: 50±1°C; Time between each readings: 4 s		

Pasting properties of barley flour was measured using a Rapid Visco Analyser (RVA-4), using the RVA General Pasting Method (Newport Scientific Pvt. Ltd., Warriewood, Australia). Samples were prepared by mixing 5% barley flour (dry solids) in 27 g of toned milk. Different types of barley flour obtained from non-germinated, imbibed and germinated barley grains were used for the preparation of sample. The samples were subject to Rapid Visco Analyser to obtain pasting curve using instrument profile indicated in **Table 3.3**. Each analysis took 35 min and conducted at least in duplicate. Results from the analysis of pasting curve obtained from Thermocline software (version 2.2,

Newport Scientific Pty. Ltd., Warriewood, Australia) included peak 1 viscosity, viscosity at 3 min, viscosity at 23 min and final viscosity at the end of test.

3.2.8.4 Effect of type of flour on rheological properties of barley-milk composite after processing and fermentation; and on the probiotic drink prepared using fermented composite

Barley-milk composite samples were prepared from three different types of flour (non-germinated, germinated and imbibed barley grains flour) in milk (selected from sub-section **3.2.8.1**) at three different levels (4%, 5% and 6%). Rheological measurements for different samples were done after processing *i.e.* heat treatment of barley-milk composite, and after fermentation of composite with 1.5 % probiotic culture and 0.5 % co-culture. Probiotic drink prepared from different fermented composites was also subjected to rheological analyses.

3.2.8.4.1 Measurement of viscosity, shear rate / shear stress data and fitting of different rheological models

A controlled-stress rheometer (Anton Par Germany GmbH) with a cone and plate (CP75-1) attachment (cone diameter 74.975 mm, cone angle 1.002°, cone truncation 149 µm) was used to determine the rheological behavior of different samples. A total of ten shear-rate/shear-stress data points were obtained for each sample at an increasing shear rate from 10 to 100 s^{-1} within a period of 60 s. Measurements were taken at five different constant temperatures (5, 10, 15, 20 and 25°C ± 0.1°C). The shear-rate and shear-stress data were fitted to some of rheological models, such as *Ostwald-De-Waele* (eq. 1), *Herschel-Bulkley* (eq. 2) and *Casson* (eq. 3) model (Mezger, 2006) using RheoPlus32 (v3.61) software.

$$\tau = \mathrm{K}.(\dot{\gamma})^{\mathrm{n}} \quad \text{------ (1)}$$

$$\tau = \tau_{\mathrm{o}} + \mathrm{K}.(\dot{\gamma})^{\mathrm{n}} \quad \text{------ (2)}$$

$$\tau^{0.5} = \mathrm{k_{oc}} + \mathrm{k_C}.(\dot{\gamma})^{0.5} \quad \text{------ (3)}$$

where,

τ = shear stress (Pa)

$\dot{\gamma}$ = shear rate (s^{-1})

n = flow behavior index

τ_{o} = yield stress (Pa)

K = consistency index ($Pa.s^n$)

τ_{c} = Casson yield stress (Pa) = $(\mathrm{k_{oc}})^2$

K_c = Casson plastic viscosity = $(k_c)^2$

The most suitable model was obtained by observing the coefficient of determination (R^2) and the agreement with the phenomenon.

3.2.8.4.2 Textural analysis of barley-milk based fermented composite

Textural attributes such as firmness, work of shear, work of adhesion and stickiness of different barley-milk based fermented composite samples obtained from 3 types of flour at 3 different levels were determined by back extrusion method using a texture analyzer, TA-XT2i (M/s Stable Micro Systems, UK) fitted with a 25 kg load cell. The texture analyzer was calibrated by using 5 kg standard dead weight prior to use. For determining the textural attributes, the barley-milk composite was prepared and processed in manner as mentioned in **Figure 3.2**. The cooled barley-milk composite was cultured with 1.5% probiotic culture and 0.5% co-culture. Cultured barley-milk composite (~150 ml) after thorough mixing was filled up to 7 cm height in pre-sterilized glass beakers of specific shape and size (10 cm height and 6 cm diameter) and incubated at 37°C till acidity reaches 0.65 (% lactic acid). Samples were then removed from incubator and kept under refrigeration condition for overnight. Before subjecting to textural measurements beakers were tempered at 20°C for 1 h prior to analysis. The probe (A/BE 35) was penetrated up to 25 mm into the probiotic curd at a cross head speed of 1.0 mm/s during test. The probe displaced the material by compression followed by back-extrusion, so that the fluid flowed upwards through the concentric annular space. From the resulting force-time curves, firmness *i.e.*, the force for compression (N), stickiness *i.e.*, the negative peak force (N) during withdrawal, work of shear (N.s) and work of adhesion (N.s) were calculated using the Texture Expert Exceed software (v 2.55) supplied by the manufacturer along with the instrument. All measurements were done in triplicate per sample.

3.2.8.5 Optimization of flour level, probiotic inoculums level and co-culture inoculum level for the manufacture of barley-milk based probiotic drink

After selection of milk and type of flour to be used in the preparation of BMFPD, levels of 3 variables namely, barley flour, probiotic inoculum and co-culture inoculum were optimized using central composite rotatable design (CCRD) of the RSM (Myers, 1971) provided in Design-Expert® version 8.0.1 (Stat-Ease Corporation, USA). Based on preliminary trials, lower and higher levels for barley flour; probiotic inoculum; and co-culture inoculum were decided as: 4% and 6%;

2% and 5%; and 0.25% and 0.75%, respectively. The experimental design matrix in terms of coded and actual factors is represented in **Table 3.4**. The design matrix consisted of six centre points with one replicate of factorial and one replicate of star points.

Table 3.4 Experimental design matrix in terms of actual and coded values for independent variables: barley flour (X_1), probiotic inoculum (X_2) and co-culture inoculum (X_3)

Standard order	Coded Values			Actual Values (%)		
	X_1	X_2	X_3	Barley flour	Probiotic inoculum	Co-culture inoculum
1	-1.000	-1.000	-1.000	4.00	2.00	0.25
2	1.000	-1.000	-1.000	6.00	2.00	0.25
3	-1.000	1.000	-1.000	4.00	5.00	0.25
4	1.000	1.000	-1.000	6.00	5.00	0.25
5	-1.000	-1.000	1.000	4.00	2.00	0.75
6	1.000	-1.000	1.000	6.00	2.00	0.75
7	-1.000	1.000	1.000	4.00	5.00	0.75
8	1.000	1.000	1.000	6.00	5.00	0.75
9	-1.682	0.000	0.000	3.32	3.50	0.50
10	1.682	0.000	0.000	6.68	3.50	0.50
11	0.000	-1.682	0.000	5.00	0.98	0.50
12	0.000	1.682	0.000	5.00	6.02	0.50
13	0.000	0.000	-1.682	5.00	3.50	0.08
14	0.000	0.000	1.682	5.00	3.50	0.92
15	0.000	0.000	0.000	5.00	3.50	0.50
16	0.000	0.000	0.000	5.00	3.50	0.50
17	0.000	0.000	0.000	5.00	3.50	0.50
18	0.000	0.000	0.000	5.00	3.50	0.50
19	0.000	0.000	0.000	5.00	3.50	0.50
20	0.000	0.000	0.000	5.00	3.50	0.50

The product was prepared by the method represented in the **Figure 3.2** and following the different combinations indicated in the design matrix. Effect of barley flour (x_1), probiotic inoculum (x_2) and co-culture inoculum (x_3) levels were investigated for probiotic count, β-glucan* content, textural* parameters, viscosity and sensory attributes of barley-milk based fermented probiotic drink (*estimated

for barley-milk based curd). All experiments were conducted in random manner, four at a time. All the experiments were conducted in triplicate.

Analytical results obtained for every response were entered into Design Expert® software and fit summary for each response was obtained, which displayed sequential F-tests, lack-of-fit tests and other adequacy measures. Process order for every response was selected as quadratic. In the next step, analysis of variance (ANOVA) tables were generated and the main effects (x_1, x_2 and x_3) and interaction between ($x_1.x_2$, $x_2.x_3$ and $x_1.x_3$) and interaction within (x_1^2, x_2^2 and x_3^2) input variables on the individual response was analysed at particular level of significance (α=0.05 or 0.01).

Adequacy of model was evaluated by its significance (F-test) and lack of fit test. A good model must be significant, while the lack-of-fit should be insignificant. Post-ANOVA analysis for the residuals (lack of fit and pure error) was also performed. Coefficients for the quadratic model (eq. 5) in terms of coded factors were obtained which could be use for the prediction of response.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}{x_1}^2 + b_{22}{x_2}^2 + b_{33}{x_3}^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad \text{------(5)}$$

where, y: response variable; x_1, x_2, x_3: input factors; b_0: constant term; b_1, b_2, b_3: coefficient of linear terms; b_{11}, b_{22}, b_{33}: coefficient of quadratic terms, and b_{12}, b_{13}, b_{23}: coefficient of interactive terms.

A coefficient of determination (R^2) value more than 0.80 for each equation obtained represents the good fit of model. Diagnostic plots were observed to statistically validate the model, which should exhibit the trends associated with good model. Model graphs (3D graphs) were generated to interpret the effect of input factors on the responses. Contour plot was used to determine the interaction between two variables on the responses.

A coefficient of determination (R^2) value more than 0.80 for each equation obtained represents the good fit of model. Diagnostic plots were observed to statistically validate the model, which should exhibit the trends associated with good model. Model graphs were generated to interpret the effect of input factors on the responses. Contour plot was used to determine the interaction between two variables on the responses.

After analysing all the responses, multiple response optimization technique was used for the optimization of input variables (x_1, x_2 and x_3) and performed by

numerical as well as graphical optimization tool provided for the purpose in software. Only three responses namely probiotic count, β-glucan content (in barley-milk based curd) and overall acceptability of BMFPD were used in optimization process. Constraints for all input variables were kept 'in-range', while the constraints for responses were 'maximized'. Out of various solutions obtained as a result of optimization process, the solution with highest desirability was selected as optimized.

3.2.8.6 Analysis of barley-milk based probiotic curd (intermediate product) obtained during optimization trials

Titratable acidity and textural attributes of intermediate product were analysed by method previously mentioned in sub-section **3.2.6.3.2** and **3.2.8.4.2**, respectively.

3.2.8.7 Analysis of barley-milk based fermented probiotic drink obtained during optimization trials

3.2.8.7.1 Probiotic count

The selective medium and method for the enumeration of probiotic count depended on the type of probiotic LAB selected (sub-section **3.2.6)**. For enumeration of *L. acidophilus*, MRS-salicin agar (**Appendix-V**) was used (Dave and Shah, 1996). MRS-salicin agar was prepared by adding 10 ml of membrane filtered sterile solutions of 10% salicin (1% final concentration of medium) per 90 ml of MRS-basal medium without dextrose just before pouring the agar medium. The plates were incubated aerobically at 37°C for 24–48 h. For the selective enumeration of *L. rhamnosus* and *L. casei*, MRS-vancomycin (**Appendix-V**) agar was prepared by adding vancomycin (Himedia) to achieve a concentration of 1 mg per litre of rehydrated MRS broth (Tharmaraj and Shah, 2003). The plates were anaerobilcally incubated at 37°C for 72 h. This medium was selective only when only one of them has been used for culturing with *S. thermophilus*. The selective differential medium designated as LPSM (*L. plantarum* selective medium) was used for enumeration of *L. plantarum* (Bujalance *et. al.*, 2006). LPSM design (**Appendix-V**) was based on the resistance of the *L. plantarum* to ciprofloxacin and its ability to produce acid from sorbitol. The medium without ciprofloxacin and sorbitol was autoclaved for 15 min at 121^{o}C (15 lbs), and cooled to 50^{o}C. Ciprofloxacin (0.22µm PES hydrophilic membrane) and sorbitol (0.45µm

PVDF membrane) was sterilized by filtration before being added to the cooled medium. When solidified, LPSM had purple color.

3.2.8.7.2 β-glucan content in barley-milk based probiotic curd

In order to minimize the interference of spices and salt in BMFPD samples during estimation of β-glucan content, it was estimated in barley-milk based probiotic curd (intermediate product). Principle and preparation of reagents for the estimation of β-glucan content has been previously mentioned in sub-section **3.2.3.10**. Few modifications in the steps for sample preparation were incorporated. Method recommended by European Brewery Convention (EBC Method 4.16.1) for estimation of β-glucan in malt samples was followed for the preparation of samples. In this preparation, sample was pre-extracted with aqueous ethanol to remove free sugars and to reduce the levels of fats and oils. Samples were then analysed for β-glucan content using streamline 995.16 method of AOAC (1995) for the estimation of β-glucan.

Accurately weighed 1.0 g sample (in duplicate) was mixed with 5.0 ml of 50% ethanol (50 % v/v) in polypropylene tube and mixed using a vortex mixer for 2 min. The tubes were incubated in a boiling water bath for 5 min followed by mixing on a vortex stirrer. Five ml of 50 % (v/v) aqueous ethanol was further added to the tubes and contents were mixed by again using vortex stirrer. The tubes were then centrifuged for 10 min at 1,000 × g and supernatant was discarded. Pellet obtained was re-suspended in 10.0 ml of 50 % (v/v) aqueous ethanol, centrifuged and again supernatant was discarded.

Samples were made wet with 0.2 ml of aqueous ethanol (50 % v/v) to aid dispersion. Four ml of sodium phosphate buffer (20 mM, pH 6.5) was added into the tubes and contents were stirred on a vortex mixer. After mixing, tubes were placed in a boiling water bath for 60 s. Again the contents were vigorously stirred on a vortex mixer and incubated at 100°C for 2 min. Tubes were then incubated at 50°C (5 min) for temperature equilibration. Lichenase (0.2 ml, 10 U) was added to the tubes and stirred well. The tubes were sealed with Parafilm® and incubated for 1 h at 50°C, with intermittent (3-4 times) vigorous stirring on a vortex mixer. Two ml of sodium acetate buffer (200 mM, pH 4.0) was added to the tubes and vigorously stirred. Tubes were allowed to equilibrate to room temperature (5 min), and centrifuged (1,000 × g, 10 min). Aliquots (0.1 ml) were carefully and accurately dispensed into the bottom of three test tubes (12 ml capacity). β-

glucosidase (0.1 ml, 0.2 U) in 50 mM sodium acetate buffer (pH 4.0) was added in two of these tubes (the reaction). To the third (the reaction blank), add 50 mM acetate buffer (0.1 ml, pH 4.0).Tubes were incubated at 50°C for 10 min. Three ml of GOPOD reagent was to each tube, and incubated at 50°C for a further 20 min. After the completion of incubation period absorbance was taken at 510 nm. With each set of determination, reagent blanks and D-glucose standards were also included and processed as discussed in sub-section 3.2.3.7. The β-glucan content of flour samples were calculated by using the following equation:

$$\beta\text{-glucan } (\%\ w/w) = \frac{\Delta A \times F \times\ 64 \times 1 \times 100 \times 162}{1000 \times W \times 180}$$

$$= \frac{\Delta A \times F \times\ 5.76}{W}$$

where,

ΔA = Absorbance after β-glucosidase treatment (reaction) minus reaction blank absorbance.

F = A factor for the conversion of absorbance values to μg of glucose.
= 100 (μg of D-glucose) / absorbance of 100 μg of D-glucose.

64 = Volume correction factor (0.1 ml out of 6.4 ml was analysed)

$\frac{1}{1000}$ = Conversion from μg to mg

$\frac{162}{180}$ = A factor to convert from free D-glucose, as determined, to anhydro-D-glucose, as occurs in β-glucan

W = Calculated dry weight of the sample analysed, in mg

Final concentration of β-glucan content in drink was calculated considering a dilution with 35 % water containing salt, spiced and stabilizer (pectin).

3.2.8.7.3 Sensory evaluation

The samples of BMFPD obtained from different combinations of barley flour, probiotic inoculum and co-culture inoculum level during optimization trails were filled in pre-sterilized PET bottles and capped tightly. Bottles and caps were sterilized in manner as described in sub-section **3.2.8.1**. Filled bottles were stored under refrigeration. Random samples of BMFPD were taken out from the refrigerator presented to the panel of sensory judges consisting 5 trained panelists from the faculty of Dairy Technology Division, National Dairy Research Institute, Karnal for the analysis of different sensory properties namely, colour

and appearance, sedimentation, consistency, flavour and overall acceptability using 9-point hedonic scale (Amerine *et al*., 1965).

3.2.8.7.4 Measurement of viscosity, shear rate / shear stress data and fitting of different rheological models

Measurements for shear rate and shear stress data were taken using the methodology discussed in sub-section **3.2.8.4.1** at two constant temperatures (5 and 20 ± 0.1°C). Viscosity at shear rate of 100 s^{-1} was used to investigate the effect of flour level, probiotic inoculum and co-culture inoculum level. The shear-rate and shear-stress data were fitted to different rheological models using RheoPlus32 (v3.61) software.

3.2.8.7.5 Textural analysis of fermented barley-milk composite

Textural properties of barley-milk based fermented composite samples obtained as an intermediate product during optimization of barley milk based fermented probiotic drink was analysed by using back extrusion test as mentioned in sub-section **3.2.8.4.2.**

3.2.9 Growth kinetics of optimized levels probiotic culture and co-culture in milk with optimized barley flour level

Three batches of toned cow milk in equal volume were heated in a thermostatically controlled water bath to reach a temperature of 70°C with continuous stirring. Two out of three batches were added with optimized level of barley flour and mixed thoroughly using electrical hand blender. Temperature of all three batches was raised to 90°C and held for 25 min, followed by cooling to 37°C in a refrigerated water bath. All the 3 batches were then inoculated with optimized level of probiotic culture. One out of the two batches containing optimized level barley flour and probiotic culture was inoculated with optimized level of co-culture. All three batches namely (i) milk with probiotic culture; (ii) barley-milk composite with probiotic culture and (iii) barley-milk composite with probiotic and co-culture were divided into properly marked sterile sample containers (100 ml) and incubated 37°C. Samples at 0, 2, 4, 6, 8 and 12 h were taken out and serial dilutions were prepared in buffered peptone water. Plating was done using appropriate dilutions by pour plating method for the enumeration of probiotic count. Samples inoculated with co-culture (*S. thermophilus* 20) were plated on ST agar (**Appendix-V**) for the selective enumeration of co-culture

(Dave and Shah, 1996) and incubated at 37°C for 24 h. Samples at different time intervals were also analysed for pH and titratable acidity.

3.2.10 Evaluation of physico-chemical, nutritional, therapeutic, microbiological and sensory properties of barley-milk based fermented probiotic drink

Optimized barley milk based fermented probiotic drink was analysed for different physico-chemical (total solids, fat, protein, ash, phytate content, colour and rheological behaviour), microbiological (probiotic, coliform, yeast and mould and non-lactic acid bacteria count), nutritional (mineral analyses, β-glucan and total dietary fibre content), therapeutic (antimicrobial, ace-inhibitory and antioxidant activity) and sensory attributes. Methods used for the analysis various parameters are described in the following subheadings.

3.2.10.1 Titratable acidity and pH

Titratable acidity and pH of the optimized product were analyzed by method previously mentioned in sub-section **3.2.6.3.2.1.**

3.2.10.2 Total solids

The total solids content of probiotic drink were estimated by gravimetric method as described for milk IS:SP:18, part XI, Handbook of food analysis (1981).

3.2.10.3 Protein

The protein content in optimized probiotic drink by micro Kjeldhal method as per the method of AOAC (1984) previously mentioned in sub-section **3.2.3.3**. One gram sample was taken for estimation of protein in probiotic drink. Percent total protein was calculated by multiplying with a factor of 6.38 (for milk protein) instead of 5.83.

3.2.10.4 Fat

The fat content of BMFPD was determined by Mojonnier method for milk fat estimation of AOAC (989.05, 1995) with few modifications. Approximately 10 g sample was weighed in to Mojonnier fat extraction flask. Weight was recorded nearest to 0.1 mg. To neutralize the acidity and dissolve protein, 2 ml of NH_4OH was added was in the tubes and shaken vigorously. Subsequently, 10 ml of ethanol (95 %) was added and shaken for 90 s. Addition of alcohol prevents gel formation. Further, 25 ml of ethyl ether was added into the tubes and again

shaken for 90 s to dissolve the lipids. Tubes were allowed to cool, added with 25 ml petroleum ether and shaken for 90 s. Petroleum ether removes moisture from the ethyl ether extract and dissolves more non-polar lipids. The contents in tubes were allowed to stand still for 30 min. Ether solution was decanted in a previously dried and weighed fat extraction beaker (supplied with SOCS PLUS, Pelican Equipments, Chennai). Second extraction was performed in manner as for the first extraction (5 ml ethanol, 15 ml ethyl ether and 15 ml petroleum ether). For the third extraction ethanol was not added (15 ml ethyl ether and 15 ml petroleum ether). However, stand still time was reduced to 15 min for second and third extractions. Solvent in the fat extraction beakers were evaporated using the SOCS PLUS (Pelican Equipments, Chennai). The beakers containing extracted fat were placed in an oven at 102°C and the contents were dried until a constant weight was achieved (1-2 h). The beaker was cooled in a desiccator and weighed along with its contents. The content left in the beaker after the extraction was estimated as fat content in the sample and expressed as per cent fat.

$$\text{Fat (\%)} = \frac{\text{Weight of beaker with fat – Weight of empty beaker}}{\text{Weight of sample}} \times 100$$

3.2.10.5 Determination of ash content in barley-milk based fermented probiotic drink

Ash content of probiotic drink samples was estimated by the gravimetric method. Ten gram of sample was weighed and transferred into a pre-weighed porcelain crucible, dried at 100°C for 10 h or overnight and weighed again. The weighed samples were ignited till smoke ceased. The crucibles were then transferred to muffle furnace maintained at 550±5°C and incinerated until light grey ash was obtained. The crucible was then cooled in desiccator and weighed. The results were reported as per cent ash.

$$\text{Ash \%} = \frac{\text{Weight of crucible after ashing – Weight of empty crucible}}{\text{Weight of sample}} \times 100$$

3.2.10.6 Colour

The surface colour of BMFBPD was measured using a ColorFlex® colorimeter supplied by HunterLab (Hunter Associates Laboratory, Inc., Reston, VA, USA) along with the software (version 4.10) and the results were expressed in terms of CIELAB system. Before test, the instrument was calibrated with standard black and white tiles as per the manufacturer instructions. The light

source was dual beam xenon flash lamp. Data was received through the software in terms of L* (lightness), ranging from 0 (black) to 100 (white), a* (redness), ranging from +60 (red) to -60 (green) and b* (yellowness), ranging from +60 (yellow) to -60 (blue) values. For this, freshly prepared BMFPD was tempered at 25°C for 30 min. Later the sample was transferred into the sample container attached with the instrument. Readings were recorded in triplicate.

3.2.10.7 Rheological behaviour of optimized barley-milk based fermented probiotic drink

Rheological behaviour of optimized barley-milk based fermented probiotic drink was analyzed by method previously mentioned in sub-section **3.2.8.4.1**. Measurements were taken at two constant temperatures (5 and 20 ± 0.1°C) and the shear-rate and shear-stress data were fitted to rheological models.

To analyze the effect of temperature, at constant shear rate (50 and 100 s^{-1}), 30 data points were obtained with a temperature ramp from 5 to 25°C within a period of 300 s with a reference temperature of -272.15°C. Activation energy was calculated by fitting Arrhenius equation to the data using RheoPlus32 (v3.61).

3.2.10.8 Phytate content

Phytate content in the probiotic drink samples was estimated by the procedure mentioned in sub-heading **3.2.3.12**. However, sample size for extraction was increased to 5 g, in place of 1 g.

3.2.10.9 β-glucan and total dietary fibre content

β-glucan and total dietary fibre (TDF) content in the optimized probiotic drink were estimated by following the procedure previously mentioned in sub-section **3.2.3.10** and **3.2.3.11**, respectively. Sample size for estimation of TDF was taken as 5 g.

3.2.10.10 ABTS and DPPH free radical scavenging activity

For the estimation of ABTS radical scavenging activity, BMFPD samples were centrifuged at 10,000 × g for 10 min. Supernatant was collected and passed through 0.45 µm syringe driven PVDF membrane filter. Filtrate obtained was directly used in the antioxidant activity estimation as per the procedure mentioned in sub-section **3.2.6.3.2.7**. For DPPH radical scavenging activity 1 ml of sample extracted with 20 ml of 60 % methanol (acidified with 0.1% conc. HCl) with

continuous stirring on a magnetic stirrer, followed by centrifugation at 3000 × g for 10 min. Supernatant obtained was used for the estimation of DPPH radical scavenging activity as per the procedure mentioned in sub-section **3.2.6.3.2.6**. Supernatant obtained was also analysed for ABTS radical scavenging activity.

3.2.10.11 Angiotensin converting enzyme (ACE) – inhibitory activity of barley-milk based fermented probiotic product

Angiotensin converting enzyme (ACE) helps in conversion of angiotensin I to angiotensin II and causes hypertension or cardiac disease. Inhibition of this enzyme is beneficial to prevent such disorders. Therefore, the product was evaluated for ACE inhibitory activity. The assay of ACE-inhibitory activity was based on the liberation of hippuric acid in the presence of ACE from the substrate hippuryl-L-histidyl-L-leucine (HHL) and measured by the method of Cushman and Cheung (1971).

Approximately 25 ml of sample was centrifuged at 6000 × g for 10 min (5°C). The supernatant was collected and pH adjusted to 8.3 using 10 M NaOH. The suspension was again centrifuged at 10000 × g (5°C) for 10 min. Supernatant was collected and filtered through 0.45 µm pore size PVDF syringe driven membrane filter (Whatman®, NJ, US). The filtrate was used for ACE-inhibitory assay.

The HHL solution (5mM) was prepared by dissolving in sodium borate buffer (100 mM, 30 mM NaCl, pH 8.3). ACE solution was prepared by mixing 1 U of ACE in 1 ml of water (HPLC grade). Twenty µl of ACE solution (1mU/ml) was diluted to 100 µl using distilled water, and used in the assay.

Table 3.5 Assay chart for ACE-inhibitory assay

	HHL	Buffer	ACE	Sample	Water	Total
Control	110 µl	100 µl	20 µl	-	20 µl	250 µl
Blank	110 µl	100 µl	-	20 µl	20 µl	250 µl
Sample	110 µl	100 µl	20 µl	20 µl	-	250 µl

In 2 ml micro centrifuge tube, 110 µl of 5mM HHL solution was mixed with 100 µl borate buffer and pre-incubated at 37°C for 3 min. Subsequently, 20 µl of sample (filtrate *i.e.* ACE-inhibitory component) was added into the tubes and reaction was initiated by adding 20 µl of ACE enzyme (4 mU). The mixture was incubated for 30 min at 37°C. Final volume was made up to 250 µl with water. Control was prepared in presence of ACE, but ACE-inhibitory component

(sample) was absent. Blank was prepared without ACE, but in presence of ACE-inhibitory component. Assay chart for ACE-inhibitory assay of control, blank and probiotic-drink samples is indicated in **Table 3.5**.

The reaction was terminated by adding 250 µL of 1N HCl. The hippuric acid liberated by the ACE was extracted by adding 1.7 ml ethyl acetate in to micro-centrifuge tubes followed by centrifugation at 3000 × g for 5 min. Upper layer was siphoned out and evaporated to dry at 95°C in water bath. The residue containing hippuric acid was then dissolved in 1 ml of deionised water and absorbance was measured at 228 nm against blank (distilled water).

$$\%\text{ACE-inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}} \times 100$$

3.2.10.12 D/L−lactic acid

D and L-Lactic acid content in BMFPD samples were estimated by following the procedure of Noll (1988) and Gawehn (1988), respectively using D/L-lactic acid kit assay (Megazyme International Ireland Limited, 2011f).

The quantification of D-lactic acid requires two enzymatic reactions. In the first reaction, D-lactate is oxidised to pyruvate by nicotinamideadenine dinucleotide (NAD^+) (eq-i), which is catalysed by D-lactate dehydrogenase (D-LDH).

$$\text{D-Lactate} + NAD^+ \overset{\text{D-LDH}}{\Longleftrightarrow} \text{pyruvate} + NADH + H^+ \qquad \text{---(i)}$$

However, since the equilibrium of reaction (eq-i) lies firmly in the favour of D-lactic acid and NAD^+, a further reaction is required to trap the pyruvate product. This is achieved by the conversion of pyruvate to D-alanine and 2-oxoglutarate, with the enzyme D-glutamate-pyruvate transaminase (D-GPT) in the presence of a large excess of D-glutamate (eq-ii).

$$\text{Pyruvate} + \text{D-glutamate} \xrightarrow{\text{D-GPT}} \text{D-alanine} + \text{2-oxoglutarate} \qquad \text{---(ii)}$$

The amount of NADH formed in the above coupled reaction is stoichiometric with the amount of D-lactic acid. It is the NADH which is measured by the increase in absorbance at 340 nm. In a similar set of reactions, L-lactic acid (L-lactate) is oxidised to pyruvate by nicotinamide-adenine dinucleotide (NAD+) and L-lactate dehydrogenase (L-LDH) (eq-iii).

$$\text{L-Lactate} + NAD^+ \xrightarrow{\text{L-LDH}} \text{pyruvate} + NADH + H^+ \qquad \text{---(iii)}$$

Pyruvate is again trapped using D-GPT in the presence of a large excess of D-glutamate (eq-ii).

Reagents provided in kit were glycylglycine buffer (0.5 M, pH 10.0, 0.5 M D-glutamate, 0.02 % sodium azide); NAD^+; D-Glutamate-pyruvate transaminase (D-GPT) suspension (1,300 U/ml); L-Lactate dehydrogenase (L-LDH) suspension (2,000 U/ml); D-Lactate dehydrogenase (D-LDH) suspension (20,000 U/ml); D/L-Lactic acid standard solution (0.15 mg/ml of each).

3.2.10.12.1 Preparation of reagents and working solution

NAD^+ (380 mg) was dissolved in in 5.5 ml of distilled water, divided into appropriate size aliquots and stored at −20°C. All other chemicals provided in kit except NAD^+ were stored at 4°C. Carrez I solution was prepared by dissolving 3.60 g of potassium hexacyanoferrate $\{K_4[Fe(CN)_6].3H_2O\}$ in 100 ml of distilled water. Carrez II solution was prepared by dissolving 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) in 100 ml of distilled water.

3.2.10.12.2 Sample preparation for D/L-lactic acid estimation

Accurately 1 g of BMFPD samples was weighed into a 100 ml volumetric flask followed by adding 60 ml of distilled water. Contents were mixed thoroughly and 2 ml of Carrez I solution was added. After mixing, 2 ml of Carrez II solution was added and mixed again. Subsequently, 4 ml of NaOH solution (100 mM) was added in to the volumetric flask and volume was made up to mark with distilled water. The contents were mixed thoroughly and filtered using Whatman No. 42 filter paper.

3.2.10.12.3 D/L-lactic acid assay procedure

For L-lactic acid, L-LDH suspension was used followed by an incubation period of 20 min, whereas for D-lactic acid, D-LDH suspension was used and incubation time was reduced to 10 min. Reactions for estimating D/L lactic acid was imitated by mixing 1.50 ml distilled water, 0.10 ml sample extract, 0.50 ml glycylglycine buffer, 0.10 ml NAD^+ solution and 0.02 ml D-GPT in disposable cuvette. Reaction blank was also prepared in similar manner without addition of sample and increasing the amount of distilled water by 0.10 ml. Contents in cuvette were mixed by inversion and absorbance (A_1) at 340 nm was taken after 3 min of incubation at ambient temperature. D-LDH (or L-LDH for L-lactic acid; 0.02 ml) was added to the cuvette and contents were mixed by inversion.

Absorbance (A_2) at 340 nm was taken after 20 min (10 min in case of L-lactic acid) of incubation at ambient temperature.

3.2.10.12.4 Calculation of D/L-lactic acid

Absorbance difference (A_2-A_1) was determined for sample and blank. The absorbance difference of the blank was subtracted from the absorbance difference of the sample, thereby obtaining $\Delta A_{D/L\text{-lactic acid.}}$ The value of $\Delta A_{D\text{-lactic}}$ acid should at least 0.100 absorbance units to achieve accurate results. The concentration of D/L-lactic acid was calculated as follows:

$$c = \frac{V \text{ x } MW \text{ x } F}{\varepsilon \text{ x } d \text{ x } v} \times \Delta A_{D/L-\text{lactic acid}} \quad [g/l\,]$$

where,

v = final volume (ml)

MW = molecular weight of D/L- lactic acid (g/mol)

ε = extinction coefficient of NADH at 340 nm = 6300 (l x mol-1 x cm-1)

d = light path (cm)

v = sample volume (ml)

F=dilution factor

$$c = \frac{2.24 \text{ x } 90.1 \text{x } F}{6300 \text{ x } 1.0 \text{ x } 0.1} \times \Delta A_{D/L-\text{lactic acid}} \quad [g/l\,]$$

3.2.10.13 Mineral analysis

The most commonly used technique for qualitative and quantitative determination of minerals in food samples is atomic absorption spectrometry (Vaughan *et al*., 1979; Zafar *et al*., 2010). The calcium (Ca), iron (Fe) and Zinc (Zn) were estimated using atomic absorption spectrophotometer (Hitachi Z-5000 Polarized Zeeman) equipped with a Zeeman background corrector and data processor using acetylene as fuel and air as oxidant following AOAC (1990) method. Specific hollow cathode lamps were used for the determination of elements.

One g sample was transferred in a pre-weighed porcelain crucible. The sample was charred over open flame till smoke ceased. The crucible was then transferred to muffle furnace maintained at 550 and incinerated until light grey ash was obtained. In cooled ash, concentrated HNO_3 was added and again digested for 15 min. Digested sample was mixed with glass rod and transferred in to 100 ml volumetric flask. Another 5 ml of concentrated HNO_3 was added and

transferred the contents in the flask and volume was made up to 100ml with distilled water, and used for the determination of minerals.

Table 3.6 Instrument setting for flame atomization

Parameters	Ca	Fe	Zn
Wavelength (nm)	422.7	248.3	213.9
Lamp current (mA)	10.0	15.0	6.5
Slit width (nm)	0.2	0.2	1.3
oxidant: air; oxidant pressure: 160; fuel: air-C_2H_2; Fuel pressure: 2.2 l/min; calculation mode: integral; calculation time: 5 s;			

The procedure described in AAS manual was followed for the determination of Ca, Fe and Zn. In the estimation of Ca, elements such as aluminum, beryllium, phosphorus, silicon, titanium and mineral acids are known to mask the response of Ca, in air acetylene flame. Therefore, 1% lanthanum chloride was added as the beam masking agent in the process of dilution of acid extract. Results were reported in ppm. Instrument setting for flame atomization is indicated in **Table 3.6**.

3.2.10.14 Antimicrobial activity

Antimicrobial activity of barley-milk based fermented probiotic drink against four test organisms, namely *Escherichia coli*, *Salmonella typhii*, *Shigella dysentriae* and *Staphylococcus aureus* was estimated using agar well diffusion assay as per method of Schillinger and Lucke (1989) with some modifications. The method is based on the principle which involves the ability of one microorganism to inhibit the growth of another, as exhibited by clear zone of inhibition. To check the antimicrobial activity, nutrient agar plates (15-20 ml) were prepared and allowed to solidify. Then the nutrient agar plates were overlaid with 7 ml of soft agar (0.7 % agar) inoculated with 50 µl of overnight grown cells of indicator strains. The soft agar was allowed to solidify in refrigerator (5°C) for 1 h. Wells of 6 mm diameter were punched out of the agar using a sterile well borer. The wells were sealed with 10 µL soft agar. The wells were filled with 50 µL cell free filter sterile supernatant of BMFPD. Cell free sterile supernatant was obtained by centrifugation of BMFPD at 10,000 × g for 10 min followed by filtration of supernatant through a syringe driven PVDF filter (45 µm, Whatman®, NJ, US). The plates were kept under refrigeration (5°C) for 3-4 h to facilitate the diffusion of supernatant into agar. The plates were then incubated at 37°C for 24

h. The diameter of zone of inhibition extending laterally around the well was measured and a clear zone of 1 mm or more was considered positive inhibition.

3.2.10.15 Probiotic count

Probiotic count of optimized barley-milk based fermented probiotic drink was estimated by procedure previously mentioned in sub-section **3.2.8.7.1**.

3.2.10.16 Coliform count

Violet Red Bile Agar (**Appendix-V**) was used for the enumeration of coliforms. This medium was not sterilized. It was only boiled for few min. Approximate dilutions of the sample in sterile buffered peptone water were pour plated in triplicates and incubated at 37^{o}C for 48 h. The red or dark pink colonies which appeared both on surface and sub-surface after incubation were counted.

3.2.10.17 Yeast and mould count

Potato dextrose agar medium (**Appendix-V**) was used to determine the yeast and mould count in the samples. The medium was sterilized by autoclaving at 121^{o}C for 15 min. The pH of the medium was adjusted to 3.5 at the time of pouring the plates with sterile 10% tartaric acid. The plates were incubated at 22^{o}C for 3-5 days and colonies of yeast and mould were counted.

3.2.10.18 Non-lactic acid bacteria count

Non-LAB count of in BMFPD was estimated using count agar sugar free (CASF) medium. CASF medium (**Appendix-V**) is suitable for enumeration of the non-LAB contaminating microflora of fermented foods (Angelidis, 2006). CASF contains peptone, sodium chloride and agar only, and, being a sugar-free medium, cannot support the growth of LAB that are strictly fermentative organisms. Non-LAB are infective (non-indigenous) microorganisms, which are not directly involved in the microbiological production of a food product, or which do not belong to its specific flora (International Dairy Federation). The medium was sterilized by autoclaving at 121°C for 15 min. Appropriate decimally diluted volumes in sterile buffered peptone water were pour-plated in triplicate. All plates were left for 2 h at room temperature and then incubated for 48 h at 35°C with subsequent additional incubation for 48 h at 20°C. All colonies formed were counted were counted and expressed in cfu/ml of barley-milk based fermented probiotic drink.

3.2.10.19 Sensory quality

Samples of optimized BMFPD were taken out from the refrigerator and presented to panel of sensory judges for analysis of different sensory attributes such as colour and appearance, sedimentation, flavour, consistency and overall acceptability using 9-point hedonic scale.

3.2.11 Evaluation of shelf life

Optimized probiotic drink was prepared and filled in pre-sterilized clear PET bottles and LDPE pouches. PET bottles and caps were sterilized by following the procedure previously mentioned in 3.2.8.1 and prepared drink was filled manually under laminar flow in bio-safety cabinet. The fill pack machine having capacity of 50 pouches per min installed at the experimental dairy of National Dairy Research Institute, Karnal was used for the packaging of BMFPD with adjustment to fill 200 ml the product per pouches. LDPE film was sterilized by exposing to UV light provided in the fill pack machine. The packed product was stored at 5±1°C. BMFPD samples were analysed for physico-chemical (pH, titratable acidity, free fatty acids content, proteolysis, whey separation and viscosity), microbiological (probiotic count, coliform count, yeast and mould count and Non-LAB count) and sensory quality (colour and appearance, sedimentation, flavour, consistency and overall acceptability) at 0, 3, 6, 9, 12 and 15 day(s).

3.2.11.1 Titratable acidity and pH

Titratable acidity and pH of the optimized product were analysed by method previously mentioned in sub-section **3.2.6.3.2**.

3.2.11.2 Total free fatty acids

The total free fatty acid (FFA) content of fresh as well as stored barley-milk based probiotic drink samples was estimated using the method described by Deeth and Fitzgerald (1976) with slight modification in the terms of expressing the results. The method involves the extraction of fat from a known quantity of sample and estimation of the FFA contained in the extracted fat by titrating against a standard alkali.

Five g BMFPD sample was taken in a 50 ml graduated and stoppered test tube. Five ml of distilled water was added followed by the addition of 10 ml of extraction mixture containing ispropanol (2- propanol), petroleum ether and 4.0 N H_2SO_4 in proportion of 40:10:1. An additional 6.0 ml of petroleum ether was then added. The test tubes were stoppered and tempered at 40°C for 10 min. The

contents were vigorously shaken and allowed to stand for few minutes (5-10 min), and an aliquot of upper layer (5 ml) was withdrawn and transferred to 50 ml conical flask. After addition of 2 drops of methanolic naphtholphthalein indicator (1 g naphtholphthalein in 100 ml methanol), the contents were titrated against 0.02 N methanolic KOH solution. The reagent blank was used to obtain background titration. The FFA content was determined using the following formula:

$$\text{FFA (µeq/g sample)} = \frac{\text{Net titre volume} \times \text{Normality of KOH}}{\text{Proportion of upper layer titrated x Weight of sample}} \times 10^3$$

Standardization of alkali: Potassium hydrogen phthalate was dried for about 2 h at 100°C and 0.03 g weighed out accurately and dissolved in 5 ml of water. The solutions are titrated with 0.02N methanolic potassium hydroxide using 5 drops of 1% methanolic phenolphthalein as indicator. Titrations were carried out in triplicate, which required 9 ml of alkali. The normality (N) of the alkali is calculated from the formula:

$$\text{FFA (µeq/g sample)} = \frac{\text{Weight of potassium hydrogen phthalate (g)}}{\text{203.23 x Titre Value}} \times 10^3$$

3.2.11.3 Measurement of proteolytic activity by *o*-phthaldialdehyde method

The extent of proteolysis in the fresh as well as stored samples of barley-milk based fermented probiotic drink was assessed by measuring liberated amino acids using the *o*-phthaldialdehyde (OPA) method described by Church *et al*., (1983). Protein substrates were prepared by mixing 5 ml of sample with 1 ml of water followed by adding 10 ml of 0.75 N trichloroacetic acid (TCA) while vortexing. Samples were then centrifuged at 10,000 × g and supernatant obtained was filtered through 0.45 µm PVDF syringe driven filter (Whatman®, NJ, US). OPA reagent was prepared fresh following the method of Goodno *et al.* (1981). OPA solution was prepared by mixing 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% sodium dodecyl sulfate (SDS), 40 mg of OPA dissolved in 1ml ethanol, 100 µl of β-mercaptoethanol and diluting the final volume up to 50 ml by distilled water. To assay the proteolysis, 50µl proteins as substrates was added directly in to 1 ml of OPA reagent in 1.5 ml quartz cuvette and mixed briefly by inversion. Solution was incubated exactly for 2 min at room temperature and absorbance was read at 340 nm on UV-VIS spectrophotometer.

The extent of proteolysis was assessed by plotting L-leucine calibration curve (from 40 to 450 µg/ml) and expressed as mg of L-leucine per gram sample.

The equation for the calibration curve (**Appendix-XIII**) was $Y = 0.0022X - 0.0045$ (where, X = concentration of L-leucine in µg/ml; Y = absorbance), and correlation coefficient was R^2 = 0.99).

3.2.11.4 Whey separation

Whey separation was measured using gravity separation. Freshly prepared sample (100 ml) in triplicate was filled up to the mark in 100 ml clear graduated measuring cylinder (Borosil). The measuring cylinders were sealed with the Parafilm® and kept undisturbed under refrigerator at 5±1°C. Separation of the serum fluid from the gel matrix was visually measured during storage at regular interval of 3 days.

3.2.11.5 Probiotic count, coliform count, yeast and mould count and non-LAB count

Probiotic count, coliform count, yeast and mould count and non-LAB count in the stored samples were estimated by procedure previously mentioned in sub-section **3.2.8.7.1**, **3.2.10.16**, **3.2.10.17** and **3.2.10.18**, respectively.

3.2.11.6 Sensory quality

Random samples of packed BMFPD were taken out from the refrigerator and presented to panel of sensory judges for analysis of different sensory attributes such as colour and appearance, sedimentation, flavour, consistency and overall acceptability using 9-point hedonic scale.

3.2.12 Statistical analysis

All the experiments were carried out in triplicate and replicated at least thrice. Results are expressed as mean ± standard deviation (SD). The mean value and standard deviation were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA). The data obtained during analyses were subjected to analysis of variance in a completely randomized design followed by Fisher's least significant difference ($p<0.05$) for multiple sample compassion as described by Snedecor and Cochran (1989). Optimization of product was carried out by Response Surface Methodology (RSM) using Design-Expert® (ver 8.0.1) software (Stat-Ease Corporation, USA).

3.2.13 Techno-economic feasibility

Success of a newly developed product exists on the economic feasibility of manufacturing process. Having established the technology for preparation of barley-milk based fermented probiotic drink, the next logical step would be to

estimate the total cost of product. Methods suggested by Peter and Timmerhaus (1968).and case studies of Singh and Kalra (1979) and Chauhan *et al.* (2006) were used with following basic assumptions for the estimation of cost for barley-milk based fermented probiotic drink in pack size of 200 ml.

1. The plant is a multi-product dairy and handles 75,000 litres of milk per day, and manufactures yoghurt as a diversified line. The quantity of product made is equal to 2500 litres.
2. The product is manufactured round the year.
3. The price of the cow milk was assumed to be Rs. 30 per kg.
4. The prices for raw materials like starter cultures, barley, PET bottles and of power and utilities like steam, refrigeration, electricity and water was based on the current market prices.
5. The handling losses of 0.5% for raw material were assumed.
6. The overhead cost would be 15% of the net manufacturing cost, which includes administrative expenses, consultancy and contingencies charges, research and developments and other general expenses.
7. The detailed process of production for BMFPD has been indicated in **Figure 3.2**. Barley flour, probiotic inoculum, co-culture inoculum were added at their optimized levels.

3.2.14 Assessment of Consumer responses

A total of 100 respondents representing potential consumers belonging to mixed age group, sex as well as from different economic group were provided with the optimized barley milk based fermented probiotic drink samples to assess their response to acceptability of the developed product. A questionnaire (**Appendix-XIV**) along with the sample was presented to the potential consumers for collecting data regarding personal information (age, sex, location and occupation), awareness regarding health benefits of fermented milk products and perception about the developed probiotic drink. Responses for perception about colour and appearance, taste, saltiness and overall liking was obtained by using 9-point hedonic scale. Willingness to purchase the developed probiotic drink was also analysed at comparable and 25 % higher cost.

CHAPTER 4

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

4.1 PROLOGUE

Barley is among the important food crop globally, which is mainly processed in the form of malt for the production of health foods and alcoholic beverages. It is an excellent source of B-complex vitamins, minerals, complex polysaccharides (Kalra and Jood, 2000) and rich source of natural antioxidants (Cook and Sammon, 1996). Barley grains are gaining renewed interest as a food component because of its soluble and insoluble dietary fiber endowed with numerous health benefits. However, presence of certain anti-nutrients like phytic acid and tannins limit the consumption of barley as food component. Phytic acid content in barley adversely affects the bioavailability of essential minerals by forming insoluble mineral-phytate complexes, thereby inhibiting their absorption in gut mucosa (Sandström and Sandberg, 1992; Bohn *et al.,* 2004), whereas, tannins reduces the biological value of food proteins by forming complexes with them (Hagerman *et al.* 1998). Moreover, the level of certain essential amino acids such as threonine, lysine and tryptophan is lesser in barley grains as compared to other food grains, rendering them less nutritious (Chavan and Kadam, 1989). Milk though referred as “complete food” lack fibre and certain micronutrients. Combining barley with milk offers enhanced nutritional value of the composite, due to the complimentary action of milk and cereal nutrients.

Use of barley as suitable substrate for the growth of probiotic microorganisms and improved functionality of colonic strains due to the presence of specific non-digestible components such as β-glucan, arabinoxylan, galacto- and fructo-oligosaccharides has been advocated by Charalampopoulos *et al.,* 2002a. β-glucan, the soluble dietary fibre of barley grain enhances the viability of probiotic lactic acid bacteria (Elsanhoty *et al.,* 2009) and contributes to various physiological health benefits including decreased cholesterol absorption, lowering in blood glucose level and improved gut microbial balance. Recently, few probiotic microorganisms have been reported to possess phytate degrading activity, which may result increased mineral absorption in the intestine (Bering *et al*, 2007; Haros *et al.*, 2009). Probiotic cultures with phytate and tannin degrading activity can be utilized for the fermentation of barley-milk composite, which offers

a newer opportunity to utilize the fermented mass for the development of health food products.

The present investigation was carried out with the aim to develop a barley-milk based fermented probiotic drink (BMFPD) by utilizing suitable probiotic lactic acid bacteria. A suitable probiotic lactic acid bacterium was screened out amongst five randomly selected lactobacilli on the basis of phytase activity, anti-nutrient reduction potential, co-culture compatibility and sensory evaluation of barley-milk based fermented probiotic composite. Probiotic products obtained using a single probiotic culture as starter have been reported to exhibit poor sensory characteristics, thus limiting consumer acceptability (Basu *et al*., 2011). Moreover, they often lack technological acceptability due to slower rate of acid production. Therefore, probiotic strain was co-cultured along with *Streptococcus thermophilus* 20 for the development of BMFPD. Various processing parameters *viz*., type of milk, type of barley flour, flour level, probiotic inoculum level and co-culture inoculum level were optimized during the development of BMFPD.

The optimized probiotic drink was evaluated for different physico-chemical, nutritional, microbiological, sensory and therapeutic properties. Shelf life of the developed product was evaluated in LDPE pouches and PET bottles, at 5±1°C. Techno-economic feasibility and consumer responses were also analysed. Results obtained for the analysis of raw materials, selection of suitable probiotic culture, optimization of processing parameters, analysis of optimized product, techno-economic feasibility and consumer responses are presented in this chapter and discussed. The tables concerning the statistical analysis are also included in the body of the text to facilitate discussion.

4.2 ANALYSIS OF MILK

4.2.1 Acidity, fat content and solid-not-fat content of milk

The acidity (% lactic acid), fat content (%) and solid-not-fat content (%) of freshly procured cow milk varied from 0.12 to 0.13, 3.9 to 4.3 and 8.65 to 9.12, respectively. Skimmed cow milk (<0.3 % fat content) was obtained by skimming using a centrifugal separator and solid-not-fat (SNF) content was adjusted to 12 percent by using skim milk powder. Fat and SNF content in double toned milk varied in range from 1.5 to 1.6 and 9.0 to 9.1 percent, respectively, whereas, the range for toned milk varied in between 3.0 to 3.1 and 8.5 to 8.7 percent. The calorie content of milk depends on the fat, protein and carbohydrate content. In

general, toned milk, double toned milk and skim milk provides an approximate energy value of 62 kcal, 48 kcal and 35 kcal per 100ml, respectively.

4.3 ANALYSIS OF BARLEY FLOUR

4.3.1 Proximate composition of barley flour obtained from non-germinated, germinated and imbibed barley grains

The flour samples prepared from non-germinated, germinated and imbibed barley grains were analysed for moisture, crude fat, protein, crude fiber, ash, total dietary fibre, β-glucan and phytate content. Compositions of barley flour obtained from non-geminated, germinated and imbibed barley grains are presented in **Table 4.1**. Barley flour obtained from non-germinated grains contained 7.37, 3.05, 11.27, 2.82 and 1.66 percent moisture, fat, protein, crude fibre and ash content, respectively.

Composition of barley flour largely varies due to differences in genotypes, primary processing intervention applied to grains and further refining of resultant flour (Pedersen *et al.*, 1989; Kong *et al.*, 1995; Marquez-Cedillo *et al.*, 2000; Fregeau-Reid *et al*., 2001). Compositional variations in crude fat, crude protein, crude fibre and ash content of barley flour prepared from hulled and hull-less barley including normal, waxy and high-amylose starch varieties have been observed by various workers, which ranged from 1.7 to 2.7, 10.5 to 16.5, 1.4 to 6.7 and 1.6 to 2.8 percent, respectively (Bhatty 1986; Bhatty, 1999; Din *et al.*, 2009; Arif *et al.*, 2011). Proximate composition of barley flour prepared from non-germinated barley grains in the present investigation were closer to findings of Helm and Francisco (2004), who reported ash, protein, starch, total dietary fibre and β-glucan in the range of 1.51 to 2.27, 12.55 to 15.92, 57.26 to 63.14, 12.37 to 17.39 and 3.70 to 5.77 percent (w/w db), respectively.

Germination of cereals increases the relative nutritional value via synthesis of vitamins (Malleshi and Desikachar, 1986; Kaukovirta-Norja *et al.*, 2004), release of minerals (Hemalatha *et al.*, 2007; Hübner *et al.*, 2010) and, enhancement in the concentration of antioxidants (Bicka *et al.*, 2011; Donkor *et al.*, 2012). Germination / sprouting have been reported to decrease anti-nutritional components such as phytic acid (Bartnik *et al.*, 1987; Egli *et al.*, 2002; Coulibaly *et al,* 2011) and tannins (Rao *et al*, 1982; Osuntogun *et al.,* 1989; Hemalatha *et al.*, 2007).

Table 4.1 Compositional and other chemical parameters for barley flour obtained from non-germinated, germinated and imbibed barley grains

Parameters	NG*	G*	IG*
Moisture (%)	7.37± 0.01	8.24±0.02	8.16±0.01
Crude fat (%, db)	3.05± 0.02^{a}	2.53±0.04^{c}	2.82±0.02^{b}
Protein (N×5.83, %, db)	11.27± 0.04^{a}	10.76±0.02^{c}	10.96± 0.03^{b}
Crude fiber (%, db)	2.82±0.02^{a}	2.98±0.03^{b}	2.89±0.03^{ab}
Total carbohydrate (%, db)	79.75±0.27^{b}	81.73±0.24^{a}	80.44±0.23^{b}
Ash (%, db)	1.66±0.02^{a}	1.57±0.02^{c}	1.51±0.01^{b}
Total Dietary Fibre (%, db)	13.24±0.08^{b}	13.98±0.04^{a}	13.76± 0.05^{a}
β-glucan (%, db)	6.83± 0.04^{a}	4.57±0.01^{c}	5.95± 0.02^{b}
Phytate (mg/g, db)	8.05±0.07^{a}	5.74±0. 03^{c}	6.96 ±0. 02^{b}
Tannin (CE^{Θ} mg/g , db)	2.97±0.04^{a}	1.53±0.01^{c}	1.99±0.01^{b}
Alcoholic acidity (%; as H_2SO_4)	0.17±0.01^{c}	0.24 ±0.02^{a}	0.21±0.01^{b}
Starch (%, db)	64.49±0.19^{a}	59.51±0.18^{c}	62.03±0.11^{b}
Reducing sugar (mg maltose/g, db)	5.43±0.09^{c}	25.95±0.09^{a}	21.74±0.12^{b}
Glucose (mg/g, db)	1.28±0.03^{c}	8.51±0.07^{b}	13.79±0.09^{a}
Total phenolics (mg GAE/g, db)	2.63±0.02^{c}	3.08±0.02^{b}	2.77±0.02^{a}
DPPH radical scavenging activity ($TEAC^{\Phi}$ mg/g, db)	1.90±0.01^{c}	2.60±0.02^{a}	2.01±0.02^{b}
ABTS radical scavenging activity ($TEAC^{\Phi}$ mg/g, db)	8.30±0.01^{c}	8.46±0.00^{a}	8.41±0.00^{b}
Free amino nitrogen (mg/g, db)	0.78±0.01^{b}	0.89±0.01^{a}	0.86±0.00^{a}

*barley flour obtained from NG: non-germinated, G: germinated, and IG: imbibed barley grains; $^{\Phi}$TEAC: trolox equivalent antioxidant capacity; $^{\Theta}$CE: catechin equivalent; Means with different superscripts in same row differ significantly ($\rho<0.05$, Fisher LSD); Values in each cell represent mean ± SE, n=3

Crude fat and protein content of barley flour were significantly ($\rho<0.05$) affected by germination and imbibition processes. Barley flour obtained from non-germinated, germinated and imbibed barley grains contained 3.05, 2.53 and 2.82 percent crude fat, respectively. Process of germination has been reported to decrease the polar lipids, triglycerides and sterol esters plus hydrocarbons content, with increase in concentration of monoglycerides; 1, 2 and 2, 3 di-glycerides; sterols; 1,3 diglycerides and free fatty acids content (Youssef *et al.*, 2012). The changes in lipids fractions might be due to hydrolysis of triglycerides and polar lipid components into simpler compounds by the action of native during germination process (Youssef *et al*, 1985).

Crude protein content in barley flour from non-germinated grains was 11.27 percent, which decreased to 10.76 percent and 10.96 percent due to germination and imbibitions process respectively. Barley flour obtained from germinated grains contained 4.52 percent lesser protein than the protein content of barley flour milled from non-germinated barley grains, whereas flour from imbibed barley contained 2.75 percent lesser protein. Arora *et al.* (2010) reported significant difference in the protein content of barley based food mixture prepared by using non-germinated and germinated barley grains. Results obtained during present study were in agreement with the findings of Akubor and Obiegbuna (1999), who reported reduction in total protein content with increased protein extractability in germinated finger millet grains. Afify *et al.* (2012) also observed significant decrease in crude protein content in germinated sorghum.

Wu (1983) studied the effect of germination on oat protein and observed a decreasing amount for globulin and prolamin fractions of oat protein, but an increasing content for non-protein nitrogen, albumin, and residue nitrogen content. Lysine content was reported to increase by germination process. Findings of previous authors were supported by Kumar and Matta (2011), who reported decrease in the levels of protein, albumins, globulins and prolamins in germinating barley seeds. Decrease in the protein content in barley flour obtained from germinated and non-germinated grains may be related to the activity of different proteinases and extent of protein degradation contributing towards the final status of a protein level. Decrease in protein content during germination might be due transfer of nitrogen to the rootlets (Taylor, 1983), which are usually removed prior to preparation of barley flour.

Crude fibre content in barley flour obtained from germinated (2.98 %) and imbibed (2.89 %) barley grains were slightly higher than the flour obtained from non-germinated (2.82 %) barley grains. The results were supported by the findings of Arif *et al.*, (2011) who studied the effect of malting on the nutrient profile of barley, and observed significant increase from 5.90 to 8.15 percent in the crude fibre content. .

Total carbohydrate content in flour obtained from non-germinated grains was 79.7 percent (db). A significant increase in the total carbohydrate was observed in flour due to germination of barley grains. However, imbibition of barley grains has not significantly influenced the total carbohydrate content in flour.

Ash content of barley flour decreased significantly ($\rho<0.05$) due to differences in the treatment of barley grains. The decrease in the ash content of barley flours in germinated and imbibed grains was observed, which was due to the solubility of minerals in water and leaching out during processing (Pawar and Machewad, 2006). The loss in ash content may also be attributed to the redistribution of ash from barley seeds to growing roots and shoots during germination process (Arif *et al.*, 2011).

Both, germination and imbibition processes have led to a significant ($\rho<0.05$) increase in the alcoholic acidity of barley flour. Alcoholic acidity of barley flour was observed to be 0.17 (% H_2SO_4), which increased to 0.24 and 0.21 by germination and imbibition process, respectively. Jood *et al*., (2012a) reported slight increase in the titratable acidity from 1.71 to 2.04 percent lactic acid for sorghum based food mixture (prepared by mixing 100 g flour with 500 ml distilled water) due to germination of grains. Increase in acidity was attributed to starch hydrolysis into simple sugars during germination, which is readily utilized by the organisms for conversion into lactic acid.

4.3.2 Effect of germination and imbibition of grains on total dietary fibre and β-glucan content of barley flour

Intake of dietary fibre has been associated with various positive health effects caused by the uptake of whole cereals grain (Marlett and Slavin, 2002; Keenan *et al.*, 2007; Ames and Rhymer, 2008, Cui and Wang, 2009). β-glucan, a cell wall structural component of barley endosperm is the most abundant source of soluble dietary fibre, which is associated with the improvement of cardiovascular health, diabetic status and enhanced immune system through fermentation by colonic microflora. Total dietary fibre and β-glucan content of barley flour obtained from non-germinated grains were 13.24 % and 6.83%, respectively, which increased slightly for dietary fibres on germination and imbibition. However, a marked decrease in β-glucan content was observed with the same treatment. Several workers reported the values of total dietary fibre and β-glucan content of barley in the range of 7.5 to 16.8% and 3.7 to 9.0 %, respectively (Vasanthan *et al.*, 2002; Helm and Francisco, 2004; Oscarsson *et al.* 1996).

A slight increase in total dietary fibre content has been previously observed by Hubner *et al.*, (2010) during germination process and they could be

attributed to the loss of starch and synthesis of fibrous compounds for the development of rootlets in growing plants. Rootlets are usually removed for the preparation of barley flour. Significant reduction of 33.1 % and 12.9 % in β-glucan content was observed as a result of germination and imbibition process, respectively (**Table 4.1**). Germination period of 48 h has been previously reported for a considerable loss of 67 % β-glucan which has been caused by the activity of beta-glucanases in barley grains (Hubner *et al.*, 2010).

Kihara *et al.,* (2007) studied the effect of 48 h imbibition (four cycles of 5 h immersion plus 7 h non-immersion at 15°C and 95% humidity) and subsequent germination up to 144 h on the changes in β-glucan and γ-aminobutyric acid (GABA) content of barley, and found 48 h imbibition process is optimal for accumulation of γ-aminobutyric with a concomitant loss of 15.7 % in β-glucan. Increase in germination time has been reported to increase GABA content in barley (Chung *et al.*, 2009; Donkor *et al.*, 2012). Results obtained in the present study for loss in β-glucan content during imbibition process are in agreement with the findings of Kihara *et al.*, (2007), who observed no change in β-glucanase activity up to 48 h imbibition, which increased rapidly during subsequent germination process. During germination and imbibition process partial solubilisation of β-glucan takes place which might have led it to leach out during processing.

4.3.3 Effect of germination and imbibition of grains on total starch, reducing sugar and glucose content of barley flour

Starch, a major storage polysaccharide of cereal grains exists in the form of discrete particles or granules (MacGregor *et al.*, 1971), which undergoes hydrolysis during germination process by the action of hydrolytic enzymes (Karki and Kharel, 2012). Starch content in barley flour was found to decrease by 7.72 % in germinated grains and 3.81 % in imbibed grains, when compared with non-germinated grains (**Table 4.1**). Holtekjolen *et al.*, (2006) analysed a large number of barley varieties for starch and non-starch polysaccharide content and reported them in the range of 51 to 64 % and 23 to 41%, respectively. Although, α-amylase synthesized by the embryo and aleurone cells during germination was believed to be responsible for starch hydrolysis (MacLeod *et al*., 1964; MacGregor *et al*., 1984), role of α-glucosidases in addition to α-amylase was reported later to be involved in the hydrolysis of starch and maltose (Sun and Henson, 1990). Currently, four enzymes namely α-amylase, β-amylase,

dextrinase and α-glucosidase have been involved in the conversion of starch to simple sugars (Kaneko *et al.*, 2002; Fox *et al.*, 2003; Bamforth, 2009).

The reducing sugar content of barley flour obtained from germinated (25.95 mg maltose/g) and imbibed (21.74 mg maltose/g) grains were significantly ($p<0.05$) higher than the flour obtained from non-germinated (5.43 mg maltose/g) barley grains. Increase in reducing sugar content could be attributed to enzymatic modification of starch into simple sugars. Results obtained were in accordance with the findings of Arif *et al.* (2011) who reported 380% increase in reducing sugar content with concomitant 33% decrease in the starch of barley on germination. A 378 % rise in the glucose content of barley flour has been observed in germinated barley grains, whereas imbibition of barley grains accounted for 300% increase in glucose content. Stanley *et al.* (2011) studied the role of α-glucosidase in germinating barley grains and noted that, it can hydrolyze both starch and maltose, besides converting maltose to glucose. Maltose has been reported to account for 50–60% of the total fermentable sugar in conventional wort, whereas maltotriose and glucose contributed around 15 to 20 and 10 to 15 % (Stewart and Russell, 1998; Stewart, 2009).

4.3.4 Effect of germination and imbibition of grains on total phenolic content of barley flour

Barley grain phenolic compounds consists polyphenols, phenolic acids, proanthocyanidins and catechins, which contributes to the antioxidant activity, may negatively influence the taste of cereal grains; and remain concentrated in the outer layers including hull, testa, and aleurone layers (Bendelow and LaBerge, 1979; Nordkvist *et al.*,1984; Mathew and Abraham, 2006). The total phenolic content (TPC) of non-germinated barley grains was 2.63 mg GAE/g. Results obtained for total phenolic contents in barley were closer to findings of Zhao *et. al.*, (2008) who analysed 14 malting varieties of barley reported TPC values in the range of 2.17 to 2.56 mg GAE/g db. However, Dvorakova *et al.*, (2008) reported comparatively lower values (0.6 to 1.5 mg GAE/g) for TPC in barley grains. Variations among the previously reported values might be due to varietal differences and method adopted for the preparation of cereal extracts (Zielinski and Troszynska, 2000; Zhou and Yu, 2004).

Germination and imbibition of barley grains resulted in a significant ($\rho<0.05$) increase in the TPC of barley flour. Donkor *et al.*, (2012) studied the influence of germination on the TPC of 7 different grains and reported significant

rise in all the grains including barley. Yang *et al.* (2001) also observed increase in the phenolic content after germination of wheat. However, Sharma and Gujral (2010) noted lowering in TPC of whole barley flour and refined barley flour upon 24 h germination. Decrease in TPC was attributed to their metabolic conversion into other compounds and leaching out in steeping water. However, significant increase in five out of eight varieties for TPC was reported for bran fractions during 24 h germination process. The increase in TPC upon germination could be attributed to the action of activated enzymes associated with synthesis and / or pathways and / or liberation of bound phenolics due to structural breakdown of cereal cell walls (Maillard *et al.*, 1996; Shibuya *et al.*, 1984; Kaukovirta-Norja, *et al.*, 2004; Katina *et. al*., 2007; Dordevic *et al.*, 2010).

4.3.5 Effect of germination and imbibition of grains on the DPPH and ABTS radical scavenging activity of barley flour

DPPH radical scavenging activity has been widely used to measure antioxidants soluble in organic solvents, especially alcohols (Arnao *et al.*, 2000). DPPH radical scavenging activity has been adopted by number of workers to determinie the antioxidant activity of different (Brindzova *et al.*, 2008; Zhao *et al.*, 2008; Biswas *et al.*, 2011; Lee *et al.*, 2011). Significant differences towards the increase in DPPH radical scavenging activity of barley grains have been observed during germination and imbibition process. The barley flour obtained from germinated barley grains exhibited highest DPPH radical scavenging activity (*i.e.* 2.60 mg TEAC/g) in comparison to flour obtained from non-germinated (1.90 mg TEAC/g) and imbibed (2.01 mg TEAC/g) barley grains. Sreeramulu *et al.*, (2009) reported DPPH radical scavenging activity of different cereals including wheat, sorghum maize and rice within the range of 0.24 to 1.39 TEAC mg/g. Increase in the antioxidant activity of cereals due to germination has been reported by Oki *et al.* (2002) and Donkor *et al.* (2012). Sharma and Gujral (2010) evaluated antioxidant activity of germinated barley and its fractions such as bran and refined flour and reported a significant rise in antioxidant activity due to 24 h germination process.

The barley flour obtained from non-germinated barley grains exhibited ABTS radical cation scavenging activity of 8.30 mg TEAC/g flour. Significant ($\rho<0.05$) increase in ABTS radical scavenging activity of flour samples was observed due to germination (8.46 mg TEAC/g) and imbibition (8.41 mg TEAC/g) of barley grains. Brindzova *et al.*, (2009) demonstrated highest values for

antioxidant capacity in barley and buckwheat amongst 13 different cereals, which ranged in between 4.64 to 6.14 and 6.58 to 7.33 mg Trolox/g, respectively and depended on genotype. Dvorakova *et al.*, (2008) reported a significant increase in the ABTS radical scavenging activity of barley due to malting process.

4.3.6 Effect of germination and imbibition of grains on the phytic acid content of barley flour

Barley is a rich source of minerals such as iron, zinc, magnesium, potassium, phosphorus and calcium (Liu *et al.*, 1975; Stewart *et al.*, 1988; Swaminathan, 1995; Hubner *et al.*, 2012). However, bioavailability of such minerals is low due to the presence of phytate, which can rapidly form insoluble complexes with such minerals (Sandberg *et al.*, 1999). Moreover, phytic acid has been reported to exert certain health benefits such as chelation of dietary Pb^{2+} (Wise and Gilber, 1981); protection against a fatty liver (Onomi *et al.,* 2004), breast and prostate cancer (Vucenik and Shamsuddin, 2003). Phytic acid content should be lowered to a level of 25 mg/100 g to obtain associated health benefits (Onomi *et al.,* 2004).

Phytic acid content in barley flour obtained from non-germinated grains was 8.05 mg/g, which was closer to the findings of Mohamed *et al.*, (2010), who reported the values in the range of 0.99 to 6.78 mg/g (db) in five barley germplasm. Comparatively higher values were reported for phytic acid content in three genotypes of barley by El-Emery and Amin, (2010), which ranged from 12.0 to 14.1 mg/g. A significant reduction up to 28.7 % and 13.5 % in the phytic acid content of barley flour was observed due to germination (5.74 mg/g) and imbibition (6.96 mg/g) of barley, respectively. Hubner *et al.*, (2010) reported a 26% decrease in the phytic acid content of barley, when the grains were germinated for 96 hours at 18°C. Centeno *et al.,* (2001) observed 60% of the original phytate was broken down after 5 days of germination period. During germination, activity of native phytases increases, which catalyzes the hydrolysis of phytate in barley thus resulting in its breakdown (Bartnik and Szafranska, 1987; Laboure *et al.*, 1993).

4.3.7 Effect of germination and imbibition of grains on the tannin content of barley flour

Tannins are the naturally occurring polyphenolic compounds which may adversely affect the nutritional quality and functionality of grains by binding proteins, carbohydrates and minerals (Salunkhe *et al.,* 1982; Salunkhe *et al.,*

1990; Butler *et al.,* 1986; Towo *et al*., 2006; Parawira and Muchuweti, 2008). Tannins have been reported to bind exogenous and endogenous proteins such as digestive enzymes, which caused poor digestibility of ingested foods specially the proteins (Asquith and Butler 1986; Griffiths, 1985; Eggum and Christensen, 1975).

Barley flour obtained from non-germinated grains contained 2.97 mg CE/g tannin, which was slightly higher than the reported values of 1.9 to 2.6 mg CE/g tannin in barley samples (Ford and Hewitt, 1979), and variation was observed due to difference in location of cultivation. Comparatively higher values (14 to 18 mg CE/g) have been reported by El-Emery and Amin (2010). Results reported by Beta *et al.*, (2007) varied in between 0.036 to 0.058 mg CE/g of barley, while Coon *et al.*, (1979) observed tannin content in the range 0.06% to 0.20%. Difference in tannin content reported by different workers could be attributed to variations in analytical techniques adopted, variety of barley and location of cultivation.

Both, germination and imbibition of grains caused a significant ($\rho<0.05$) decrease in the tannin content of barley flour, which caused 48.5 % and 32.9 % reduction in germinated and imbibed barley grains, respectively (**Table 4.1**). Bressani and Elias (1980) reported decrease in the tannin content of legume seeds during germination. Tannin content in different has been reported to decrease significantly by processes such as decortication, soaking and germination (Rao and Deosthale, 2006). Idris *et al.*, (2005) investigated the effect of germination on tannin content of sorghum cultivars and observed reduction up to 61% and 34% in two different varieties, when the grains were germinated for 48 h.

Reduction in tannin content due to germination and imbibition process can be attributed to increased activity of polyphenol oxidases (Khandelwal *et al.*, 2010), hydrophobic association of tannins with proteins and enzymes (Rusydi and Azina, 2012) and binding of polyphenols with other organic substances such as carbohydrate or protein (Jood *et al.*, 1987; Sharma and Sehgal, 1992; Saharan *et al.*, 2002). In addition, loss of tannins during germination and imbibition was also attributed to the leaching of tannins into the water. Hydrolysable tannins go under hydrolysis into sugars and recognizable phenolic carboxylic acids, whereas condensed tannins cannot be hydrolysed in the similar manner (Marquardt, 1989).

4.3.8 Effect of germination and imbibition of grains on the free amino nitrogen content of barley flour

In brewing science, free amino nitrogen (FAN) content is a measure of individual amino acids, small peptides, and ammonium ions formed from the proteolysis during the process of malting and mashing, which is utilised by yeast for cell growth and proliferation (Taylor and Boyd, 1986; Pugh *et al.*, 2005; Lekkas *et al.*, 2007). Free amino acids, peptides and other nitrogenous substances serve as food for microorganisms. Studies carried out have indicated positive influence of FAN concentration in malt from different cereals on the growth and viability of probiotic cultures (Charalampopoulos *et al.*, 2002a; Charalampopoulos *et al.*, 2003; Patel *et al.*, 2004).

Significant ($\rho<0.05$) influence towards the increase in FAN content has been observed due to process of imbibition (10.2%) and germination (14.1%) if compared with barley flour obtained from non-germinated grains. Approximately 30% increase in the FAN content of malted barley has been previously reported (Goode *et al.*, 2005). Afify *et al*., (2012) observed increase in free amino acids with an overall decrease in majority of non-essential amino acids and increased protein solubility during germination of sorghum grains. Agu and Palmer (1997) observed lower germination temperatures of 17°C yielded higher levels of free amino nitrogen along with peptides. A net increase in the free amino nitrogen during germination is derived partly from the breakdown of protein reserves by native proteases. Enari and Mikola (1977) observed enhanced activities of proteinases and peptidases during germination of barley grains.

4.4 CULTURE COLLECTION AND MAINTENANCE

Standard cultures as mentioned in **Table 3.1** were procured from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute (NDRI), Karnal and from the Ultrastructure Lab of Dairy Microbiology Division, NDRI, Karnal. Procured cultures were maintained as per the procedure mentioned in **3.2.5.**

4.5 PRELIMINARY EXAMINATION OF BACTERIAL CULTURES

4.5.1 Morphological examination

Microorganisms grow and divide on solid medium in specific patterns of growth which form discrete units called colonies. The morphology of which is characteristic of that microbial species. Lactic acid bacterial cultures were streaked on their respective media and incubated at prescribed temperatures for

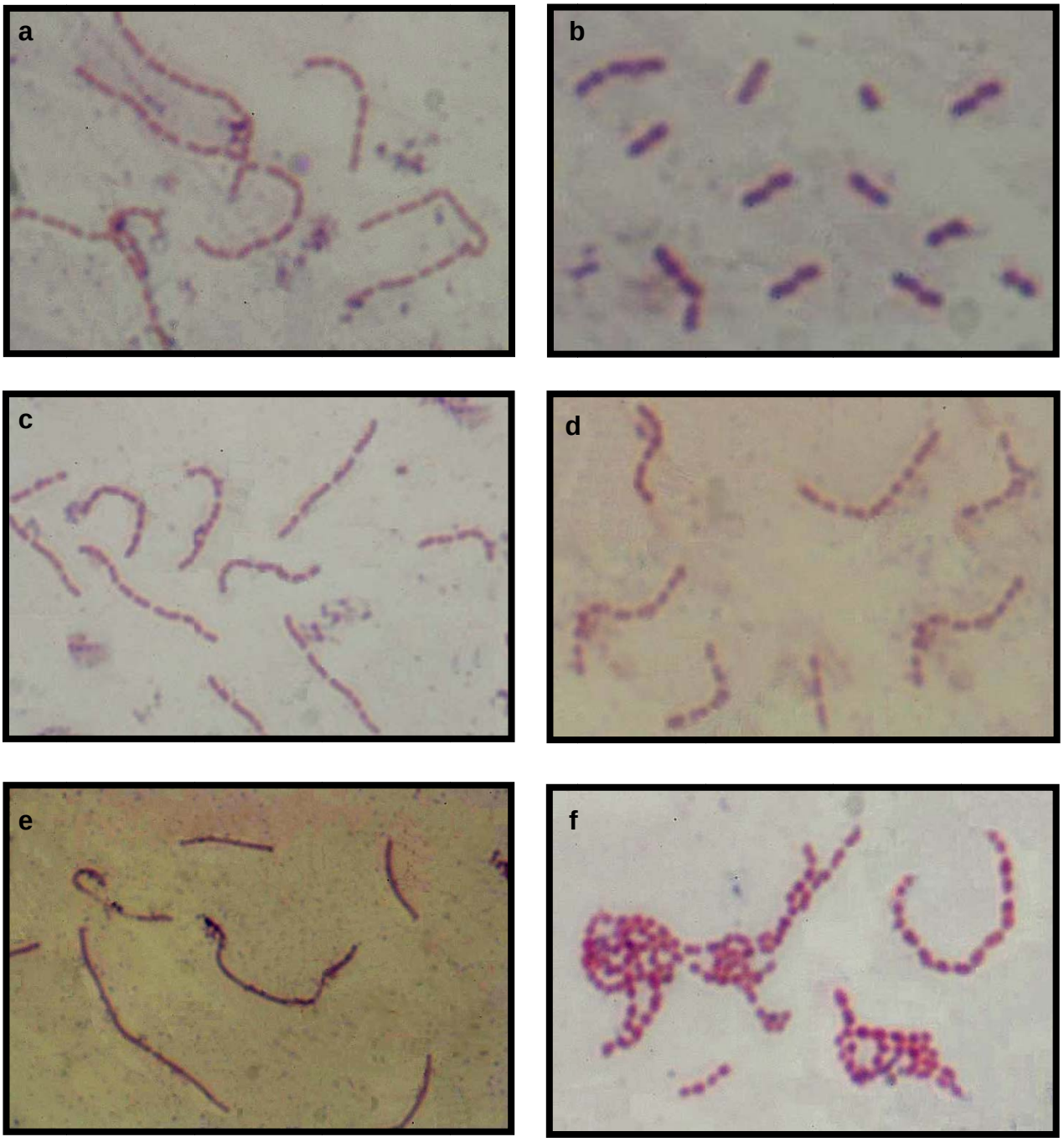


Plate IV: (a) *L. rhamnosus* RSI3 (b) *Lactobacillus plantarum* 344 (c) *L. casei* 297 (d) *L. casei* 299 (e) *L. acidophilus* 13 (f) *S. thermophilus* 20

the period ranging from 24 to 48 h. *S. thermophilus* 20 was grown on ST agar, whereas, *L. rhamnosus* RSI3, *L. acidophilus* NCDC13, *L. casei* NCDC299, *L. casei* NCDC297 and *L. plantarum* NCDC344 were streaked on MRS Agar. *Lactobacillus plantarum* cultivated on MRS agar appeared as whitish, uniform and rounded colonies. All the pure cultures of Lactobacilli indicated whitish, regular, large, spherical, convex, smooth colonies with diameter ranging in between 2 to 3 mm after 24 h of incubation on MRS agar.

L. rhamnosus colonies were Gram positive and appeared medium thin rods in long chains, *L. casei* with Gram positive small rods in long chains, *L. acidophilus* NCDC13 with Gram positive long slender rods and *L. plantarum* with Gram positive, short rods in short chian and depicted in **Photoplate IV**. *S. thermophilus* 20 indicated Gram positive cocci in long chains. When examined the indicator organisms, *S. aureus* were found Gram positive cocci in bunches, *S. typhi* were Gram negative medium rods, *E.coli* were Gram negative short cocco-bacilli and *S. dysentriae* were Gram negative rods.

4.5.2 Catalase Test

Catalase, an extracellular enzyme secreted by certain microorganisms, is a characteristic feature of pathogenic bacteria hence, it can serve as an important diagnostic tool. The catalase catalyses the breakdown of hydrogen peroxides to produce molecular oxygen that generates vigorously with effervescence. Mixing of microbial cultures with an equal volume of 3% hydrogen peroxide followed by generation of effervescence indicate catalase positive. In the present study, mixing of LAB cultures did not produce any visible effervescence, hence could be termed as catalase negative. On the other side all indicator organisms were found to be catalase positive except *Shigella dysentriae*.

4.6 SELECTION OF SUITABLE PROBIOTIC LACTIC ACID BACTERIA FOR THE MANUFACTURE OF BARLEY-MILK BASED FERMENTED PROBIOTIC DRINK (PHASE-I)

4.6.1 Phytase plate assay

Phytate, an anion, myo-Inisitol 1, 2, 3, 4, 5, 6 – hexakis dihydrogen phosphate has capability to bind minerals like iron, calcium, zinc, manganese, *etc*. thus making stable complexes and rendering them unavailable for intestinal absorption (O'Deli and Savage, 1960; Lopez *et al.*, 2002). Increase of phytic acid in diet is associated with decrease the mineral uptake (Reinhold *et al.*, 1973).

Hydrolysis of phytic acid during food processing operations can be accomplished by activation of intrinsic phytase of cereals through germination or soaking process, or by applying microbial phytase obtained from microbial sources (Marklinder *et al.*, 1995; Harland and Harland, 1980; Poutanen *et al.*, 2009). Breakdown of phytic acid using phytase from various sources may increases the bioavailability of minerals.

The incorporation of probiotic lactic cultures for traditional food fermentation process has been widely applied by several researchers for the manufacture of different categories of fermented food products. Cereals, such as barley and oat are gaining importance as an alternative for the production of probiotic foods owing to their better nutritional and sensory values. Besides being beneficial to the consumers, coarse cereals also offer suitable growth medium for the probiotic strain due to availability of non-digestible polysaccharides and assimilable nutrients. They have capability to degrade anti-nutritional factors like phyate and tannins.

Table 4.2 Relative phytase activity of some lactic acid bacteria

Culture	Zone of clearance	Phytase activity (FTU/ml)	Inorganic phosphate (µmol)
L. acidophilus NCDC13	8 mm	0.0573 ± 0.00^{c}	0.172 ± 0.00^{c}
S. thermophilus 20	--	--	--
L. casei NCDC297	10 mm	0.0588 ± 0.00^{b}	0.177 ± 0.00^{b}
L. casei NCDC299	11 mm	0.0602 ± 0.00^{ab}	0.181 ± 0.00^{ab}
L. plantarum NCDC344	12 mm	0.0614 ± 0.00^{a}	0.184 ± 0.00^{a}
L. rhamnosus RSI3	12 mm	0.0606 ± 0.00^{a}	0.182 ± 0.00^{a}

Means with different superscripts in same column differ significantly ($p<0.05$, Fisher LSD); Values in each cell represent mean ± SE, n=3

During the present investigation, six lactic cultures namely *L. plantarum* NCDC344, *L. casei* NCDC297, *L. casei* NCDC299, *L. acidophilus* NCDC13 and *L. rhamnosus* RSI3 were subjected to phytase plate assay. Five out of six strains tested for phytase assay on solid medium were able to hydrolyse hexacalcium phytate and formed clear halo zones around the spots, diameter of which ranged in between 8 to 12 mm (**Photoplate V, Table 4.2**). Highest phytase activity on solid medium was observed for *L. plantarum* and *L. rhamnosus* (12 mm each), followed by *L. casei* NCDC299 (11 mm), *L. casei* NCDC297 (10 mm) and *L. acidophilus* NCDC13 (8 mm). *S. thermophilus* 20 was unable to hydrolyze hexacalcium phytate thus did not produced any clear zone.

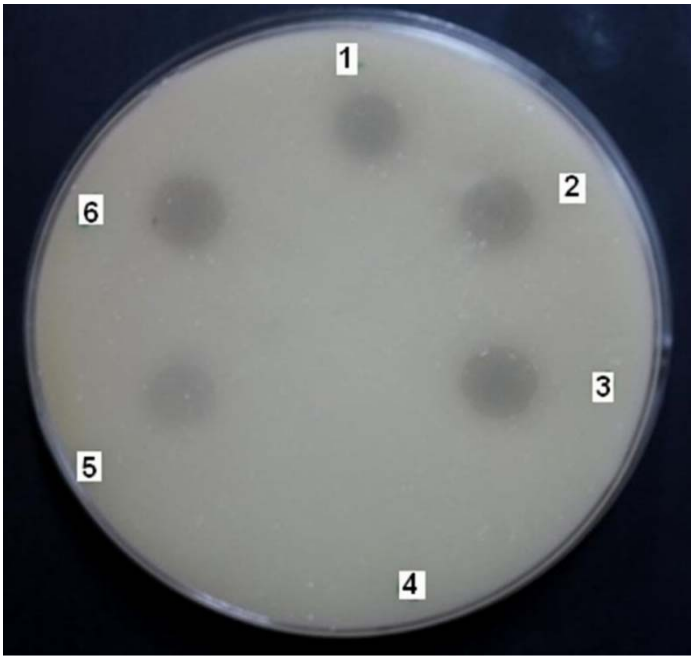


Plate V: Plate of modified chalmer agar containing hexacalcium phytate showing zones of clearance produced by 1: *L. casei* 297 (11mm); *L. casei* 299 (11mm); *L. plantarum* 344 (12mm); *S. thermophilus* 20 (no zone); *L. acidophilus* 13 (8mm); *L. rhamnosus* RSI3 (12mm)

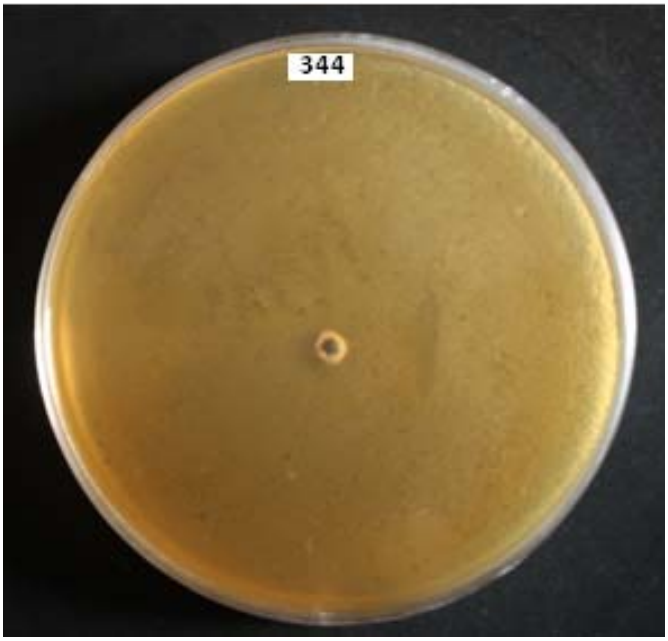


Plate VI: Co-culture compatibility assay indicating no zone of inhibition for *L. plantarum* 344 against supernatant of *S. thermophilus* 20

Anastasio *et al.* (2010) tested 150 lactic cultures isolated from sour dough, pizza dough and sausages for phytate degrading enzyme on a solid medium containing hexacalcium phytate, out of which 29 were able to produce clear halo zones of 3 to 13 mm. *L. plantarum* H5 and *E. faecium* A86 were reported to exhibit highest phytase activity. Phytase positive strains of LAB were reported to improve the bioavailability of essential minerals like iron, manganese and zinc.

4.6.2 Phytase assay

All phytase positive lactobacilli in the plate assay were subjected for quantitative estimation of phytase enzyme in liquid medium. Among the five strains tested for phytase activity, maximum phytase activity (**Table 4.2**) was obtained for *L. plantarum* NCDC344 (0.0614 FTU/ml) followed by *L. rhamnosus* RSI3 (0.0606 FTU/ml), *L. casei* (0.0602 FTU/ml) and *L. acidophilus* NCDC13 (0.0573 FTU/ml). There was no significant ($\rho<0.05$) difference in the phytase activity of three LAB namely *L. casei* NCDC299, *L. plantarum* NCDC344 and *L. rhamnosus* RSI3, however, phytase activity was significantly higher than that of *L. acidophilus* NCDC13 and *L. casei* NCDC297. Zamudio *et al.* (2001) reported *L. plantarum* as highest extracellular phytase producer among the six strains of LAB, which was associated with non-specific acid phosphatases exhibiting higher hydrolysis rates with monophosphorylated compounds. The intracellular phytase activity of 50 LAB was reported in the range of 0.3 to 5.7 mU/ml, whereas no extracellular phytase activity was reported (Reale *et al*., 2007).

Haros *et al*., (2008) studied phytase producing capacity of different LAB isolated from wide range of sources including dairy products, human and plant products. The evaluated species included *L. acidophilus*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, *L. plantarum*, *L reuteri*, *L. rhamnosus* and *L. salivarius.* Highest phytase activity was reported for *L. casei* DSM 20011and *L. plantarum* JBPRS, whereas *L. acidophilus* BS showed lowest phytase activity. Khodaii *et al*. (2013) investigated phytase activity of 38 lactic cultures isolated from different dairy and pharmaceutical probiotic products, and reported more of dairy product isolates as high phytase producer than the isolates of pharmaceutical origin. Microbial strains isolated from plant sources naturally posses phytase activity that assist them in utilizing a wide range of food constituents including phosphate rich phytate efficiently. In present investigation, *L. plantarum* and *L. rhamnosus* were isolated from milk and plant sources, respectively and showed better phytase activity. Since microbial phytase activity is less pH dependent as reported for

majority of plant phytases, they could be effective in minimizing the phytate levels in wide range of food substrates.

4.6.3 Anti-nutrient reduction potential of different lactic acid bacteria in barley-milk composite model

After investigating the phytase activity, the potential of probiotic lactobacilli as well as *Streptococcus thermophilus* 20 was estimated to degrade anti-nutritional factors (phytate and tannins) present in barley grains following the methods mentioned in sub-section **3.2.6.3**. For this, a barley-milk composite model was prepared and separately fermented with different LAB; which were analysed for pH, titratable acidity, phytate and tannin content. The effect of fermentation on the total phenolic content and antioxidant activity was determined.

4.6.3.1 Effect of fermentation by different lactic acid bacteria on the pH and acidity of barley-milk composite model

The rate of decrease in the pH and rise in acidity levels after 12 h fermentation of barley-milk composite laboratory model was found to be associated with the strain used for fermentation process. The composite model fermented with *L. casei* NCDC297 attained lowest pH values with maximum acidity levels, whereas, samples fermented with *S. thermophilus* 20 resulted in minimum acidity levels. The pH of samples fermented with different lactic cultures ranged in the range of 4.30 and 4.45. The initial pH of 6.34 in control samples (barley-milk composite model without added culture) decreased to 5.83 after 12 h of natural fermentation at 37°C. Significant ($\rho<0.05$) variations in pH and acidity levels of different samples were observed after 12 h fermentation (**Table 4.3**). The decrease in pH and increase in acidity levels during fermentation was mainly due to the production of lactic acid and acetic acid by added lactic cultures and native microflora of added ingredients. A negative correlation between pH and acidity levels existed.

Charalampopoulos *et al*., (2002b) monitored the growth characteristics of probiotic strains of *L. fermentum*, *L. reuteri*, *L. plantarum* and *L. acidophilus* in wheat, barley and malt based natural food substrate and observed malt medium could provide better nutrients probably sugars and free amino nitrogen than wheat and barley based mediums. *L. plantarum* and *L. fermentum* were reported to reach higher counts than *L. reuteri* and *L. acidophilus* in all mediums. The rate of acid production is influenced by the strains of lactic cultures, their metabolic

rate, ability to ferment carbohydrates and actively prevailing environmental conditions. The lactic cultures used differ in above-mentioned attributes, which was reflected as difference in pH and acidity levels.

4.6.3.2 Effect of fermentation by different lactic acid bacteria on phytic acid content

A marked decrease up to 50.3% in the phytic acid content of barley-milk composite model was observed, when the composite was inoculated with *L. plantarum* NCDC344 and fermented for 12 h at 37°C. Phytic acid content of barley-milk composite laboratory model fermented with different lactic cultures ranged from 1.38 to 2.03 mg/g (**Table 4.3**).

Table 4.3 Effect of fermentation by different lactic acid bacteria on the pH, acidity, phytic acid content and tannin content of barley-milk composite

Description	pH	Acidity %LA	Phytic acid mg/g	Tannin CE* mg/g
Barley Flour	-	-	7.50 ± 0.04	2.76 ± 0.08
Control (0 h)	6.34 ± 0.00^{a}	0.35 ± 0.01^{g}	2.78 ± 0.07^{a}	0.95 ± 0.02^{a}
Control (12 h)	5.83 ± 0.00^{b}	0.56 ± 0.00^{f}	2.38 ± 0.08^{b}	0.79 ± 0.06^{b}
L. acidophilus NCDC13	4.39 ± 0.01^{d}	1.32 ± 0.01^{d}	1.74 ± 0.03^{d}	0.25 ± 0.03^{d}
L. casei NCDC297	4.30 ± 0.01^{g}	1.46 ± 0.01^{a}	1.51 ± 0.02^{ef}	0.30 ± 0.07^{c}
L. casei NCDC299	4.31 ± 0.01^{f}	1.44 ± 0.01^{b}	1.45 ± 0.01^{f}	0.28 ± 0.02^{c}
L. plantarum NCDC344	4.36 ± 0.01^{e}	1.34 ± 0.01^{c}	1.38 ± 0.01^{g}	0.25 ± 0.02^{d}
L. rhamnosus RSI3	4.32 ± 0.01^{f}	1.44 ± 0.01^{b}	1.57± 0.03^{e}	0.27 ± 0.02^{c}
S. thermophilus 20	4.45 ± 0.01^{c}	1.27 ± 0.01^{e}	2.03 ± 0.03^{c}	0.33 ± 0.02^{c}

*CE: catechin equivalent; Means with different superscripts in same column differ significantly ($\rho<0.05$, Fisher LSD); Values in each cell represent mean ± SE, n=3

The initial phytic acid content of 2.78 mg/g in control samples decreased only to 2.38 mg/g when fermented for 12 h at 37°C. The difference in final phytic acid levels in fermented food system was similar as observed in phytase assay. Strains exhibiting higher phytase activity caused lower phytate content in fermented food system model. Similar findings on the reduction of phytic acid content have been reported upon fermentation using strains of LAB by various workers (Marklinder *et al.* 1995; Toufeili *et al.*, 1999; Ganguly *et al.*, 2014). Reduction of phytic acid content in the fermented cereals and cereal based products has been attributed to the activity of native cereal phytase and phytase from the fermenting microflora (Zotta *et al.*, 2007; Marfo *et al.*, 1990).

In a study, 126 *Lactobacilli* were isolated from Nigerian fermented foods, and screened for few enzymes (amylase, phytase, linamarase) and bacteriocin production. On the basis of findings, Olukoya (1995) identified *L. plantarum*, *L. pentosum* and *L. fermentum* as strains that can be used as starter in production of cassava or cereal based fermented products. Lactic acid fermentation of cereal flours has been reported for 100% reduction in phytate content for rye, 95-100 reduction in wheat and 39-47% reduction for oat within 24 h (Reale *et. al*., 2007). Phytate reduction in cereals was reported to be positively correlated with their native phytase activity.

Effect of LAB sourdough on phytic acid content of flat bread was investigated by Didar (2011), who observed application of *L. plantarum* PTCC1058 sourdough addition at 30% rate could cause 45% reduction in the phytic acid content. Phytase activity from Lactobacillus ssp. including *L. acidophilus* (ATCC4962, 33200, 4356 and 4561), *L. casei* ASCC290, *L. plantarum* ASCC276 and *L. fermentum* VRI-003 in calcium fortified soymilk was studied by Tang *et al*. (2010), who reported up to 91% increase in the phytase activity by *L. acidophilus* ATCC4160, however, no degradation in phytate content was reported. Parameters affecting enzymatic degradation of phytate depend on phytase activity, particle size of flour, acidity, temperature, period of fermentation and water content (Harinder *et al.*, 1998; De Angelis *et al.*, 2003). Most of phytate degrading lactic cultures act on calcium phytate, which is most abundant phytate in cereals and legumes (Raghwendra and Halami, 2009).

4.6.3.3 Effect of fermentation by different lactic acid bacteria on tannin content

Significant reduction in tannin content of barley-milk composite laboratory model have been observed due to natural fermentation as well fermentation by different lactic cultures. A reduction of 16.84 % in the tannin content was observed when control samples were incubated at 37ºC for 12 h, whereas efficiency of different LAB to degrade tannin content ranged from 65.26 to 73.68 %. The initial mean tannin content of 0.95 mg CE /g reduced to 0.25 mg CE /g in the samples fermented with *L. acidophilus* NCDC13 and *L. plantarum* NCDC344, indicating their best potential ($p<0.05$) among the evaluated lactic cultures to degrade tannin content (**Table 4.3**). More than 60 % reduction in the tannin content of sorghum cultivars during fermentation using previously fermented

dough as inoculum has been reported (Hassan and Tinay, 1995; ELKhier and ALRaheem, 2011). Romo-Parada *et al.* (1985) reported up to 92% reduction in tannin content of high tannin sorghum cultivar as a result of fermentation. Effect of traditional fermentation process on the tannin content in two sorghum cultivars was studied by ELKhier and ALRaheem (2011) who observed 72 % and 97 % reduction in Dabar and Tabat variety of sorghum, respectively. The reduction in the tannin content could be possibly owing to liberation and activation of native tannase by lactic cultures or metabolic transformation of tannins into other compounds. Tannase catalyzes the hydrolysis of hydrolysable tannins and gallic acid esters, liberating glucose and gallic acid (Lekha and Lonsane, 1997). *L. plantarum* an isolate from olive waste has been reported for its extracellular tannase activity (Ayed and Hamdi, 2002). Nishitani et al., (2004) studied 77 lactobacilli isolated from human feces and fermented foods belonging to the species of *L. plantarum*, *L. paraplantarum* and *L. pentosus* for intensities of tannase production, and observed a marked variation ranging from <0.1 to 5.99 mU/ml of tannase among the strain. Kannan *et al*., (2011) studied culture conditions for extracellular tannase production by *L. plantarum* MTCC 1407 which was associated with the growth of added culture.

4.6.4.4 Effect of fermentation by different lactic acid bacteria on total phenolics

Phenolic compounds are principle group of phytochemicals that contribute to the antioxidant activity of cereal grains (Mathew and Abraham, 2006). Phenolics present in coarse cereals and millets have been thoroughly investigated for their antioxidant potential. The total phenolic content (TPC) of barley flour used in the preparation of barley-milk composite model was 2.38 mg GAE/g flour. Similar results has been reported for TPC of different malting variety of barley by Zhao *et al.*, (2008) which ranged in between 2.17 to 2.56 mg GAE/g, whereas Dvorakova *et al.*, (2008) reported comparatively lower values (0.6 to 1.5 mg GAE / g) for TPC in ten barley cultivars. Variations among the previously reported results have been observed due to differences in barley cultivars as well as variation in extraction and estimation methods.

Zhou and Yu (2004) attributed the variation in TPC of barley and wheat cultivars to differences in the extraction methods. Fermentation of barley-milk composite laboratory model by different lactic cultures led to increase in the TPC

values. Highest value for TPC was observed in the samples fermented using *L. acidophilus* NCDC13, whereas lowest values were noted for samples fermented with *L. plantarum* NCDC344 (**Table 4.4**). Dordevic *et al.*, (2010) established positive influence of fermentation by *L. rhamnous* A71 and *S. cerevisiae* on TPC values and antioxidant activity of barley, however, the degree of which varied with the type of cultures used. The increase in TPC during fermentation, as observed in the present study, is consistent with the findings of previous investigators (Katina *et al.*, 2007; Moktan *et al.*, 2008; Haron and Raob, 2014). Katina *et al*., (2007) studied changes in the nutritional value of native or germinated rye meals when fermented using either single strain of *L. plantarum*, *L. brevis*, *S. cerevisiae* and in combination of all. Highest increase in the TPC was reported for the rye samples fermented with yeast (*S. cerevisiae*), whereas, lowest increase was reported for *L. plantarum.*

Table 4.4 Effect of fermentation by different lactic acid bacteria on the total phenols and antioxidant activity of barley-milk

Description	Total Phenols[$]	DPPH[†]	ABTS[†]
Barley Flour	2.38 ± 0.06	1.76 ± 0.06	7.68 ± 0.03
Control (0 h)	1.06 ± 0.01^{e}	0.67 ± 0.04^{e}	6.25 ± 0.01^{c}
Control (12 h)	1.13 ± 0.01^{d}	0.73 ± 0.03^{d}	6.31 ± 0.01^{e}
L. acidophilus NCDC13	1.37 ± 0.02^{a}	0.90 ± 0.03^{a}	6.63 ± 0.02^{a}
L. casei NCDC297	1.21 ± 0.01^{c}	0.73 ± 0.01^{d}	6.43 ± 0.02^{b}
L. casei NCDC299	1.23 ± 0.02^{c}	0.84 ± 0.01^{b}	6.45 ± 0.02^{b}
L.plantarum NCDC344	1.15 ± 0.02^{d}	0.79 ± 0.03^{c}	6.37 ± 0.01^{d}
L. rhamnosus RSI3	1.23 ± 0.02^{c}	0.78 ± 0.04^{c}	6.43 ± 0.02^{b}
S. thermophilus 20	1.32 ± 0.01^{b}	0.73 ± 0.01^{d}	6.36 ± 0.02^{d}

[$]Gallic acid equivalent (mg/g); [*]Trolox Equivalent Antioxidant Capacity (mg/g); Means with different superscripts in same column differ significantly (ρ<0.05, Fisher LSD); Values in each cell represent mean ± SE, n=3

Moktan *et al*., (2008) reported 144 % increase in the TPC while prepared *kinemma*, a soybean fermented food popular in Nepal when cooked non-fermented soybean was fermented using *Bacillus subtilis*. Haron and Raob, (2014) also noted significant increase in the TPC by fermentation during preparation of soy tempeh. The increase in TPC might be due to hydrolysis of cell wall constituents by the enzymes secreted by LAB or native cereal enzymes, which may lead to liberation of bound phenolics and synthesis of new phenolic compounds (Katina *et al.*, 2007; Dordevic *et al.* 2010). Extent of positive influence

of fermentation on the TPC depends on the species and strain of microbial culture used in fermentation process (Dordevic *et al.*, 2010).

4.6.4.5 Effect of fermentation by different lactic acid bacteria on DPPH radical scavenging activity

DPPH radical scavenging activity has been widely used to measure antioxidants soluble in organic solvents, especially alcohols (Arnao *et al.*, 2000) and used for the assessment of antioxidant activity for plant based products. DPPH radical scavenging activity of fermented barley-milk composite model ranged from 0.73 to 0.90 mg TEAC/g sample, with highest value for the samples fermented with *L. acidophilus* NCDC13 (**Table 4.4**). Other authors have also reported positive influence of fermentation on the antioxidative capacity of cereals and legumes such as barley, buckwheat, wheat, rye, soybean *etc*. (Moktan *et al.*, 2008; Dordevic *et al.*, 2010; Wang *et al*., 2014). Sourdough fermentation of flour prepared from various cereal such as wheat, rye, spelt, oat, rice, kamut, barley and maize by a mixture of LAB including *L. alimentarius*, *L. brevis*, *L. sanfranciscensis* and *L. hilgardii* have been reported (Coda *et al.*, 2011) to generate antioxidant peptides through the proteolysis of native cereal flour proteins. Findings of Abubakr *et al*., (2012) indicated that whey from skim milk fermented for 24 h with the different LAB isolates exhibited scavenging activity in the range of 14.7 to 48.9%, among them LAB isolates having proteolytic activity showed better antioxidant potential.

4.6.4.6 Effect of fermentation by different lactic acid bacteria on ABTS radical scavenging activity

Although DPPH scavenging is a widely used method for the assessment of antioxidant activity of natural products, it has certain limitations during evaluation of hydrophilic antioxidants (Şanlıdere and Öner, 2011). In contrast to DPPH, ABTS can be solubilized in aqueous as well as organic solvents; thus, radical scavenging activities of hydrophilic compounds can also be measured (Tang *et al.*, 2010). As shown in **Table 4.4**, ABTS radical scavenging activity of examined fermented samples were the maximum for composite samples fermented with *L. acidophilus* NCDC13. Significant increase in antioxidant activity of barley-milk composite laboratory model is observed owing to natural fermentation as well as fermentation of composite mixes with different lactic cultures, when compared with control at 0 h. The barley flour used for the

preparation of barley-milk model exhibited ABTS radical activity of 7.68 mg TEAC/g flour. Brindzova *et al.*, (2009) demonstrated highest values for antioxidant capacity for barley and buckwheat among various analysed coarse and pseudocereals. The increase in antioxidant activity of fermented composite laboratory model could be attributed to the release of bound phenolics, generation of antioxidant peptides and LAB mediated transfer motion of phenolics.

4.6.5 Co-culture compatibility

LAB are the most important group of bacteria used in food fermentation processes. Apart from numerous specific demands technological effectiveness and economics should be considered, when selecting strains with desired attributes for production of fermented foods (Leroy and de Vuyst, 2004; Hati *et al.*, 2013). The selection of suitable starter strains takes into account for their interactions with mixed cultures, along with the consideration for behaviour of these strains under defined conditions, and within the food substrate. Important factors to be taken in consideration during selection of starter cultures includes viability, antagonism against pathogens and spoilage organism, synergism among mixed cultures, rate of acid production, organoleptic improvement, anti-nutrient reduction potential, and probiotic attributes (Holzapfel, 1997; Holzapfel, 2002). Probiotics are considered as slow acid producing microorganisms and exhibit slow growth when inoculated within a suitable food base. It limits their usage as starter in preparation of value added products. Basu (2011) isolated *L. rhamnosus* RSI3 from a cereal-milk based traditional product and selected as a starter culture on the basis of its excellent phytase activity and promising antifungal activity. However, the isolated culture was not able to ferment pearl millet-milk composite that may produce organoleptically acceptable acidic taste even after fermentation period of 12 to 14 h, therefore, it was co-cultured with *S. thermophilus* 20 to enhance the product acceptability.

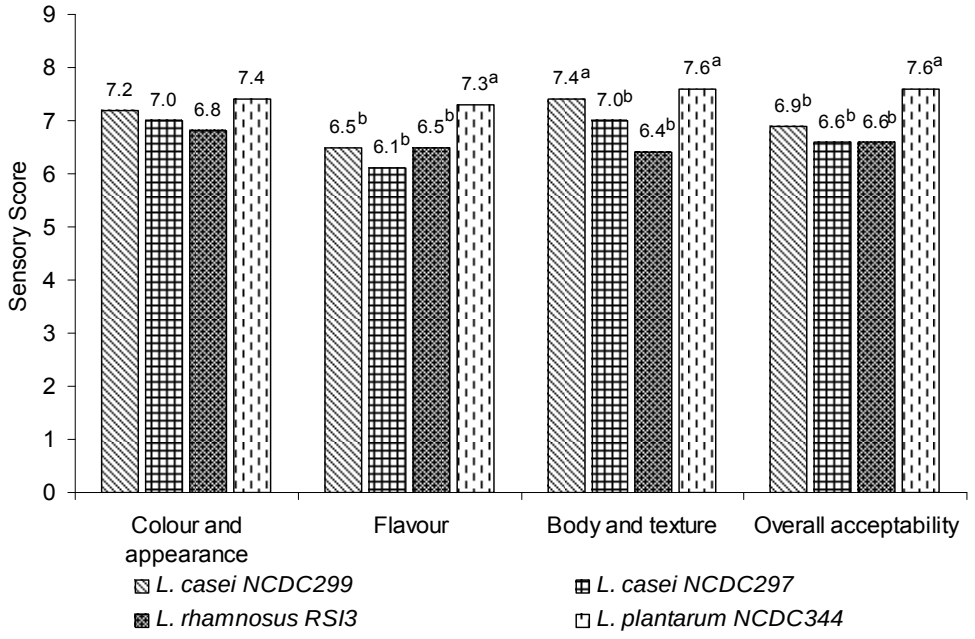
During present study, *S. thermophilus* 20 was used as a co-culture to improve the technological suitability by reducing the fermentation time. In preliminary trials, *S. thermophilus* 20 was found to improve the sensory attributes of barley-milk based fermented probiotic curd, when cultured with other probiotic organisms. However, it is considered that added co-culture may influence the growth of probiotic organism to be used for production of barley-milk based fermented probiotic curd by producing antimicrobials as their metabolite.

Therefore, the compatibility of co-culture with the probiotic cultures having potential degrade anti-nutritional factors was checked. No zone of inhibition was observed when cell free supernatant of *S. thermophilus* 20 was tested against *L. plantarum* NCDC344, *L. rhamnosus* RSI3, *L. casei* NCDC299 and *L. casei* NCDC297, which exhibited maximum anti-nutritional reduction ability. This indicates that no antagonistic compound was excreted into the broth which may have ability to inhibit the growth of probiotic lactic cultures. In this case, all tested lactobacilli with capability to degrade anti-nutritional factors were found compatible with *S. thermophilus* 20, with no zone of inhibition as shown in Photoplate VI. Vinderola *et al*., (2001) studied interactions among lactic acid starter and probiotic lactobacilli to establish satisfactory combinations of strains, and observed a variety of interactions among them. Probiotic bacteria were reported to exhibit more inhibition towards lactic acid starter than vice versa, since the lactic acid starter did not exert any effect on the growth of the probiotic in most of studied interactions. Hati (2012) studied compatibility of *L. casei* C6 with dahi culture NCDC 323 and observed no zone of inhibition. Basu (2011) observed no antagonism between *L. rhamnosus* RSI3 and *S. thermophilus* 20 during development of pearl millet-milk composite based fermented product.

4.6.6 Sensory quality of barley-milk based fermented probiotic composite

Sensory characteristics of any food product are the ultimate criterion for its acceptability by the consumers. The sensory quality of a food is generally considers its attributes related to colour and appearance, aroma, flavour and textural properties. Textural attributes are important features for semisolid food products, and are dependent on their composition and relative interactions in the food matrix (Pereira *et al.* 2003; Majchrzak *et al.*, 2010). The texture of fermented dairy product largely vary and depends on type of milk and its fat content, microflora used for fermentation and applied technology during production process (Cooper 1987; Folkenberg and Martens 2003). Unless the food product meets the desired aroma, taste, flavour, body and texture, the consumers will not accept the product. Therefore, effect of probiotic strain on the sensory quality of barely-milk based fermented curd was studied. After confirmed compatibility with *S. thermophilus* 20, phytase producing probiotic strains *L. plantarum* NCDC344, *L. casei* NCDC297, *L. casei* NCDC299 and *L. rhamosus* RSI3 were individually co-cultured to develop the barley-milk based probiotic curd (as discussed in

3.2.6.5) and compared for different sensory attributes. Significant differences in flavour, body and texture and overall acceptability scores were observed due to differences in probiotic strains used for fermentation process (**Figure 4.1**).



Means with different superscript within grouped column differ significantly (Fisher's LSD Test, ρ<0.01)

Figure 4.1 Effect of phytase producing LAB on the sensory quality of barley-milk based fermented probiotic composite

Highest scores for all the analysed sensory parameters were obtained for *L. plantarum* NCDC344 co-cultured with *S. thermophilus* 20. Curd made with *L. plantarum* NCDC344 co-cultured with *S. thermophilus* 20 was significantly superior in terms of flavour, colour & appearance, body & texture, and overall acceptability followed by curd made with *L. casei* NCDC299. Hence, *L. plantarum* NCDC344 was selected as suitable probiotic culture to develop barley-milk fermented probiotic drink.

4.6.7 Selection of suitable culture with desired attributes

The probiotic LAB, *L. plantarum* NCDC344 indicated its highest potential to degrade anti-nutritional factors *i.e.* phytic acid, tannins in barley-milk composite laboratory model. The culture was also found compatible with *S. thermophilus* 20. Barley-milk based probiotic curd produced by using *L. plantarum* NCDC344 co-cultured with *S. thermophilus* 20 obtained maximum sensory scores, and therefore *L. plantarum* NCDC344 was selected as suitable probiotic culture for the development of barley-milk based fermented probiotic curd and drink in subsequent experiments.

4.6.8 Acidity and pH profile of barley-milk based fermented probiotic curd

Barley-milk composite samples were prepared by using the method **3.2.6.5** and fermented by *L. plantarum* NCDC344 and *S. thermophilus* 20 either alone (1% inoculation) or in combination (1% inoculation of each) at 37°C**.** The drop in pH values and development in titratable acidity of barley-milk composite samples during fermentation were recorded at the interval of 2 h up to 12 h and results have been represented in **Figure 4.2.**

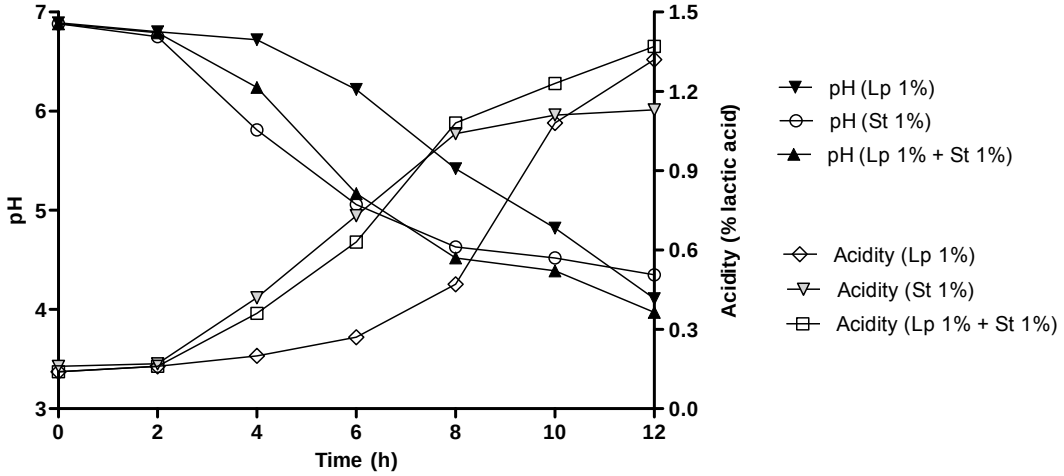


Fig. 4.2 Changes in the pH and acidity of barley-milk composite inoculated with *L. plantarum* NCDC344 (Lp) and *S. thermophilus* 20 (St) either alone or in combination with varying incubation period

All the bacterial combinations tested produced lactic acid and exhibited lowering of pH with progressing fermentation period. It was noted that higher lactic acid was produced when *L. plantarum* NCDC344 was co-cultured with *S. thermophilus* 20. Lowest lactic acid production was obtained for the samples fermented only with *S. thermophilus* 20. However, the samples inoculated with *L. plantarum* NCDC344 alone showed slower rate of acid production, if compared with those fermented using *S. thermophilus* 20 in the initial 6 h. Low pH value in a shorter course of time could be beneficial as it may reduce fermentation period and inhibits the growth of undesirable contaminating microflora. Shorter fermentation period is another important factor, which might be crucial in its ability to proliferate on a large scale. Wang *et al.*, (2005) observed that maximum population of starter organism could be obtained in shorter period of fermentation, when mixed cultures (bifidobacteria with either S*. thermophilus* or *Lb. acidophilus)* were used. Active synergism between the mixed strains has been reported by previous authors as well (Garro *et al.*, 2004; Altieri *et al.*, 2008; Belkaaloul *et al.*, 2010). In yoghurt, a classical example of microbial synergism,

S. thermophilus grow faster in the initial stages of fermentation and reduces the pH of system, provides folic acid and formic acid for the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* (Sieuwerts *et al*., 2010). The initial rapid proliferation of *S. thermophilus* in milk is favoured by shorter lag period.

4.6.9 Probiotic count

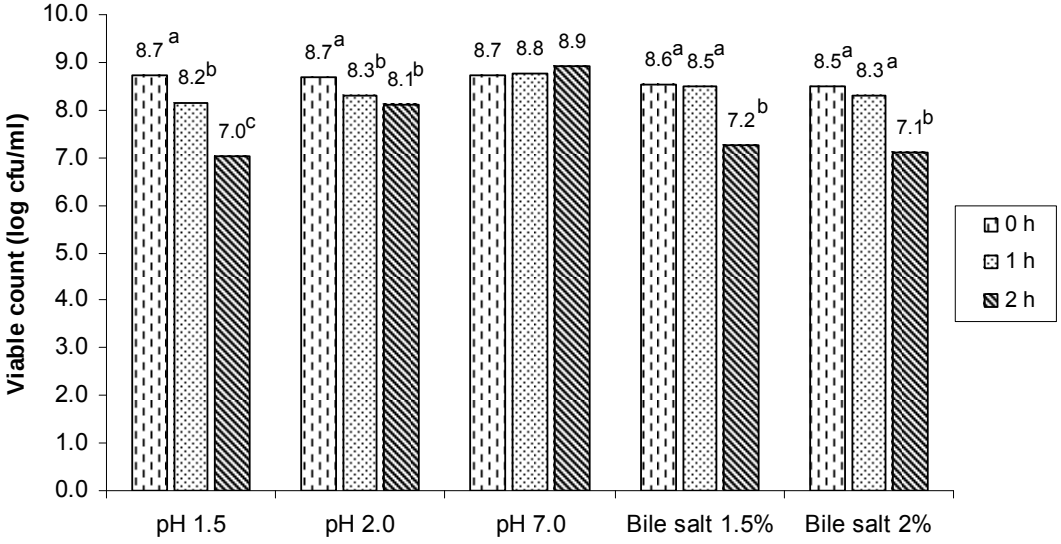
A selective and differential medium for *Lactobacillus plantarum* (Bujalance *et al.*, 2006) was used for accurate enumeration of viable probiotic cells. The developed barley-milk based probiotic curd had probiotic count of 8.10 ± 0.02 log cfu/g on the selective medium. Higher probiotic count in barley-milk fermented composite indicated the positive influence of barley constituents including polysaccharides, free amino acids, peptides, minerals and other growth factors. *L. plantarum* NCDC344 exhibited highest anti-nutrient reduction potential, co-culture compatibility and yielded barley-milk based probiotic curd with most desirable sensory characteristics. Based on the above mentioned findings, for the development of barley-milk based fermented probiotic drink, *L. plantarum* NCDC344 was selected.

4.7 Evaluation of *in vitro* low pH, bile salt tolerance and cell surface hydrophobicity of selected probiotic lactic acid bacteria (confirmatory test)

L. plantarum NCDC344 is a potential probiotic strain isolated from indigenous origin and proven for its probiotic attributes (Kaushik *et al.*, 2009; Ashutosh, 2006) as per the guidelines of FAO/WHO (2002). Expression of *atpD* gene in probiotic *L. plantarum* lp9 under *in vitro* acidic conditions and its relative gene expression of bile salt hydrolase activity *in vitro* gut conditions has been studied by Duary *et al.*, (2010) and Duary *et al.*, (2012), respectively. Duary *et al.*, (2011) assessed the adhesion of putative indigenous probiotic *L. plantarum* strains to human colonic epithelial cells. Achuthan *et al.* (2012), studied antioxidative potential of indigenous lactobacilli including *L. plantarum* lp9. Before using the selected probiotic strain for product development confirmatory tests for its acid and bile salt tolerance were conducted to assess the ability of bacteria to survive in biological barriers encountered in the stomach and small intestine during transit. Furthermore, adhesion ability was also estimated to ascertain the probiotic potentialities of the bacteria under *in vitro* conditions.

4.7.1 *In vitro* tolerance to low pH

The gastric juice in stomach of pH 1.5-2.0 causes destruction of the most of the microorganisms ingested (Charteris *et al.*, 1998). In this sense, resistance to human gastric transit is an important selection criterion for probiotic organisms.



Means with different superscript within grouped column differ significantly (Fisher's LSD test, ρ<0.01)

Figure 4.3 Low pH and bile salt tolerance of *L. plantarum* NCDC344

Acid tolerance of the culture was studied in adjusted pH MRS broth of 1.5 and 2.0 as per the method discussed in **3.2.7.1**, and its tolerance to low pH has been depicted in **Figure 4.3.** Significant (ρ<0.05) differences in viability of cells were observed due to time intervals at pH 2.0 and pH 1.5. A reduction of 1.7 log cycle was observed when the broth of pH value 1.5 was incubated for 2 h. The rate of decrease in viability of cells were comparatively lower at pH 2.0 as observed in case of pH 1.5 However, an increase in viability of probiotic strain was observed at neutral pH of MRS broth. The results obtained to survive low pH were similar to the findings of Kaushik *et al.*, (2009) for the same probiotic strain. In similar type of work by Conway *et al.* (1987), it was observed that *L. rhamnosus* NDFM had potential to tolerate pH 3.0 but a rapid decline in viability with reduction of 5 log count was observed at pH 1.0 after 1h, suggesting that the strain had the ability to survive short-term acid stress but not long-term exposure to acidic environments.

4.7.2 Tolerance to high bile concentrations

Gastrointestinal systems have varying concentrations of bile ranging from 0.5% to 2.5% in the first hour of digestion and the levels may decrease further in

subsequent hours. Moreover, tolerance to bile salts is considered to be a main prerequisite for growth, colonization and metabolic activity of bacteria in the host's gut. The resistance to bile salts varies a lot among the lactic acid bacteria species and even between strains themselves (Xanthopoulos, 1997; Mourad and Nour-Eddine, 2006). The bile salt tolerance pattern of the selected probiotic culture is represented in **Figure 4.3.** The culture had survived and tolerated bile salts (1.5-2%) quite effectively. The culture tolerated 1.5% bile with viability being reduced from 8.5 to 7.2 log count after 2 h of inculabtion, however, viable counts differs significantly ($P<0.05$) in comparison to control. At higher concentration (2%), bile tolerance of the strain decreased marginally as compared to 1% bile after 2 h. The culture had shown slight growth in control (media with no bile) with viability of 8.9 log cfu ml^{-1} after 2 h. The difference in the level of bile tolerance in the present study probably indicate their ability to grow and colonize in the intestinal tract.

4.7.3 Cell surface hydrophobicity

Adhesion to host gut epithelial cells and intestinal mucus is an important property of a probiotic strain for temporary colonization of the gastrointestinal tract and stimulation of beneficial health effects. The culture exhibiting higher cell surface hydrophobicity could be better performers in terms to adhere the intestinal epithelial cells, thus enhancing their useful property in competitive exclusion of pathogens (Perez *et al.*, 1998). Hydrophobicity to different hydrocarbons has been established as a valid *in vitro* biochemical marker and qualitative phenomenological approach to assess the colonization potential of the organism (Rosenberg *et al.*, 1980; Kiely and Olson, 2000). The selected probiotic culture was evaluated for its cell surface hydrophobicity towards *n*-hexadecane, *n*-octane and *o*-xylene. The strain exhibited high hydrophobicity value towards all the three hydrocarbons and indicated higher levels of adhesion (**Table 4.5**).

Table 4.5 Cell surface hydrophobicities of *L. plantarum* NCDC344 to selected hydrocarbons

Hydrocarbons	% Hydrophobicity[1]
n-Hexadecane	33.60 ± 0.27
n-Octane	31.38 ± 0.21
o-Xylene	38.24 ± 0.33

[1]the decrease in absorbance of aqueous phase was taken as measure of cell surface hydrophobicity (H%); studied at 600nm, ± standard error of three replicates

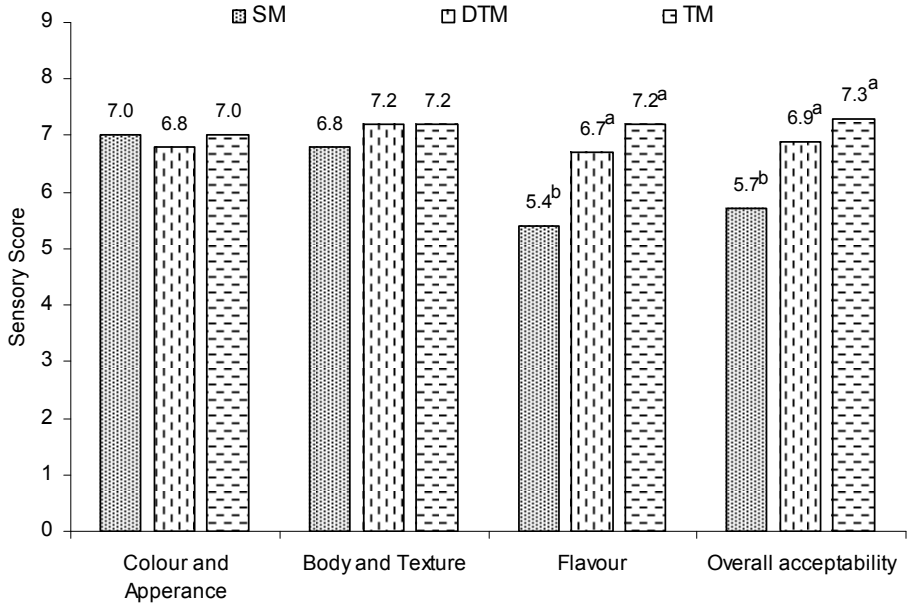
The higher cell surface hydrophobicity favors the colonization at mucosal surfaces and plays a role in the adhesion of bacteria to epithelial cells (Schillinger *et al.*, 2005) by preventing wash-out especially in the small intestine where flow rates are relatively higher. It was observed from the results that the culture exhibited maximum adherence towards xylene and lowest towards n-octane. The variation in hydrophobicity to solvents has been reported in other probiotic cultures and explained by the fact that adhesion depends upon the origin of strains as well as surface properties (De Ambrosini *et al.*, 1998). Kaushik *et al.*, (2009) examined percent hydrophobicity in the presence of n-hexadecane and xylene for *L. acidophilus* LA7 and *L. plantarum* lp9 which ranged in between 56-58% and 37-38%, respectively for the selected strains. However, % H values of some strains of *L. rhamnosus* has been reported as low as 2–5% (Schillinger, 2005). The large differences in the cell surface hydrophobicity could be due to variation in the level of expression of cell surface proteins among strains of a species as well as due to environmental conditions which could affect the expression of surface proteins.

4.8 OPTIMIZATION OF PROCESSING PARAMETERS FOR BARLEY-MILK BASED FERMENTED PROBIOTIC DRINK (PHASE II)

4.8.1 Selection of type of milk

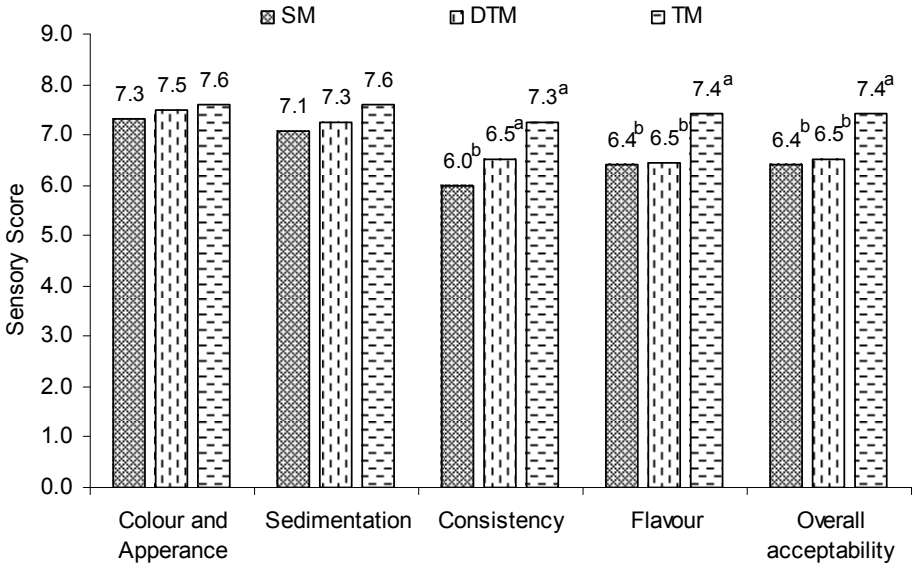
Barley-milk based probiotic curd as well as probiotic drink using skim milk, double toned milk and toned milk prepared by the procedure **3.2.8.1** were subjected to sensory evaluation by a panel of semi-trained judges. The scores based on a 9-point hedonic scale for different sensory parameters are represented in **Figure 4.4** and **4.5**. Flavour score for the barley-milk based curd and probiotic drink varied greatly in between 5.4 to 7.2 and 6.4 to 7.4, respectively. Highly significant ($\rho<0.01$) differences in flavour and overall acceptability of the barely-milk based probiotic curd as well as probiotic drink was observed due to difference in type of milk used in their preparation. Overall acceptability score for probiotic drink obtained from toned milk was significantly higher than the drink obtained from double toned and skim milk. Effect of fat level on sensory properties of a fermented dairy product '*misti dahi*' has been studied by Raju and Pal (2009), who observed significant influence of fat content on the flavour and overall acceptability score of *misti dahi*. Fat content of milk imparted richness to the flavour of milk (Tuorila, 1986; Tuorila, 1987; Phillips *et al*., 1995;

Frost *et al*., 2001). Derndorfer (2006) reported that a higher fat content in yoghurt is not only relevant for desired consistency; it also causes a sweeter taste and creamy flavour and lasting mouthfeel. On the other hand, yoghurt with a low fat content was reported to have often experience of astringent and sour flavour.



Means with different superscript within grouped column differ significantly (Fisher's LSD test, $\rho<0.01$)

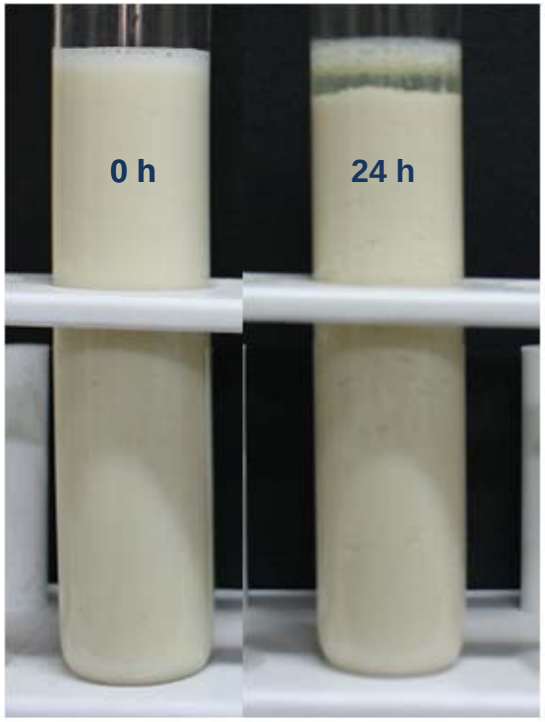
Figure 4.4 Effect of type of milk on the sensory quality of barley-milk based probiotic composite



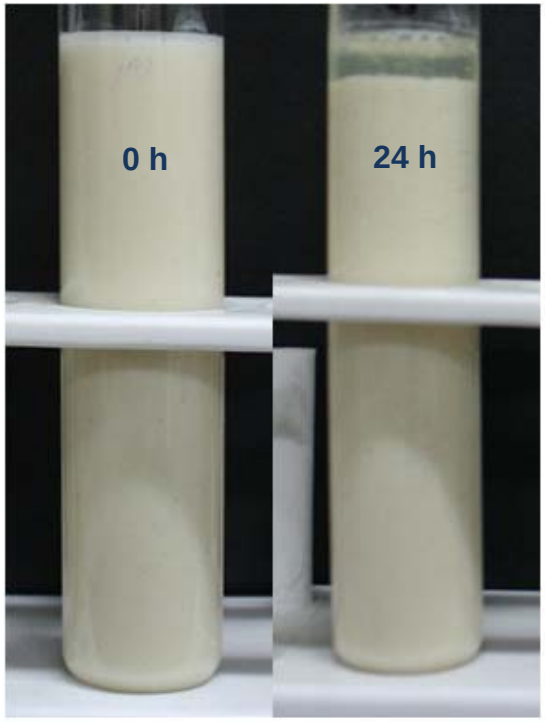
Means with different superscript within grouped column differ significantly (Fisher's LSD test, $\rho<0.01$)

Figure 4.5 Effect of type of milk on the sensory quality of barley-milk based probiotic drink

Beverage obtained from Skim Milk



Beverage obtained from Double Toned Milk



Beverage obtained from Toned Milk

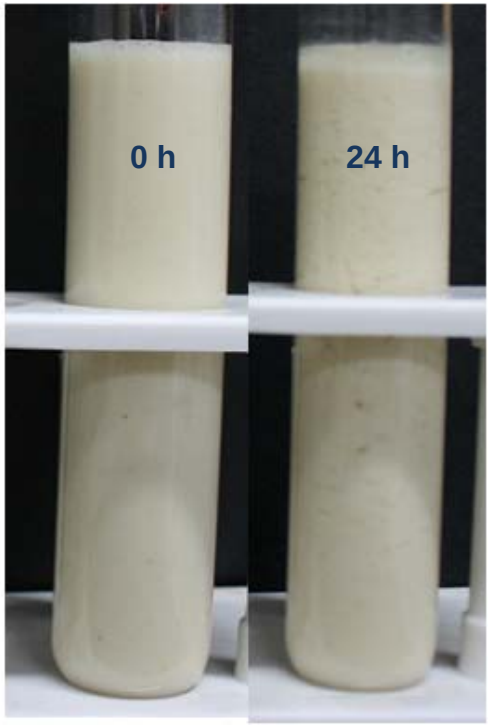
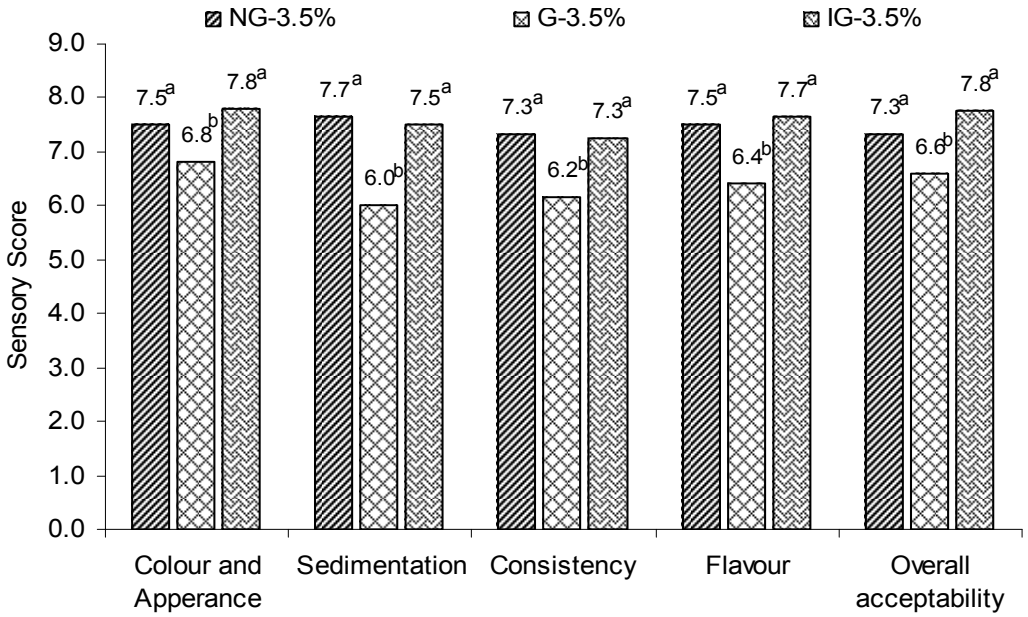


Plate VII: Barley-milk based fermented probiotic drink obtained from different types of milk at 0 h and after 24 h of storage

Folkenberg and Martens (2003) also observed that the fat content affected the textural properties of yoghurt and other sensory attributes like odor, flavour and taste. In another experiment, equal volume (50 ml) of probiotic drink obtained from three different types of milk were transferred in (25 mm dia; 20 cm long), transparent glass test tubes, and left undisturbed for a period of 24 h to examine the extent of serum separation. Serum separation observed for the samples prepared from skim milk and double toned milk were higher than the samples prepared with toned milk (**Photoplate VII**). Due to significantly higher flavour and overall acceptability scores of probiotic curd and drink prepared from toned cow milk than skimmed and double toned cow milk, it was selected for the use in the subsequent experiments.

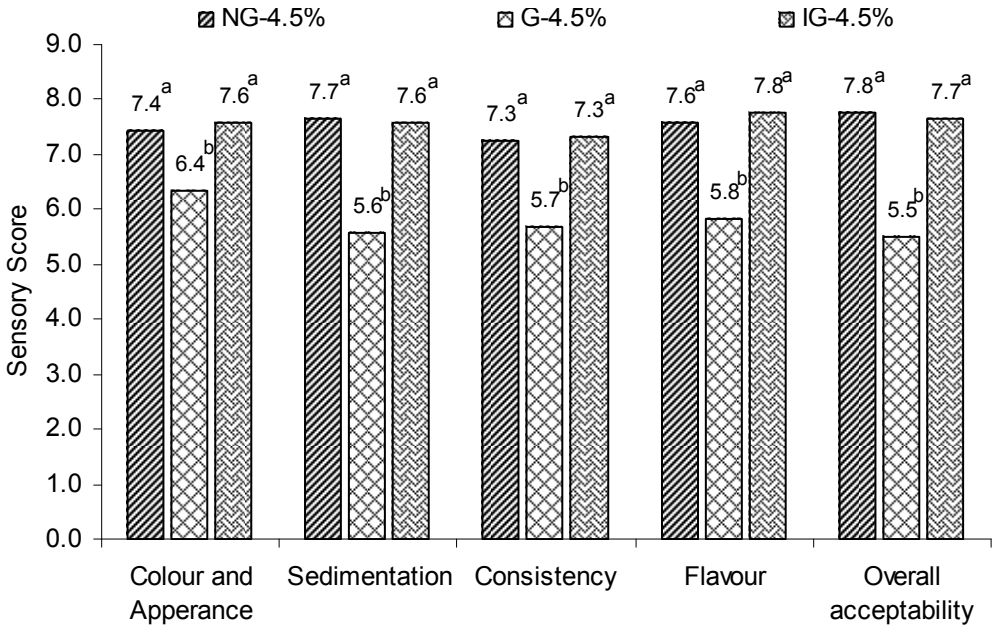
4.8.2 Selection of type of flour

Three different types of flour prepared using non-germinated, germinated and imbibed barley grains as described in section **3.2.8.2** were used at two different levels, *i.e.* 3.5% and 4.5 % to prepare barley-milk based fermented probiotic drink. Prepared drinks were subjected to sensory evaluation by a panel of semi-trained judges. Differences in the sensory parameters due to type of flour have been depicted in **Figure 4.6** and **Figure 4.7**.



Means with different superscript within grouped column differ significantly (Fisher's LSD test, $\rho<0.01$)

Figure 4.6 Effect of type of flour on the sensory quality of barley-milk based fermented probiotic drink when incorporated at the rate of 3.5% (NG: non-germinated; G: germinated; IG: imbibed barley flour)

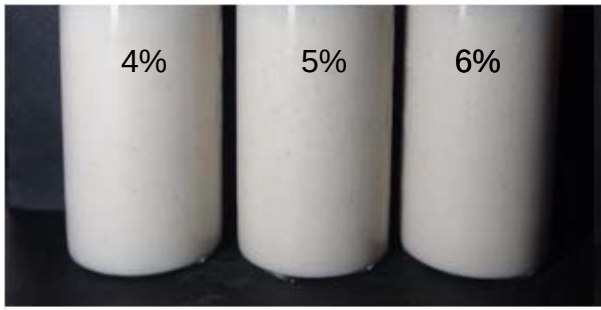


Means with different superscript within grouped column differ significantly (Fisher's LSD test, $\rho<0.01$)

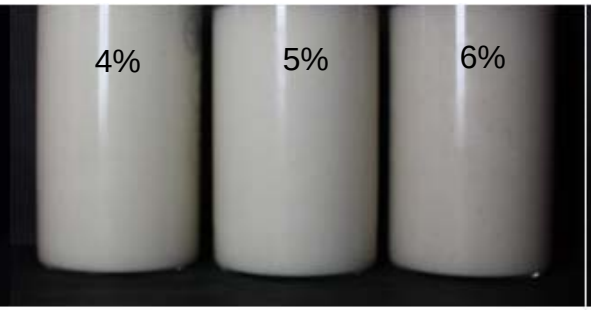
Figure 4.7 Effect of type of flour on the sensory quality of barley-milk based fermented probiotic drink when incorporated at the rate of 4.5% (NG: non-germinated; G: germinated; IG: imbibed barley flour)

Flavour profile of barley-milk based probiotic drink significantly ($\rho<0.01$) varied due to flour type thus influencing the acceptability of the drink. Separation of serum in the barley-milk composite after fermentation was observed when germinated flour was used for the preparation of barley-milk composite (**Photoplate VIII**).

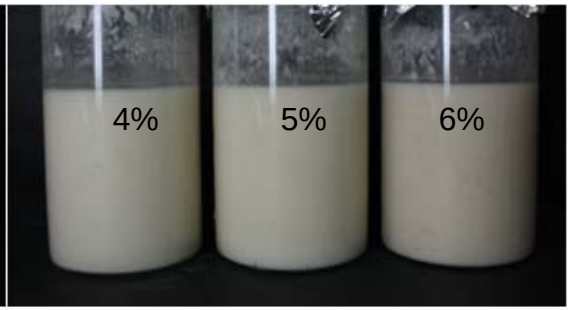
The sedimentation characteristics of the beverage greatly influenced sensory scores. No serum separation in fermented barley-milk composite and corresponding probiotic drink was observed when non-germinated and imbibed barley grain flour were used in their preparation. Increased acidity of flour from germinated barley grains and differences in protein-polyphenol interaction of cereal flour and milk proteins, might be the reasons for separation of serum during fermentation of composite. Higher phenolic content and increased acidity of germinated barley flour (**Table 4.1**) might have contributed towards decreased water holding capacity of milk-protein matrix. Process of germination led to starch hydrolysis, and water binding and gel forming characteristics of starch hydrolysates are usually poor as compared to native starch molecules.



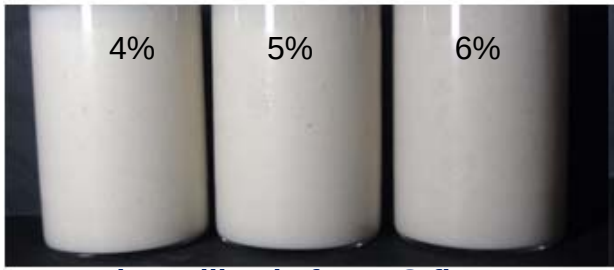
Barley-milk mix obtained from NG flour (after heating and cooling)



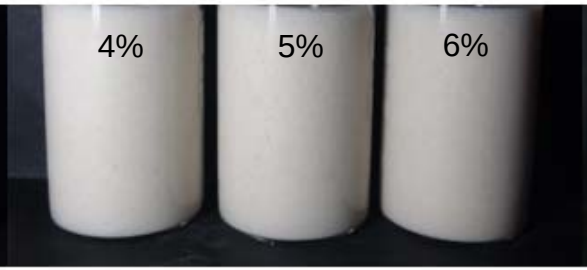
Barley-milk mix from NG flour after fermentation



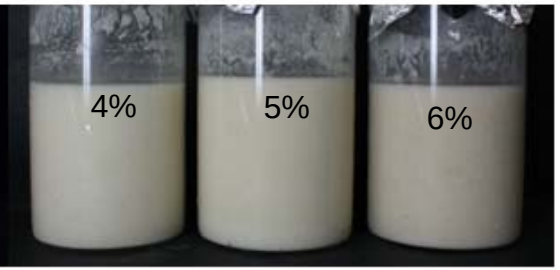
Probiotic drink prepared by using Barley-milk based curd from NG flour



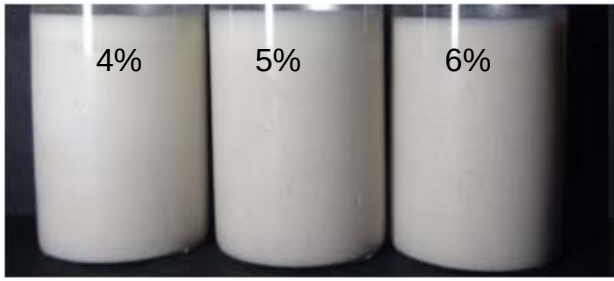
Barley-milk mix from IG flour (after heating and cooling)



Barley-milk mix from IG flour after fermentation

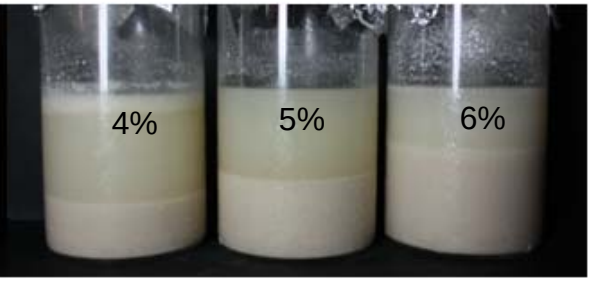


Probiotic drink prepared by using barley-milk based curd from IG flour



Barley-milk mix from G flour (after heating and cooling)

Barley-milk mix from G flour after fermentation



Probiotic drink prepared by using barley-milk based curd from G flour

Plate VIII: Effect of type of flour on the appearance of barley-milk mix after heating and cooling; after fermentation; and on the appearance of the drink obtained (NG: Non-germinated barley grain; IG: Imbibed barley grain; G: germinated barley grain; 4, 5, 6% are the concentration of barley flour in milk)

4.8.3 Effect of type of flour on the probiotic count of barley-milk based fermented probiotic composite

Significant differences in viability of probiotic cells were observed due to the pre-treatment of barley grains. Flour obtained from germinated barley grains provided excellent growth medium for *L. plantarum* NCDC344 in barley-milk composite, resulting in maximum viability of probiotics in the fermented barley-milk composite. Comparatively lower viability of probiotic cells were observed in the fermented composite obtained using flour from imbibed barley grains, followed by the composite prepared using non-germinated barley grain flour. (**Table 4.6**).

Table 4.6 Effect of type of flour on the viability of probiotic cells in barley-milk based probiotic curd

Time (h)	Viable cell count (log cfu/ml)		
	NG 4.5%	G 4.5%	IG 4.5 %
0h	6.11 ± 0.15^{a}	6.31 ± 0.19^{a}	6.18 ± 0.11^{a}
12 h	7.19 ± 0.11^{a}	8.23 ± 0.12^{c}	7.92 ± 0.12^{b}

Values in each cell represent mean ± SE, n=3; Means with different superscript within row differ significantly (Fisher's LSD test, $\rho<0.01$)

Germination of barely grains has been previously reported to improve the nutritional quality (Pedersen *et al.*, 1989) and enhanced the lactobacilli count by 1 log cycle in a food mixture comprised of germinated barley flour, whey powder and tomato pulp (Arora *et al.*, 2010). However, germination of barley grains led to decrease (**Table 4.1**) in the therapeutically important constituent *i.e.* β-glucan in barley grain (Kihara *et al.*, 2007; Hubner *et al.*, 2010). Increase in the free amino nitrogen, reducing sugar and glucose content (**Table 4.1**) in the flour due to germination and imbibiton of barley grains could have contributed towards enhanced viability of probiotic microorganisms in the barley-milk based fermented composite. Charalampopoulos *et al*., (2002) studied growth kinetics of probiotic LAB in barley, wheat and malt mediums, among which malt medium supported the better growth of lactobacilli, which was attributed to the high sugar and free amino nitrogen content.

Ganguly and Sabikhi, (2012) analysed fermentation dynamics of an indigenous probiotic strain *L. acidophilus* NCDC-13 in a composite dairy-cereal substrate composed of whey-skim milk with either germinated pearl millet flour or liquid barley-milk extract. Formulation containing whey-skim milk with liquid-barley-milk extract was reported as better substrate than formulation containing germinated pearl millet flour. However, inclusion of barley malt extract at the rate

of 3 % and with germinated pearl millet flour (5%) proved as best for the growth rate of probiotic organism.

4.8.4 Effect of flour type and concentration on the appearance of barley-milk composite after processing and fermentation, and on the probiotic drink prepared from the fermented composite

Barley-milk composites were prepared by using flour milled from non-germinated, imbibed and germinated barley grains at three different levels, *i.e.* 4%, 5% and 6%. The prepared composite was processed and fermented by using 1.5% probiotic culture and 0.5% co-culture as discussed in **Figure 3.2**. Appearance of barely-milk composite obtained after processing, fermentation and the probiotic drink obtained from the fermented composite are represented in **Photoplate VIII**.

Flakes of a fat rich layer after heating, holding and cooling of barley-milk composite was observed when germinated barley flour was used in the preparation. Separation of milk in three distinct layers occurred if fermentation of composite containing germinated barley grain flour was carried out. Top most layer consisted of fat rich portion, middle portion represented whey like liquid and bottom layer consisted of dense cereal-milk protein mix. Barley-milk composite obtained using flour form non-germinated and imbibed barley grains remained stable during processing and fermentation.

At levels of more than 6.5 % for germinated barley flour, coagulation of milk occurred during heating and holding of the composite. Disturbed salt balance due to addition of germinated barley flour, profound protein-polyphenol interactions probably caused reduction in heat stability of milk proteins. At 6% level gelatinization of starch in mix prevented separation of serum during fermentation. However, probiotic drink prepared using fermented barley-milk composite prepared from germinated barley flour resulted separation in two layers (**Photoplate VIII**).

Hydrogen bonding between the phenolic hydroxyl groups, and the NH- and CO- groups of protein has been reported to be involved in protein-phenolic interactions (Haslam, 1974; Haslam *et al.*, 1999; Ali, 2002). Hydrophobic attraction between proline rich caseins and phenolics are stabilized by H-bonding between phenolic ring and the *bis*-alkyl substituted amide nitrogen (Fox, 2001; Yuskel *et al.*, 2010). The existence of electrostatic attraction or repulsion between charged milk proteins and phenolic compounds, are pH dependent (Ali, 2002).

Thus, the amount of protein-phenolic complex precipitated depends on the phenolic content in mixture and its pH value (Ali, 2002; Ozdal *et al*., 2013).

In light of above-mentioned discussion, it could be suggested that phenolic compounds present in barley might have interacted with milk proteins, resulting in their precipitation. The interaction might have promoted by increasing concentration of phenolics and increasing temperature.

4.8.5 Effect of type of flour and its concentration on the rheological properties of barley-milk composite after processing and fermentation; and on the probiotic drink prepared from the fermented composite

Quality attributes such as texture, consistency and flow properties have been recognized as major determinant related to sensory characteristics and can be used for the improvement of acceptability for any beverage. According to Benezech and Marigonnat (1992), *Ostwald-De-Waele*, *Herschel-Bulkley* and *Casson* models are often used to describe flow behaviour of cultured milk. Many studies have been carried out to evaluate the rheological behavior of stirred yoghurt samples were using a viscometer or a rheometer (Skriver *et al.*, 1993; van Marle *et al.*, 1999; Afonso and Maia, 2000; Haque *et al.*, 2001; Lee and Lucey, 2004). Samples of yoghurt were loaded after agitating by spoon or a mixer. Hassan *et al.*, (2003) gently stirred fermented milk samples 10 times by spoon prior to rheological analyses. Purpose of the mixing or stirring is to break the gel and create free flow. During mixing and loading steps samples were subjected to shear, that led to structural changes in the stirred yoghurt, affecting its flow properties. Any kind of shear resulted in a reduction of yield stress as well as the viscosity. Structural changes were reported to be partially restored after shearing is stopped, which is a time-dependent phenomenon (Lee and Lucey, 2010), and affects the apparent viscosity of yoghurts.

Samples to analyze rheological properties of barley-milk composite were prepared and subjected to rheological measurements as mentioned in **3.2.8.4**. Barley-milk composite based set curd in beakers having 200 g sample size, were stirred 5 times in clockwise and 5 times in anticlockwise direction with polypropylene stick (8 mm diameter) and subjected to rheological analyses. Data pertaining to shear rate *vs*. shear stress at 5, 10, 15, 20 and 25°C for different samples of barley-milk composite before and after fermentation; and that for corresponding probiotic drink are depicted in **Figures 4.8 to 4.22.**

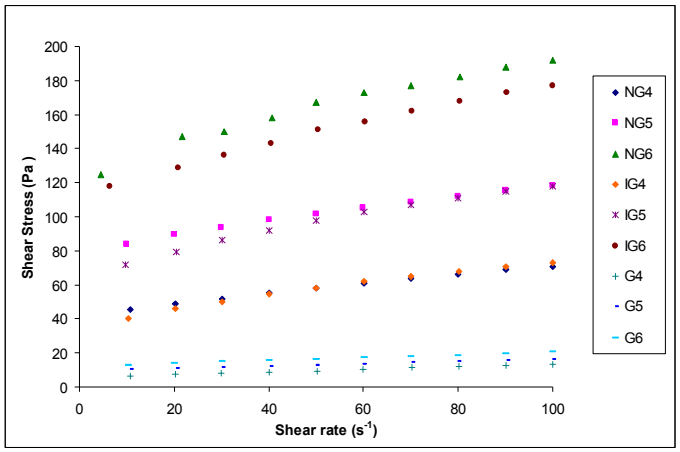


Figure 4.8 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour before fermentation at 5°C (4, 5 and 6 represents the concentration of flour in milk)

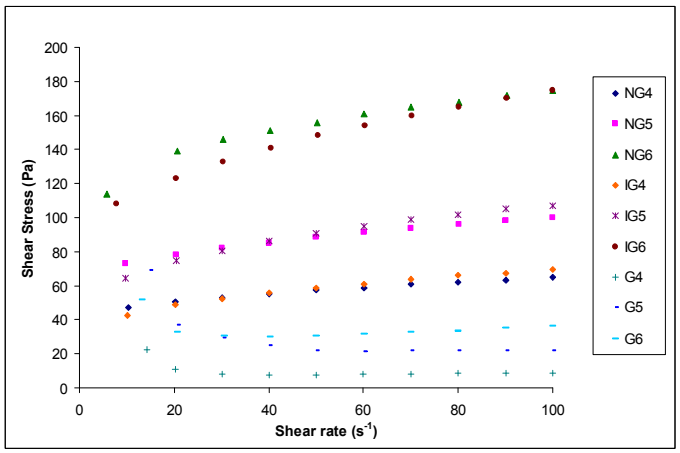


Figure 4.9 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour after fermentation at 5°C (4, 5 and 6 represents the concentration of flour in milk)

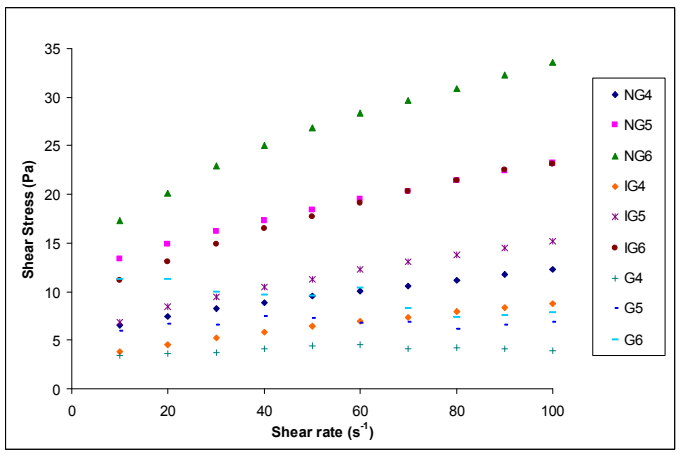


Figure 4.10 Rheogram of barley-milk based fermented probiotic drink obtained using fermented barley-milk composite from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour at 5°C (4, 5 and 6 represents the concentration of flour in milk)

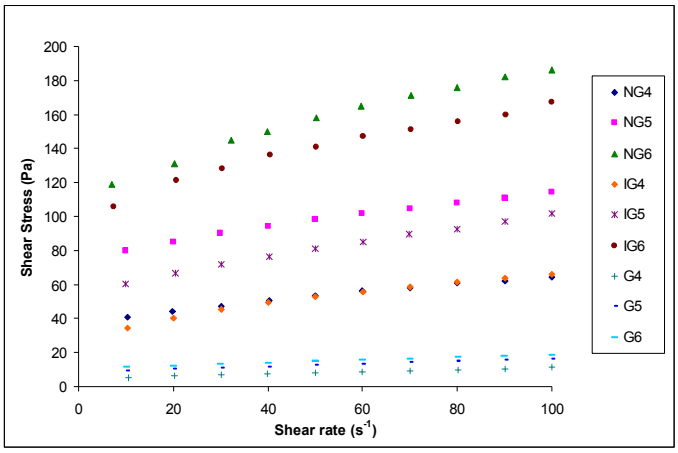


Figure 4.11 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour before fermentation at 10°C (4, 5 and 6 represents the concentration of flour in milk)

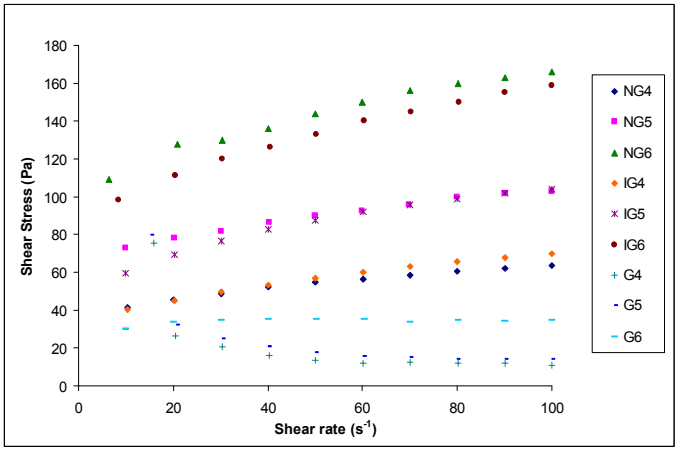


Figure 4.12 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour after fermentation at 10°C (4, 5 and 6 represents the concentration of flour in milk)

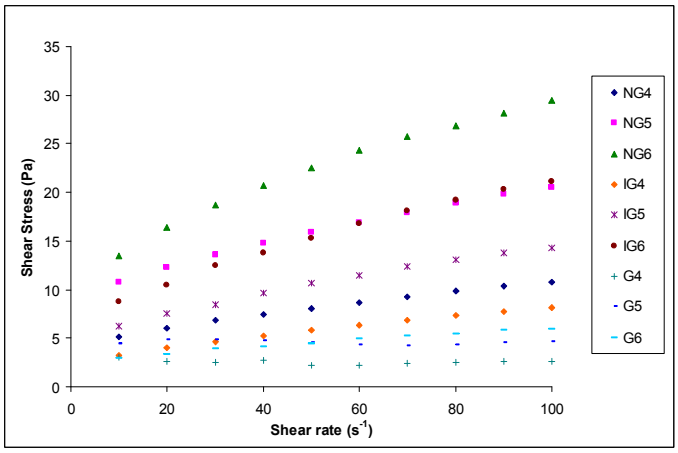


Figure 4.13 Rheogram of barley-milk based fermented probiotic drink obtained using fermented barley-milk composite from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour at 10°C (4, 5 and 6 represents the concentration of flour in milk)

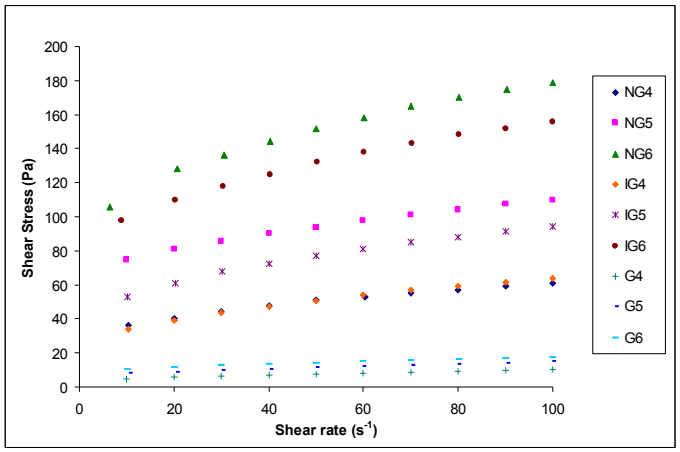


Figure 4.14 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour before fermentation at 15°C (4, 5 and 6 represents the concentration of flour in milk)

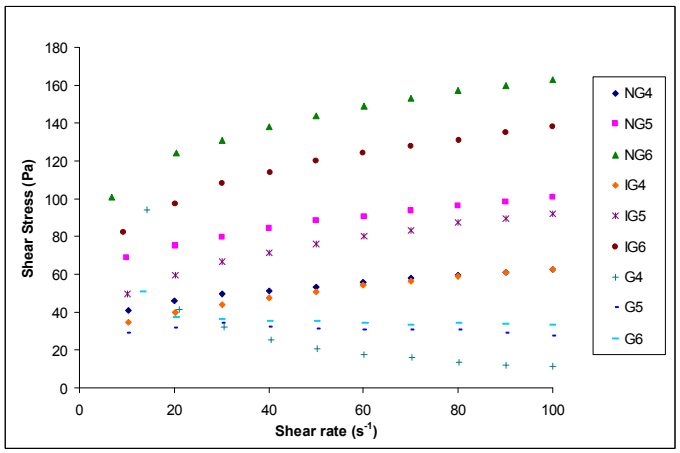


Figure 4.15 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour after fermentation at 15°C (4, 5 and 6 represents the concentration of flour in milk)

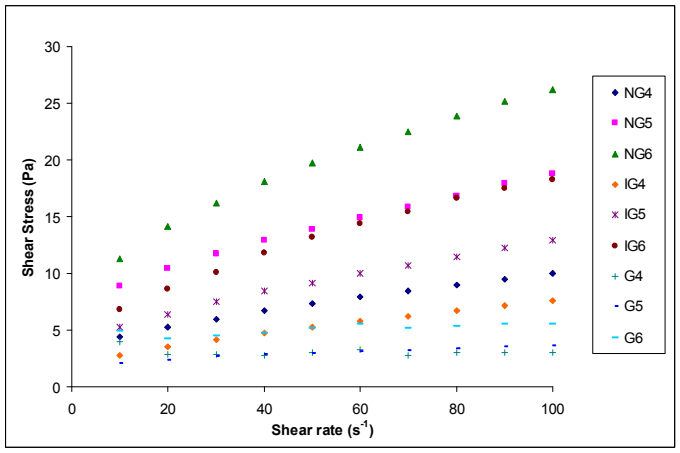


Figure 4.16 Rheogram of barley-milk based fermented probiotic drink obtained using fermented barley-milk composite from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour at 15°C (4, 5 and 6 represents the concentration of flour in milk)

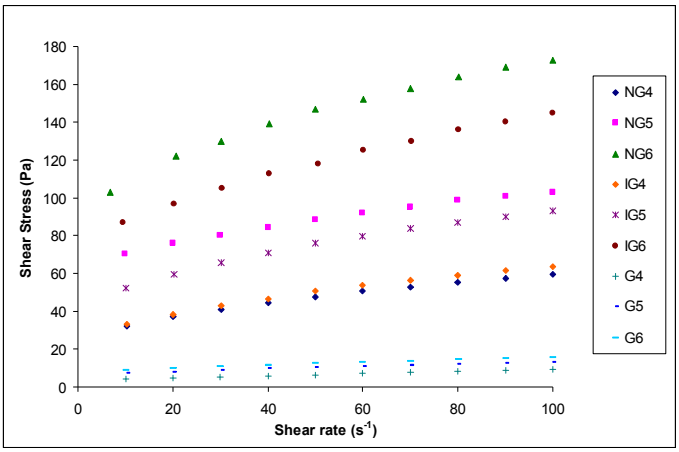


Figure 4.17 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour before fermentation at 20°C (4, 5 and 6 represents the concentration of flour in milk)

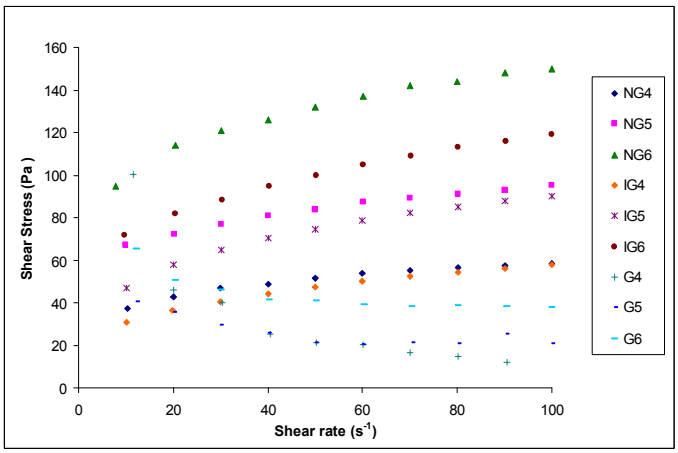


Figure 4.18 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour after fermentation at 20°C (4, 5 and 6 represents the concentration of flour in milk)

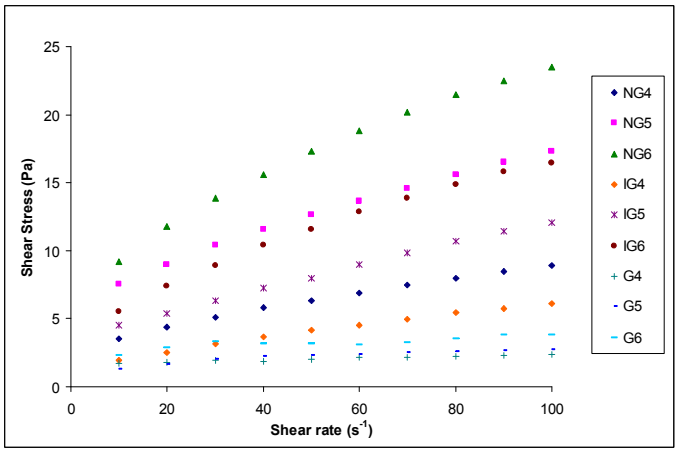


Figure 4.19 Rheogram of barley-milk based fermented probiotic drink obtained using fermented barley-milk composite from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour at 20°C (4, 5 and 6 represents the concentration of flour in milk)

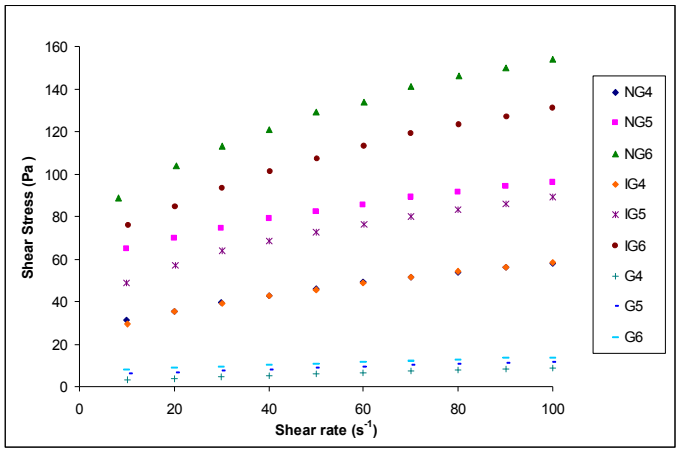


Figure 4.20 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour before fermentation at 25°C (4, 5 and 6 represents the concentration of flour in milk)

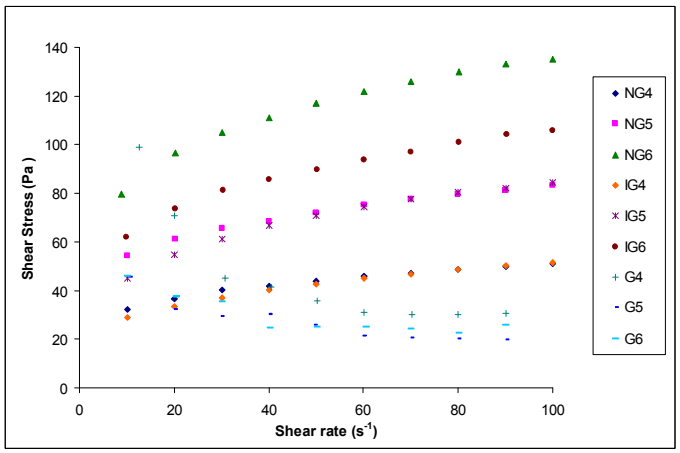


Figure 4.21 Rheogram of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour after fermentation at 25°C (4, 5 and 6 represents the concentration of flour in milk)

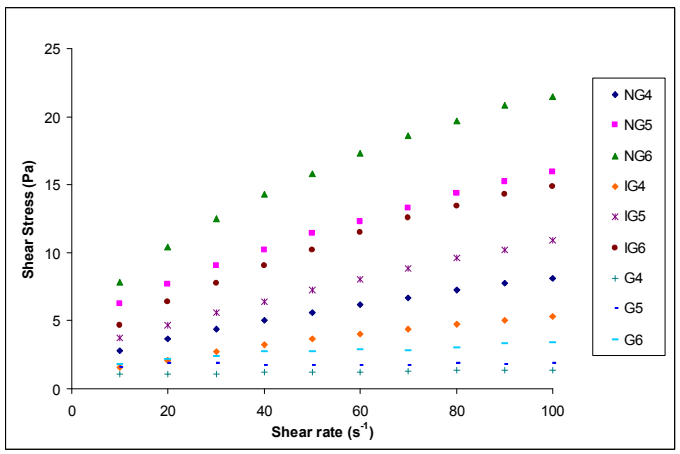


Figure 4.22: Rheogram of barley-milk based fermented probiotic drink obtained using fermented barley-milk composite from NG: non-germinated; IG: imbibed grain; and G: germinated barley grain flour at 25°C (4, 5 and 6 represents the concentration of flour in milk)

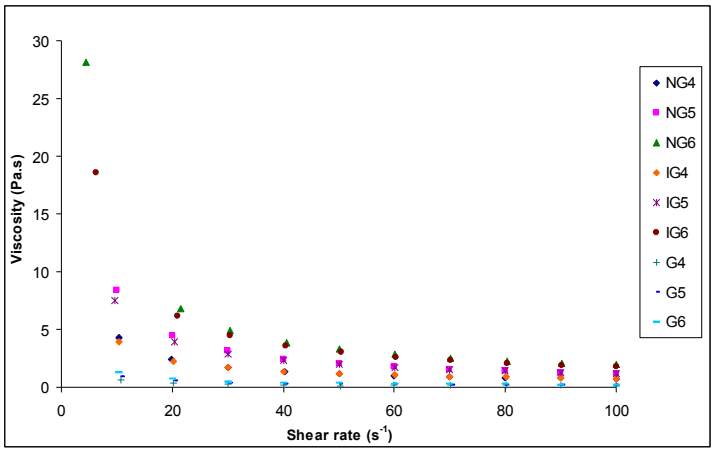


Figure 4.23 Variable shear-rate *vs.* viscosity of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated grains flour before fermentation at 5°C (4, 5 and 6 are the concentration of flour in milk)

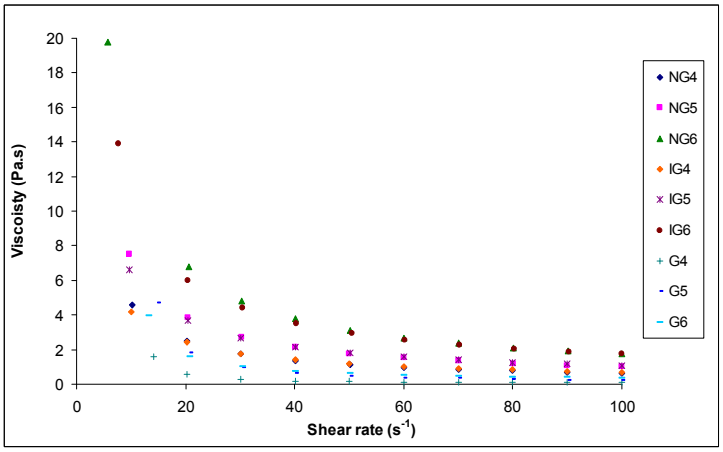


Figure 4.24 Variable shear-rate *vs.* viscosity of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated grains flour after fermentation at 5°C (4, 5 and 6 are the concentration of flour in milk)

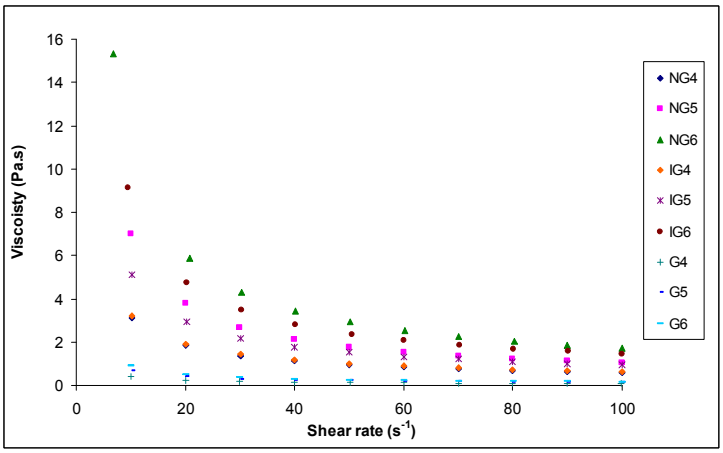


Figure 4.25 Variable shear-rate *vs.* viscosity of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated grains flour before fermentation at 20°C (4, 5 and 6 are the concentration of flour in milk)

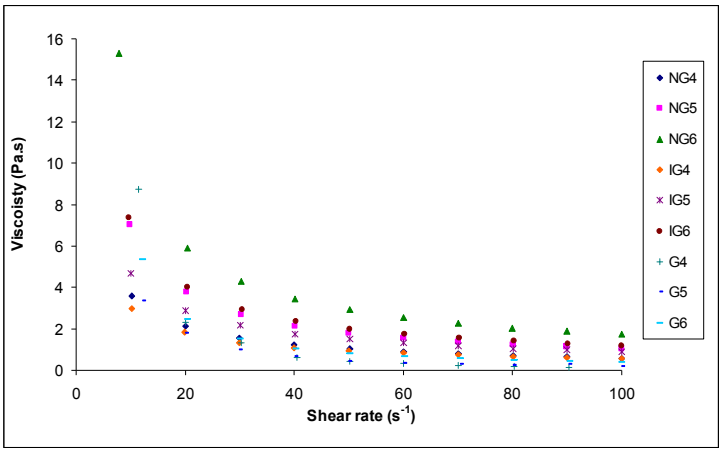


Figure 4.26 Variable shear-rate *vs.* viscosity of barley-milk composite obtained from NG: non-germinated; IG: imbibed grain; and G: germinated grains flour before fermentation at 20°C (4, 5 and 6 are the concentration of flour in milk)

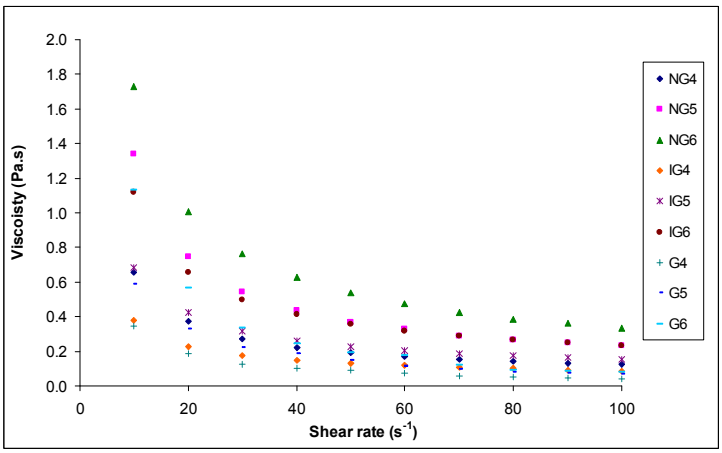


Figure 4.27 Variable shear-rate *vs.* viscosity of barley-milk based fermented probiotic drink obtained from NG: non-germinated; IG: imbibed grain; and G: germinated grains flour before fermentation at 5°C (4, 5 and 6 are the concentration of flour in milk)

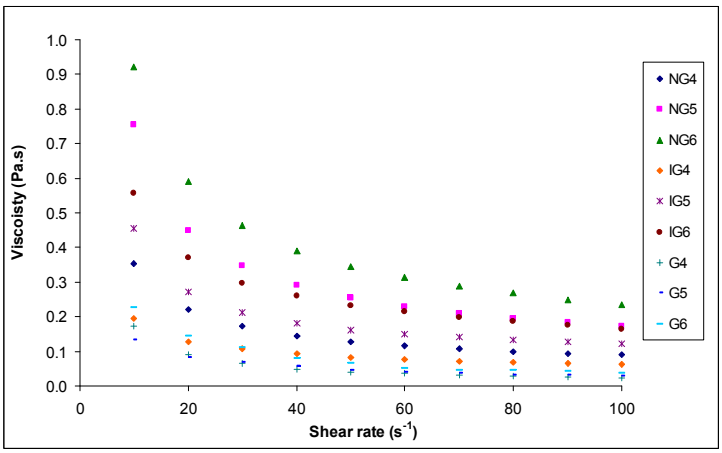


Figure 4.28 Variable shear-rate *vs.* viscosity of barley-milk based fermented probiotic drink obtained from NG: non-germinated; IG: imbibed grain; and G: germinated grains flour before fermentation at 5°C (4, 5 and 6 are the concentration of flour in milk)

In general, plot of shear stress *vs*. shear rate is known as rheogram. It can be observed from the figures that viscosity of products decreased on increasing the shear rate and all the samples exhibited Non-Newtonian behavior. Data related to shear rate *vs*. shear stress were computed to different rheological models *viz.*, *Ostwald-De-Waele*, *Herschel-Bulkley* and *Casson* model using RheoPlus32 (v3.61) software and parameters obtained for different models are depicted in **Table 5-15**. Changes in viscosity with increasing shear rate at 5 and 20°C for barley-milk composite samples and their corresponding probiotic drink samples obtained using different types of flour are also indicated in the form of graphs from **Figure 4.23 to 4.29**.

4.8.5.1 Power law model

One of the most widely used models is called Power law or *Ostwald-De-Waele* model for approximation of shear rate and shear rate data. The model is simpler and contains only two parameters consistency coefficient (K) and flow behavior index (n), as defined in sub-section **3.2.8.3**. The results in the tables showed reasonably good fitting of power law model to shear stress and shear rate data with coefficient of determination (R^2) values ranging in between 0.941 to 0.999 for the different samples analysed. The samples exhibited pseudoplastic in nature (Holdsworth, 1993), with shear thinning flow behaviour where flow behavior index (n) was less than '1'. Majority of fluid foods are reported to have pseudoplastic nature, which exhibits shear thinning behaviour as indication of structural breakdown of food molecules due to the hydrodynamic forces applied as shear (Rao, 1999; Nindo *et al.*, 2007). Yoghurt can be categorized as pseudoplastic material which showed a yield stress that has to be exceeded for flow to be initiated. It can be considered either as viscoelastic fluid, if we are dealing with stirred or drinking yoghurt (Lee and Lucey, 2010). Yoghurt exhibits time-dependent shear thinning behavior since structural breakdown due to shear and is not completely reversible once the shear stops. Vercet *et al.*, (2002) subjected yoghurt to a controlled stress rheometer at constant temperature with variable shear rate between 10 s^{-1} and 290 s^{-1} (upward and downward). Data obtained for upward and downward parts of the plots were separately fitted to the power law equation, in which K indicated consistency index and n is the power law index which expresses the flow behavior as Newtonian (n is close to 1) or non-Newtonian (n is far from 1).

Table 4.7 Fitting of different rheological models on the shear rate *vs*. shear stress data for barley-milk composite before fermentation and obtained using non-germinated barley grain flour

Flour Level	*Ostwald-De-Waele* Model			*Casson* Model			*Herschel Bulkley* Model			
	K_o	n_o	R^2	τ_c	k_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
					5°C					
4%	26.518	0.2065	0.9654	34.134	0.06505	0.9958	40.194	0.7581	0.8063	0.9990
5%	55.976	0.1577	0.9665	69.324	0.06387	0.9975	74.335	2.1204	0.6565	0.9994
6%	98.096	0.1384	0.9499	109.87	0.11425	0.9963	113.16	5.1313	0.5942	0.9963
					10°C					
4%	23.281	0.2168	0.9811	30.985	0.06046	0.9978	35.324	0.9570	0.7475	0.9978
5%	52.722	0.1634	0.9745	65.727	0.66689	0.9992	69.445	2.4660	0.6282	0.9995
6%	77.440	0.1860	0.9690	96.752	0.14750	0.9960	100.51	5.5820	0.5939	0.9949
					15°C					
4%	20.453	0.2337	0.9919	27.050	0.07036	0.9960	31.043	1.1790	0.7085	0.9966
5%	47.973	0.1763	0.9811	60.733	0.07258	0.9998	63.818	2.6581	0.6207	0.9999
6%	71.607	0.1948	0.9861	87.083	0.17201	0.9965	77.755	11.901	0.4663	0.9993
					20°C					
4%	17.949	0.2520	0.9741	23.980	0.07773	0.9959	27.828	1.1165	0.7260	0.9966
5%	44.565	0.1791	0.9821	56.718	0.07038	0.9990	58.966	2.7896	0.6028	0.9995
6%	68.508	0.1964	0.9853	83.718	0.16648	0.9974	77.261	10.061	0.4902	0.9996
					25°C					
4%	16.502	0.2743	0.9933	22.512	0.09073	0.9979	25.881	1.4207	0.6982	0.9996
5%	40.364	0.1861	0.9846	50.912	0.07019	0.9959	53.383	2.8346	0.5933	0.9994
6%	53.425	0.2266	0.9920	68.397	0.18017	0.9981	58.33	11.303	0.4654	0.9994

Table 4.8 Fitting of different rheological models on the shear rate *vs.* shear stress data for barley-milk composite after fermentation and prepared using non-germinated barley grain flour

Flour Level	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	k_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
					5°C					
4%	33.159	0.1417	0.9832	39.933	0.03022	0.9993	39.830	2.108	0.5368	0.9995
5%	51.742	0.1397	0.9793	61.896	0.04653	0.9991	62.802	2.803	0.5639	0.9992
6%	87.983	0.1481	0.9980	102.74	0.10446	0.9660	101.58	7.339	0.4995	0.9767
					10°C					
4%	25.644	0.1944	0.9906	32.720	0.05289	0.9969	29.972	3.687	0.4817	0.9988
5%	49.136	0.1578	0.9709	59.928	0.06023	0.9969	60.928	4.012	0.5238	0.9968
6%	79.148	0.1571	0.9701	93.278	0.10910	0.9898	90.019	7.551	0.5044	0.9908
					15°C					
4%	26.408	0.1841	0.9928	33.388	0.04680	0.9944	27.057	5.055	0.4170	0.9979
5%	45.415	0.1702	0.9909	56.328	0.06651	0.9962	52.273	5.525	0.4729	0.9987
6%	71.585	0.1788	0.9995	87.118	0.13076	0.9699	80.365	10.03	0.4655	0.9850
					20°C					
4%	23.378	0.2012	0.9976	30.185	0.05184	0.9717	24.089	5.454	0.4072	0.9887
5%	45.988	0.1554	0.9908	56.183	0.05306	0.9954	50.914	5.834	0.4410	0.9986
6%	65.576	0.1798	0.9988	80.810	0.11702	0.9759	71.032	10.91	0.4361	0.9903
					25°C					
4%	20.155	0.2004	0.9987	25.947	0.04461	0.9842	17.325	6.768	0.3495	0.9972
5%	34.651	0.1888	0.9961	43.998	0.06583	0.9929	32.748	9.204	0.3709	0.9992
6%	49.397	0.2205	0.9994	63.857	0.14806	0.9751	60.591	17.22	0.3618	0.9946

Table 4.9 Fitting of different rheological models on the shear rate *vs*. shear stress data for barley-milk composite before fermentation and prepared using germinated barley grain flour

Flour Level	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	k_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
					5°C					
4%	2.491	0.3536	0.9680	3.857	0.02663	0.9900	5.460	0.1219	0.8904	0.9957
5%	5.367	0.2324	0.9480	7.341	0.01649	0.9864	8.956	0.1575	0.8268	0.9972
6%	7.645	0.2267	0.9472	9.322	0.02005	0.9859	11.53	0.2317	0.7934	0.9966
					10°C					
4%	2.022	0.3608	0.9616	3.160	0.02299	0.9890	4.256	0.1566	0.8141	0.9964
5%	4.084	0.2810	0.9647	6.212	0.02283	0.9909	7.856	0.1919	0.8171	0.9959
6%	5.970	0.2375	0.9665	8.150	0.01988	0.9961	9.254	0.3766	0.6870	0.9981
					15°C					
4%	1.857	0.3641	0.9725	2.837	0.02250	0.9945	3.759	0.1793	0.7758	0.9966
5%	3.682	0.2933	0.9694	5.330	0.02253	0.9941	6.715	0.1933	0.8069	0.9981
6%	5.644	0.2378	0.9752	7.628	0.01924	0.9965	8.451	0.4318	0.6540	0.9982
					20°C					
4%	1.216	0.4381	0.9787	1.975	0.02709	0.9941	2.658	0.2017	0.7579	0.9962
5%	3.240	0.2989	0.9825	4.733	0.02091	0.9974	5.650	0.2525	0.7375	0.9979
6%	4.559	0.2627	0.9715	6.392	0.02022	0.9962	7.061	0.4281	0.6486	0.9971
					25°C					
4%	0.844	0.5061	0.9861	1.397	0.03197	0.9974	1.854	0.2306	0.7375	0.9969
5%	2.359	0.3436	0.9975	3.538	0.02304	0.9943	4.250	0.2763	0.7104	0.9983
6%	3.931	0.2625	0.9717	5.476	0.01745	0.9968	5.912	0.0434	0.6172	0.9964

Table 4.10 Fitting of different rheological models on the shear rate *vs*. shear stress data for barley-milk composite before fermentation and prepared using imbibed barley grain flour

Flour Level	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	k_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
					5°C					
4%	20.728	0.2687	0.9873	28.130	0.10744	0.9987	29.550	2.5522	0.6179	0.9990
5%	41.910	0.2201	0.9818	54.183	0.12537	0.9980	57.190	3.5507	0.6198	0.9982
6%	83.824	0.1542	0.9409	97.627	0.11469	0.9948	99.579	5.3260	0.5748	0.9995
					10°C					
4%	17.100	0.2903	0.9958	23.614	0.11128	0.9967	25.531	2.8866	0.5879	0.9985
5%	34.064	0.2276	0.9693	44.428	0.11131	0.9972	44.235	4.1129	0.5646	0.9952
6%	72.279	0.1743	0.9786	87.672	0.12383	0.9974	85.036	7.3603	0.5183	0.9976
					15°C					
4%	16.737	0.2866	0.9925	23.003	0.10524	0.9984	21.680	3.3060	0.5541	0.9997
5%	27.578	0.2607	0.9941	37.211	0.13066	0.9966	32.132	6.2308	0.4961	0.9994
6%	61.046	0.2005	0.9866	77.037	0.14300	0.9981	74.292	7.3241	0.5260	0.9992
					20°C					
4%	16.129	0.2945	0.9933	22.296	0.11001	0.9983	20.574	3.4863	0.5477	0.9998
5%	28.642	0.2556	0.9954	38.513	0.12798	0.9965	30.562	7.7507	0.4580	0.9999
6%	50.319	0.2238	0.9828	65.178	0.15796	0.9994	60.473	7.9530	0.5107	0.9986
					25°C					
4%	14.240	0.3024	0.9942	19.574	0.10502	0.9975	16.903	3.7795	0.5206	0.9995
5%	26.079	0.2641	0.9980	35.276	0.12824	0.9931	26.678	7.9210	0.4498	0.9997
6%	40.674	0.2505	0.9903	54.499	0.17112	0.9979	48.630	8.0960	0.5049	0.9989

Table 4.11 Fitting of different rheological models on the shear rate *vs*. shear stress data for the barley-milk composite after fermentation obtained using imbibed grain barley flour

Flour Level	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	k_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
					5°C					
4%	25.577	0.2143	0.9965	33.239	0.06893	0.9934	26.563	6.0794	0.4249	0.9989
5%	38.562	0.2205	0.9977	50.228	0.11343	0.9919	38.572	10.299	0.4138	0.9924
6%	70.116	0.1937	0.9851	86.814	0.15678	0.9904	71.546	14.872	0.4189	0.9984
					10°C					
4%	21.568	0.2512	0.9879	28.902	0.09168	0.9981	19.944	7.4909	0.4098	0.9961
5%	33.230	0.2480	0.9993	44.447	0.13552	0.9881	29.167	12.288	0.3961	0.9987
6%	61.628	0.2008	0.9809	77.337	0.14870	0.9984	60.375	15.322	0.4011	0.9952
					15°C					
4%	18.153	0.2667	0.9942	24.651	0.09162	0.9967	14.583	7.9297	0.3911	0.9984
5%	24.745	0.2819	0.9995	33.983	0.14684	0.9796	17.283	12.623	0.3846	0.9966
6%	51.016	0.2117	0.9989	66.082	0.14333	0.9730	46.803	15.716	0.3866	0.9920
					20°C					
4%	15.583	0.2842	0.9984	21.429	0.09502	0.9921	10.515	8.2749	0.3801	0.9996
5%	26.372	0.2716	0.9996	35.971	0.13998	0.9879	14.220	16.067	0.3443	0.9997
6%	42.015	0.2240	0.9941	54.796	0.13000	0.9951	33.039	16.987	0.3519	0.9985
					25°C					
4%	15.603	0.2578	0.9965	21.047	0.07114	0.9929	10.045	8.2784	0.3497	0.9989
5%	23.804	0.2772	0.9985	32.64	0.13374	0.9767	12.215	14.951	0.3461	0.9971
6%	36.75	0.2298	0.9986	48.46	0.12005	0.9826	24.909	17.718	0.3321	0.9975

Table 4.12 Fitting of different rheological models on the shear rate *vs*. shear stress data for barley-milk based fermented probiotic drink prepared using non-germinated barley grain flour

Flour Level	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	k_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
					5°C					
4%	3.039	0.2942	0.9815	4.449	0.01092	0.9992	5.094	0.2960	0.6915	0.9999
5%	6.899	0.2574	0.9764	9.601	0.02904	0.9985	10.659	0.5879	0.6626	0.9993
6%	8.471	0.2958	0.9963	11.70	0.05866	0.9941	11.905	1.4127	0.5926	0.9966
					10°C					
4%	2.127	0.3480	0.9885	3.202	0.02241	0.9993	3.687	0.2975	0.6898	0.9999
5%	4.965	0.3035	0.9843	7.218	0.03408	0.9995	8.004	0.6049	0.6577	0.9996
6%	5.901	0.3453	0.9958	8.340	0.06689	0.9970	7.907	1.4773	0.5839	0.9993
					15°C					
4%	1.644	0.3873	0.9902	2.541	0.02462	0.9999	2.904	0.3073	0.6810	0.9999
5%	3.619	0.3512	0.9854	5.486	0.03905	0.9993	6.037	0.6292	0.6497	0.9992
6%	4.488	0.3811	0.9971	6.795	0.06513	0.9977	5.802	1.5013	0.5680	0.9998
					20°C					
4%	1.198	0.4327	0.9932	1.884	0.02641	0.9999	2.103	0.3023	0.6772	0.9999
5%	2.804	0.3908	0.9908	4.338	0.04385	0.9997	4.607	0.6575	0.6413	0.9993
6%	3.302	0.4257	0.9983	5.056	0.07060	0.9967	3.709	1.5115	0.5609	0.9999
					25°C					
4%	0.817	0.4964	0.9962	1.286	0.02999	0.9996	1.325	0.3121	0.6693	0.9999
5%	2.047	0.4428	0.9934	3.211	0.04897	0.9996	3.288	0.6685	0.6398	0.9996
6%	2.713	0.4516	0.9993	4.091	0.07295	0.9993	2.404	1.5242	0.5542	0.9993

Table 4.13 Fitting of different rheological models on the shear rate *vs.* shear stress data for barley-milk based fermented probiotic drink prepared using imbibed barley grain flour

Flour Level	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	k_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
					5°C					
4%	1.425	0.3897	0.9922	2.195	0.02440	0.9974	2.765	0.185	0.7593	0.9990
5%	2.985	0.3475	0.9919	4.212	0.03464	0.9990	4.754	0.478	0.6715	0.9991
6%	4.743	0.3423	0.9921	7.071	0.04787	0.9987	7.722	0.764	0.6573	0.9994
					10°C					
4%	1.091	0.4332	0.9937	1.712	0.02421	0.9993	2.114	0.211	0.7306	0.9997
5%	2.296	0.3955	0.9918	3.542	0.03721	0.9979	3.919	0.489	0.6669	0.9986
6%	3.021	0.4205	0.9930	4.716	0.06047	0.9985	5.072	0.787	0.6569	0.9991
					15°C					
4%	0.839	0.4744	0.9931	1.340	0.02566	0.9999	1.615	0.223	0.7142	0.9993
5%	2.009	0.3952	0.9877	2.863	0.3595	0.9975	3.010	0.488	0.6502	0.9992
6%	2.176	0.4617	0.9965	3.390	0.06160	0.998	3.351	0.770	0.6471	0.9991
					20°C					
4%	0.758	0.5303	0.9973	0.816	0.02519	0.9987	0.879	0.213	0.6967	0.9992
5%	1.489	0.4436	0.9765	2.110	0.04008	0.9960	2.105	0.470	0.6582	0.9939
6%	1.684	0.4959	0.9985	2.576	0.06305	0.997	2.211	0.772	0.6368	0.9995
					25°C					
4%	0.429	0.5472	0.9983	0.633	0.02378	0.9953	0.619	0.198	0.6920	0.9972
5%	0.977	0.5195	0.9918	1.584	0.04116	0.9992	1.596	0.445	0.6546	0.9963
6%	1.305	0.5305	0.9983	1.966	0.06330	0.9972	1.381	0.780	0.6229	0.9993

Yoghurt can be classified as pseudoplastic material which contains a yield stress that has to be exceeded for flow to be initiated. It can be considered either as viscoelastic fluid if we are dealing with stirred or drinking yoghurt (Lee and Lucey, 2010). Yoghurt exhibits time-dependent shear thinning behavior since structural breakdown due to shear is not completely reversible once the shear stops. Lee and Lucey (2006) found that the rheological properties of stirred yoghurts were greatly influenced by the physical properties of the original intact yoghurt gels. Chin *et al.*, (2009) reported a consistent decrease in the consistency index (K) with increasing flow behavior index (n) of pummelo juice concentrates having 20, 30 and 50% total soluble solids when temperature was increased from 6 to 75°C, and parameters were obtained for power law model. Increase in the total soluble solids of pummelo juice was reported to increase the consistency index which varied with the change in temperature. A consistent decrease in the K and consequent increase in n was observed with the increase in temperature from 5 to 25°C. Vandresen *et al.*, (2009) observed increase in the flow behavior index and decrease in consistency index in *Ostwald-De-Waele* model when temperature of carrot juice increased from 8°C to 25°C.

Fermentation of barley-milk composite samples consisting 4% flour from non-germinated and imbibed grain showed rise in consistency index, whereas samples having 5% and 6% flour level indicated decrease in the consistency index. Rise in consistency index due to fermentation could be attributed to the formation of firm curd matrix by coagulation of milk protein during fermentation process. However, for the samples having 5% and 6% flour level, gelatinized starch which majorly contributed towards increased viscosity of barley-milk composite samples might have gone under acid or microbial hydrolysis during fermentation process, leading to decrease in consistency index. Fermentation of barley-milk composite samples prepared using germinated flour samples were coagulated representing clear whey separation from cereal-casein matrix during fermentation, therefore, not subjected to rheological analyses after fermentation.

Treatments of barley grains such as imbibtion and germination were the major factors which influenced the viscosity of barley-milk composite samples. Samples containing barley flour from non-germinated barley grains showed highest viscosity when compared with the samples prepared using flour from imbibed barley grains and germinated barley grains. High viscosity of samples containing non-germinated barley flour was due to higher starch content

compared with the other two types of flour. Samples prepared using germinated barley grain flour were not gelatinized properly due to starch hydrolysis during gerimation process thus exhibited lowest viscosity during processing among the three types of flour. Germination and imbibitions of barley grains have led to partial hydrolysis of starch due to activation of cereal enzymes. Wen *et al.*, (2012) estimated flow behavior index, consistency coefficient and regression coefficient in power law model for the set yoghurt samples prepared using skim milk, skim milk with horseradish peroxidase, skim milk with ferulic acid and horseradish peroxides. Significant differences were reported for consistency index of different samples. Ferulic acid and horseradish peroixdase were added to improve textural properties of yoghurt.

4.8.5.2 *Herschel-Bulkley* model

This rheological model has been generally used for non-Newtonian fluids and is appropriate for many liquid foods (Ahmed, 2004). The *Herschel-Bulkley* model describes materials which combine power law and Bingham behavior. Above the yield stress the rheogram is non-linear and may display either shear thinning or shear thickening. Parameters for *Herschel Bulkley* models for different samples have been represented in Table **4.7** to **4.13**.

Hassan *et al.*, (2003) recorded the shear stress with increasing shear rates from 0.00185 to 116 s^{-1} followed by decreasing shear rates at 5°C. The *Herschel-Bulkley* model have been previously proven to give significantly better fits to the upward flow curves than the power law and *Casson* models (Skriver *et al.*, 1993). Yield stress (T_o) for pasteurized carrot juice in *Herschel-Bulkley* model was reported to decrease from 1.351 Pa to 0.219 Pa with increase in temperature from 8°C to 35-85°C. Flow behavior index (n) for carrot juices was reported to decrease from 0.931 to 0.638 with temperature increase from 8 to 25°C. In relation to the increase in temperature and shear rate, the viscosity decreased as these two parameters increased for all type of samples. According to Hassan and Hobani (1998), increase in the thermal energy due to increase in temperature leads to an increase in the molecular distances due to reduction in the intermolecular forces. Also, with a temperature increase, the shear stress is enhanced, causing rearrangement of the molecules in parallel directions and breakdown into smaller particles, which can flow more easily due to decrease intra-patricle interactions (Vandresen *et al.*, 2009).

Keshani *et al.*, (2012) studied the rheological behavior of pomelo juice at different concentrations (20-60%) and temperatures (23-60°C) using different rheological models which included power law model, *Casson* model, *Herschel Bulkley* model and Bingham plastic model. Silva (2000) reported that the value of the behavior index (n) indicates the degree of pseudoplasticity for fruit juices and pulps. Ramaswamy and Basak (1991) characterized the rheological behavior of commercial stirred yoghurt and found that the nonreversible structural breakdown can be expressed in a three-cyclic shearing sequence, where upward flow curves generally followed the *Herschel-Bulkley* model and downward curves followed a linear relationship. Falguera and Ibraz (2010) studied the flow behavior of concentrated orange juice between -12 to +6°C with intervals of 3°C and +10 to 350°C with an interval of 5°C. No particular pattern for σ_o and n were reported with the changes in temperature from -12°C to 30°C. However, decrease in the value of consistency index (K) was reported when temperature range varied from 0 to 30°C.

4.8.5.3 *Casson* model

The *Casson* model is widely used in the range of food products such as molten chocolate, fruit purees, gums, fruit juice concentrate and tomato products (Rao, 1999; Holdsworth, 1993). *Casson* model are closely related with the effect of particle size distribution on the flow behavior of pigment oil suspensions (Casson, 1959). There are two parameter determined from *Casson* model, namely *Casson* yield, and the *Casson* Plastic viscosity. *Casson* model parameters are tabulated in **Table 4.7** to **4.13**. The *Casson* equation has been adopted by the International Office of Cocoa and Chocolate for interpreting chocolate flow behavior.

Yield stress in all types of models fitted was found decreasing with the increase in temperature from 5 to 25°C. No particular pattern of increasing or decreasing for *Casson* plastic viscosity was observed in the fermented as well as non-fermented barley-milk composite samples. Danagala (2008) prepared set yoghurt from goat, cow and sheep milk and subjected fresh and two weeks old samples for sensory and rheological analyses. Three different models *viz.*, *Ostwald-De-Waele*, *Herschel-Bulkley* and *Casson* models were fitted to shear stress data obtained in variable shear rate from 1 to 437.4 s^{-1} and 437.4 to 1s^{-1} (Danagala, 2008). Highest R^2 values were reported for *Ostwald-De-Waele* model, followed by R^2 values for Herschel Bulkely model for the different samples.

Casson model were reported to have lower R^2 values when compared with other two models. Similar shapes of flow curves to the ones obtained in present study were also represented by rheological investigation of others (Hassan, 2003; Jaros *et al.* 2003; Lee and Lucey, 2010). Rheological properties of gellan and gellan-pectin fluid gels in fermented dairy drinks have been evaluated by Kiani *et al.*, (2010) using viscometric measurements. Author suggested the use of Herchel-Bulkey and *Casson* model for the estimation of yield stress. Gellan and gellan-pectin mixture were reported to reduce sedimentation behavior of colloidal protein particles as well as added dried and grinded basil leaves.

In addition, composition of milk has important influence on the consistency and texture of cultured milk (Tamime and Robinson, 1999). Texture of yoghurt is influenced several processing factors during, before and after fermentation. Denaturation of whey protein initiates the interactions among β-lactoglobulin, κ-casein, and α-lactalbumin, thus produces stronger and more stable gel. The type and concentration of yoghurt inoculum influence gelation and final texture of yoghurt, by altering the start and onset of gelation (Jumah *et al.*, 2001).

4.8.6 Pasting properties of barley flour in toned milk

Gelatinization of starch is responsible for its change in various physical properties during the preparation and processing of food. Starch gelatinization is disruption of the starch granules resulting irreversible changes in various properties such as swelling, crystalline melting and loss of birefringence and solublisation. The point of initial gelatinization and range over which it occurs is governed by starch concentration, method of observation, treatments given to grains and heterogeneity within the starch granules.

Table 4.14 Summary of the results from RVA over the instrument profile to demonstrate the differences in properties among different barley-milk composite

	Peak	Visc. at 3 min	Visc. at 23 min	Final Visc.	Delta
NG	131.35±1.13	12.5±1.13	471±3.53	1409±45.03	458.5±4.58
IG	73.5±0.35	30±0.79	96±0.79	307±35.39	66±1.46
G	19.5±0.35	5±1.41	4.5±1.56	10.5±2.51	6.5±1.49

Each value represents mean±SE of three trials

Pasting is known as phenomenon of gelatinization which involves granular swelling, exudation of molecular components from the granule and eventually, total disruption of the granules. Pasting properties of different types of barley flour were determined in toned milk using Rapid Visco Analyser (RVA) and method discussed in **3.2.8.4**. Tests were done to see the changes in viscosity at 90ºC for

a period of 20 minutes at constant shear rate of 100 s^{-1}. Results obtained for pasting properties of different types of barley flour in toned milk are presented in **Table 4.14**. The pasting curves of the composites were obtained by RVA and expressed as viscosity (cP) (**Figure 4.29)** with respect to time and changing temperature. Barley-milk composite obtained from non-germinated showed highest final viscosities than the composites obtained by mixing of flour obtained from germinated and imbibed barley grains.

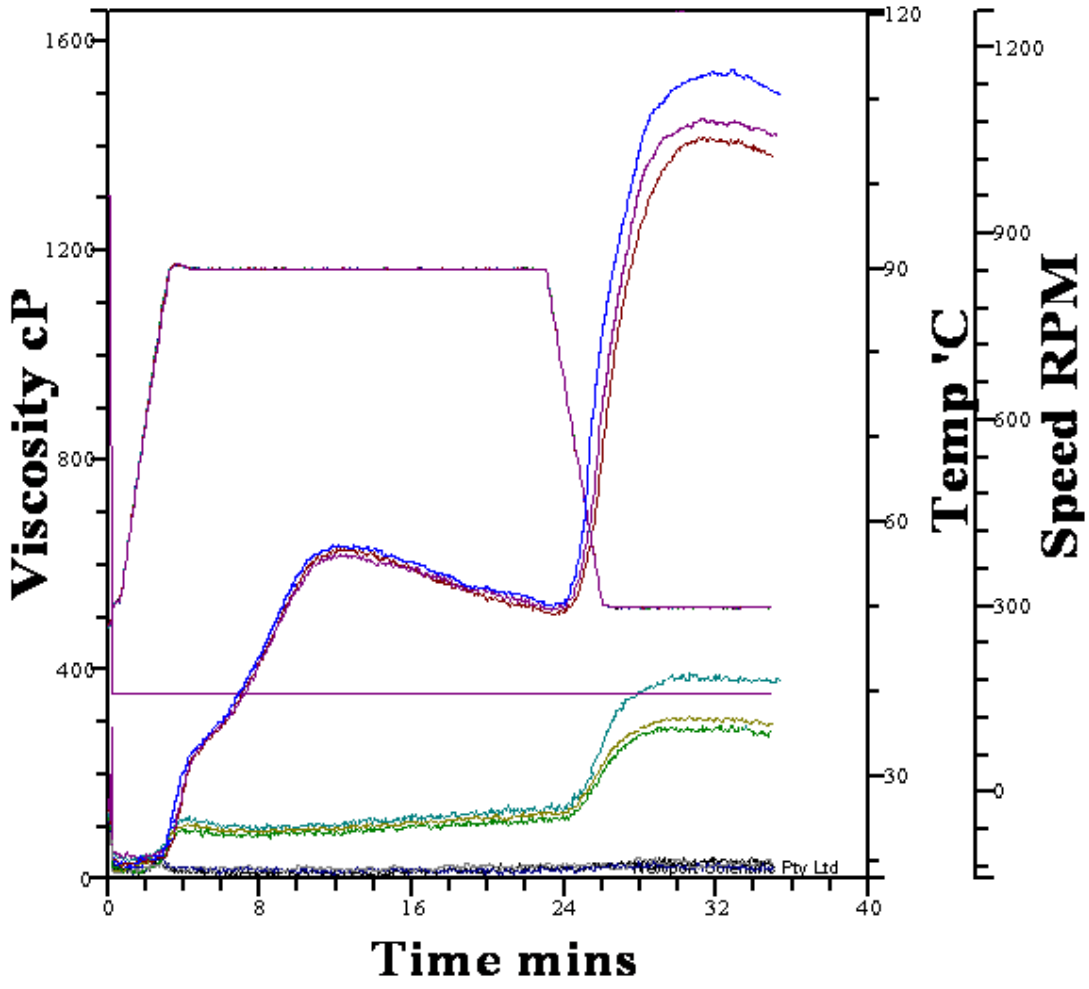


Figure 4.29 Result of the viscosity measurements with RVA to determine the viscosity of the barley-milk composite during heating and continuous stirring

The pasting curve of germinated barley flour and milk composite exhibited very low pasting viscosity with a flat curve. However, continuous stirring of samples led to decrease in viscosity for the composites obtained using non-germinated and imbibed barley-grain flour. Higher final viscosity at 90°C of barley-milk composite samples led to difficulty in processing of the composite while preparing for the fermentation purposes. High viscosity of composites interferes with the proper mixing of starter cultures. Final peak viscosity at the

end of test was significantly increased with increasing the levels of barley flour in the composite samples prepared using non-germinated and imbibed barley grain flour. Barley-milk composites obtained from non-germinated and imbibed barley grains exhibited structural stability under heat and shear. It may be due to that uncooked *β*-glucan in the barley resulted in an entanglement of molecules immediately after cooking and during cooling indicated the formation of a matrix with greater stability under heat and shear (Inglett *et al.*, 2013). It suggests that the barley-milk composites exhibited greater structural stability under heat and shear, which may be due to gelatinization of barley starch and *β*-glucan content in the barley grain. Swelling of starch molecules after cooking and during cooling indicated the formation of a matrix with greater stability. Both germination and imbibition treatment of grains has led to decrease in final viscosity of the barley-milk composite. However, incorporation level of flour in milk is limited due to the high viscosity of the composite, which causes difficulty in processing of composite and also decreases the sensory acceptability while developing a beverage like product.

Data obtained during RVA provided useful information for viscosity changes during processing. Improvement in the textural properties of food using *β*-glucan has been previously reported (Inglett *et al.*, 2013; Lee *et al.*, 2009). Viscosities of all the samples at 3 min and 23 min were significantly different from each other. Final viscosity of at 5 % level of flour were found highest for the composites obtained using non-germinated barley flour which was followed by viscosity of samples prepared using imbibed barley grain flour and germinated barley grain flour. Barley-milk composites prepared using germinated barley flour were failed to show any significant rise in the viscosity when continuously heated at 90°C for 20 min.

4.8.7 Effect of types of flour and its level on the textural properties of fermented barley-milk composite

The back extrusion test can be used as a quality control tool to ensure consistency and monitoring the effects during formulation or new product development. Textural attributes such as firmness and stickiness were determined by back extrusion method using a texture analyzer, TA-XT2i (M/s Stable Micro Systems, UK) following the methods discussed in **3.2.8.5**. A typical force-deformation curve of 'back extrusion' test of fermented barley-milk composite is given in **Figure 4.30**. Back extrusion test has been studied

previously to the fermented milk gels because it is not affected by free whey on the surface of the samples (Pereira *et al.*, 2003; Raju and Pal, 2009).

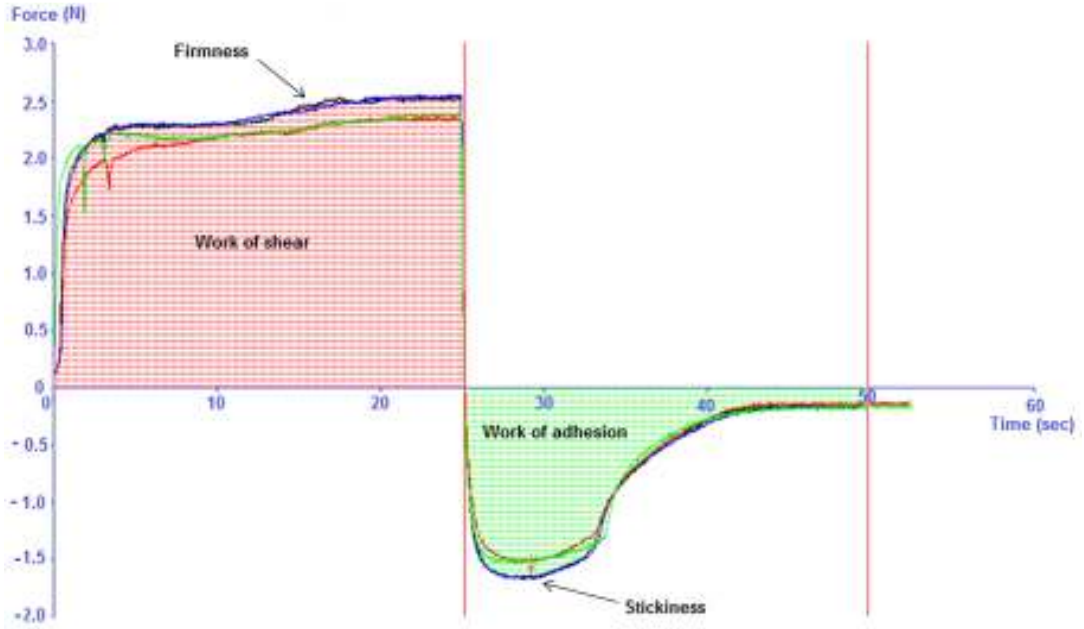


Figure 4.30 A Typical force-deformation curve of fermented barley-milk composite obtained using texture analyzer

Firmness, which is defined as the peak force obtained during penetration of the probe, increased with increasing the flour level from 4% to 6% in the composite samples prepared using non-germinated and imbibed grain flour. The increase in firmness value of fermented barley-milk composite samples could be due to increased total solids content in composite samples.

Table 4.15 Effect of type of flour and its level on the textural attributes of fermented barley-milk composite

Description	Firmness (N)	Work of Shear (N.s)	Stickiness (N)	Work of Adhesion (N.s)
NG-4%	1.87±0.02^{bc}	42.65±1.58^{c}	-1.25±0.02^{cd}	-13.52±0.32^{d}
NG-5%	2.88±0.06^{d}	57.25±1.16^{f}	-1.94±0.06^{ab}	-17.59±0.38^{b}
NG-6%	3.35±0.10^{e}	76.28±2.54^{h}	-2.52±0.09^{a}	-26.37±0.55^{a}
G-4%	0.47±0.08^{a}	4.33±0.44^{a}	-0.44±0.36^{e}	-0.59±0.12^{f}
G-5%	0.39±0.08^{a}	5.09±0.43^{a}	-0.73±0.43^{de}	-0.77±0.19^{f}
G-6%	1.57±0.30^{b}	25.06±0.86^{b}	-0.85±0.19^{de}	-5.07±0.80^{e}
IG-4%	2.15±0.04^{c}	49.92±0.87^{d}	-1.16±0.03^{cd}	-14.39±0.50^{cd}
IG-5%	2.53±0.05^{d}	55.48±1.24^{e}	-1.51±0.03^{bc}	-15.29±0.52^{c}
IG-6%	2.87±0.03^{d}	63.85±1.37^{g}	-1.67±0.03^{bc}	-18.00±0.38^{b}

Values in each cell represent mean ± SE, n=4; Means with different superscripts (a, b, c) in the same column indicate significant difference (Fisher's LSD test, $\rho<0.05$)

Mistry and Hassan (1992) observed increase in the firmness value of yoghurt when fortified with non-fat dry milk and high protein milk powder. Separation of clear whey and fat rich layer from protein rich cereal-milk composite have been observed in the samples obtained using germinated barley flour, which has led to large variation among the results obtained during textural analyses. Separation in three distinct layers has led it unsuitable for the preparation of drink. Firmness value for the samples using 4% of imbibed grain barley flour was comparatively higher than the firmness value of fermented barley-milk composite prepared using non-germinated barley flour at 4% level. However, differences observed were non-significant ($p>0.05$). But, when the flour level was increased from 4 to 5 and 6%, firmness value for the samples prepared using non-germinated barley flour were found greater than that of samples prepared using imbibed grain barley flour.

The work of shear, *i.e.* the area under the penetration cycle, representing the amount of energy required to perform the shearing process, increased with increasing flour level. The value for work of shear was highest for the samples prepared using non-germinated barley flour, when compared with other two types of flour at similar level. Significant ($p<0.05$) differences in work of shear was observed due to differences in flour type and levels except for samples prepared using 4% and 5% germinated barley flour. Increased work of shear in samples prepared using non-germinated barley flour is due to increased viscosity of the fermented barley-milk composite which may be due to higher starch and β-glucan content as compared to imbibed and germinated barley grain flour.

Stickiness is the negative peak force which is obtained during the up-stroke of the probe. Maximum stickiness was observed for the fermented composite samples prepared using non-germinated barley grains flour when compared with samples prepared using imbibed or germinated barley grains flour. However, after fermentation of composite samples prepared using germinated barley flour, separation of fat rich layer was observed on the top, middle layer represented whey and bottom layer was consisted of casein and flour rich portion. Therefore, stickiness and work of adhesion was lower in the samples prepared using germinated barley grain flour at 6% level, when compared with the samples prepared using non-germinated and imbibed barley grain flour at same level. Very low stickiness and work of adhesion was observed for the composite samples prepared using 4% and 5% level of germinated barley

flour, because probe could not touch the casein and flour rich bottom layer portion, since it was penetrated only up to 25 mm into the barley-milk fermented composite during the test. The work of adhesion is the area under withdrawal of probe after penetration (up stroke), representing the amount of energy required to perform the shearing action during withdrawal of proble. Similar pattern for the changes in work of adhesion was observed as for stickiness among all the samples prepared using different types of flour. Processes like germination and imbibition have been previously reported to decrease the starch content by converting it into simple sugars, which also affects the gelatinization properties of starch molecules (Kaneko *et al.*, 2002; Fox *et al.*, 2003; Bamforth, 2009). Changes in milk composition, especially fat content have been previously reported to affect the textural properties of cultured milk products (Akalin *et al.*, 2008; Raju and Pal, 2009).

4.8.8 Optimization of flour level, probiotic inoculum level and co-culture inoculum level for the formulation of barley-milk based fermented probiotic drink

Based on preliminary trials, imbibed barley grain flour, *Lactobacillus plantarum* NCDC344 (probiotic) and *Streptococcus thermophilus* 20 (co-culture) were selected as critical processing variables for the manufacture of barley-milk based fermented probiotic drink. The barley-milk based fermented probiotic drink prepared from the various combinations of these processing variables was evaluated for sensory parameters, probiotic count, β-glucan content and rheological attributes. However, the level of barley flour, concentration of probiotic inoculum and co-culture inoculum were noticed to have major influence on the organoleptic characteristics and therapeutic profile of developed beverage. To optimize the levels of these variables a central composite rotatable design (CCRD) of response surface methodology (RSM) was adopted. The design matrix consisting of 3 factors CCRD was used for the optimization (Singh *et al.*, 2008; Nair and Thompkinson, 2008) of barley-milk based fermented probiotic drink and has been indicated in previous sub-section **3.2.8.5**.

4.8.8.1 Effect of different levels of flour, probiotic inoculum and co-culture inoculum on the sensory quality of barley-milk based fermented probiotic drink

Probiotic drinks prepared using different levels of barley flour, probiotic culture and co-culture inoculum as outlined in **Table 3.4** were presented to the

trained panel of judges along with structured score card (**Annexure XII**) and were asked to evaluate samples for colour and appearance, consistency, sedimentation, flavour and overall acceptability. The average sensory scores obtained for each sample is presented in **Table 4.16**. The data of CCRD were fitted with a second order quadratic function through regression analysis. The models were generated from the response surface analysis of the data and evaluated for their fitness. The dependence of these responses with respect to independent variables and in the form of correlations is presented in **Table 4.16 and 4.18,** respectively. The model adequacy was checked and goodness of fit was expressed by the coefficient of determination (R^2).

The coefficients of determination values for the models obtained for sensory parameters varied in the range of 0.76 to 0.80 (**Table 4.18**). Coefficient of determination reflects the percentage variability in the responses due to independent variables, and higher values indicate better suitability of the regression equation to predict the response. According to Henika (1982) R^2 value of more than 0.70 indicates the significance of model and may be successfully used to predict the responses for barley-milk based fermented probiotic drink. Furthermore, lack of fit was non-significant for all the five sensory responses that confirmed the fitness of model terms (**Table 4.17**). The low coefficient of variation suggested that models were precise and reliable. Regression coefficients of full second order model to predict sensory quality of barley-milk based fermented probiotic drink has been presented in **Table 4.17**.

4.8.8.1.1 Colour and appearance of barley-milk based fermented probiotic drink

The colour and appearance score of barley-milk based fermented probiotic drink ranged from 7.50 to 7.80. Differences in colour and appearance score due to barley flour concentration, probiotic inoculum and co-culture inoculum level was non-significant at linear level (**Table 4.17**). However, at quadratic level barley flour concentration had a significant ($p<0.05$) influence on the colour and appearance of barley-milk based fermented probiotic drink. It is evident from contour graph (**Figure 4.31**) that, at lower levels of barley flour, colour and appearance scores were higher, but a gradual decrease was observed by increasing the concentration of barley flour.

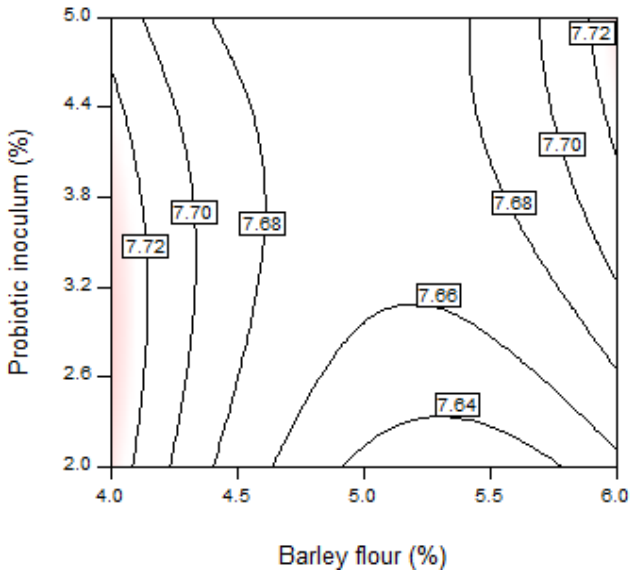


Figure 4.31 Effect of barley flour concentration and probiotic inoculum level on the colour and appearance of barley-milk based fermented probiotic drink

The negative sign for the coefficient of estimates indicated that barley flour and co-culture inoculum had adverse effect on the colour and appearance score (**Table 4.18**). Barley flour at higher levels resulted probiotic drink which was more viscous and creamier in colour as compared to the drink prepared with lower amount of barley flour. Modha and Pal (2011) reported positive influence of flour on the colour and appearance of pearl-millet based *rabadi* like product. The results reported were contrary to our findings, which could be attributed low viscosity of the beverage which ranged in between 32 to 62 cP ($\dot{\gamma}$: 100s^{-1} at 25°C).

4.8.8.1.2 Sedimentation score of barley-milk based fermented probiotic drink

Sedimentation either due to denaturation of milk proteins under acidic conditions or because of gravitational forces is considered as major quality in fermented milk beverages. Sedimentation scores for the fermented probiotic drink as rated by the panellists varied from 7.2 to 7.9. Highest sedimentation score was achieved for the drink prepared using 6.68 % barley flour (**Table 4.16**). Among the variables probiotic concentration influenced sedimentation score at quadratic level ($\rho<0.01$) and interactive level ($\rho<0.05$) with co-culture concentration. Barley flour concentration in the formulation had positive highly significant ($\rho<0.01$) effect. Higher barley flour in probiotic drink might have provided more starch, which form firmer gel on heating and minimized the tendency of sedimentation.

Table 4.16 Sensory scores, probiotic count and β-glucan content of barley-milk based fermented probiotic drink prepared during optimization trials

Standard order	Actual factors (%)			Coded values			Sensory scores (based on 9-point hedonic scale)					Probiotic count (log cfu/ml)	β-glucan* (g/100g)
	x_1	x_2	x_3	x_1	x_2	x_3	CA	Sedimentation	Consistency	Flavour	OA		
1	4.00	2.00	0.25	-1.00	-1.00	-1.00	7.70	7.50	7.65	7.46	7.54	8.10	0.14
2	6.00	2.00	0.25	1.00	-1.00	-1.00	7.70	7.60	7.50	6.40	6.60	8.01	0.25
3	4.00	5.00	0.25	-1.00	1.00	-1.00	7.60	7.40	7.40	7.36	7.41	8.25	0.14
4	6.00	5.00	0.25	1.00	1.00	-1.00	**7.80**	7.40	6.94	6.96	7.04	8.15	0.23
5	4.00	2.00	0.75	-1.00	-1.00	1.00	7.70	7.30	7.00	7.10	7.20	8.11	0.14
6	6.00	2.00	0.75	1.00	-1.00	1.00	**7.50**	7.40	6.90	6.90	7.10	8.05	0.23
7	4.00	5.00	0.75	-1.00	1.00	1.00	**7.80**	7.60	7.70	7.15	7.06	8.48	0.14
8	6.00	5.00	0.75	1.00	1.00	1.00	7.60	7.50	6.60	6.80	6.80	8.21	0.23
9	3.32	3.50	0.50	-1.68	0.00	0.00	**7.80**	7.60	7.60	7.30	7.44	8.05	**0.12**
10	6.68	3.50	0.50	1.68	0.00	0.00	**7.80**	**7.90**	**6.20**	**6.30**	**6.38**	**7.45**	**0.25**
11	5.00	0.98	0.50	0.00	-1.68	0.00	7.60	**7.20**	7.60	7.20	7.22	8.10	0.19
12	5.00	6.02	0.50	0.00	1.68	0.00	7.60	7.50	7.50	7.14	7.24	**8.81**	0.18
13	5.00	3.50	0.08	0.00	0.00	-1.68	**7.50**	7.40	7.50	7.50	7.50	8.27	0.19
14	5.00	3.50	0.92	0.00	0.00	1.68	**7.50**	7.40	7.30	6.90	6.90	8.41	0.18
15	5.00	3.50	0.50	0.00	0.00	0.00	7.70	7.40	7.40	7.16	7.19	8.32	0.19
16	5.00	3.50	0.50	0.00	0.00	0.00	7.60	7.50	7.10	7.30	7.31	8.35	0.19
17	5.00	3.50	0.50	0.00	0.00	0.00	7.60	7.50	7.40	7.10	7.30	8.28	0.19
18	5.00	3.50	0.50	0.00	0.00	0.00	7.60	7.60	7.00	7.30	7.50	8.39	0.20
19	5.00	3.50	0.50	0.00	0.00	0.00	**7.80**	7.60	**7.90**	**7.70**	**7.88**	8.30	0.19
20	5.00	3.50	0.50	0.00	0.00	0.00	7.70	7.40	7.34	7.12	7.33	8.20	0.18

*estimated in barley-milk based fermented probiotic curd; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum; CA: colour and appearance; OA: overall acceptability

Table 4.17 Analysis of variance (ANOVA) for sensory quality, probiotic count and β-glucan content of barley-milk based fermented probiotic drink

Source of Variation	d.f.	Sum of squares						
		Colour and appearance	Sedimentation	Consistency	Flavour	Overall acceptability	Probiotic count	β-glucan
Model	9	0.157394*	0.320306*	2.450972*	1.742889*	1.775642*	1.153863**	0.026537**
x_1	1	0.002948	0.02668	1.268588**	0.996964**	0.871886**	0.170707**	0.026211**
x_2	1	0.002929	0.026761	0.024478	0.006996	0.00068	0.29703	0.000116**
x_3	1	0.002929	0.000732	0.193679	0.11242	0.151641	0.024247	3.8E-05
x_1x_2	1	0.005	0.01125	0.214513	0.032513	0.021013	0.00605	6.61E-05
x_1x_3	1	0.045*	0.00125	0.043512	0.103513	0.112813	0.00245	4.51E-05
x_2x_3	1	0.005	0.06125*	0.183013	0.032512	0.070312	0.0072	2.81E-05
x_1^2	1	0.045816*	0.093358**	0.44512*	0.443029**	0.466574**	0.563229**	9.6E-06
x_2^2	1	0.002973	0.053555*	0.042167	0.028593	0.0643	0.038394	2.85E-06
x_3^2	1	0.035624*	0.026998	1.61E-05	0.016597	0.086342	0.00173	2.54E-05
Residual	10	0.050606	0.085194	0.721683	0.430886	0.462578	0.107632	0.00046
Lack of fit	5	0.017273	0.045194	0.22935	0.181286	0.157094	0.086499	0.000353
Pure error	5	0.033333	0.040000	0.492333	0.2496	0.305483	0.021133	0.000107

**significant at $\rho<0.01$, *significant at $\rho<0.05$, x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Table 4.18 Regression coefficients of full second order model to predict sensory quality, probiotic count and β-glucan content in barley-milk based fermented probiotic drink

Model term	Coefficient estimate†						
	Colour and appearance	Sedimentation	Consistency	Flavour	Overall acceptability	Probiotic count	β-glucan
Intercept	7.6652	7.5012	7.3589	7.2809	7.4184	8.3068	0.1879
x_1	-0.0147	0.0442	-0.3048	-0.2702	-0.2527	-0.1118	0.0438
x_2	0.0146	0.0443	-0.0423	0.0226	-0.0071	0.1475	-0.0029
x_3	-0.0146	-0.0073	-0.1191	-0.0907	-0.1054	0.0421	-0.0017
x_1x_2	0.0250	-0.0375	-0.1638	0.0638	0.0513	-0.0275	-0.0029
x_1x_3	-0.0750	-0.0125	-0.0737	0.1138	0.1188	-0.0175	-0.0024
x_2x_3	0.0250	0.0875	0.1513	-0.0637	-0.0937	0.0300	0.0019
x_1^2	0.0564	0.0806	-0.1759	-0.1755	-0.1801	-0.1979	-0.0008
x_2^2	-0.0144	-0.0610	0.0541	-0.0445	-0.0668	0.0516	-0.0004
x_3^2	-0.0497	-0.0433	0.0011	-0.0339	-0.0774	0.0110	-0.0013
R^2	0.76	0.79	0.80	0.77	0.79	0.91	0.98
R^2_{adj}	0.54	0.60	0.62	0.57	0.61	0.84	0.97
APV$^{\Phi}$	7.17	8.41	8.14	7.48	7.38	15.56	30.72

$^{\Phi}$ adequate precision value, † for final equation in terms of coded factors; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

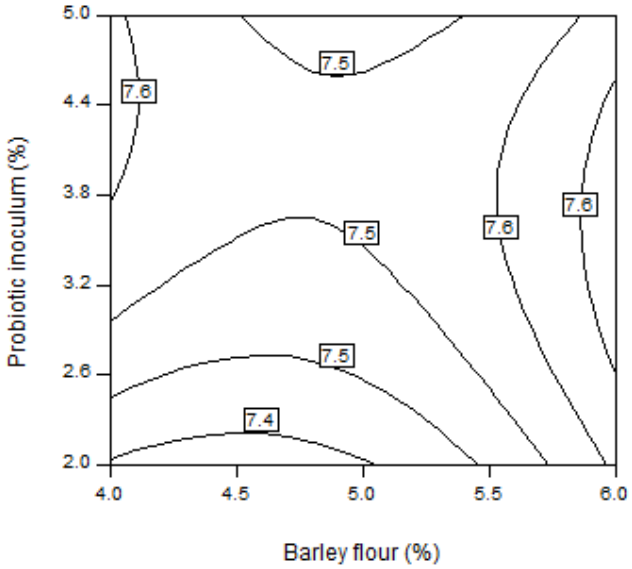


Figure 4.32 Effect of barley flour concentration and probiotic inoculum level on the sedimentation score of barley-milk based fermented probiotic drink

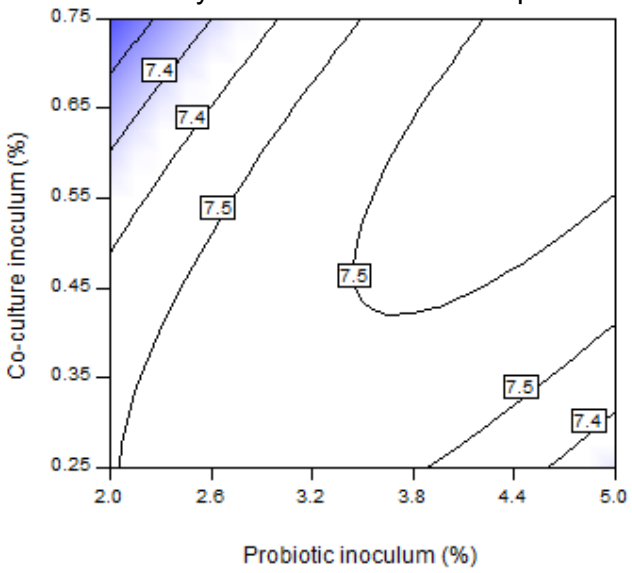


Figure 4.33 Effect of probiotic inoculum and co-culture inoculum level on the sedimentation score of barley-milk based fermented probiotic drink

Interaction effect of probiotic and co-culture inoculum indicated a positive significant influence on the sedimentation score of the drink. Non-linear effects were more pre-dominant than linear ones. Increasing probiotic and co-culture inoculum level have indicated a slight increase in the sedimentation score (**Figure 4.33**).

4.8.8.1.3 Consistency of barley-milk based fermented probiotic drink

The consistency score of barley-milk based probiotic drink varied from to 6.2 to 7.9. Concentration of barley-flour had a highly significant ($\rho<0.01$) influence on the consistency of probiotic drink. A very slight decrease in consistency score

were observed with increase in flour level from 4 to 5 %, however, further increase in flour level have adversely affected the consistency score (**Figure 4.34 - 4.35**). With increasing flour level a significant ($\rho<0.01$) rise in the viscosity of drink was observed (**Table 4.19**) which may have adversely affected the consistency of drink. This can be explained by thickening of drink due to thickening of composite owing to gelatinization of starch and increased β-glucan content with enhancing barley flour level. Highly viscous and heavy bodied beverages are not usually preferred by the consumers as it remains for longer duration in the buccal cavity and may also affect the swallowing.

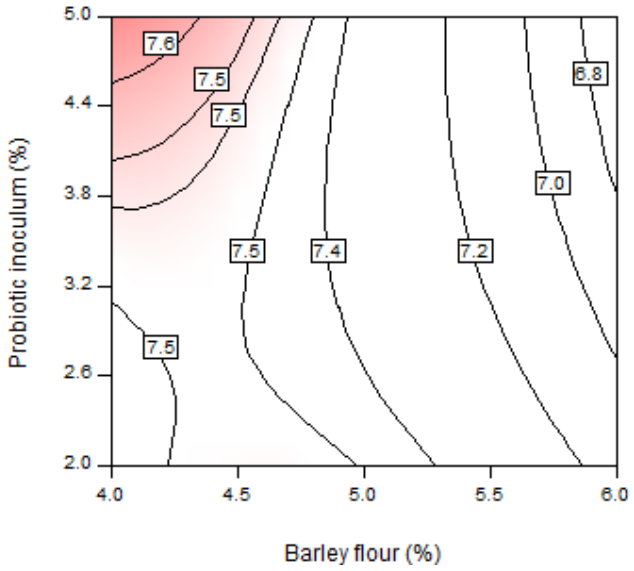


Figure 4.34 Effect of barley flour concentration and probiotic inoculum level on the consistency score of barley-milk based fermented probiotic drink

A rich mouthfeel has been reported to enhance the acceptability of many beverages like malted milk beverages (Singh *et al.*, 2008) and whey based lassi-like beverage (Nair and Thompkinson, 2008), viscosity of these beverages have been reported in the range of 4.8 to 12 cP and 13 to 35 cP at 20°C. During the present investigation viscosity of the drink obtained during optimization trials were in the range 36 to 214 cP at 20°C, which is comparatively much higher than the earlier reported values for malted milk beverage and whey-based lassi like beverage. Heavy mouthfeel of the probiotic drink were not liked by the sensory panellists. Mouthfeel attribute is associated with both consistency and perception in buccal cavity. Both concentration of probiotic and co-culture inoculum did not influence the consistency of the probiotic drink. However, increasing the inoculum concentration resulted in lower consistency score of the probiotic drink.

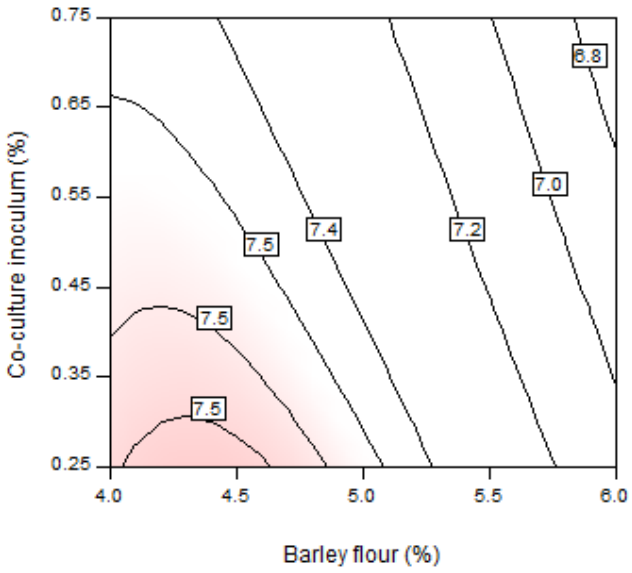


Figure 4.35 Effect of barley flour concentration and co-culture inoculum level on the consistency score of barley-milk based fermented probiotic drink

All the three variables namely barley flour concentration, probiotic and co-culture inoculum level have indicated a negative correlation with the consistency of the probiotic drink.

4.8.8.1.4 Flavour of barley-milk based fermented probiotic drink

The flavour score of the barley-milk based fermented probiotic drink obtained during optimization trials varied in the range of 6.30 to 7.70 (**Table 4.16**). Highest flavour score was obtained for the drink prepared using 5% barley flour, 3.5% probiotic inoculum and 0.5% co-culture inoculum level, whereas probiotic drink prepared using 6.68% barley flour with similar levels of probiotic and co-culture inoculum, was rated lowest for flavour score. Flavour score of freshly prepared sorghum based fermented milk beverage was 7.7 on a 9-point hedonic scale (Hussain *et al.*, 2014).

Among the 3 variables studied, barley flour level in the formulation was the most flavour determining variable, which significantly ($\rho<0.01$) influenced the flavour of probiotic drink in linear and quadratic terms. Coefficient of estimates for probiotic and co-culture inoculums indicated positive and negative influence on flavour of probiotic drink, respectively; however effect of both microbial starters was insignificant on flavour characteristics (**Table 4.18**). The contour graph showed that up to 5% level, there was a slight decrease in the flavour scores, but increasing the level to 6% drastically reduced the flavour score (**Figure 4.36**).

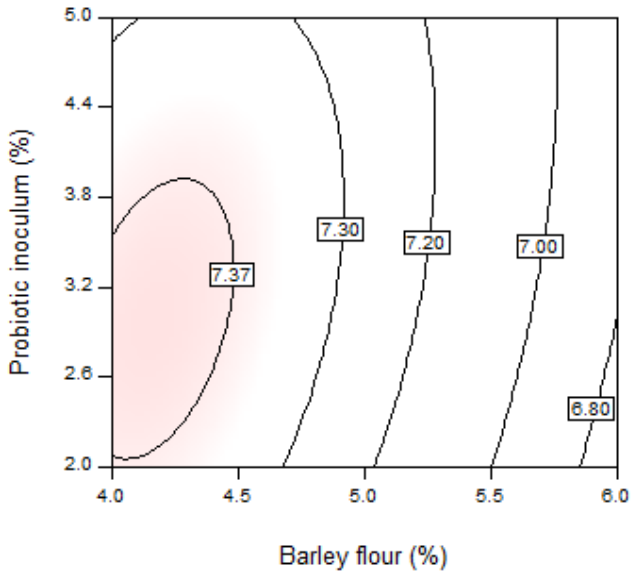


Figure 4.36 Effect of barley flour concentration and probiotic inoculum level on the flavour of barley-milk based fermented probiotic drink

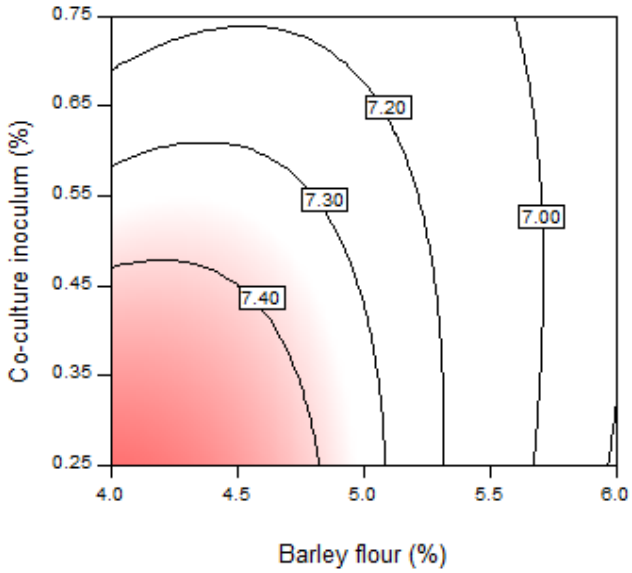


Figure 4.37 Effect of barley flour concentration and co-culture inoculum level on the flavour of barley-milk based fermented probiotic drink

At higher barley flour levels, the floury and starchy flavour predominated resulting in lowering of flavour score. Increased viscosity of the beverage due to presence of gelatinized starch and higher β-glucan concentration might have decreased the perceivable intensity of the flavour, which could be attributed to coating of tongue with viscous probiotic drink. Highly viscous drink while swallowing could have masked the other flavour metabolites produced during fermentation metabolism. Similar trend for the flavour have been reported for pearl-millet based fermented milk drink, indicating significant decrease in flavour score by increasing the flour concentration (Modha and Pal, 2011).

4.8.8.1.5 Overall acceptability of barley-milk based fermented probiotic drink

Overall acceptability of probiotic drink indicated relative preference of the panellists based on flavour consistency, sedimentation and colour and appearance. Overall acceptability of barley-milk based fermented probiotic drink ranged from 6.38 to 7.88 (**Table 4.16**). Concentration of barley flour during optimization process of probiotic drink indicated highly significant ($\rho<0.01$) effect on overall acceptability at linear and quadratic levels (**Table 4.17**).

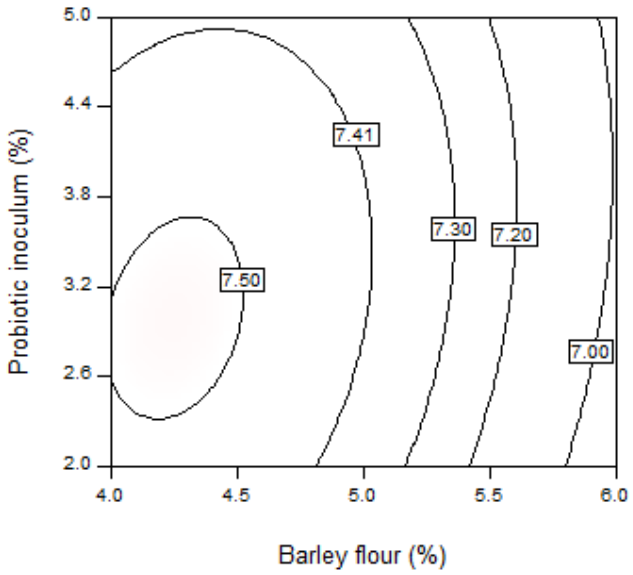


Figure 4.38 Effect of barley flour concentration and probiotic inoculum level on the overall acceptability of barley-milk based fermented probiotic drink

From the contour plot it is evident that probiotic drinks prepared with barley flour in the range of 4.0 to 5.0%; and *S. thermophilus* 20 inoculum level varied from 0.25 to 0.55% were highly acceptable. Beyond these levels overall acceptability of the drink decreased considerably. Interaction plot between *L. plantarum* NCDC344 (probiotic) and *S. thermophilus* 20 (co-culture) showed that using 3.0 to 5.0% probiotic concentration and 0.25 to 0.45% co-culture level yielded probiotic drinks of higher sensory score. Coefficients of estimates for all the variables used in optimization process indicated their negative influence on the acceptability of barley-milk based probiotic drink at linear as well as on quadratic level (**Table 4.18**). However, interaction effect of barley flour with probiotic inoculum and co-culture inoculum improved the acceptability of the probiotic drink.

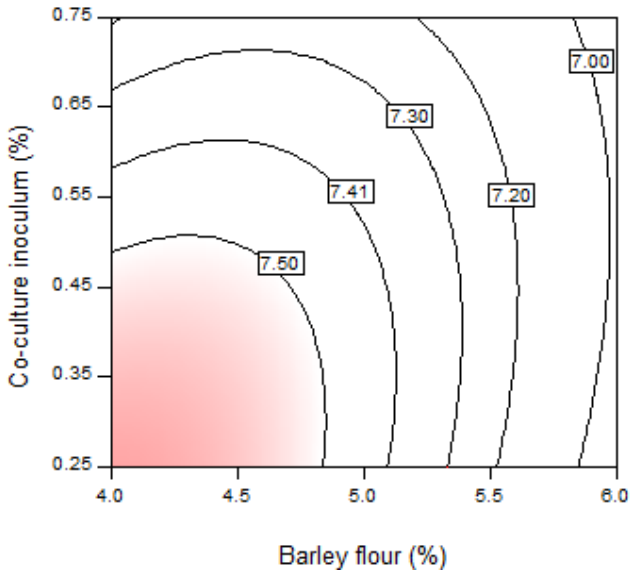


Figure 4.39 Effect of barley flour concentration and co-culture inoculum level on the overall acceptability of barley-milk based fermented probiotic drink

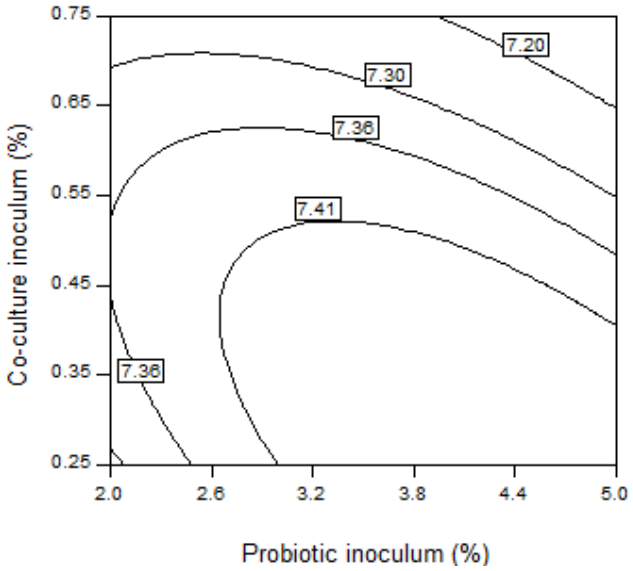


Figure 4.40 Effect probiotic inoculum and co-culture inoculum level on the overall acceptability of barley-milk based fermented probiotic drink

The contour plots for the effect of barley flour concentration, probiotic and co-culture inoculum level are shown in **Figure 4.39** and **4.40**. Overall acceptability of the pearl-millet based *rabadi* like beverage has been previously reported to depend largely on flour concentration (Modha and Pal, 2011). Overall acceptability score of a barley-buttermilk based fermented beverage, *rabadi* has been reported in range from 6.35 to 8.36, which varied due to variation in preiod and temperature of incubation (Gupta *et al.*, 1992). Plot of predicted *vs.* actual score of barley-milk based fermented probiotic drink is indicated in **Figure 4.41**.

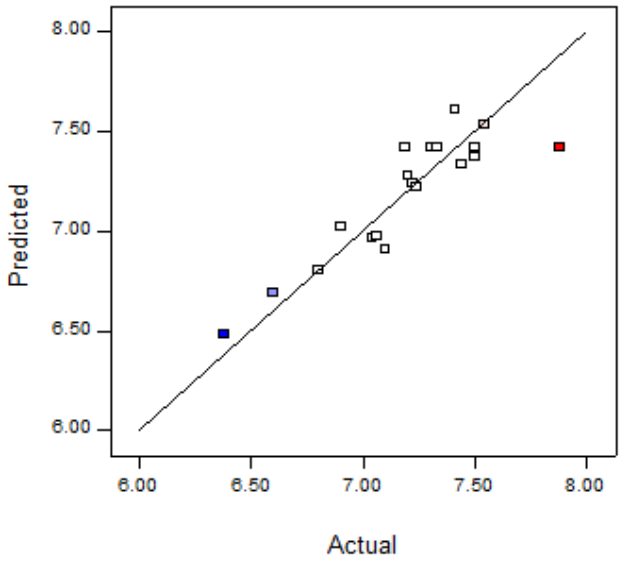


Figure 4.41 Plot of predicted *vs.* actual score for overall acceptability of barley-milk based fermented probiotic drink

4.8.8.2 Probiotic count of barley-milk based fermented probiotic drink

Regression coefficients of 3 factor second order polynomial equation to predict the probiotic count has been presented in **Table 4.18**. The analysis of variance (**Table 4.17**) showed that regression model developed was highly significant ($\rho<0.01$) and had coefficient of determination (R^2) value of 0.91. Lack of fit test for the developed model was non-significant. The low co-efficient of variation (1.26%) suggested that the model was precise and reliable for predicting the probiotic count. Plot of predicted *vs.* actual probiotic count is represented in **Figure 4.42**.

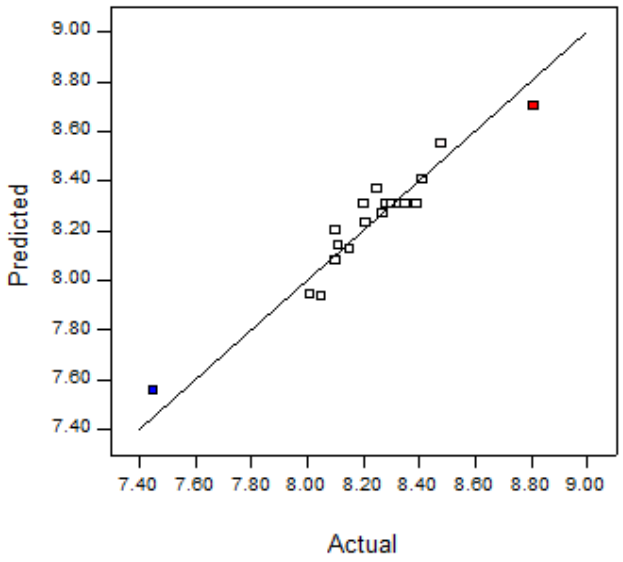


Figure 4.42 Plot of predicted *vs.* actual value of probiotic count in barley-milk based fermented probiotic drink

Probiotic (*L. plantarum* NCDC344) count of the fermented drink prepared from barley-milk composite varied from 7.45 to 8.81 log cfu/ml, which is above the recommended concentration probiotic to impart health benefit to the hosts (**Table 4.16**). Among the 3 variables studied, barley flour concentration and probiotic inoculum level were the determining variables which significantly ($\rho<0.01$) affected the probiotic count of the fermented drink. The linear terms of both the variables significantly affected the probiotic count. At quadratic level, only barley flour concentration had significant ($\rho<0.01$) influence on the probiotic count (**Table 4.17**). Co-efficient of estimates for the independent variables in the quadratic equation at linear level indicated that barley flour concentration negatively affected probiotic count, which implies that at higher barley flour level counts decreased significantly. As expected, probiotic inoculum and co-culture inoculum have indicated their positive influence on the probiotic count (**Table 4.18**). Interaction of barley flour with probiotic and co-culture inoculum as well as its effect at quadratic level adversely affected the probiotic count of the drink prepared using fermented barley-milk composite.

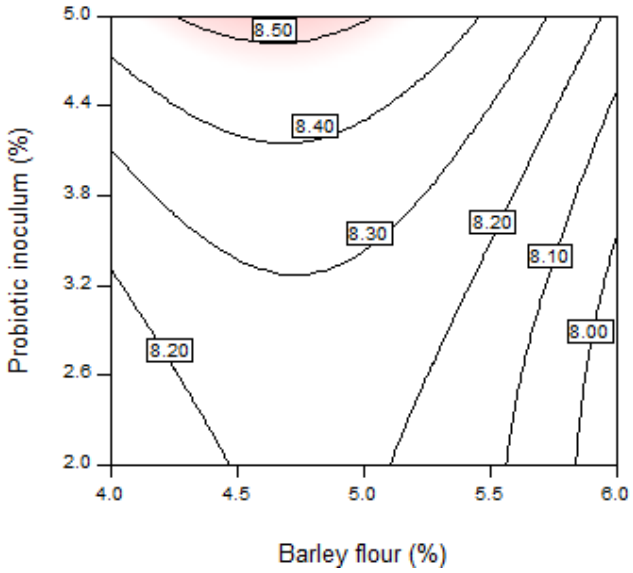


Figure 4.43 Effect barley flour concentration and probiotic inoculum level on the probiotic count of barley-milk based fermented probiotic drink

Figure 4.43 indicates significant increase in the probiotic count with increasing the probiotic inoculum level. **Figure 4.44** clearly showed there was a slight increase in the probiotic count with increasing flour levels from 4 to 5%. Further increase in the flour levels resulted decrease in the probiotic count thus indicating a negative co-efficient of estimate for flour concentration in linear and

quadratic terms. The increase in *L. plantarum* NCDC344 count could be achieved with by raising the concentration of probiotic; however, enhancement in *S. thermophilus* 20 did not show any significant increase in probiotic counts. This might be because of relative ability of two inoculums used to utilize wide range of available carbohydrates in barley-milk composite for their growth (*Charalampopoulos* et al., 2002b). The carbohydrate utilization spectrum of *L. plantarum* is more diverse as compared to *S. thermophilus* (Patil *et al*., 2010; Erkus *et al.* 2014).

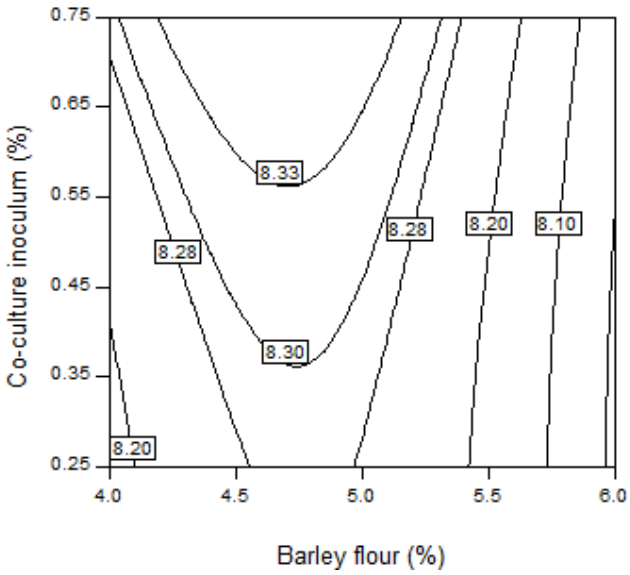


Figure 4.44 Effect barley flour concentration and co-culture inoculum level on the probiotic count of barley-milk based fermented probiotic drink

Interaction plot between the two inoculums (**Figure 4.45**) reflected that simultaneous increase in concentration of inoculum led to enhancement in the population of *L. plantarum* NCDC344. It confirmed synergistic relationship between the two LAB species. *S. thermophilus* is one of the most widely applied dairy starters worldwide. It dominates the initial stages of fermentation and produce metabolites which create conclusive environment for the growth of *Lactobacillus* spp. (Sieuwerts *et al*., 2010).

An oat based functional beverage using lactic acid fermentation by *L. plantarum* ATCC 8014 has been optimized for the formulation containing oat flour (range: 4-7%), sugar (sugar: 1-2%) and inoculum (range: 1-10%) by Gupta *et al.*, (2010). Oat flour concentration has been reported to have highly significant ($\rho<0.01$) influence on the probiotic count of the lactic acid fermented beverage at linear as well as quadratic level. Gupta *et al.*, (2010) also observed oat flour as a synergistic factor for the probiotic only up to a certain level (5.5%), further

increasing its level had adverse influence on the growth of probiotic organism. The growth of lactic acid bacteria in barley-milk based fermented probiotic drink has been supported the findings of previous researchers who worked with barley and other cereal grains (Gupta *et al.*, 2010; Kedia *et al.*, 2007; Charalampopoulos *et al.*, 2002b; Arora *et al.*, 2010; Charalampopoulos and Pandiella, 2010). Barley β-glucan has been previously proven to extend the viability of *L. acidophilus* LA-5 and *Bifidobacterium lactis* Bb-12 (Elsanhoty *et al.,* 2009). A milk plant based composite provide an ideal growth environment by supplying the diverse groups of macro and micro-nutrients, such as sugars, complex polysaccharides, free amino acids, peptides, B-vitamins and minerals.

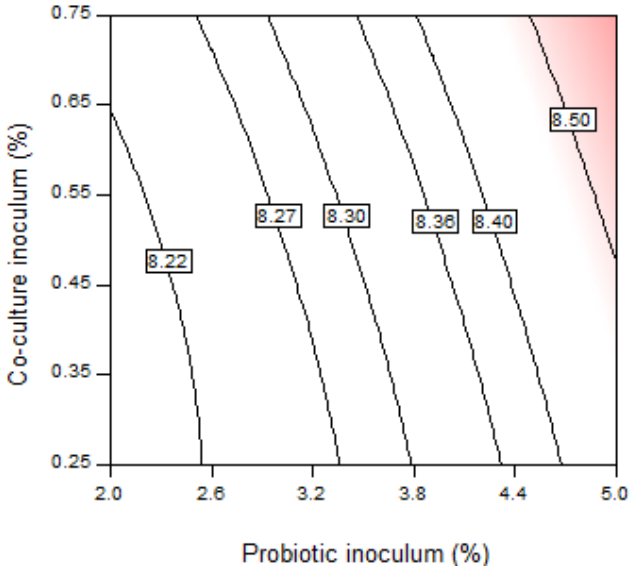


Figure 4.45 Effect probiotic inoculum and co-culture inoculum level and on the probiotic count of barley-milk based fermented probiotic drink

Application of 5.0% or 10% inoculums have been reported to reach the desired pH levels of 4.0 to 4.5 in shorter time of 8 and 4 h as compared to 1.0% inoculum level (Angelov *et al.*, 2006). Use of 5.0% starter culture concentration was recommended by Angelov *et al.*, (2006), which resulted probiotic count of 9.3×10^{9} cfu ml^{-1} in 6h of fermentation period. No significant difference was reported in the probiotic count of fermented oat mash containing 4.0 to 5.5% oat flour concentration, therefore, 5.5 % oat flour concentration was recommended to obtain high β–glucan content in fermented products (Angelov *et. al.*, 2006). Ganguly and Sabikhi (2012) observed positive effect of increasing germinated pearl millet flour level and liquid barley-malt extract on the fermentation dynamics, acidification rate and the viability of probiotic culture. Highest growth of probiotic culture was reported for the formulation containing whey-skim milk blend

in combination with 5% germinated pearl millet flour and 3% liquid barley-milk extract (Ganguly and Sabikhi, 2012).

4.8.8.3 β-glucan content of barley-milk based fermented probiotic curd

β-glucan is most important group of dietary fibre present in wide range of food grains. It is most important component of barley grain which exerts various physiological health benefits including cholesterol lowering effect (McIntosh *et al.,* 1991, Newman *et al.,* 1989), regulating blood glucose level and insulin response in diabetics (Cavallero *et al.,* 2002) and reducing risk of cancer (Jacobs *et al.,* 1998). β-glucan level during development of any food product should not be affected by the processing treatments involved during their manufacture. Gupta *et al.*, (2010) found β-glucan content as matter of great concern during the development of oat based lactic acid fermented beverages and therefore estimated it for various combinations during the optimization of formulation to see the effects of heating and fermentation on the β-glucan level. Processing of barley grain into food involves a wide range of techniques including milling, hydrothermal treatments, extrusion cooking, fermentation *etc.* either alone or in combination which may affect the properties of barley β-glucan (Wood, 2002; Lazaridou and Biliaderis, 2007, Andersson *et al.,* 2007, Tosh, 2007).

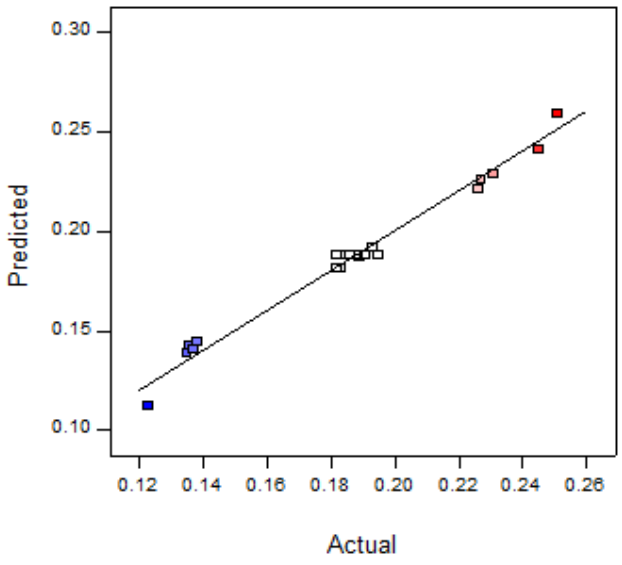


Figure 4.46 Plot of predicted *vs.* actual value of β-glucan content in barley-milk based fermented composite (g/100g)

A three factor second order polynomial regression equation was developed to predict the response. The analysis of variance (**Table 4.17**) showed that regression model developed was highly significant ($\rho<0.01$) with coefficient of determination (R^2) value of 0.98, indicating 98% variability can be predicted for

the response using the regression equation. Furthermore, lack of fit was non-significant which exhibited the fitness of model terms. The low co-efficient of variation (3.64%) suggested that the model was precise and reliable. Regression coefficients of full second order model to predict β-glucan content of barley-milk based fermented probiotic composite has been presented in **Table 4.18**. Plot of predicted *vs.* actual value of β-glucan content has been indicated in **Figure 4.46**.

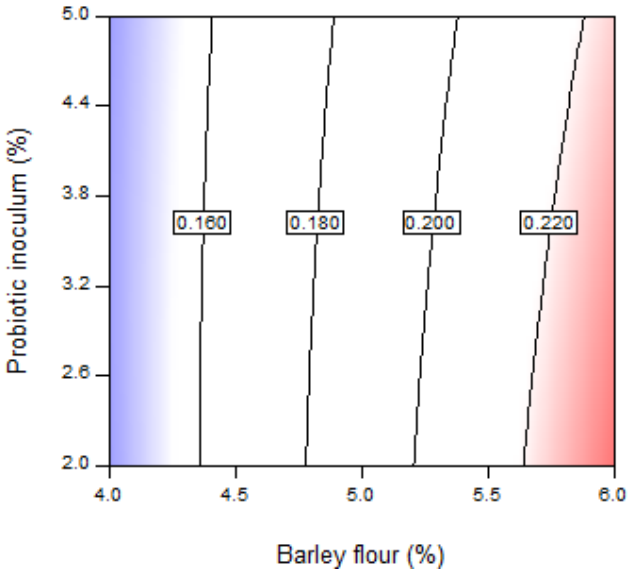


Figure 4.47 Effect barley flour concentration and probiotic inoculum level on the β-glucan content of barley-milk based fermented probiotic curd

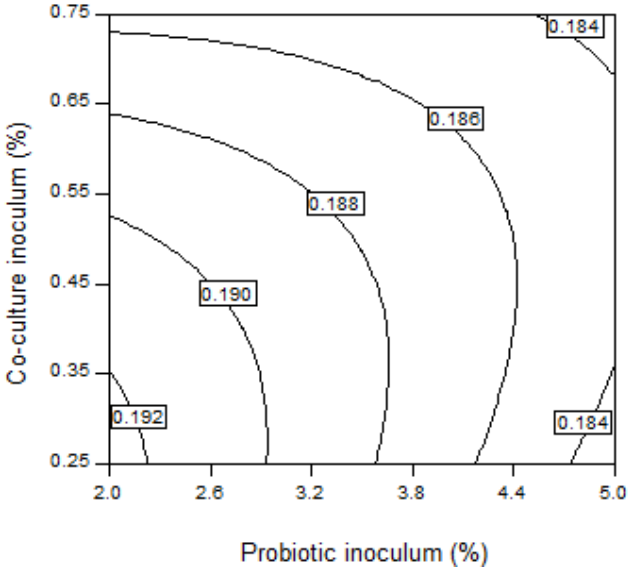


Figure 4.48 Effect probiotic inoculum and co-culture inoculum level on the β-glucan content of barley-milk based fermented probiotic curd

β-glucan of barley-milk based fermented composites obtained as intermediate during optimization of probiotic drink were in the range from 0.12 to 0.25 (g/100g). Barley flour concentration had highly significant ($\rho<0.01$) positive

influence (**Figure 4.47**) on the β-glucan content of the fermented composite at linear level (**Table 4.17**), whereas, it remained unaffected with the varying levels of probiotic and co-culture inoculum level (**Figure 4.48**). However, increasing the inoculum levels showed decrease in β-glucan content at linear and quadratic levels as indicated by the co-efficient of estimates (**Table 4.18**). Circular contour plots indicate that the interactions between the variables are negligible, whereas elliptical contour plots indicates the evidence of interaction (Gupta *et al.*, 2010). Gupta *et al.*, (2010) observed non-significant ($\rho>0.05$) differences in the β-glucan content due to heating and fermentation during preparation of the lactic acid fermented oat based beverage. Lambo *et al.*, (2005) reported reduction in β-glucan level due to 20 h fermentation by lactic acid bacteria.

4.8.8.4 Effect of different levels of barley flour, probiotic inoculum and co-culture inoculum on the textural attributes of barley-milk based fermented probiotic curd

Results for textural attributes such as, firmness, work of shear, stickiness and work of adhesion for the barley-milk based fermented curd were obtained by back extrusion method (3.2.8.5) using TA-XT2i (M/s Stable Micro Systems, UK). A typical force-deformation curve of "back extrusion" test of barley-milk based fermented probiotic curd is given in **Figure 4.30**. Barley-milk based fermented probiotic curd was obtained as intermediate product to prepare probiotic drink. The data for CCRD were fitted with a quadratic function through regression analysis and generated models were evaluated for their fitness using ANOVA analysis. The dependence of these responses with respect to independent variables in form of correlations is presented in **Table 4.20** and **Table 4.21,** respectively.

The coefficients of determination values for the generated regression models to predict firmness, work of shear, stickiness and work of adhesion were 0.93, 0.98, 0.88 and 0.91, respectively (**Table 4.22**). Furthermore, lack of fit for all responses related to textural attributes was non-significant which exhibited the fitness of model terms. The low co-efficient of variation (for firmness: 6.73%; work of shear: 3.45%; stickiness: 10.51% and work of adhesion: 7.17%) suggested that the models were reliable. Regression coefficients of full second order model to predict textural attributes of barley-milk based fermented probiotic curd are presented in **Table 4.22**.

Table 4.19 Rheological properties of barley-milk based fermented probiotic curd prepared during optimization trials

Standard order	Actual factors (%)			Coded values			Textural attributes				Viscosity* (cP)	
	x_1	x_2	x_3	x_1	x_2	x_3	Firmness (N)	Work of Shear (N.s)	Work of Adhesion (N.s)	Stickiness (N)	5°C	20°C
1	4.00	2.00	0.25	-1.00	-1.00	-1.00	2.09	49.53	-14.23	-1.18	87	57
2	6.00	2.00	0.25	1.00	-1.00	-1.00	2.65	61.42	-19.23	-1.72	249	186
3	4.00	5.00	0.25	-1.00	1.00	-1.00	2.04	49.65	-14.13	-1.26	99.5	59.3
4	6.00	5.00	0.25	1.00	1.00	-1.00	2.88	66.18	-18.29	-1.63	286	187
5	4.00	2.00	0.75	-1.00	-1.00	1.00	2.22	49.89	-14.39	-1.2	86.7	57.4
6	6.00	2.00	0.75	1.00	-1.00	1.00	2.95	73.74	-19.88	-1.82	288	196
7	4.00	5.00	0.75	-1.00	1.00	1.00	2.28	53.50	-15.92	-1.33	99.7	59.8
8	6.00	5.00	0.75	1.00	1.00	1.00	3.09	75.56	-20.76	-1.94	270	166
9	3.32	3.50	0.50	-1.68	0.00	0.00	**1.87**	**46.35**	**-13.25**	**-1.11**	**66.3**	**36.4**
10	6.68	3.50	0.50	1.68	0.00	0.00	**3.83**	**85.59**	**-26.48**	**-2.56**	**429**	**214**
11	5.00	0.98	0.50	0.00	-1.68	0.00	2.18	51.9	-15.24	-1.31	152	120
12	5.00	6.02	0.50	0.00	1.68	0.00	2.59	54.69	-17.84	-1.6	200	149
13	5.00	3.50	0.08	0.00	0.00	-1.68	2.11	49.56	-15.41	-1.29	150	105
14	5.00	3.50	0.92	0.00	0.00	1.68	2.84	61.99	-19.28	-1.85	187	141
15	5.00	3.50	0.50	0.00	0.00	0.00	2.33	55.12	-15.99	-1.34	174	129
16	5.00	3.50	0.50	0.00	0.00	0.00	2.45	55.46	-18.06	-1.63	157	112
17	5.00	3.50	0.50	0.00	0.00	0.00	2.59	54.69	-17.8	-1.6	192	137
18	5.00	3.50	0.50	0.00	0.00	0.00	2.29	52.64	-16.15	-1.37	185	132
19	5.00	3.50	0.50	0.00	0.00	0.00	2.46	56.05	-17.12	-1.61	188	130
20	5.00	3.50	0.50	0.00	0.00	0.00	2.51	56.34	-17.11	-1.55	155	111

*estimated for barley-milk based fermented probiotic drink at shear rate 100 s^{-1}; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Table 4.20 Analysis of variance (ANOVA) for rheological parameters of barley-milk based fermented probiotic curd

Source of Variation	d.f.	Sum of squares					
		Firmness	Work of shear	Stickiness	Work of adhesion	Viscosity$^{\$}$ (5°C)	Viscosity$^{\$}$ (20°C)
Model	9	0.395994**	1915.309**	1.908395**	154.3637**	140057.8**	47557.16**
x_1	1	2.846227**	1440.836**	1.534308**	127.5109**	129494.2**	46889.23**
x_2	1	0.083761	16.48008	0.038777	2.41477	1148.256	43.85186
x_3	1	0.32529**	160.4773**	0.152217*	9.816503*	530.6122	186.3278
x_1x_2	1	0.0162	1.015313	0.00405	0.277512	5.28125	141.9612
x_1x_3	1	0.00245	38.23751*	0.0128	0.171112	66.70125	17.70125
x_2x_3	1	5E-05	0.037812	0.00845	1.487813	371.2813	119.3512
x_1^2	1	0.260033*	248.4682**	0.111521	9.601445*	7190.117	28.72261
x_2^2	1	0.012997	1.549461	0.030936	1.856535	127.3848	52.04091
x_3^2	1	4.62E-05	4.342531	0.000464	0.079569	455.949	67.59015
Residual	10	0.028623	40.12605	0.263705	15.43721	3958.074	2106.686
Lack of fit	5	0.044749	31.35125	0.181371	11.91333	2675.241	1507.853
Pure error	5	0.012497	8.7748	0.082333	3.523883	1282.833	598.8333

**significant at ρ<0.01, *significant at ρ<0.05; $^{\$}$estimated for barley-milk based fermented probiotic drink; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Table 4.21 Coefficients of variables in the model to predict rheological parameters of barley-milk based fermented probiotic curd ($drink)

Source of Variation	Coefficient estimate†					
	Firmness	Work of shear	Stickiness	Work of adhesion	Viscosity$ (5°C)	Viscosity$ (20°C)
Intercept	2.4400	55.0042	-1.5204	-17.0660	175.6637	125.3853
x_1	0.4566	10.2737	-0.3353	-3.0563	97.3971	58.6080
x_2	0.0783	1.0985	-0.0533	-0.4205	9.1695	1.7919
x_3	0.1543	3.4279	-0.1056	-0.8478	6.2332	3.6937
x_1x_2	0.0450	0.3563	0.0225	0.1863	-0.8125	-4.2125
x_1x_3	0.0175	2.1863	-0.0400	-0.1463	2.8875	-1.4875
x_2x_3	0.0025	0.0688	-0.0325	-0.4313	-6.8125	-3.8625
x_1^2	0.1344	4.1557	-0.0880	-0.8169	22.3554	-1.4129
x_2^2	-0.0300	-0.3279	0.0463	0.3589	-2.9730	1.9003
x_3^2	0.0018	0.5489	0.0057	0.0743	-5.6247	-2.1656
R^2	0.93	0.98	0.88	0.91	0.96	0.96
R^2_{adj}	0.86	0.96	0.77	0.83	0.92	0.92
APV$^{\Phi}$	7.17	13.93	10.63	12.52	23.52	19.20

$^{\Phi}$ adequate precision value, † for final equation in terms of coded factors; $estimated for barley-milk based fermented probiotic drink; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Among the three formulation variables namely, barley flour concentration, probiotic and co-culture inoculum level, level of barley flour was observed to have major influence on textural attributes of the fermented mass obtained after fermentation. Linear term of barley flour level was having a profound significant ($\rho<0.01$) effect on all the textural and rheological parameters investigated. Increasing the concentration of barley flour in formulation caused linear increase in the values of textural parameters and viscosity of fermented mass (or curd). Formulation containing 3.32 % barley flour, 3.50% probiotic and 0.50% co-culture inoculum exhibited lowest values of firmness (1.87N), work of shear (46.35N.s), stickiness (-1.11N) and work of adhesion (-13.25N.s). However, the fermented mass (or curd) containing 6.68% of barley flour at 3.50% probiotic and 0.50% co-culture inoculum level had maximum values for all parameters (firmness: 3.83N; work of shear: 85.59N.s; stickiness: -2.56N; and work of adhesion: -26.48N.s) as obtained during the back-extrusion textural analysis (**Table 4.19**). Barley flour contain appreciable amount of starch and polysaccharides specially β-glucan which are have excellent water holding and gelling properties characteristics.

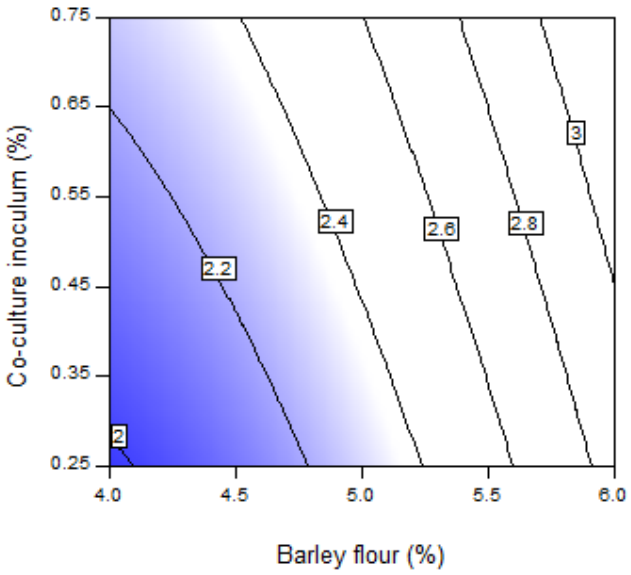


Figure 4.49 Effect of barley flour concentration and co-culture inoculum level on the firmness of barley-milk based fermented probiotic curd

Firmness value is the peak force obtained during penetration of the probe in curd (**Figure 4.30**) obtained during back-extrusion test. A significant ($\rho<0.01$) rise in the firmness of barley-milk based fermented probiotic curd was noticed on increasing the barley and enhancement in co-culture inoculum level (**Figure 4.50**). At quadratic level, only flour concentration significantly ($\rho<0.05$) influenced the firmness of barley-milk based probiotic curd. However, interaction effects for

all the variables on the firmness were non-significant (ρ>0.05). Positive sign of quadratic term indicated that firmness fermented mass increased significantly at both at lower or higher levels of barley-flour.

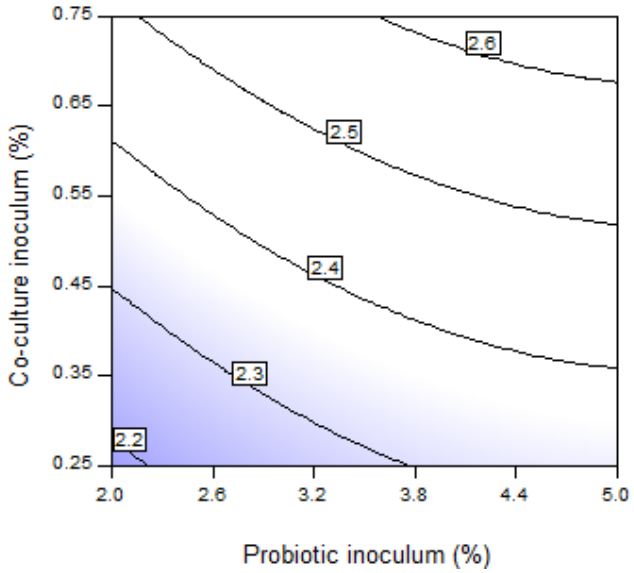


Figure 4.50 Effect of probiotic inoculum and co-culture inoculum level on the firmness of barley-milk based fermented probiotic curd

At lower levels, the rise in firmness could be attributed to enhancement in the level of milk solids particularly milk-solids-not-fat content. Raju and Pal (2009), reported increase in the firmness value of misti dahi, a traditional fermented and sweetened yoghurt with increasing milk solid-not-fat content (MSNF).

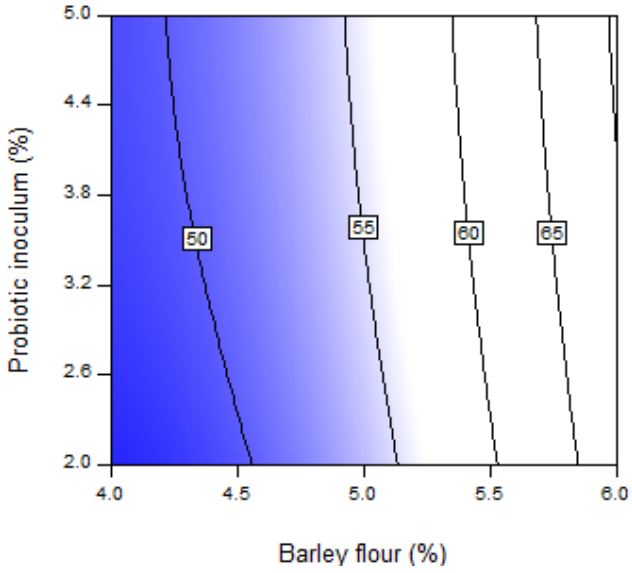


Figure 4.51 Effect of barley flour concentration and probiotic inoculum level on the work of shear for barley-milk based fermented probiotic curd

The work of shear is the area under the penetration cycle, representing the amount of energy required to perform the shearing process by overcoming the various forces binding curd constituents cohesively. Work of shear increased with increasing flour level (**Figure 4.51**) because of the formation of strong gel particularly due to gelatinization of barley starch, interaction between barley and milk constituents and gelation of milk proteins under acidic conditions. Similar trend were observed in quadratic terms ($\rho<0.01$) and interactive terms with co-culture inoculum ($\rho<0.05$). Co-culture inoculum level also exerted significant effect (**Table 4.20**) in linear terms ($\rho<0.01$). Among the starter cultures used *Streptococcus thermophilus* 20 (co-culture) was fastidious acid producer and acidified gel characteristics depends on the rate of acidification.

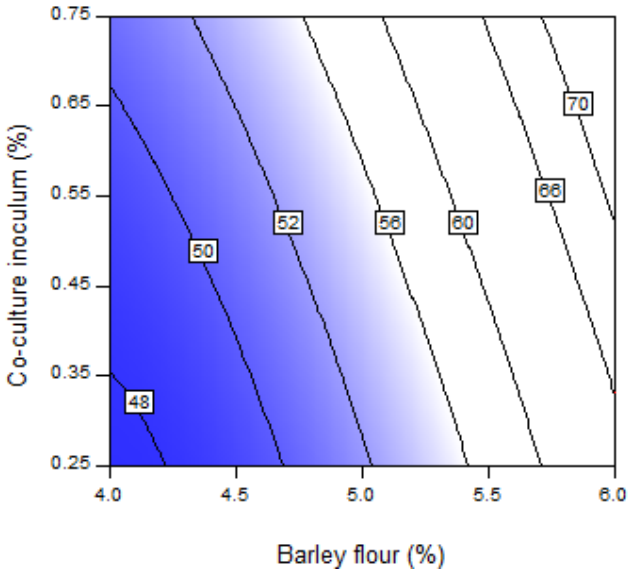


Figure 4.52 Effect of barley flour concentration and co-culture inoculum level on the work of shear for barley-milk based fermented probiotic curd

Barley-flour concentration and co-culture inoculum had positive significant influence on work of adhesion during back extrusion test for texture analysis in linear terms. Contour plot indicated that increase in probiotic inoculum and co-culture inoculum increased work of shear for barley-milk based probiotic curd (**Figure 4.52**), which might be because of the development of firmer gel on fermentation.

Stickiness is the negative peak force which is obtained during the up stroke of the probe and work of adhesion represents the area during withdrawal of probe after penetration cycle, which indicates the amount of energy required to perform the shearing action during withdrawal. Increasing flour level significantly

(ρ<0.01) increased stickiness and work of adhesion for fermented barley-milk fermented mass (**Table 4.20**).

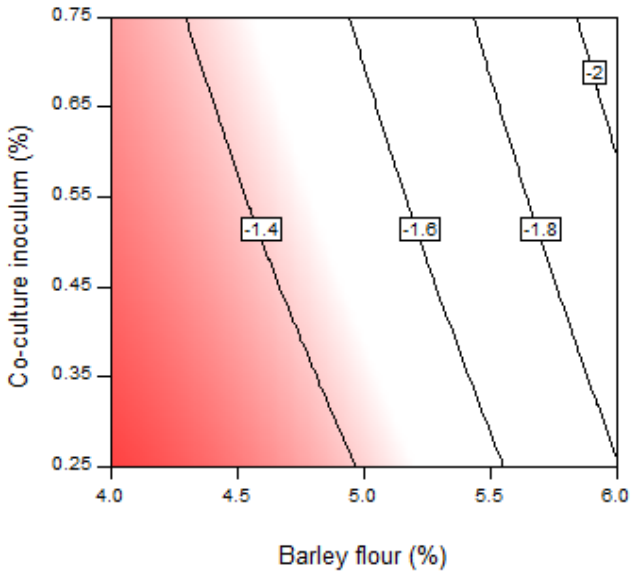


Figure 4.53 Effect of barley flour concentration and co-culture inoculum level on the stickiness for barley-milk based fermented probiotic curd

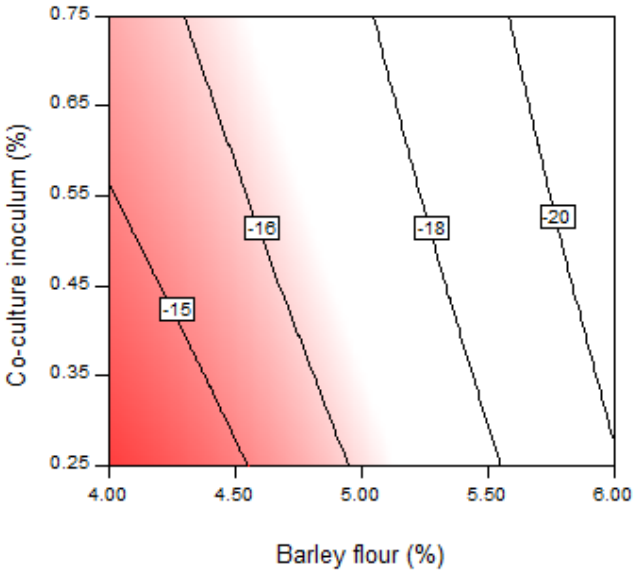


Figure 4.54 Effect of barley flour concentration and co-culture inoculum level on the work of adhesion for barley-milk based fermented probiotic curd

Increase in the co-culture inoculum level also had significant negatively influence on the stickiness and work of adhesion values (**Table 4.21**). Negative influence indicates increase in stickiness and work of adhesion, due to negative force applied on the withdrawal of probe.

4.8.8.5 Effect of different levels of barley flour, probiotic inoculum and co-culture inoculum on the viscosity of barley-milk based fermented probiotic drink

Viscosity of any beverage has a close relationship with its consistency and mouthfeel during consumption (Mathur and Mathur, 2003). Different barley-milk based fermented probiotic drink samples obtained during optimization trials were subjected to measurement of shear rate / stress data at 5 ± 0.1°C and 20 ± 0.1°C using method discussed in subsection **3.2.8.4**. Data related to variable shear-rate *vs.* viscosity and variable shear rate *vs.* shear stress for barley-milk based fermented probiotic drink obtained during optimization trials has been depicted in the form of graph in **Figure 4.55 to 4.58**.

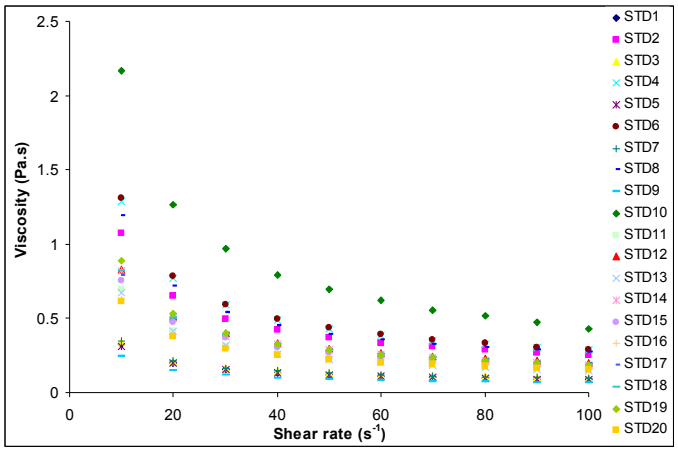


Figure 4.55 Variable shear-rate *vs.* viscosity of barley-milk based fermented probiotic drink for different RSM trials at 5°C

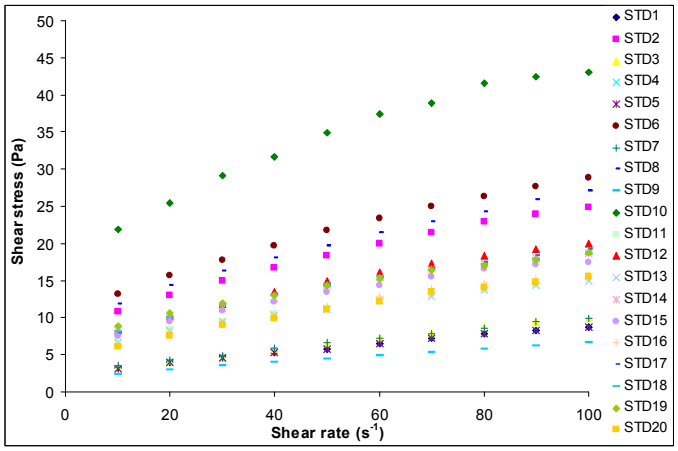


Figure 4.56 Rheogram of barley-milk composite based fermented probiotic drink obtained during different RSM trials at 5°C

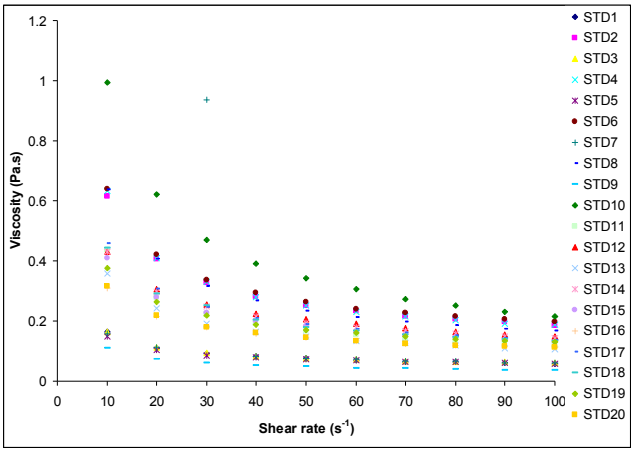


Figure 4.57 Variable shear-rate *vs.* viscosity of barley-milk based fermented probiotic drink for different RSM trials at 20°C

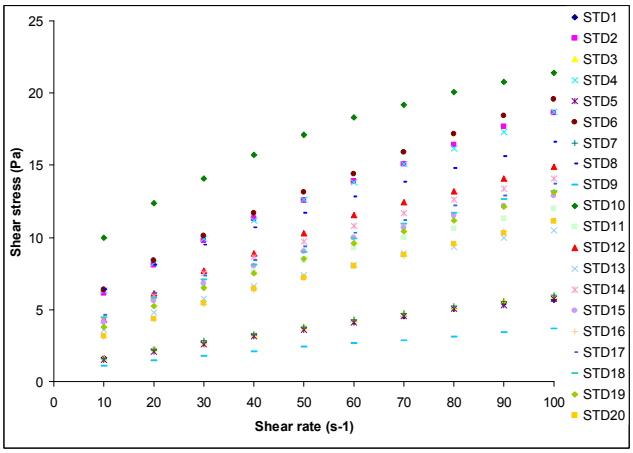


Figure 4.58 Rheogram of barley-milk composite based fermented probiotic drink obtained during different RSM trials at 20°C

Viscosity data at $\dot{\gamma}$ = 100s^{-1} were used to evaluate the effects of formulation variables on the viscosity of probiotic drink. Viscosity of the barley-milk based fermented probiotic drink was in the range of 66.3cP to 429cP at 5°C and 36.4cP to 214cP at 20°C.

ANOVA for viscosity data indicated highly significant ($\rho<0.01$) influence of barley flour concentration on the viscosity of barley-milk based fermented probiotic drink, at linear level (**Table 4.20**). The highest viscosity was recorded for the sample containing 6.68% barley flour, whereas lowest viscosity was recorded for the sample containing 3.32% flour (**Table 4.19**). However, the formulation containing 6.68% barley flour had lowest flavour score as well as lowest overall acceptability score. From the results, it can be concluded that increasing flour level had a prominent effect (**Figure 4.60**) on the viscosity of barley-milk based fermented probiotic drink, however it also reduced the sensory acceptability of the product.

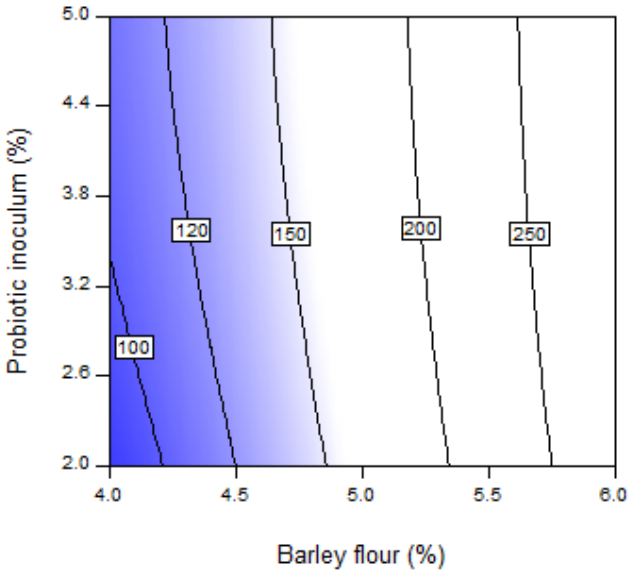


Figure 4.59 Effect of barley flour concentration and probiotic inoculum level on the viscosity (5°C, $\dot{\gamma}$ = 100s^{-1}) of barley-milk based fermented probiotic drink

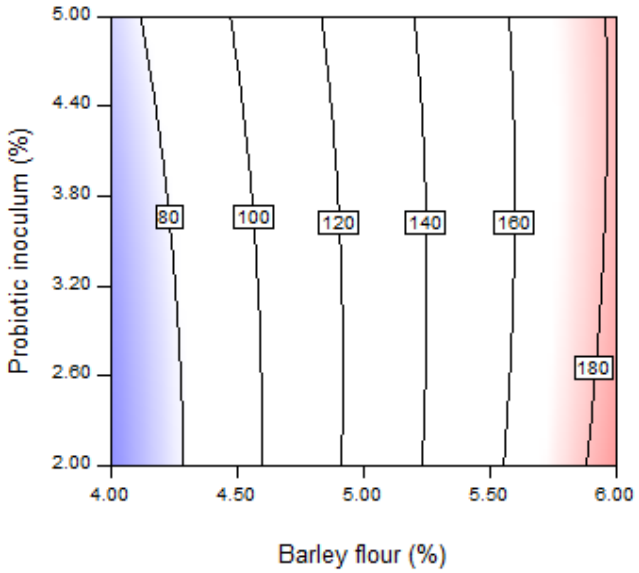


Figure 4.60 Effect of barley flour concentration and probiotic inoculum level on the viscosity (20°C, $\dot{\gamma}$ = 100s^{-1}) of barley-milk based fermented probiotic drink

Increase in probiotic inoculum and co-culture inoculum level showed increase (**Figure 4.60** and **4.61**) in the viscosity, which is evident from the positive co-efficient of regression equations (**Table 4.21**), at linear level. However, increase in the viscosity was not significant due to increase in inoculums level. This can be attributed to increase in the polysaccharide content which has ability to gelatinize and come into close contact with each other (Nair

and Thompkinson, 2008). The polysaccharides hinder with mass deformation in the product and causes increase in the viscosity (Zecher and van Coillie, 1992).

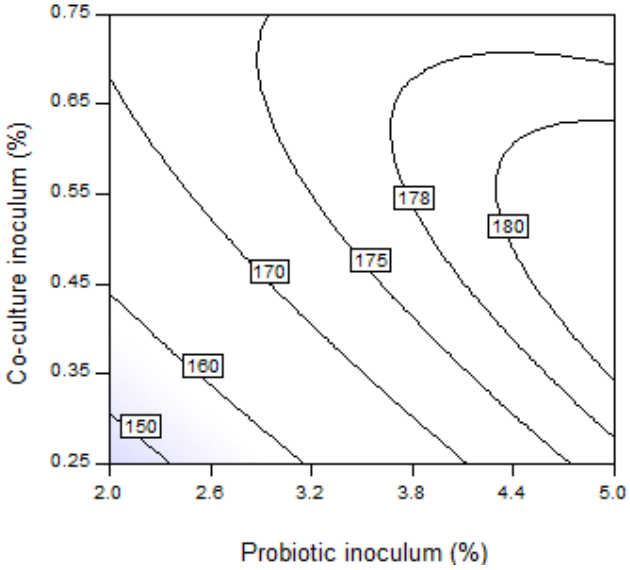


Figure 4.61 Effect of probiotic inoculum and co-culture inoculum level on the viscosity (5°C, $\dot{\gamma}$ = 100s^{-1}) of barley-milk based fermented probiotic drink

Increase in viscosity may also be attributed to the positive effects on total solids content of probiotic drink. Interaction of barley flour x probiotic inoculum and probiotic inoculums x co-culture inoculum showed non-significant decrease in the viscosity at 5°C as well as 20°C (**Table 4.20**). However, interaction of probiotic inoculum with co-culture inoculum indicated slight increase in the viscosity at 5°C but decrease at 20°C. Significant ($\rho<0.05$) increase in the viscosity of fermented milk-pearl millet based beverage was previously reported with increasing flour level (Modha and Pal, 2011), which varied in between 32 to 62 cP, with varying levels of germinated pearl millet flour and water proportion.

4.8.8.6 Effect of different levels of barley flour, probiotic inoculum and co-culture inoculum on the consistency index, flow behavior index and yield stress of barley-milk based fermented probiotic drink

Samples of barley-milk based fermented probiotic drink obtained during optimization trials were subjected to the measurement of shear rate / shear stress data at 5 and 20 ± 0.1°C and fitted to some of rheological models, such as *Ostwald-De-Waele*, *Herschel-Bulkley* and *Casson* model using RheoPlus32 (v3.61) software as per the method mentioned in **3.2.8.4**. Data pertaining to shear rate *vs*. shear stress and viscosity *vs*. shear rate at 5 and 20°C of different samples are previously represented in **Figure 4.55** to **4.58**.

Table 4.22 Fitting of different rheological models on shear rate *vs.* shear stress data at 5°C for barley-milk based fermented probiotic drink obtained during optimization trials

Standard order	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	K_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
1	0.927	0.4827	0.9839	1.522	0.02945	0.9959	2.101	0.1953	0.7633	0.9973
2	3.957	0.3977	0.9930	6.109	0.06698	0.9989	6.695	0.8720	0.6633	0.9995
3	0.837	0.5328	0.9872	1.388	0.38652	0.9974	2.080	0.2026	0.7919	0.9981
4	5.143	0.3685	0.9931	7.805	0.06589	0.9993	8.559	0.9487	0.6637	0.9995
5	1.017	0.4585	0.9868	1.442	0.03077	0.9977	2.019	0.1788	0.7889	0.9979
6	5.914	0.3714	0.9926	7.836	0.06765	0.9985	8.616	0.9666	0.6623	0.9995
7	0.901	0.5164	0.9861	1.490	0.03686	0.9965	2.181	0.2051	0.7860	0.9969
8	4.472	0.3861	0.9894	6.914	0.06620	0.9997	7.728	0.8901	0.6671	0.9997
9	0.666	0.4932	0.9857	1.099	0.02289	0.9977	1.495	0.1564	0.7536	0.9983
10	9.895	0.3222	0.9924	14.42	0.08341	0.9907	13.12	2.3797	0.5593	0.9960
11	2.816	0.3614	0.9916	4.266	0.33745	0.9997	4.798	0.4618	0.6770	0.9998
12	3.014	0.4103	0.9968	4.623	0.05626	0.9975	5.038	0.6906	0.6743	0.999
13	2.737	0.3663	0.9939	4.149	0.03460	0.9977	4.598	0.4599	0.6817	0.9972
14	3.142	0.3879	0.9924	4.790	0.04818	0.9951	5.287	0.6291	0.6725	0.9977
15	3.010	0.3839	0.9958	4.531	0.04520	0.9932	4.992	0.5564	0.6883	0.9950
16	2.215	0.4277	0.9944	3.402	0.48004	0.9944	3.841	0.5074	0.6954	0.9976
17	3.112	0.3936	0.9985	4.706	0.05081	0.9956	5.112	0.6271	0.6834	0.9952
18	3.337	0.3727	0.9972	5.001	0.04533	0.9945	5.025	0.7613	0.6327	0.9978
19	3.382	0.3709	0.9969	5.080	0.04499	0.9959	5.328	0.6880	0.6519	0.9974
20	2.041	0.4389	0.9939	3.189	0.04774	0.9978	3.539	0.5347	0.6784	0.9982

Table 4.23 Fitting of different rheological models on shear rate *vs.* shear stress data at 20°C for barley-milk based fermented probiotic drink obtained during optimization trials

Standard order	*Ostwald-De-Waele* model			*Casson* Model			*Herschel Bulkley* model			
	K_o	n_o	R^2	τ_c	K_c	R^2	τ_{HB}	K_{HB}	n_{HB}	R^2
1	0.373	0.5917	0.9963	0.568	0.02729	0.9991	0.594	0.20100	0.7018	0.9980
2	1.723	0.5140	0.9958	2.715	0.07204	0.9996	2.355	0.86554	0.6347	0.9994
3	0.362	0.6062	0.9968	0.545	0.02938	0.9966	0.572	0.20374	0.7094	0.9988
4	1.815	0.5004	0.9941	2.887	0.06807	0.9996	2.582	0.85778	0.6308	0.9990
5	0.312	0.6915	0.9976	0.448	0.03024	0.9993	0.592	0.15098	0.7670	0.9995
6	1.732	0.5233	0.9951	2.743	0.07723	0.9999	2.606	0.82086	0.6550	0.9993
7	0.352	0.6132	0.9990	0.515	0.03028	0.9995	0.594	0.18049	0.7374	0.9998
8	2.147	0.4397	0.9950	3.362	0.05049	0.9994	2.588	1.00200	0.5689	0.9993
9	0.264	0.5651	0.9957	0.411	0.01598	0.9997	0.493	0.10910	0.7272	0.9997
10	4.483	0.3412	0.9988	6.543	0.04623	0.9885	3.293	2.43099	0.4406	0.9988
11	1.363	0.4685	0.9967	2.162	0.04101	0.9993	1.799	0.62876	0.6027	0.9995
12	1.211	0.5460	0.9990	1.747	0.06760	0.9939	1.001	0.78686	0.6274	0.9997
13	1.119	0.4851	0.9986	1.718	0.03853	0.9971	1.353	0.54286	0.6150	0.9997
14	1.234	0.5294	0.9995	1.835	0.06022	0.9958	1.221	0.77490	0.6148	0.9995
15	1.216	0.5126	0.9995	1.826	0.05226	0.9958	1.335	0.65816	0.6253	0.9995
16	0.733	0.5875	0.9969	1.105	0.05253	0.9992	0.905	0.46079	0.6698	0.9994
17	1.350	0.5003	0.9969	2.111	0.05135	0.9991	1.612	0.72535	0.6088	0.9997
18	1.269	0.5067	0.9965	1.990	0.05049	0.9993	1.685	0.63521	0.6285	0.9994
19	0.962	0.5622	0.9972	1.465	0.05794	0.9988	0.994	0.63827	0.6341	0.9996
20	0.778	0.5729	0.9973	1.184	0.05036	0.9994	0.950	0.47855	0.6591	0.9995

Table 4.24 Analysis of variance (ANOVA) for the parameters of different rheological models fitted on the shear rate *vs*. shear stress data for barley-milk based fermented probiotic drink obtained during optimization trials at 5°C

Source of Variation	d.f.	Sum of squares						
		Ostwald-De-Waele Model		*Casson* Model		*Herschel Bulkley* Model		
		K_o	n_o	τ_c	K_c	τ_{HB}	K_{HB}	n_{HB}
Model	9	81.1418**	0.0494*	166.5411**	0.0894	144.7523**	3.9199**	0.0518*
x_1	1	71.8183**	0.0417**	149.6975**	0.0010	133.8814**	3.2212**	0.0469**
x_2	1	0.0012	0.0023	0.1215	0.0009	0.1693	0.0128	0.0001
x_3	1	0.3294	0.0000	0.2745	0.0077	0.3766	0.0069	0.0000
x_1x_2	1	0.0003	0.0019	0.0925	0.0167	0.0872	0.0001	0.0001
x_1x_3	1	0.1602	0.0001	0.0828	0.0153	0.1434	0.0003	0.0000
x_2x_3	1	0.8805	0.0003	0.7418	0.0154	0.8250	0.0023	0.0001
x_1^2	1	6.5830*	0.0028	12.6663*	0.0081	8.6696**	0.4616*	0.0005
x_2^2	1	0.3689	0.0005	0.7879	0.0106	0.0675	0.0620	0.0023
x_3^2	1	0.3300	0.0001	0.7295	0.0112	0.0515	0.0850	0.0025
Residual	10	6.9374	0.0129	13.9894	0.2409	6.9411	0.5689	0.0125
Lack of fit	5	5.2652	0.0087	10.6327	0.0845	4.1222	0.5206*	0.0095
Pure error	5	1.6721	0.0041	3.3567	0.1564	2.8189	0.0483	0.0029

**significant at $\rho<0.01$, *significant at $\rho<0.05$; $^{\$}$estimated for barley-milk based fermented probiotic drink; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Table 4.25 Coefficients of variables in the model to predict different parameters of rheological models for barley-milk based fermented probiotic drink at 5°C

Source of Variation	Coefficient estimate†						
	Ostwald-De-Waele Model		*Casson* Model		*Herschel Bulkley* Model		
	K_o	n_o	τ_c	K_c	τ_{HB}	K_{HB}	n_{HB}
Intercept	2.8774	0.3963	4.3607	0.1191	4.6650	0.6205	0.6699
x_1	2.2937	-0.0553	3.3115	-0.0084	3.1317	0.4858	-0.0586
x_2	-0.0094	0.0129	0.0943	-0.0082	0.1113	0.0307	0.0019
x_3	0.1553	-0.0009	0.1418	-0.0238	0.1661	0.0224	0.0005
x_1x_2	-0.0062	-0.0153	0.1075	-0.0457	0.1044	-0.0042	-0.0026
x_1x_3	0.1415	0.0040	0.1018	0.0437	0.1339	0.0063	-0.0022
x_2x_3	-0.3318	0.0065	-0.3045	-0.0439	-0.3211	-0.0168	-0.0034
x_1^2	0.6764	0.0139	0.9383	-0.0237	0.7763	0.1791	0.0060
x_2^2	-0.1600	0.0062	-0.2338	0.0271	-0.0684	-0.0656	0.0127
x_3^2	-0.1513	0.0031	-0.2250	-0.0279	-0.0598	-0.0768	0.0132
R^2	0.92	0.79	0.92	0.27	0.95	0.87	0.81
R^2_{adj}	0.85	0.61	0.85	-0.38	0.91	0.76	0.63
APV$^{\Phi}$	13.65	7.32	13.61	2.29	17.88	10.91	7.89

$^{\Phi}$ adequate precision value, † for final equation in terms of coded factors; $^{\$}$estimated for barley-milk based fermented probiotic drink; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Table 4.26 Analysis of variance (ANOVA) for the parameters of different rheological models fitted on the shear rate *vs.* shear stress data for barley-milk based fermented probiotic drink obtained during optimization trials at 20°C

Source of Variation	d.f.	Sum of squares						
		Ostwald-De-Waele Model		*Casson* Model		*Herschel Bulkley* Model		
		K_o	n_o	τ_c	K_c	τ_{HB}	K_{HB}	n_{HB}
Model	9	14.6823**	0.0080	33.3696**	0.0040*	12.3306**	3.8227*	0.0674
x_1	1	12.5842**	0.0595**	29.1088**	0.0030**	11.4153**	3.2998**	0.0604**
x_2	1	0.0058	0.0001	0.0014	0.0000	0.0974	0.0163	0.0004
x_3	1	0.0157	0.0012	0.0221	0.0001	0.0002	0.0127	0.0002
x_1x_2	1	0.0286	0.0001	0.0698	0.0001	0.0066	0.0025	0.0006
x_1x_3	1	0.0212	0.0031	0.0533	0.0000	0.0070	0.0037	0.0023
x_2x_3	1	0.0175	0.0033	0.0360	0.0001	0.0061	0.0058	0.0018
x_1^2	1	1.7899*	0.0033	3.6754*	0.0007*	0.7737*	0.3734	0.0002
x_2^2	1	0.0144	0.0002	0.0157	0.0000	0.0480	0.0205	0.0008
x_3^2	1	0.0719	0.0002	0.1326	0.0000	0.0045	0.0437	0.0008
Residual	10	2.1802	0.0031	4.0489	0.0011	0.9203	0.8712	0.0249
Lack of fit	5	1.8325*	0.0048	3.1494	0.0010*	0.3184	0.8152*	0.0223*
Pure error	5	0.3477	0.0015	0.8995	0.0000	0.6020	0.0559	0.0026

**significant at ρ<0.01, *significant at ρ<0.05; $^{\$}$estimated for barley-milk based fermented probiotic drink; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Table 4.27 Coefficients of variables in the model to predict different parameters of rheological models for barley-milk based fermented probiotic drink at 20°C

Source of Variation	Coefficient estimate†						
	Ostwald-De-Waele Model		*Casson* Model		*Herschel Bulkley* Model		
	K_o	n_o	τ_c	K_c	τ_{HB}	K_{HB}	n_{HB}
Intercept	1.0688	0.5380	1.6369	0.0524	1.2463	0.6110	0.6352
x_1	0.9601	-0.0660	1.4603	0.0148	0.9145	0.4917	-0.0665
x_2	0.0205	-0.0022	0.0100	0.0012	-0.0844	0.0345	-0.0052
x_3	0.0339	0.0095	0.0403	0.0020	0.0040	0.0305	0.0038
x_1x_2	0.0598	-0.0042	0.0934	-0.0041	0.0286	0.0176	-0.0085
x_1x_3	0.0515	-0.0198	0.0816	-0.0020	0.0296	0.0216	-0.0169
x_2x_3	0.0468	-0.0204	0.0671	-0.0031	-0.0276	0.0270	-0.0149
x_1^2	0.3527	-0.0152	0.5054	-0.0068	0.2319	0.1611	-0.0036
x_2^2	-0.0316	0.0039	-0.0330	0.0014	0.0577	-0.0378	0.0074
x_3^2	-0.0706	0.0039	-0.0959	-0.0003	0.0177	-0.0551	0.0074
R^2	0.87	0.70	0.89	0.79	0.93	0.81	0.73
R^2_{adj}	0.75	0.42	0.79	0.60	0.87	0.65	0.49
APV$^{\Phi}$	10.27	6.61	11.29	8.04	14.34	8.34	6.57

$^{\Phi}$ adequate precision value, † for final equation in terms of coded factors; $^{\$}$estimated for barley-milk based fermented probiotic drink; x_1: barley flour; x_2: probiotic inoculum, x_3: co-culture inoculum

Results obtained for different model terms after fitting of models are shown in **Table 4.22** and **4.23**. *Herschel-Bulkley* model gave best fit in terms of consistently high coefficient of determination values (R^2) when compared with other two models namely, *Ostwald-De-Waele* and *Casson* model. The data related to parameters obtained for different models for CCRD were fitted with a quadratic function through regression analysis and generated models were evaluated for their fitness using ANOVA analysis (**Table 4.24** and **4.26**).

The dependence of model terms with respect to independent variables in the form of correlations is presented in **Table 4.25** and **4.27.** The model adequacy was checked and goodness of fit was in terms of the coefficient to determination (R^2). The coefficients of determination values for K_o, n_o, τ_c, k_c, τ_{HB}, K_{HB} and n_{HB} for the models generated at 5°C were 0.92, 0.79, 0.92, 0.27, 0.95, 0.87 and 0.81, respectively (**Table 4.25**). The co-efficient of variation for different model parameters namely K_o, n_o, τ_c, k_c, τ_{HB}, K_{HB} and n_{HB} at 5°C were 26.64, 8.70, 25.23, 151.63, 16.31, 36.94 and 5.11%, respectively; and at 20°C were 37.66, 10.43, 33.60, 21.43, 20.83, 44.88 and 7.76%, respectively. Low co-efficient of variation is indication of model preciseness. Regression coefficients of full second order model to predict rheological model parameters of barley-milk based fermented probiotic curd are presented in **Table 4.25** and **4.27**.

Among three independent variables namely, barley flour concentration, probiotic inoculum level and co-culture inoculum level, barley flour concentration was most vital component which significantly ($\rho<0.01$) affected all parameters of different rheological models (*Ostwald-De-Waele* model, *Casson* model and *Herschel-Bulkley* model) developed for barley-milk based fermented probiotic drink at 5 and 20°C (**Table 4.24** and **4.26**).

ANOVA of rheological models terms indicated that quadratic models generated for barley-milk based fermented probiotic drink at 5°C were non-significant ($\rho>0.05$) for K_c, whereas, at 20°C were non-significant ($\rho>0.05$) for n_o and n_{HB}. Lack of fit test for generated quadratic models to predict rheological parameters for probiotic drink at 5°C was found significant ($\rho<0.05$) for K_{HB}, whereas, quadratic models to predict rheological parameters for probiotic drink at 20°C was significant ($\rho<0.05$) for K_o, k_c, K_{HB} and n_{HB} (**Table 4.24** and **4.26**).

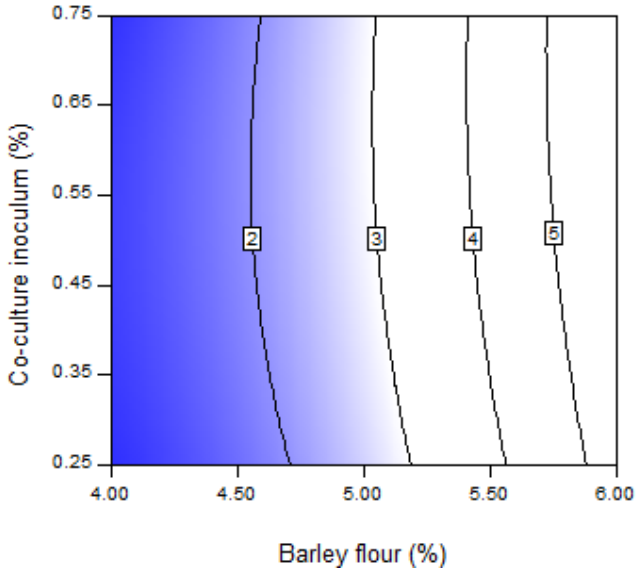


Figure 4.62 Effect of barley flour concentration and co-culture inoculum level on the consistency index in *Ostwald-De-Waele* model (K_o) for barley-milk based fermented probiotic drink at 5°C

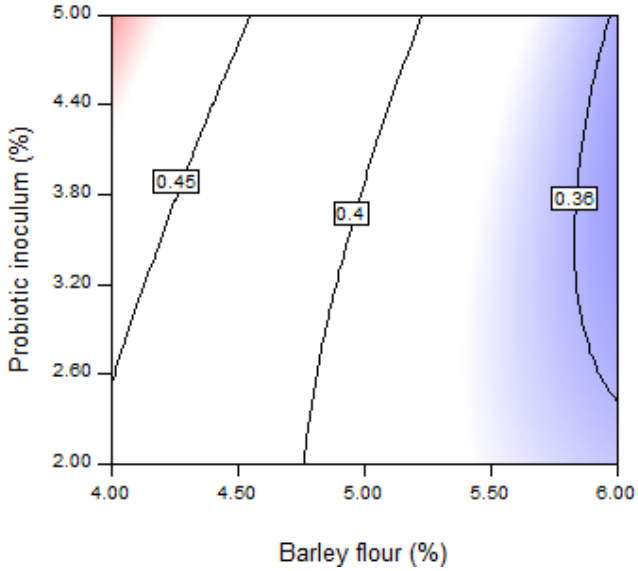


Figure 4.63 Effect of barley flour concentration and probiotic inoculum level on the flow behaviour index in *Ostwald-De-Waele* (n_o) for barley-milk based fermented probiotic drink at 5°C

Consistency index for *Ostwald-De-Waele* (K_o) increased (**Figure 4.62**) with increasing barley flour concentration in the formulation, whereas, a consistent decrease (**Figure 4.63**) in the flow behavior index of barley-milk based fermented probiotic drink was noticed.

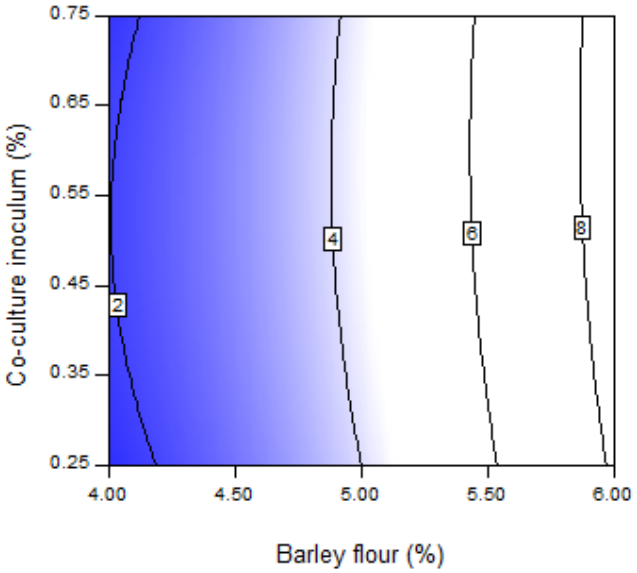


Figure 4.64 Effect of barley flour concentration and co-culture inoculum level on the yield stress (τ_c) in *Casson* model for barley-milk based fermented probiotic drink at 5°C

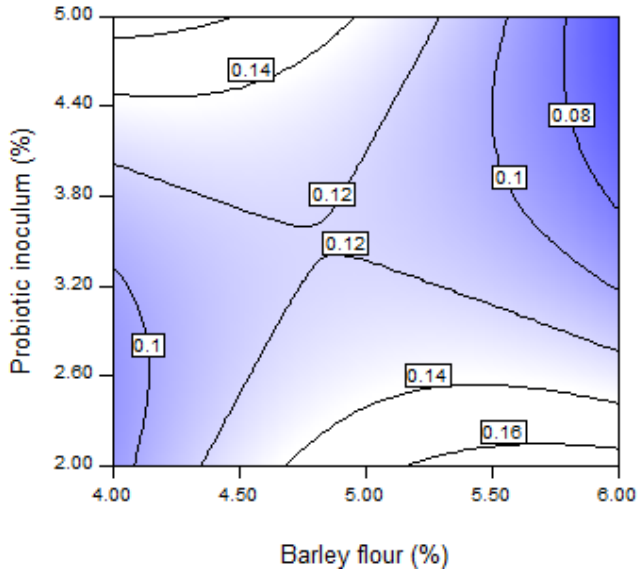


Figure 4.65 Effect of barley flour concentration and probiotic inoculum level on the *Casson* plastic viscosity (K_c) for barley-milk based fermented probiotic drink at 5°C

Increase in the barley flour concentration has significantly affected *Casson* yield stress (τ_c) and *Casson* plastic viscosity (K_c) at linear level ($\rho<0.01$) as well as on quadratic level ($\rho<0.05$). An increasing trend was found for τ_c with increasing flour level (**Figure 4.64**). Circular contour plots (**Figure 4.64**) indicated that interactions between the variables are negligible. Whereas, elliptical ones (**Figure 4.65**) indicate the evidence of interactions. Increase in barley flour level and probiotic inoculum level showed increase in K_c.

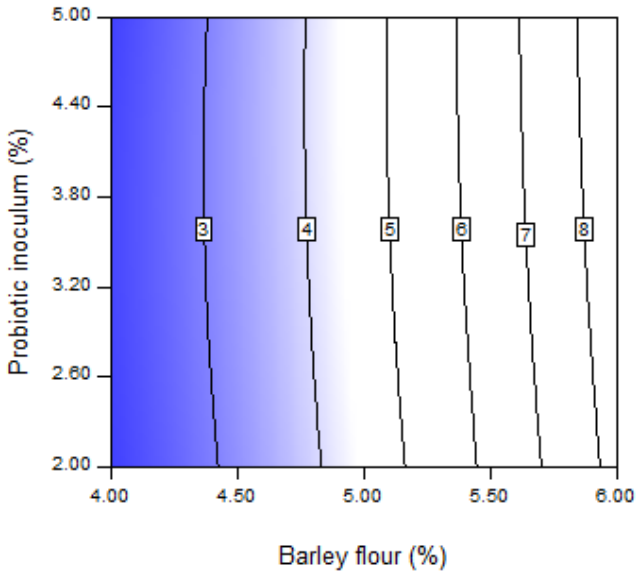


Figure 4.66 Effect of barley flour concentration and probiotic inoculum level on the *Herschel-Bulkley* yield stress (τ_{HB}) for barley-milk based fermented probiotic drink at 5°C

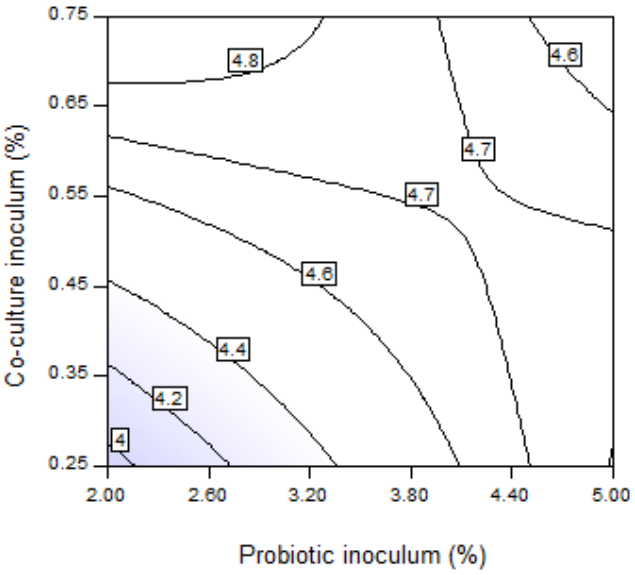


Figure 4.67 Effect of probiotic inoculum and co-culture inoculum level on the yield stress in *Herschel-Bulkley* (τ_{HB}) model for barley-milk based fermented probiotic drink at 5°C

Barley flour concentration has led to a significant ($\rho<0.01$) positive influence on the *Herschel-Bulkley* yield stress value (**Figure 4.66**) and consistency index (K_{HB}) (**Figure 4.68**), whereas, flow behavior index in *Herschel-Bulkley* model was found decreasing (**Figure 4.69**). Interaction of probiotic inoculum and co-culture inoculum showed positive influence on the yield stress value (**Figure 4.67**).

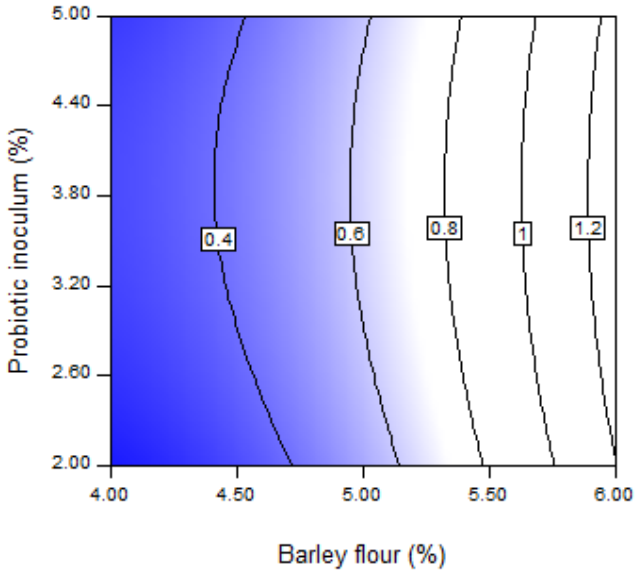


Figure 4.68 Effect of barley flour concentration and probiotic inoculum level on the consistency index (K_{HB}) in *Herschel-Bulkley* model for barley-milk based fermented probiotic drink at 5°C

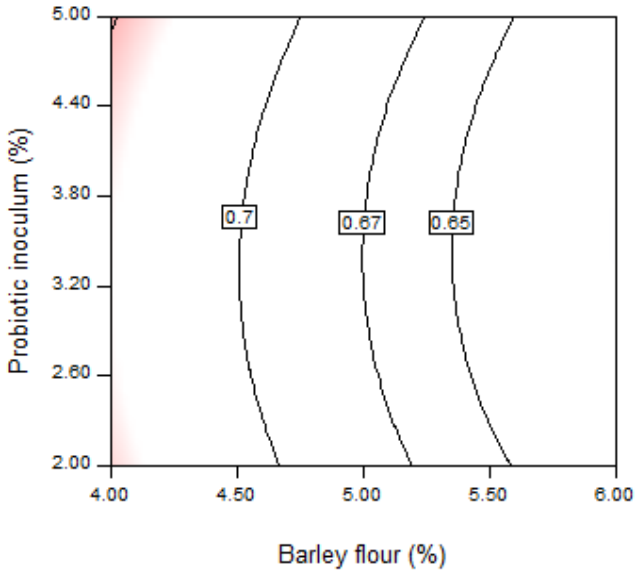


Figure 4.69 Effect of barley flour concentration and probiotic inoculum level on the flow behaviour index (n_{HB}) in *Herschel-Bulkley* model for barley-milk based fermented probiotic drink at 5°C

4.8.8.7 Optimization of the levels for barley flour concentration, probiotic inoculum level and co-culture inoculum level

For optimization of the formulation of barley-milk composite based fermented probiotic drink, three critical responses namely, overall acceptability, probiotic count and β-glucan content were selected. Contour plots were generated to investigate the interactions among independent variables on overall acceptability; probiotic count and β-glucan content have been discussed

previously. The above mentioned responses had direct effect on the acceptability and functionality of barley-milk composite based fermented probiotic drink.

Table 4.28 Constraints used for the optimization of barley-milk based fermented probiotic drink

	Goal	Lower Limit	Upper Limit	Importance
Variables				
Barley flour	in range	4	6	+++
Probiotic inoculum	in range	2	5	+++
Co-culture inoculum	in range	0.25	0.75	+++
Responses				
Probiotic count	maximize	7.45	8.81	+++
Overall acceptability	maximize	6.38	7.88	+++
Beta glucan content	maximize	0.123	0.251	+++

Numerical multiple response optimization technique was used for the optimization of independent variables. Various constraints used in the optimization of levels for independent variables are given in **Table 4.28**. Goal for the selected responses (probiotic count, β-glucan content and overall acceptability) were 'maximized', whereas, for the independent variables (barley flour concentration, probiotic inoculum level and co-culture inoculum level) kept 'in range'. The numerical optimization provided optimum formulation as 5.24% barley flour, 5.00% probiotic (*L. plantarum* NCDC344) and 0.34% co-culture (*S. thermophilus* 20) inoculum level.

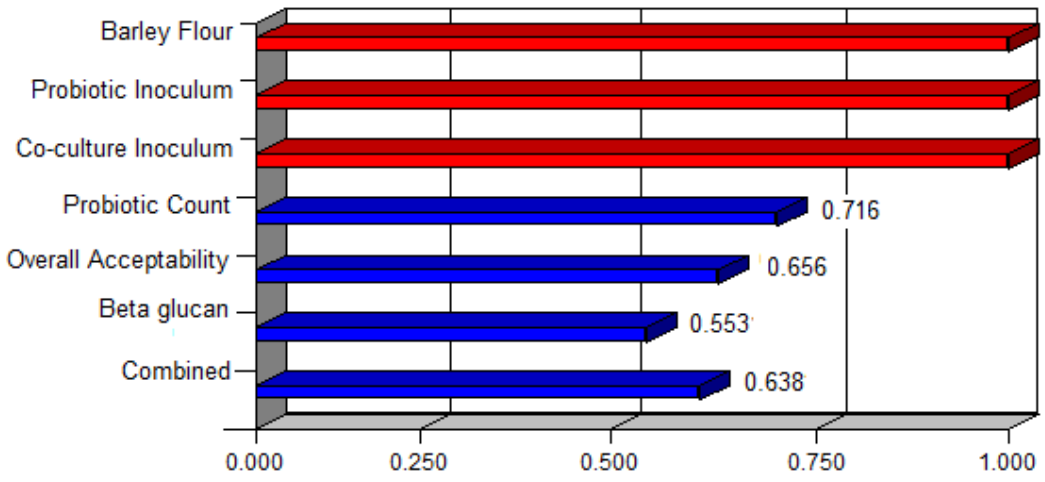


Figure 4.70 Desirability bar graph for the solutions of multiple responses optimization

Under optimum formulation, analysis indicated the predicted values for overall acceptability, probiotic count and β-glucan content as 7.37 (based on 9-

point hedonic scale), 8.40 (log cfu/ml) and 0.194 (g/100 g), respectively. Desirability bar graph for individual and combined responses for the selected formulation is represented in (**Figure 4.70**).

Verification of the results for optimization was accomplished by preparation of barley-milk based fermented probiotic drink and comparing the experimental results with predicted ones. Probiotic drink was analyzed for sensory acceptability, probiotic count and β-glucan and it was having overall acceptability score of: 7.80±0.17; probiotic count: 8.59±0.09 (log cfu/ml) and β-glucan*: 0.195±0.00 (g/100g; *estimated in barley-milk based fermented composite). Gupta *et al.*, (1992) attempted preparation of a barley-butter milk based fermented traditional beverage popularly called as *rabadi* at different time-temperature combinations. The beverage was reported to have overall acceptability score in range 6.35 to 8.36 (Gupta *et. al*., 1992), which depended on time and temperature of incubation.

Arora *et al.*, (2010) reported 8.88 log cfu/g of *L. acidophilus* in fermented mixture prepared with germinated barley flour, whey powder and tomato pulp. Comparatively higher probiotic count (10.4 log cfu/ml) of *L. plantarum* ATCC 8014 was reported in 8 h fermented oat based probiotic drink containing 5.5% of oat flour, 1.25% sugar and 5% of probiotic inoculum level (Gupta *et al.*, 2010). Viability of *L. plantarum* B28 in a probiotic oat based beverage was reported in the range of 9.96 to 10.87 log cfu/ml count, that influenced by the probiotic inoculum level (Angelov *et al.*, 2006). As per International Dairy Federation (1997), in probiotic foods the specific microorganisms shall be viable, active and abundant at the level of at least 10^7 cfu/g in the product (Ouwehand and Salminen, 1998). It has been suggested that approximately 10^9 cells per day consumption of probiotics is necessary to elicit health promoting effects to the host. Based on daily consumption of 100 gram of a probiotic food, it has been suggested that a product should contain at least 10^7 cells per gram or milliliter, a level suggested by current Japanese recommendations (Ishibashi and Shimamura, 1993).

Cereal β-glucans demonstrates prebiotic properties owing to their ability to pass undigested through the upper part of gastro-intestinal tract and reach in the colon region, where it is selectively utilized by beneficial LAB and assist in their proliferation (Gibson *et al.*, 2004). Presence of 2-substituted-(1,3)-β-D-glucan of non-dairy bacterial origin has been reported to promote adhesion of the probiotic

Lactobacillus plantarum WCFS1 to human intestinal epithelial cells (Russo *et al.*, 2012). The use of β-glucans derived from cereals as a source of fermentable substrates for the growth of probiotics has been reviewed by Bigliardi and Galati (2013). Lam and Cheung (2013) extensively reviewed non-digestible long chain β-glucans as novel prebiotics.

Optimized formulation of the three variables of the three variables resulted 0.195 g/100g of β-glucan content in the fermented barley-milk composite. Since, during preparation of barley-milk based fermented probiotic drink, 35% water-spice mix (pasteurized water containing roasted cumin powder, black pepper, salt and pectin) is added (**Figure 3.2**), hence the final probiotic drink contained 0.144 g/100 g β-glucan content due to dilution of the fermented barley-milk composite. According to FDA (2006), recommended level of β-glucan in a probiotic based functional drink should be 0.75 g, which leads to 3 g per day in four servings. However, drink prepared using optimal combination of barley flour yielded only 0.144 g/100 g β-glucan content in the drink. Consumption of 250 ml of the optimized barley-milk based fermented probiotic drink will yield only 0.36 g of β-glucan content in single serving. Furthermore, increasing the levels of barley flour beyond 5.24% have adversely affected the acceptability of the drink as well as showed significant decrease in the probiotic count. An oat based probiotic drink was developed by Angelov *et al.*, (2006) by using 4.0, 5.5 and 7.0% oat flour, which had 0.23% 0.36% and 0.41% β-glucan, respectively. No significant changes in concentration of β-glucan were reported during fermentation of oat mashes. However, there are certain apprehensions that LAB may utilize β-glucan during the fermentation process.

4.8.9 Growth kinetics of *L. plantarum* NCDC344 in milk containing optimized levels of barley flour concentration and co-culture inoculum level.

The development of a barley-milk based fermented product containing probiotics will require a strain having ability to grow in the composite along with ability to compete or synergize between co-cultured strains. Litreature availiable on the growth of probiotic in the form of mixed culture using barley as cereal-substare are scantly (Blandino *et al.*, 2003; Hassan *et al.*, 2012; Rathore *et al.*, 2012, Vasudha and Mishra, 2013). Although some data are available on mixed lactobacilli cultures, little is known about the growth characteristics of *Lactobacillus* strains with *S. thermophilus*, one of the most widely employed dairy starter.

Table 4.29 Growth characteristics of probiotic *L. plantarum* NCDC344 in milk; barley-milk composite; and barley-milk composite inoculated with co-culture *S. thermophilus* 20

Time (h)	Probiotic count (log cfu/ml)			Mean	pH			Mean
	Milk	Barley-milk composite	Barley-milk composite with St 20		Milk	Barley-milk composite	Barley-milk composite with St 20	
0	7.30	7.29	7.25	7.28^{a}	6.38	6.34	6.38	6.37^{a}
2	7.55	8.1	8.25	7.97^{b}	5.63	5.72	5.52	5.62^{b}
4	8.19	8.44	8.58	8.40^{c}	4.71	4.79	4.67	4.72^{c}
6	8.28	8.59	8.64	8.50^{c}	4.28	4.35	4.19	4.27^{d}
8	8.41	8.68	8.77	8.62^{c}	3.88	3.99	3.95	3.94^{e}
12	8.25	8.61	8.65	8.50^{c}	3.79	3.91	3.89	3.86^{e}
Mean	8.00^{A}	8.29^{B}	8.36^{B}		4.78^{AB}	4.85^{A}	4.77^{B}	

Means with different superscripts (a, b, c) in the same column indicate significant difference (Fisher's LSD test, $\rho<0.05$) from each other.
Means with different superscripts (A, B) in a row indicate significant difference (Fisher's LSD test, $\rho<0.05$).

Table 4.30 Analysis of variance for growth characteristics of *L. plantarum* NCDC344 in milk; barley-milk composite; and barley-milk composite inoculated with co-culture *S. thermophilus* 20

Source of variation	df	Mean sum of square	
		Probiotic count	pH
Time intervals	5	0.781**	3.020**
Type of substrate	2	0.218**	0.012
Error	10	0.016	0.003

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

To study the growth kinetics of optimized levels of probiotic culture (5.00%) in different substrates namely toned cow milk; milk with optimized level of barley flour (5.24%); and milk with optimized levels of barely flour (5.24%) along with co-culture inoculum (0.35%) following the procedure mentioned in sub-section **3.2.9**. Growth kinetics was monitored in terms of pH and viable probiotic counts (*L. plantarum* NCDC344). Results obtained for pH and viability can be depicted from the **Table 4.30** and presented graphically as well (**Figure 4.71).**

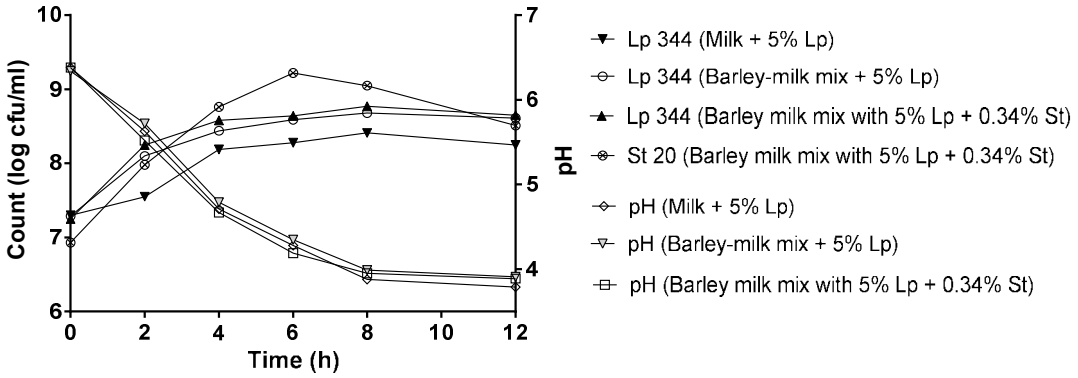


Figure 4.71 Growth kinectics of *L. plantarum* NCDC344 (Lp 344) either alone and in combination with *S. thermophilus* 20 (St 20) in milk and barley-milk composite

All samples have attained a pH value of less than 4.0 within a short fermentation period of 8 h. The initial probiotic count of *L. plantarum* NCDC344 at 0 h in milk, barley-milk composite, and barley-milk composite incoulated with co-culture was 7.30, 7.29 and 7.25 (log cfu/ml), respectively which exhibted an increasing trend upto 8 h of incubation and recorded as 8.41, 8.68 and 8.77 (log cfu/ml), respectively. The pH of different substrate at the end of 8 h were 3.88, 3.99 and 3.95, respectively. Further incubation upto 12 h resulted lowering in the probiotic counts. Initial count for co-culture *S. thermophilus* 20 was 6.93, which attained its maximum number of cells at 6 h. Viability of *S. thermophilus* 20 decreased on extending the incubation period. *L. plantarum* NCDC344 indicated significantly ($\rho<0.01$) higher grwoth rate in barley-milk composite if compared with milk alone (**Table 4.30**). However, inoculation of co-culture in barley-milk composite indicated non-sginifcant ($\rho>0.05$) increase in the viability of probiotic count. *L. plantarum* NCDC344 inoculation in milk alone reflected slow growth rate and manifested as lowest probiotic count at the end of ferementation period. Probiotic count in the samples fermented with *L. plantarum* NCDC344 along with *S. thermophilus* 20 was maximum among three substrates investigated. All types of samples exhibited similar pattern for the changes in pH values (**Table 4.29**).

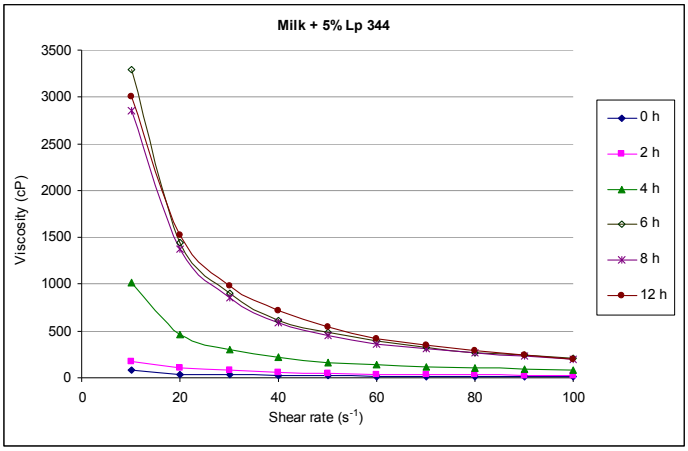


Figure 4.72 Shear rate *vs.* viscosity for toned cow milk inoculated with 5% *L. plantarum* NCDC344 with increasing fermentation time

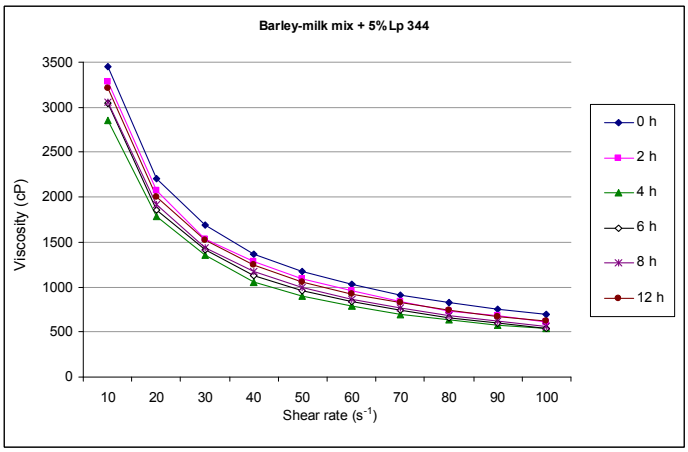


Figure 4.73 Shear rate *vs.* viscosity for barley-milk composite inoculated with 5% *L. plantarum* NCDC344

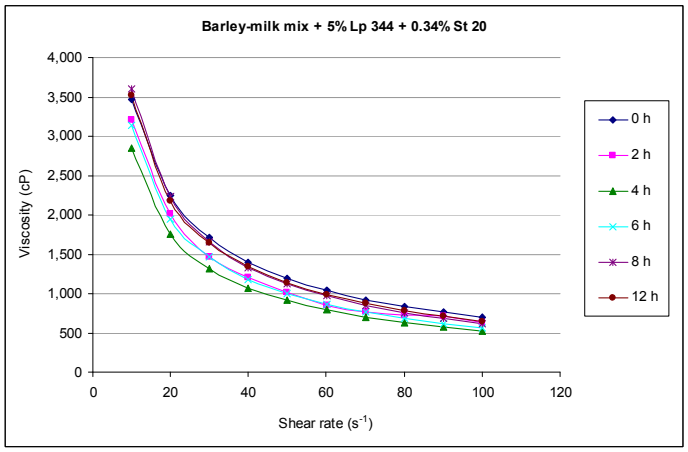


Figure 4.74 Shear rate *vs.* viscosity for barley-milk composite inoculated with 5% *L. plantarum* NCDC344 and 0.34 %

Cereal substrate might have contributed towards buffering capacity of fermented composite (Charalampopoulos *et al.*, 2003; 2010). Therefore, small difference in pH values for milk and barley-milk based fermented samples was obseved in spite of faster growth rate for probiotic cultures. Probiotic culture has resulted in faster acidification rates, when co-cultured with *S. thermophilus* 20, suggesting synergestic relationship between them.

Shear rate *vs.* viscosity data were also obtained for 5.00% *L. plantarum* NCDC344 in milk; barley-milk composite; barley-milk mix inoculated with 0.34% *S. thermophilus* 20 during the fermentaion and depicted in **Figure 4.72 to 4.74**. Fermentation of milk with *L. plantarum* NCDC344 resulted increase in the viscosity ($\dot{\gamma} = 100$) of milk, whereas, viscosity of barley-milk composite decreased during initial 4 h of fermentaion, after which viscosity increased up to 12 h. Increase in the viscosity of milk could be attributed to gelation of casein due to acidification of composite, thereby formation of firm curd matrix. Decrease in the viscosity of barley-milk composite in intital stages of fermenation could be attributed to hydrolysis of starch by microbial amylolytic enzymes.

4.9 PHYSICOCHEMICAL, NUTRITIONAL, THERAPEUTIC, MICROBIOLOGICAL AND SENSORY ATTRIBUTES OF OPTIMIZED BARLEY-MILK BASED FERMENTED PROBIOTIC DRINK

Barley-milk based fermented probiotic drink prepared as per the method illustrated in **Figure 3.2** using optimized levels of barley flour (5.24%), probiotic inoculum (5.00%) and co-culture inoculum (0.34%) was analyzed for physico-chemical properties in terms of acidity, pH, total solids, protein, fat, ash content, colour and viscosity (**Table 4.31**) following the analytical procedures mentioned in sub-section **3.2.10**.

The acidity and pH of probiotic drink was 0.72 % (as % lactic acid) and 4.74, respectively. The total solids of content of the probiotic drink was 14.11% which included 2.96% protein, 2.83% fat, 1.22% ash content and remaining solids included starch, simple sugars and complex polysaccharides. Hussain *et al.*, (2014) prepared a sorghum malt based fermented milk beverage, acidity of which was around 0.76 % of lactic acid. Comparatively lower acidity for a salted pearl millet milk based beverage was reported by Kakde (2010), which varied in the range of 0.57 to 0.61.

Table 4.31 Physico-chemical properties of barley-milk based fermented probiotic drink

Characteristics	Mean ±SE
Acidity (% LA)	0.72 ± 0.01
pH	4.74 ± 0.01
Total Solids (%)	14.11 ± 0.09
Protein (%)	2.96± 0.04
Fat (%)	2.83 ± 0.03
Ash (%)	1.22± 0.02
L*	77.21
a*	0.42
b*	16.63
Viscosity (cP, 5°C, $\dot{\gamma}$ =100)	228 ± 2.31

Total solids, protein, fat and ash content of sorghum malt based fermented milk beverage were reported as 9.83, 2.55, 2.26 and 1.1 %, respectively (Hussain, 2008). Slightly lower values for the total solids (8.74%), protein (1.84%), fat (2.05%) and ash (1.08%) was reported by Kakde (2010). Differences in the physico-chemical properties among the above-mentioned composite mix based fermented beverages results were observed due to variation in the formulations. Around 69% water containing spices was added by Hussain (2008) for the preparation of sorghum malt based fermented milk beverage. Similar rate of addition for water was also used by Kakde (2010), for the preparation of salted pearl millet based fermented milk based beverage. During the preparation of barley-milk composite based fermented probiotic drink, only 35% water-spice mix was added. Moreover, variation in proximate composition could be related to the differences in the composition of cereal-millet flour used, thus levels in formulation and starter culture utilized. Viscosity of developed probiotic drink was much higher than other previously reported cereal / millet and milk based composite beverages, and may be because of the lower proportion of added water, higher proportion of barley flour in the formulation, differences in gelatinization properties of cereal starches and presence of high molecular weight β-glucan of barley grains.

Nutritional and therapeutic qualities of the developed probiotic drink was measured in terms of phytate, β-glucan, total dietary fibre, antioxidant capacity,

ACE-inhibitory activity, lactate (D/L), mineral content (**Table 4.32**) and antimicrobial properties against common pathogens. Methods to evaluate nutritional and therapeutic quality are detailed in sub-section **3.2.10**.

Barley is a rich source of certain minerals such as iron, zinc, magnesium, potassium and calcium (Swaminathan, 1995; Hubner *et al.*, 2012). However, bioavailability of such minerals is low owing to phytic acid content which has ability to form insoluble complexes with minerals (Sandberg *et al.*, 1999). However, recent findings suggested certain health benefits of phytic acid such as chelation of dietary Pb^{2+} (Wise and Gilber, 1981), protection against a fatty liver (Onomi *et al.,* 2004), lowering of serum cholesterol and triglyceride levels (Jariwalla *et al.*, 1990), protection against breast and prostate cancer (Vucenik and Shamsuddin, 2003). Onomi *et al.,* (2004) advocated that phytic acid content should be lowered to a level of to about 0.035% of the phytate in foods, to demonstrate above sighted health benefits. Phytic acid content in barley-milk based fermented probiotic drink was 0.02 (g/100g), which is lower than the suggested value. The lower levels of phytic acid was related to the degradation of phytic acid by L. plantarum NCDC344

Table 4.32 Nutritional and therapeutic quality of barley-milk based fermented probiotic drink

Characteristics	Mean ±SE
Phytate (g/100g)	0.02± 0.00
β-glucan (g/100g)	0.14 ± 0.00
Total dietary fibre (g/100g)	2.3 ± 0.04
ABTS (TEAC) (mg/ml)	0.40±0.01
DPPH (TEAC) (mg/ml)	0.79±0.02
ACE-inhibitory activity	14.1%
L-lactate (g/l)	10.33
D-lactate (g/l)	3.13
Fe (ppm)	6.19± 0.04
Zn (ppm)	4.29 ±0.05
Ca (ppm)	872±15.11

β-glucan is most important and functional ingredient of barley grain which has been reported to exert various physiological effects including cholesterol lowering (McIntosh *et al.,* 1991), regulating blood glucose level (Cavallero *et al.,*

2002) and reducing risk of cancer (Jacobs *et al.,* 1998). Optimized barley-milk based fermented probiotic drink contains 0.14 g/100 g of β-glucan content.

Iron, zinc and calcium contents of the developed drink are indicated in **Table 4.32**. The level of calcium, iron, manganese, copper, zinc and phosphorus in unfermented raw barley flour-buttermilk mixture was reported as 762, 5.01, 1.25, 0.63, 2.25 and 847 mg/100 g, respectively (Gupta *et al.*, 1992). The calcium, iron and zinc content in probiotic drink were on higher side and could serve as a suitable source in diet.

L(+)-lactic acid and D(-)-lactic acid are optical isomers of 2-hydroxypropanoic acid. The two optical isomers can occur in the pure form or as their racemic mixture as DL-lactate (Gawehn, 1988). In mammals, L(+)-lactic acid is formed through the reduction of pyruvate by lactate dehydrogenase (LDH). Mammalian tissues have only L-LDH, which cannot metabolise the D(-)-lactic acid. D(-)-lactate normally present in blood at nano-molar concentrations (Brandt *et al.*, 1980), which is derived either through methyl-glyoxal metabolic pathway (Thornalley, 1990 and Kondoh *et al.*, 1992) or from the gastrointestinal tract where it is produced by the normal commensal bacterial flora (Connolly and Lonnerdal, 2004). LAB can produce both stereoisomers of lactic acid or as its racemic mixture. L(+) and D(-) lactate content in barley-milk based fermented probiotic drink were 10.33 and 3.13 g/l, respectively. Previous reports on total lactic acid content in yoghurt showed a value of 800 mg/100 g product, out of which 46% was D(-) isomer and 54% was L(+) isomer (Alm, 1982). A higher amount of L-lactate in barley-milk based probiotic drink is another its positive health attributes.

Trolox equivalent DPPH and ABTS radical scavenging activity of barley-milk based fermented probiotic drink was 0.40 and 0.79 mg TEAC/ml, respectively. Antioxidant activity of probiotic drink may be due to the presence of barley polyphenols, and owing to release of anti-oxidative peptides by starter proteases. Antioxidant activity with ABTS assay of *L. lactis* fermented milk samples were reported as lowest (0.16 mmol trolox equivalent per litre) among 25 different LAB fermented milk samples, whereas milk fermented with *Lactobacillus jensenii* was reported to exhibit highest (0.70 mmol trolox equivalent per litre) antioxidant activity (Virtanen *et al.*, 2007). In another study, Jiménez *et al.*, (2008) reported the TEAC activity of strawberry fortified yoghurt as 10 μM using ABTS method. Trolox equivalent ABTS radical scavenging

capacity for commercial yoghurts were reported in the range from 7.697 to 8.739 mM Trolox/g, and in traditional yoghurts from 10.115 to 13.182 mM Trolox/g (Şanlıdere and Öner, 2011). This difference may be because of the proteolytic activity of individual cultures, which regulates the release of antioxidative peptides.

The ACE-inhibitory activity of barley-milk based fermented probiotic drink was 14.1%. ACE-inhibitory of cow milk fermented by a combination of *L. plantarum* and *L. casei* was much higher i.e. 52% (Praveesh *et al.*, 2011). They reported maximum ACE-inhibitroy activity at lower inoculum concentration (1%), and observed decreease ACE inhibitory activity with increasing the inoculum levels up to 3%. The low levels of inhibition may indicate that fermented milk do not produce aprreciable amount of ACE-inhibitory peptides. Minervini *et al.*, (2009) fermented goat milk with selected multiple starters and reported ACE-inhibitory activity in the range of 37.7 to 82.0%. Goat milk fermented with *L. casei* LC01 and *L. plantarum* 1288 reflected low ACE-inhibitory activity of 34.3 and 37.7%, respectively. However, goat milk fermented with *L. acidophilus* did not possess any ACE-inhibitory activity (Minervini *et al.*, 2009). The type of starter may be considered as one of the major factors that influences the synthesis of bioactive peptides in dairy products (Gobbetti *et al.*, 2002), thus influencing ACE-inhibitory activity.

Table 4.33 Antimicrobial properties of barley-milk based fermented probiotic drink

Pathogen	Zone of Inhibition (mm)
Escherichia Coli	22±0.58
Salmonella typhii	20±0.67
Shigella dysentriae	16±0.33
Staphylococcus aureus	12±0.33

The antimicrobial activity of probiotic is considered to be major selection criteria for competitively excluding or inhibiting the activities of pathogenic intestinal microflora rendering the host safer against the invasion of harmful microorganisms. The inhibition of the growth of pathogens can be through the production of antimicrobial compounds like organic acid, hydrogen peroxide, bacteriocins etc. (Jin *et al.,* 1996; Zarate and Nader-Macias, 2006). Lactobacilli are the natural inhabitants of human gastrointestinal tract along with other microflora and these fermentative organisms produce organic acids (*i.e*. acetic

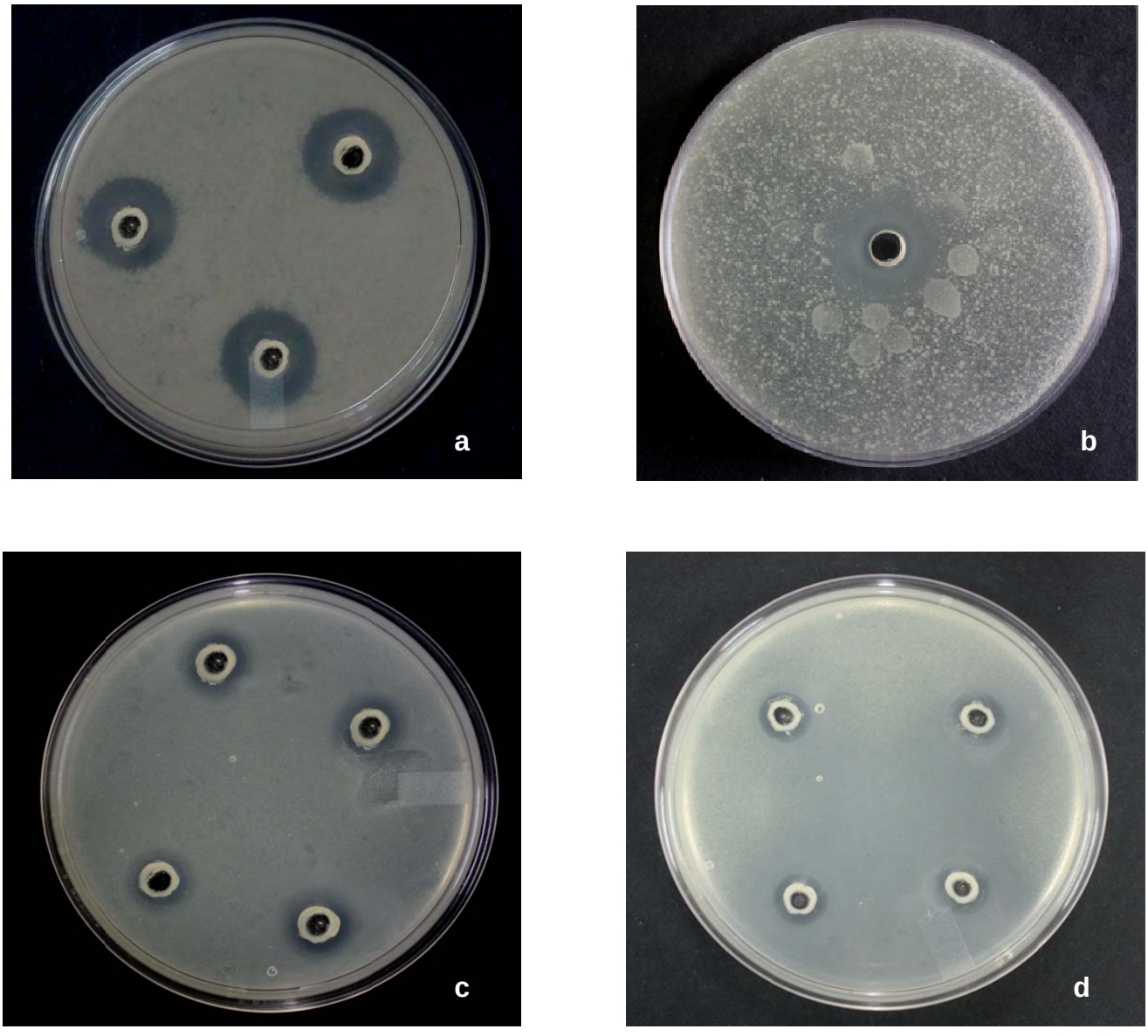


Plate IX: Antimicrobial activity of barley-milk based fermented probiotic drink against (a) *E. coli* (b) *Salmonella typhii* (c) *S. dysentriae* (d) *S. aureus*

and lactic acids), that tend to lower the intestinal pH thus inhibiting the poliferation of harmful microbes in the microecological environment (Musikasang *et al.*, 2009). Probiotic microorganisms can co-aggregate with pathogens or attach to enterocytes (competitive exclusion) and thus inhibit the binding of enteric pathogens to the intestinal mucosa.

The optimized barley-milk based fermented probiotic drink was studied for its antimicrobial activity against various test organisms by agar well diffusion assay as described in sub-section **3.2.10**. Antimicrobial activity was determined by measuring the diameter of zone of inhibition (**Table 4.34** and **Photoplate IX**), indicating that the cultures used in the production of barley-milk based probiotic drink had shown inhibitory activity against the test pathogens. The zones of inhibition of indicator organisms tested were ranged from 12 mm to 22 mm in diameter. The highest zone of inhibition was observed for gram negative *E. coli* (22 mm), whereas lowest activity was observed against gram positive *Staphylococcus aureus* (12 mm). Antagonistic activity against *Shigella* and *Salmonella* was 16 mm and 20 mm by optimized probiotic drink. From all these data, it could be inferred that observed that the inhibition shown by developed barley-milk based fermented probiotic drink was maximum against *E. coli*. This antimicrobial activity could be attributed to the presence of lactic acid, antimicrobial peptides, and bacteriocins which may have inhibitory effect against indicator organisms as mentioned by many workers (Jin *et. al.,* 1996).

Table 4.34 Microbiological quality of barley-milk based fermented probiotic drink

Count (log cfu/ml)	Mean ±SD
Probiotic count (Bujalance *et. al.*, 2006)	8.59± 0.09
Coliform count	Nil
Yeast and mould count	1.12±0.00
S. thermophilus	9.02±0.15
Non-LAB (Angelidis, 2006)	1.22±0.00

Developed barley-milk based fermented probiotic drink was also evaluated for its microbiological quality (**Table 4.34**) and sensory quality (**Table 4.35**). Optimized probiotic drink contained 8.59 log cfu/ml of probiotic cells. The growth of LAB in barley-milk based fermented probiotic drink was comparable with the results obtained by previous worker in oat based fermented probiotic drink (Gupta *et al.*, 2010). Result obtained for probiotic count was supported with the finding of Arora *et al.*, (2010) who reported 8.88 log cfu/g of *L. acidophilus* NCDC16 in

fermented mixture formulated using germinated barley flour, whey powder and tomato pulp. However, comparatively higher probiotic count (10.4 log cfu/ml) of *L. plantarum* ATCC 8014 was reported in 8 h fermented oat based probiotc drink (Gupta *et al.*, 2010). Angelov *et al.*, (2006) reported viability of *L. plantarum* B28 in a probiotic oat based beverage in the range of 9.96 to 10.87 log cfu/ml.

Coliform count in the developed probiotic drink was found nil when first dilution was plated on VRBA agar. Non-LAB in barley-milk based fermented probiotic drink was 1.22 log cfu/ml, which represents the extent of contaminating microflora which depends on the microbial quality of raw materials, purity of starter cultures and hygiene during manufacturing conditions, and may be due to post processing contamination. These microorganisms may belong to heterogeneous bacterial genera and are capable of growing using peptides and amino acids as a sole carbon and energy source (Angeledis *et al.*, 2006). Non-LAB on count agar sugar free for different types of cheese samples were reported in the range of 4.85 to 6.55 log cfu/ml (Angeledis *et al.*, 2006).

Sensory scores of optimized barley-milk based fermented probiotic drink for colour and appearance, sedimentation, consistency, flavour and overall acceptability score were 8.4, 8.1, 7.9, 7.8 and 7.8, respectively (**Table 4.35**). Results for the overall acceptability of barley-milk based fermented probiotic drink was in accordance with the findings of previous authors (Modha and Pal, 2011; Hussain *et al.,* 2014; Kakde, 2010) who worked on millet-milk based fermented beverages.

Table 4.35 Sensory quality of barley-milk based fermented probiotic drink

Characteristics	Mean ±SD
Colour and Appearance	8.4± 0.11
Sedimentation	8.1± 0.18
Consistency	7.9± 0.18
Flavour	7.8± 0.15
Overall acceptability	7.8± 0.17

Colour and appearance, consistency, flavour and overall acceptability scores for pearl-millet based fermented milk beverage were 7.4, 7.1, 7.2 and 7.3, respectively (Modha and Pal, 2011). Overall acceptability of a sorghum-malt based fermented beverage was reported in the between of 7.5 to 7.8 (Hussain *et al*., 2014).

4.10 Changes in viscosity of the barley-milk composite during heating and continuous stirring at optimized level of barley-flour

Change in the viscosity of barley-milk composite at optimized level of barley flour in toned milk was determined using Rapid Visco Analyser (RVA) following the method discussed in **3.2.8.4**. Tests were done to see the changes in viscosity at 90ºC for a period of 20 minutes at constant shear rate of 100 s^{-1}. The pasting curve of the composite was obtained by RVA and expressed as viscosity (cP) (**Figure 4.75)** with respect to time and changing temperature. Viscosity at the start of test *i.e.* at 3 min for barley-milk composite was 51 cP, which increased to 127 cP at 23 min due to continuous heating at 90°C. Final viscosity of the barley-milk composite was 401 cP. Data obtained during RVA provided useful information for changes during processing.

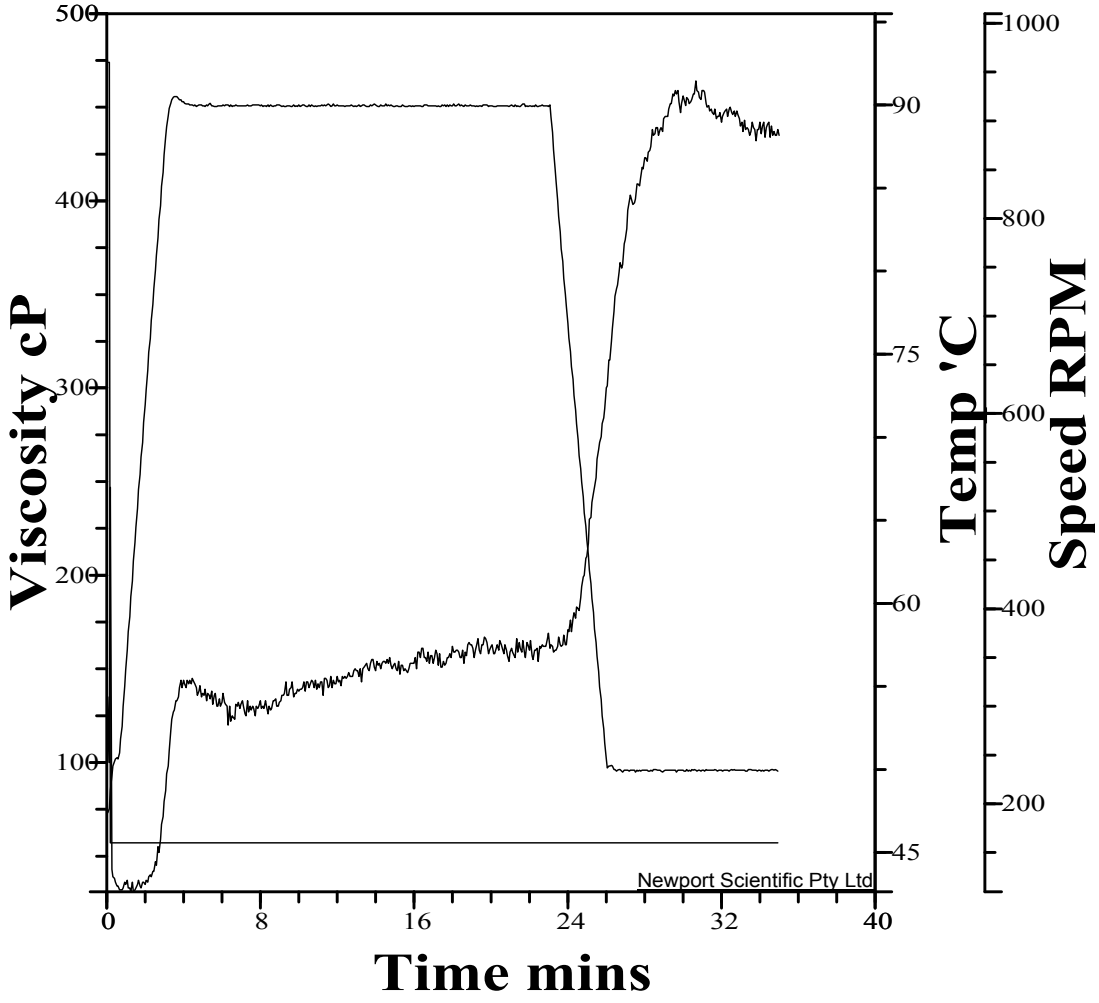


Figure 4.75 Result of the viscosity measurements with RVA to determine the viscosity of the barley-milk composite during heating and continuous stirring

4.11 Effect of temperature on the viscosity of optimized barley-milk based fermented probiotic drink

Temperature has an important role on viscosity of any liquid or semisolid food products. The viscosity of a solution is a function of intermolecular forces and water solute interactions that restrict the molecular motion. These forces are affected by change in concentration of solute and dependent on temperature. Decrease in viscosity with increase in temperature is due to increase in the thermal energy resulting intermolecular distances (Constenla *et al.*, 1989; Hassan and Hobani, 1998). Changes in viscosity with the rise in temperature a three different shear rates is represented in **Figure 4.76**.

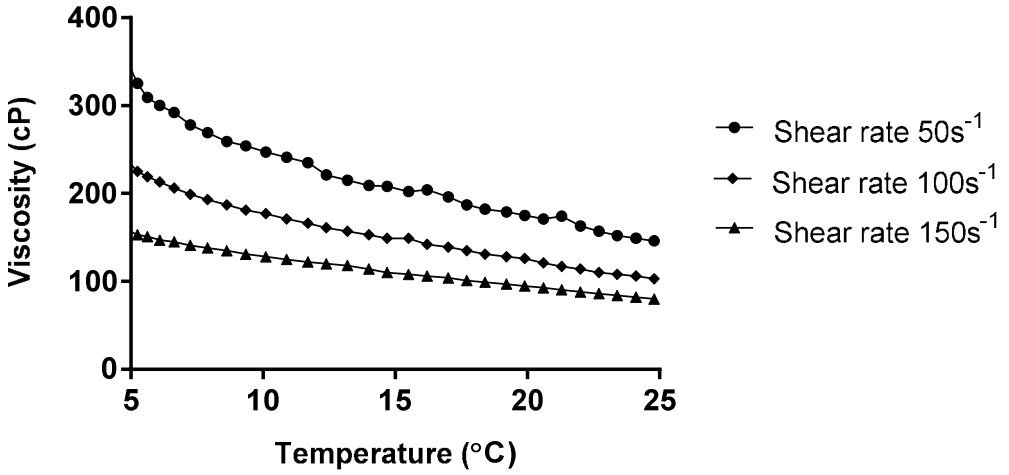


Figure 4.76 Effect of temperature on the viscosity of barley-milk based fermented probiotic drink at different shear rate

The Arrhenius equation has been successfully used to predict the dependence of rheological behavior on temperature in fruit juices (Kaya and Sözer, 2005; Vandresen *et al.*, 2009; Goula and Adamopoulos, 2011). The effect of temperature on the viscosity of barley-milk based fermented probiotic drink was determined at specified shear rate. The effect of temperature on viscosity was studied by fitting the rheological data to the Arrhenius equation (Equation 4.1). Data obtained for changes in viscosity with temperature ramp were fitted to the equation 2 for calculation of activation energy using RheoPlus32 (v3.61) software. Data obtained for changes in viscosity with temperature ramp were fitted to the Equation 4.2 for calculation of activation energy using RheoPlus32 (v3.61) software.

$$\eta_a = \eta_o e^{\left(\frac{E_a}{RT}\right)} \quad (4.1)$$

where, η_a= empirical constant (Pa.s)

η_o= apparent viscosity (Pa.s)

E_a= activation energy (kg/mol)

R = Universal gas constant (J/mol.K)

T= absolute temperature (K)

$$y = a.e^{b.\left(\frac{1}{x+c} - \frac{1}{x_o - c}\right)} \quad (4.2)$$

where,

x = temperature (°C)

y = viscosity (cP)

x_o= reference temperature

$a = y(x_o)$

$b = y\left(\frac{y}{\lambda}\right)$ with R=8.314

with, $x + c > 0$; $x_o + c > 0$; $x > 0$; $x_o > 0$;

Arrhenius equation can also be used to describe the temperature dependency at the zero shear viscosity.

Table 4.36 Activation Energy of barley-milk based fermented probiotic drink

Shear rate	Regression parameters				Activation energy (E_a)	R^2
	A	b	c	X_o		
50	Infinite	3174.3	-0.99482	-272.15	26.393 kJ/mol	0.9835
100	Infinite	3143.4	-0.98203	-272.15	26.136 kJ/mol	0.9958
150	Infinite	3215.3	27.611	-272.15	26.733 kJ/mol	0.9985

Regression parameters along with activation energy and their corresponding R^2 values are presented in **Table 4.36.** The activation energy did not present any statistical difference (ρ>0.05) when estimated at 3 different shear rates 50, 100 and 150 s^{-1}.

According to Lopes *et al.* (1994), the greater the E_a, the greater is the effect of temperature on viscosity. Vandersan *et al.* (2009) observed no significant difference in the E_a of carrot juice due to pasteurization treatment. However, a slight decrease from 3.66 to 3.07 kcal/mol in E_a and 2.87 to 2.14 in η_o has been reported. Total solids content of unpasteurized and pasteurized juices were 8.94 and 8.90, respectively. Retnowati *et al.* (2008) studied the rheological properties of tropical fruit juices containing 5 to 65% total solids at temperature range between 30 to 60°C. Activation energy and Arrhenius constant for

concentrated fruit juices having 35% total solids were 21.924 kJ/mol and 2.051 x 10^{-6} Pa.s, respectively. Viscosity of barley-milk based fermented probiotic drink samples was constantly decreased with the rise in temperature as well as with increasing shear rate. Falguera and Ibraz (2010) observed an increase in activation energy from 28 to 32 kJ/mol, with increase in shear rate from 10 to 150s^{-1}. However, no change in E_a was reported when shear rate varied in between 100 to 150 s^{-1}. Vandresen *et al.* (2009) observed a constant decrease (0.1191 to 0.0104) in k with a significant increase in n from 0.528 to 0.762 when temperature of carrot juices raised from 8°C to 35 - 85°C at variable shear rate from 0 to 1600 s^{-1}.

4.12 Effect of refrigeration storage and type of package on the physico-chemical, microbial and sensory quality of barley-milk based fermented probiotic drink

Optimized probiotic drink was prepared and filled in pre-sterilized clear PET bottles (**Photoplate X**) and LDPE pouches (**Photoplate XI**). PET bottles and caps were sterilized by following the procedure previously mentioned in **3.2.8.1** and prepared drink was filled manually under laminar flow in bio-safety cabinet. The barley-milk based fermented probiotic drink samples were analysed to determine physico-chemical quality (pH, titratable acidity, free fatty acids content, proteolysis, whey separation and viscosity; microbial quality (probiotic count, non-LAB count and, yeast & mould count); and, sensory quality (colour and appearance, consistency, sedimentation, flavour and overall acceptability score) quality during storage at 5±1°C. The samples were analysed as per the methods discussed in sub-section **3.2.11**. Each experiment was conducted in three separate trials. The data obtained during analysis were subjected to analysis of variance (ANOVA) in a completely randomized design with intervals of storage and types of package as main effect. ANOVA was followed by Fisher's least significant difference test (Snedecor and Cochran, 1989) for multiple sample comparisons ($\rho<0.05$).

4.12.1 pH and acidity

The initial titratable acidity (% lactic acid) of 0.69 ± 0.00 in packed samples of barley-milk based fermented probiotic drink increased to 1.16 ± 0.00 and 1.15 ± 0.00, respectively in PET bottles and LDPE pouches, after 15 days of storage (**Table 4.37**), indicating that the product packed in PET bottles and LDPE pouches did not had any significant difference in terms of increase in the acidity.

Plate X: Optimized barley-milk based fermented probiotic drink packed in PET bottle

Plate XI: Optimized barley-milk based fermented probiotic drink packed in LDPE pouches

Table 4.37 Chemical changes in barley-milk based fermented probiotic drink during storage

Storage intervals	Acidity (% LA)		pH		FFA (μeq/g)		Proteolysis (L-leucine mg/g)	
	PET	LDPE	PET	LDPE	PET	LDPE	PET	LDPE
0	0.69±0.00^{a}	0.69±0.00^{a}	4.77±0.01^{a}	4.80±0.01^{a}	5.16±0.19^{a}	4.97±0.09^{a}	1.05±0.00^{a}	1.05±0.00^{a}
3	0.80±0.00^{b}	0.78±0.00^{b}	4.69±0.01^{b}	4.70±0.02^{b}	7.47±0.11^{b}	7.38±0.15^{b}	1.12±0.01^{b}	1.12±0.01^{b}
6	0.90±0.00^{c}	0.88±0.01^{c}	4.57±0.02^{c}	4.61±0.01^{c}	8.70±0.14^{c}	8.49±0.09^{c}	1.19±0.00^{c}	1.18±0.00^{c}
9	0.99±0.00^{d}	0.97±0.00^{d}	4.45±0.01^{d}	4.49±0.02^{d}	10.33±0.30^{d}	9.98±0.45^{d}	1.22±0.00^{d}	1.22±0.00^{d}
12	1.08±0.01^{e}	1.06±0.01^{e}	4.31±0.02^{e}	4.35±0.02^{e}	12.80±0.37^{e}	12.16±0.37^{e}	1.24±0.00^{e}	1.23±0.00^{e}
15	1.16±0.00^{f}	1.15±0.00^{f}	4.16±0.02^{f}	4.19±0.02^{f}	13.65±0.21^{f}	13.23±0.22^{f}	1.25±0.00^{f}	1.24±0.01^{e}

Means with different superscripts in same column differ significantly (ρ<0.05, Fisher LSD); Values in each cell represent mean ± SE, n=3

Table 4.38 ANNOVA for Physico-chemical changes during storage

Source of variation	df	Mean sum of square					
		Acidity	pH	FFA	Proteolysis	Viscosity	Whey separation
				PET			
Storage intervals	5	0.093**	0.162**	31.317**	0.018**	2041.867**	8.785**
Error	12	0.00002643	0.00036	0.170	0.00002092	70.722	0.018
				LDPE			
Storage intervals	5	0.089**	0.156**	28.270**	0.017**	1556.589**	-
Error	12	0.00001649	0.00037	0.212	0.00001728	114.056	-
				PET *vs.* LDPE			
Type of package	1	0.003	0.009	0.901	0.00024	51.361	-
Error	34	0.027	0.047	8.898	0.005	594.40	-

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

The pH of samples in the PET bottles and LDPE pouches decreased from 4.77±0.01 to 4.16±0.02 and 4.80±0.01 to 4.19 ± 0.02 after 15 days of storage. Decrease in pH value from 4.73 to 4.39 and increase in acidity 0.24 to 0.40 g/100 ml of a symbiotic fermented drink during 21 days of storage at 6°C has been previously reported by Dias *et al.*, (2013). A decrease in the pH value from 4.93 to 4.64 with subsequent increase in titratable acidity (0.89 to 1.32%) of probiotic dahi has been reported by Yadav *et al.*, (2007).

From the view point of increase in acidity with consequent decrease in the pH for packed samples of barley-milk based fermented probiotic drink intervals of storage had highly significant ($\rho<0.01$) influence. The decreased pH and increased titratable acidity may be due to production of lactic acid by lactic acid bacteria and spoilage organisms during storage.

4.12.2 Free fatty acids

Free fatty acids (FFA) value is often used as an indicator for degree of fat hydrolysis (Smirnoff, 1995). The FFA content of 5.16 ± 0.19 and 4.97 ± 0.09 (meq/g) of barley-milk based fermented probiotic drink packed in PET bottles and LDPE pouches, respectively increased to 13.65±0.21 and 13.23±0.22 after 15 days of storage at 5±1°C, revealing 164.5% increase in FFA for the samples packed in PET bottles and 166.2% increase for the samples packed in LDPE pouches (**Table 4.37**).

The influence of storage intervals on the FFA values was highly significant ($\rho<0.01$), whereas types of packages have non-significant ($\rho>0.05$) influence on the FFA of barley-milk based fermented probiotic drink during storage **(Table 4.38**). These observations are in accordance with the findings of Abdul *et al.*, (2012), who reported significant influence of storage period on the development of FFA in sorghum malt based fermented milk beverage during storage. Consistent increase in the FFA of salted pearl millet lasssi during storage has also been established by Kakde (2010). Sahan *et al.*, (2008) prepared yoghurt samples fortified with β-glucan and observed a small degree of lipolysis during storage at 4°C up to 15 days. The increase in FFA value during storage could be attributed to the action of microbial lipase on the triglycerides, which might have caused varied degree of lipolysis.

4.12.3 Proteolysis

Proteolysis is the breakdown of large and complex proteins into the smaller and simple peptides due to activity of proteinase and peptidases.

Proteolysis in fermented milk products causes some desirable as well as undesirable effects. Lactic acid bacteria are considered to render several health beneficial effects like antimicrobial, immunomodulatory, anti-cancer activity, anti-thrombiotic, anti-hypertensive and cholestrol lowering properties etc. During fermentation, proteins are hydrolysed by the extracellular proteinases of LAB resulting an increase in bioactive peptides (Shahidi and Zhong, 2008). Detrimental effects of proteolysis on sensory quality of fermented milk products during storage may also result from the enzymatic hydrolysis of milk proteins which produces peptides containing hydrophobic amino acid (Clife and Law, 1990). The *o*-phthaldialdehyde (OPA) spectrophotometric method (Church *et al.*, 1983; Donkor *et al.*, 2007; Shihata and Shah, 2000) was used to assay proteolysis in barley-milk based fermented probiotic drink during storage. Spectrophotometric absorbance forms the basis of OPA values relating to the release α-amino groups resulting from the proteolysis of milk proteins. The initial concentration of 1.05 ± 0.00 leucine mg/g of barley-milk based fermented probiotic drink, which is an index of proteolysis, increased to 1.25 ± 0.00 mg/g in both the packages (PET and LDPE) after 15 days of storage at 5 ± 1°C. The statistical analysis showed that, intervals of storage had significant ($\rho<0.01$) influence on the proteolysis of barley-milk based fermented probiotic drink (**Table 4.38**). However, non-significant variations in proteolytic activity was observed due to differences in package type. Amirdivani and Baba (2011) observed increase in the OPA value of plain and herbal yoghurt samples up to 7 days which started decreasing gradually on further storage up to 28 days. Increase in the OPA value up to 7 days was attributed to the metabolic avtivity of *L. acidophilus* and *S. thermophilus* at 4 °C (Papadimitriou *et al.*, 2007), whereas decrease in OPA value was reported due to decrease in α-amino groups reacting with β-mercaptoethanol rendering lower aborption at 340 nm (Amirdivani and Baba, 2011). Sahan *et al.*, (2008) reported significant increase in the concentration of tyrosine (an index of proteolysis) in plain yoghurt and β-glucan fortified yoghurt samples during storage. The increase in tyrosine content was attributed to the protelytic action of yoghurt cultures which caused enzymatic hydrolysis of milk proteins and subsequent degradation of peptides to act as nutrient during their metabolism (Tamime and Robinson, 1999; Sahan *et al.*, 2008). Shori and Baba (2011) also reported an increasing trend for OPA values in *Allium sativum* (garlic) yoghurts prepared from cow and camel milk during storage up to 21 days at 4°C.

4.12.4 Whey separation

Fermented milk based curd is a protein network formed by casein micelles entrapping serum and fat globules. In cultured dairy products, the physical characteristics of curd are of primary importance with reference to its quality, texture, and body. This casein framework is relatively weak, therefore, the distribution of the rest of the components has an important effect on the rheological behavior of the curd (Tamime *et al.*, 1999). Whey separation or wheying off is defined as the expulsion of whey from the curd matrix which then becomes visible as surface whey. Phenomenon of wheying off negatively affects acceptability of the product by consumers (Lee and Lucey, 2010). In case of drink type cultured products phase separation negatively affects its consistency thereby reducing the acceptability. The phase separation phenomena in any kind of beverages depends on the overall balance of all forces acting on a particle, which should be equal to zero if particle stability is targeted. Four possible forces gravitational, buoyancy, hydrodynamic and intermolecular has been reported to act on a solid particle suspended in a liquid medium (Kiani *et al.*, 2010). Stabilizers like pectin, gelatin, sodium alginate, carboxy methyl cellulose have been reported to prevent wheying off in milk-cereal based beverages (Hussain, 2008; Kakde *et al.,* 2010).

Table 4.39 Physical changes in barley-milk based fermented probiotic drink during storage

Storage intervals	Whey separation (ml/100ml)	Viscosity (cP)	
		PET	LDPE
0	0.00±0.00^{a}	242.67±8.41^{a}	236.67±10.39^{a}
3	1.60±0.10^{b}	201.67±5.24^{b}	205.00±4.72^{b}
6	2.27±015^{c}	185.00±2.31^{c}	187.00±5.03b^{c}
9	2.93±15^{d}	174.33±3.18^{c}	192.33±4.81b^{c}
12	4.10±20^{e}	180.33±1.86^{c}	179.67±3.52^{d}
15	4.70±.20^{f}	176.00±4.95^{c}	173.67±6.06^{d}

Means with different superscripts in same column differ significantly (ρ<0.05, DMRT); Values in each cell represent mean ± SE, n=3

Separation of whey in yoghurt is related to an unstable network (Lucey *et al.*, 1998), rearrangement which may be related to dynamics, and relaxation of the protein-protein bonds (van Vliet *et al.*, 1997; Lucey, 2001). Syneresis in fermented milk occurs as a result of an open structure allowing the flow of serum out from the acid-induced protein network (Puvanenthiran *et al.*, 2002).

Authors (Harwalkar and Kalab, 1983; Guirguis *et al.*, 1984) have used centrifugation method or drainage of whey from screen for the measurements of whey expulsion from set type fermented milk products. Lucey *et al.*, (1998) formed gels in glass volumetric flasks which were used to quantify spontaneous whey separation in fermented milk.

Whey separation in barley-milk based fermented probiotic drink was estimated by quiescent storage of 100 ml drink in measuring flask under refrigeration and observing the whey separation at an interval of 3 days for a period of 15 days. Results obtained for wheying off during storage are indicated in **Table 4.39**. Intervals of storage had highly significant ($\rho<0.01$) influence on whey separation during storage of barley-milk based fermented probiotic drink. This may be due to breakdown of starch (Cronk *et al.* 1977) due to microbial hydrolysis and subsequent decrease in the viscosity during storage. Addition of pectin in the fermented milk based beverage has been reported to decrease wheying off during storage (Kakde, 2010).

4.12.5 Viscosity

Viscosity of any beverage has a close relationship with its consistency and mouthfeel during consumption. Therefore, changes in the viscosity of barley-milk based fermented probiotic drink was monitored during storage. Consistent decrease in the viscosity of barley-milk based fermented probiotic drink packaged in PET bottles and LDPE pouches has been observed during storage at 5±1 °C **(Table 4.39**). The initial viscosity of 243 cP and 236 cP for the samples of barley-milk based fermented probiotic drink packed in PET bottles and LDPE pouches decreased to 176 cP and 174 cP, respectively on 15 days of storage under refrigeration. Changes in viscosity vs. shear rate data during storage for barley-milk based fermented probiotic drink are depicted in the **Figure 4.77** and **7.75**. Intervals of storage indicated highly significant ($\rho<0.01$) influence on the viscosity of barely milk based fermented probiotic drink during storage. Rate of decrease in the viscosity of drink was higher in first six days of storage, which decreased gradually during further storage. Modha and Pal (2011) optimized pearl millet based rabadi like beverage and stored at 5-7°C. During storage, slight increase in the viscosity of beverage was reported up to 3 days of storage, which decreased further on storage up to 7 days. However, reported changes in the viscosity of pearl millet based rabadi like beverage were non-significant.

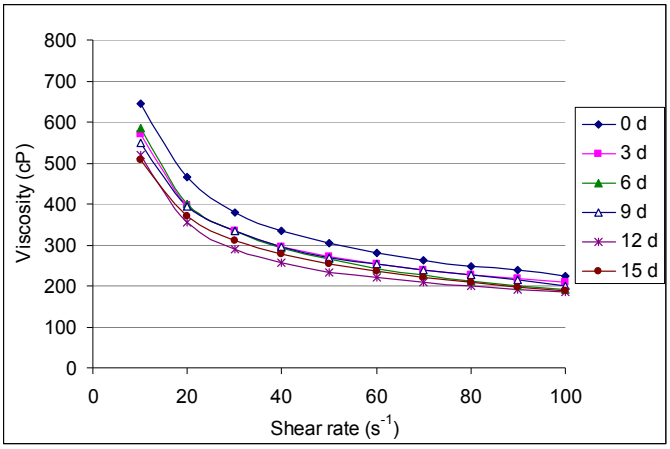


Figure 4.77 Changes in viscosity *vs.* shear rate during storage of barely-milk based fermented probiotic drink packed in PET bottles and stored at 5±1°C

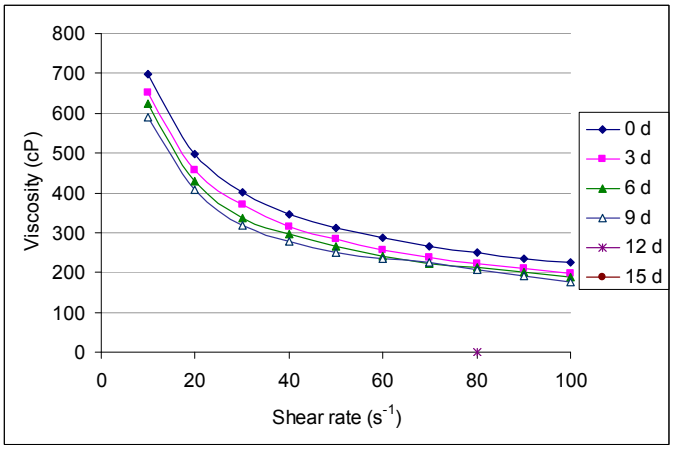


Figure 4.78 Changes in viscosity *vs.* shear rate during storage of barely-milk based fermented probiotic drink packed in LDPE pouches stored at 5±1°C

Hussain *et al.*, (2014) observed increase in the viscosity of sorghum malt based fermented milk beverage during 0 to 7 days of storage, which started decreasing gradually during further storage up to 42 days storage. Increase in the viscosity of sorghum malt based fermented beverage was attributed to the interaction of pectin with water molecules leading to enhanced structure enforcement (Oliveira *et al.*, 2001; Hussain *et al.*, 2014). Hanif *et al.*, (2012) observed constant gradual decrease in the viscosity of commercial, buffalo milk and cow milk yoghurt samples during storage up to 15 days, whereas, Fetahagic *et al.*, (2004) observed increase in the viscosity from 2779 to 3790 mPas of yoghurt up to 5 days of storage which decreased to 3388 mPas when estimated on 10 days of storage. Slight but non-significant increase in the viscosity of rice; rice and soy; rice and barley; rice and emmer; rice and oat yoghurt like beverages was observed by Coda *et al.*, (2012) during 8 h of fermentation by *L.*

plantarum 6E and M6. However, final viscosities of the beverages were reported to decrease significantly after 30 days of storage at 4°C. The decrease in viscosity could be attributed to the hydrolysis of starch by the microorganisms during storage. Breakdown of starch into mono, di or oligosaccharide leads to decrease in the water holding capacity of starch (Cronk *et al.* 1977), thus supports in decreasing the viscosity of beverage.

4.12.6 Colour and appearance

The "first taste is almost always with the eye" (Imram, 1999). The colour and appearance of a food can have the effect of appetite stimulation or depression, which may result in joy or depression (Hutchings, 1994). Visual cues, such as a food colour may modify the perception of a food flavour by influencing the gustatory and olfactory attributes (Spence *et. al*., 2010). The initial average scores for colour and appearance of barley-milk based fermented probiotic drink packed in PET bottles and LDPE pouches decreased from 8.10 to 7.80 and 8.05 to 7.80, respectively on 15th days of storage at 5±1°C (**Table 4.40**). Data obtained for changes in colour and appearance were in accordance with the findings of Modha and Pal (2011), who reported consistent decrease in colour and appearance score with the increase in storage period. However, observed differences in colour and appearance scores during storagee and due to difference in packages were non-significant ($\rho>0.05$). Decrease in the colour and appearance scores may be due to decrease in the viscosity and whey separation during storage.

4.12.7 Sedimentation

Sedimentation shows the amount of settled particles in a beverage. Sedimentation score for barley-milk based fermented probiotic drink exhibited decreasing trend with increasing storage period (**Table 4.40**). The initial score of 7.90 was decreased to 7.40 in PET bottles and 7.50 in LDPE pouches on 15th day of storage. Intervals of storage had significant ($\rho<0.05$) influence on the sedimentation score for the samples packed in PET bottles (**Table 4.41**), whereas the effect of storage intervals for samples packed in LDPE pouches were non-significant ($\rho>0.05$), which could be due to differences in see through properties of packaging material. Towler (1984) reported that pectin is effective in preventing sedimentation in the fermented beverage base, which was added at the rate of 0.3, which inhibited of whey separation during storage.

Table 4.40 Changes in sensory quality of barley-milk based fermented probiotic drink during storage

Storage intervals	Colour & appearance		Sedimentation		Consistency		Flavour		Overall acceptability	
	PET	LDPE	PET	LDPE	PET	LDPE	PET	LDPE	PET	LDPE
0	8.10±0.10	8.05±0.05	7.90±0.10^{a}	7.90±0.18	7.73±0.20	7.93±0.16	7.57±0.20^{a}	7.67±0.17^{a}	7.62±0.19^{a}	7.77±0.15^{a}
3	8.15±0.15	8.10±0.10	7.50±0.13^{b}	7.85±0.18	7.90±0.12	7.90±0.19	7.75±0.20^{a}	7.60±0.21^{a}	7.70±0.26^{a}	7.70±0.25^{a}
6	8.10±0.18	8.10±0.10	7.45±0.19^{b}	7.65±0.15	7.90±0.12	7.90±0.10	7.40±0.31^{a}	7.50±0.24^{a}	7.50±0.27^{a}	7.60±0.22^{a}
9	7.90±0.10	7.85±0.11	7.75±0.11^{ab}	7.60±0.18	7.45±0.23	7.50±0.24	7.45±0.20^{a}	7.30±0.33^{ab}	7.50±0.13^{a}	7.25±0.28^{a}
12	7.85±0.11	7.85±0.18	7.35±0.11^{b}	7.60±0.16	7.50±0.17	7.40±0.24	7.00±0.20^{a}	6.65±0.21^{bc}	6.95±0.26^{ab}	6.95±0.26^{ab}
15	7.80±0.13	7.80±0.13	7.40±0.15^{b}	7.50±0.15	7.50±0.21	7.65±0.26	6.25±0.34^{b}	6.25±0.40^{c}	6.25±0.34^{b}	6.35±0.38^{b}

Means with different superscripts in same column differ significantly (ρ<0.05, DMRT); Values in each cell represent mean ± SE, n=5

Table 4.41 ANOVA for sensory attributes of barley-milk based fermented probiotic drink during storage

Source of variation	df	Mean sum of square				
		Colour and appearance	Sedimentation	Consistency	Flavour	Overall acceptability
				PET		
Storage intervals	5	0.227	0.474*	0.431	2.953**	3.109**
Error	54	0.173	0.179	0.329	0.619	0.632
				LDPE		
Storage intervals	5	0.194	0.247	0.529	3.354**	2.986**
Error	54	0.142	0.282	0.388	0.734	0.712
				PET *vs.* LDPE		
Type of package	1	0.019	0.469	0.075	0.169	0.008
Error	118	0.162	0.242	0.369	0.886	0.873

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

4.12.8 Consistency

Consistency explains about the physical nature of the substance with respect to thick or thin, smooth or coarse. As storage period progressed, a consistent decrease in the consistency score of barley-milk based fermented probiotic drink was noticed in both samples (**Table 4.40**). The initial consistency score on 0 day for samples packed in PET bottles and LDPE pouches decreased from 7.73 to 7.50 and 7.93 to 7.65, respectively on 15th day of storage. Modha and Pal, (2011) also observed slight but non-significant decrease in the consistency score of rabadi like fermented milk beverage during storage for 7 days. Similar findings has also been reported by Abdul, (2008) for sorghum malt based fermented milk beverage during storage. However, changes in consistency score during storage was non-significnant ($\rho>0.05$).

4.12.9 Flavour

Flavour is the sensory impression of a food product, and is determined mainly by the chemical senses of taste and smell. It is a complex combination of the olfactory, gustatory, and trigeminal sensations perceived during tasting (ISO, 1992). Flavour is most important attribute for measuring the quality of a product, which determines its acceptability. Flavour score for barley-milk based fermented probiotic drink decreased from 7.57 to 6.25 and 7.67 to 6.25 when packed in PET bottles and LDPE pouches (**Table 4.40**), respectively and stored for 15 days at 5±1°C. Differences in flavour score observed up to 12th day of storage was non-significant ($p>0.05$) for barley-milk based probiotic drink packed in PET bottles, whereas, probiotic drink packed in LDPE pouches indicated non-significant difference only up to 9th day of storage. Flavour of the product was significantly affected due to increase in acidity of beverage during storage period. Results obtained for flavour score of barley-milk based fermented probiotic drink during storage was in accordance with the findings of Modha and Pal (2011), who reported consistent decrease in flavour score with developing acidity during storage. Gradual decrease in the flavour score of sorghum malt based fermented beverage during storage has also been reported by Hussain *et al.*, (2014). Clover honey aroma, buttermilk aroma, butter aroma, sweetness, sourness, chalky mouthfeel and viscosity were reported as significant attributes in the prebiotics fortified yoghurt drinks (Allgeyer *et al*., 2010).

4.12.10 Overall acceptability

Overall acceptability is the indicative parameter of sensory quality of a product in totality. The initial overall acceptability score for barley-milk based fermented probiotic drink packed in PET bottles and LDPE pouches decreased from 7.62 to 6.25 and 7.77 to 6.35, respectively on 15th day of storage at 5 ±1°C. The barley-milk based fermented probiotic drink packed in PET bottles and LDPE pouches were acceptable with sensory scores of 7.50 and 7.25, respectively on 9th day of storage. The scores for overall acceptability of probiotic drink went down below 7.00 on 12th day of storage (**Table 4.40**). Significant differences ($p<0.05$) were observed in overall acceptability scores during storage which could be attributed to whey separation, decrease in viscosity and increase in the acidity of the beverage during storage.

ANOVA of the data indicated non-significant ($p<0.05$) influence of packaging material on overall acceptability score of probiotic drink (**Table 4.41**). Decrease in overall acceptability of sorghum malt and pearl millet -milk based fermented beverage have been reported by Abdul *et al.*, (2014) and Modha and Pal (2011), respectively. Overall acceptability score for pearl millet based rabadi like beverage was reported to decrease from 7.2 to 7.0 on the 5th day of storage (Modha and Pal, 2011). Sorghum malt based fermented milk beverage containing potassium sorbate as a preservative, and thermized samples were reported to be acceptable up to 14 days as compared 7 days acceptability of control samples (Hussain, 2008). Probiotic dahi containing *L. acidophilus* NCDC14, *L. casei* NCDC19 and *L. lactis* ssp. *lactis* biovar *diacetylactics* NCDC60 was reported to be acceptable up to 8 days of storage at 7°C.

4.12.11 Probiotic count

Table 4.42 shows the viability of *L. plantarum* NCDC344 during storage of barley-milk based fermented probiotic drink at 5±1°C. The results obtained for probiotic microflora showed that counts decreased during refrigeration storage. The initial count of 8.59±0.07 and 8.57±0.07 log cfu/ml in PET bottle and LDPE pouches, respectively for *L. plantarum* NCDC344 in barley-milk based fermented probiotic drink decreased to 7.24±0.08 and 7.23±0.07 log cfu/ml on 15th day of storage at 5±1°C.

Table 4.42 Changes in microbial quality of barley-milk based fermented probiotic drink during storage

Storage intervals	Probiotic count (log cfu/ml)		Non-LAB count (log cfu/ml)		Yeast and mould count (log cfu/ml)	
	PET	LDPE	PET	LDPE	PET	LDPE
0	8.59±0.07^{a}	8.57±0.07^{a}	1.27±0.08^{d}	1.46±0.08^{c}	0.00±0.00^{a}	1.22±0.09^{e}
3	8.52±0.07^{a}	8.47±0.05^{a}	1.50±0.07^{c}	1.68±0.08^{c}	1.06±0.06^{b}	1.57±0.06^{d}
6	8.18±0.03^{b}	8.14±0.05^{b}	1.62±0.05^{b}	2.06±0.14^{c}	1.22±0.00^{c}	1.95±0.09^{c}
9	7.83±0.08^{c}	7.75±0.09^{c}	1.69±0.03^{b}	2.24±0.03^{b}	1.39±0.03^{d}	2.10±0.06^{b}
12	7.46±0.08^{d}	7.48±0.07^{d}	1.71±0.06^{b}	2.31±0.02^{ab}	1.57±0.06^{e}	2.32±0.01^{a}
15	7.24±0.08^{e}	7.23±0.05^{e}	1.92±0.05^{a}	2.41±0.07^{a}	1.81±0.06^{f}	2.38±0.06a

Means with different superscripts in same column differ significantly (ρ<0.05, DMRT); Values in each cell represent mean ± SE, n=3

Table 4.43 ANOVA for microbiological characteristics of barley-milk based fermented probiotic drink during storage

Source of variation	df	Mean sum of square		
		Probiotic count	Non-LAB count	Yeast and mould count
			PET	
Storage intervals	5	0.933**	0.142**	1.200**
Error	12	0.005	0.004	0.008
			LDPE	
Storage intervals	5	0.881**	0.433**	0.614**
Error	12	0.004	0.006	0.007
			PET *vs.* LDPE	
Type of package	1	0.008	1.487**	5.060**
Error	34	0.270	0.088	0.271

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

At the end of storage the counts for probiotic counts were higher than the suggested levels of 10^6 cfu/ml. With respect to dairy products, a therapeutic dose of minimum 10^9 cfu/day or consumption of 100 g or ml/day should translate to a food containing at least 10^7 cells per g or ml (Ishibashi and Shimamura, 1993; Jayamanne and Adams, 2006; Raeisi *et al.*, 2013). The minimum effective dose for therapeutic cultures has been reported at daily intake level of 10^6-10^7 viable cfu (Lee and Salminen, 1995; Minelli and Benini, 2008; Ghoddusi and Robinson, 1996; Cruz *et al.*, 2010). Tannock (2003) suggested an optimal dose of 10^9 cfu/day, based on the appearance of the probiotic organism in the faeces of the majority of human subjects.

On the 9th day of storage, when overall acceptability score was more than 7.00, *i.e.* 7.50±0.13 in PET bottles and 7.25±0.28 in LDPE pouches, the counts for *L. plantarum* NCDC344 were 7.83±0.08 and 7.75±0.09 log cfu/ml, respectively. Considering a minimum consumption of 200 ml of barley-milk based fermented probiotic drink at a time once in a day will deliver around 1.12 to 1.35 x 10^{10} cfu/day. However, consumption of 100 ml of same will deliver 5.62 to 6.76 x 10^9 cfu/day. Significant ($\rho<0.01$) decrease in the viability of probiotic cells have been observed during storage of barley-milk based fermented probiotic drink at 5±1°C (**Table 4.43**). Gupta *et al.*, (2010) optimized an oat based functional beverage based on lactic acid fermentation of oats and stored at 4°C up to 25 days. The initial viability of 10.4 log cfu/ml for *L. plantarum* in oat based functional beverage was reported to decrease by 0.9 log cfu/ml during the storage period. In our case, decrease of 1.35 log cfu/ml was observed during 15 days storage of barley-milk based fermented probiotic drink. However, on 9th day of storage, a reduction of 0.76 log cfu/ml and 0.82 log cfu/ml in PET bottles and LDPE pouches, respectively was observed for the viability of *L. plantarum* NCDC344. Factors such as permeability of the package, oxygen level in the products during refrigerated storage, and sensitivity to substances produced by the bacteria can cause loss for the viability of probiotic microorganisms in dairy products (Ojansivu *et al*., 2011). Yadav *et al.*, (2007), who prepared probiotic dahi containing *L. acidophilus* NCDC14, *L. casei* NCDC19 and *L. lactis* ssp. *lactis* biovar *diacetylactics* NCDC60 reported a decrease of 0.4 log cfu/ml in the lactobacilli during storage up to 8 days at 7°C. A decrease of 2 to 3 log cycles decrease in the survival of probiotics has been reported during 30 days storage of prebiotics fortified yoghurt drinks (Allgeyer *et al*., 2010). Though the viable counts of

probiotic bacteria remained significantly higher than suggested range throughout storage period but as the sensory acceptibilty decreased below 7 on the 12th day of storage, we suggested our product to be consumed up to 9th day of storage.

4.12.12 Non-lactic acid bacteria count

Non-LAB count in ready to eat foods indicates contaminating microflora which are infective microorganisms, *i.e*. those organisms which are not directly involved in microbiological production of those food products, or which do not belong to its specific microflora (International Dairy Federation, 1991). Angelidis *et al.*, (2006) suggested that, provision of standards besides for pathogens in ready to eat foods *i.e*. aerobic plate counts (HPD 9/1989, 1989) would be a meaningless representation in fermented dairy and meat products because of their natural-LAB flora. Contaminating microflora in such fermented products can be selectively enumerated by using peptone based sugar free medium (IDF, 1991). Non-LAB in barley-milk based fermented probiotic drink was estimated by plating on count agar sugar free medium (Angelidis *et al.*, 2006). Counts for contaminating microflora were found increasing during storage period (**Table 4.42**). Storage intervals and type of package significantly ($\rho<0.01$) affected non-LAB count in barley-milk based fermented probiotic drink (**Table 4.43**). The initial count of 1.27 and 1.46 log cfu/ml in PET bottles and LDPE pouches increased to 1.92 and 2.41 log cfu/ml, respectively, indicating better microbial quality in PET bottles. The magnitude of contaminating microflora depends on microbiological quality of starting materials, good manufacturing practices applied during manucature and hygienic conditions of cold storage. Non-LAB count in various varieties of cheeses was reported in the range of 4.85 to 6.55 log cfu/g (Angelidis *et al.*, 2006).

4.12.13 Yeast and mould count

Data pertaining to yeast and mould count in barley-milk based fermented probiotic drink revealed that initial yeast and mould counts of 0.00 log cfu/ml and 1.22 log cfu/ml for the product packed in PET bottles and LDPE pouches, respectively, were increased to 1.81 and 2.38 log cfu/ml on 15th day of storage at 5±1°C. On 9th day of storage, when overall acceptability of probiotic drink was more than 7.0, yeast and mould counts for the drink packed in PET bottles and LDPE pouches were 1.39 and 2.10 log cfu/ml, respectively. However, coliform counts were absent in barley-milk based fermented probiotic drink throughout the

storage period. George *et al.*, (2010) studied physico-chemical, microbial and sensory quality of sweetener / sweetener blends in lassi during storage and observed that yeast and mould count for different sweetener blends on 0^{th} day varied in between 1.03 to 1.49 log cfu/ml. The counts for yeast and moulds were reported to increase during 15 days of storage at 6-8°C, with final count in the range of 2.78 to 3.04 log cfu/ml. Hussain *et al.*, (2014) observed the growth of yeast and mould in sorghum malt based salted lassi individually containing potassium sorbate, Nisin, MicroGARD and reported a lower rate of increase in samples containing potassium sorbate compared to others additives. Observations for yeast and moulds in barley-milk based fermented probiotic drink were closer to the findings of Hussain (2008) who reported yeast and mould count in the range of 1.08 to 2.49 log cfu/ml in samples individually containing potassium sorbate, nisin and MicroGARD during 42 days of storage at 5-7°C.

4.13 Techno economic feasibility for barley-milk based fermented probiotic drink

The marketing success of a new product exists on the techno-economic feasibility of manufacturing process. After optimizing the process of production for barley-milk based fermented probiotic drink, total cost of production was estimated by method discussed in **3.2.13**. The requirement in terms of building and equipment and their approximate cost at current rates are presented in the form of tables in **Appendix I.** Utilities consumption rate and their charges; raw materials; manpower cost for barley-milk based fermented probiotic drink based are presented in **Appendix II**. Annual depreciation on capital investment was worked out at the rate of 5% for civil construction and 7.5% for plant and machinery. Annual income from ghee obtained from excess of fat is given in **Annexure III**. The detailed break-up for the total cost of production of barely-milk based fermented probiotic drink has been shown in **Annexure IV***.* The total cost per 200 ml of barley-milk based fermented probiotic drink in PET was estimated to be 12.09 ₹.

4.14 Consumer's acceptability of barley-milk based fermented probiotic drink

Assessment of consumer responses was done by using method as described in sub-section **3.2.14** by presenting the questionnaire given in Appendix XIV. The study was conducted near milk parlour of National Dairy

Research Institute, Karnal, India. A total of 100 respondents representing potential consumers belonging to mixed age group and sex as were provided with freshly prepared barley-milk based fermented probiotic drink along with a questionnaire for collecting data regarding their personal information (age, sex and location or residence) and perception about the developed probiotic drink. Out of 100, 58 respondents were male and 42 respondents were female. Most of the respondents (74%) belonged to urban area of residence, whereas, 16% belonged from sub-urban and 10 % from rural area. Distribution of respondents on the basis of their age and location of residence has been depicted in **Figure 4.79.**

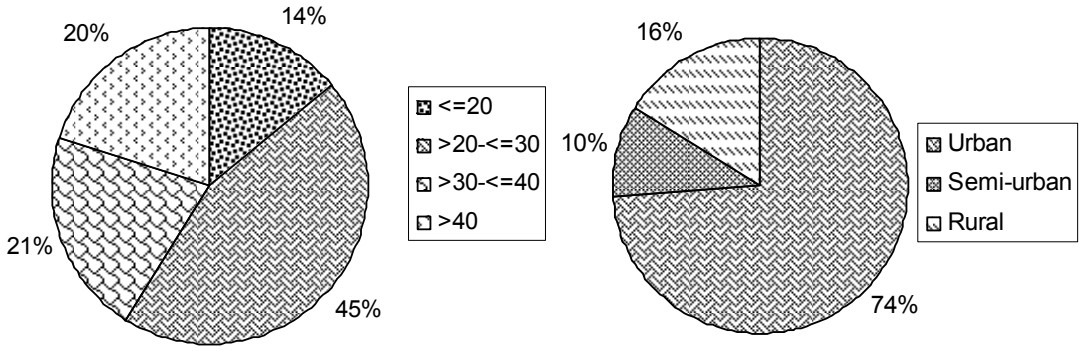


Figure 4.79 Distribution among the respondents based on their age and location of residence

Responses for perception of sensory quality in terms of colour and appearance, taste, saltiness and overall liking were obtained from the consumers by using 9-point hedonic scale and summarized in **Figure 4.80**.

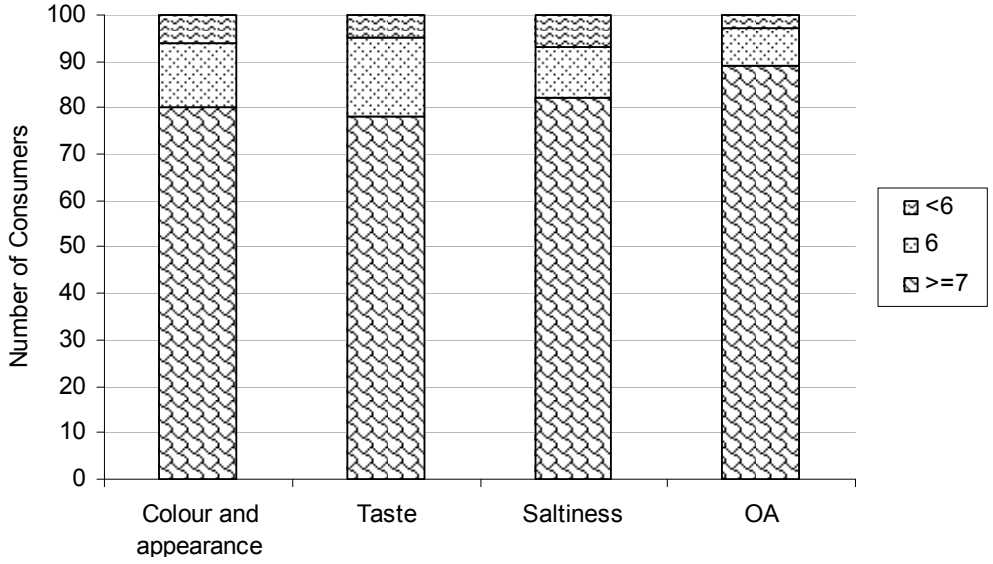


Figure 4.80 Acceptability of developed barley-milk based fermented probiotic drink among the respondents

Among 100 consumers, 89 rated overall acceptability score of barley-milk based fermented probiotic drink more than or equals to 7, which was nearer to 'liked extremely' region of hedonic scale. Average scores of 100 respondents for colour and appearance, taste, saltiness and overall liking were 7.41, 7.49, 7.46 and 7.76, respectively on 9-point hedonic scale.

Figure 4.81 Acceptability of developed barley-milk based fermented probiotic drink among the male respondents

Figure 4.82 Acceptability of developed barley-milk based fermented probiotic drink among the female respondents

When compared for overall liking of barley-milk based fermented probiotic drink in man *vs.* female, 90 % of male respondents and 88 % of female respondents rated the drink with score of '>=7'. The mean score for overall liking of bajra lassi, a pearl millet-milk based fermented beverage was reported to be 7.55 among urban consumers and 6.97 among the rural consumers, representing its more acceptability in urban consumers (Jadoun *et al.*, 2011).

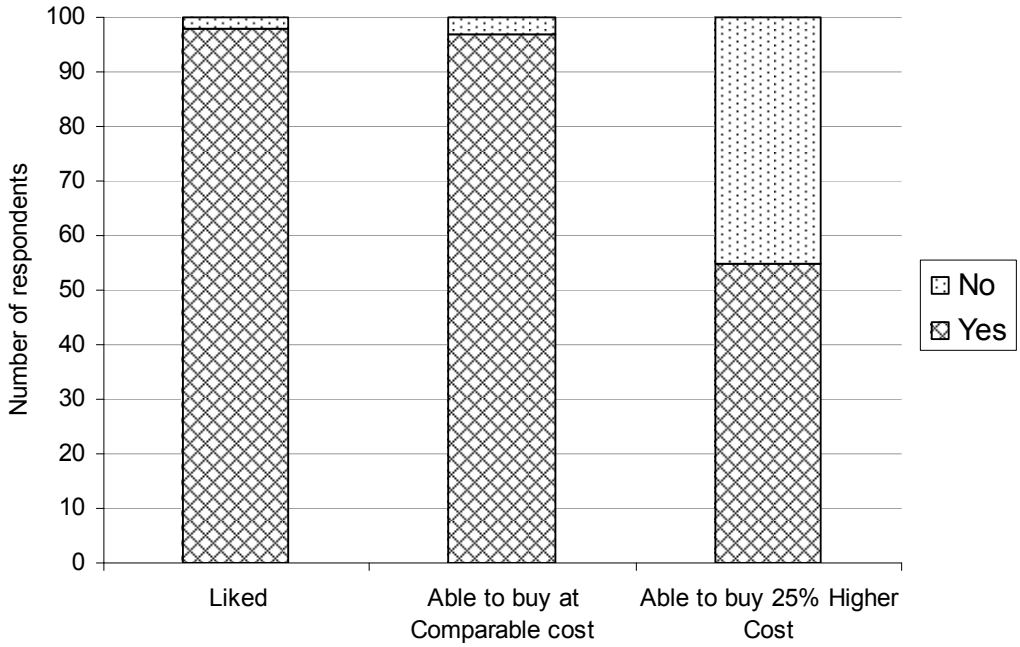


Figure 4.83 Desirability among the respondents to purchase barley-milk based fermented probiotic drink

However, 98% of total respondents liked barley-milk based fermented probiotic drink, and 97% respondents showed their willingness to purchase the product at comparable cost of conventional lassi. Among 100 consumers, 55 indicated their willingness to purchase product at 25% higher cost. Around 98% of consumers were agreed or strongly agreed that fermented milk and milk products are nutritious and should be included in daily diets. A total of 94 among the100 also agreed or strongly agreed about the therapeutic and micro-nutrient of barley and oat cereal grains. Majority (58%) of consumers were daily consumer of fermented milk product, whereas 30 % of total respondents indicated lower frequency (once or twice in a week) for consumption of fermented milk based products. Only 13%, 16% and 10% were daily, weekly and fortnightly consumer of rabadi, a pearl millet milk based fermented milk beverage, respectively. These findings reflect the acceptability and willingness of consumers to purchase barley-milk based fermented probiotic drink.

CHAPTER 5

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSIONS

5.1 SUMMARY

The salient findings of the present investigation encompassing the development of barley-milk based fermented probiotic drink are summarized in this section.

1. Barley-milk based fermented probiotic drink was developed using fermented mass of barley-milk composite.
2. A suitable probiotic lactic acid bacteria (LAB) was selected amongst five randomly selected strains of probiotics, namely *L. acidophilus* NCDC13, *L. casei* NCDC297, *L. casei* NCDC299, *L. plantarum* NCDC344 and *L. rhamnosus* RSI3 for the fermentation of barley-milk composite.
3. The suitable probiotic LAB was selected on the basis of anti-nutrient reduction potential, compatibility with co-culture and sensory acceptability of barley-milk based fermented probiotic curd or drink. Anti-nutrients like phytates and tannins are known to decrease absorption of minerals and decrease the biological value of proteins, respectively.
4. Toned cow milk was selected among skimmed cow milk, double toned cow milk and toned cow milk based on the acceptability of barley-milk based fermented probiotic curd and drink.
5. Three different types of flour were obtained from non-germinated, germinated and imbibed barley grains. Effect of type of flour was studied on the probiotic count and sensory attributes of barley-milk composite based probiotic drink.
6. Flour samples were analysed for compositional, nutritional (β-glucan, total dietary fibre, phytic acid and free amino acid) and functional attributes (total phenols and anti-oxidant activities). Germination and imbibition of grains were found to increase the DPPH and ABTS radical scavenging activity of flour samples; decrease β-glucan content and decrease the anti-nutritional factors present in barley grains, however, slight increase in the total dietary fibre content was observed.
7. Flour from imbibed barley grain was selected on the basis its positive influence on the growth of *L. plantarum* NCDC344 and exhibiting acceptable sensory attributes of barley-milk based fermented probiotic drink.

8. Effect of fermentation was studied on the rheological properties of barley-milk composite, and rheological models such as *Ostwald-de-waele*, *Herschel-Bulkley* and *Casson* model were fitted on shear rate–shear stress data. *Herschel-Bulkley* model gave highest co-efficient of determination values. Thus, can be used to predict yield stress.
7. Concentration of barley flour, probiotic inoculum and co-culture inoculum were optimized using central composite rotatable design of response surface methodology. Probiotic count, overall acceptability and β-glucan content were used as critical responses during optimization.
8. Effect of barley flour concentration, probiotic inoculum level and co-culture inoculum level on various sensory attributes, probiotic count, β-glucan content, viscosity, textural attributes and on the parameters of different rheological models was analysed.
9. Optimized levels of barley flour, probiotic inoculum and co-culture inoculum were 5.24%, 5.00% and 0.34%, respectively. Desirability for selected formulation was 0.638. Verification of the results for optimized formulation was accomplished by the preparation of barley-milk based fermented probiotic drink and comparing the experimental results with predicted ones.
10. Barley-milk based fermented probiotic drink prepared using the optimized levels of barley flour, probiotic inoculum level and co-culture inoculum level gave an overall acceptability score of 7.80; probiotic count: 8.59 log cfu/ml and β-glucan content: 0.144 g/100g.
11. Optimized barley-milk based fermented probiotic drink samples were also estimated for its physico-chemical, microbiological, therapeutic and sensory attributes.
12. β-glucan and total dietary fibre content of optimized barley-milk based fermented probiotic drink were 0.14 g/100g and 2.3 g/100 g, respectively. Iron, zinc and calcium content of developed probiotic drink were found to be 6.19, 4.29 and 872 ppm respectively. ACE-inhibitory activity of the developed probiotic drink was less than %.
13. Trolox equivalent DPPH and ABTS radical scavenging activity of barley-milk based fermented probiotic drink were 0.79 and 0.40 mg/ml, respectively.
14. Antimicrobial activity against test pathogens were analysed by measuring the zone of inhibition, which were found to be 22 mm for *E. coli*; 20 mm for

Salmonella typhii; 16 mm for *Shigella dysentriae*; and 12 mm for *Staph. aureus.*

15. Shelf life of barley-milk based fermented probiotic drink was estimated in LDPE pouches and PET bottles at 5±1°C. PET bottles were found as better packaging material in terms of significantly lower count of non-LAB and yeast and moulds as compared to LDPE pouches. Shelf life barley-milk based fermented probiotic drink was found to be 9 days at 5±1°C, with an overall acceptability score of 7.50 and 7.25 in PET bottles and LDPE pouches, respectively. On the 9^{th} day of storage probiotic count in PET bottles and LDPE pouch was 7.83 and 7.75 log cfu/ml, respectively.
16. Estimated cost of production for barley-milk based fermented probiotic drink was 12.08 ₹ for 200 ml in PET bottles.
17. Consumer acceptability of barley-milk based fermented probiotic drink was tested among 100 respondents, out of which 89 consumers rated more than or equals to '7' on a 9-point hedonic scale.

5.2 CONCLUSIONS

Incorporation of barley flour in milk indicated positive influence on the count of probiotic LAB up to a certain level (4 to 5%). Optimized barley-milk based fermented probiotic drink contained 0.144g/100g of β-glucan and 8.59 log cfu/ml of probiotic *L. plantarum* NCDC344. Considering a serving of 200 ml of probiotic drink at a time in a day will provide only 0.28 g of β-glucan and around 10^{10} cells of *L. plantarum* NCDC344. The developed probiotic drink exhibited good antioxidant potential; and high anti-microbial activity against common pathogens such as *E. coli* and S. *typhii*. The developed probiotic drink harness the nutritional and therapeutic virtues of under-utilized plant food *i.e.* barley, milk and probiotic *Lactobacillus plantarum* NCDC344 and can also assist processors to diversify their product profile towards the "functional" dairy beverages. Large scale consumer acceptance studies also indicated readily acceptability of probiotic drink among the masses. However, further exploration related to validation of health benefits of probiotic drinks through *in vitro* or *in vivo* investigations will pave the way for its rapid commercialization. In future projections, developed barley-milk based fermented probiotic drink can be evaluated for its anti-diabetic properties in clinical studies.

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APPENDICES AND ANNEXURES

APPENDIX I

CHEMICALS AND THEIR SOURCES USED IN THE STUDY

Chemical / Media / Kit	Make	Catalog / CAS No.
2-mercaptoethanol	SRL	1327198
3.5-Dinitrosalicylic acid	Merck	80014101001730
ABTS (3-ethlylbenzo-thiazoleine-6-sulfonic acid)	Sigma-Aldrich	A1888-2g
Acetic acid glacial extrapure	SRL	129168
Acetone	SRL	129135
Agar-Agar	Merck	61931205001730
Ammonia solution	SRL	129189
Ammonim sulfate	SRL	7783-20-2
Ammonium Metavandate MW 116.98	Fisher Scientific	CAS 7803-55-6
Ammonium molybdate MW 1235.86	Fisher scientific	CAS 12054-85-2
Ammonium sulphate	SRL	144138
Anaerogas Pack 3.5L	Himedia	LE002A
Angiotensin Converting Enzyme from rabbit lung	Sigma-Aldrich	A6778-1UN
Beakers (tall form without spout)	Borosil	1040021
Beef extract	Himedia	RM-002-500G
Bile salts	Merck	61931805001730
Borax (sodium tetraborate)	SRL	1303-96-4
Borick acid	SRL	249133
Brain Heart Infustion broth	Himedia	M210-100g
Bromocresol purple	Merck	61802500051730
Buffered peptone water	Himedia	M-1275
Calcium chloride dihydrate	SRL	349152
Calcium phytate	Himedia	RM3255-5g
Catechin hydrate	Sigma	C1251-5G
Celite (acid washed)	Sigma	C8656
Ciprofloxacin	Sigma Aldrich	17850-25g-F
Cobalt chloride	SRL	347205
Copper sulfate extrapure AR	SRL	349158
Count agar sugar free	Merck	1.10878.0500
D (-) Tartaric acid pure	SRL	2048171
D/L lactic acid assay kit	Megazyme	K-DLATE
D+ Glucose 016-500ganhydrous	Himedia	RM016-500g
di-potassium hydrogen ortho-phosphate	Qualigens	26735
DPPH (2,2'-diphenyl-1-pikryl-hydrazyl)	Sigma-Aldrich	D913-2
Ethanol	Merck	1.00983.0511
Ethyl acetate	SRL	052947 (141-78-6)

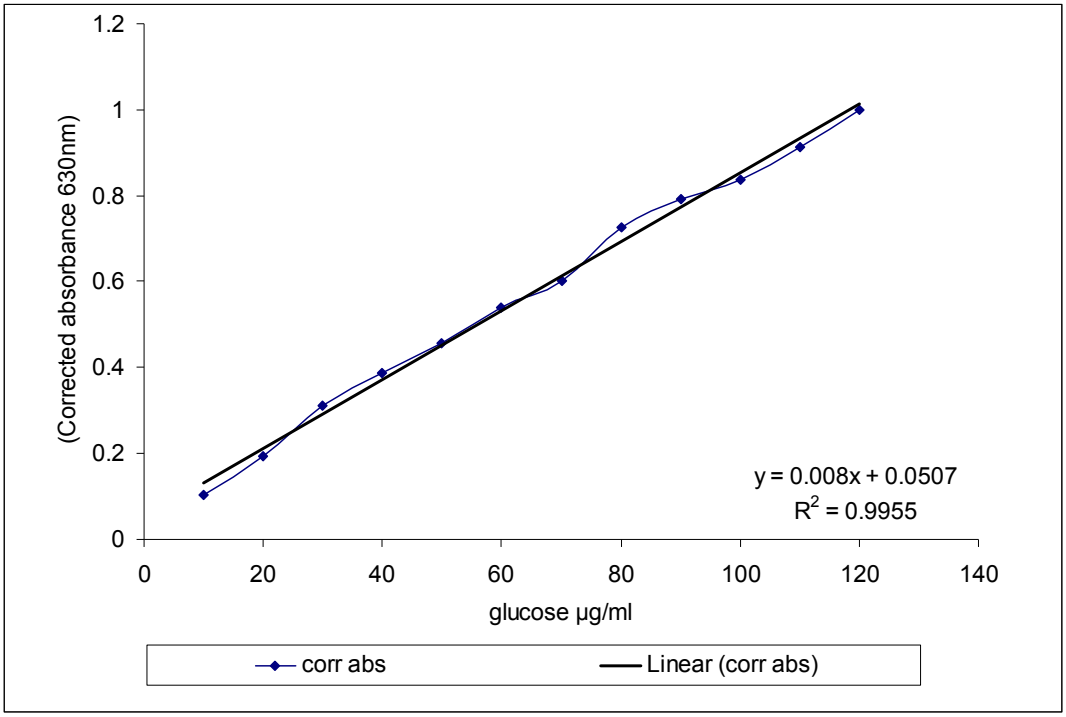
Ferrous sulphate extrapure AR	SRL	64963
Folin & Ciocalteu's phenol reagent	SRL	62015
Formaldehyde	Merck	61780805001730
Gallic Acid	Sigma-Aldrich	G7384-500g
Glacial acetic acid	SRL	12885
Glycerol	SRL	72762
Hippuryl-His-Leu acetate salt	Sigma-Aldrich	H4884-500MG
Hydrochloric acid	Merck	61762505001730
Hydrogen peroxide (50%)	Merck	61765305001730
Iodofor	Merck	61832510001730
Iso propyl alcohol	SRL	92956
Lactobacillus MRS broth	Merck	61942405001730
Lactose	SRL	64044-51-5
L-ascorbic acid	SRL	19100
L-Leucine	Himedia	RM054-25G
M17-Agar Base	Himedia	M929-500g
Magnesium Sulfate heptahydate	Merck	61777005001730
Manganese (II) sulfate monohydtae	Merck	61754805001730
Methanol >= 99.6%	Sigma-aldrich	17,995-7-1L
Methyl Red Indicator	SRL	134953
Methylene Blue	SRL	1340162
Nigrosine stain	Himedia	S025-100ML
Nitric acid	Merck	61762605001730
Nutrient Broth	Himedia	M002-100g
Pectin	Merck	9000-69-5
Petroleum ether 40-60	Thomas Baker	125021-55
Phenolpthalein indicator	SRL	1649188
Phthaldialdehyde	Sigma	P1378-25g
Phytic acid assay kit	Megazyme	K-PHYT
Phytic acid dodecasodium salt hydrate	Sigma	P0109-10g
Potassium per sulfate	SRL	1647204
Potassium permanganate	SRL	7722-64-7
Potassium phosphate dibasic anhydrous pure	SRL	1648212
Potassium standard for AAS	Merck	1.70230.0100
Potassium Sulfate	Sigma-Aldrich	12658-1kg
Potato dextrose agar	Himedia	M096-500g
Proteose Peptone	Himedia	RM-005-500g
Selenium dioxide	SRL	1948285
Sodium acetate trihydrate	SRL	1949141
Sodium azide	Thomas baker	142750

Sodium carbonate anhydrous	SRL	1949157
Sodium dihydrogen orthophosphate dehydrate AR	SD Fine-Chem Ltd	20245K05
Sodium Hydroxide (0.1N)	Merck	60914105001730
Sodium Hydroxide Pellets extrapure AR	SRL	1949181
Sodium Lauryl Sulfate	SRL	1949181
Sodium phosphate dibasic anhydrous	SRL	7558-79-4
Sorbitol	Himedia	RM-109-500G
Sterile disposable petri plates	Himedia	PW001
Sulfuric acid (98%)	Merck	61762705001730
Syringe filter (PVDF 0.45µm, 25mm Φ)	Whatman	6900-2504
Syringe-driven filter (PES 0.22µm 13mm Φ)	Himedia	SF13-1x75No.
Total dietary fibre assay kit	Megazyme	K-TDFR
Total starch assay kit	Megazyme	K-TSTA
Tri-Ammonium citrate	Himedia5	RM-1204-500g
Trichloro-acetic-acid	SRL	204842
Tween 80	Himedia	RM-159-500g
Vancomycin supplement	Himedia	FD261
Vanillin GR	Merck	61911701001830
Violet Red Bile Broth	Himedia	M458-500g
Water for chromatography	Merck	61765010001730
Xylene extrapure AR	SRL	242921
Yeast extract	Himedia	RM668-500G
Zinc standard for AAS	Merck	1.19806.0100
α-Naphtholphthalein FW 418.45	Thomas baker	1632
B-glucan-assay kit	Megazyme	K-BGLU

SRL- Sisco Research Laboratory, Mumbai
Merck Specialities Pvt Limited, Mumbai
Sigma-Aldrich Chemicals Pvt Limited,Bangalore, India
Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India
Megazyme International Ireland Ltd., 2011
SD Fine-Chem Ltd
Thomas Baker, Mumbai
Whatman Schleicher & Schuell, NJ 07932, US

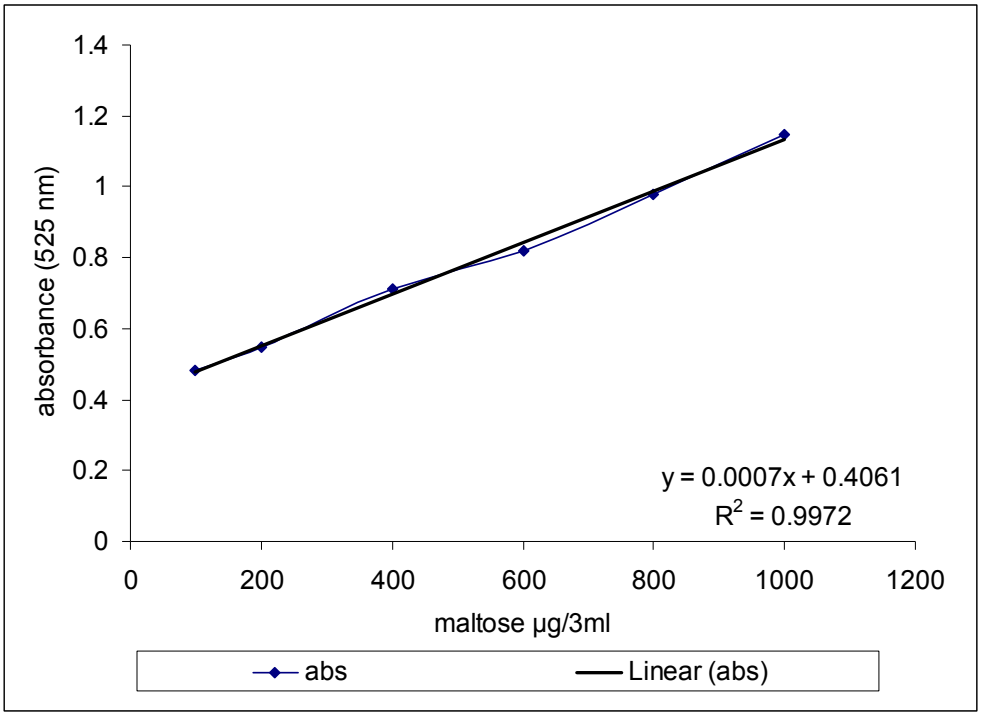
Appendix-II

Standard curve for glucose for the estimation of total carbohydrate



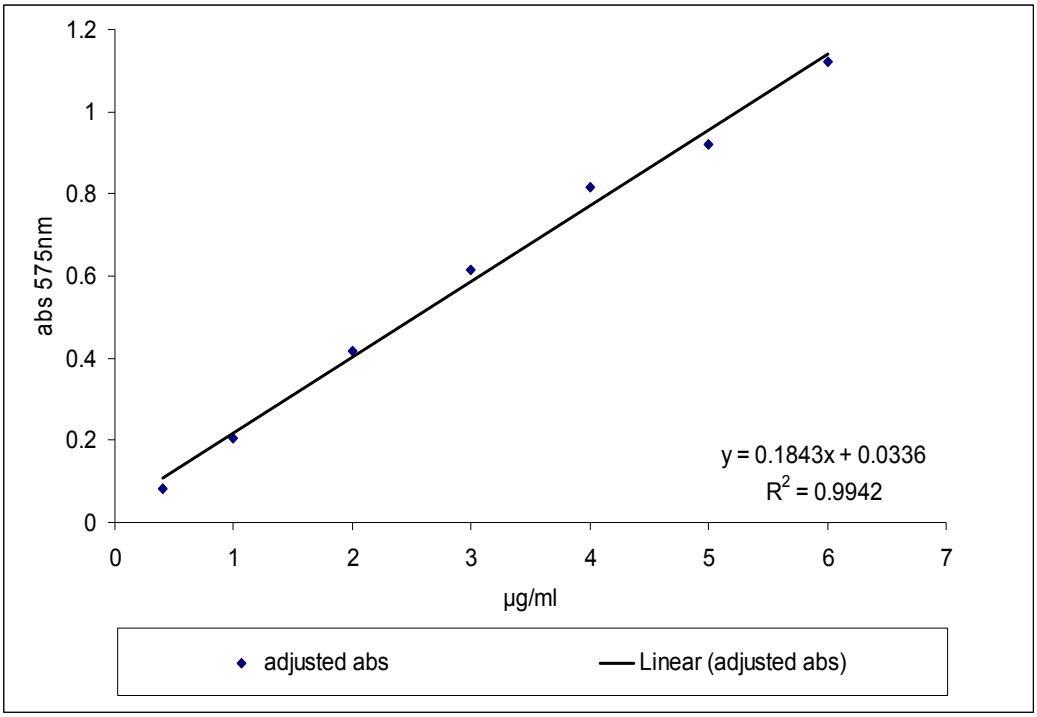
Appendix-III

Standard curve for maltose for the estimation of reducing sugar content



Appendix-IV

Standard curve for glycine for the estimation of free amino nitrogen content



APPENDIX-V

COMPOSITION OF DIFFERENT MICROBIOLOGICAL MEDIA

1. deMan Rogosa Sharpe Medium (deMann *et al.*, 1960)

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Dextrose	20.0 g
Dipotassium hydrogen phosphate	2.0 g
Sodium acetate	5.0 g
Ammonium citrate	2.0 g
Magnesium sulfate	0.1 g
Manganese sulfate	0.05 g
Tween 80	1.0 g
Distilled water	1000 ml

pH 6.5 $\pm$ 0.2

2. M17 Medium (Terzahi and Sandine, 1975)

Tryptone	5.0 g
Peptone	5.0 g
Yeast extract	2.5 g
Beef extract	5.0 g
Lactose	5.0 g
Sodium-β-glycerophosphate	19.0 g
Ascorbic acid	0.5 g
Magnesium sulfate	0.25 g
Distilled water	1000 ml

pH 7.0 $\pm$ 0.1

3. Modified Chalmers broth (Anastasio *et al.*, 2010)

Proteose peptone	5 g
Beef extract	5 g
Yeast extract	5 g
Dextrose	20 g
Lactose	20 g
Distilled water	1000 ml

pH 6.0±0.1

4. MRS-salicin agar (Dave and Shah., 1996)

Peptone	10 g
Beef extract	10 g
Yeast extract	5 g
Salicin*	10 g
Sodium acetate trihydrate	5 g
Tween 80 (polysorbate 80)	1 g
di-Potassium hydrogen phosphate	2 g
tri-ammonium citrate	2 g
Magnesium sulfate heptahydrate	0.2 g
Manganese sulfate tetrahydrate	0.05 g
Agar	15 g
Water	1000 ml

pH 6.5 $\pm$ 0.2

5. MRS-vancomycine agar (Tharmaraj and Shah, 2003)

Peptone	10 g
Beef extract	10 g
Yeast extract	5 g
Dextrose	20 g
Sodium acetate trihydrate	5 g
Tween 80 (polysorbate 80)	1 g
di-Potassium hydrogen phosphate	2 g
tri-ammonium citrate	2 g
Magnesium sulfate heptahydrate	0.2 g
Manganese sulfate tetrahydrate	0.05 g
Agar	15 g
Vancomycin*	1 mg
Water	1000 ml

pH 6.5 $\pm$ 0.2

*added after sterilization of medium below 50°C just before pouring into plates and sterilized by using syringe driven membrane filter sterilization technique

6. ST agar (Dave and Shah, 1996)

Casein enzymic hydrolysate	7.5 g
Yeast extract	7.5 g
Sucrose	5.0 g
Dipotassium phosphate	2.0 g
Agar	15.0 g
Distilled water	1000 ml

pH 6.8 ± 0.1

7. *Lactobacillus plantarum* Selective Medium (LPSM) (Bujalance *et al*., 2006)

Peptone	10 g
Beef extract	10 g
Yeast extract	5 g
Sorbitol*	20 g
Sodium acetate trihydrate	5 g
Tween 80 (polysorbate 80)	1 g
di-Potassium hydrogen phosphate	2 g
tri-ammonium citrate	2 g
Magnesium sulfate heptahydrate	0.2 g
Manganese sulfate tetrahydrate	0.05 g
Agar	15 g
Ciprofloxacin*	4 mg
Water	1000 ml

pH 6.2 $\pm$ 0.1

8. Violet Red Bile Agar (VRBA) (HIMEIDA- M0496)

Peptic digest of animal tissue	7.0 g
Yeast extract	3.0 g
Lactose	10.0 g
Bile salts mixture	1.50 g
Sodium chloride	5.0 g
Neutral red	0.03 g
Crystal violet	0.002 g
Agar	15.00 g
Distilled water	1000 ml

pH 7.4 $\pm$ 0.1

*added after sterilization of medium below 50°C just before pouring into plates and sterilized by using syringe driven membrane filter sterilization technique

9. Potato Dextrose Agar (Himedia- M096)

Potatoes infusion from 200.0 g	4 g
Dextrose	20.0 g
Agar	15.0 g
Distilled water	1000 ml

pH 5.6 ± 0.2

The pH of the medium was adjusted to 3.5 at the time of pouring the plates with sterile 10% tartaric acid.

10. Count agar sugar free (Merck-1.10878)

Peptone from gelatin	7.5 g
peptone from casein	7.5 g
sodium chloride 5.0;	5.0 g
Agar	15.0 g
Distilled water	1000 ml

pH 7.5 ± 0.2

11. Brain Heart Infusion Broth (Himedia-M210)

Proteose peptone	10 g
Calf brain, infusion from 200 g	7.5 g
Beef heart, infusion from 250 g	10 g
Dextrose	2 g
Sodium chloride	5 g
Disodium phosphate	2.5 g

pH 7.4±0.2

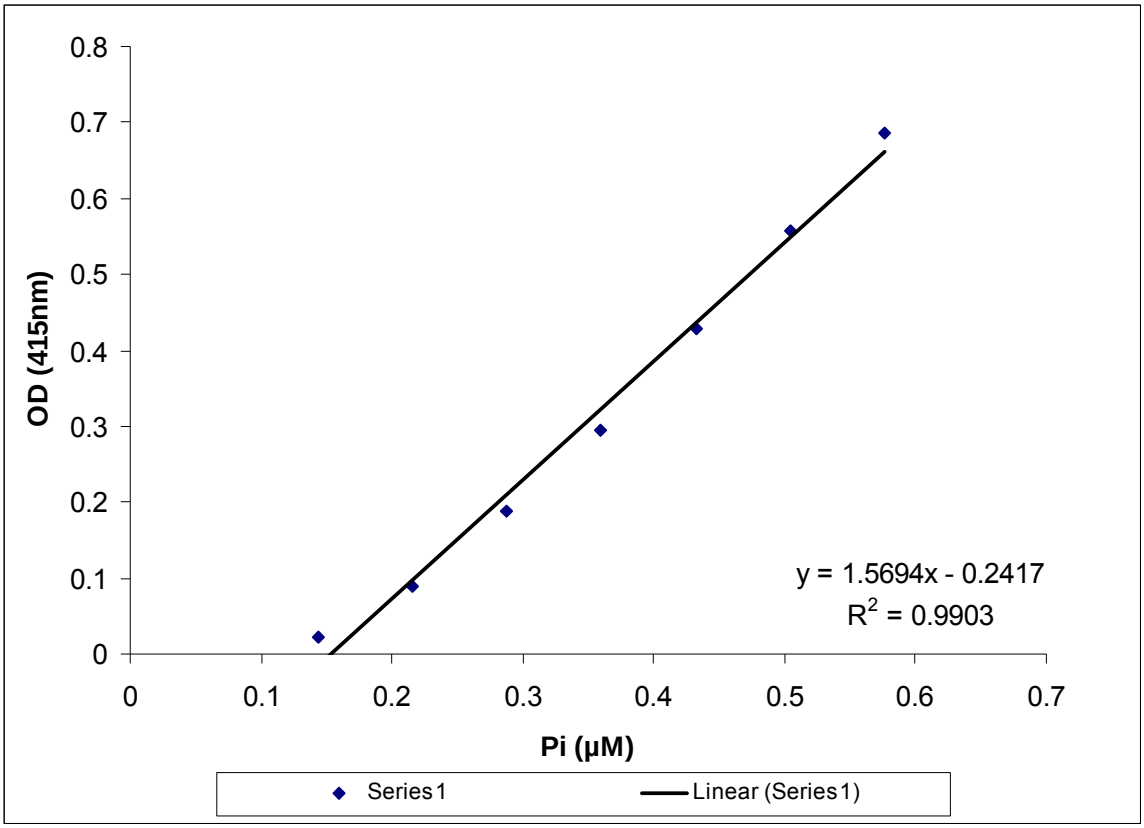
12. Buffered peptone water (Himedia-M614)

Proteose peptone	10 g
Sodium chloride	5 g
Disodium phosphate, anhydrous	3.5 g
Disodium phosphate, anhydrous	1.5 g

pH 7.2±0.2

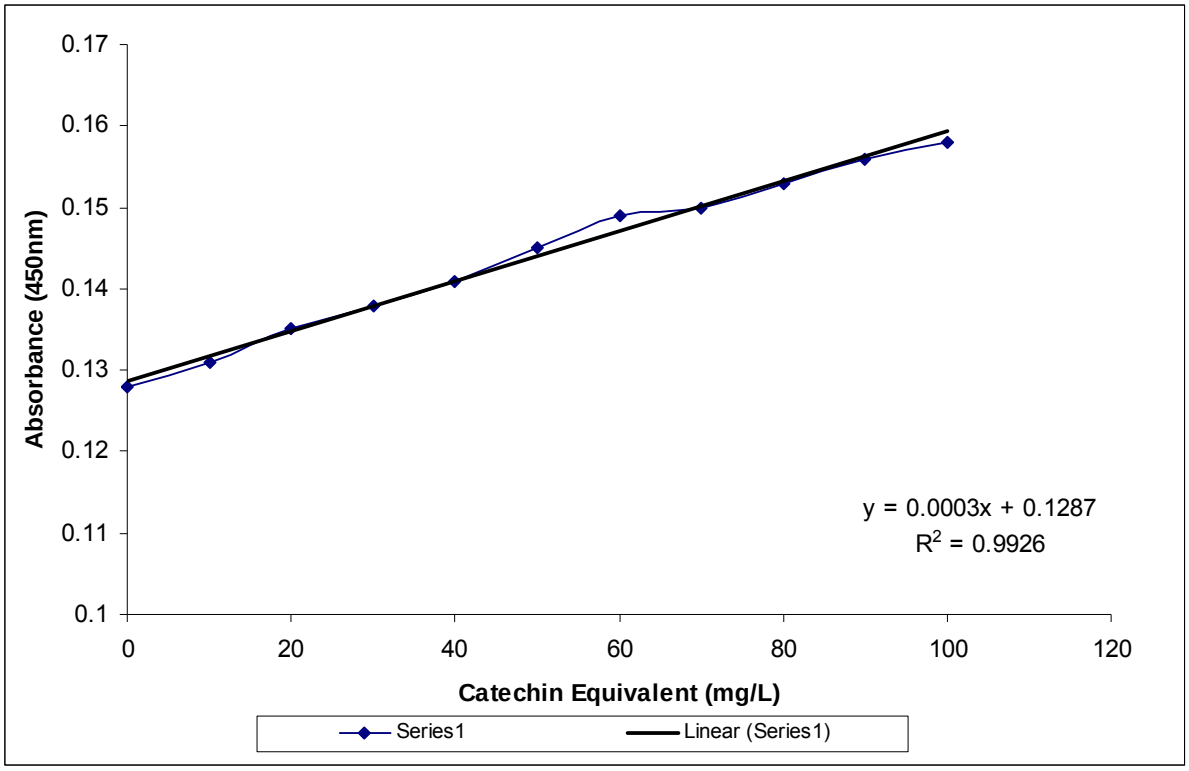
Appendix-VI

Phosphate calibration curve



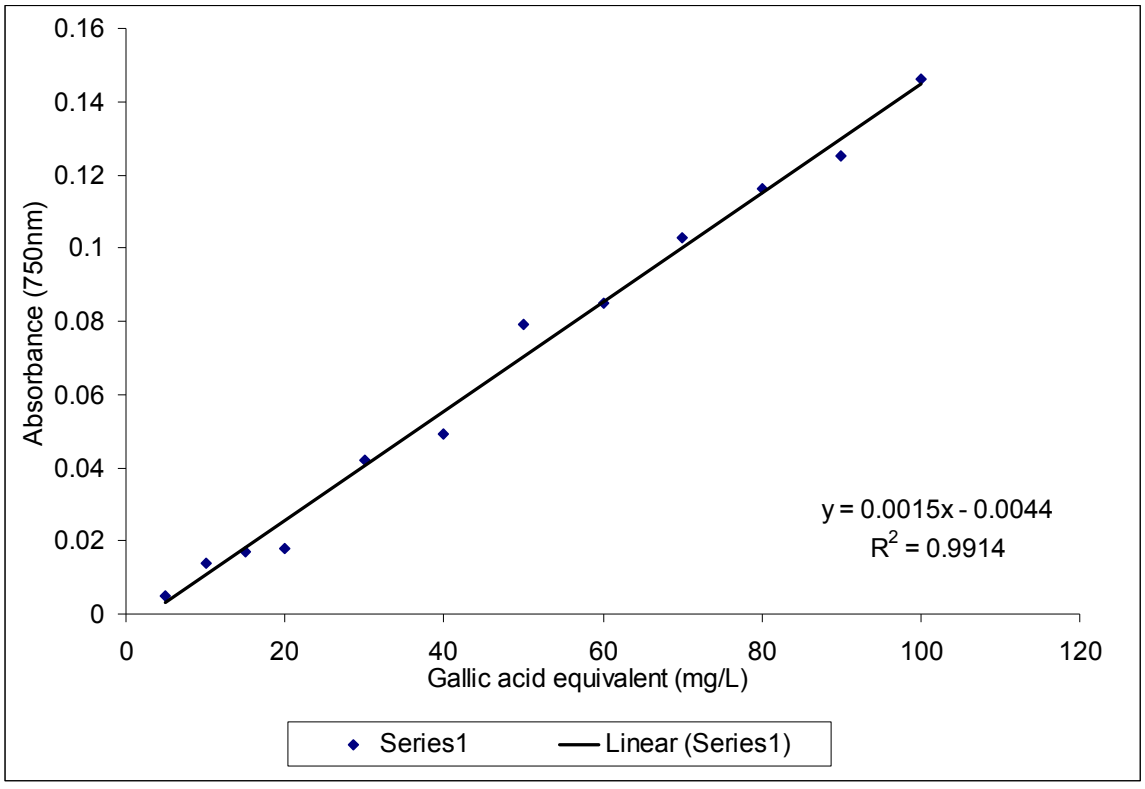
Appendix-VII

Standard curve for catechin equivalent for the estimation of tannin content



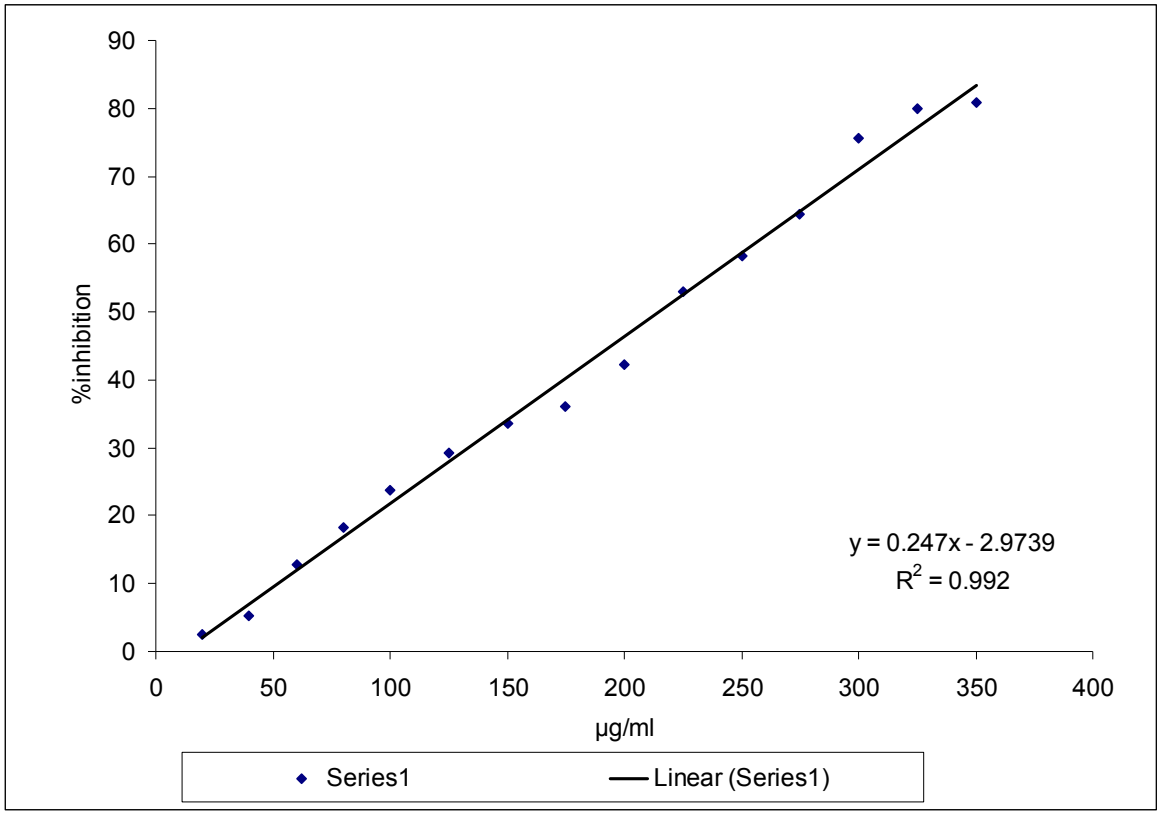
Appendix-VIII

Standard curve for gallic acid equivalent for the estimation of total phenolics content



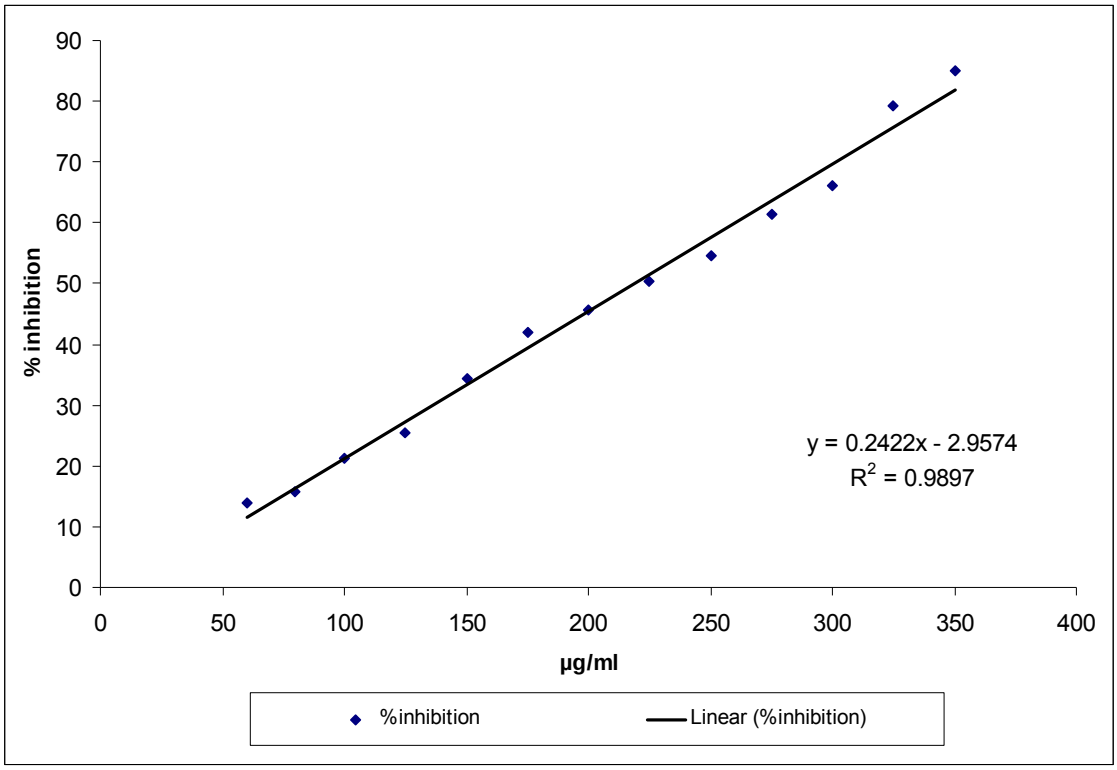
Appendix-IX

Standard curve for trolox equivalent DPPH radical scavenging activity



Appendix-X

Standard curve for trolox equivalent ABTS radical scavenging activity



APPENDIX-XI

Division of Dairy Technology
NATIONAL DAIRY RESEARCH INSTITUTE
Karnal (Haryana)

Score Card for Sensory Evaluation of Barley Milk based Fermented Probiotic Curd

Product Particulars:_____ **Date:**_____

Kindly evaluate the given samples of Barley-Milk based Fermented Probiotic Curd for colour and appearance, body and texture, flavour and overall acceptability using the 9-point Hedonic Scale and enter the score for each sample in the space provided below.

Hedonic Ratings

Score Preference	Code
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Sensory Attributes	Sample Code			
Color and Appearance				
Body and Texture				
Flavour				
Overall acceptability				

Remarks (If any)

Signature:_____

Name: _____

APPENDIX XII

Division of Dairy Technology
NATIONAL DAIRY RESEARCH INSTITUTE
Karnal (Haryana)

Score Card for Sensory Evaluation of Barley Milk based Fermented Probiotic Drink

Product Particulars:_____ **Date:**_____

Kindly evaluate the given samples of Barley Milk based Fermented Probiotic Drink for colour and appearance, sedimentation, consistency, flavour and overall acceptability using the 9-point Hedonic Scale and enter the score for each sample in the space provided below.

Hedonic Ratings

Score Preference	Code
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

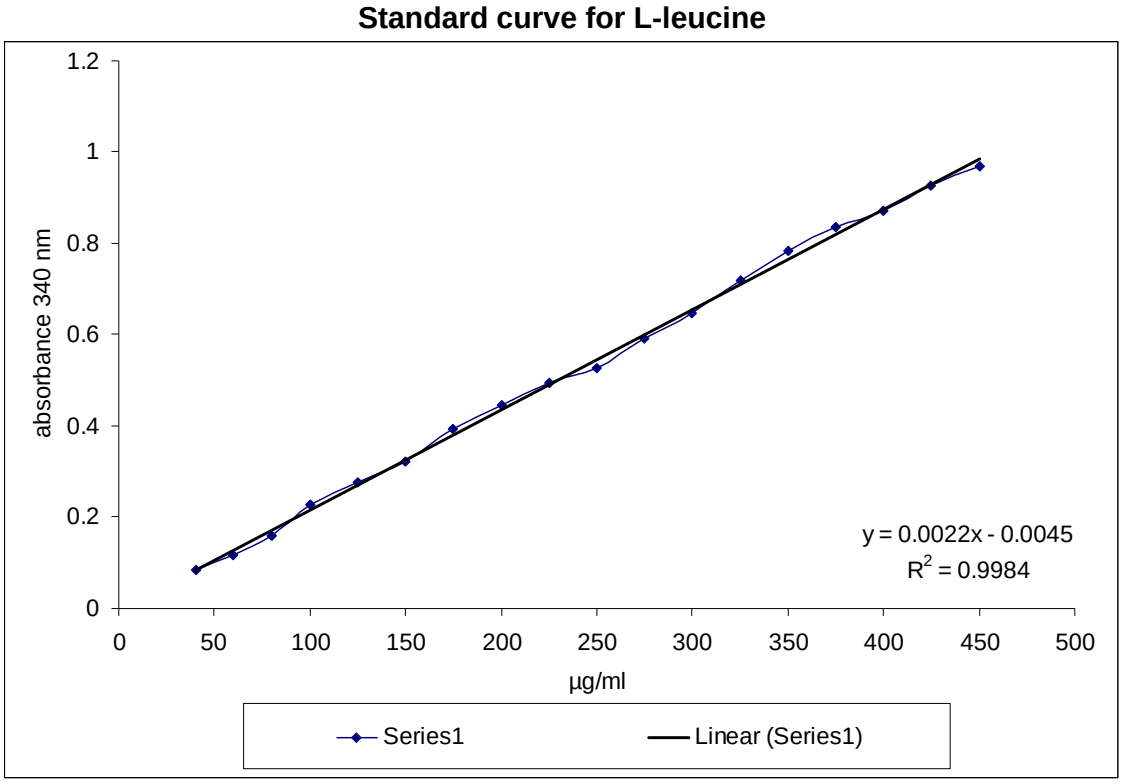
Sensory Attributes	Sample Code			
Color and Appearance				
Sedimentation				
Consistency				
Flavour				
Overall acceptability				

Remarks (If any)

Signature:_____

Name: _____

Appendix- XIII



APPENDIX-XIV

CONSUMER SURVEY OF BARLEY- MILK BASED FERMENTED PROBIOTIC DRINK

Dear Consumer,

We have developed Barley-Milk based Fermented Probiotic Drink, which contains healthful fiber, proteins, micronutrients and probiotic attributes. You are requested to taste it and give your honest opinion about the product. It will help us to decide its acceptability.

Please provide the following information:

1. Name of the consumer _____Age_____Sex (M/F)_____

2. Occupation/Designation_____

3. Location: Urban ☐ Periurban ☐ Rural ☐

4. Awareness regarding Nutritional and therapeutic status of foods:

   a. Fermented milk and milk products are nutritious and should be included in diet.
   ☐ Strongly agree ☐ Agree ☐ Neither agree nor disagree
   ☐ Disagree ☐ Strongly disagree ☐ No idea

   b. Coarse cereals grains such as barley and oat contains various micronutrients and therapeutic components.
   ☐ Strongly agree ☐ Agree ☐ Neither agree nor disagree
   ☐ Disagree ☐ Strongly disagree ☐ No idea

   c. Probiotic foods help in restoration of gastro intestinal micro flora?
   ☐ Strongly agree ☐ Agree ☐ Neither agree nor disagree
   ☐ Disagree ☐ Strongly disagree ☐ No idea

   d. How often you consume fermented milk products?
   Daily ☐ Weekly ☐ Fortnightly ☐ Occasionally ☐ Rarely ☐ Never ☐

   e. Have you heard of “Rabadi” or any other cereal buttermilk based fermented product
   Yes ☐ No ☐

   f. Do you consume cereal or millet based beverage (Rabadi)? If yes then indicate frequency of consumption?
   Daily ☐ Weekly ☐ Fortnightly ☐ Occasionally ☐ Rarely ☐ Never ☐

   g. Do you consume any probiotic food / beverage? If yes then indicate frequency of consumption?
   Daily ☐ Weekly ☐ Fortnightly ☐ Occasionally ☐ Rarely ☐ Never ☐

5. **Please taste the probiotic drink, and circle your response for the parameters below.**

Colour and Appearance								
1	2	3	4	5	6	7	8	9
Dislike Extremely			Neither Like nor dislike				Like Extremely	

Taste

1	2	3	4	5	6	7	8	9
Dislike Extremely			Neither Like nor dislike				Like Extremely	

Saltiness

1	2	3	4	5	6	7	8	9
Dislike Extremely			Neither Like nor dislike				Like Extremely	

Overall Liking

1	2	3	4	5	6	7	8	9
Dislike Extremely			Neither Like nor dislike				Like Extremely	

6. Did you like this Barley-Milk based Fermented Probiotic Drink? Yes ☐ No ☐

7. Would you like to buy Barley-Milk based Probiotic Drink in place of conventional *Lassi*, if it is available at a:

Comparable cost?	Yes	☐	No	☐
25% Higher cost?	Yes	☐	No	☐

8. What factors will influence your choice?

Price:	Most Affected	☐	Least Affected	☐	Not Affected	☐
Flavour:	Most Affected	☐	Least Affected	☐	Not Affected	☐
Health:	Most Affected	☐	Least Affected	☐	Not Affected	☐
Advertising:	Most Affected	☐	Least Affected	☐	Not Affected	☐
Availability:	Most Affected	☐	Least Affected	☐	Not Affected	☐
Colour:	Most Affected	☐	Least Affected	☐	Not Affected	☐
Packaging:	Most Affected	☐	Least Affected	☐	Not Affected	☐

9. Would you like to make any suggestions for further improvement?

_____

_____

Date:

Place: **Signature**

Appendix-XV

Measuring System Data Sheet
Messsystem-Datenblatt
CP75-1

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Geometry Data	Geometrie-Daten		Unit
Serialnumber	Seriennummer	23957	
Cone, Diameter, D	Kegel, Durchmesser, D	74,975	mm
Cone, Angle, α	Kegel, Winkel, α	1,002	°
Cone, Truncation, d	Kegel, Spitzenabnahme, d	149	µm

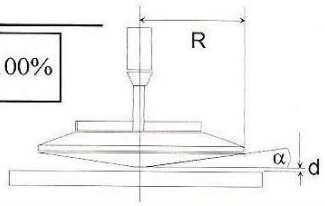
Description	Beschreibung
Cone & Plate Measuring System	Kegel-Platte Messsystem
Basis of Calculation: ISO 3219	Basis der Berechnung: ISO 3219

$$CSD_{SI-Units} = \frac{\gamma}{\varphi} = \frac{1}{\tan(\alpha)}\left[\frac{1}{rad}\right]$$

$$CSR_{SI-Units} = \frac{\dot{\gamma}}{\omega} = \frac{1}{\tan(\alpha)}\left[\frac{1}{rad}\right]$$

$$\frac{°}{rad} = \frac{2\pi}{360} \qquad \frac{\omega}{n} = \frac{2\pi}{60} \qquad 1 = 100\%$$

$$CSS_{SI} = \frac{\tau}{M} = \frac{3}{2\pi \cdot R^3}$$



Variable	Variable	SI-Unit
τ ...shear stress	τ ...Schubspannung	Pa
M...torque	M...Moment	Nm
γ ...strain	γ ...Deformation	1
φ...deflection angle	φ...Auslenkwinkel	rad
$\dot{\gamma}$ ..shear rate	$\dot{\gamma}$ ..Scherrate	s^{-1}
n...speed	n...Geschwindigkeit	s^{-1}
R...cone radius	R...Kegelradius	m
α...cone angle	α...Kegelwinkel	rad
ω...angular velocity	ω...Winkelgeschwindigkeit	rad/s
d...cone truncation	d...Kegelspitzenabnahme	m

Dimensions	Abmessungen		Unit
Cone, Radius	Kegel, Radius	37,488	mm
Cone, Angle	Kegel, Winkel	1,002	°
Cone, Truncation	Kegel, Spitzenabnahme	149	µm
Measuring Position, Gap	Messposition, Messspalt	0,149	mm

Geometry Data	Geometriedaten		Unit
Sample Volume, approximate	Probenmenge, ungefähr	1,94	ml
Active Length	Aktive Länge	100	mm
Positioning Length	Positionierungslänge	100	mm

Conversion Factors	Umrechnungsfaktoren		Unit
Conversion Factor C_{SS} (3)	Umrechnungsfaktor C_{SS} (3)	9,0472	Pa / mNm
Conversion Factor C_{SR} (3)	Umrechnungsfaktor C_{SR} (3)	5,9525	s^{-1} / min^{-1}
Average Factor $C_{SS,AVG}$ (1)	Mittelungsfaktor $C_{SS,AVG}$ (1)	0,99823	-
Average Factor $C_{SR,AVG}$ (2)	Mittelungsfaktor $C_{SR,AVG}$ (2)	0,99417	-
$C=C_{ISO}*C_{AVG}$	$C=C_{ISO}*C_{AVG}$		

(1) $C_{SS,AVG}$ factor for cone truncation
(2) $C_{SR,AVG}$ factor for cone truncation
(3) Rheoplus 3.21 or higher

(1) $C_{SS,AVG}$ Faktor für Kegelspitzenabnahme
(2) $C_{SR,AVG}$ Faktor für Kegelspitzenabnahme
(3) Rheoplus 3.21 oder höher

Measuring System Data Sheet
Messsystem-Datenblatt
CP75-1

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...Additional Data	...Zusätzliche Daten		Unit
Cone angle [SI-Unit]	Kegelwinkel [SI-Einheit]	0,017488	rad
Conversion Factor C_{SD}	Umrechnungsfaktor C_{SD}	5,684213	% / mrad
Conversion Factor C_{SD} [SI-Unit]	Umrechnungsfaktor C_{SD} [SI-Einheit]	56,84213	1 / rad
Conversion Factor C_{SS} [SI-Unit]	Umrechnungsfaktor C_{SS} [SI-Einheit]	9047,2	Pa / Nm
Conversion Factor C_{SR} [SI-Unit]	Umrechnungsfaktor C_{SR} [SI-Einheit]	357,1496	s^{-1} / s^{-1}

...Additional Data	...Zusätzliche Daten		Unit
Conversion Factor C_{SS} (4)	Umrechnungsfaktor C_{SS} (4)	9,0632	Pa / mNm
Conversion Factor C_{SR} (4)	Umrechnungsfaktor C_{SR} (4)	5,9874	s^{-1} / min^{-1}

(4) Factors for Rheoplus < 3.21 | (4) Faktoren für Rheoplus < 3.21

Calculation / Conversion Factors	Berechnung / Umrechnungsfaktoren
C_{SS}	$\tau = C_{SS} \cdot M$
C_{SD}	$\gamma = C_{SD} \cdot \varphi$
C_{SR}	$\dot{\gamma} = C_{SR} \cdot n$

Remark	Bemerkung
Standard Cone & Plate	Standard Kegel-Platte

Compliance	Nachgiebigkeit		
Compliance Measuring System	Nachgiebigkeit Messsystem	0,000000	rad/Nm
G' maximum (ideal conditions)	G' maximum (ideale Bedingungen)	0,1	MPa
G' maximum (safe range, low relative error)	G' maximum (sicherer Bereich, kleiner Mesefehler)	0,0	MPa
The measuring system should not be used above the given limitations for G'. For measuring higher G' values -> use a smaller plate	Das Messsystem sollte nicht über den angegebenen Begrenzungen für G' verwendet werden. Zur Messung höherer G'-Werte -> verwenden Sie eine kleinere Platte		

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Annexure I

A.I.1 Capital investment for civil works of dairy plant (A)

Description	Amount (₹)	Amount for BMFPD (₹)	Additional (₹)
Cost of land	80,00,000	1600000	64,00,000
Cost of civil construction including production block with cold store, general store, laboratory, administrative block, road, hard park, internal electrification	1,50,00,000	5000000	1,00,00,000
Total cost	230,00,000	6600000	164,00,000

A.I.2: Capital investment for plant and machinery items (B)

Item	Capacity	Quantity
1. Milk Reception Section		
Can tipping bar		1
Milk weighing machine (elect.)	500 kg	1
SS dump tank	1000 L	1
Inline strainer	STD	1
Milk pump	10 KLPH	1
Tanker unloading hose pipe	50 mm x 5 m	1
Tanker unloading pump	10 KLPH	1
Milk chiller (30°C-4°C)	10 KLPH	1
Milk can scrubber (SS body)	450 L	1
Raw milk storage tank	15000 L	1
Total cost (₹)		30,00,000
2. Milk Processing Section		
Milk transfer pump	5 KLPH	1
Milk pasteurizer	5 KLPH	1
Cream separator	5 KLPH	1
Pasteurized milk storage tank	15,000 L	3
Multipurpose tank	5000 L	3
CIP equipment	1000 L	1 set
SS pipe & fitting for entire plant		1 set
Total cost (₹)		1,50,00,000
3. Milk Packaging Section		
Milk transfer pump	10 KLPH	1
Balance tank with float	500 L	3
Milk pouch filling machine (mechanical)	5,000 pouches/h	3
Crate washer	1000 crates/h	1
Packing table	..	6
Plastic crates	10 L	7,500
Crate transporter	..	6
Total cost (₹)		1,25,00,000
4. Fat Handling Equipment		

Cream pasteurizer	1,000 L	1
Cream storage tank	3 KL	2
Cream transfer pump	2 KLPH	1
Butter churn	2000 L	1
Butter trolley	500 L	2
Ghee boiler	500 L	1
Ghee balance tank & pump	100 L	1 set
Ghee settling tank	500 L	2
Ghee storage tank	5000 L	1
Ghee clarifier	800 L/h	1
Ghee pump	2KLPH	1
Ghee pouch filling machine	800 pouches/h	1
Total cost (₹)		80,00,000
5. BMFPD production Section		
Bottle filling and sealing machine	150 bottle/min	1
Grain soaking tank (double jacketed with aeration facility)	5000 l	1
Temperature / humidity cabinet / room	10x10 m^2	1
Tray drier	5x5m^2	2
Pearling machine	-	2
Milling machine	-	2
Cup cooling cabinet	10000 kg	2
Double jacketed vat with agitator	5000 kg	2
Total cost (₹)		95,00,000
6. Service Equipment Section		
Refrigeration plant including cold store equipment	..	1 set
Steam boiler (husk/oil-fired) and other accessories	1,000 kg/h	1 set
Electricals including LT panel, MCC, cable, cable trays, earthing etc.	..	1 set
Water supply equipment including tube well	..	1 set
Pipes and fittings (MS/GI) for steam, water, chilled water, insulation and cladding	..	1 lot
Plant erection and commissioning	..	1 lot
Total cost (₹)		180,00,000
7. Miscellaneous equipment		
Laboratory equipment and glassware	..	1 set
Fire fighting equipment	..	1 set
Steam water mixing batteries	..	3
Cans for plant use	..	25
Furniture and fixtures, computers		
Total cost (₹)		28,00,000
Total cost (₹) (1 to 7)		688,00,000

TOTAL PROJECTED COST (A+B)	(₹)
A. Cost of civil works	230,00,000
B. Cost of plant and machinery	688,00,000
TOTAL CAPITAL INVESTMENT	918,00,000

Annexure II

A.II.1 Utilities' consumption rate

Water Liquid milk plant Product dairy	 1-1.5 L/L of milk 1.5-2 L/L of milk
Power	1 kw/20 L milk
F.O./LSHS	1 L/100 L of milk
Steam	1 kg/8 L of milk
Refrigeration Liquid milk plant Product dairy	 1 TR/1000-1200 L of milk 1 TR/600-800 Liters of milk

A.II.2 Charges on power and utilities for BMFPD (2500 litre milk basis)

Item	Requirement		Rate (₹)	Annual cost (₹)
	Daily	Annual		
Steam (kg)	375+110	145500	800/1000 kg	116400
Electricity (kWh)	250	75000	8/unit	600000
Water (L)	6,150	1845000	6/1000 L	11070
Electricity for Refrigeration (kWh)	285	85500	8/unit	684000
			Total utilities cost	1411470

A.II.3 Requirement and cost of raw materials for Probiotic drink

S.No.	Item	Requirement (kg)		Rate (₹/kg)	Annual cost (₹)
		Daily	Annual		
1	Cow whole milk	2500	750000	23	17250000
2	Starter culture	125	37500	5	562500
3	Pectin	7.5	2250	915	2058750
4	Cumin	12.5	3750	155	562500
5	Salt	13.75	4125	15	61875
6	Barley flour	133.5	40050	12.5	500625
7	Black pepper	0.75	225	325	70875
8	Water	875	262500	0.006	1575
				Total	21068700
	Product Losses (@ 2% milk basis)	50	15000		
	Product retained	3325	997500	~ (2500-50+875)=3325	

A.II.4 Requirement and cost of packaging material for Probiotic drink

Requirement		Rate (`/pc)	Annual Cost (₹)	Extra (1%) (for losses) (₹)	Total cost(₹)
Daily	Annual				
Bottle: 3325x5=16625	4987500	2.40	119,70,000	1,54,612.5	156,15,863
Cap: 3325x5=16625	4987500	0.70	34,91,250		

A.II.5 Manpower cost for Probiotic drink

Operating Labourers (A)							
S. No.	Staff	For Probiotic plant	Additional	Total	Monthly salary (₹)	Total monthly cost (₹)	Annual cost (₹)
1	Labourers	9	34	43	6000	2,58,000	28,80,000
2	Skilled worker	4	10	14	8000	11,2000	13,44,000
3	Mechanics/ electrician	0	3	3	10000	30,000	3,60,000
4	Boiler operator	0	4	4	8000	32,000	3,84,000
5	Lab analysts	0	4	4	10000	40,000	4.80,000
6	Lab attendant	0	4	4	7000	28,000	3,36,000
			Sub Total			4,77,000	60,00,000
Operational supervisors (B)							
7	Shift supervisors	0	2	2	15,000	30,000	360000
Administrative expenses (C)							
1	Plant Manager	0	1	1	50,000	50,000	6,00,000
2	Manager (P+QC)	0	1	1	45,000	45,000	5,40,000
3	Clerk/Accountant	0	2	2	8000	16,000	1,92,000
4	Store keeper	0	1	1	16,000	16,000	1,92,000
5	Office attendant	0	2	2	7000	14,000	1,68,000
6	Security staff	0	6	6	7000	42,000	5,04,000
			Sub Total			1,83,000	21,96,000
			Grand Total (A+B+C)				85,56,000
	Manpower cost specially for BMFPD					1032000	
	Manpower cost additional					7524000	
	Working capital (3 months pay of personnel) (for BMFPD)					258000	
	Working capital (3 months pay of personnel) (additional)					150480	
	Total					408480	

Annexure III: Income from sale of ghee

Annual income from ghee obtained from excess fat during preparation of BMFPD

	kg/day	kg/year	1% extra packaging material for losses	Total	Rate	Annual amount (₹)
Amount of ghee	22.72	6816	--	6816	300	2044800
Packaging material for ghee	24.58	7374	73.74	7447.74	10	74477.4
Ghee losses @0.5%					34.08 kg	
Net ghee					6781.9 kg	
NET income after losses					1960098.6	

Annexure IV

Detailed break-up cost for barley-milk based fermented probiotic drink (capacity 2500 kg/day)

A. MANUFACTURING COST (in ₹)					
a	Fixed charges		For BMFPD	Additional	Total
	i	Interest on total fixed capital investment (12% of total fixed capital and working capital)	1962960	728164.61	2691124.6
	ii	Depreciation on capital Investment (@5% of civil works, @7.5% of plant and machinery)	962500	395800	1358300
	iii	Insurance and taxes (2% of total fixed capital investment)	322000	121120	443120
			Sub total	4492544.6	
b	Direct production cost		For BMFPD	Additional	Total
	i	Raw materials cost	21068700		21068700
	ii	Packaging material (anuual requirement + 1%)	15615863		15615863
	iii	Manpower cost	1032000	601920	16,33,920
	iv	Power and utilities	1411470		1411470
	v	Maintenance and repairs (5% of fixed capital)	805000	302800	1107800
	vi	Laboratory charges (0.2% of raw mat. cost)	42137.4		42137.4
	vii	Cleaning & sanitizing material (@ 0.1% of raw material cost)	21068.7		21068.7
			Sub total	40900959	
c	Overhead expenses @ 15% of (a+b)			6809025.6	
d	Income from sale of ghee @ ₹ 300/kg			1960098.6	
A	NET MANUFACTURING COST (a+b+c-d)			50242431	
B. MARKETING and DISTRIBUTION COST (in ₹)					
@ 20% of Manufacturing cost				10048486.1	
Cost of product/annum = 60290916.8					
Cost/kg=₹ 60.44					
Cost/200g = ₹ 12.088					