

**TECHNOLOGY DEVELOPMENT FOR THE
MANUFACTURE OF *Aloe vera* SUPPLEMENTED
PROBIOTIC ICE CREAM**



**THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF**

**DOCTOR OF PHILOSOPHY
IN**

DAIRY TECHNOLOGY

BY

**VIDHU YADAV
M.Sc.**

**DIVISION OF DAIRY TECHNOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R)**

KARNAL – 132001 (HARYANA), INDIA

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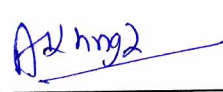
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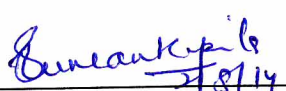

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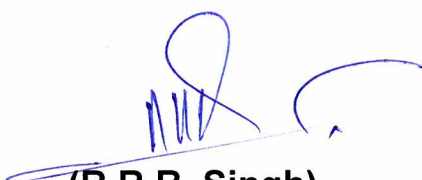
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This is to certify that the thesis entitled, **“Technology development for the manufacture of *Aloe vera* supplemented probiotic ice cream”** submitted by **Ms. Vidhu Yadav** towards the partial fulfillment for the award of the degree of **Doctor of Philosophy** in **Dairy Technology** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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Dedicated To

*My Husband
&
Family Members*

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Dated:

(Vidhu Yadav)

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ABBREVIATIONS

Symbol/short form	Abbreviation
%	Percent
°C	Degree Celsius
<i>et al.</i>	and co-workers
g	Gram
i.e.	That is
ml	Millilitre
cfu	Colony forming unit
h	Hour
MRS	deMan Rogosa sharpe medium
rpm	Revolutions per minute
sec	Second
cm	Centimeter
kg	Kilogram
LAB	Lactic acid bacteria
NCDC	National Collection of Dairy Cultures
\$	US Dollar
SPC	Standard Plate Count
APL	<i>Aloe vera</i> supplemented probiotic <i>lassi</i>
psi	Pounds per square inch
FFA	Free fatty acid
N	Normality
M	Molarity
nm	Nanometer
Pa.S.	Pascal Second
LDPE	Low density polyethylene
µm	Micro meter
ANOVA	Analysis of variance
% LA	Percent lactic acid
lit	Liters
viz	Namely
GRAS	Generally Recognized as Safe
FAO	Food and Agricultural Organization
WHO	World health organization
snf	Solids- not-fat

ABSTRACT

Ice cream is a delicious dairy product consumed all over the world by people of all ages. Ice cream with a combination of health enhancing ingredients viz. *Aloe vera* and probiotics can fulfill the consumer demand for healthy ice cream. The present study was therefore aimed at producing ice cream with enhanced biofunctional attributes using two nutraceuticals viz., *Aloe vera* juice and probiotic culture. *Aloe vera* supplemented ice cream (ASIC) was first standardized using Response Surface Methodology (RSM) with *Aloe vera* juice, Whey Protein Concentrate (WPC) and fat as three critical parameters and the obtained data was modelled using non-linear multiple regression technique. After analysis of sensory, physico-chemical and other parameters, the optimum level of the variables was found out to be *Aloe vera* juice: 20%, WPC (as percentage of Solids-not-fat): 25% and Fat: 8% with a desirability quotient of 0.84. Four probiotic cultures from National Collection of Dairy Cultures (NCDC), Karnal, were procured and screened for their probiotic attributes and low temperature tolerance ($-20 \pm 2^{\circ}\text{C}$). Out of the four probiotic cultures, NCDC 627 was selected, as it possessed better acid tolerance, antimicrobial activity, antibiotic susceptibility, highest % cell surface hydrophobicity and better low temperature tolerance. *Aloe vera* supplemented probiotic ice creams (ASPIC) were developed by incorporating probiotic cultures (NCDC-627 and DVS culture separately) into the optimized ASIC. Both the ice creams were evaluated for their *in vivo* immunomodulatory potency using cyclophosphamide (10 mg/kg per body weight for 13 days and 10 mg/kg body weight for 3 days) induced immunosuppression model (swiss albino female mice as experimental animal). Feeding ASPIC enhanced immunity in mice as shown by higher macrophage count, lymphocyte count, phagocytic activity, phagocytic index, lymphocyte proliferation index, IgA content and blood parameters. On storage for 3 months ($-20 \pm 2^{\circ}\text{C}$), non-significant difference in sensory attributes was observed. Even though *Lactobacilli* count was significantly ($p < 0.01$) decreased by $0.88 \log_{10}$ cfu/g during storage, their viability was maintained above the recommended minimum limits of 10^6 cfu/g. Consumer survey with 150 respondents showed high acceptability for the product indicating that it could be successfully launched. The cost of production of the ASPIC was estimated at Rs. 78.24 (with NCDC culture) and Rs. 96.74 (with DVS culture) per liter. The present study revealed that ASPIC with acceptable consumer quality and better immunoprotective effects could be manufactured at reasonable cost.

एलो वेरा पूरित प्रोबिओटिक आइसक्रीम के विकास के लिए प्रोद्योगिकी

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आइसक्रीम एक स्वादिष्ट दुग्ध उत्पाद है, जिसका सेवन विश्व भर में सभी उम्र के लोगों द्वारा किया जाता है। स्वास्थ्य वर्धक घटकों अर्थात एलो वेरा और प्रोबिओटिक से मिश्रित आइसक्रीम उपभोक्ताओं की स्वस्थ आइसक्रीम की मांग को परिपूर्ण कर सकती हैं। इसलिए वर्तमान अध्ययन का उद्देश्य २ न्यूट्रास्युटिकल्स अर्थात एलो वेरा और प्रोबिओटिक के प्रयोग से वर्धित जैव कार्यात्मक गुणों से परिपूर्ण आइसक्रीम विकसित करना था। एलो वेरा पूरित आइसक्रीम का मानकीकरण प्रत्युत्तर तल प्राविधि (आर एस एम) के केंद्रीय मिश्रित रूपरेखा (सी सी डी) द्वारा, तीन प्रमुख घटकों- एलो वेरा रस , दुग्ध वसा एवं सांद्रित दूध प्रोटीन (वसा रहित दुग्ध पाउडर के भाग के रूप में) के निर्धारण से किया गया। संवेदी, भौतिक-रासायनिक एवं अन्य मापदंडों के विश्लेषण के उपरान्त अनुकूलित सूत्र में २०% एलो वेरा रस, २५% सांद्रित दूध प्रोटीन एवं ८% दुग्ध वसा, ०.८४ वांछनीयता के साथ, पाया गया। एन.सी. डी.सी. से प्राप्त किये गए ४ प्रोबिओटिक जीवाणुओं को प्रोबिओटिक गुण एवं कम तापमान सहिष्णुता के लिए जांचा गया। बेहतर अम्ल सहिष्णुता, रोगाणुरोधी गतिविधि, एंटीबायोटिक संवेदनशीलता, प्रतिशत कोशिका सतह ह्यूड्रोफोबिसिटी और कम तापमान सहिष्णुता से युक्त होने के कारण ४ प्रोबिओटिक जीवाणुओं में से एन.सी.डी.सी. ६२७ का चयन किया गया। एलो वेरा पूरित आइसक्रीम को प्रोबिओटिक जीवाणुओं (डी.वी.एस. और एन, सी.डी.सी. ६२७) से किण्वित किया गया और फिर उससे दो अलग-२ एलो वेरा पूरित प्रोबिओटिक आइसक्रीम विकसित की गयी। दोनों आइसक्रीम को स्विस् अल्बिनो चूहियों को खिलाया गया और साइक्लोफॉस्फेमाईड द्वारा प्रेरित प्रतिमान से प्रतिरक्षा प्रणाली का विश्लेषण किया गया। प्रतिरक्षा प्रणाली का अवरोध करने के लिए साइक्लोफॉस्फेमाईड का दो खुराक स्तरों पर सभी मापदंडों के लिए प्रयोग किया गया। लघु पशु अध्ययन से प्रतिरक्षा में बढ़ोतरी पाई गयी, जैसा की बढ़े हुए मेक्रोफेज गणना, लिम्फोसाइट प्रसार अनुक्रमणिका, IgA स्तर और रक्त मापदंडों और में देखा गया। भंडारण स्थिरता का ३ महीने के लिए अध्ययन करके संवेदी स्वीकार्यता में नगण्य अंतर देखा गया तथापि लैक्टोबैसिल्लाए गणना में ०.८८ लॉग का सार्थक अंतर पाया गया लेकिन भंडारण के तीन महीने की अवधि में आइसक्रीम में जीवाणुओं की संख्या 10^6 सी एफ यु/ग्राम की संस्तुत सीमा से ज्यादा पाई गयी। निर्मित सूत्र की कुल संरचना एफ एस एस ऐ आई-२०११ द्वारा तय मापदंडों के अनुसार मध्यम वसा आइसक्रीम के अनुरूप है। विकसित उत्पादों और बाजार से आदर्श वनिला आइसक्रीम की तुलना में संवेदी तुलनीय परिणाम पाए गये। १५० उत्तरदाताओं के उपभोगता सर्वेक्षण में उच्च स्वीकृति देखी गयी, जिसने दर्शाया की दोनों विकसित उत्पादों का बाजार में सफलतापूर्वक शुभारम्भ किया जा सकता है। एलो वेरा पूरित प्रोबिओटिक आइसक्रीम की उत्पादन लागत ७८.२४ रुपए प्रति लीटर (एन. सी. डी. सी प्रोबिओटिक जीवाणुओं के साथ) एवं ९६.७४ रुपए प्रति लीटर (डी. वी. एस. प्रोबिओटिक जीवाणुओं के साथ) अनुमानित की गयी। विकसित उत्पाद का प्रयोग प्रतिरक्षा वर्धक गुणों वाले कार्यात्मक उत्पाद की तरह किया जा सकता है।

शोधकर्त्री मुख्य मार्गदर्शक

Chapter 1

Introduction

INTRODUCTION

With the start of this millennium, we witness a new era in the field of food science and nutrition with an increasing importance given to the interaction of food and medicine. The outcome of this area of study is known as “Functional Foods”, which involves food components as essential nutrients required for optimum health and as non nutritional component which contribute to the prevention or the delay of onset of chronic illnesses. The latest market surveys show that, in India, there is a great margin for such value-added products as well as health promoting food products.

The health virtues and growing consumer consciousness about probiotics have grabbed the interest of the food industry. According to WHO, probiotics is defined as a live microorganism that, when consumed in adequate quantities, confers a health benefit to the host (Dhewa *et al.*, 2011). Some of the main health benefits associated with probiotics include: anti-microbial activity, prevention and treatment of diarrhea, relief of symptoms caused by lactose intolerance, anti-mutagenic and anti-carcinogenic activities, and stimulation of the immune system (Shah, 2007). The dairy industry, in particular, has found probiotic cultures as valuable component for the development of new functional products.

Aloe comes from a family of its own called *Aloaceae*. There are four species of over 360 known ones that have medicinal properties-*Aloe arborescens* Miller; *Aloe perryi* Baker; *Aloe ferox* Miller o *Aloe capensis*; and *Aloe barbadensis* Miller (Atherton, 1998). Scientific investigations on *Aloe vera* have gained more attention due to its medicinal properties. *Aloe* has multiple pharmacological effects on constipation, insomnia, stomach disease, pain, hemorrhoids, itching, headache, hair loss, gum disease, healing wounds, burns and frostbite, kidney disease, blisters, sunburn and more (Lee, 2006).

Ice-cream is a delicious, wholesome and nutritious frozen dairy product, which is widely consumed in different parts of world. With an average annual

growth rate of 15% by volume and 20% by value, Indian ice cream market is about 350 million liters per annum valued at about Rs. 21,000 million of which about 65% is with the organized sector. However, still per capita ice cream consumption is just 0.25 liters as compared to 12 liters per annum in USA (Saxena, 2007). It indicates that there is still a vast untapped potential for expansion of ice cream market in India. Ice cream and frozen dairy desserts among various dairy products could be used as vehicles to deliver probiotics. These products have the advantage of storage at low temperatures that minimizes the temperature abuse and raises the viability at the time of consumption (Cruz *et al.*, 2009). However, it has not met with overwhelming success. There are many technological issues that need to be addressed for successfully using ice cream as matrix for incorporating probiotics for delivering desired health benefits. The major challenges are the survival in sufficient numbers of the specific strain as a function of pH of the product and also the different additives used in the product formulation. The processing parameters viz., form and stage of addition of the probiotic culture, freezing time/temperature combinations, storage conditions etc. do have bearing on the survival of the probiotics. Addition of *Aloe vera* may affect the survival and performance of the selected probiotics as it is likely to alter the composition and microenvironment in the product. It is therefore important that incorporation of the two nutraceuticals together is evaluated for use in ice cream. In view of the above justification, the present study entitled “Technology development for the manufacture of *Aloe vera* supplemented probiotic ice cream” has been proposed with the following objectives:

- Optimization of level of *Aloe vera* juice for incorporation in ice cream
- Selection of probiotic bacteria and evaluation of their survivability in ice cream containing *Aloe vera* juice
- Incorporation of selected probiotic bacteria in *Aloe vera* juice supplemented ice cream
- Evaluation of immune response of *Aloe vera* juice supplemented probiotic ice cream

Chapter 2

Review of Literature

REVIEW OF LITERATURE

Today, the link between diet and health is an integral part of healthy lifestyle. The role of diet and specific foods in the prevention and treatment of disease is being highlighted. Consumers are now fascinated in their personal health, and look ahead the food to be healthy and competent of preventing illness (Mattila-Sandholm *et al.*, 2002). Functional foods comprise some bacterial strains and products of plant and animal source with physiologically active compounds favorable for human health and lower risk of chronic diseases (Grazek *et al.*, 2005). Worldwide, the demand for consumption of functional foods is growing rapidly due to the increased awareness of the consumers from the impact of food on health. By 2014, the international functional foods market is expected to reach a value of about \$29.75 billion (Singh *et al.*, 2012).

The development of functionality in dairy-based products involves modification and/or enrichment of the primary base (Khurana and Kanawjia, 2007). Dairy industry has found probiotic cultures as a tool for the promotion of functional products (Champagne *et al.*, 2005). Dairy products with incorporated probiotic bacteria consist of approximately 65% of the world's functional food market (Agrawal, 2005). There is rise in world dairy food's consumption owing to cultured dairy products, with 2005 retail sales close to \$ 4.8 million (Mavhungu, 2006). *Aloe vera* has been used for many centuries for its curative and therapeutic properties. The present functional food market is full of health foods with *Aloe vera* as a functional ingredient (Chauhan *et al.*, 2007). Several *Aloe vera* products for oral consumption are available in the market in various forms like capsules, gel and juice. Frozen products from *Aloe vera* juice could provide an alternative product for consumers, apart from the commercially available Aloe drinks that are generally unacceptable due to off flavor. Ice-cream matrix might be a good vehicle for probiotic cultures owing to its composition, which includes milk proteins, fat and lactose. The addition of probiotic cultures to ice-creams, in

addition to adding value to the product, provides it with the advantage of being functional.

Ice cream with a combination of health enhancing ingredients viz. *Aloe vera* and probiotics can fill a gap in the market and fulfill consumer demand by providing a healthful solution to majority of the people with multiple health problems. Considering these facts the present investigation has been planned to develop *Aloe vera* supplemented probiotic ice cream which can meet the demand of modern health conscious consumer.

2.1 ALOE VERA

Aloe vera is a xerophytic, cactus like clump forming perennial plant with spikes. It has thick fibrous root that usually produces large 12-16 basal leaves per plant usually. The plant has a life span of about 12 years and gets matured when it is about 4 years old. A native of Africa, it is also known as “lily of the dessert”, “plant of immortality”, “medicine plant”, “burn plant” or “first aid plant”. *Aloe* is known as ‘Ghee-kuvar’ in Hindi. It has been used for its therapeutic value for several thousand years. The records of ancient cultures of India, Egypt, China, Greece and Rome have depicted its application. In biblical times the Chinese called it their elixir of youth and the Egyptians referred *Aloe vera* as the plant of immortality (Ahlawat and Khatkar, 2011). The name was derived from the Arabic word “Alloeh” meaning shining bitter substance because of the bitter liquid found in the leaves. *Aloe vera* leaf is composed of three layers: 1) An inner clear gel that contains 99 percent water and rest is made of glucomannans, amino acids, lipids, sterols and vitamins. 2) The middle layer of latex which is the bitter yellow sap and contains anthraquinones and glycosides. 3) The outer thick layer of 15-20 cells called as rind which has protective function and synthesizes carbohydrates and proteins (Eshun and He, 2004).

Aloe vera is known by a number of names in the literature i.e. *Aloe barbadensis* Mill, *Aloe chinensis* Bak, *Aloe vera* L. var. *chinensis* Berger, *Aloe elongate* Murray, *Aloe vera* L. var. *littoralis* Konig ex Bak, *Aloe indica* Royale, *Aloe officinalis* Forsk, *Aloe perfoliata*, *Aloe rubescens* DC and *Aloe vulgaris* Lam.

Aloe barbadensis Mill has been regarded as the correct name in most of the reference books and *Aloe vera* (L.) Burm f. as a synonym. However, *Aloe vera* (L.) Burm f. is the legitimate name of this species according to the International Rules of Botanical Nomenclature (Tucker *et al.*, 1989; Newton 1979). The *Aloe*, which comes from Barbados, named *barbadensis* according to Miller, or *Aloe vera* (Vera means true or genuine) by Linnaeus, or yet still called *Aloe vulgaris* by Lamark, are one and the same botanical species. *Aloe* comes from a family of its own called Aloaceae (Reynolds, 1985). This plant is related to the Liliaceae family, which is known to have medicinal properties, such as onion, garlic, and asparagus, (Lawless and Allan, 2000). Most of these plants originated in the dry regions of Africa, Asia, and Southern Europe (Urch, 1999).

Over 250 species of *Aloe* are grown around the world, of which only two species are grown commercially with *Aloe barbadensis* Miller and *Aloe arborescens* being the most dominant. *Aloe perryi* Baker and *Aloe ferox* are the two other species that have medicinal value. According to the use, the *Aloe* species are classified into two groups, one group for production of extract as crude drugs and the other group for the production of gel as an ingredient in functional foods. The species used for the production of crude drugs are *Aloe vera* L. (*A. Barbadensis* Miller), *A. ferox* Miller, *A. perryi* Baker, etc., and species used in functional foods formulation include *Aloe vera* L., *A. arborescens* Miller, *A. saponaria*, etc. A dwarf species which is only a few centimeter in diameter and is a popular house plant is *Aloe variegata*. Most *Aloe* plants are non-toxic but a few are extremely poisonous containing a hemlock like substance (Atherton, 1998).

Aloe vera gel production is an emerging industry for making cosmetics, functional food, and drugs due to the numerous beneficial effects attributed to *Aloe vera* gel (Eshun and He, 2004). The herbal movement initiated by naturopaths, holistic healers, yog gurus and alternative medicine promoters has led to the increased use of *Aloe vera* (Ahlawat and Khatkar, 2011). The main producers of *Aloe*, include Mexico, Latin America, China, Thailand, and the United States. The estimated size for *Aloe* raw material industry is about 125

million dollars. Whereas, the retail market value of the finished product containing *Aloe* is estimated at around \$ 110 billion (Rodriguez, 2004). A recent market analysis report showed that in 2008 Americans had spent almost 40 billion dollars on functional foods, drinks and supplements for the improvement of their appearance as well as to provide energy and nutrition to handle various health issues and *Aloe vera* products are one among these popular applications (Ahlawat and Khatkar, 2011).

In India, *Aloe vera* wildy grows in Tamil Nadu and Maharashtra and the states which are commonly known for its cultivation include Andhra Pradesh, Gujarat and Rajasthan. The first company supplying organic *Aloe* juice to European countries is Brihans Natural Products from Tamil Nadu. The major areas of *Aloe* production are Alwar in Rajasthan, Satnapalli in Andhra Pradesh and dry areas of Maharashtra and Tamil Nadu. The estimated production in India is about 1,00,000 tonne per year. The Indian pharmaceutical companies annually consume 200 tonnes of *Aloe* extract which is met from the wild sources from states of Maharashtra and Tamil Nadu and the ayurvedic pharmacies are only using 1% of the total production from India. The price of dried *Aloe* in India ranges from Rs. 600 to 1000 per kg depending upon the aloin content. However, the rate of fresh *Aloe* leaves varies from Rs. 40 to 55 per kg. The fresh leaves are used as a source of gel whereas; the dried leaves are used in Unani medicines (Chauhan *et al.*, 2007).

2.1.1 Chemical composition of *Aloe vera*

Most of the whole leaf of *Aloe* is water, making up more than 99 percent of the content and there are more than 200 chemical substances in the remaining 1 percent of the dry matter constituting the leaf. Therefore, the concentration of these components is relatively low (Luta and McAnalley, 2005). However, these solids constitute a marvellously diverse mixture of antibiotics, pain inhibitors, cell growth stimulators, inflammation fighters, burns healers, capillary dilators, vasoconstrictor inhibitors and moisturizers with a remarkable degree of penetration. Thus, *Aloe vera* is a complete drug store of medically useful

ingredients (Waller *et al.*, 1978). The composition of the *Aloe vera* juice/gel depends on factors like species/subspecies or varieties of *A. vera* used (Sacc`u *et al.*, 2001), annual seasons (Eshun and He, 2004), exposure to light (Paez *et al.*, 2000), the climate and the land (Grindlay and Reynolds, 1986), cultivation methods and the age of the plant (Pandhair *et al.*, 2011). However, the preservation methods used can modify the chemical composition and physicochemical characteristics of the *Aloe vera* products. The post-harvest processing methods used can also influence the chemical composition of the *Aloe vera* products (Femenia *et al.*, 2003; Simal *et al.*, 2000). All the commercial *Aloe vera* products may not contain quantifiable amounts of mucilaginous polysaccharide or other bioactive ingredients (Bozzi *et al.*, 2007).

The chemical compounds in the leaf of *Aloe* can be divided into two groups: Exudate compounds and Gel compounds. Both the components have different chemical composition, particularly in terms of polysaccharides. Therefore, different products with different chemical composition can be obtained depending on the processing technique employed to obtain the gel. The yellow exudate (latex) is rich in anthraquinones which are phenolic compounds (Reynolds, 1985). They have been used for their purgative effects and as a bitter agent in the preparation of alcoholic drinks for centuries (Sacc`u *et al.*, 2001). According to most of the authors, the beneficial effect of the plant is due to the gel. The therapeutic properties attributed to the gel are disproportionate in relation to the contents of the substances that they contain, as the constituent active principles, act synergically in the prevention and cure of several disorders and illnesses (Rodriguez *et al.*, 2010). The chemical composition of *Aloe vera* gel from the raw leaf without preservation processes is presented in Table 2.1.

The highest concentration in the dry matter is comprised by the carbohydrates. The part of the leaf used in the preparation decides the composition of these carbohydrates (Femenia *et al.*, 1999; Ni *et al.*, 2004). Free sugars, soluble polysaccharides and fibers constitute major fractions in the *Aloe* gel. The Acemannan forms the majority carbohydrate of the *Aloe* gel (Manna and McAnalley, 1993), possessing important therapeutic properties (Zhang and

Tizard, 1996) and is an acetylated polymer of mannose that is isolated from the *Aloe vera* gel (McDaniel, 1987). It is composed of 93 percent mannose and the remaining part of the molecule is composed of glucose, galactose, arabinose and other sugars (McAnalley, 1990; Talmadge *et al.*, 2004). The polysaccharides of the *Aloe* gel can be acylated, partially acylated, or not acylated. *Aloe* gel polysaccharides are lineal polymers without any branching and different proportions of single sugars are linked through β -glycosidic 1-4 linkages (Gowda, 1980; Manna and McAnalley, 1993). Xylose, rhamnose, galactose, arabinose and uronic acids have also been identified in minor quantities in *Aloe* gel (Mandal and Das, 1980). The free monosaccharides represent approximately 25 percent of the dry gel (Femenia *et al.*, 1999). Paez *et al.* (2000) indicated the presence of other free sugars like fructose and galactose, in significant amounts, but in smaller quantities than the glucose.

Lipids (2-5% of the dry matter) and proteins (6-8% of the dry matter) are in larger quantities in the gel than in the exudate of the plant (Femenia *et al.*, 1999). Analysis of extract of whole leaves detected 17 amino acids in a free state with arginine representing approximately 20% of total amino acids (Waller *et al.*, 1978). Enzymes like bradykinase, cellulase, carboxypeptidase, catalase, amylase, and oxidase exist in the *Aloe* gel (Meadows, 1980). It was observed that *A. vera*, *A. arborescens*, and *A. saponaria* had a total of 12, 9, and 12 major polypeptides, respectively. The presence of glycoproteins with biological or enzymatic activity has also been described in *A. vera*. During the inflammatory process, the enzyme bradykinase produces pain associated with vasodilatation. Carboxypeptidase is an important enzyme which inactivates bradykinase at site of wound in body and produces pain relieving and anti-inflammatory effect. A glycoprotein with anti-allergic properties, called alprogen has been isolated from *Aloe* gel recently (Chauhan *et al.*, 2007).

Table 2.1 Chemical composition of *Aloe vera* gel (Rodriguez *et al.*, 2010)

A. NUTRITIVE COMPOUNDS		
1.	Water/moisture	98.5-99.5%
2.	Carbohydrates	0.25% (25-50% of dry matter)
•	Soluble polysaccharides	Glucomannans (acemannan)
3.	Nitrogen fraction	N ₂ PROTEIN (0.013%)
•	Amino acids	18(7 essential; 20% Arg)
•	Enzymes	Bradykinase, Catalase, Peroxidase
4.	Vitamins	Ascorbic acid, Thiamin, Riboflavin, Niacin, Folic acid, Carotenoids, Tocopherols
5.	Minerals and trace elements	24-25% of dry matter Mineral and electrolytes K, Cl, Ca, Mg, P Trace Elements Fe, Cu, Zn, Mn, Al, Se, Cr
B. NON-NUTRITIVE COMPOUNDS		
1.	Organic acids	Salicylic, Malic, Lactic, Acetic, Succinic acids
2.	Phenolic compounds	Anthraquinones, Aloin AyB, Aloe-emodin, Aloesin, Aloenin, Aloeresin
3.	Phytosterols	β – sitosterol, campesterol
4.	Other compounds	Aliphatic hydrocarbons/esters long chain volatile compounds.

Anthraquinones are the phenolic compounds present in the sap or yellow exudates of leaf or *Aloe vera* latex, the most prominent being aloin A and aloin B. Anthraquinones are formed by oxidation of low molecular weight components such as aloin, which is a glycoside derivative of *Aloe-emodin*. The largest aloin quantity was observed in *A. arborescens*, and the levels were low or very low in *A. vera* and *A. saponaria*, respectively (Li & Li, 2003). The bitter aloes (dried yellow exudates) consists of free anthraquinones and their derivatives i.e. barbloin-10-(1151-anhydroglucosyl)-aloe emodin-9-anthrone, isobarbloin, anthrone-C- glycosides and chromones. These compounds exerts a powerful purgative effects when ingested in large amounts but when low in concentration,

they appear to aid absorption from the gut and are potent antimicrobial and powerful analgesic agents. Isolation and structure determinations of these chromones from the *Aloe vera* leaves were also studied and these compounds were identified to be 8-C-glycosyl-7-O methyl-(S) aloesol, isoaloeresin D and aloeresin E (Sacc`u *et al.* 2001). Besides the phenolic compounds, the exudate of the leaf contains small quantities of polysaccharides and free sugars, especially glucose, as well as volatile and aliphatic compounds (Rebecca *et al.*, 2003; Sacc`u *et al.*, 2001).

Other biologically active components include phenolic compounds, organic acids and amino acids, certain vitamins and minerals, and volatile compounds (Luta and McAnalley, 2005). Among the organic acids, malic acid has the highest content (Paez *et al.*, 2000) and is absent in the exudate of the plant and it has been proposed as one of the markers for the recognition of *Aloe* in commercial products (Luta and McAnalley, 2005). Other organic acids, such as lactic or succinic, can be present as a consequence of microbiological or enzymatic alteration of the product (IASC, 2004). Certain vitamins such as the A, C, E, B1 (thiamine), B3 (niacin), B2 (riboflavin), and folic acid, many of them with an antioxidant capacity, have been identified (Atherton, 1998; Lawless and Allan, 2000). Some investigators observed trace amounts of vitamin B12, which is usually only available in foods of animal origin (Atherton, 1998; Lawless and Allan, 2000; Urch, 1999). However, this finding has not been confirmed in recent studies. The concentrations of chlorine and potassium are high, whereas the sodium content is smaller than the usual content in plants. There are other minor essential minerals such as Fe, Cu, Zn, Mg, Mn, P, Cr, Si, and Ni, and small amounts of toxic elements such as Al, B, Ba, Sr, Cd, and Pb (Femenia *et al.*, 1999; Sahito *et al.*, 2003; Yamaguchi *et al.*, 1993; Yang *et al.*, 2004). A novel anti-inflammatory compounds, C-glycosyl chromones, has also been isolated from aloe gel. Saponins are the soapy substances that form 3% of the gel and are general cleansers, having antiseptic properties. Comperterol, β - sitosterol and lupeol includes the sterols present. Salicylic acid is an aspirin like compound

possessing pain relieving properties. Table 2.2 depicts the components of *Aloe vera* along with the health benefits.

Table 2.2 Novel components of *Aloe vera* along with their health benefits

Chemical component	Health benefits
Acemannan	Accelerate wound healing, modulate immune system, Antineoplastic and antiviral effects
Alprogen	Anti-allergic
C-glycosyl chromone	Anti-inflammatory
Bradykinase	Anti-inflammatory
Magnesium lactate	Anti-allergic
Salicylic acid	Analgesic, Anti-inflammatory

(Source: Peng *et al.* 1991)

2.1.2 Biological activities *Aloe vera*

The use of *Aloe* dates from biblical times, and it has been and is still used in traditional medicine for the treatment of numerous illnesses. In the first century AD, Dioscorides gave the first detailed description of the pharmacological effects of *Aloe* is recorded in “The Greek Herbal”. According to that literature, *Aloe* has multiple pharmacological effects on constipation, insomnia, stomach disease, pain, hemorrhoids, itching, headache, hair loss, gum disease, healing wounds, burns and frostbite, kidney disease, blisters, sunburn and more (Lee, 2006). The polysaccharides in *Aloe vera* gel have therapeutic activity such as immunostimulation, anti-inflammatory effects, anti-diabetic, anti-viral, anti-fungal, anti-bacterial, anti-oxidant effects, skin and wound healing, anti-cancer effects, promotion of radiation damage repair, etc. (Talmadge *et al.*, 2004; Ni and Tizard, 2004). The health benefits of *Aloe vera* have been credited mainly to the polysaccharides and especially to Acemannan. However, some authors credit the biological activities of *Aloe vera* to the synergistic action of a variety of

compounds, rather than to a single component (Hamman, 2008). Recently, five phytosterols with antidiabetic properties have been isolated from *Aloe vera* (Tanaka *et al.*, 2006) and a polysaccharide called *Aloeride* having potent immunostimulatory activity compared to Acemannan has also been identified (Pugh *et al.*, 2001). *Aloe* polysaccharides (approx. MW 50,000 to 1,000,000 daltons) can be utilized better by human colonic bacteria Sinnott *et al.* (2007).

However, there are several clinical reports that have found *Aloe vera* not effective in above mentioned therapeutic activities or even observed undesirable effects. These conflicting results could be attributed to the use of plants from different locations with varying chemical composition and also due to the use of different isolation techniques for the extraction of compounds from *Aloe* leaf pulp (Hamman, 2008). Some of the biological activities of *Aloe vera* are briefly discussed below with a special focus on the immunomodulatory activities of *Aloe vera*.

2.1.2.1 Skin and Wound Healing

Wound healing is the response to injured tissue that results in the restoration of tissue integrity. In several studies, topical and systemic administration of aloe gel has shown to improve wound healing while some studies claimed no effect or even a delay in wound healing. These conflicting results may be explained by the stability of active ingredients at the time of treatment, as conditions after harvesting plays an important role in determining the activity of *Aloe vera* (Hamman, 2008). The various mechanisms that have an effect on wound healing include the ability of *Aloe vera* to keep the wound moist, increased epithelial cell migration, rapid maturation of collagen and reduction in inflammation (Reynolds and Dweck, 1999). It can be used successfully in treatment of skin ulcers, including mouth ulcers, leg ulcers, and simple herpes. This is attributed to an anti-viral effect of the *Aloe* gel in concentrations of 80% (Eshun and He, 2004). Some studies have indicated that the *A. barbadensis* gel accelerates the cure of wounds in diabetic rats due to its ability to stimulate the synthesis and maturation of collagen in fibroblasts (Chithra *et al.*, 1998).

2.1.2.2 Anti-diabetic effect

Several pre-clinical (in animals) and clinical (in humans) studies showed a reduction in blood glucose levels by the use of *Aloe vera* gel preparations in different forms viz., juice, powder etc. While there are other studies that indicate no change in blood glucose level. This difference in results could be attributed to the difference in the way the aloe mucilaginous gel was isolated and separated from the exudates anthraquinones (Hamman, 2008). A number of studies have suggested that *Aloe vera* extract reduces blood glucose levels by enhancing glucose metabolism (Hamman, 2008). In a study on streptozotocin-induced diabetic rats, it was suggested that *Aloe vera* extract reduced blood glucose levels by enhancing glucose metabolism. It was further proposed that the glucose lowering effect could be explained by an antioxidant mechanism as it attenuated oxidative damage in the brains of streptozotocin-induced mice and reduced peroxidation levels in the kidneys of rats (Bourdreau and Beland, 2006). There are studies on the effects of *Aloe vera* in diabetic humans. Oral use of *Aloe vera* gel decreased fasting blood glucose (by more than 100 mg/100 ml) level in three studies of people with type II diabetes (Ghannam *et al.*, 1986).

2.1.2.3 Immunomodulatory Effects

The Latin term “immunis” meaning “exempt” is the source of the English word immunity, meaning the state of protection from infectious disease. From birth, we are exposed to a continuous stream of micro-organisms that have the ability to wreak havoc on our bodily processes. The state of protection from infectious diseases, immunity, has both non specific (innate immunity) and specific (adaptive) components. The study of the association between nutrition and immunity is gaining interest due to the hypothesis that intake of some specific foods may lower down susceptibility for the development and/or progression of immunological diseases (Kapila *et al.*, 2009). Conventional therapy (radiotherapy and chemotherapy) impairs immune systems’ ability to defend the body against foreign or abnormal cells (James, 2007). Modulation of immune responses, by various plant materials for disease alleviation is therefore

an interesting approach and a basic concept in *Ayurveda* (Gokhale *et al.*, 2003). *Aloe* contains more than 200 substances which are responsible for its different types of health and nutritional benefits. There are many claims regarding *Aloe vera* gel's ability to support and enhance the immune system. Acemannan isolated from *Aloe vera* gel has been shown to increase the response of lymphocytes to antigens in *in-vitro* studies highlighting the anti-viral effect of acemannans. The anthraquinones present in *Aloe* latex, has direct virucidal effects. It inactivates various enveloped viruses such as herpes simplex and influenza. Some studies indicate that *Aloe vera* can be used in the treatment of HIV-AIDS. This can be attributed to the anti-viral and immuno-modulating properties of acemannan which directly affects the immune system by activating and stimulating the macrophages, monocytes, antibodies and T-cells. Chandu *et al.*, (2011) studied the immunomodulatory activity of saline extracts of leaves of *Aloe vera* Linn. (Family: Liliaceae) on the albino mice using pyrogallol induced immunosuppression and concluded that *A. vera* extract produces stimulatory effect on the humoral and cell mediated immune response in the experimental animals and suggested its therapeutic usefulness in immunological disorders. Madan *et al.*, (2008) carried out an experiment to study immunomodulatory properties of *Aloe vera* gel in mice and concluded that *Aloe vera* gel extract may act as a potential candidate in several immuno-suppressed clinical conditions. They observed proliferation of stem cells, as seen from an increase in total white blood cells, stimulation of humoral immunity as shown by increase in Plaque-Forming Cells and circulating antibody titre and stimulation of phagocytic activity.

Macrophages are cells produced by the differentiation of monocytes in tissues. Macrophages function in both non-specific defense (innate immunity) as well as help initiate specific defense mechanisms (adaptive immunity) of vertebrate animals. A number of studies indicated immunomodulating activities of the polysaccharides in *A. vera* gel, and suggested that these effects occur via activation of macrophage cells to generate nitric oxide, secrete cytokines and present cell surface markers (Zhang and Tizard, 1996, Chow *et al.*, 2005, Im *et al.*, 2005). Yagi *et al.*, (1987) extracted two neutral amino acid fractions from *Aloe*

arborescens var. *natalensis* which also have shown enhanced phagocytosis. Immunoglobulins function as antibodies, the antigen-binding protein that are present on the B cell membrane and also are secreted by plasma cells. Types of antibodies include IgG, IgM, IgA, IgE and IgD. Among these, IgG is the most important class in serum, which constitutes about 80% of total serum Ig. IgA constitutes only 10-15% of total Ig in serum. Daily production of secretory IgA is greater than that of any other Ig class. *Aloe vera* peel extract possesses immunomodulatory effects *in vivo* that include the elevated production of IgG and IgA and the promotion of anti-inflammatory cytokines (Kwon *et al.*, 2011). *Aloe vera*, a rich depository of many biological compounds has also been reported for its effect on haematological parameters. Oguwike *et al.* (2009) carried out a study to check the effect of *Aloe vera* on the haemostatic mechanism of albino Wistar rats and observed no increase in the haemoglobin concentration, and packed cell volume of rats but increased the white blood cell count, platelet count, without increasing the bleeding time, clotting time, pro-thrombin time, and partial thromboplastin time.

2.1.2.4 Hypolipidemic Effect

The oral administration of *Aloe vera* can be useful for reducing lipid levels in patients with hyperlipidemia, hepatic cholesterol and oxidative status in aged rats (Lim *et al.*, 2003). The oral consumption of *Aloe vera* gel (10–20 ml/day) for 12 weeks can reduce low density lipoprotein (LDL) cholesterol by about 18%, total cholesterol by about 15%, and triacylglycerides by 25–30% in patients with hyperlipidemia (Shapiro and Gong, 2002).

2.1.2.5 Anti-Cancer Effect

Aloe extracts have been tested in the treatment of cancer and positive effects have been observed in inhibiting the growth of tumors. The two fractions from aloes that are claimed to have anti-cancer effects include glycoproteins (lectins) and polysaccharides (Reynolds and Dweck 1999). The anti-tumor activity of *Aloe vera* polysaccharides, especially acemannan has been investigated in many *in vitro* models and different animal models. Several studies

indicate anti-tumor activity for *Aloe vera* gel in terms of reduced tumor burden, tumor shrinkage and tumor necrosis (Hamman, 2008). Stimulation of immune response is one of the proposed mechanism of action for anti-cancer effect of *Aloe* polysaccharide (Zhang and Tizard, 1996). *Aloe vera* has also shown chemopreventative and anti-genotoxic effects on benzo(alpha) pyrene-DNA adducts (Boudreau and Beland 2006)

2.1.2.6 Antioxidant Effects

Several authors have reported that different fractions of *Aloe vera* and also the unfractionated whole gel possess anti-oxidant effects. *Aloe vera* extracts in an equivalent amount have a stronger anti-oxidant effect than butyl hydroxianisol (BHA) and α -tocopherol, which could be useful in designing functional foods, cosmetics, and medications (Hu *et al.*, 2003). Glutathione peroxidase activity, superoxide dismutase enzymes and phenolic anti-oxidant present in *Aloe vera* gel, are supposed to be responsible for anti-oxidant effects (Hamman, 2008). The *Aloe vera* gel in 1 in 50 concentration inhibited production of prostaglandin E₂ from inflamed colorectal biopsies without having any effect on thromboxane B₂ release (Langmead *et al.*, 2004).

2.1.2.7 Antimicrobial Effects

Nowadays, antibiotic resistant bacterial species have become a great risk to human kind. Researchers are trying to develop effective antimicrobial drugs from natural origin. *Aloe vera* peel extracts may act as an economic source of alternative supplementary agents for antimicrobials in the treatment of bacterial infections. This could reduce the use of antimicrobials. Different plant fractions viz. juice, inner gel, leaf and ethanolic, methanolic, acetone, aqueous and other extracts of *Aloe vera* have been studied for their antimicrobial activities against several microorganisms. Components namely anthraquinones (aloin, *Aloe* emodin, etc.), proteins, etc. with potent antimicrobial activity and their respective mechanisms of action were proposed by Lawrence *et al.*, 2009. Habeeb *et al.* (2007) studied the antimicrobial activity of *Aloe vera* inner leaf gel against several antibiotic resistant bacterial species namely *Shigella flexneri*, Methicillin-

Resistant *Staphylococcus aureus* (MRSA), *Enterobacter cloacae* and *Enterococcus bovis*. The workers reported that *Aloe vera* had antimicrobial effect against all the tested bacterial species. Banu *et al.* (2012) also observed the antimicrobial activity of *Aloe vera* gel against multi drug resistant bacteria. Devi *et al.* (2012) studied the antimicrobial activity of dimethyl sulfoxide (DMSO) crude extracts of *Aloe barbadensis* Miller gel against the selected bacterial and fungal pathogens namely *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus niger*, *Candida albicans* and *Penicillium sps.* The workers found the maximum zone of inhibition (16mm) in case of *S. aureus* and minimum (10mm) in case of *E. coli* and *Penicillium ssp.* Antifungal activity of the *Aloe vera* gel (at a concentration of 0.35 percent) was also reported by Sitara *et al.* (2011) and it was ascribed to a protein of 14 kDa present in the leaf gel (Das *et al.*, 2011)

2.1.3 Effect of processing on *Aloe vera*

The preparation of *Aloe vera* products involve some type of processing viz., heating, dehydration, concentration, etc. Processing may result in irreversible modifications of the active components that can affect their original structure and may cause changes in the proposed physiological and pharmacological properties of these components (Thibault *et al.*, 1992). Femenia *et al.* (2003) evaluated the physico-chemical modifications due to the heat treatment and dehydration at different temperatures (30-80°C) on a bioactive polysaccharide of *Aloe barbadensis* Miller (acemannan) and reported a significant modification of acemannan when dehydration was performed above 60°C. The molecular weight of acemannan was 45 kDa in fresh *Aloe* which increased to 75 and 81 kDa in samples dehydrated at 70 and 80°C, respectively. These structural modifications resulted in the significant changes in the functional properties like swelling, water retention capacity, and fat adsorption capacity, which exhibited a significant decrease as the temperature of dehydration increased. Further, dehydration promoted significant modification of the main type of cell wall polysaccharides present within the *Aloe* parenchyma tissues. Chang *et al.* (2006)

carried out a study to evaluate the thermal stabilities of polysaccharides substances and barbaloin, present in *Aloe vera* gel juice. The workers observed that the polysaccharide from *Aloe vera* exhibited a maximal stability at 70°C decreasing either at higher or lower temperatures. Heating promoted a remarkable decrease in barbaloin content depending on temperature and time. Miranda *et al.* (2009) evaluated the effect of drying temperature on rehydration ratio, water holding capacity, texture, microstructure and total polysaccharide content of *Aloe vera* gel powder. The results indicated that a drying temperature of 60-70°C resulted in minor alterations in the structural properties and total polysaccharide content of the *Aloe* gel powder.

2.1.4 Applications of *Aloe vera*

As the incidences of various diseases are on the rise throughout the world, people have become conscious about their diet. To fill a gap in the market and fulfill consumer demand without torturing their taste buds studies are going on for developing technology for the manufacture of products with medicinal herbs. In the food industry, *Aloe vera* has been used as a source of functional foods and as an ingredient in other food products (Hamman, 2008). Some people in Rajasthan consume *Aloe vera* along with fenugreek seeds. In Tamil Nadu, some people prepare a curry using *Aloe vera* which is taken along with Indian bread or rice. In Mexico fruit smoothies made out of *Aloe vera* are fairly common. Drinks made from or containing chunks of *Aloe* pulp are popular in Asia as commercial beverages and as a tea additive; this is notably true in Korea.

Recently, ice creams with *Aloe vera* as one of the constituent have been prepared and subjected to sensory evaluation for consumer acceptance. Manoharan *et al.*, (2012) incorporated *Aloe vera* juice at different levels (5, 10, 15, 20, 25, 30, 35, 40 and 45 %) in ice cream mix, prepared by using ingredients viz., buffalo milk, butter, skimmed milk powder, stabilizers (gelatin) and emulsifiers (Glycein-monostearate) and cane sugar (sucrose). The authors concluded that the inclusion of *Aloe vera* juice in the ice cream significantly altered the organoleptic scores of the ice cream samples. *Aloe vera* juice at 20

% inclusion level, among the different inclusion levels (5-45%), had the maximum scores, without much affecting its acceptability. Srisukh *et al.*, (2006) also prepared pandan-flavored, coconut-flavored and orange-flavored *Aloe* ice creams using fresh *Aloe vera* pulp and carried out sensory evaluation of the developed *Aloe* ice creams. The *Aloe* leaf pulp in 3 forms viz., *Aloe* gel, fresh *Aloe* leaf pulp and fresh *Aloe* leaf pulp coated with icing sugar was used for the preparation of *Aloe* ice cream. The three forms of the *Aloe* preparation, at 20% w/w, were then separately mixed with an ice cream base of pandan-flavoured ice cream. The gel form was selected for further adjustment. The concentration of the gel was then adjusted from 20%w/w to 25%w/w to increase the flavor of the *Aloe* gel in the ice cream. The latter concentration was chosen for further development. Sensory evaluation of the *Aloe* ice cream formulae showed that pandan-flavored *Aloe* ice cream obtained the highest average score followed by Coconut-flavored and orange-flavored *Aloe* ice creams.

The *Aloe vera* juice finds wide application in other food products also like production of ready to serve drink, health drink, soft drink, laxative drink, *Aloe vera* lemon juice, sherbet, aloe sports drink with electrolyte, diet drink with soluble fiber, hangover drink with B vitamin, amino acids and acetaminophen, healthy vegetable juice mix, tropical fruit juice with *Aloe vera*, *Aloe vera* yoghurts, *Aloe vera* mix for whiskey and white bread, cucumber juice with *Aloe vera* (Eshun and He 2004; Hamman 2008; Grindlay and Reynolds 1986). Wei *et al.* (2004) prepared a health beverage from fresh *Aloe vera* leaves. The leaves were washed, pulped, sterilized and filtered, then mixed with different concentrations of Dangshen, Maidong juices and Chinese herbs. Effects of processing conditions e.g. temperature, pH, sucrose, vitamin C and citric acid on the stability of colour and gelatinoids in *Aloe vera* juice were studied and it was concluded that the stability was negatively affected by increasing sucrose and citric acid concentrations while vitamin C and sodium chloride at low concentrations improved the stability. Do-sang *et al.* (1999) prepared vinegar from *Aloe vera* juice using *Acetobactor* sp. The fleshy portion can also be converted into candies, squash, jam, jellies, bar, munch, etc. (Chauhan *et al.*, 2007). Hu *et al.*,

(2003) described the technology for making *Aloe* jelly by addition of 1% carageenan, 0.6% citric acid, 19.6% sugar and 17.83% glucose. Healthy baby infant formula beverage and healthy baby toddler formula beverage have been prepared from cow's milk, refined sugar, goat's milk, rice milk along with *Aloe vera* juice and water (Benward and Benward, 2000).

In another study use of pure *Aloe vera* juice as a main fermentation broth has resulted in higher viable counts (1×10^9 cfu/ml for *L. plantarum* and 1×10^{11} cfu/ml for *L. casei*) of two probiotic bacterial strains namely *Lactobacillus plantarum* (NCIMB 11718) and *L. casei* (NRRL 1445) compared with those achieved in standard medium (MRS) for these strains (González *et al.*, 2008). The authors suggested that *Aloe vera* can be used as a prebiotic ingredient in functional foods. Recently, Panesar and Shinde (2011) prepared *Aloe vera* juice fortified probiotic yoghurt. The probiotic species used were *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Counts of probiotic bacteria remain more than suggested value of more than 10^7 throughout the storage period (28 days). The workers suggested that *Aloe vera* fortified probiotic yoghurt could be used as an adequate carrier of probiotic bacteria. Singh *et al.* (2012) prepared *Aloe vera* juice supplemented *lassi* to improve the health benefits of the *lassi*. The workers reported that good quality *Aloe vera* juice added *lassi* could be prepared by incorporation of 15 percent *Aloe vera* juice to medium (3.0 percent) as well as low fat (0.5 percent) milk with shelf life up to 23 days. Although *Aloe vera* gel is best known for its therapeutic effect, the product is nowadays introduced as an additive to fruits and vegetable products (ingredient, coatings, etc.) (Miranda *et al.*, 2009). The *Aloe vera* gel has been found to be useful in extending the shelf life of grapes (Valverde *et al.*, 2005) and sweet cherries (Martinez-Romero *et al.*, 2006) when applied as a edible coating. Vacuum impregnation of vegetables using a solution with 30 g/l of *Aloe vera* powder at 20°C not only enhanced the functionality of vegetables but also increased their shelf life by decreasing their respiration rates (Sanzana *et al.*, 2011). *Aloe vera* contains several beneficial long-chain polysaccharides. The process of blending the gel in a mix might cause some of the polysaccharides to be broken. Adams

(2007), therefore, suggested that it's best to add the *Aloe vera* as the last ingredient so that any potential shredding of the polysaccharides can be minimized. Whether *Aloe vera* is used as raw *Aloe vera* gel or a dried *Aloe vera* powder, all other ingredients must be blended first followed by *Aloe vera* as the last ingredient, blending *Aloe* for the shortest time necessary.

Other areas of *Aloe vera* application include cosmetic and toiletry industries which could be attributed to its valuable moisturizing effect. *A. vera* gel has become an important selling point in cosmetic products. A large range of moisturizing creams, cleansers, shampoos, and soaps are important cosmetic formulations available. *Aloe* extracts have been incorporated into shaving creams and lotions to promote the healing of cuts. *Aloe vera*'s application in the pharmaceutical industry is also highly significant. It is used in the manufacture of medical products, such as burn treatments, ointments, and medicated creams and lotions for topical applications to fight various skin disorders. *Aloe vera* gel polysaccharides were also used as potential drug absorption enhancing agents (Chen *et al.*, 2009; Subramanian *et al.*, 2010). The *Aloe vera* gel powder was also shown to be used as dissolution enhancer for improving the drug absorption of water insoluble drugs (Rahman *et al.*, 2012).

2.1.5 Safety aspects of *Aloe vera* products

Regarding the safety of *Aloe vera* products, the scientific community is divided into two groups. According to one group *Aloe vera* is quite safe for human consumption. While the other group warns to use it with caution to avoid contamination of aloin from the yellow exudates, as aloin is reported to damage DNA and cause cancer (Lachenmeier *et al.* 2005). On the contrary, scientists have reported that anthroquinones present in *Aloe vera* leaf, including aloin, have beneficial effect when used in small quantity, although this small quantity is not well defined (Sydiskis *et al.* 1991). However, it is reported that *Aloe vera* gel is safe for external use and allergies are rare with no adverse reactions with other medications. *Aloe* should not be used internally during pregnancy, lactation or childhood and by persons suffering from abdominal pain, appendicitis or

intestinal obstruction (Kemper and Chiou 1999). *Aloes* are considered as menstruation promoting agents. Such agents might result in abortion of the foetus from uterus. It is also believed that *Aloe* creates abnormalities in the fetus if taken orally during the first forty days of pregnancy. Although, the emmenagogue activities of *Aloes* have been documented, further research is needed to substantiate these assumptions (Chauhan *et al.*, 2007).

In the clinical trials, no serious adverse reactions were reported following *Aloe vera* administration. Severe adverse effects have been associated with the oral application of *Aloe vera* only in rare cases. Due to the possible contamination by anthraquinones, oral aloe gel may cause symptoms of abdominal cramps and diarrhoea. There have also been several reports of *Aloe vera* gel lowering plasma glucose in laboratory animal and in human (Ghannam *et al.* 1986). In animal studies, life-long *Aloe vera* gel ingestion (contributing 1 percent of total diet) in rats was demonstrated to produce no harmful effects or deleterious changes (Ikeno *et al.*, 2002). In contrast, chronic ingestion of 100 mg/kg *Aloe vera* (extracted in ethanol) given orally in rats produced reproductive toxicity, significant sperm damage, inflammation, and mortality compared to control animals (Shah *et al.* 1989). *Aloe vera*-derived ingredients were not found to be toxic in acute oral studies using mice and rats. In mice, the LD₅₀ was >200 mg/kg and >80 mg/kg in parenteral and intravenous studies, respectively, whereas in rats the corresponding LD₅₀ values were >50 mg/kg and >15 mg/kg, respectively. No significant toxicity was seen with acemannan given intravenously or intraperitoneally at 4-day intervals over 30 days at maximum dose levels of 200 mg/kg in mice and 50 mg/kg in rats (Cosmetic Ingredient Review Expert Panel, 2007). The no observed adverse effect level (NOAEL) for whole-leaf *Aloe vera* powder was 87.7 and 109.7 mg/kg/day in male and female rats, respectively (Matsuda *et al.*, 2007). The Natural Standard Research Collaboration further concluded that the oral use of *Aloe vera* gel for its potential hypoglycemic effects and the short-term use of oral *Aloe* latex as a laxative are possibly safe; however, prolonged use of the latex is likely to be unsafe due to a theoretical risk of dehydration and electrolyte imbalance (Ulbricht *et al.*, 2008).

The toxicity profile of the methanol extract of the *Aloe vera* (*Aloe barbadensis*) gel was studied in Wistar rats. A multiple oral administration of the extract at single dose of 4, 8, 16 g/kg body weights for 6 weeks did not produce significant toxic effect in rats during acute and sub-acute treatment in rats (Saritha and Anilakumar, 2010).

2.1.6 Investigation of authenticity

The major concern for the *Aloe vera* market is the adulteration of *Aloe vera* products with fillers such as maltodextrin, glucose, glycerin, and malic acid (Bozzi *et al.*, 2007). To make sure that one is buying a product containing *Aloe* and paying a reasonable price, it is important to identify and quantify the *Aloe* gel used in the preparation of the commercial product, as well as to recognize possible adulterations or dilutions. Therefore, it is advisable to see logotype of International *Aloe* Science Council (IASC). IASC is a non profit making international organization, founded in 1981 with members in over sixty countries. Its mission is to improve and standardize the *Aloe* industry through the certification of *Aloe* raw material and *Aloe* finished products. For IASC certification, finished product which contains the tag 'with aloe' must contain minimum 15% *Aloe vera* in the final product to greatly magnify the benefits to the end use customers (IASC, 2004).

2.2 PROBIOTICS

According to WHO, probiotics is defined as a live microorganism that, when consumed in adequate quantities, confers a health benefit to the host (Lacono *et al.*, 2011; Dhewa *et al.*, 2011). The concept of probiotics is very old and they have been consumed for thousands of years by human beings in the form of fermented foods (Kopp-Hoolihan, 2001). The therapeutic potential of probiotics has been long known; with several scientists reported that fermented milk could cure disorders of the digestive system (Ranadheera *et al.*, 2010). There is a mention of use of probiotics in treating body ailments even in biblical scriptures (Lourens-Hattingh and Viljoen, 2001). The Russian scientist Metchnikoff in his fascinating treatise "The Prolongation of Life' propounded that

the longevity of Bulgarians was in part due to their consumption of fermented milks containing *Lactobacilli*. Now it is accepted that daily intake of these probiotics contribute to improvement and maintenance of well balanced intestinal flora and prevention of gastrointestinal disorders (Lavermicocca, 2006). *Lactobacillus* and *Bifidobacterium* are common species of bacteria used as probiotics for the production of dairy products (Fuller, 1989). Different strains of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii subsp. Bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, *Lactobacillus helveticus*, *Lactobacillus lactis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium lactis*, *Bifidobacterium adolescentis*, *Bifidobacterium essensis*, *Bifidobacterium laterosporus* and other species like *Escherichia coli* Nissle, *Saccharomyces boulardii*, *Streptococcus thermophilus*, *Enterococcus francium*, *Propionibacterium*, *Pediococcus* and *Leuconostoc* are considered as important species used as probiotics (Senok *et al.*, 2005; Shah 2007). Regardless of the diversity of these micro-organisms, lactic acid bacteria (e.g. *Lactobacilli*, *Streptococci* and *Bifidobacteria*) are important probiotics in probiotic preparations currently available in the market. These lactic acid bacteria are important normal constituents of the human gastrointestinal microflora and produce lactic acid as major metabolic product (Penner *et al.*, 2005). Both viable and nonviable probiotic microorganisms have probiotic properties, but viable cultures have better effects (Ouweland and Salminen, 1998). The most common forms for probiotics are dairy products and probiotics fortified foods. However, tablets, capsules, and sachets containing the bacteria in freeze-dried form are also available.

2.2.1 The history of probiotics

There is a mention of cultured dairy products in the Bible and the sacred books of Hinduism and their origin dates back to the dawn of civilization. The development of many of the traditional soured milk or cultured dairy products viz., kefir, koumiss, leben and dahi must have been favoured by climatic conditions

(Hosono, 1992). Many of these products are still widely consumed and had often been used therapeutically before the existence of bacteria was recognized (Shortt, 1999). At the beginning of the 20th century, the role of gut flora was unknown. The winner of Nobel prize, Ilya Ilyich Metchnikoff, in 1908 linked the health and longevity to ingestion of bacteria present in yoghurt (Metchnikoff and Mitchell, 1910; Metchnikoff, 2004). In 1907, he postulated that the bacteria viz., *Lactobacillus bulgaricus* and *Streptococcus thermophilus* that were involved in yoghurt fermentation suppress the putrefactive-type fermentations of the intestinal flora and the consumption of these yoghurts played a role in maintaining health. The discovery of bifidobacteria in breast-fed infants by Tissier also played a key role in establishing the concept that specific bacteria take part in the maintenance of health. Tissier in 1906 reported clinical benefits from modulating the flora in infants suffering from intestinal infections (Tissie, 1906). At the time, many others had a doubt about the concept of bacterial therapy and questioned whether the yoghurt bacteria (*L. bulgaricus*) were able to survive intestinal transit, colonize and convey benefits (Kulp, 1924). In the early 1920s, *L. acidophilus* milk was documented to have a settling effect on the digestion process (Cheplin and Rettger, 1922). It was believed that the ability of the microorganism to colonize and grow in the gut were essential for their efficacy, and therefore, the use of intestinal isolates was supported. In the early 1930s in Japan, Shirota carried out a research on the selection of the strains of intestinal bacteria that could survive passage through the gut and on the use of such strains to develop fermented milk for distribution in his clinic. His first product containing *L. acidophilus* Shirota that was subsequently named *L. casei* Shirota was the basis for the establishment of the company Yakult Honsha. Intestinal microflora had several functions became clear only at the end of the century (Guarner and Malagelade, 2003). Now, the health benefits derived from the consumption of foods with *Lactobacillus acidophilus*, *Bifidobacterium* and *L. casei* are well documented. Yoghurt starter cultures viz., *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* offer health benefits; however, they are not natural inhabitants of the intestine. Therefore, to consider yoghurt as

a probiotic product, *L. acidophilus*, *Bifidobacterium* and *L. casei* are incorporated as dietary adjuncts (Shah, 2007). The guidelines that are required for a product to be called a probiotic were published by FAO/ WHO in 2002. They require that strains must be designated individually and retain a viable count in the product formulation at the end of their shelf life. The probiotic definition requires that the efficacy and safety of probiotics be verified and thus, this assessment is an important part of their characterization for human use (Isolauri *et al.*, 2004).

2.2.2 Criteria for probiotic selection

The right selection and application of a probiotic strains in food materials exhibits fundamental impacts on qualitative aspects of final products, namely safety (related to the mentioned strains), health benefits (related to probiotics), sensory attributes and even, the price. Therefore, selecting the adequate probiotic strains is the first prerequisite for designing a specific probiotic food product. The incorporation of incorrectly identified probiotic bacteria in functional food products clearly has public health implications, by undermining the efficiency of probiotics and by affecting public confidence in functional foods (Huys *et al.*, 2006). Figure 2.1 highlights the main points that must be kept in mind before selecting a probiotic microorganism for food products.

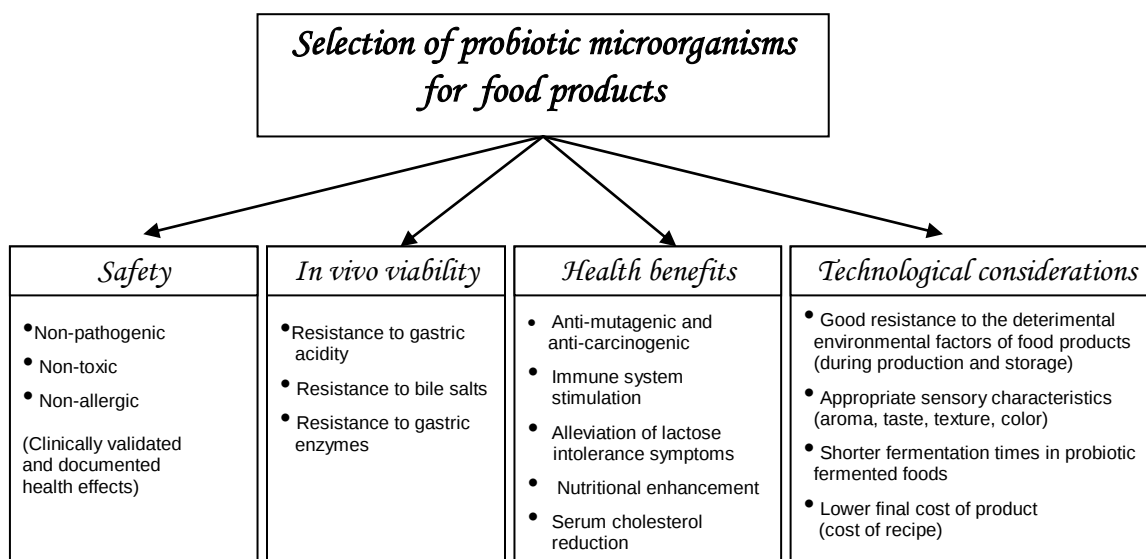


Fig. 2.1 Main criteria for selection of probiotic strains in food products (Source: Mortazavian *et al.*, 2012)

There is a large scientific consensus that, in order to assess the properties of probiotic bacterial strains, it is mandatory to perform a preliminary *in vitro* assessment (FAO/WHO, 2001, 2002). Strains belonging to species normally inhabiting the human gut have been shown to behave better when assayed for their *in vitro* resistance to low pH or to simulated gastric juice (Conway *et al.*, 1987). There is an ambiguous situation among the enteric species of lactobacilli, strains with a documented ability to colonize the human gut showed poor performance in the *in vitro* assay (Charteris *et al.*, 1998; Mishra and Prasad, 2005) but the same strains showed excellent results when analysed *in vivo* (Fonden *et al.*, 2000). This disagreement between *in vitro* and *in vivo* results points out the need to refine these kinds of tests. In India there were no regulatory guidelines for probiotic foods. In the absence of any such standards and guidelines, the chances for spurious products with false claims being marketed are high. It is therefore essential that these products fulfill some prerequisite conditions before being labeled as a 'probiotic product'. A holistic approach is therefore needed for formulating guidelines and regulations for the evaluation of safety and efficacy of probiotics in India which should be in consonance with current international standards. Keeping in view the above situation, a Task Force was constituted by ICMR, comprising of experts from varied fields to develop guidelines for evaluation of probiotics in food in India. The Task Force took into consideration the guidelines available in different parts of the world and deliberated on the various aspects to be covered. The guidelines by ICMR-DBT deal with the use of probiotics in food and provide requirements for assessment of safety and efficacy of the probiotic strain and health claims and labeling of products with probiotics. Various steps for evaluation of candidate probiotic strains include: (1) genus, species and strain identification; (2) *in vitro* tests to screen potential probiotic strains; (3) *in vivo* safety studies in animal models; (4) *in vivo* efficacy studies in animal models; (5) evaluation of safety of probiotics for human use; (6) evaluation of efficacy studies in humans; (7) effective dosage of probiotic strain / strains; (8) labeling

requirements; and (9) manufacturing and handling procedures. The critical *in vitro* parameters for probiotic selection are discussed briefly as under.

2.2.2.1 Acid tolerance

The variations in the pH of gastric acid occurs in the range of about 1.5-4.5 during a period of 2h , depending on the entering time and the type of gastric contents (Verdenelli *et al.*, 2009). Most of the microorganisms ingested get destroyed by the gastric juice (pH 2.0) in the stomach (Charteris *et al.* 1998). Minimum 90 min incubation time in acidic broth is essential because the time from entrance to release from the stomach is 90 min. However, the passage time is increased by further digestive processes (Cebeci and Gurakan., 2003). Therefore, resistance to human gastric transit is an important selection criterion for probiotic organisms. Recent development in new delivery systems and use of specific foods, clearly demonstrates that acid sensitive strains can be buffered through the stomach. It is, therefore, expected that the relatively much acid tolerant strains of *Lactobacillus* would show better gastric survival, if consumed along with fermented milk products or within a food matrix. Several studies have been conducted in the past to evaluate the effect of pH on the survivability of microorganism. *Lactobacilli casei* showed higher survival at pH 3.0 than at pH 2.0 (Mishra and Prasad, 2005). Mourad and Nour-Eddine (2006) screened eleven strains of *L. plantarum* for *in vitro* resistance to low pH. All tested strains survived an incubation period of 2h to 6h at pH 2.0 and pH 3.0 with decrease in survival percentage when the exposure time progressed. Cebeci and Gurakan (2002) studied 13 strains of *Lactobacillus plantarum* and they observed that none of the strains showed a dramatic decrease in viable counts after 90 min in MRS broth at pH 3.5. They then transferred all the strains to MRS agar (pH 3.5) and monitored the growth at 37 C for 24-96h and observed that out of the 13 strains only 10 were able to grow in acidified MRS agar (pH 3.5) after 96 h indicating good acid tolerance of *Lactobacillus plantarum*.

2.2.2.2 Bile Tolerance

Ingested microorganisms must survive numerous environmental extremes in the human gastrointestinal tract, as the liver secretes as much as a litre of bile into the intestinal tract each day therefore exposure to bile represents an important challenge. Bile is a digestive secretion that plays a major role in emulsification of lipids, also affects phospholipids and proteins of cell membranes. Therefore, for survival and subsequent colonization of gastro intestinal tract, the ability of pathogens and the desirable strains to tolerate bile is very important (Burns *et al.*, 2008). The ability of bacteria to survive depends on their resistance to bile, once they reach the small intestinal tract (Gilliland *et al.*, 1984), as bile entering the duodenal section of the small intestine has been reported to reduce the survival of bacteria by causing destruction of the susceptible lipids and fatty acids of cell membranes by its detergent activity (Jin *et al.*, 1998). According to Haung and Adams (2004) small intestine tolerance is of potentially more importance than gastric survival in probiotic selection. There is no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentration of bile salts in the small intestine is between 0.2 and 2% (Gunn, 2000). It was reported by Jamaly *et al.* (2011) that the bile resistance of *L. paracasei* strains varied from 3-65 percent at 1 percent bile concentration. Sieladie *et al.* (2011) isolated *L. plantarum* strains from cow milk and tested them for bile tolerance. They observed good survivability of *L. plantarum* strains at 0.4% bile concentration after 24h.

2.2.2.3 Cell Surface Hydrophobicity (CSH)

The adhesion ability of probiotics to intestinal epithelial cells is considered as one of the most important selection criteria. Several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells (Brassart *et al.*, 1994). The hydrophobic nature of the outermost surface of microorganism has been implicated in the attachment of bacteria to host tissue as this property could confer a competitive advantage, important for bacterial maintenance in human GI

tract (Keiley and Olson, 2000). Thus, a high cell surface hydrophobicity is considered an advantage in the colonization to mucosal surfaces (Ljungh and Wadstom, 1982). This adhesion ability of good bacteria to intestinal epithelial cells gives competition to the coming intruders inside host cell. Higher CSH may favour the colonization of mucosal surfaces and play a role in the adhesion of bacteria to epithelial cells by preventing wash-out especially in the small intestine where flow rates are relatively higher (Schillinger *et al.*, 2005). The ability to adhere can give information about the possibility of probiotics to colonize and may modulate the host immune system (Klayaung *et al.*, 2008). The variation in hydrophobicity to solvents reported by probiotic bacteria has been explained by the fact that adhesion depends upon the origin of strains as well as surface properties (Ambrosini *et al.*, 1998). The large differences in the cell surface hydrophobicity could be due to 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.

2.2.2.4 Antibiotic Susceptibility

One of the crucial criteria for the safety point of view of potential probiotics is antibiotic susceptibility, since bacteria used as probiotics may serve as host of antibiotic resistant genes, which can be transferred to pathogenic bacteria. (Klayaung *et al.*, 2008). As per the European Union (EU) Scientific Committee on Animal Nutrition (SCAN) guidelines, bacteria used in feeds should not contain any acquired antibiotic resistances (SCAN, 2002). Vancomycin is a glycopeptide antibiotic and has been reserved as a drug of “last resort” in the past, as it was used only after treatment with other antibiotics had failed. However, vancomycin resistance is now a widespread phenomenon among Lactobacilli. Some LAB strains of *L. casei*, *L. plantarum*, *Enterococcus* spp., *Pediococcus* spp. and *Leuconostoc* spp. have been reported to be resistant to vancomycin. (Mishra and Prasad, 2005). Out of the 13 *L. plantarum* strains studied by Cebeci and Gurakan (2003) for antibiotic susceptibility, all the strains were susceptible to erythromycin, clindamycin and cephazolin and four cultures showed resistance to penicillins. Verdenelli *et al.* (2009) carried out antibiotic susceptibility test of two probiotic

cultures viz., *Lactobacillus rhamnosus* and *Lactobacillus paracasei* and observed that both the strains were resistant to vancomycin, gentamicin.

2.2.2.5 Antimicrobial Activity

Antimicrobial activity is thought to be an important means for probiotic bacteria to competitively exclude or inhibit activities of harmful or pathogenic intestinal microbes. Inhibition of the growth of pathogen can be through production of antimicrobial components such as organic acid-Lactic acid, hydrogen peroxide and bacteriocins (Jin *et al.*, 1996). Lactobacilli are natural components of human intestinal microbial flora and these fermentative organisms produce organic acids such as acetic and lactic acids, which tend to lower the intestinal pH which, inhibit the multiplication of harmful or pathogenic microorganisms. Jamaly *et al.* (2011) isolated *L. paracasei* and *L. plantarum* strains from Moroccan fermented dairy foods and evaluated their antimicrobial activity. The workers observed that both the probiotic cultures had strong inhibitory against some common food borne pathogens. Several workers have reported inhibitory activity of lactobacilli against *Escherichia coli*, *Salmonella*, *Shigella*, *S. aureus*, *E. faecalis* and *B. cereus* etc. (Oyetayo, 2004; Reddy *et al.* 2006; Savadogo *et al.* 2004). Gaudana *et al.* (2010) observed strong inhibitory activity of *L. rhamnosus* CS25 strain (isolated from faeces of human child) against both Gram-positive and Gram-negative bacteria.

2.2.3 Health benefits of probiotics

The health benefits accredited to functional food are increasing and the probiotics are one of the fastest growing categories within food for which therapeutic evidence have been demonstrated in scientific researches (Soccol *et al.*, 2010). Several therapeutic applications of the probiotics include the alleviation of constipation, protection against traveller's diarrhoea, reduction of hypercholesterolaemia, protection against colon and bladder cancer, prevention of osteoporosis, food allergy and prevention of urogenital diseases (Lourens-Hattingh and Viljoen, 2001). The various health benefits mentioned above are discussed briefly as under.

2.2.3.1 Prevention of intestinal infections

Intestinal infection caused by the intake of pathogenic microorganisms with the contaminated water and food are the main causes of death. Under this circumstance, probiotics can assist in part the foodborne problematic situation, as it is demonstrated in several studies. The intestinal barrier maintains the epithelial integrity protecting the organism against bacterial or food antigens that could induce inflammatory processes leading to intestinal disorders (Hooper et al, 2001). Probiotics exert antagonistic effects on the growth of pathogens such as *Staphylococcus aureus*, *Salmonella typhimurium*, *Yersinia enterocolitica* and *Clostridium perfringens* (Gilliland and Speck, 1977). Probiotic bacteria enhance resistance against intestinal pathogens via antimicrobial mechanisms viz. competitive colonization and production of organic acids, such as lactic and acetic acids, bacteriocins and other primary metabolites, such as hydrogen peroxide, carbon dioxide and diacetyl (Mishra and Lambert, 1996). By competitive colonization, probiotic bacteria inhibit the adhesion of gastrointestinal pathogens to the intestinal mucosa (Conway, 1996). Production of organic acids, such as lactic and acetic acids, by probiotic bacteria lowers intestinal pH and thereby inhibits the growth of pathogens. These organic acids also increase peristalsis, thereby indirectly removing pathogens by accelerating their rate of transit through the intestine (Laroia and Martin, 1990). Numerous bacteriocins, such as nisin, lactobrevin, acidophilin, acidolin, lactobacillin, lactocidin and lactolin, have been reported to be produced by lactobacilli (Shahani and Chandan, 1979).

2.2.3.2 Immune enhancement

The ability of probiotic bacteria to modulate immunity and to improve the microbial balance of commensal enteric microorganisms offers the consumer a more biologically effective alternative to better health than the consumption of therapeutic drugs (Chin and Kailasapathy, 2000). *In vivo* and *in vitro* indices of immunity in healthy mice fed with *Lactobacillus rhamnosus* (HN001, DR20), *L. acidophilus* (HN017) and *B. lactis* (HN019, DR10) were examined by Gill et al.

(2000) and the results suggested that supplementation of the diet with these strains was able to enhance several indices of natural and acquired immunity. Christina (2008) reported that daily feeding of 1×10^8 colony-forming units (CFU) of the probiotic *Lactobacillus paracasei* ssp. *paracasei* strain F19 (LF19) to healthy term infants from 4 to 13 months of age resulted in maturation of adaptive immune responses. Rizzardini *et al.* (2011) conducted a randomised, double-blind, placebo-controlled, parallel-group study to investigate the immunomodulatory effects of a dairy drink containing *Lactobacillus paracasei* ssp. *paracasei* (*L. casei* 431). The workers reported that the intake of probiotic dairy drink significantly improved immune function by augmenting systemic and mucosal immune responses. The probiotics exert their immunity enhancing effects by augmenting both specific (antibody production, lymphocyte proliferation) and non-specific (phagocytic function) host immune response. The infant's immature intestinal immune system develops as it comes into contact with dietary and microbial antigens in the gut. The evolving indigenous intestinal microbiota have a significant impact on the developing immune system (Rautava and Isolauri, 2002). Many probiotic strains can influence innate defense mechanisms such as phagocytosis. Key players in the innate immune response include the phagocytic cells like neutrophils, monocytes and macrophages. Supplementation with *L. rhamnosus*, *L. acidophilus* or *B. lactis* resulted in a significant increase in the phagocytic activity of peripheral blood leucocytes and peritoneal macrophages compared with the control mice (Gill *et al.*, 2000). Kapila, (2004) also demonstrated that the feeding of probiotic culture *Lactobacillus casei* 19 and milk fermented with *Lactobacillus casei* 19 activated the peritoneal macrophages by increasing phagocytic activity. Salaria (2012) demonstrated significantly higher phagocytic activity in the mice group fed with probiotic supplemented infant formula than control and commercial formula fed group. Kapila *et al.*, (2012) depicted that feeding of probiotic fermented milk enhances phagocytic activity of the macrophages by conducting a comparative evaluation of oral administration of probiotic Lactobacilli-fermented milks on macrophage function. Enhancement in non-specific (β -galactosidase, β -

glucuronidase and phagocytic activity) immune system was observed by Yadav (2012) in mice fed with soy based probiotic yoghurts.

Gram-positive commensal strains of *Lactobacilli* *Lactobacillus*, *L. acidophilus* and *L. casei*, found in the human and mouse gastrointestinal tracts have been considered as probiotics with beneficial health effects, including enhanced lymphocyte proliferation (Gill *et al.*, 2000; Kirjavainen *et al.*, 1999). Feeding probiotics led to enhanced lymphocyte proliferative indices. Seven days pre-feeding with probiotic dahi significantly increased anti-*S. enteritidis* sIgA (secretary IgA) antibodies and lymphocyte proliferation in *S. enteritidis* infected mice (Shalini, 2008). Paturi *et al.*, (2008) observed that the proliferative responses of splenocytes to concanavalin A and lipopolysaccharide were significantly higher in mice fed *L. acidophilus* LAFTI L10 after the 14-day feeding trial. Statistical analysis carried out to evaluate efficiency of probiotic infant formula indicated that the probiotic infant formula showed enhanced lymphocyte proliferation assay compared to control and commercial group. The mitogen LPS showed the response of B-cells which were related to humoral immunity. The proliferative response of T-cells to Con A indicated cell mediated autoimmunity (Salaria, 2012).

Many probiotic strains are able to stimulate the production of IgA, which help maintain intestinal humoral immunity by binding to antigens, thereby limiting their access to the epithelium. Vandana (2011) reported enhancement in specific immune system (IgA) in mice groups fed with probiotic whey drinks. Gopalrao, (2011) also reported increased level of IgA by evaluation of probiotic attributes of *Lactobacillus reuteri* strain using animal model. Salaria, (2012) reported a statistically significant increase in the level of IgA than that of control group and commercial formula fed group after 35 days of dietary intake of probiotic infant formula. Enhancement in specific (increased IgA) immune system was observed by Yadav (2012) in mice fed with soy based probiotic yoghurts.

2.2.3.3 Prevention of cancer

The antitumour action of probiotics may be due to: (i) inhibition of carcinogens and/or procarcinogens; (ii) inhibition of bacteria that convert procarcinogens to carcinogens; (iii) activation of the host's immune system; (iv) reduction of intestinal pH to reduce microbial activity; and (v) alteration of colonic motility and transit time (McIntosh, 1996). Further work is needed to assess the long-term effects of probiotics on the host's immunity in relation to anti-carcinogenesis. Immune-based anticancer therapies have not yet demonstrated their efficacy because few clinical trials have been done (Commane *et al.*, 2005). *Lactobacillus* and *Bifidobacteria* strains and *E. coli* strain Nissle 1917 have shown anti-mutagenic activities *in vitro*, probably due to their capacity to metabolize and inactivate mutagenic compounds (Geire *et al.*, 2006). Roller *et al.*, (2004) correlated the inhibition of carcinogenesis in rats with changes in the immune activity, in response to probiotic consumption. Studies in animal models also suggest that increasing natural killer cell activity by probiotic consumption may have potential effects on delayed tumour development.

2.2.3.4 Mycotoxicosis

Some bacterial species are recognised for their capacity to prevent or limit mycotoxinogenic mould growth such as *Lactobacillus* (Sathe *et al.*, 2007; Garez *et al.*, 2009), *Lactococcus* (Florianowicz, 2001), *Pediococcus* (Mandai *et al.*, 2007) and *Leuconostoc* (Suzuki *et al.*, 1991). It has been reported that lactic acid bacteria are able to bind aflatoxin B1 *in vitro* and *in vivo* (Kankaanpaa *et al.*, 2000), but this property seems to depend on bacterial strain (Shah and Wu, 1999). In the study conducted by Gratz *et al.* (2006) rats received doses of aflatoxin B1 and were fed with oral gavage containing *Lactobacillus rhamnosus* strain GG (ATCC 53013). After administration, an increase in the aflatoxin B1 in fecal excretion was observed due to bacterial binding. The probiotic treatment prevented weight loss and reduced the hepatotoxic effects of the aflatoxin B1.

2.2.3.5 Cholesterol reduction

Rising evidence has indicated that lactobacilli and bifidobacteria could cause, when ingested, a significant reduction in serum cholesterol. This is because cholesterol synthesis mainly occurs in the intestines, hence the gut microflora promote effects on lipid metabolism (Socol et al., 2010). Some studies demonstrated that probiotics could promote a decrease in the blood cholesterol levels and increase the resistance of low-density lipoprotein to oxidation, therefore leading to a reduced blood pressure (Goel et al., 2006). Liong and Shah (2005), using *in vitro* experiments, reported that cholesterol could be removed from a medium by *L. acidophilus* not only through assimilation during growth, but also through binding of cholesterol to the cellular surface. This mechanism was proposed when both non-growing cells and dead cells were also found to remove cholesterol. Nguyen et al. (2007) evaluated *L. plantarum* PH04 as a potential probiotic with cholesterol-lowering effect in mice. Kaushik et al. (2009) demonstrated that the indigenous *L. plantarum* Lp9 exhibited cholesterol-lowering properties. Tanida et al. (2008) noted that long-term ingestion of *Lactobacillus paracasei* ST11 (NCC2461) reduced body and abdominal fat mass. Their results suggest that *L. paracasei* NCC2461 has an anti-obese action, and in this mechanism, autonomic nerves may function to facilitate the lipolytic and thermogenic responses in rats.

2.2.3.6 Hypertension

Recent studies have also suggested that probiotics could have beneficial effects beyond some metabolic disorders such as hypertension. Primary hypertension is caused by various factors and the predominant causes include hypercholesterolemia (Lye et al., 2009). Probiotics convert milk protein into bioactive peptides, which have antihypertensive effect. Milk peptides may exert antihypertensive effects also through other mechanisms, such as inhibition of the release of endothelin-1 by endothelial cells, stimulation of the bradykinin activity, enhancement of the endothelium-derived nitric oxide production and enhancement of the vasodilatory action of binding to opiate receptors.

Angiotensin I-converting enzyme (ACE), a dipeptidyl carboxypeptidase, catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II and plays an important physiological role in regulating blood pressure and fluid and salt balance in mammals (Soccol *et al.*, 2010).

2.2.4 Probiotic food products

Probiotic food is a food product containing viable probiotic microorganisms in sufficient populations incorporated in a suitable carrier (Gibson and Roberfroid, 1995). Probiotic microorganisms are available in three different types for direct or indirect human consumption: 1) culture concentrate to be added to a food (dried or deep-freeze form) for industrial or home uses, 2) food products (fermented or non-fermented), and 3) dietary supplements (drug products in powder, capsule or tablet forms) (Tannis, 2008). Consumption of probiotic cells via food products are the most popular and widespread way. For humans to develop beneficial effects, ingestion of 10^6 to 10^9 viable cells per day is necessary (Lee and Salminen, 1995). The viability and metabolic activity must be maintained in all the steps of the food processing operation and they must be able to survive in the gastrointestinal tract to confer health benefits to the host (Sanz, 2007). Different types of food matrices have been used as carrier of probiotics such as various types of cheese, ice creams, milk-based desserts, powdered milk for newborn infants, butter, mayonnaise, powder products or capsules and fermented food of vegetable origin (Tamime *et al.*, 2005).

2.2.4.1 Dairy products

Dairy products are considered as ideal vehicle for the delivery of probiotic bacteria to the human gastrointestinal tract. Cheese, yoghurt, ice cream and other dairy products are the frequently used matrices. Ice cream and frozen dairy desserts among various dairy products are the vehicles that could be used to deliver probiotics. These products have the advantage of storage at low temperatures that minimizes the temperature abuse and raises the viability at the time of consumption (Cruz *et al.*, 2009). The most important advantage is that they are consumed by people of all ages and are composed of milk proteins, fat and lactose as well as other compounds that are required for bacterial growth.

Table 2.3 Commercial probiotic dairy products on the European market

Type of product	Trade name	Probiotic microorganism
Fermented milk with high viscosity	Bifisoft, Bifidus, Bioghurt, Biofit, Biola, Biologic bifidus, Cultura Dofilus, Aktiv, RELA, Verum, Vifit Vitamel, Vitality, Weight Watchers, Yogosan Milbona, ProViva, probiotisches Joghurt, Gefliac, Dujat Bio Aktiv, BiofardePlus, Fit&Aktiv, Ekologisk Jordgubbs, LC 1, Fjall Yoghurt	<i>L. acidophilus</i> , <i>L. acidophilus</i> La5, <i>L. casei</i> L19, <i>L. rhamnosus</i> (LGG, LB21 and 271), <i>L. casei</i> , <i>L. johnsonii</i> , <i>L. plantarum</i> 299v, <i>L. reuteri</i> , <i>Lactococcus lactis</i> ssp. <i>lactis</i> L1A, <i>B. bifidum</i> , <i>B. animalis</i> ssp. <i>lactis</i> BB-12, <i>B. animalis</i> ssp. <i>animalis</i> ,
Fermented milk with low viscosity (e.g. cultured buttermilk, dairy drink, yoghurt drink)	A-fil, Actimel, Aktifit, AB-piima, Bella Vita, Bifidus, Biofit, Biola, Casilus, Cultura, Emmifit, Everybody, Fit&Aktiv, Fundo, Gaio, Gefilac, Kaiku Actif, LC 1 Gol, LGG+, Onaka, Oresundsfil, Philura, Probiotic drink, ProViva, Pro X, Verum, ViktVaktarna, Vitality, Le'Vive, Yakult, Yoco Acti-Vit	<i>L. acidophilus</i> , <i>L. acidophilus</i> La5, <i>L. casei</i> (F19, 431, Imunitas, Shirota), <i>L. rhamnosus</i> (LGG, LB21 and 271), <i>L. johnsonii</i> , <i>L. plantarum</i> 299v, <i>L. reuteri</i> , <i>L. fortis</i> , <i>Lactococcus lactis</i> ssp. <i>lactis</i> L1A, <i>B. bifidum</i> , <i>B. animalis</i> ssp. <i>lactis</i> BB-12, <i>B. animalis</i> ssp. <i>animalis</i> , <i>B. longum</i> BB536.
Non-fermented dairy products (e.g. milk, ice cream)	Gefilus, God Hals, RELA, Vivi Vivo	<i>L. rhamnosus</i> LGG, <i>L. plantarum</i> 299v, <i>L. reuteri</i>

(Source: Tamime *et al.*, 2005)

However, some probiotic species showed a reduction in the viability during the manufacture and freezing of ice cream (Alamprese *et al.*, 2002). For the efficient production of probiotic ice cream, selection of the oxygen-resistant strains is important since the incorporation of air (overrun) in the mixture occurs in the production process, that is harmful to microaerophilic and anaerobic strains such as *Lactobacillus* sp. and *Bifidobacterium* sp. The addition of whey protein owing to their buffering power may also enhance the viability of some probiotics. In addition, the incorporation of prebiotics in yoghurt formulations could stimulate the growth and activity of probiotics. In this regard, fructooligosaccharides proved effective in maintaining the probiotic viability (Capela *et al.*, 2006).

The concept of probiotic is not new to even Indian consumers. Dahi is one of the five elixirs (Panchamrita) often used in Hindu rituals. The fermented milk or dahi is beneficial and is already widespread in India, because, traditionally these products have been used since vedic times for the treatment of conditions such as stomach upsets, especially diarrhea. Probiotic food concept has just come in light with the introduction of some dairy products. In India, leading probiotic players are GCMF Ltd, Amul (AMUL ProLife – Dahi, Ice cream and Lassi); Nestle, India (Nesvita-Dahi); and Yakult-Danone, India (Yakult – Probiotic drink) (Table 2.4).

Various studies in the field of probiotics have been conducted and can be summarized as under: World Health Organization recommends use of yoghurt in the management of acute diarrhoeal disorders based on evidence in human intervention studies (Boudraa *et al.*, 1990). Dahi alongwith usual diet is known to reduce number of episodes as well as duration of diarrhoea (Ullman and Korzenik, 1998). Yoghurt consumption helps in improving immune response in immunocompromised elderly population (Meydani and Ha, 2002). Agarwal and Bhasin (2002) showed that Indian Dahi controls as well as prevents diarrhea. At National Dairy Research Institute, Karnal, Vibha (2004) developed probiotic dahi with two combinations viz *L.acidophilus* (NCDC 14) @ 1%, *L.casei* (NCDC 19) @ 0.4% and *L.lactis* ssp. *Lactis biovar. diacetylactis* (NCDC 60) @ 0.6% and *L.casei* (NCDC 19) @ 1.0% and mixed dahi culture NCDC 167 @ 0.6% were

found better in terms of physico-chemical and sensory qualities with probiotic count more than 10^7 CFU/ml. Rajpal (2006) developed Acidophilus-bifidus dahi (AB dahi) using *L.acidophilus*, *Bifidobacterium bifidum* and mixed mesophillic cultures *L.lactis ssp. cremoris* and *L.lactis ssp. Lactis biovar. diacetylactis*. Kumar (2009) has worked on synbiotic ice-cream using *L.acidophillus* (NCDC 13) and reported that the viability of the culture was more than 10^7 cfu/ml after a storage period of 120 days. Yadav (2012) and Salaria, (2012) developed a soy based probiotic yoghurt and probiotic infant food formulation, respectively.

Table 2.4 Probiotic products and their manufacturers in India

PRODUCTS	COMPANY/MANUFACTURER
Probiotic curd	Heritage Foods (India) Ltd.
'b-Activ' probiotic curd (<i>L. acidophilus</i> and <i>B. lactis</i> strain BB12)	Mother Dairy
'Nesvita' probiotic yoghurt	Nestle
Probiotic ice creams, 'Amul Prolife' 'Prolite' and 'Amul Sugarfree'	Amul (Brand of Gujarat Cooperative Milk Marketing Federation Ltd.)
Yakult	Yakult Danone India (YDI) Private Limited
Probiotic drugs	Ranbaxy (Binifit)
Probiotic drugs	Dr. Reddy's Laboratories
Probiotic drugs	Zydus Cadila
Probiotic drugs	Unichem
Probiotic drugs	JB Chem
Probiotic drugs	GlaxoSmithKline
Fructo-Oligo Saccharides, Probiotic drugs	Glenmark Alkem Labs
Probiotic curd with <i>L. casei</i> strain <i>Shirota</i>	Yakult Danone India (YDI) Private Limited

(Source: Bhadoria and Mahapatra, 2011)

2.2.4.2 Non-dairy products

The presence of allergens and requirement of cold environments are important limitations of using dairy products as delivery matrix for probiotics. Other claims related to probiotic products are lactose intolerance and fat content. This fact has led to the use of non-dairy matrices for the development of new products with probiotics. Some of the matrices used in the development of non-dairy probiotic products includes fruits, vegetables, legumes and cereals. Fruits and vegetables can be considered good matrices as they are rich in nutrients such as minerals, vitamins, dietary fibres, and antioxidants. Studies have been conducted on the development of different probiotic fruit juices (Yoon *et al.*, 2004). However, the protection against acid conditions must be considered before the incorporation of probiotics in fruit juices. This limitation can be overcome by microencapsulation technologies, which entrap the cells into matrices with a protective coating. Various vegetable gum and gelatin have demonstrated a good protection for acid-sensitive *Bifidobacterium* and *Lactobacillus* (Sultana *et al.*, 2000; O’Riordan *et al.*, 2001; Chandramouli *et al.*, 2004). Encapsulation processes in milk protein have also been studied by Heidebach *et al.*, 2009). Species belonging to *Lactobacillus* and *Leuconostoc* genera i.e. *L. plantarum*, *L. casei* and *L. delbrueckii* are the probiotic strains usually found in vegetable materials. They were able to grow in cabbage juice without nutrient supplementation and reached 10^8 CFU/mL after 48 h of incubation at 30 °C (Yoon *et al.*, 2006) and also in beet juice (Kyung *et al.*, 2005). In cereals, the fermentation with probiotic microorganisms could be beneficial due to the decrease of nondigestible carbohydrates (poly- and oligosaccharides), the improvement of the quality and level of lysine, increased availability of the B group vitamins, degradation of phytates and release of minerals (e.g. manganese, iron, zinc, and calcium) (Blandino *et al.*, 2003). Oat-based substrates facilitate the growth of *L. reuteri*, *L. acidophilus* and *B. bifidum* (Martenson *et al.*, 2002). An enhancement of 14 and 11 % in thiamine and niacin contents, respectively, when food mixture based on germinated barley flour with whey powder and tomato pulp were autoclaved and fermented by *L. acidophilus*

was observed by Arora *et al.* (Arora *et al.*, 2010). Also, non-germinated and germinated mixture showed an increase of 31 and 34 % in lysine content, respectively, after autoclaving and fermentation, showing the importance of the germination and fermentative process on the bioavailability and improvement of the nutritional quality of foods.

2.2.5 Challenges in addition of probiotics in food products

Probiotic cultures are increasingly being used in health-promoting food products. However, to develop a functional food product with probiotics, there exist a number of technological challenges that must be addressed. Some of the important challenges that must be considered before addition of probiotics in food products when developing functional foods include: 1) type or form of probiotic to be used; 2) level of addition required to have a beneficial effect; 3) toxicity; 4) effect of processing steps on viability; 5) determination of the cell populations added in the product; 6) stability during storage; 7) changes in sensory properties of the food (Champagne *et al.*, 2005)

Viability of probiotic microorganisms (the number of viable and active cells per g or mL of probiotic food products) at the time of consumption is a critical value of these products as it determines their medicinal efficacy (Khorbekandi *et al.*, 2011; Tamime *et al.*, 2005). There is no world-wide agreement on the minimum viable probiotic cells per gram or milliliter of probiotic product, however, the concentrations of 10^6 and 10^7 - 10^8 cfu mL⁻¹ (cfu g⁻¹), respectively, have been accepted as the minimum and satisfactory levels generally. In order to have a positive effect in the intestinal tract some specific requirements regarding food products should be fulfilled. First, probiotics need to resist the manufacturing process; second, they should remain viable during the storage period in the commercial products until the end of the shelf-life. Many factors influence the viability of probiotic microorganisms in food products during production and storage periods. Figure 2.2 implies main factors affecting the viability of probiotics in food products and during delivery through gastrointestinal tract.

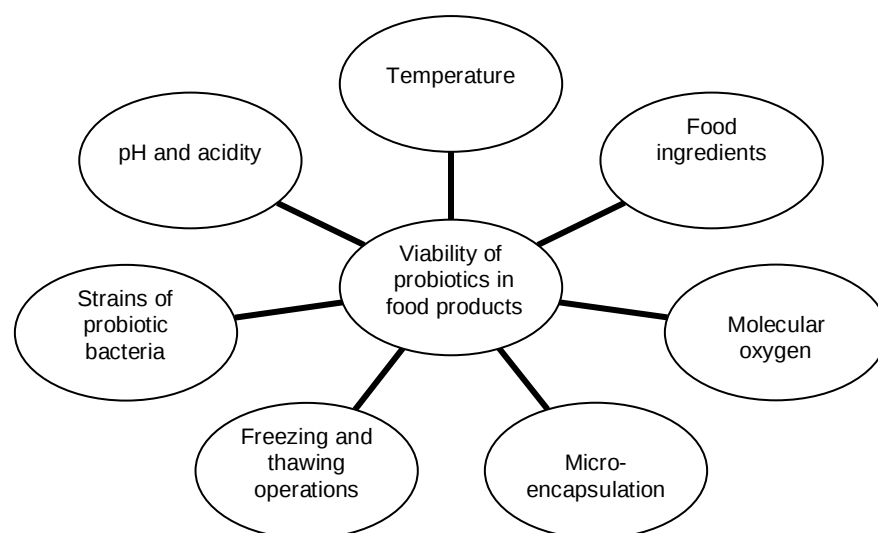


Fig. 2.2 Main factors affecting the viability of probiotics in food products
(Source: Mortazavian *et al.*, 2012)

The selection of a probiotic strain to be added is a crucial step in probiotic based functional food development (Mattila-Sandholm *et al.*, 2002). Every bacterial strain cannot be easily produced industrially because of low yields in the medium of growth and poor survivability during freezing and freeze-drying (Saxelin *et al.*, 1999). The strain must reach high numbers in fermented milks and may produce health promoting metabolites such as peptides even if they do not grow in the gastrointestinal tract (Champagne *et al.*, 2005). The probiotics can be selected on the basis of general microbiological criteria that refer to safety, technology, performance, and health benefits (Gibson and Fuller, 2000). No single strain has demonstrated all the health properties ascertained for lactic acid bacteria (Salminen and Playne, 2001). Some strains are effective in enhancing immune system while others can reduce lactose assimilation. Therefore, strain selection must be made considering the health claims that will be made for the target consumer group (Champagne *et al.*, 2005). The biomass yield at large scale and ease of concentration and survival to freezing and drying are the critical technological attributes that the strain should possess (Mercenier *et al.*, 2002). In the strain selection process, it is not only the strain that is

important, but also the method by which it is prepared is critical. Aspects of fermentation technology, drying technology, and microencapsulation will influence the functionality of probiotics significantly (Mattila-Sandholm *et al.*, 2002).

Initially, the standards for addition level have been adopted to provide bacterial concentrations that were technologically attainable and cost effective, rather than to achieve a specific health effect in humans (Sanders *et al.*, 1996). With an increase in availability of data on bacterial population requirement, it becomes clear that numbers vary as a function of the strain and the health effect desired (Champagne *et al.*, 2005). Till now, it is not clear how many cells are required in the gastrointestinal tract to significantly affect the environment of the gut, but it is believed to be between 10^6 and 10^8 CFU/g of intestinal contents (Richardson, 1996). However, the number of cells is not the only aspect to consider, but it is believed that the food carrying the cultures also influence the level of activity (Salminen and Playen, 2001). Ideally, the decision for the level of addition of probiotic the decision should be based on scientific data obtained for a given food, with a given number of cells of a specific strain (Champagne *et al.*, 2005).

According to several workers, probiotic cultures from human origin are preferable and is logical at first glance. However, lactic acid bacteria are very common in nature and strains isolated from plant material could prove to be excellent probiotics (Champagne *et al.*, 2005). Richardson (1996) suggested another approach to demand that the strain should have a long history of occurrence in foods for human consumption. However, there is currently insufficient evidence to suggest that the use of lactic acid bacteria in food fermentation poses any danger. The strain selection process must take into account various safety features in addition to the technological properties including: GRAS (generally recognized as safe) status, possession of a desirable antibiogram profile, nonpathogenic (even to immunocompromised hosts) and noninflammation - causing microorganisms (Collins *et al.*, 1998).

In the processing of foods a number of technological operations are used and many of them have an effect on the growth and survival of probiotics in the food product. The type of substrate, heating and freezing are the critical steps affecting survival of probiotics in the food (Champagne *et al.*, 2005). The food matrix formulation is a major technological factor influencing functionality of probiotics (Mattila-Sandholm *et al.*, 2002). According to several workers, non-viable probiotics can have beneficial effects in the host. Therefore, it might not be necessary to maintain high viability during storage for several strains and good growth during manufacturing process could be sufficient for beneficial effects on health. Studies suggest that probiotic cultures can grow in many types of milk. However, most studies have been performed on cow's milk, data is available on buffalo (Murad *et al.*, 1997) and goat (Gomes and Malcata, 1998). Many probiotic cultures cannot multiply in pure milk; supplementation of milk with yeast extract can solve this problem (Saxelin *et al.*, 1999). Yeast extract can be replaced by a combination of amino acids, minerals and ribonucleotides (Elli *et al.*, 1999) but there are chances of ineffectiveness of this strategy (Gomes *et al.*, 1995). The strategy for improving the growth of probiotic cultures by the addition of novel ingredients has led to development of new products. Such novel dairy blends include tomato juice (Prajapati *et al.*, 1987), peanut milk (Murad *et al.*, 1997), soy milk (Murad *et al.*, 1997) and rice (Rani and Khetarpal, 1999). These studies suggest that plant-based supplements are valuable in enhancing growth of probiotics (Mital and Garg, 1992; Mital *et al.*, 1974; Murti *et al.*, 1993).

Starters are typically added to fermented products for technological purposes like acidification, texture, flavor, etc. When manufacturing fermented products, the addition of probiotic cultures to the normal starter generally results in slower growth of the probiotic strains than if they were added alone in milk (Samona and Robinson, 1994 and Roy *et al.*, 1995). This could be partially related to the production of bacteriocins or other inhibitors produced by the starter culture (Mattila-Sandholm *et al.*, 2002). However, faster growth of typical starter, rapid acidification and shorter fermentation times in the presence of starter culture are probably the dominant factor for limiting the growth of

probiotics during manufacture (Champagne *et al.*, 2005). Heating in processing of food also affects the survival of probiotics in foods. Temperatures below 45°C are usually not detrimental to probiotics. Heating of products over 45°C will destroy at least a fraction of the population, depending on the temperature (Sakai *et al.*, 1987) and the strain. Food processes that include a heating step above 65 °C are highly detrimental to probiotic cultures (Champagne *et al.*, 2005). The freezing process does not seem to affect the cells' sensitivity to bile salts (Tamime *et al.*, 1995). However, the effect of freezing on the subsequent sensitivity to stomach acid remains to be determined. Apart from the above factors and supplementation of milk with various growth supplements, several changes in processing steps have been proposed to promote the growth of the probiotic cultures viz., changes in temperature of incubation, addition of enzymes, adding compounds that affect the redox conditions of the medium and encapsulation (Champagne *et al.*, 2005).

The determination of the viable population in functional foods is an important feature to assure the consumer that the product they are purchasing follows certain norms. Several methods could be applied for the detection of probiotics in food products, but plate count methods are preferable for quality control measurements in food products. It is, therefore, necessary to have a growth medium that selectively promotes different probiotic bacteria while suppressing the other bacteria. The characterization of probiotic bacteria is important for food industries to identify particular strain required for product manufacture. The new techniques and approaches of molecular biology would make it possible to clarify species of probiotic strain (Champagne *et al.*, 2005).

Since, the ability of probiotics to survive during processing and storage are not linked (Prajapati *et al.*, 1987), it is necessary to specifically examine factors that affect survival during storage. The biological effect of probiotic culture is related to the strain used and form and number consumed. pH and oxygen conditions have significant effects. Acid pH tolerance in probiotic bacteria is strain dependent, and Bifidobacteria strains are more sensitive than Lactobacillus strains (Godward *et al.*, 2000). Even though this specific mechanism is still not

well established, reports suggest the influence of a particular enzyme, H^+ - ATP-ase (Takahashi *et al.*, 2007). To prevent the adverse effect of pH on the quality of the product, the simplest measure is to select strains resistant to low pH values. The application of sub-lethal stress conditions, such as prolonged exposures of probiotic bacteria to very low pH values (2.0–3.0) for a determined time present good results and lead to the generation of a stable and highly acid resistant strain (Collado and Sanz, 2005). This treatment also introduces phenotypic changes which improve biological properties, as higher fermentative ability and enzymatic activities. The other possibility is the addition of chemical compounds – carbonate, and citrate salts – at acceptable levels before or during the incubation, in order to eliminate acidic stress through chemical reactions. The resultant products would be metabolized in a subsequent step, with consequent provision of a favorable condition for probiotic bacterial growth (Zhao and Li, 2008). This finding deserves more investigation.

Oxygen also affects the probiotic cultures in two ways. The first is a direct toxicity to cells. Certain probiotic cultures are very sensitive to oxygen and die in its presence (Dave and Shah, 1997) presumably due to the intracellular production of hydrogen peroxide. The second way through which oxygen affects the probiotic cultures is indirect. When oxygen is in the medium, certain cultures, particularly *Lb. delbrueckii* (Villegas and Gilliland, 1998) excrete peroxide in the medium. A synergistic inhibition of bifidobacteria by acid and hydrogen peroxide has been demonstrated (Lankaputhra *et al.*, 1996). Last but not the least, a successful market introduction of the functional food is linked to good flavor and texture of the food. Ice cream and ice milk appear to be good products for the delivery of probiotic bacteria. When small quantities of concentrated cultures are introduced, the sensory properties are not effected (Kebary *et al.*, 1998).

2.2.6 Safety

Theoritically, probiotics may be responsible for four types of side effects in susceptible individual's viz., systemic infections, deleterious metabolic activities, excessive immune stimulation, and gene transfer (Marteau, 2001; Marteau and

Seksik, 2004). However, lactobacilli and bifidobacteria rarely cause infections in humans (Salminen *et al.*, 1998; Borriello *et al.*, 2003; Ishibashi and Yamazaki, 2001). This lack of pathogenicity extends across all age groups including immunocompromised individuals (Cohendy, 1906). Traditional lactic acid bacteria (LAB) strains have a long history of safe use and different species of *Lactobacillus* and *Enterococcus* have been consumed daily since humans started consuming fermented milk as food. However, the safety aspects must be considered and possible adverse effects be continuously evaluated. Strains of *Lactococcus* and *Lactobacillus* are most commonly given the GRAS status while the members of the genera *Streptococcus*, *Enterococcus* and some other genera of LAB are considered opportunistic pathogens. The safety of probiotics has been considered and clinical reports have drawn attention to isolate cases of human bacteraemia (Gasser, 1994; Aguirre and Collins 1993; Saxelin *et al.*, 1996). The safety of commercial LAB has been supported by surveillance studies (Gasser, 1994; Saxelin *et al.*, 1996; Adams and Marteau, 1995). No harmful effects have been observed in controlled clinical studies with lactobacilli and bifidobacteria as indicated by available data (Salminen *et al.*, 1998). Three approaches can be used to assess the safety of a probiotic strain: 1.) studies on the intrinsic properties of the strain; 2.) studies on the pharmacokinetics of the strain (survival, activity in the intestine, dose–response relationships, faecal and mucosal recovery); 3.) studies searching for interactions between the strain and the host. The Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food recognized the need for guidelines to set out a systematic approach for the evaluation of probiotics in food, to substantiate the health claims. Consequently, a Working Group was convened by FAO/WHO to generate guidelines and recommend criteria and methodology for the evaluation of probiotics, and to identify and define what data need to be available to accurately substantiate health claims. The aims of the Working Group were to identify and outline the minimum requirements needed for probiotic status. The guidelines were prepared in 2002 to meet this objective. These guidelines are available in: Joint FAO/WHO Working Group Report on

Drafting Guidelines for the Evaluation of Probiotics in Food (FAO/WHO 2002). In India there are no regulatory guidelines for probiotic foods. A Task Force was constituted by ICMR, comprising of experts from varied fields to develop guidelines for evaluation of probiotics in food in India. The Task Force took into consideration the guidelines available in different parts of the world and deliberated on the various aspects to be covered (ICMR, 2011).

2.3 ICE CREAM

Ice cream (glaces a la crème in French; Eiskrem in German; helado in Spanish; Morozhenoe in Russian; Roomijs in Dutch; Fledies in Danish; Gelato in Italian; Sorvetes de crème in Portugese) is a frozen dairy product prepared by freezing a mix with agitation, for proper incorporation of air in the mix and uniformity of consistency. Ice-cream is a highly acceptable product by children, adolescents, and adults, as well as by the elderly public. Due to its refreshing traits, it is consumed more in the summer, but nowadays it is consumed throughout the year. Because of recent advances in production and rapidly developing technology, ice cream industry has become a profitable industry. Ice cream has become a high quality food due to the diverse ingredients and methods of freezing used in its manufacture and thus resulted in more than 200 different types of ice cream.

Food Safety and Standards Regulations defines Ice Cream as the product obtained by freezing a pasteurized mix prepared from milk and /or other products derived from milk, with or without the addition of nutritive sweetening agents, fruit and fruit products, eggs and egg products, coffee, cocoa, chocolate, condiments, spices, ginger and nuts and it may also contain bakery products such as cake or cookies as a separate layer and/or coating. The said product is frozen hard, has a pleasant taste and smells free from off flavor and rancidity, may contain food additives permitted in these regulations and must conform to the compositional and microbiological requirements specified.

2.3.1 Historical background

The origins of ice cream are known to reach back the second century B.C., however no specific date of origin nor the inventor has been irrefutably credited with its discovery. Alexander the Great enjoyed snow and ice flavored with honey and nectar. Biblical references show that King Solomon was also fond of iced drinks. Nero Claudius Caesar (A.D. 54-86), during the Roman Empire used to send runners frequently into the mountains to bring snow, which was then flavored with fruits and juices. Around thousand years later, Marco Polo returned to Italy from the Far East with a recipe that resembled what is now called sherbet. It was sometime in the 16th century, according to historians' estimate, that this recipe evolved into ice cream. England seems to have discovered ice cream around the same time or even earlier than the Italians. It was called as "Cream Ice" and appeared regularly at the table of Charles I during the 17th century. It was in 1553 that France was introduced to similar frozen desserts by the Italian Catherine de Medici when she became the wife of Henry II of France. Until 1660 ice cream was not available to the general public.

The first official account of ice cream came from a letter written in 1744 by a guest of Maryland Governor William Bladen. The first advertisement for ice cream in United States of America appeared in the *New York Gazette* on May 12, 1777, when confectioner Philip Lenzi announced that ice cream was available "almost every day." Records kept by a Chatham Street, New York, merchant show that President George Washington spent approximately \$200 for ice cream during the summer of 1790. The first ice cream parlor in America opened in New York City in 1776. American colonists were the first to use the term "ice cream". The name came from the phrase "iced cream" that was similar to "iced tea". The name was later abbreviated to "ice cream" the name we know today.

In 1813, Dolley Madison served a magnificent strawberry ice cream creation at President Madison's second inaugural banquet at the White House. Until 1800, ice cream was a rare and exotic dessert enjoyed mostly by the elite.

Around 1800, insulated ice houses were invented. In 1846, Nancy Johnson patented a hand-cranked freezer that established the basic method of making ice cream still used today. William Young patented the similar "Johnson Patent Ice-Cream Freezer" in 1848. In 1851, manufacturing ice cream became an industry in America, pioneered by a Baltimore milk dealer named Jacob Fussell. The production of ice cream increased because of technological innovations, including steam power, mechanical refrigeration, the homogenizer, electric power and motors, packing machines, and new freezing processes and equipment. Alfred Cralle patented an ice cream mold and scooper used to serve on February 2, 1897. The treat became both distributable and profitable with the introduction of mechanical refrigeration. Around 1926, the first commercially successful continuous process freezer for ice cream was invented by Clarence Vogt that enabled the production of frozen products on a faster and larger scale. In 1940, the first Dairy Queen ice cream shop was opened near Chicago, Illinois.

In India, the growth of ice cream is traced back to a restaurant at Calcutta called "Magnolia" run by an Italian caterer who was the first one to prepare and serve ice cream on western bases. Later, in the year 1956, ice cream machinery was imported by Kwaliti Ice-cream Company, which was the beginning of commercial production and marketing of the product (Lamba, 1998).

2.3.2 Legal standards in India

According to Food Safety and Standards (Food Products Standards and Food Additives) Regulations, Ice Cream shall conform to the microbiological requirements specified in Table 2.6 and to the following requirements (Table 2.5), namely:—

Table 2.5 Requirements of ice cream according to FSSAI

Requirement	Ice cream	Medium Fat Ice cream	Low Fat Ice cream
Total Solid	Not less than 36.0 percent	Not less than 30.0 percent	Not less than 26.0 percent
Wt/Vol (g/l)	Not less than 525	Not less than 475	Not less than 475
Milk Fat	Not less than 10.0 percent	More than 2.5 percent but less than 10.0 percent	Not more than 2.5 percent
Milk Protein (Nx6.38)	Not less than 3.5 percent	Not less than 3.5 percent	Not less than 3.0 percent

Table 2.6 Microbiological Standards for ice cream according to FSSAI

S.No.	Requirements	Sampling Plan	Ice cream, frozen dessert, milk lolly, ice cream candy
1.	Total Plate Count	m	2,00,000/g
		M	2,50,000/g
2.	Coliform Count	m	50/g
		M	100/g
3.	E.coli	M	Absent/g
4.	Salmonella	M	Absent/25g
5.	Staph. aureus (coagulase +ve)	M	Less than 10/g
6.	Yeast and mold count	M	Less than 10/g
7.	Listeria monocytogenes	M	Absent/g

m = represents an acceptable level and values above it are marginally acceptable in terms of the sampling plan.

M = values above M are unacceptable in terms of the sampling plan and detection of one or more samples exceeding this level would be cause for rejection of the lot.

2.3.3 Ice cream market

According to Paul Thachil, CEO – Dairy & Foods, Mother Dairy Fruit & Vegetable, the ice cream industry has grown at a healthy rate of 12% year-on-year. The growth in Ice cream industry has been primarily due to strengthening of distribution network and cold chain infrastructure. Mobile Vending Units have been increasing every year to reach out to a larger set of consumers. Nowadays, consumers also have the choice of trying out varied product offerings from different brands to keep them excited.

Ice cream market in India is valued at Rs. 2,400 crore per annum of which the organized sector contributes to Rs. 950 crore (Bhusan, 2007). In India, GCMF's Amul, NDDB's Mother Dairy, Hindustan lever's Kwality Walls, Vadilal, Baskin Robbins are the major players and the regional brands accounts for the rest. Almost all the state co-operative Dairy Federations in the country are also manufacturing and marketing the same under their own brand names. The non-organized sector accounts for 60% of Indian ice cream market, but it is now decreasing considerably in urban areas. The market for organized sector is limited to metropolitan cities.

India's per capita consumption of ice cream is just 500ml compared to 30 litres in the US and 1.2 litres in China. In India, there is scope for more growth than the 8-10% observed over the past few years. The western region of the country has a higher consumption than the all India average. Almost 35% of the ice cream sold in the country is consumed in the western region with Mumbai being the major city followed by 30% in north, 20% in south and 15% in eastern and central India. Delhi and Gujarat together contributes for 30% of country's ice cream market. In the eastern region kolkata is the major market for ice cream.

Legal requirement for minimum percentage of fat, refrigerated transport, taxes, absence of nation wide efficient and reliable cold chain system, etc. are the reasons for three times higher prices than those prevailing in America. Strong distribution network, cold storage and freezer cabinets are must for healthy growth of ice cream industry. In 2008-2009, percentage market share of different

ice cream brands in India was: Amul: 38%, Kwality Walls: 14%, Vadilal: 12% and Mother Dairy: 8% (Soni, 2009).

2.3.4 Physico-chemical properties of ice cream

The physico-chemical properties of ice cream depend on the quality and quantity of ingredients and processing conditions used for its preparation. Some of the important properties include acidity and pH, specific gravity, viscosity, overrun, meltdown time and firmness. Acidity of ice cream is related to the composition of ice cream mix. The natural or normal acidity of mix is due to the milk proteins, minerals and dissolved gases (Marshall and Arbuckle, 1996). A high percent acidity is undesirable as it contributes to excessive mix viscosity, decreases whipping rates, contributes to inferior flavor and less stable mix resulting in possible coagulation during processing stages (Arbuckle, 1966). Reddy *et al.*, (1987) used liquid channa whey as a substitute for milk solids not fat in ice cream mixes, and observed that increase in the level of replacement for solids not fat increased titrable acidity. The milk solid not fat (MSNF) content of the ice cream mix has a remarkable effect on the pH of the ice cream mix. As the MSNF portion of a mix increases, the normal acidity increases and pH decreases (Arbuckle, 1986). The pH of an ice cream mix can be used as an indicator of mix quality. The normal pH of ice cream mix is about 6.3 (Marshall and Arbuckle, 1996). Lee and White (1991) used ultrafiltration retentate (concentrated to three times) and whey protein concentrate to replace different levels of solids not fat in vanilla ice cream (12% fat) at 25, 50, 75 or 100 percent, respectively. pH of the ice cream mixes were evaluated and it was found that pH increased as percentage of ultrafiltration retentate substitution increased but decreased as percentage of substitution of whey protein concentrate increased.

Arbuckle (1966) reported that specific gravity of ice cream mix varies from 1.0544 to 1.2232. However, it was greatly influenced by the proportion of ingredients used for its preparation. Jain and Verma (1970) formulated two ice cream mixes (i) containing 10 percent fat, 12 percent SNF, 15 percent sugar, 0.4% percent gelatin and 37.4 percent total solids and (ii) 12 percent fat, 10

percent serum solids, 15 percent sugar, 0.4 percent gelatin and 37.4 percent total solids. The ice cream mixes were homogenized at varying pressure (110-210 kg/cm²) and it was reported that specific gravity of mix decreased by increasing the amount of fat and decreasing the amount of solids not fat and also with the homogenization pressure irrespective of type of ice cream.

Viscosity is an important property of the ice cream mix and a definite amount of it is essential for proper whipping and retention of air. It is the internal friction that tends to resist the sliding of one part of the fluid over another. Among the various ingredients, milk fat and stabilizer influence the viscosity more. On the other hand, sugar decreases the viscosity of the ice cream mix. Ice cream mix has both apparent and true viscosity. The true viscosity of the ice cream mix may range from 50 to 300 cp. The higher the viscosity of a mix greater is the power required to freeze mix (Marshall and Arbuckle, 1996). Nickerson and Pongborn (1961) observed that the 10 to 12 percent increase in the milk solids not fat content did not increase the viscosity of the mix appreciably but the stabilizer addition substantially increased it. Olenov and Bdulenko (1968) reported that the addition of dried dairy products to mixes increased the viscosity while liquid products decreased it. They further observed that the viscosity of mix did not change significantly in temperature range of 90 and 60 °C, but with further reduction in the temperature, particularly between 40 and 20 °C the viscosity increased significantly. Patel and Mathur (1982) reported that lower viscosity was observed in ice cream made from 5.5 percent hydrolysed lactose whey (HLW). Thompson *et al.*, (1983) substituted succinated whey protein concentrate for dried skim milk in ice cream mix and observed increased viscosity of ice cream mix by the use of whey protein concentrate. Schmidt and Smith (1989) observed that ice cream mix treated with 4000-500 psi homogenization pressure had lesser viscosity than reference mix sample (2000-500 psi). At the homogenization pressure of 4000-500 psi fat globules had smaller diameter. The authors inferred that lowered viscosity resulted due to lower internal resistance in smaller fat globules than in large fat globules. Salem and Zeidan (1993) added dried skim milk, sodium caseinate, whey protein concentrate or soy protein

isolate to whipping cream at 1, 2 or 3 percent and observed increased viscosity by dried skim milk and sodium caseinate of whipping cream more than whey protein and soy proteins and lowest whipping time was required when whey protein concentrate and sodium caseinate were added together. They also reported that use of either whey protein concentrate or soy protein isolate improved foam stability. Singh (2007) found that with the increase in milk fat in the mix of low fat, fibre enriched fruit ice cream, there was a decrease in viscosity of the ice cream mix and attributed this finding to the insignificant crystallization / clumping of fat globules during ageing of mix, which resulted into non significant increase in viscosity. However, Marshall *et al.* (2003) observed opposite results, he reported an increase in viscosity with the increase in fat levels. Lee and White (1991) used whey protein concentrate to replace different levels of solids not fat in vanilla ice cream (12% fat) at 25, 50, 75 or 100 percent respectively and observed that viscosity of the ice cream mixes decreased as percentage of substitution of whey protein concentrate increased. Tirumalesha and Jayaprakasha (1998) also studied the effect of an admixture of spray dried WPC and buttermilk powder on physico-chemical and sensory characteristics of ice cream and observed a decrease in viscosity of the mix with increased level of an admixture of spray dried WPC and buttermilk powder.

Overrun in ice cream means the increase in the volume of ice cream over the volume of mix used, which is achieved by incorporation of air. The overrun in ice cream is important because it influences the quality and is responsible for profitability. Dleuzewski *et al.*, (1981) revealed that overrun increased with the increase in fat content and decreased with the increase in solid not fat content. Thompson *et al.*, (1983) substituted whey protein concentrate (WPC) for dried skim milk (DSM) in ice cream and observed reduction in overrun in ice cream by the use of WPC. Anon (1991) enumerated the factors which affected the overrun in ice cream such as proportion of total solids in mix; homogenization pressures, type and quality of fat, quality of emulsifier and stabilizers. Whereas, Schmidt and Smith (1989) observed no effect of homogenization treatment on overrun, if ice cream was made in a continuous freezer with constant freezer speeds. To obtain

the required overrun in ice cream air incorporation is essential. The overrun defines the structure of the final product, as the presence of air gives the ice cream a light texture and influences the physical properties of the final product (Sofjan and Hartel, 2004). Oxygen plays an important role in the poor survival of probiotic bacteria (Brunner *et al.*, 1993) and oxygen tolerance in probiotic bacteria is strain dependent (Kawasaki *et al.*, 2006). Overrun results in partial coalescence and destabilization of the fat of the mixture with the formation of an internal lipid structure, imprisoning air bubbles (Bolliger *et al.*, 2000). However, the addition of the probiotic microorganism did not cause any modification in the overrun or any alteration in firmness of the ice-cream supplemented with *Lactobacillus johnsonii* La1 (Alamprese *et al.*, 2002).

The melt-down rate of ice cream is affected by many factors, including the amount of air incorporated, the nature of the ice crystals, and the network of fat globules formed during freezing. How the ice cream melts down (melt-down) is one of the factor affecting appearance of the product, either adversely as a curdled, wheyed-off melted product, or favorably as an especially smooth, creamy, rich-appearing melted product. Flack (1988) emphasized that consumers regard ice creams having minimum drip loss and good shape retention upon melting as good ice creams.

According to Pelan *et al.*, 1997, it is the stability of air cells that slows down the meltdown rate of ice cream. Arbuckle (1966) reported that in general, increase in viscosity of mix, increases the resistance to melting and smoothness but decreases whipping rate. The melting properties of the ice cream were improved because of the association of the emulsifier with the proteins at the interface of fat and water (Berger, 1990). The emulsifiers ensure a finer dispersion of the air cells stabilized agglomerated fat (Mahdi and Bradley, 1970). Agglomerated fat forms a network surrounding the air cells and causes a resistance to meltdown. An insulating effect provided by the fine dispersion also tends to retard meltdown (Flack, 1988). Prindville *et al.*, (1999) formulated low fat ice cream by using whey proteins and polydextrose as fat replacers, containing 0.5 percent, 4 percent, 6 percent, 9 percent fat and concluded that milk fat at

concentration of 9 percent and 6 percent produced more creaminess and smoothness. Bray (1991) described a process to manufacture low fat ice cream based on thermal denaturation of a mixture of lactose free whey protein concentrate and defatted milk, which forms an aggregate resulting in a gel stable under freezing. This gel could be combined with the normal ingredients of ice cream and processed conventionally. Roland *et al.* (1999) who observed that increasing fat content improved melting property. Ohmes *et al.*, (1988) also reported that ice creams containing fat would be expected to melt more slowly than non fat ice creams containing similar amounts of total solids and stabilizers / emulsifiers. Apart from fat content in the ice cream formulation, source of milk fat is also vital from melting quality point of view as observed by Abu Lehia *et al.*, (1989). He observed that ice cream made by using camel milk fat (melting point, 42 °C) was more resistant to melting than ice cream made with bovine milk fat (melting point, 31.5 °C). . Tirumalesha and Jayaprakasha (1998) studied the effect of an admixture of spray dried WPC and buttermilk powder on characteristics of ice cream and observed that the melting resistance decreased with increasing level of the admixture in to the mix.

The firmness of ice cream is related to its structure. The air cells of ice cream structure are essentially spherical although some distortion due to fat and ice crystal formation is there (Prentice, 1992). The material surrounding these air cells is a non-Newtonian fluid containing small ice crystals and clumps of fat (up to 80%). In ice creams with low fat content, the rheology of the composite fluid surrounding the air cells will be altered due to the reduction in the fat clumps which predominate the composite fluid of conventional ice cream structure. The firmness of ice cream is affected by such factors as the overrun, ice crystal size, ice phase volume, and extent of fat destabilization. An inverse relationship between hardness and overrun has been noted by many researchers (Tanaka *et al.*, 1972; Goff *et al.*, 1995; Wilbey *et al.*, 1998). Generally, it is observed that ice cream firmness is inversely related to the fat content of ice cream. Guinard *et al.* (1997) also reported that ice cream firmness as measured instrumentally, was inversely related to fat. Aime *et al.* (2001) used two types of probe viz., cylindrical

probe/plunger attachment and a knife attachment to measure the firmness of ice cream. The only difference with the knife attachment was that with the fat free (0.4% fat) ice cream sample had a lower firmness value than the regular fat (10% fat) ice cream. Specter and Sester (1994) also conducted tests using a plunger attachment on an Instron Universal Testing Machine and observed a three-fold increase in firmness in fat free (0% fat) sample than regular ice cream (12% fat). Tirumalesha and Jayaprakasha (1998) studied the effect of an admixture (50:50) of spray dried WPC and buttermilk powder on physico-chemical and sensory characteristics of ice cream and observed that the hardness of ice cream decreased with increasing level of the admixture in to the ice cream.

2.3.5 Ice cream as a probiotic carrier

Ice cream and frozen desserts have potential as carrier of probiotics but the effect of stress due to freezing on the viability of probiotics during manufacture and extended storage must be considered. Alteration of cell membrane permeability and the intracellular dehydration caused due to ice crystal formation are the main causes of microbial inactivation during freezing (Moss and Specks, 1963). Freezing and thawing may result in severe damage to the cells that may have a lethal effect or can cause inhibition of multiplication and/or interruption of metabolic activity (Davies and Obafemi, 1985), defeating the potential advantage of probiotics. Among the dairy products, ice cream might act a carrier for *L. acidophilus* and is a better vehicle than milk (Duthie *et al.*, 1981).

Development of probiotic ice-cream with good sensorial quality is possible (Vardar and Oksuz, 2007). Probiotic cultures do not modify the sensory properties of the products to which they are added intensely (Champagne *et al.*, 2005). *L. acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* – are homofermentative and produce lactic acid from lactose metabolism, without gas production. Non-fermented product like probiotic ice-creams do not normally present problems resulting from the microbial metabolism, since they are stored at very low temperatures (<18 °C), minimizing

the probiotic microorganisms biochemical reactions. Vardar and Oksuz (2007) reported that artisan strawberry ice-cream supplemented with *L. acidophilus* showed good sensory acceptance and the incubation of the mix at pH 5.6 led to better results in terms of flavor and taste. Addition of acidic fruit to ice-cream might be useful in masking the sour taste resulting from the metabolism of probiotic cultures.

Addition of probiotic culture in ice cream adds value to the product and also provides it with the advantage of being functional. Each stage of the production process of probiotic ice cream must be optimized so as to guarantee the functional properties of the product. There are two ways of addition of cultures to an ice cream, one by using DVS (Direct Vat Set) type culture for the direct addition to the product during its manufacture and in the other method using the milk as a substrate for fermentation. In the second case, it is essential to control the pH to prevent any undesirable effect during the fermentative process and also the control of temperature during storage is important. Increased sensitivity of probiotic strains to low pH values (4.0–4.5) and negative effects on sensory acceptance of the product may be observed. Probiotic ice creams with fermentation as one of the step in production are more acidic due to the production of lactic acid during the fermentation process. These changes might be undesirable, since traditionally ice-creams are not characterized as high acidic food products. However, to overcome this problem, fermentation can be stopped at pH values ranging from 5.0 to 5.5 (Vardar and Oksuz, 2007). Several workers have carried out studies to develop probiotic ice creams in the past and they also checked the viability of the probiotic microorganisms in ice cream as affected by the freezing process and storage. Some of the studies are mentioned below.

In a study, the strains of *L.acidophilus* and *Bifidobacterium bifidum* inoculated into pasteurized fermented ice cream mixes, survived at levels of more than 1×10^6 CFU/g after freezing and during storage for eight weeks (Laroia and Martin, 1990). The frozen storage at -5 °C for 6 hr had no adverse effect on the bile salt sensitivity of the organism (Holocomb *et al.*, 1991). The

ability of lactobacilli to withstand even longer periods of freezing has also been reported (Laroia and Martin, 1990) with probiotic viability maintained at approx. 10^6 CFU/g for up to 52 weeks at -20 °C. A good quality probiotic ice cream can be manufactured by simply mixing commercial *L.acidophilus* and *Bifidobacterium bifidum* cultured milk with unfermented ice cream mix (Christiansen *et al.*, 1996). Some micro organisms such as *L. reuteri* or *Bifidobacterium bifidum*, produce a slightly acid flavor due to fermentation. Therefore, manufacturing conditions that limit fermentation may be adopted to minimize such flavors (Hagen and Narvhus, 1999).

Alamprese *et al.*, (2002) carried out a study with ice-cream supplemented with *Lactobacillus johnsonii* La1 and prepared four formulations containing different amounts of fat (5% and 10% w/v) and sugar (15% and 22% w/v). For each formulation, ice-creams with and without La1 were prepared, and stored at temperatures of -16 °C and -28 °C. High survival rates of the probiotics during 8 months of storage of the ice-creams were observed without any decrease in the population inoculated initially (7 log CFU per g). The addition of the probiotic microorganism did not cause any modification in the overrun or any alteration in firmness of the finished product. Survival of freeze dried *L. bulgaricus* was enhanced during storage at -20°C over 10 months, by growing the cells in the presence of fructose, lactose, or mannose, or when glucose, fructose, monosodium glutamate or sorbitol (Carvalho *et al.*,2004). Alamprese *et al.*, (2005) carried out another experiment under the same conditions tested before, but using *L. rhamnosus* GC. The results obtained were similar with this microorganism, with no influence on the physical properties of the ice-cream.

Basygjit *et al.*, (2006) investigated the viability of human-derived probiotic lactobacilli in ice cream prepared with sucrose and aspartame. For manufacturing ice cream, all ingredients were mixed with milk and the mixes were pasteurized at 95°C for 20 min. The mixes were then homogenized, cooled and stored at 4°C. After aging at 4°C for 24h, each mix was divided into two parts, one with sucrose and the other with aspartame and each of these were then divided into three additional parts. The probiotic cultures were directly mixed

with the ice cream mix to obtain a high initial cell count between 10^6 and 10^8 CFU/g. Akalin and Erisir, (2008) studied the effects of supplementation of oligofructose or inulin on the rheological characteristics and survival of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 in low-fat ice cream stored for 90 days at $-18\text{ }^{\circ}\text{C}$. For ice cream manufacture, the mixes were pasteurized at $68\text{ }^{\circ}\text{C}$ for 30 min. *L. acidophilus* La-5 and *B. animalis* Bb-12 cultures were added to the mixes (@ 0.3%) after cooling to $40\text{ }^{\circ}\text{C}$, to achieve approximately 10^8 CFU/g, mixed well, and fermented for approximately 4 h at $40\text{ }^{\circ}\text{C}$ until the desired pH of 5.5 was reached. Turgut (2009) prepared and investigated probiotic ice cream using probiotics in the form of fermented milk, representing 10% of the total milk (culture inoculation @ 4%) being added to the mix after ageing the ice cream mix at $4 \pm 1\text{ }^{\circ}\text{C}$ for 18h. The ice cream mix were stirred with simple mixer for 5 min and then frozen.

2.4 CYCLOPHOSPHAMIDE AS AN IMMUNOSUPPRESSANT

Cyclophosphamide is an alkylating agent widely used in anti-neoplastic therapy. Cyclophosphamide acts on both cyclic and intermitotic cells, resulting in general depletion of immune-component cells. It is a potent immunosuppressive agent, capable of inhibiting both humoral (Santos, 1967) and cell-mediated immune responses (Maguire *et al.*, 1961). Many studies have been carried out using cyclophosphamide as an immunosuppressive agent.

Hou *et al* (2007) carried out a study to identify the immunosuppressive effects of cyclophosphamide (a known immunosuppressant) in male Wistar rats at which the immunosuppressive effect is identified. The results showed that 10 mg/kg cyclophosphamide treatment induced decreases in body weight, body weight gain, relative weight of spleen and thymus, antibody plaque-forming cells, delayed-type hypersensitivity reaction, natural killer cell activity, lipopolysaccharide-induced B-cell proliferation, and Concanavalin A-induced T-cell proliferation. But, no significant clinical symptoms of toxicities and stress were observed and no rats died. So, it was concluded that the dose of 10 mg/kg

of cyclophosphamide orally for 30 days in male Wistar rats could be used as the dose of positive control group for chemicals immunotoxicity assessment tests.

Cyclophosphamide has been used as an immunosuppressant by several workers for the evaluation of immunomodulatory potential of several plant materials or extracts in animal models. Vigila and Baskaran (2008) investigated the immunomodulatory properties of coconut protein in Swiss albino mice using cyclophosphamide (CP) as an immunosuppressant. Orally administered coconut protein in CP treated animals showed increased levels of RBC, WBC and Platelet counts. Gupta *et al.*, (2010) investigated immunomodulatory effect *Moringa oleifera* Lam. extract on cyclophosphamide induced toxicity in mice using cyclophosphamide (30 mg/kg b.w, orally) once for three days as a standard immunosuppressant drug. Sultana *et al.*, (2011) used cyclophosphamide (30mg/kg body weight, i.p route, once for three days) induced immunosuppression model to study the immunomodulatory activity of methanol extracts of fruits of *Solanum xanthocarpum* (Solanaceae) on Swiss albino mice. The extent of protection against immunosuppression caused by Cyclophosphamide was evaluated after 14 days of drug administration, by estimating hematological parameter and neutrophil adhesion test. A study was also carried out by Gaikwad and Mohan (2011) to find out immunomodulatory potential of methanolic extract of leaves of *Thespesia populnea* using cyclophosphamide (30 mg/kg b.w, p.o) once for three days as a standard immunosuppressant drug. All the above mentioned studies showed the effectiveness of cyclophosphamide as an immunosuppressant in animal model.

2.5 RESPONSE SURFACE METHODOLOGY (RSM)

RSM is a collection of statistical and mathematical techniques and has important application in the design, development and formulation of new products, as well as in the improvement of existing product design. The effect of the independent variables either alone or in combination, on the processes can be defined using this tool. This experimental methodology generates a mathematical model which describes the chemical or biochemical processes,

along with analyzing the effects of the independent variables (Myers and Montgomery, 1955; Anjum *et al.*, 1997). This statistical tool considers interactions among the test factors and can be used to determine how the product changes in the factor levels (Thompson, 1982; Desai *et al.*, 1992). The graphical perspective of the mathematical model has led to the term Response Surface Methodology (Bas and Boyaci, 2007).

RSM is a four step process. Identification of the critical factors which account for most of the variation in the quality of the product under study is the first step. Defining factor levels is the second step. A range of factor levels which encircle the product quality are defined in this step. These factor levels must be carefully selected considering feasibility, cost and government regulations if any. In the third step, selection of the test samples is carried out by the proper statistical design, from among all the possible combinations to be tested. After selecting the samples, experiments are conducted and related data on the product quality are obtained and subjected to appropriate statistical analysis. In the final step data analysis is carried out. An appropriate computer programme analyzes the data so obtained, which are further interpreted by those who have participated in the data collection. The conclusions drawn from the above analysis are then be confirmed by follow-up experiments with optimum product (Desai *et al.*, 1992).

Chapter 3

Scope, Objectives & Plan of Work

SCOPE, OBJECTIVES AND PLAN OF WORK

3.1 SCOPE

With the changing trends, ice cream is becoming an all season product rather than a summer treat only and so the ice cream consumption is increasing. Some of the reasons that have triggered the growth of ice cream consumption are: growing urbanization, improved disposable income, increasing deep freezer penetration, growth of modern format / convenience stores, improved cold chain both in terms of quality and availability. Today, consumers are more open to accept exotic and natural flavors as compared to earlier trend when the basic flavors used to contribute the maximum in overall sales. Global production of ice cream is increasing constantly and the rate of growth in production is enormous. In India, there is scope for more growth than 8-10% observed over the past few years (Soni, 2009).

The immune system of the body provides protection to the host from invading pathogens and elimination of disease (Sultana *et. al.* 2011). The investigation for the potential immunomodulation agents from natural products is increasing as the consumers are becoming more aware about the impact of food on health. Several studies in the past have reported the role of probiotics and plant material in enhancing the natural resistance of the body against infection and illness.

Probiotic bacteria are known as GRAS (generally regarded as safe) microorganisms which induce mucosal immunity and influence intestinal physiology by modulating the endogenous flora (Wei *et. al.*, 2007). The importance of carrier for the delivery of probiotics to the human body goes with the saying "The journey is as important as the destination." The choice of right vehicle is very critical as it can make or break that journey. Without the probiotic surviving the journey, probiotic benefit claims are just an empty promise. Food substrate is one of the factors that regulates the colonization of micro-organisms in the gastrointestinal tract. Ice cream and frozen dairy desserts owing to their

lower storage temperature and less risk of temperature abuse during storage results in higher viability of probiotic bacteria at the time of consumption.

Plants play an essential role in complementary and alternative medicine, due to this they develop the ability to form secondary metabolites like flavonoids, phenolic substances, proteins, alkaloids and steroids which are then used for restoration of health and elimination of disease (Sultana *et. al.* 2011). Aloe has been used therapeutically since Roman times and perhaps long before (Srisukh *et al.*, 2006). A number of studies have indicated immunomodulatory activities of the polysaccharides in *Aloe vera* gel (Hamman, 2008). Frozen products from *Aloe vera* juice could provide an alternative product for consumers, apart from the commercially available Aloe drinks that are generally unacceptable due to off flavor. Producing an ice cream with medicinal herb like *Aloe vera* can fill a gap in the market and fulfill consumers' demand for an ice cream with health benefits.

Incorporation of *Aloe vera* and probiotics together in an ice cream can help the consumption of the two nutraceuticals in a delightful way by people of all the ages. The present study was aimed at developing *Aloe vera* supplemented probiotic ice cream (ASPIC) and investigating the immunomodulatory potency of combination *Aloe vera* and probiotics in ice cream using cyclophosphamide induced immunosuppression model by evaluating the effect on specific and non-specific immune response in Swiss albino female mice.

3.2 OBJECTIVES

Keeping in view the above facts, the present work was thus envisaged with the following objectives:

- Optimization of level of *Aloe vera* juice for incorporation in ice cream
- Selection of probiotic bacteria and evaluation of their survivability in ice cream containing *Aloe vera* juice
- Incorporation of selected probiotic bacteria in *Aloe vera* juice supplemented ice cream

- Evaluation of immune response of *Aloe vera* juice supplemented probiotic ice cream

3.3 PLAN OF WORK

To fulfill the objectives of the study the following plan of work was followed.

3.3.1 Optimization of level of *Aloe vera* juice for incorporation in ice cream

3.3.1.1 *Ice cream preparation*

Ice cream was prepared using the standard procedure with some modifications, maintaining 15% sugar, 0.4% emulsifier and stabilizer and 40% total solids. Pasteurized *Aloe vera* juice was added to ice cream mix, prepared by using buffalo milk, cream, SMP (spray dried), WPC, sugar, emulsifier and stabilizer, flavor and color.

3.3.1.2 *Milk solid not fat (MSNF)*

MSNF content of ice cream was maintained using combination of skim milk powder (SMP) and whey protein concentrate-35 (WPC-35). WPC has been included in ice cream mix formulations as it contributes to favorable sensory and textural qualities (Tirumalesha and Jayaprakasha, 1998).

3.3.1.3 *Selection of flavor*

Amount and type of flavor was decided on the basis of sensory evaluation of ice cream mix prepared by incorporation of 30% *Aloe vera* juice with different flavors.

3.3.1.4 *Optimization of Aloe vera juice supplemented ice cream (ASIC)*

To obtain optimum quality of ASIC using Central Composite Rotatable Design (CCRD) of Response Surface Methodology (RSM), following critical factors were used for optimization.

- | | |
|------------------------------------|------------------------|
| a) <i>Aloe vera</i> juice (20-30%) | b) Fat content (8-12%) |
| c) WPC (0-25% of MSNF) | |

3.3.1.5 Analysis of the ASIC mix and ASIC

The ASIC mix was analyzed for titrable acidity, pH, specific gravity, viscosity and instrumental color parameters and the ASIC was analyzed for sensory attributes, % melt per hour, overrun and firmness.

3.3.2 Selection of probiotic bacteria and evaluation of their survivability in ice cream containing Aloe vera juice

3.3.2.1 Probiotic strains

Four probiotic lactic acid bacteria (LAB) strains viz., NCDC-624 (*Lactobacillus plantarum*), NCDC-625 (*Lactobacillus plantarum*), NCDC-626 (*Lactobacillus rhamnosus*) and NCDC-627 (*Lactobacillus paracasei* ssp. *paracasei*) were procured from National Collection of Dairy Cultures (NCDC) of Dairy Microbiology Division, National Dairy Research Institute, Karnal and one commercial DVS culture.

3.3.2.2 Selection of a single desirable NCDC probiotic strain

Selection of a single NCDC probiotic strain was done on the basis of the following preliminary tests:

(a) Probiotic activity verification of the cultures

All the above mentioned NCDC probiotic strains were tested for the following parameters viz. acid tolerance (Clark *et al.*,1997 with modification), bile tolerance (Gilliand *et al.*,1984 with modification), antibiotic susceptibility (CLSI;Wayne,USA), antimicrobial activity (Ulhman *et al.*,1992) and cell surface hydrophobicity (Rosenberg *et al.*,1980 with modification) to examine their in-vitro probiotic potential.

(b) Survivability of probiotic strain in presence of Aloe vera at freezing temperature (-20±2°C)

Different probiotic cultures (NCDC-624, NCDC-625, NCDC-626, and NCDC-627) @ 1% were added to sterile reconstituted skim milk (10% TS) samples containing the 20% *Aloe vera* and were incubated at 37 °C for 48h. After 48h of incubation, samples with probiotic cultures were placed in deep freezer

maintained at $-20\pm 2^{\circ}\text{C}$ for 40 days. Enumeration of probiotic count was done on 40th day of storage to check their tolerance and survivability at freezing temperature.

3.3.2.3 Selection of best probiotic strain

The best probiotic strain was selected on the basis of the results of the preliminary tests (3.3.2.1 and 3.3.2.2).

3.3.3 Incorporation of selected probiotic bacteria in Aloe vera juice supplemented ice cream

Fermented milk prepared by incorporation of selected NCDC probiotic culture was added @ 4% and 8% separately in to the ice cream mix samples and ice cream mix samples were frozen. The probiotic count of each sample was enumerated to decide the level of probiotic fermented milk that provide the desired minimum level of probiotic count in the final product

3.3.3.1 Ice cream manufacture

Manufacture of ASPIC was done according to the standard procedure (Fig. 3.1). Optimized levels of ingredients (Section 3.3.1) and desired level of probiotic fermented milk (Section 3.3.3) were used to prepare the ice cream mix.

3.3.4 Evaluation of immune response of ASPIC

3.3.4.1 Immunosuppression of mice

Cyclophosphamide induced immunosuppression model was used in the present study. Intraperitoneal injection of cyclophosphamide (10 mg/kg body weight) was used for the immunosuppression of mice (25-30 g). Studies were carried out in two batches. In first batch, cyclophosphamide injection (10mg/kg body weight) was given only for first three days and the feeding was continued till 13th day. In the second batch, cyclophosphamide injection (10mg/kg body weight) was given daily for 13 and animals were then sacrificed on 14th day.

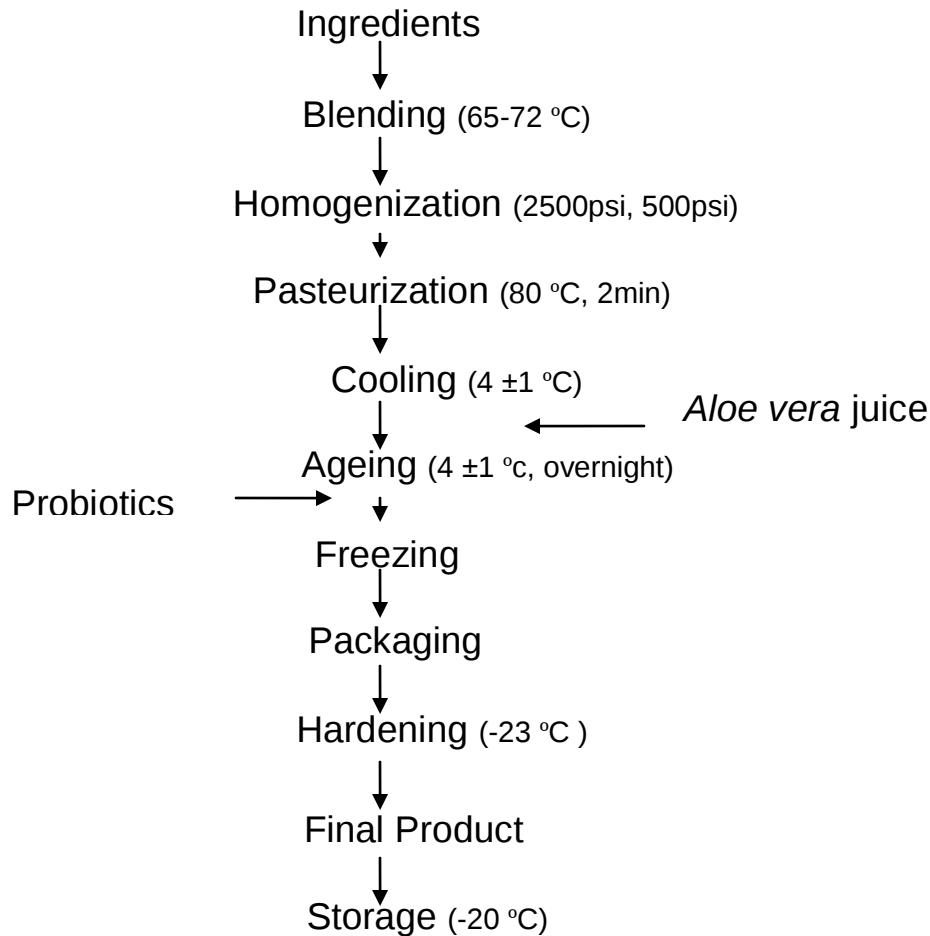


Fig 3.1 Flow diagram for the preparation of ASPIC

3.3.4.2 Oral feeding of mice

Mice in both the batches were fed orally continuously for 13 days according to the following grouping

- Group A : Control diet (CD)
- Group B : Control ice cream (CIC)
- Group C : ASPIC with NCDC 627 probiotic culture (NCDC)
- Group D : ASPIC with commercial DVS probiotic culture (DVS)

3.3.4.3 Evaluation of immune response

After 13 days of continuous feeding mice were sacrificed on 14th day and evaluated for immune response in terms of the following parameters viz.,

phagocytic activity (Hay and Westwood, 2002). lymphocyte proliferation (Mosmann, 1983), antibody response (IgA) (Engwall and Perlmann,1971 and modified by Perdigon *et al.*, 1991), haematological parameters and organ weights.

3.3.5 Storage study of the developed product

ASPIC was stored at $-20 \pm 2^{\circ}\text{C}$ for 3 months and was analyzed after every 15 day interval for sensory attributes and microbial counts.

3.3.6 Packaging & storage

Product was filled into High Impact polystyrene cups (100 ml) and LDPE coated cardboard boxes (1 Ltr.) that were procured from experimental dairy, NDRI and local market, Karnal respectively and were stored at $-20 \pm 2^{\circ}\text{C}$.

3.3.7 Consumer response study and cost analysis of the final product

The developed product was given to people of different age groups of either sex and was evaluated for its consumer response. Cost of the product was calculated by the standard procedure.

3.3.8 Statistical analysis

Optimization of the product was carried out by CCRD design of Response Surface Methodology (RSM). Data obtained from various experiments were subjected to statistical analysis to arrive at meaningful inferences using SAS Enterprise Guide 4.3 software (Welch's variance - weighted ANOVA test, Post hoc analysis was done by Duncan's multiple range test).

Chapter 4

Materials and Methods

MATERIALS AND METHODS

This chapter deals with the materials and methods employed in the present investigation as well as instruments and analytical techniques adopted for physico-chemical, sensory, microbiological and biochemical evaluation of the product. The manufacturing of ice cream was carried out at the Experimental Dairy of National Dairy Research Institute, Karnal.

To fulfill the objectives mentioned earlier, the investigation was carried out in four phases. In the first phase, optimization of level of *Aloe vera* juice for its incorporation into *Aloe vera* supplemented ice cream (ASIC) was carried out using Response Surface Methodology (RSM). In the second phase, selection of potential probiotic bacteria (based on the probiotic attributes and low temperature tolerance) and the subsequent incorporation of the selected probiotic bacteria in ASIC was carried out. In the third phase, *Aloe vera* juice supplemented probiotic ice cream (ASPIC) was evaluated for immune response in animal model. In the last phase, storage study, consumer response survey and cost analysis of the developed product were carried out.

4.1 MATERIALS

4.1.1 Buffalo milk

Fresh buffalo whole milk obtained from Experimental Dairy of the National Dairy Research Institute, Karnal was used for the preparation of ice cream.

4.1.2 Cream

Fresh cream was procured from Experimental Dairy of the National Dairy Research Institute, Karnal for the preparation of ice cream.

4.1.3 Skim milk powder (SMP)

Spray dried skim milk powder (medium heat classified; WPNI: 3-4 g as per manufacturer's report) was procured from M/s Modern Dairies Ltd., Karnal with the following composition: 1.4 % fat, 3.5% moisture and 95% milk solids-not-fat.

4.1.4 Whey protein concentrate 35 (WPC-35)

WPC-35 with the following composition: 3 % fat, 3.5% moisture and 93.5% milk solids-not-fat was procured from M/s Modern Dairies Ltd., Karnal.

4.1.5 *Aloe vera* (*Aloe barbadensis* Miller) juice

Food grade *Aloe vera* juice was procured from M/s Mehta Herbs and Spices, Coimbatore, in 20 ltr. cans and had following specifications as per the test report provided alongwith (analysed by ATOZ Pharmaceuticals Pvt Ltd., Chennai).

Aloin content	:	0.012%
Mucopolysaccharides	:	20,000 molecules in length
pH	:	4.51
Specific gravity	:	0.8091
TS	:	0.9782 %
Coliforms	:	negative
Salmonella	:	negative
Total yeast & molds	:	negative

4.1.6 Sugar

Commercial grade white crystalline sugar drawn from the store of Experimental Dairy, National Dairy Research Institute, Karnal was used in all the trials.

4.1.7 Stabilizer and emulsifier

Commercial grade Sodium alginate stabilizer emulsifier blend and GMS (Glycerol monostearate) was drawn from Experimental Dairy, National Dairy Research Institute, karnal.

4.1.8 Flavor

Eight commercial grade flavors from M/s Sonarom, Banglore and one vanilla flavor from Bush Boak Allen India Ltd, Chennai were used for the selection of flavor during the preliminary trials.

4.1.9 Packaging material

High impact polystyrene cups (100ml) procured from the store of Experimental Dairy, National Dairy Research Institute, Karnal and 1 litre paperboard cartons lined with LDPE were procured from local market Karnal were used for the packaging of the product .

4.1.10 Starter Culture

Four probiotic LAB strains viz., NCDC-624 (*Lactobacillus plantarum*), NCDC-625 (*Lactobacillus plantarum*), NCDC-626 (*Lactobacillus rhamnosus*) and NCDC-627 (*Lactobacillus paracasei* ssp. *paracasei*) were procured from National Collection of Dairy Cultures (NCDC), Dairy Microbiology Division, National Dairy Research Institute, Karnal and one commercial DVS culture.

4.1.11 Culture maintenance and propagation

Probiotic cultures obtained from the NCDC were maintained by sub-culturing fortnightly into sterile MRS broth and incubating for 16 to 18 h at 37°C. At the end of the incubation, the cultures were stored under refrigeration in-between subculturing transfers. Also, a stock culture of all the cultures was preserved in 20% glycerol stock medium at -20°C. The cultures were activated prior to use by subculturing thrice in sterile MRS broth. The commercial DVS culture was stored in deep freezer at -20°C.

4.1.12 Tissue culture related materials

RPMI-1640 medium (cell culture tested; with L-glutamine and 25mM HEPES, without bicarbonate), Delbecco modified eagle medium-HamF12 (DMEM-F12), Fetal calf serum, (FCS), LPS (From *Escherichia coli* serotype 055:B5 L-2880), concanavalin A (from *Canavalia ensiformis*) and streptomycin sulfate (cell culture tested, sterile γ -irradiated), penicillin-G (cell culture tested),

TMB and MTT (3-[4, 5-dimethyl thazol-2yl]-2, 5-diphenyl tetrazolium bromide), N-2 hydroxyethyl piprazine-N-2 ethanesulphonic acid (HEPES) buffer, L-glutamine, Penicillin G sodium salt and nitroblue tetrazolium (NBT) were procured from Sigma-Aldrich Chemical Co. St. Louis, MO, USA. Mouse IgA ELISA core kits (pink-ONE) was procured from KOMABIOTECH, Seoul, Korea.

4.1.13 Microbiological media

For microbiological analysis, dehydrated media viz., plate count agar (PCA), potato dextrose agar (PDA), violet red bile agar (VRBA), MRS agar and MRS broth were procured from Hi-Media Laboratories, Mumbai.

4.1.14 Chemicals and reagents

The analytical grade or reagent grade chemicals and reagents, used in the present study, were procured from different manufacturers like SRL Ltd., Loba Chemie Pvt Ltd., S.D. Fine Chemical Ltd., Mumbai (India) and Sisco Research Laboratories Pvt. Ltd., Mumbai.

4.2 EQUIPMENTS

4.2.1 Heating / Pasteurization Vat

A 20 litre capacity stainless steel jacketed, round bottomed, open pan provided with steam line and steam control valve was used for heating and pasteurization of mix.

4.2.2 Hand grinder

Hand grinder (Type: DX 505, Max. rpm: 12000, make: Leema Industries, India) was used to mix stabilizer, emulsifier, WPC and sugar into the liquid portion (milk and cream mix).

4.2.3 Homogenizer

Two stage homogenizer (Crepaco Food Equipment & Refrigeration, APV company, Chicago, ILL USA) was used for the homogenization of ice cream mix.

4.2.4 Fryma grinder

Fryma grinder (ML-150 Fryma maschinen, Switzerland) was used to make uniform ice cream mix.

4.2.5 Plunger

A stainless steel plunger with total length of 34 cm and diameter of the disc of the plunger 15 cm with 6 holes was used to stir the ice cream mix before freezing.

4.2.6 Hardening unit

Hardening room of experimental dairy and vest frost deep freezer (Biogen Scientific) were used for hardening and storage of ice cream at -20 ± 2 °C.

4.2.7 Ice cream freezer

'Gusti' batch type ice cream freezer (Sigma Sales Services) was used for freezing of different batches for RSM trials. Continuous freezer (Hoyer manufacturing company, Aarhus, Denmark) was used for the bulk production of ice cream.

4.2.8 Hunter Color lab

Color measurements were conducted using Colorflex (Hunter Associates Laboratory, Inc., Reston VA, USA).

4.2.9 Viscometer

Apparent viscosity of ASIC was measured at 20°C using a rotational viscometer (VISCO STAR plus, FUNGILAB, Spain) with a fixed outer cylinder and rotating spindle.

4.2.10 Texture analyzer

The samples of ASIC were evaluated for its firmness, using Texture Analyzer TAXT2 (Stable Micro systems, Godalming, Surrey, UK) fitted with a 25 kg load cell. Experiments were carried out using warner blade.

4.2.11 Inverted microscope

Inverted microscope (OLYMPUS CK-40, DSS IMAGETECH Pvt. Ltd., New Delhi, India) was used to count the cells for biochemical studies.

4.2.12 High speed refrigerated centrifuge

High speed refrigerated centrifuge (Model: Supra 22-K, Hanil Science Industrial Co Ltd., Korea) was used for the microbiological and biochemical studies.

4.2.13 ELISA plate reader

ELISA plate reader (Tecan infinite F200 PRO, Tecan Austria GmbH, Austria) was used for taking ELISA plate readings.

4.2.14 Complete blood count analyzer

Complete blood count of animal blood was carried out by using cell counter (Mindray, BC2800).

4.3 METHODS

4.3.1 Selection of form and level of *Aloe vera*

Two forms of *Aloe vera* viz., gel and juice were used for the preparation of ice cream. Ice cream samples obtained were evaluated for its sensory characteristics. Out of the two forms of *Aloe vera* viz., gel and juice, the one which gave sensorily better ice cream was selected for further trials to choose minimum and maximum levels for RSM trials.

4.3.2 Flavour selection for *Aloe vera* ice cream

To select a desirable flavor for ASIC, ice cream mix was prepared with highest level of *Aloe vera* juice selected for RSM trials and then ten different flavors (raspberry, chocolate, kesar elaichi, mango, rose, vanilla, pineapple, banana, cardamom and one sample without any added flavor) @ 0.1% level were added in ice cream mix to select the best flavor compatible with ice cream mix containing *Aloe vera* juice. Sensory evaluation of the ASIC mix samples with different flavors was carried out by sensory panel.

4.3.3 Effect of heat treatment on *in vitro* lymphocyte proliferation of *Aloe vera* juice

An *in vitro* lymphocyte proliferation experiment was carried out using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay to check the effect of heat on immunostimulatory activity of *Aloe vera* juice. *Aloe vera* juice samples subjected to different time-temperature combinations viz. 70°C/10 min, 80°C/10 min, and 121°C/15 min, were tested for lymphocyte proliferation against control *Aloe vera* juice (no heat treatment) sample. Two positive controls namely Concanvalin A and LPS were also used in this experiment.

(i) Preparation of basal culture medium (RPMI-1640): Following ingredients were added to one liter of autoclaved milliQ water.

RPMI-1640	:	10.4 g (one vial)
NaHCO ₃	:	2.10 g
Sodium pyruvate	:	110.1 mg
HEPES	:	5.96 g (50 mM)
Penicillin	:	61 mg (100 U/ ml)
Streptomycin	:	100 mg (100 µg/ml)

The pH of the resulting solution was adjusted to pH 7.4 and sterilized through 0.22 µm PVDF membranes using positive pressure. The prepared medium distributed in small aliquots was stored at 5°C.

(ii) Preparation of culture medium: The basal culture medium was supplemented with following constituents for its use as culture medium.

- a) β-Mercaptoethanol - 5 µM solution
- b) Fetal calf serum (FCS) - 10 percent heat inactivated at 56°C for 30 min
- c) Mitogens - Lipopolysaccharide - 50 µg/ml
- Concanavalin A - 2.5 µg/ml

(iii) Collection of spleen of mice: The male albino mice were sacrificed by cervical dislocation. The peritoneum was opened and spleen was taken out and placed into sterile tubes containing tissue culture medium. It was washed and made free from connective tissue and adipose tissue.

(iv) Preparation of spleenocytes: The collected spleens were subsequently transferred to sterile petridish containing RPMI-1640 basal culture medium. They were teased gently using a sterile needle and forceps to release cells from spleen. It was allowed to stand for 2 min for the sedimentation of clumps. Then, using 1 ml pipette the upper portion of medium containing spleenocytes was transferred to a 15 ml sterile centrifuge tube and again left to stand for 2 min (in ice) and transferred to another sterile centrifuged tube. This cell suspension now devoid of clumps and larger particles was centrifuged at 1500 rpm for 5 min. The cells were washed once again in basal culture medium and then to the pellet, 1 ml of erythrocyte lysis buffer was added. After few seconds, 5 ml of culture medium was added to this and centrifuged at 1500 rpm for 5 min.

The lymphocyte cell suspension was washed twice with cold culture medium to remove traces of lysis buffer and finally suspended in 1 ml of 10 percent FCS and β - mercaptoethanol containing medium. A small aliquot of cell suspension was taken for cell count and checking cell viability.

(v) Cell counting: The viability and counting of cells was carried out by the method of Hay and Westwood (2002). The Neubauer ruling hemocytometer was used for counting of lymphocyte cells. After placing a cover slip on hemocytometer, a small aliquot (~20 μ l) of mixture was transferred to both chambers using pipette. Counting of cells was done within 2 min of mixing. The observation was made at 40 percent objective magnification. Cell count was done in one of the four peripheral, single ruled areas and total viable cells were calculated using following formula:

$$\text{Total no. of cells/ml} = \text{Average count of cells} \times 10^4 \times \text{Dilution factor}$$

(vi) Dispensing and culturing of lymphocytes: Cell suspension containing 1×10^7 viable cells/ml was made in culture media (containing 1 μ M solution of β -mercaptoethanol, 10 percent FCS). 100 μ l cell suspension was dispensed in each well of 96 well flat bottomed tissue culture plates and then added with 5 μ l of *Aloe vera* juice subjected to different heat treatments. Lymphocytes were cultured in above medium at 37°C for 48 h in a 95 percent relative humidity air atmosphere with 5 percent CO₂ in a CO₂ incubator. Positive controls LPS and Con A were used for comparison.

(vii) Colorimetric MTT assay: The cell proliferation was determined using the colorimetric MTT assay originally described by Mosmann (1983). MTT (3-[4, 5-dimethyl thazol-2yl]-2, 5-diphenyl tetrazolium bromide) was dissolved in RPMI-1640 at 5 mg/ml and filter sterilized. At the end of 48 h of incubation, 10 μ l of MTT solution was added to all wells and plates were incubated for 4 h at 37°C. During this period crystals of MTT formazon were formed at the bottom of each well. The spent media along with suspension of cultured cells was decanted. 100 μ l of DMSO (di-methyl sulfoxide) was added to wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature the plates were read using a plate analyzer. Plates were read within 1 h after adding DMSO. Data is expressed as Stimulation index (SI) and calculated using the following equation:

$$\text{Stimulation Index (SI)} = \frac{\text{OD with } \textit{Aloe vera} \text{ juice}}{\text{OD without } \textit{Aloe vera} \text{ juice}} \times 100$$

4.3.4 Optimization of *Aloe vera* juice supplemented ice cream (ASIC)

The levels of *Aloe vera* juice, milk fat and WPC for ASIC were optimized with the help of Response Surface Methodology (RSM) using CCRD design. The experimental design consisted of three variables with 20 runs. The minimum and maximum levels of ingredients/variables taken for application in RSM were: 8 to 12% milk fat, 20 to 30% *Aloe vera* juice and 0 to 25% WPC (as % milk solids-not-fat). All the analysis were performed with Design Expert version 7.5.1 software.

Responses observed for ASIC optimization were: sensory parameters (color and appearance, body and texture, flavor, sweetness, creaminess, melting quality and overall acceptability), pH, acidity, specific gravity, instrumental color parameters (L^* , a^* and b^* values), viscosity, overrun, % melt per hour and firmness.

4.3.5 Ice cream preparation

Ice cream was prepared using the standard procedure with some modifications (Fig. 4.1), maintaining 15% sugar, 0.4% emulsifier and stabilizer and 40% total solids. Pasteurized *Aloe vera* juice was added to ice cream mix, prepared by using buffalo milk, cream, SMP (spray dried), WPC, sugar, emulsifier and stabilizer and flavor.

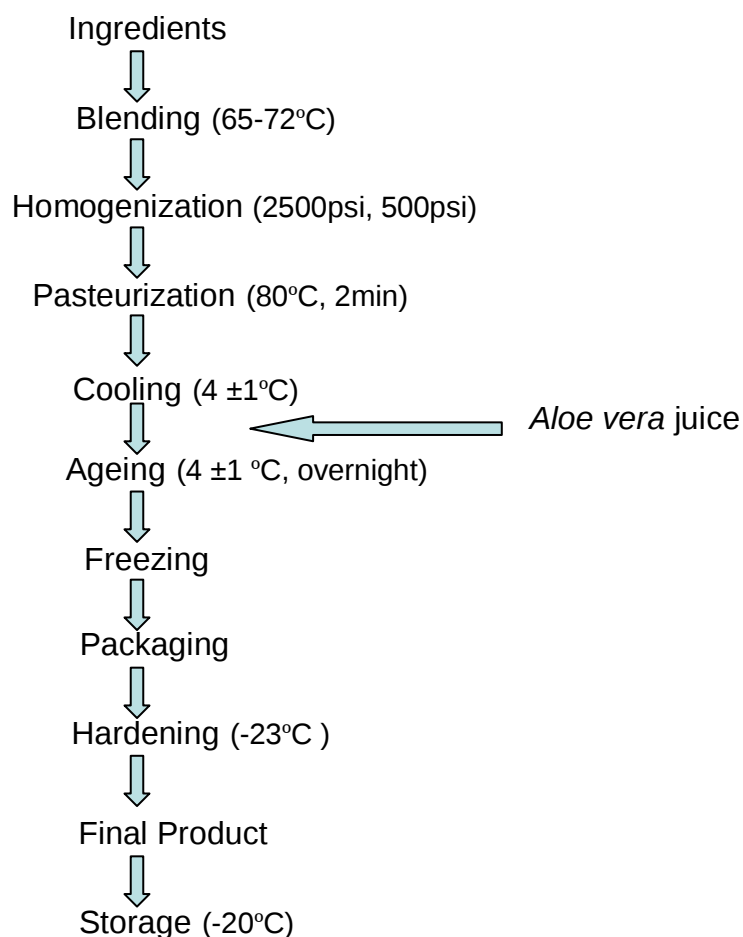


Fig 4.1 Flow diagram for the preparation of ASIC

4.3.6 Analysis of ice cream mix and ice cream

All the samples of ASIC were analyzed for their physico-chemical, rheological and sensory characteristics.

4.3.6.1 Sensory evaluation

Sensory evaluation of all the samples of ASIC was carried out using 9-point hedonic scale (Stone *et al.*, 1974). Sensory evaluation panel consisted of ten judges having adequate knowledge about the sensory evaluation methods and product characteristics were chosen from the Dairy Technology Division of NDRI, Karnal. The samples were drawn from the refrigerator just before serving to the panelists. The sensory score cards presented to the panelists during the sensory evaluation of the ASIC samples is presented in Annexure I.

4.3.6.2 Physico-chemical analysis

Various tests conducted for physico-chemical analysis of the ASIC samples are described in the following section.

(a) pH

The pH of ASIC mix samples was measured using a pH meter (pH Tutor, EUTECH Instruments, Malaysia) at 20°C using a combined glass electrode fitted in association with a temperature probe. Before starting the experiment, the pH meter was standardized using standard buffers of pH 4.0 and 9.0 at 20°C.

(b) Titratable acidity

Titrate acidity (as % lactic acid) of ASIC mix samples was estimated as per ISI (1981). A 10 g of ASIC mix sample was taken in a beaker and an equal amount of distilled water (10 ml) was added to it. The content was mixed well followed by addition of few drops (2-3) of phenolphthalein indicator. The sample was titrated against 0.1N NaOH till the appearance of light pink tinge, which persisted for 30 seconds in the solution. The titrate acidity was calculated by the following formula and expressed as percent lactic acid.

$$\text{Acidity (\% Lactic acid)} = \frac{9 \times N \times V}{W} \times 100$$

Where, V = Volume of 0.1 N NaOH required for titration

N = Normality of NaOH solution

W = Weight of sample taken for the titration

(c) *Overrun*

The overrun of the ice cream was determined by using the given formula:

$$\text{Overrun (\%)} = \frac{W1 - W2}{W2} \times 100$$

Where, W1: Weight per unit volume of the mix

W2: Weight for same volume of frozen ice cream.

(d) *% Melt/h*

The % melt/h was measured by the method of Specter and Sester (1994). The sample of known weight was placed on a wire mesh, which in turn was placed on a pre-weighed measuring cylinder (100 ml capacity) with a glass funnel (10 cm dia). The whole assembly was kept undisturbed for 1h. The weight of the melted sample was determined by the formula:

$$\% \text{ Melt/h} = \frac{\text{Wt. of the melted ice cream}}{\text{Initial wt. of ice cream}} \times 100$$

4.3.6.3 Instrumental analysis

(a) *Color*

Color measurements were conducted using Colorflex (Hunter Associates Laboratory, Inc., Reston VA, USA) color measurement system equipped with dual beam xenon flash lamp and universal software. The instrument was calibrated prior to sample measurements with standard black and white tile as prescribed by the supplier.

The results were represented by the L*, a* and b* notation. It is a 3-D color presentation method in which L* is the lightness of color and equals 0 for

black and 100 for white. The a^* is the amount of red (0 to 60) or green (0 to -60) while b^* is the yellowness (0 to 60) or blueness (0 to -60).

(b) Viscosity

Apparent viscosity of ASIC was measured at 20°C using a rotational viscometer (VISCO STAR plus, FUNGILAB, Spain) with a fixed outer cylinder and rotating spindle TL7 at 200 rpm. Sample volume loaded into the outer cylinder using a pipette was 9.5 ml. Values of viscosity were recorded after 15 sec of the start of the experiment. Before starting the measurement, the viscometer was correctly leveled with the help of bubble level indicator fitted at the back side of the viscometer. All the measurements were obtained in cP (centi Poise) units.

(c) Firmness

The samples of developed ice cream were evaluated for its firmness, using Texture Analyzer TAXT2 (Stable Micro systems, Godalming, Surrey, UK) fitted with a 25 kg load cell. Experiments were carried out using warner blade. The ice cream samples were carefully filled up in 1 ltr ice cream bricks (of similar dimensions) so that no air pockets remained within the sample. It was then stored at $-20 \pm 2^\circ\text{C}$ temperature for 48 h. The probe i.e., warner blade used to be kept at $-20 \pm 2^\circ\text{C}$ for half an hour before starting the experiment, to reduce any variations due to temperature difference between the probe and the ice cream sample. The experiments were carried out after maintaining room temperature at approx. 20°C using air conditioners. After maintaining all the conditions, the carton or brick was removed carefully with cutter to avoid any damage to the shape and size of the brick. For all the trials, the ice cream brick's width was subjected for the application of probe. Triplicate measurements were made for each sample. The test conditions maintained were as under:

Mode	: Measuring force in compression
Option	: Return to start
Pre-test speed	: 25 mm/sec

Test speed : 1mm/sec
 Post-test speed : 10 mm/sec

4.3.6.4 Proximate composition analysis

(a) Fat

The fat percent of buffalo milk was estimated by the Gerber method described in ISI (1981). The fat content of ASIC mix was determined by Mojonnier method with certain modifications. Approximately 2 g sample was weighed in to a 50 ml beaker, added with 2 ml of alcohol and stirred to mix with the entire sample, so as to prevent lumping on subsequent addition of acid. 10 ml of HCl (25 parts diluted with 1 parts of water) was added into the beaker and mixed well. The beaker was held in a water bath at 70 to 80°C for 30 min with intermittent stirring of the contents. Then, 10 ml of alcohol was added and the mixture was cooled to room temperature. It was then transferred to Mojonnier fat extraction tube with 25 ml diethyl ether, added in three portions. The tube was stoppered with a cork and shaken vigorously for 1 min. Then 25 ml petroleum ether (B.P., 40-60°C) was added to the tube and the tube contents were again shaken vigorously for 1 min. The tube was allowed to stand until the upper layer was clear. The clear solution was decanted (without disturbing the surface of the bottom layer) into a dried and tared aluminum dish. The extraction step was repeated twice taking each time only 15 ml of ether. Ether from the extract was slowly evaporated initially on a hot plate and then in an oven at 100°C to a constant weight. The beaker was allowed to cool and then weighed. Fat content was calculated as under:

$$\% \text{ Fat} = \frac{W_1 - W_2}{W} \times 100$$

Where, W_1 = Weight of empty tared aluminum dish (g)
 W_2 = Weight of aluminum dish with residual fat (g), and
 W = Weight of sample (g).

(b) *Total Solids*

Total solids content in the samples was determined as per the BIS method ISI (1981).

(c) *Protein*

The protein content was determined after multiplying percent nitrogen by the factor 6.38. The total nitrogen content was determined by the micro-Kjeldahl method (AOAC, 1970).

(d) *Ash*

The ash content of ASIC samples was estimated by the method described in ISI (1981).

4.3.7 Probiotic activity verification of the cultures

All the above mentioned probiotic strains were tested *in vitro* for the probiotic activity using the following parameters:

4.3.7.1 *Acid tolerance*

Resistance to acidic conditions was tested by the method described by Iyer *et al.* (2010) with some modifications. All four *Lactobacillus* probiotic bacteria were grown in MRS broth overnight at 37°C. The actively grown cells were suspended in MRS broth with pH adjusted to pH 2.0, pH 3.0, pH 4.0 with 1 M HCl and in MRS broth with pH 7.0 as control. Survival was evaluated by determining the viable counts of the samples serially diluted in saline after 0, 30, 60, 120 and 180 min, which was subsequently plated on MRS agar and incubated at 37°C for 48h.

4.3.7.2 *Bile tolerance*

Bile tolerance of all four *Lactobacillus* bacteria was tested according to the method of Gilliland *et al.*, 1984 with modification. All four *Lactobacillus* bacteria were grown in MRS broth overnight at 37°C. The actively grown cells were suspended in MRS broth supplemented with 1%, 2% w/v ox bile (Himedia Laboratories Pvt. Ltd, India) and without supplement as a control. Survival was

evaluated by plate count on MRS agar, after 0, 1 and 3h of incubation in MRS broth containing bile salts and the plates were incubated at 37°C for 48h.

4.3.7.3 Antibiotic susceptibility

Disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA) was used to study the pattern of resistance/susceptibility to antibiotic of probiotic. A total of 14 antibiotic discs (HiMedia Ltd, Mumbai, India) of Amikacin (30mcg), Ampicillin (10mcg) Co-Trimoxazole (25mcg), Tetracyclin (30mcg), Gentamycin (10mcg), Chloramphenicol (30 mcg), Cefazolin (30mcg), Cefuroxime (30mcg), Ofloxacin (1mcg), Erythromycin (15mcg), Clindamycin (2mcg), Vancomycin (30mcg), Penicillin (10 units), Cephalothin (30mcg) were used. MRS agar was poured in to plates and allowed to solidify. These were subsequently overlaid with 5 ml of MRS soft agar tempered at 45°C and seeded with 200 µL of active cultures. Petriplates were allowed to stand at room temperature for 15 min and then the Himedia antibiotic discs were dispensed onto agar using forceps under aseptic conditions. The agar plates were incubated at 37°C for 24 h. Diameter (mm) of zone of inhibition was measured and results were expressed in terms of resistance, moderate susceptibility or susceptibility by comparing with the interpretative zone diameters given by Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI, 2007) for disc diffusion antibiotic susceptibility test.

4.3.7.4 Antimicrobial activity

Lactobacillus isolates were screened for their antibacterial activity and inhibitory spectra against a broad range of Gram-positive and Gram-negative strains (from NCDC) viz. *Escherichia coli*, *Escherichia faecalis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Shigella spp.* and *Salmonella typhi* by agar well diffusion assay given by Anand *et al.* (1984) with some modification. Nutrient agar containing 0.1 percent Tween-80 was seeded with 100 ml of 24h old culture of indicator organism's, stirred gently and poured into the glass plates. These

were then allowed to solidify. The plates were marked into five different zones to represent four lactobacilli cultures and one for the control in central region. Five wells (6 mm diameter) were bored at equal distance on the solidified agar medium in each of those plates. A sterile cylindrical hollow stainless steel gel-cutter (6 mm diameter) was used for this purpose. Then holes were filled (except central hole) with 50 µl of the 24h old culture of *lactobacilli* grown in MRS broth. The central hole was used as a control, filled with sterile water. The plates were kept at room temperature until the liquid was absorbed, then incubated at 37°C for 48h and the diameter (mm) of inhibition zone measured.

4.3.7.5 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 of all four probiotic bacteria was determined according to the method described by Rosenberg *et al.*,1980 with modification using n-Hexadecane, n-Octane and Xylene. Cultures of the strains were grown in MRS broth overnight at 37°C. The cells were harvested by centrifugation at 12,000 x g for 5 min at 5°C, washed twice and resuspended in 5 ml phosphate urea magnesium (PUM) buffer (pH 6.5) and the cell suspension was adjusted to an absorbance value (A_{610}) of approx. 0.8 - 1.0. Three ml of the bacterial suspension were put in contact with 1 ml of each of the hydrocarbons. The cells were pre-incubated at 37°C for 10 min and then vortexed for 120 s. The suspension was then kept undisturbed at 37°C for 1h to allow phase separation and the hydrocarbon layer was allowed to rise completely. After 1h, aqueous phase was removed carefully and the absorbance (A_{610}) was measured using Spectrophotometer (Jenway Genova, Jenway Ltd. Gransmore Green, Felsted, Dunmow, UK). The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (%H) calculated with the equation (2).

$$\% \text{ Hydrophobicity} = \frac{\text{OD}_{\text{initial}} - \text{OD}_{\text{final}}}{\text{OD}_{\text{initial}}} \times 100$$

Where, OD_{initial} and OD_{final} are the absorbance (at 610nm) before and after extraction with the three hydrocarbons.

4.3.8 Survivability of probiotic strain with *Aloe vera* at freezing temperature ($-20\pm 2^{\circ}\text{C}$)

Different probiotic cultures @ 1% were added to sterile reconstituted skim milk (10% TS) samples containing the *Aloe vera* juice (previously pasteurized) at 20% level and were incubated at 37°C for 48 hrs. After 48 h of incubation period, cultured samples were placed in deep freezer maintained at $-20\pm 2^{\circ}\text{C}$ for forty days. Enumeration of probiotic count was done to check the low temperature tolerance and survivability of the probiotic strains at freezing temperature.

4.3.9 Incorporation of selected probiotic bacteria in ASIC

The ASIC mixes were inoculated by addition of fermented milk representing 4% and 8% of the total milk of the formulation. The fermented milk was prepared by culturing sterilized milk (4% and 8% of total milk separately) with NCDC 627 @ 2% rate and incubating it at 37°C for 16h. The fermented milk was then mixed in to the ASIC mix and was stirred with a plunger for 5 min. After mixing, the ice cream mix was frozen and number of viable probiotic bacteria was determined immediately after freezing. For ice cream with commercial DVS culture, same percentage addition, as selected for NCDC-627 culture was selected. Milk was inoculated with the commercial DVS culture @ 2% and incubated at 37°C for 2h (activation period). Manufacture of ASPIC was done according to the standard procedure given in Fig. 4.2.

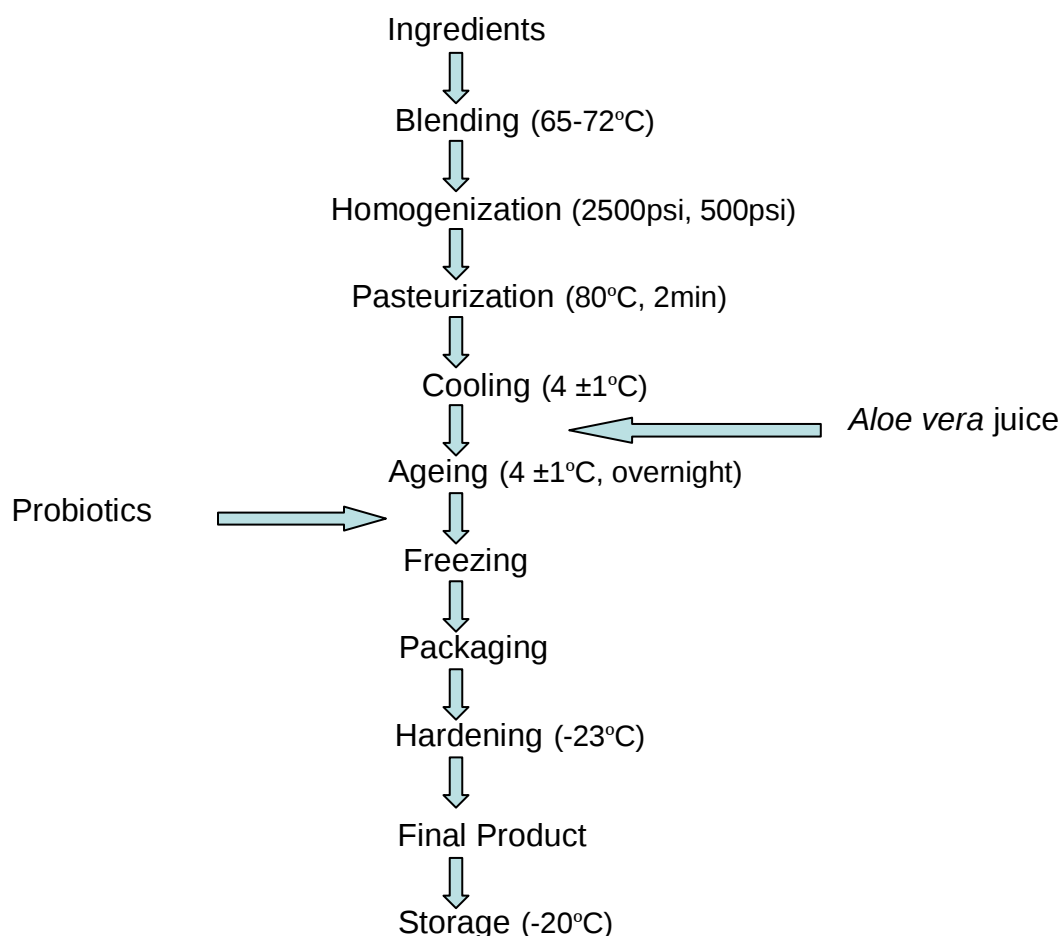


Fig. 4.2 Flow diagram for the preparation of ASPIC

4.3.10 Validation of immunomodulatory activity

The animal trials were conducted at Small Animal House of the National Dairy Research Institute under the guidance, supervision of an experienced animal expert and animal caretaker. The study was approved by the Ethical Committee for Animal Experiments (ECAE) of NDRI, Karnal.

4.3.10.1 Model

The immunomodulatory activity of ASPIC was validated by cyclophosphamide induced immunosuppression model.

4.3.10.2 Experimental animals

Adult female albino mice (25-30 g) were procured from the small animal house of National Dairy Research Institute (NDRI), Karnal, Haryana, India. The animals were housed in plastic cages per group under controlled temperature ($25 \pm 2^{\circ}\text{C}$) and were maintained in accordance with the guidelines of ECAE of the Institute.

4.3.10.3 Drug

Cyclophosphamide (Sigma, life science) was used as standard immunosuppressant. The drug was suspended in phosphate buffered saline (PBS, pH 7.2). The solution was administered intraperitoneally at a dose of 10 mg/kg body weight.

4.3.10.4 Grouping of animals

The study was carried out into two batches according to the dose of cyclophosphamide given (Fig. 4.3). In one batch (Batch- 1) intraperitoneal injection of cyclophosphamide (10 mg/kg body weight) was given only on 1st, 2nd and 3rd day. In other batch (Batch– 2) intraperitoneal injection of cyclophosphamide (10 mg/kg body weight) was given continuously for 13 days. In both the batches animals were sacrificed on 14th day.

❖ Batch- 1 (Cyclophosphamide injection on 1st, 2nd and 3rd day)

A total number of 24 mice were randomized into 4 groups consisting of 6 mice each on the basis of their body weight and age so that mean body weight and mean age of four groups did not differ significantly ($p>0.05$) at the beginning of experiment. Grouping of mice was carried out according to the feed given to the animal and animals were fed on respective diets for 13 days.

- Group I (Control diet) – CD-3
- Group II (Control ice cream) – CIC-3
- Group III (ASPIC with NCDC 627) NCDC-3
- Group IV (ASPIC with Commercial DVS culture) – DVS-3

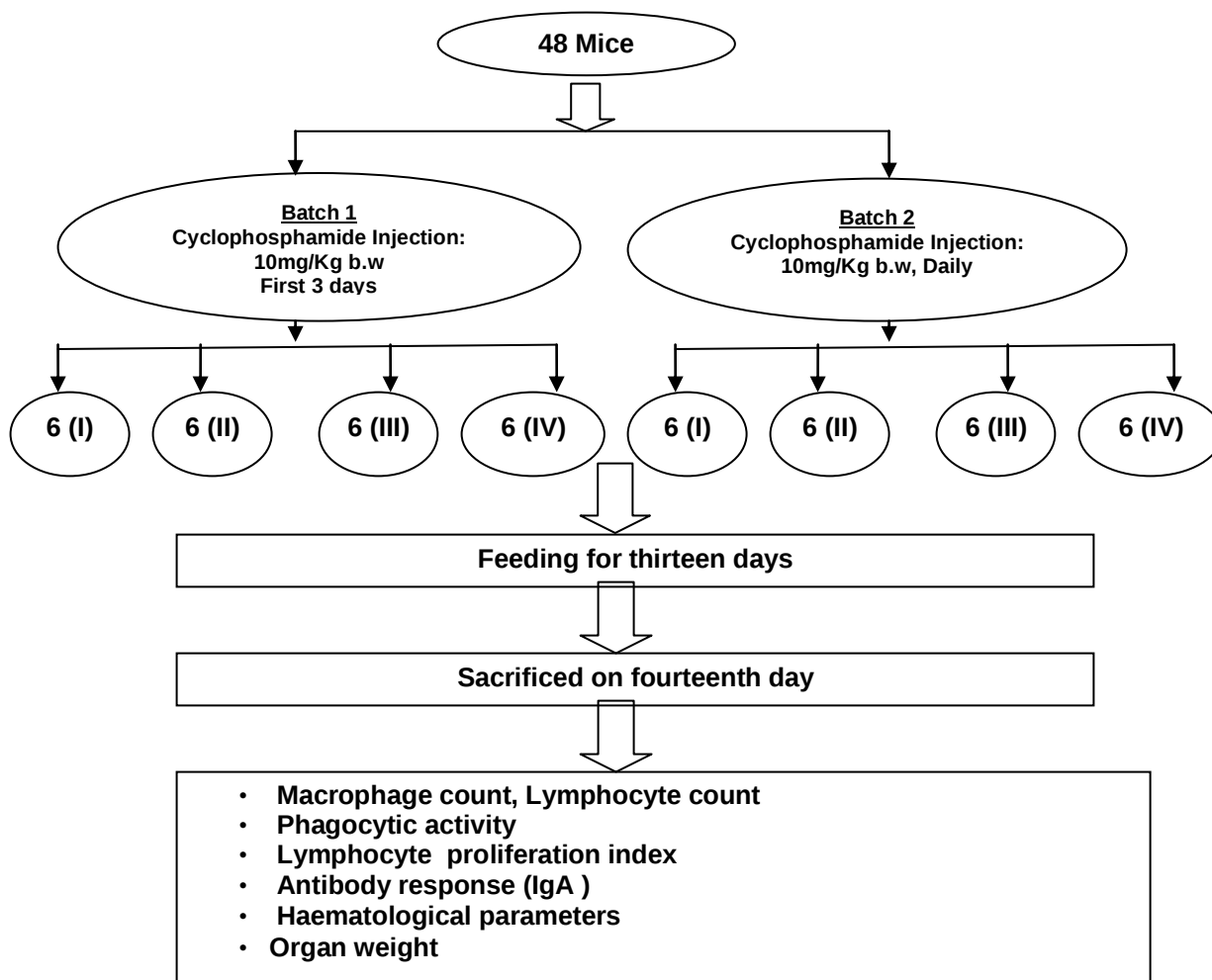


Fig. 4.3 Schematic representation of *in vivo* study

❖ *Batch- 2 (Daily cyclophosphamide injection till 13th day of feeding)*

A total number of 24 mice were randomized into 4 groups consisting of 6 mice each on the basis of their body weight and age so that mean body weight and mean age of four groups did not differ at the beginning of experiment. Grouping of mice was carried out according to the feed given to the animal and animals were fed on respective diets for 13 days.

- Group I (Control diet)– CD-D
- Group II (Control ice cream) – CIC-D
- Group III (ASPIC with NCDC 627) – NCDC-D
- Group IV (ASPIC with Commercial DVS culture) – DVS-D

4.3.10.5 Feeding schedule

The mice were allowed a 7 day adaptation period to remove the effect of stress possibly experienced by the animals due to separation from the main stock. All groups were fed *ad libitum* on basal diet and water. Subsequently, for both the batches, at the end of adaptation period, Group I was fed on basal diet only, Group II was fed on control ice cream, Group III was fed on ASPIC containing NCDC 627 probiotic culture and Group IV was fed on ASPIC containing Commercial (DVS) probiotic culture. Basal diet (in form of chalk) was crushed into powder and was mixed with melted ice cream in the ratio 50:50 (weight basis). Melted ice creams were also kept in a bowl along with the mixed diet in animal cages. The mice were maintained at this diet for whole experimentation period and had free access to water during the experimental period. The day on which the feeding of the diets started was referred to as 1st day. Animals were fed for 13 days and sacrificed on 14th day. Before sacrificing, the animals were kept on overnight fasting.

4.3.10.6 Monitoring of animals and sampling design

Animals were monitored for any abnormalities in activities, behaviours and general health status. As food was provided *ad libitum*, no measure of individual intake could be calculated. The individual weight of the mice was monitored once a week. Mice were anaesthetized using Diethyl ether and were sacrificed by cervical dislocation on 14th day of post feeding. For macrophage count and phagocytic activity peritoneal fluid, for lymphocyte count and Lymphocyte Proliferation Index spleen, for IgA small intestine and blood for blood analysis were collected. Liver, kidney, spleen and small intestine were also sampled to check the difference in weight.

4.3.10.7 Macrophage count and in vitro phagocytosis assay

Binding (adherence) of foreign particle to a phagocytic cell followed by ingestion of former by the later are the two stages of phagocytosis process. Phagocytosis assay was performed using the method described by Hay & Westwood (2002) using yeast cells as foreign particles. Dried Bakers' yeast

(*Saccharomyces cerevisiae*) cells were obtained from Dairy Microbiology Division, NDRI. Yeast beads were dissolved in RPMI media and sonicated gently to disrupt clumps, diluted to 10^8 cells per ml and autoclaved.

(a) *Collection of peritoneal fluid*

RPMI media was used to collect peritoneal fluid. The pH of the medium was adjusted to 7.4 using 1 N HCl or 1 N NaOH and then filter-sterilized through 0.22 μ Millex-GV disposable filter unit (Millipore). Animals were sacrificed by cervical dislocation and the abdominal skin was swabbed with alcohol (70%). The skin was carefully removed, leaving the peritoneum intact. RPMI (5 ml) was injected into peritoneal cavity followed by gentle messaging of the abdomen and then peritoneal exudate (approx. 4 ml) was collected.

(b) *Macrophage count*

Macrophage cells in peritoneal exudates were counted using Neubauer chamber. Cell suspension was diluted with culture medium. After placing a coverslip on hemocytometer, a small aliquot (~20 μ l) of mixture was transferred to both chambers using pipette. Counting of viable cells (unstained) and dead cells (stained) was done within 2 min of mixing. The observation was made at 40% objective magnification. Cell count was done in one of the four peripheral, single ruled areas and total viable cells were calculated using following formula:

$$\text{Total number} = \text{Average count of viable} \times 10^4 \times \text{Dilution factor of viable cells/ml}$$

(c) *In vitro phagocytosis assay*

Aliquots of peritoneal macrophages (10^5 cells/ml) were incubated at 37°C for 2 h in 35 mm culture plates. Non-adherent cells were removed by decantation and fresh medium (1 ml) poured in the culture plates, and again incubated at 37°C for 2 h. The macrophages were then incubated with 100 μ l of yeast cell suspension (10^8 cells / ml) for 1 h in a humidified atmosphere (5% CO₂) at 37°C. The medium was removed and the cells washed twice gently with culture

medium. The cells were dried in air and then stained with May-Grunwald stain freshly diluted with Giemsa buffer (1:2) for 2 min. The extra stain was removed by washing cells with Giemsa buffer. The phagocytosis was observed at 1000x magnification under oil immersion, and the following observations were recorded:

Percentage phagocytosis = $\frac{\text{Number of macrophages with yeast cell internalized}}{\text{per 100 macrophages}}$

4.3.10.8 Lymphocyte count

For the lymphocyte count following method was used:

(a) *Collection of spleen of mice:* The mice were sacrificed by cervical dislocation. The peritoneum was opened and spleen was taken out and placed into sterile tubes containing tissue culture medium. It was washed and made free from connective tissue and adipose tissue.

(b) *Preparation of spleenocytes:* The collected spleens were subsequently transferred to sterile petridish containing RPMI-1640 basal culture medium. They were teased gently using a sterile needle and forceps to release cells from spleen. It was allowed to stand for 2 min for the sedimentation of clumps. Then, using 1 ml pipette the upper portion of medium containing spleenocytes was transferred to a 15 ml sterile centrifuge tube and again left to stand for 2 min (in ice) and transferred to another sterile centrifuged tube. This cell suspension now devoid of clumps and larger particles was centrifuged at 1500 rpm for 5 min. The cells were washed once again in basal culture medium and then to the pellet, 1 ml of erythrocyte lysis buffer was added. After few seconds, 5 ml of culture medium was added to this and centrifuged at 1500 rpm for 5 min.

The lymphocyte cell suspension was washed twice with cold culture medium to remove traces of lysis buffer and finally suspended in 1 ml of 10 percent FCS and β - mercaptoethanol containing medium. A small aliquot of cell suspension was taken for cell count and checking cell viability.

(c) *Cell counting:* The viability and counting of cells was carried out by the method of Hay and Westwood (2002). The Neubauer ruling hemocytometer was

used for counting of lymphocyte cells. After placing a cover slip on hemocytometer, a small aliquot (~20µl) of mixture was transferred to both chambers using pipette. Counting of cells was done within 2 min of mixing. The observation was made at 40 percent objective magnification. Cell count was done in one of the four peripheral, single ruled areas and total viable cells were calculated using following formula:

$$\text{Total no. of cells/ml} = \text{Average count of cells} \times 10^4 \times \text{Dilution factor}$$

4.3.10.9 Lymphocyte proliferation assay

Lymphocyte proliferation assay was carried out using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay to check the effect of feeding on lymphocyte proliferation index in mice.

(a) *Preparation of basal culture medium (RPMI-1640)*: Following ingredients were added to one liter of autoclaved milliQ water.

RPMI-1640	:	10.4 g (one vial)
NaHCO ₃	:	2.10 g
Sodium pyruvate	:	110.1 mg
HEPES	:	5.96 g (50 mM)
Penicillin	:	61 mg (100 U/ ml)
Streptomycin	:	100 mg (100 µg/ml)

The pH of the resulting solution was adjusted to pH 7.4 and sterilized through 0.22 µm PVDF membranes using positive pressure. The prepared medium distributed in small aliquots was stored at 5°C.

(b) *Preparation of culture medium*: The basal culture medium was supplemented with following constituents for its use as culture medium.

a) β-Mercaptoethanol - 5 µM solution

b) Fetal calf serum (FCS) - 10 percent heat inactivated at 56°C for 30 min

c) Mitogens - Lipopolysaccharide - 50 µg/ml

- Concanavalin A - 2.5 µg/ml

(c) *Collection of spleen of mice:* The mice were sacrificed by cervical dislocation. The peritoneum was opened and spleen was taken out and placed into sterile tubes containing tissue culture medium. It was washed and made free from connective tissue and adipose tissue.

(d) *Preparation of spleenocytes:* The collected spleens were subsequently transferred to sterile petridish containing RPMI-1640 basal culture medium. They were teased gently using a sterile needle and forceps to release cells from spleen. It was allowed to stand for 2 min for the sedimentation of clumps. Then, using 1 ml pipette the upper portion of medium containing spleenocytes was transferred to a 15 ml sterile centrifuge tube and again left to stand for 2 min (in ice) and transferred to another sterile centrifuged tube. This cell suspension now devoid of clumps and larger particles was centrifuged at 1500 rpm for 5 min. The cells were washed once again in basal culture medium and then to the pellet, 1 ml of erythrocyte lysis buffer was added. After few seconds, 5 ml of culture medium was added to this and centrifuged at 1500 rpm for 5 min.

The lymphocyte cell suspension was washed twice with cold culture medium to remove traces of lysis buffer and finally suspended in 1 ml of 10 percent FCS and β - mercaptoethanol containing medium. A small aliquot of cell suspension was taken for cell count and checking cell viability.

(e) *Cell counting:* The viability and counting of cells was carried out by the method of Hay and Westwood (2002). The Neubauer ruling hemocytometer was used for counting of lymphocyte cells. After placing a cover slip on hemocytometer, a small aliquot (~20 μ l) of mixture was transferred to both chambers using pipette. Counting of cells was done within 2 min of mixing. The observation was made at 40 percent objective magnification. Cell count was done in one of the four peripheral, single ruled areas and total viable cells were calculated using following formula:

$$\text{Total no. of cells/ml} = \text{Average count of cells} \times 10^4 \times \text{Dilution factor}$$

(f) *Dispensing and culturing of lymphocytes*: Cell suspension containing 1×10^7 viable cells/ml was made in culture media (containing 1 μ M solution of β -mercaptoethanol, 10 percent FCS). 100 μ l cell suspension was dispensed in each well of 96 well flat bottomed tissue culture plates. In positive control wells 10 μ l of mitogen (LPS or Con A) was added. Lymphocytes were cultured in above medium at 37°C for 48 h in a 95 percent relative humidity air atmosphere with 5 percent CO₂ in a CO₂ incubator.

(g) *Colorimetric MTT assay*: The cell proliferation was determined using the colorimetric MTT assay originally described by Mosmann (1983). MTT (3-[4, 5-dimethyl thazol-2yl]-2, 5-diphenyl tetrazolium bromide was dissolved in RPMI-1640 at 5 mg/ml and filter sterilized. At the end of 48 h of incubation, 10 μ l of MTT solution was added to all wells and plates were incubated for 4 h at 37°C. During this period crystals of MTT formazon were formed at the bottom of each well. The spent media along with suspension of cultured cells was decanted. 100 μ l of DMSO (di-methyl sulfoxide) was added to wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature the plates were read using a plate analyzer. Plates were read within 1 h after adding DMSO. Data is expressed as Stimulation index (SI) and calculated using the following equation:

$$\text{Proliferation Index} = \frac{\text{OD with mitogen}}{\text{OD without mitogen}} \times 100$$

4.3.10.10 Measurement of intestinal fluid antibodies

Secretory immunoglobulin anti-pathogen response was studied in the intestinal fluid by ELISA. ELISA was performed as described by Perdigon *et al.* (1991; 1991a) who modified the procedure given by Engwall and Perlmann (1971).

(a) Collection of intestinal fluid

The procedure used for the collection of the intestinal fluid was a modification of procedure described by Lim *et al.* (1981). The intestine from

gastro-duodenal to ileocaecal junctions was carefully removed and the contents were washed out with 5 ml phosphate buffered saline (PBS, pH 7.2) and then centrifuged at 2000 g for 30 min.

(b) Reagents

(i) Mouse IgA ELISA Core kit, pink-ONE

Mouse IgA ELISA Core kit, pink-ONE (Catalogue No. K0231081P) was obtained from KOMABIOTECH, Seoul, Korea was used for the estimation of IgA from intestinal fluid.

(ii) Kit contents:

Component	Description	Concentration	Working dilution (recommended by manufacturer)	Working dilution (used in the present study)
Coating antibody	Affinity purified Goat anti-Mouse IgA	1 mg/ml	1:1000	1:2000
Detection antibody	HRP conjugated Goat anti-Mouse IgA	1 mg/ml	1:5000-1:20000	1:100000
Standard protein	Mouse IgA calibrator	4.71 mg/ml	1000-15.625 ng/ml	10000-15.625 ng/ml
Pre-stained colour development reagent	Pink-ONE TMB solution	-	Ready for use	-

(iii) Coating buffer: 50 mM carbonate-bicarbonate buffer (pH 9.6)

For the preparation of coating buffer, 530 mg of Na_2CO_3 and 420 mg of NaHCO_3 were dissolved in 100 ml distilled water separately. Na_2CO_3 solution was added to NaHCO_3 solution and the final pH was adjusted to 9.6.

(iv) Assay diluent

Assay diluent was prepared by dissolve Bovine serum albumin (BSA) @ 1 percent in Phosphate buffer saline (PBS) and adjust the pH to 7.4.

(v) Washing solution

Washing solution was prepared by mixing Tween 20 @ 0.05 percent into PBS and the final pH was adjusted to 7.4.

(vi) Blocking solution

Blocking solution was prepared by dissolve BSA @ 1 percent in PBS and the final pH was adjusted to 7.4.

(vii) 2M H₂SO₄

2 M H₂SO₄ was prepared by diluting 11.1 ml of conc. H₂SO₄ to 100 ml using distilled water.

(viii) ELISA Protocol

❖ *Coating*

1. Coating antibody was diluted at a ratio of 1:2000 with coating buffer and each well of microplate was coated with 100 µl of diluted coating antibody
2. Incubated for overnight at 4°C
3. The wells were aspirated to remove liquid and the plate was washed 3-5 times with washing solution

Note: completely remove the liquid at each step for good performance and do not dry the well completely and so immediately go on next step

❖ *Blocking*

1. 200 µl of blocking solution was added to each well
2. Incubated at room temperature for at least 1 hour
3. The wells were aspirated to remove liquid and the plate was washed 3-5 times with washing solution

❖ *Standards and samples addition*

1. The standards were diluted in assay diluent at 1:2 serial dilutions to get different concentrations viz. 10000 ng/ml, 1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml and 15.625 ng/ml
2. The samples was also diluted in assay diluents based on the expected concentration of the analyte to fall within the concentration range of standards
3. 100 µl of standard or sample was transferred to assigned cells
4. 100 µl of assay diluents was transferred to empty well to be used as standard blank
5. Incubated at room temperature for at least 1 hour
6. The wells were aspirated to remove the liquid and the plate was washed 3-5 times with washing solution

❖ *Detection antibody addition*

1. Detection anti body was diluted in assay diluent @ 1: 100000 (1 part detection antibody in 100000 parts assay diluents)
2. 100 µl of this diluted detection antibody was transferred to each well
3. Incubated at room temperature for at least 1 hour
4. The wells were aspirated to remove the liquid and the plate was washed 3-5 times with washing solution

❖ *Colour reaction and reading*

1. 100 µl of pink-ONE TMB color development reagent was added to each well. Incubated at room temperature for proper colour development (pink-ONE TMB produces a deep blue colour during the enzymatic degradation of H₂O₂ by peroxidase)
2. After sufficient colour development (2-12 min at room temp.), 100 µl of stop solution (2 M H₂SO₄) was added to each well

- Using a microtiter plate reader, the plate readings were taken at the wavelength that is appropriate for the substrate used (450 nm for TMB)

❖ *Calculation of results*

- Blank readings were subtracted from each of the standard, control, and sample readings
- Duplicate (or triplicate) readings of each standard, sample and control were averaged
- The known conc. of IgA was converted to Log (IgA) values and a standard curve (Fig. 4.4) was obtained by plotting absorbance (OD 450nm) values on Y-axis and Log (IgA) concentration values on X-axis. Ensure that R^2 value should be more than 0.85
- Sample's OD readings were placed in the regression equation ($y=0.4846x-0.677$) to get the IgA concentration of the sample as Log (IgA). Antilog was applied for sample Log (IgA) values to get the sample's IgA concentration.

Table 4.1 Preparation of standard curve for IgA values (ng/ml)

IgA Concentration (ng/ml) (X-axis)	10000	1000	500	250	125	62.5	31.25	15.625
Absorbance (OD) values (Y-axis)	1.245	0.887	0.689	0.402	0.242	0.115	0.039	0.019

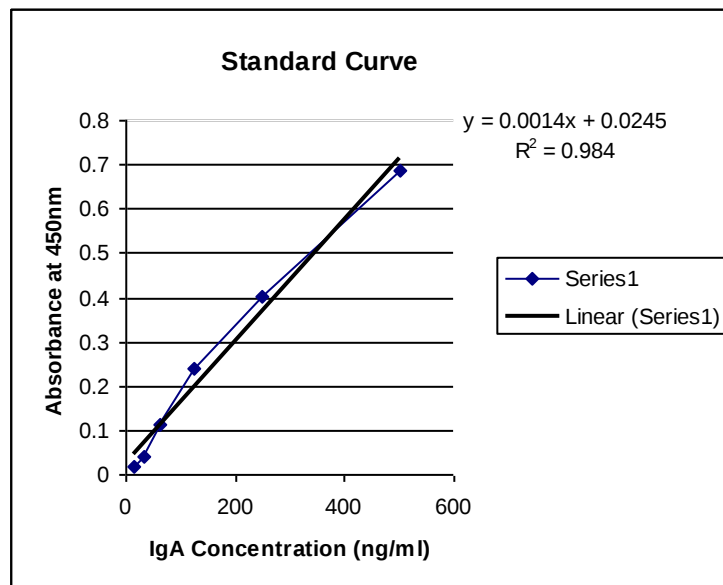


Fig.4.4 Standard curve for IgA value

4.3.10.11 Hematological parameters

Blood sample was carefully collected from the animals' heart immediately after opening the peritoneum and was transferred to an appendoff containing EDTA. Blood analysis was outsourced from Sikka laboratories, Karnal.

4.3.10.12 Organ Weights

Weight of vital organs such as spleen, liver, kidney, small intestine and large intestine were recorded to observe difference in weight of organs as affected by the feed on the same day after sacrificing the animals.

4.3.11 Storage study

The ice cream packed in polystyrene cups (100 ml) and paperboard cartons lined with LDPE (1000 ml) were stored for a period of 90 days at -20 ± 2 °C. During the storage period of 90 days, the products were evaluated for its sensory quality, microbiological quality and viability of the probiotic microorganisms at regular interval of 15 days.

4.3.11.1 Sensory evaluation

Same as in section 4.3.6.1

4.3.13.2 Standard plate count

Enumeration of the SPC of optimized product was performed by the pour plate method as described by Houghtby et al. (1993). Plating in triplicates of serially diluted ice cream sample was done and plates were incubated at 37 °C for 48 h. Colonies were counted and expressed as log cfu/g of ice cream sample.

4.3.11.3 Coliform Count

The coliform count was estimated using Violet Red Bile Agar media autoclaved at 15 psi (121°C) by pour plate method as described by Houghtby et al. (1993). Plates in triplicate were incubated at 37°C for 48 h. Colonies with dark red coloration were counted and expressed as log cfu/g of ice cream sample.

4.3.11.4 Yeast and mold count

Yeast and mold counts of optimized product was enumerated with the help of standard procedure suggested by Houghtby et al. (1993) using Potato Dextrose Agar medium. Triplicate plates were incubated at 25°C for 24 to 48h. Colonies were expressed as log cfu/g of ice cream sample.

4.3.11.5 Probiotic Lactobacilli count

MRS agar medium was used for the count of *Lactobacilli* at 37°C for 48h and expressed as log cfu/g of ice cream sample.

4.3.12 Consumer response study

A consumer survey was conducted to evaluate the acceptability of both the developed formulations. A heterogeneous group of consumers with different educational background and age from either sex were selected from the faculty members and staff from different divisions of NDRI, students of NDRI and general public visiting NDRI. Each sample was given to 150 respondents and comments were received through the score card provided with the sample (Annexure- II).

4.3.13 Cost analysis of the final product

The prevailing cost of each ingredient, equipment, land & building, manpower and other utilities was used in estimating the cost of ice cream. The quantity of raw material required per day was estimated according to the composition of the ice cream and assuming 100% overrun. In order to work out the cost of production of the ice cream, the following assumptions were made:

- 1) Plant capacity would be 500 kg ice cream mix per day.
- 2) Plant would be operated in single shift and 300 working days in a year.
- 3) Milk and cream to be procured through a contractor and to be delivered at the factory site in chilled condition.
- 4) *Aloe vera* juice would be procured ready-made in bulk.
- 5) The finished product would be packaged in ice cream cups and 1ltr. ice cream bricks.
- 6) Losses would be 0.5%

4.3.14 Statistical analysis

Optimization of the product was carried out by Response Surface Methodology (RSM) using CCRD design. Data obtained from various experiments were subjected to statistical analysis (one way ANOVA) to arrive at meaningful inferences using SAS Enterprise Guide 4.3 software (Welch's variance- weighted ANOVA test, Post hoc analysis was done by Duncan's multiple range test).

Chapter 5

Results and Discussions

RESULTS & DISCUSSION

5.1 INTRODUCTION

Ice cream is one of the delicious dairy products relished by all age groups of people throughout the world. Global production of ice cream is increasing constantly and the growth rate of production is tremendous. Earlier, ice cream was considered merely as a dessert or a special food that was taken for enjoyment. But, nowadays with the increasing consumption of ice cream, the nutritional properties become more important. Incidence of diabetes and coronary diseases are on the rise worldwide and hence people have become conscious about their diet. Ice cream being a product relished by people from all age groups and also because of its characteristic low temperature production and storage, can serve as a matrix for many desirable, high temperature sensitive nutraceuticals. Recently, there has been a growing interest in the field of herbal medicines research and search for their potential as immunomodulatory agents. Herbal drugs are believed to enhance natural resistance of the body against infection and their immunomodulatory have been reported in several studies but due to their undesirable and bitter taste their health benefits cannot be utilized, especially in case of children. Therefore, a matrix like ice cream, with its chilling exciting taste, can help in utilizing the potential therapeutic properties of herbs. Along with herbs, the probiotic approach to intestinal health and well being had led to new dimensions and opportunities for health based foods in today's consumer conscious era. However, in the development of functional foods, the technological suitability of probiotic strains poses a serious challenge since their survival and viability may be adversely affected by processing conditions as well as by the product environment and storage conditions. In case of probiotics too, ice cream can serve as a desirable base for the incorporation of same because of its low temperature storage. Thus, producing an ice cream with nutraceuticals viz. a herb like *Aloe vera* and probiotics can fill a gap in the market and fulfill consumer demand.

The present investigation was undertaken to standardize the process for the manufacture of *Aloe vera* supplemented probiotic ice cream and to protect the probiotic nature of selected organism and assessment of its survival during processing and ingestion. The effect of supplementation of *Aloe vera* on the sensory quality of the product and other important parameters was also evaluated. Attempts were made to assess the functional efficacy of *Aloe vera* supplemented probiotic ice cream in establishing the possible health outcomes using animal model. The results pertaining to these studies are discussed hereunder.

5.2 PRELIMINARY STUDIES

5.2.1 Selection of form and level of *Aloe vera*

For a new product development, sensory acceptability of the product is utmost important. To prepare an ice cream with a herb like *Aloe vera*, which possess an off-taste and off-flavor, selection of form i.e., gel or juice and level (percentage of addition) was carried out.

Aloe vera gel and juice from different brands were procured. Depending upon the IASC certification and availability, *Aloe vera* gel of forever living brand, available from local pharmacist and *Aloe vera* juice from (M/s Mehta Herbs and Spices, Coimbatore) were selected for preparation of *Aloe vera* supplemented ice cream.

Aloe vera gel was comparatively thicker in consistency than *Aloe vera* juice due to presence of additional stabilizer (xanthan gum @ 10%). Ice cream prepared using 25% *Aloe vera* gel was gummy in taste and was unacceptable. However, ice cream prepared with *Aloe vera* juice @ 25% was quite acceptable. Therefore, out of the two forms of *Aloe vera* i.e., gel and juice, *Aloe vera* juice was selected.

To select the level of *Aloe vera* juice for incorporation in ice cream, ice creams were prepared with higher levels of *Aloe vera* juice. Keeping in view the technical limitations of addition of *Aloe vera* juice at higher levels in ice cream

and minimum 15% requirement of Aloe for health benefits as per IASC guidelines, 20-30% addition level of *Aloe vera* juice was selected for development of *Aloe vera* supplemented ice cream.

5.2.2 Flavour selection for *Aloe vera* ice cream

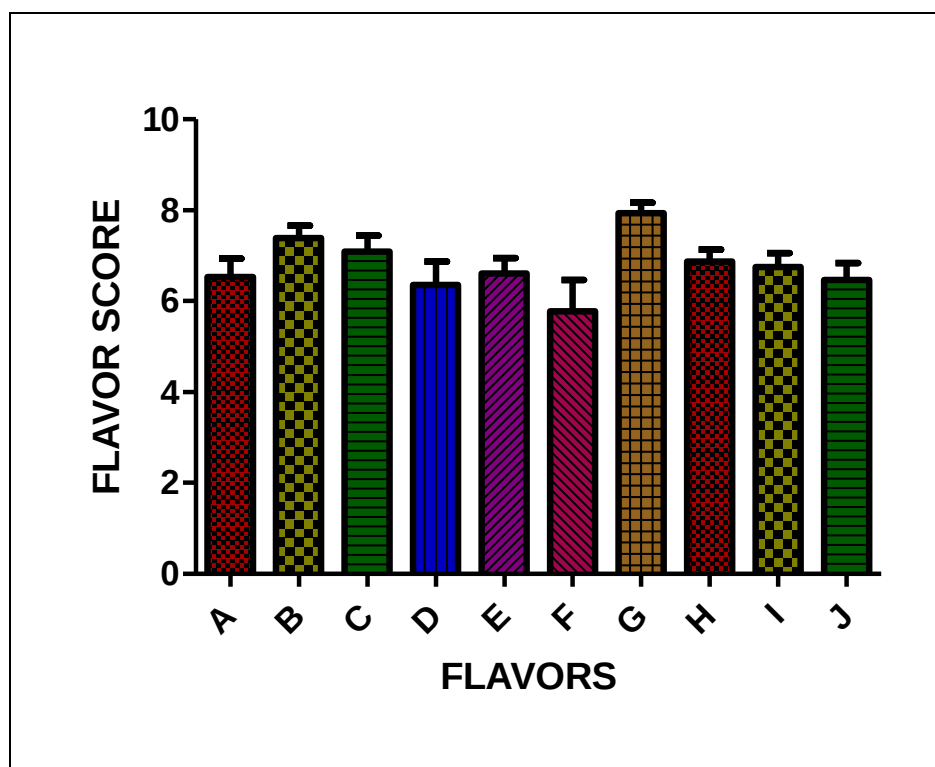
Flavor selection plays a critical role in development of ice cream and especially in case of a herbal ice cream in which masking of any undesirable herbal flavor is very critical. To select a desirable flavor for *Aloe vera* supplemented ice cream, ice cream mix was prepared with highest level of *Aloe vera* juice selected for RSM trials i.e., 30% and then ten different flavors (@ 0.1%) were added in ice cream mix to select the best flavor compatible with *Aloe vera*. Sensory evaluation of the *Aloe vera* supplemented ice cream mix samples with different flavors was carried out by sensory panel.

The ice cream mix with vanilla flavor obtained highest mean score of 7.94. It was suggested by the members of sensory panel to increase the level of vanilla flavor from 0.1% to 0.15% to make the product better. Therefore, vanilla flavor @ 0.15% addition was selected for the preparation of *Aloe vera* ice cream.

Table 5.2.1 Average sensory scores* for different flavor

FLAVOUR	AVERAGE SCORE
A - RASPBERRY	6.53 ± 0.41
B - CHOCOLATE	7.39 ± 0.27
C - KESAR ELAICHI	7.08 ± 0.36
D - MANGO	6.36 ± 0.51
E - CONTROL	6.61± 0.33
F - ROSE	5.78 ± 0.68
G - VANILLA	7.94 ± 0.22
H - PINEAPPLE	6.88 ± 0.26
I - BANANA	6.75 ± 0.31
J - CARDAMOM	6.47 ± 0.36

*(Mean ± S.E.)



A - J are different flavors

Fig. 5.2.1 Sensory scores for different flavors

5.2.2 Immunomodulatory activity of *Aloe vera* using *in-vitro* MTT assay

Generally, heat treatment or other processes are applied to enhance the shelf-life of commercial food products and also for the destruction of any potential harmful pathogens. As the *Aloe vera* juice was procured from Coimbatore in bulk and was stored at lower temperature in cold store, heating of *Aloe vera* juice was essential for destruction of any potential harmful microorganism and also to increase the shelf life of the developed product. However, Chang *et al.*, (2006) showed that the heating process promotes thermal degradation of polysaccharide of *Aloe vera* juice at higher temperature ranging from 80 °C to 90 °C, and mainly enzymatic hydrolysis at lower temperature from 50 °C to 60 °C, the maximal stability of the polysaccharide occurred at 70 °C.

Since, one of the objectives of the study is to validate the immunomodulatory potential of the developed ice cream, checking the effect of

heat on the immunomodulatory properties of *Aloe vera* juice was carried out using lymphocyte proliferation index by *in-vitro* MTT method. *Aloe vera* juice heated to different temperatures (70°C/10 min, 80°C /10 min, 121°C/10 min, and control (no heat treatment)) was used in MTT assay to check the effect of heat on immunomodulatory properties of *Aloe vera*. Results showed non significant ($p > 0.05$) decrease in immunomodulatory activity of *Aloe vera* upon heating. However, to avoid any destruction of polysaccharides of *Aloe vera* juice at higher temperatures, heating treatment of 70°C/10 min was selected as a heat pretreatment of *Aloe vera* juice before addition into ice cream mix.

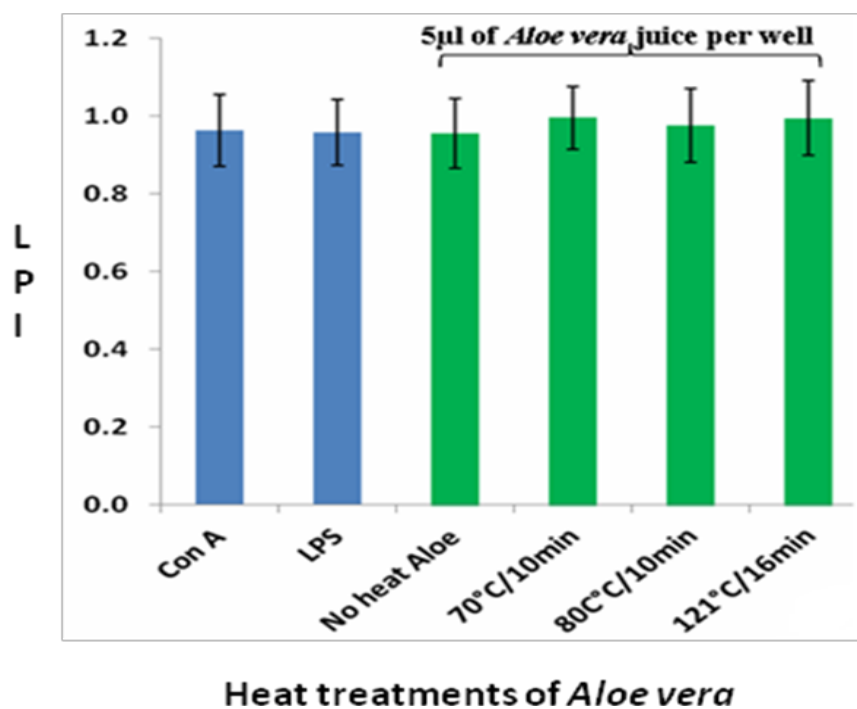


Fig 5.2.2 Effect of heat treatment on lymphocyte proliferation index (LPI) of *Aloe vera* juice

5.3 OPTIMIZATION OF *Aloe vera* SUPPLEMENTED ICE CREAM (ASIC) FORMULATION

RSM (Response Surface Methodology) experiment was framed to examine the effects of the selected ingredients of ASIC on the selected responses together with interactions among them so that an optimum

combination of variables could be worked out. ASIC samples were prepared from different levels of *Aloe vera* juice, WPC (percentage SMP substitution) and fat in ice cream mix. A five-level three-factor Central Composite Rotatable Design (CCRD) was adopted for the optimization. The coded levels of the three factors (design factors) are given in Table 5.3.1 and design matrix representing different combinations of the three factors are presented in Table 5.3.2.

Table 5.3.1 Coded levels of *Aloe vera* juice, WPC and fat

Factor	Axial point	Factorial point	Centre coordinate	Factorial point	Axial point
	-1.682	-1	0	+1	+1.682
A: <i>Aloe vera</i> juice	16.59	20	25	30	33.41
B: WPC (% SMP substitution)	-8.52 (0.00)*	0	12.5	25	33.52
C : Fat	6.64	8	10	12	13.36

*Value actually used in RSM trial

Table 5.3.2 The design matrix for three independent variables: *Aloe vera* juice, WPC and fat levels

Run	AV	WPC	FAT	Coefficient assessed by
1	20.00	0.00	8.00	Factorial
2	25.00	12.50	10.00	Centre
3	20.00	0.00	12.00	Factorial
4	30.00	0.00	12.00	Factorial
5	20.00	25.00	12.00	Factorial
6	25.00	12.50	10.00	Centre
7	25.00	12.50	6.64	Axial
8	25.00	12.50	10.00	Centre

9	30.00	0.00	8.00	Factorial
10	16.59	12.50	10.00	Axial
11	25.00	-8.52	10.00	Axial
12	25.00	12.50	13.36	Axial
13	25.00	12.50	10.00	Centre
14	25.00	33.52	10.00	Axial
15	30.00	25.00	8.00	Factorial
16	25.00	12.50	10.00	Centre
17	30.00	25.00	12.00	Factorial
18	25.00	12.50	10.00	Centre
19	20.00	25.00	8.00	Factorial
20	33.41	12.50	10.00	Axial

The responses generated in terms of sensory attributes, instrumental colour parameters, physico-chemical properties and rheological parameters are described below.

5.3.1 Effect of different levels of *Aloe vera* juice, WPC and Fat on sensory characteristics of ASIC

The organized sensory evaluation of dairy products has been an important factor in the development of the dairy industry. The ultimate enjoyment of ice cream is an experience that is the result of perceptions provided by the simultaneous application of multiple senses.

The quadratic models for various sensory attributes namely color and appearance, body and texture, sweetness, flavor, creaminess, melting quality and overall acceptability were obtained through successive regression analysis. The sensory scores of ASIC and partial coefficients of regression of these responses with respect to the levels of selected three factors in the form of correlations are presented in Tables 5.3.3 and Table 5.3.4 respectively.

Table 5.3.3 Sensory scores* of ASIC with different levels of *Aloe vera* juice, WPC and fat.

Run	AV	WPC	FAT	C&A	B&T	SWEETNESS	FLAVOR	CREAMINESS	MQ	O.A
1	20.00	0.00	8.00	7.88	7.8	7.60	7.25	7.40	7.40	7.38
2	25.00	12.50	10.00	7.94	7.64	7.78	7.69	7.67	7.53	7.53
3	20.00	0.00	12.00	7.75	7.50	7.80	7.45	7.65	7.70	7.64
4	30.00	0.00	12.00	7.75	7.20	7.58	7.48	7.80	7.67	7.45
5	20.00	25.00	12.00	7.74	7.70	7.67	7.40	7.25	7.30	7.50
6	25.00	12.50	10.00	7.50	7.20	7.70	7.50	7.60	7.20	7.35
7	25.00	12.50	6.64	7.47	7.83	7.61	7.50	7.25	7.55	7.63
8	25.00	12.50	10.00	7.81	7.56	7.69	7.59	7.44	7.47	7.50
9	30.00	0.00	8.00	7.52	7.00	7.50	7.32	6.80	7.23	7.20
10	16.59	12.50	10.00	7.81	7.63	7.66	7.63	7.72	7.56	7.41
11	25.00	-8.52	10.00	7.63	7.25	7.81	7.16	7.37	7.56	7.36
12	25.00	12.50	13.36	7.74	7.88	7.69	7.69	7.78	7.69	7.93
13	25.00	12.50	10.00	7.75	7.40	7.80	7.54	7.30	7.40	7.40
14	25.00	33.52	10.00	7.81	7.32	7.69	7.53	7.44	7.41	7.64
15	30.00	25.00	8.00	7.60	7.20	7.53	7.73	7.40	7.50	7.54
16	25.00	12.50	10.00	7.80	7.50	7.70	7.60	7.50	7.30	7.50
17	30.00	25.00	12.00	7.80	7.55	7.56	7.60	7.75	7.30	7.58
18	25.00	12.50	10.00	7.75	7.40	7.65	7.60	7.40	7.50	7.50
19	20.00	25.00	8.00	7.63	7.92	7.75	7.58	7.75	7.71	7.80
20	33.41	12.50	10.00	7.84	7.13	7.52	7.63	7.65	7.39	7.50

* Scored on a 9- point hedonic scale.

Table 5.3.4 Regression coefficients and ANOVA of the quadratic model for sensory characteristics of ASIC as influenced by *Aloe vera* juice, WPC and fat.

Factor	C & A	B & T	Sweetness	Flavor	Creaminess	Melting Quality	Overall Acceptability
Intercept	7.76	7.45	7.72	7.59	7.49	7.40	7.47
A-A V	-0.020	-0.21**	-0.062**	0.033	-0.031	-0.051	-0.029**
B-WPC	0.013	0.072	-9.652E-003	0.100**	0.045	-0.032	0.089
C-Fat	0.063	8.354E-003	0.030	0.027	0.15**	0.027	0.055
AB	0.049	0.029	-0.021	0.031	0.075	-1.250E-003	0.024
AC	0.056	0.13*	-0.021	1.250E-003	0.20**	0.044	0.041
BC	0.026	0.029	-0.061*	-0.084**	-0.17**	-0.170**	-0.096 *
A ²	0.022	-0.030	-0.058**	6.196E-003	0.057	0.014	-0.019*
B ²	-0.015	-0.064	-1.242E-003	-0.095**	-0.042	0.018	-2.665E-003
C ²	-0.055	0.140**	-0.037	-6.179E-003	-2.695E-003	0.066*	0.096**
R-Squared	0.5470	0.8693	0.7803	0.8881	0.9003	0.7914	0.8007
Adeq Precision	4.793	8.837	6.926	11.860	13.851	7.051	8.850
Model F value	1.34 ^{ns}	7.39 **	3.95*	8.81**	10.03**	4.21 *	4.46 **
Lack of fit	ns	ns	ns	ns	ns	Ns	ns

** Highly Significant ($p \leq 0.01$)

* Significant ($p \leq 0.05$)

^{ns} Non-significant

5.3.1.1 Effect of process variables on color & appearance score

When it comes to food, color and appearance are the first impressions, even before one's olfactory sense is tickled with a pleasing aroma. Color and appearance is a typical and basic sensory perception of any food product that appeals to the consumer and affects its acceptability. Ideally, the surface of ice cream should be homogeneous in appearance and show a color that is consistent with its declared flavor. The color and appearance ratings of the ASIC varied from 7.47 to 7.94 (Table 5.3.3). Lowest score was obtained in formulation prepared with 25% *Aloe vera* juice, 12.5% WPC and 6.64% fat. The formulation having 25% *Aloe vera* juice, 12.5% WPC and 10% fat showed maximum score implying that *Aloe vera* juice and WPC had no role in change of color and appearance score of the product.

The regression coefficient model estimates revealed that none of the ingredients in any formulation had significant impact on the sensory color and appearance score of ASIC (Table 5.3.4). Singh *et al.*, (2012) also reported that color and appearance of *Aloe vera* juice containing *lassi* remained unaffected by the incorporation of *Aloe vera* juice probably because the transparent pale green color of *Aloe vera* juice which masked by the milk color.

5.3.1.2 Effect of process variables on body & texture score

The term "body" refers to the cohesiveness of the product as it is being served and consumed. It is an indication of the overall structure of the product. "Texture" refers to the perceived relative smoothness of ice cream as it is being manipulated in the mouth. Texture is directly related to the size of crystalline material present. All ice cream contains these crystals, either as a result of the freezing of water into ice or through the crystallization of some components of the sugars present. The loss of quality is directly related to the size of the crystals - the larger the particles, the poorer the quality. The score for body & texture of the ASIC varied from 7.00 to 7.92 in the present study (Table 5.3.3). Maximum score (7.92) was obtained for the product with the following combination of variables:

20% *Aloe vera* juice, 25% WPC and 8% fat. The minimum score was observed in products prepared with 30% *Aloe vera* juice, 0% WPC and 8% fat.

Among the ingredients, *Aloe vera* juice and fat had highly significant effect on body and texture score of ASIC ($p \leq 0.01$), the former having essentially a linear impact whereas the later showed a quadratic relationship (Table 5.3.4). As the *Aloe vera* juice level increased from 20 to 30%, the body and texture score decreased proportionately at all the levels of fat and WPC (Fig.5.3.1a,b). Manoharan *et al.*, (2012) carried out organoleptic evaluation of herbal ice cream with 5 to 45% levels of added *Aloe vera* juice and observed a significant decrease in average body and texture score of ice cream with increase in the *Aloe vera* juice content. The decrease in sensory score for body and texture with increased *Aloe vera* juice concentration in *lassi* incorporated with *Aloe vera* juice has also been reported by Singh *et al.*, (2012).

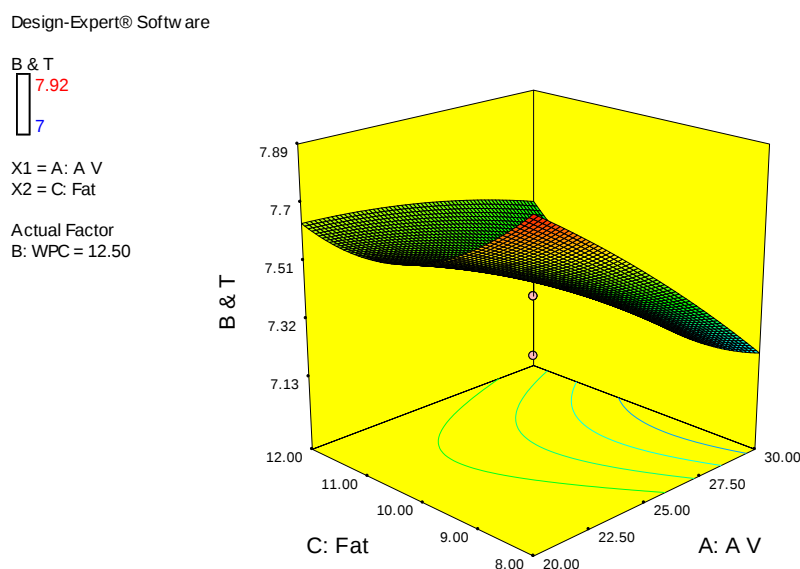


Fig. 5.3.1(a) Response surface plot relating to body and texture score as influenced by the levels of fat and *Aloe vera* juice

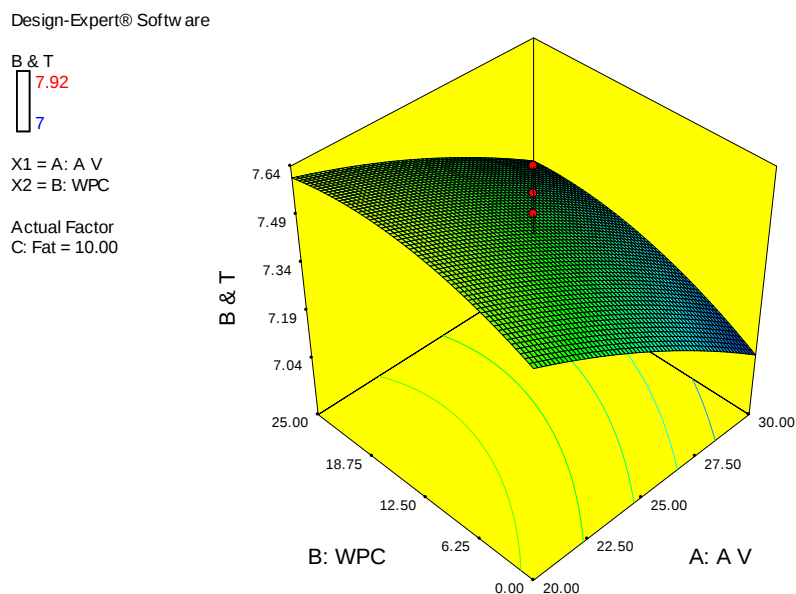


Fig. 5.3.1(b) Response surface plot relating to body and texture score as influenced by the levels of WPC and *Aloe vera* juice

Although fat level did not significantly influence the body and texture score at linear level, the interaction effect of fat with *Aloe vera* juice was significant ($p \leq 0.05$) (Table 5.3.4). As depicted in Fig. 5.3.1(a), the highest body and texture score was observed at 8% fat and 20% *Aloe vera* juice. The body and texture score decreased slightly when fat level increased to around 10% and *Aloe vera* juice level increased to 25%. However, the body and texture score increased with further increase in fat and *Aloe vera* juice levels. This increase in score with increase in fat could be attributed to the fact that at higher fat levels body and texture improves. However, the decrease in body and texture score from 8% to 10% could be due to the impact of other ingredients where fat could not show significant effect. The quadratic term of fat being significant ($p \leq 0.01$), the response- surface was concave upward (Fig.5.3.1a,c).

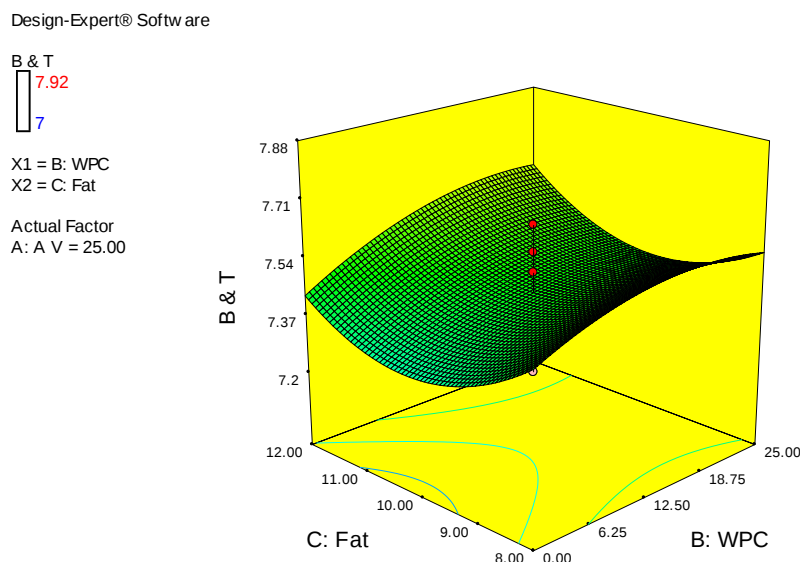


Fig.5.3.1(c) Response surface plot relating to body and texture score as influenced by the levels of fat and WPC

The R^2 value for the polynomial model predicting texture score was 0.87 suggesting that about 87 percent variation in the score could be explained by it within the factor range studied. The model could be given as under:

$$\text{Body \& Texture score} = 14.61617 - 0.12004 * AV - 6.99041E-003 * WPC - 1.03271 * Fat + 4.60000E-004 * AV * WPC + 0.013375 * AV * Fat + 1.15000E-003 * WPC * Fat - 1.21240E-003 * AV^2 - 4.08945E-004 * WPC^2 + 0.034407 * Fat^2$$

5.3.1.3 Effect of process variables on sweetness score

Sweetness is one of the five basic tastes and is almost universally regarded as a pleasurable experience. For an ice cream, sugar is an essential ingredient which not only imparts sweetness to the product but also affects palatability, body and texture, freezing point and storage properties of the ice cream. Sweetness score of ASIC ranged from 7.50 to 7.81. Maximum sweetness score was observed in the formulation with 25% *Aloe vera* juice, 0% WPC and 10% fat and minimum sweetness score was obtained in the formulation added with 30% *Aloe vera* juice, 0% WPC and 8% fat, indicating that the sweetness

score decreased with *Aloe vera* juice concentration increasing from 25% to 30% in the formulation (Table 5.3.3).

The coefficients of estimates of the relevant polynomial model (Table 5.3.4) showed that *Aloe vera* juice had a negative significant ($p \leq 0.01$) effect on the sweetness score of ASIC at both linear and quadratic levels. Fig.5.3.2(a) and Table 5.3.4 depict the decrease in sweetness score of ASIC with increasing *Aloe vera* juice level in the formulation. This decrease in sweetness score of the developed product could be attributed to the undesirable taste of *Aloe vera* juice, as it possess some bitter compounds. Interaction of WPC and fat with each other showed a significant ($p \leq 0.05$) negative effect on the sweetness score of ASIC (Table 5.3.4) and Fig.5.3.2(b).

The regression analysis of the data presented in Table 5.3.4 further shows that the coefficient of determination (R^2) was 0.78 and the “lack of fit test” was not significant, indicating that the model is sufficiently accurate for predicting the sweetness of the ASIC made with any combination of the factors within the range evaluated. The adequate precision was found out to be 6.93 appreciably higher than the minimum desirable i.e. 4 (for high prediction ability).

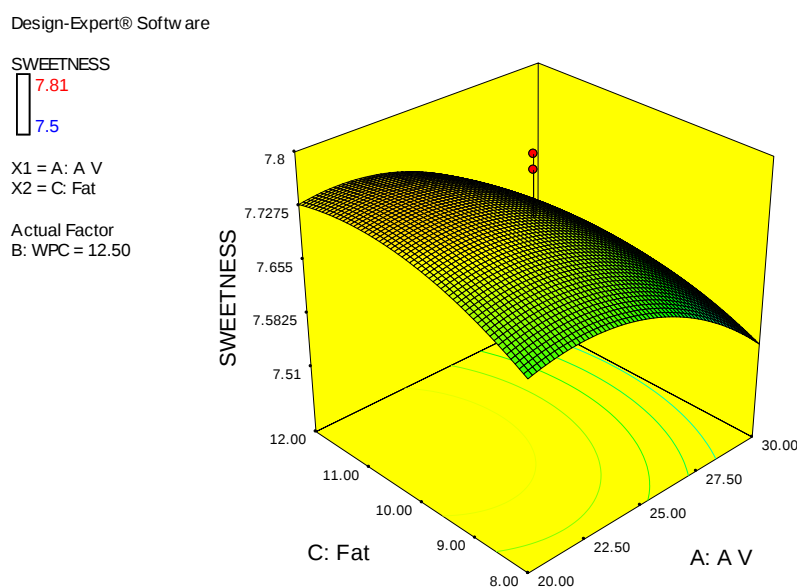


Fig. 5.3.2(a) Response surface plot relating to sweetness score as influenced by the levels of fat and *Aloe vera* juice

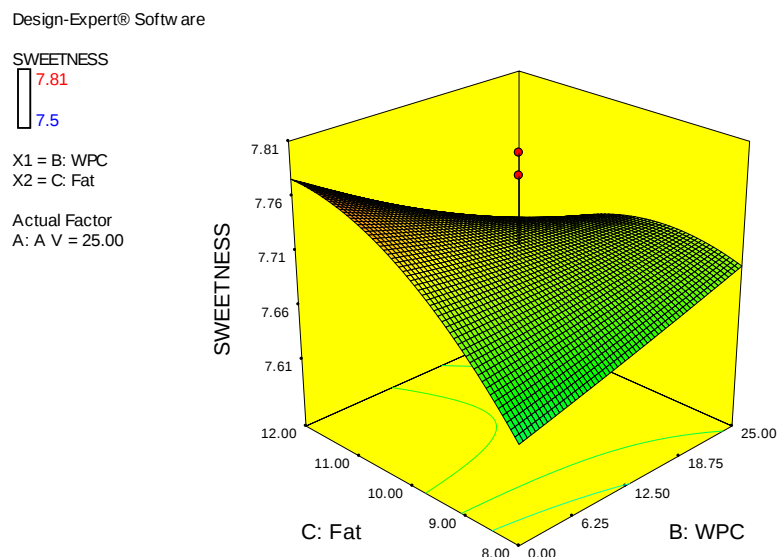


Fig. 5.3.2(b) Response surface plot relating to sweetness score as influenced by the levels of fat and WPC

The relevant quadratic model (for actual values) given below could account for 78% variation in the sweetness score:

$$\begin{aligned} \text{Sweetness score} = & 4.58772 + 0.12874 * AV + 0.032426 * WPC + 0.28155 * \text{Fat} \\ & - 3.40000\text{E-}004 * AV * WPC - 2.12500\text{E-}003 * AV * \text{Fat} - 2.45000\text{E-}003 * WPC * \\ & \text{Fat} - 2.31240\text{E-}003 * AV^2 - 7.94585\text{E-}006 * WPC^2 - 9.14922\text{E-}003 * \text{Fat}^2 \end{aligned}$$

5.3.1.4 Effect of process variables on flavor score

Flavor is the most important positive attribute of ice cream. The type and intensity of flavor in ice cream are the two important flavor characteristics. Mild flavor like vanilla tends to mask background flavor only to a limited extent; whereas, chocolate masks other flavors well (Marshall *et al.*, 2003). In the present study, *Aloe vera* juice did not impart any characteristic undesirable flavor to ice cream. Addition of vanilla flavor @ 0.15% was sufficient to mask the characteristic herb like flavor of *Aloe vera* juice in ice cream as shown by the not significant effect of *Aloe vera* juice on flavor of ice cream at all the three levels viz., linear, quadratic and interaction. The flavor score of the ASIC varied from 7.16 to 7.73 (Table 5.3.3). Lowest flavor score was observed in the formulation with 25% *Aloe vera* juice, 0% WPC and 10% fat and the maximum flavor score

was received by the product added with *Aloe vera* juice, WPC and fat at 30%, 25% and 8% levels respectively. The coefficient estimates of the relevant polynomial model (Table 5.3.4) showed that WPC level significantly ($p \leq 0.01$) affected the flavor score of ASIC. At linear level, WPC showed a positive effect on flavor score of ASIC. However at quadratic level the effect was negative. This decrease in flavor score at quadratic level indicates that higher level of WPC adversely affects the flavor of ASIC (Fig.5.3.3a). Similar results were observed by Kerdchouay and Surapat (2008) who studied the effect of whey protein concentrate on the qualities of low fat coconut milk ice cream and reported that the ice cream with WPC possessed undesirable flavour owing to the heat sensitivity of WPC, causing lower sensory acceptability.

Fat did not show any significant effect on the flavor score of ASIC at linear level (Table 5.3.4). However, the interaction between fat and WPC showed a negative significant ($p \leq 0.01$) effect which indicated that simultaneous increase in fat and WPC decreased the flavor score of ASIC. This could be due to the processing steps (homogenization and pasteurization) involved in the preparation of ASIC as a concentrated mix was processed and the *Aloe vera* was added at the end that might have altered the fat and WPC structure. From Fig. 5.3.3(b), it is evident that after 12.5% level of WPC and 10% level of fat, the flavor score of ASIC decreased.

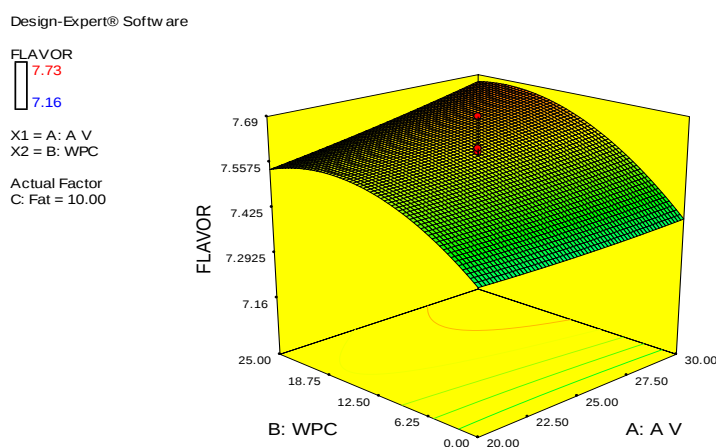


Fig. 5.3.3(a) Response surface plot relating to flavor score as influenced by the levels of WPC and *Aloe vera* juice

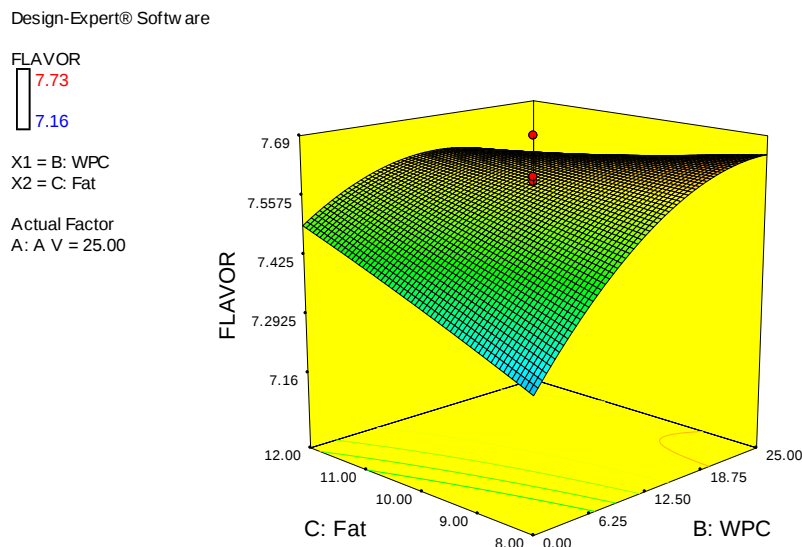


Fig. 5.3.3(b) Response surface plot relating to flavor score as influenced by the levels of fat and WPC

The regression analysis data presented in Table 5.3.4 highlighted that the coefficient of determination (R^2) was 0.89 and the “lack of fit test” was not significant, indicating that the model is sufficiently accurate for predicting the flavor of ASIC made with any combination of the factors within the range evaluated. The adequate precision was found out to be 11.86, appreciably higher than the minimum desirable i.e. 4 (for high prediction ability).

The flavor score of ASIC could be predicted by the equation (for actual values of the variables) given below:

$$\text{Flavor score} = 6.85775 - 0.013301 * AV + 0.044521 * WPC + 0.083173 * \text{Fat} + 5.00000E-004 * AV * WPC + 1.25000E-004 * AV * \text{Fat} - 3.35000E-003 * WPC * \text{Fat} + 2.47826E-004 * AV^2 - 6.05229E-004 * WPC^2 - 1.54468E-003 * \text{Fat}^2$$

5.3.1.5 Effect of process variables on creaminess score

Creaminess is a desired characteristic of ice cream determining the acceptability of the product. The creaminess score of ASIC ranged between 6.80

and 7.80. The minimum creaminess score was recorded in the formulation having 30% *Aloe vera* juice, 0% WPC and 8% fat and the maximum score was observed in the formulation with 30% *Aloe vera* juice, 0% WPC and 12% fat.

The coefficient of estimates of the relevant polynomial model (Table 5.3.4) showed that fat level significantly ($p \leq 0.01$) affected the creaminess score of ASIC at the linear level as well as during its interaction with *Aloe vera* juice and WPC. However, there was no significant effect of fat at quadratic levels. An increasing fat level gradually increased the creaminess score of ASIC (Fig.5.3.4a,b). From Table 5.3.4, it is evident that *Aloe vera* juice had no significant effect on the creaminess score of ASIC. However, the interaction of fat with *Aloe vera* juice showed a significantly ($p \leq 0.01$) positive effect on the creaminess score of ASIC (Fig.5.3.4b). WPC also did not significantly influence the creaminess score at linear level but showed a negative significant ($p \leq 0.01$) effect on the creaminess score on interaction with fat. Fig. 5.3.4(a) shows that the interaction of fat with WPC at levels higher than 12.5% WPC resulted in lower creaminess score.

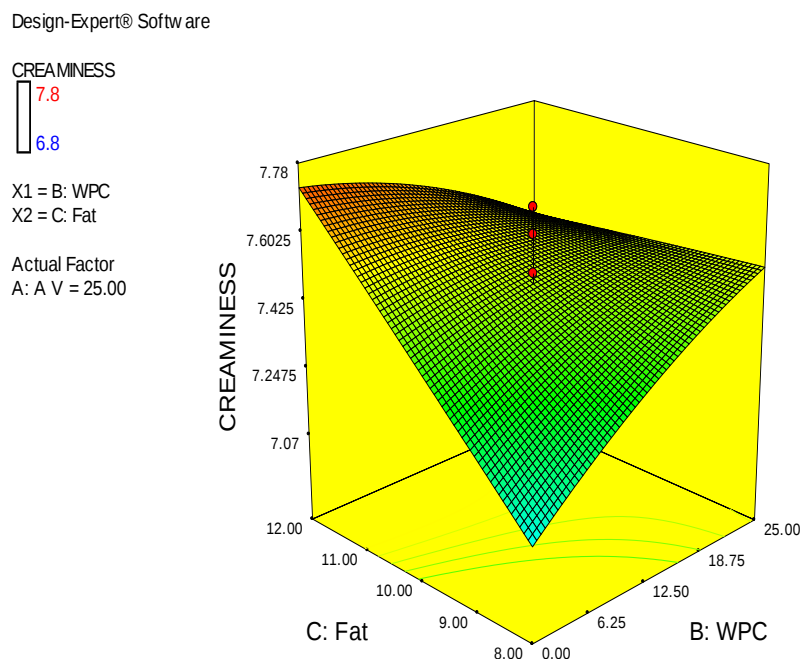


Fig.5.3.4(a) Response surface plot relating to creaminess score as influenced by the levels of fat and WPC

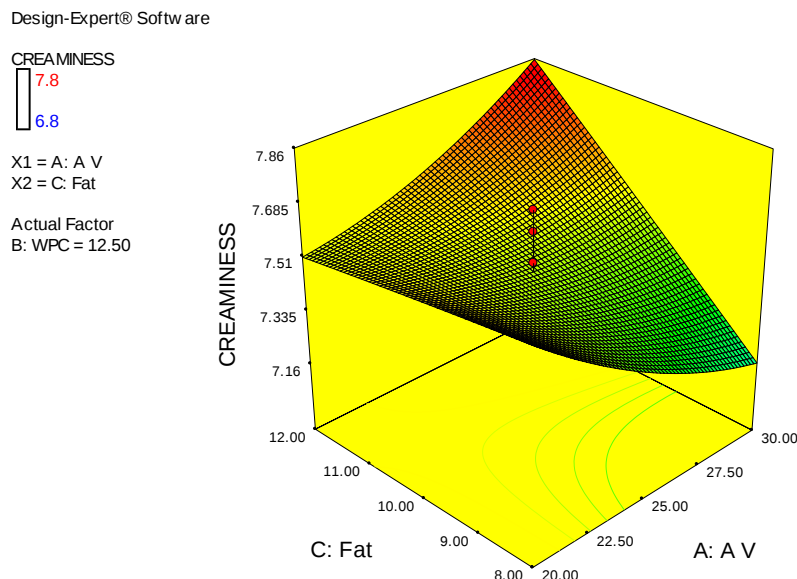


Fig. 5.3.4(b) Response surface plot relating to creaminess score as influenced by the levels of fat and Aloe vera juice

The regression analysis of data presented in Table 5.3.4 indicated that coefficient of determination (R^2) was 0.90 implying that 90% variation in the creaminess score could be explained by the three factors studied. The “lack of fit test” was not significant, indicating that the model was sufficiently accurate for predicting the creaminess score of ASIC made with any combination of the factors. The adequate precision was found to be 13.85, which is considerably higher than the minimum desirable i.e. 4. The creaminess score of ASIC could be predicted by the equation (for actual values of the variables) given below:

$$\begin{aligned} \text{Creaminess score} = & + 12.69195 - 0.33594 * AV + 0.050272 * WPC - 0.32612 * \\ & \text{Fat} + 1.20000E-003 * AV * WPC + 0.020000 * AV * \text{Fat} - 7.00000E-003 * WPC * \\ & \text{Fat} + 2.29637E-003 * AV^2 - 2.66149E-004 * WPC^2 - 6.73720E-004 * \text{Fat}^2 \end{aligned}$$

5.3.1.6 Effect of process variables on melting quality

Melting quality of ice cream relates to the behavior and appearance of a portion of ice cream as it melts. Ideally, the melted product should be smooth and homogeneous, resembling ice cream mix before freezing. The melting

quality score of the ASIC ranged between 7.20 and 7.71. Lowest score was obtained for the formulation with 25% *Aloe vera* juice, 12.5% WPC and 10% fat and the highest score was recorded for the formulation with the corresponding values of 20%, 25% and 8% for *Aloe vera* juice, WPC and fat (Table 5.3.3).

The coefficient estimates of the melting quality model (Table 5.3.4) showed that none of the three ingredients had any significant effect on the melting quality of ASIC at linear level. However, at quadratic level, fat had a significantly positive ($p \leq 0.05$) effect on the melting quality of ASIC. Roland (1999) studied the effect of fat content on the melting quality of ice cream and reported that the ice cream samples with higher fat percentage took longer to melt and were softer than the ice creams with lower fat content. The interaction of fat and WPC showed a significant ($p \leq 0.01$) negative impact on the melting quality (Table 5.3.4). It is evident from Fig. 5.3.5(a) that upon increasing WPC above 12.5% level and fat above 10% level, the melting quality decreased gradually. This decrease in the melting quality of the ASIC due to the interaction between fat and WPC could be attributed to increasing levels of WPC where the positive impact of fat might have been masked. Moreover, the heating of concentrated mix during pasteurization might have altered the structure of WPC that resulted in the negative behavior of WPC. Pandiyan *et al.*, (2010) prepared ice cream by replacing skimmed milk powder with whey protein concentrate and observed a decrease in melting time of ice cream with increased WPC. Similar results were observed by Rong-rong *et al.*, (2007) who studied the effect of fat replacers from whey protein on quality of ice cream and reported a decrease in melting resistance when the ratio of the replacement increased. In contrary, Kerdchouay and Surapat (2008) observed a reduction in melting rate of the ice cream on replacement of skim milk powder with WPC in low-fat coconut milk ice cream and attributed it to the water holding capacity of WPC.

In our study no effect of *Aloe vera* juice incorporation on the melting property of ice cream was observed. However, Manoharan *et al.* (2012) carried out an investigation to find the acceptable level of *Aloe vera* pulp inclusion in the ice cream. The authors subjected the prepared ice cream to sensory evaluation

and observed significantly lower average scores for melting quality as compared to the control samples.

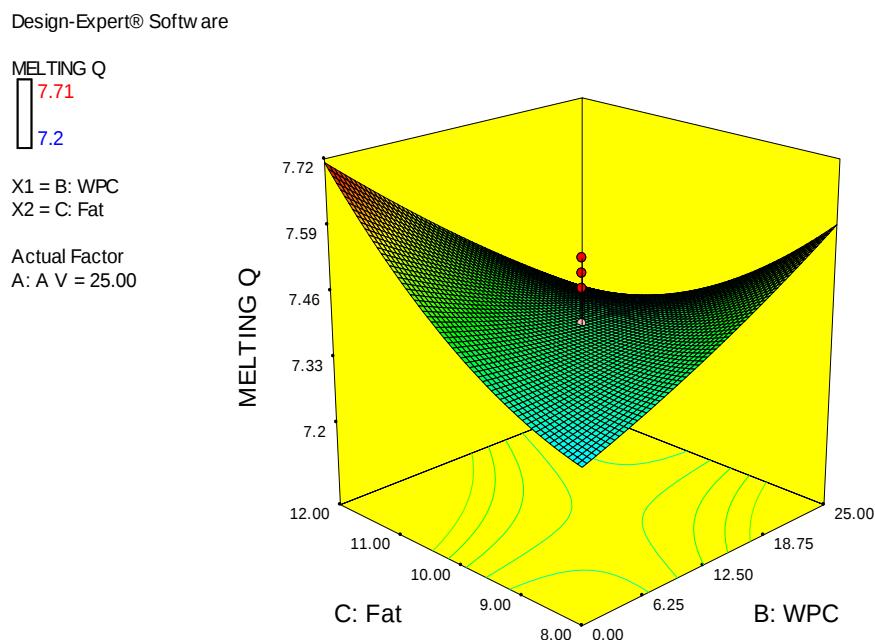


Fig.5.3.5(a) Response surface plot relating to melting quality score as influenced by the levels of fat and WPC

The melting quality score of ASIC could be predicted by the equation (for actual values of the variables) given below:

$$\begin{aligned} \text{Melting quality score} = & + 9.81121 - 0.082203 * AV + 0.062563 * WPC - 0.33923 * \\ & \text{Fat} - 2.00000\text{E-}005 * AV * WPC + 4.37500\text{E-}003 * AV * \text{Fat} - 6.75000\text{E-}003 * \\ & WPC * \text{Fat} + 5.70238\text{E-}004 * AV^2 + 1.13865\text{E-}004 * WPC^2 + 0.016380 * \text{Fat}^2 \end{aligned}$$

5.3.1.7 Effect of process variables on overall acceptability score

The overall acceptability score represents a comprehensive picture of the sensory attributes of the product as perceived sensorily. The score for overall acceptability of ASIC varied from 7.20 to 7.93. The product prepared in Trial no. 9 registered lowest score of 7.2, while that from Trial no. 12 was highest at 7.93 (Table 5.3.3).

Addition of *Aloe vera* juice had a negative effect on overall acceptability score of ASIC and the effect was significant at both linear and quadratic levels at 1% and 5% levels of significance (Table 5.3.4 and Fig.5.3.6a). In one of the related study, Hussain (2013) developed *Aloe vera* supplemented probiotic dahi and *lassi* and reported that increase in *Aloe vera* juice level resulted in lower overall acceptability score for both the products. Fat had no significant effect on overall acceptability at linear level, however, it significantly ($p \leq 0.01$) increased the overall acceptability score at quadratic level (Table 5.3.4). Interaction of WPC with fat had a significant ($p \leq 0.05$) negative effect on overall acceptability score of ASIC (Table 5.3.4 and Fig.5.3.6b).

Design-Expert® Software

O.A.

 7.93
 7.2

X1 = A: A V
 X2 = B: WPC

Actual Factor
 C: Fat = 10.00

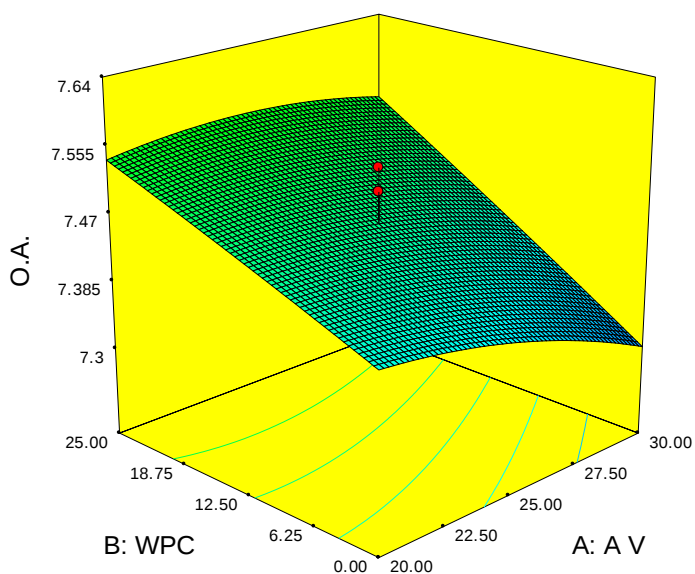


Fig.5.3.6(a) Response surface plot relating to overall acceptability score as influenced by the levels of WPC and *Aloe vera* juice

Design-Expert® Software

O.A.
 7.93
 7.2

X1 = B: WPC

X2 = C: Fat

Actual Factor

A: A V = 25.00

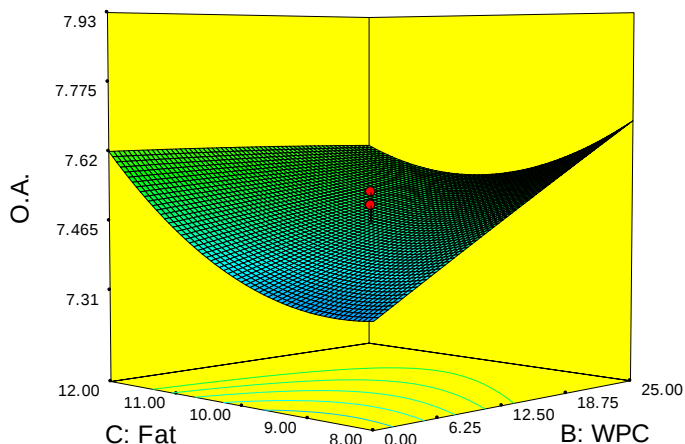


Fig.5.3.6(b) Response surface plot relating to overall acceptability score as influenced by the levels of fat and WPC

Organoleptic evaluation of herbal ice cream added with 5 to 45% levels of *Aloe vera* juice was carried out by Manoharan *et al.*, (2012) and the authors reported a significant decrease in overall acceptability score of ice cream with increasing level of *Aloe vera* juice. Singh *et al.*, (2012) also observed a decrease in overall acceptability score of *lassi* with increased levels of *Aloe vera* juice addition.

The regression analysis of data presented in Table 5.3.4 highlighted that the coefficient of determination (R^2) was 0.80 and the adequate precision was found to be 8.8, appreciably higher than the minimum desirable i.e. 4 required for reasonably good prediction. The non-significant “lack of fit test” indicated that the respective quadratic models could be used to predict the effect of the ingredients level on the overall acceptability of ice cream. The regression equation (for actual values) indicated that 80% variation in the overall acceptability score of ASIC could be accounted for by the factors studied:

$$\text{Overall acceptability score} = 9.85600 - 0.014689 * AV + 0.036578 * WPC - 0.50903 * Fat + 3.80000E-004 * AV * WPC + 4.12500E-003 * A V * Fat - 3.85000E-003 * WPC * Fat - 7.42983E-004 * AV^2 - 1.70538E-005 * WPC^2 + 0.024083 * Fat^2$$

5.3.2 Effect of ingredient levels on instrumental color parameters of ASIC mix

The lightness of the product is basically a surface characteristic and it depends on the presence of the substances that reflect or absorb light. The L* value which indicates lightness (or whiteness) of the product varied from 79.51 to 83.96. Lowest L* value was obtained in the ASIC mix with 25% *Aloe vera* juice, 33.52% WPC and 10% fat and the maximum L* value was recorded for the formulation containing 30% *Aloe vera* juice, 25% WPC and 12% fat (Table 5.3.5). The a* value indicating greenness/redness of ASIC mix varied from -3.21 to 0.91 (Table 5.3.5) suggesting that for some combinations of the factors, the product displayed redness (a) and for the rest greenness (-a). The minimum value was obtained when 20% *Aloe vera* juice, 0% WPC and 8% fat were used in the formulation and the maximum value was observed in the product prepared with 16.59% *Aloe vera* juice, 12.50% WPC and 10% fat. The b* value indicating yellowness (positive values) or blueness (negative values) of ASIC varied from 11.46 to 15.34. Minimum b* value was noted in the formulation containing 20% *Aloe vera* juice, 0% WPC and 12% fat while the formulation with 25% *Aloe vera* juice, 12.50% WPC and 10% fat showed maximum yellowness value (Table. 5.3.5).

The regression coefficient model estimates showed that none of the ingredients in any formulation had significant impact on the L*, a* and b* values of the ASIC mix (Table 5.3.6). The results are in agreement with observations made for sensory color and appearance score which also was not affected significantly by the three variables (Table 5.3.4).

Table 5.3.5 Effect of *Aloe vera* juice, WPC and fat on color (L*, a* and b* values) characteristics of ASIC mix

Run	AV	WPC	FAT	L*	a*	b*
1	20.00	0.00	8.00	83.12	-3.21	13.73
2	25.00	12.50	10.00	82.69	-1.52	12.48
3	20.00	0.00	12.00	83.57	0.80	11.46
4	30.00	0.00	12.00	83.67	-0.10	13.19
5	20.00	25.00	12.00	83.80	-1.26	14.41
6	25.00	12.50	10.00	83.19	-3.07	15.34
7	25.00	12.50	6.64	82.55	-2.03	13.46
8	25.00	12.50	10.00	80.83	-1.57	13.86
9	30.00	0.00	8.00	81.86	0.76	12.08
10	16.59	12.50	10.00	82.98	0.91	11.62
11	25.00	-8.52	10.00	80.49	-1.69	14.51
12	25.00	12.50	13.36	83.34	-1.02	13.08
13	25.00	12.50	10.00	82.68	-1.52	12.53
14	25.00	33.52	10.00	79.51	0.11	12.89
15	30.00	25.00	8.00	82.56	-2.97	15.28
16	25.00	12.50	10.00	82.68	-1.54	12.94
17	30.00	25.00	12.00	83.96	-1.63	13.35
18	25.00	12.50	10.00	82.60	0.60	13.53
19	20.00	25.00	8.00	82.72	-0.57	13.83
20	33.41	12.50	10.00	80.21	-1.60	14.03

Table 5.3.6 Regression coefficients and ANOVA of quadratic model for instrumental color parameters of ASIC as influenced by *Aloe vera* juice, WPC and fat levels

Factor	L*	a*	b*
Intercept	82.39	-0.92	13.44
A-A V	-0.43	-0.29	0.33
B-WPC	-0.061	-0.12	0.27
C-Fat	0.44	0.40	-0.23
AB	0.15	-0.73	0.039
AC	0.21	-0.36	0.11
BC	0.028	-0.31	-0.024
A ²	0.058	0.18	-0.19
B ²	-0.51	0.018	0.12
C ²	0.54	-0.24	-0.028
R-Squared	0.49	0.29	0.19
Adeq Precision	4.353	2.387	2.031
Model F Value	1.06 ^{ns}	0.46 ^{ns}	0.27 ^{ns}
Lack of fit	ns	ns	Ns

^{ns} Non-significant (p>0.05)

5.3.3 Effect of Aloe vera juice, WPC and fat on acidity, pH and specific gravity of ASIC mix

The quadratic model for acidity, pH and specific gravity were obtained through successive regression analysis. The acidity, pH and specific gravity values of ASIC and partial coefficients of regression of these responses with

respect to the levels of the three factors in the form of correlation is presented in Table 5.3.7 and Table 5.3.8, respectively.

Table 5.3.7 Effect of *Aloe vera* juice, WPC and fat on acidity, pH and specific gravity of ASIC mix

Run	AV	WPC	FAT	Acidity	pH	Specific gravity
1	20.00	0.00	8.00	0.51	6.20	1.0957
2	25.00	12.50	10.00	0.42	6.23	1.1117
3	20.00	0.00	12.00	0.32	6.33	1.0625
4	30.00	0.00	12.00	0.31	6.38	1.0730
5	20.00	25.00	12.00	0.27	6.62	1.0935
6	25.00	12.50	10.00	0.43	6.17	1.1220
7	25.00	12.50	6.64	0.49	6.17	1.0495
8	25.00	12.50	10.00	0.34	6.33	1.0888
9	30.00	0.00	8.00	0.45	6.22	1.0515
10	16.59	12.50	10.00	0.38	6.32	1.0671
11	25.00	-8.52	10.00	0.39	6.27	1.0954
12	25.00	12.50	13.36	0.36	6.21	1.0991
13	25.00	12.50	10.00	0.41	6.20	1.0320
14	25.00	33.52	10.00	0.36	6.37	1.0905
15	30.00	25.00	8.00	0.47	6.14	1.1180
16	25.00	12.50	10.00	0.43	6.19	1.0460
17	30.00	25.00	12.00	0.32	6.24	1.0775
18	25.00	12.50	10.00	0.40	6.17	1.0370
19	20.00	25.00	8.00	0.39	6.52	1.1053
20	33.41	12.50	10.00	0.36	6.33	1.1021

Table 5.3.8 Regression coefficients and ANOVA of fitted quadratic model for acidity, pH and specific gravity of ASIC mix

Factor	Acidity	pH	Specific gravity
Intercept	0.41	6.21	1.07
A-A V	1.930E-003	-0.049*	1.601E-003
B-WPC	-0.014	0.041	7.568E-003
C-Fat	-0.060**	0.041	1.422E-003
AB	0.025	-0.10**	3.800E-003
AC	2.500E-003	3.750E-003	3.250E-003
BC	7.500E-003	-0.011	-5.075E-003
A ²	-0.014	0.049*	4.132E-003
B ²	-0.013	0.047*	7.084E-003
C ²	5.153E-003	1.517E-003	4.903E-004
R-Squared	0.84	0.79	0.15
Adeq Precision	9.493	6.877	1.658
Model F value	5.80 **	4.10 *	0.20 ^{ns}
Lack of fit	ns	ns	Ns

** Highly Significant ($p \leq 0.01$) * Significant ($p \leq 0.05$) ^{ns} Non-significant

5.3.3.1 Effect of process variables on acidity

Acidity of ice cream is related to the composition of ice cream mix. The natural or normal acidity of mix is due to the milk proteins, minerals and dissolved gases (Marshall and Arbuckle, 1996). A high percent acidity is undesirable as it contributes to excessive mix viscosity, decreased whipping rates, inferior flavor and less stable mix resulting in possible coagulation during processing stages (Arbuckle, 1966). The percentage acidity of ASIC mix varied from 0.27 to 0.51 (%)

lactic acid) (Table 5.3.7). Lowest acidity was observed in the ice cream mix having 20% *Aloe vera* juice, 25% WPC and 12% fat. The maximum acidity (% lactic acid) was recorded in the product added with 20% *Aloe vera* juice, 0% WPC and 8% fat (Table 5.3.7).

Fat was the only factor that had a significant negative effect ($p \leq 0.01$) on the acidity of ASIC mix and the relationship was linear in nature (Table 5.3.8). Thus, the acidity decreased gradually with increasing fat at all levels of the other factors viz., *Aloe vera* juice and WPC (Fig.5.3.7a,b). Singh (2007) also reported that with increase in the fat level, acidity of the low fat, fibre enriched fruit ice cream decreased.

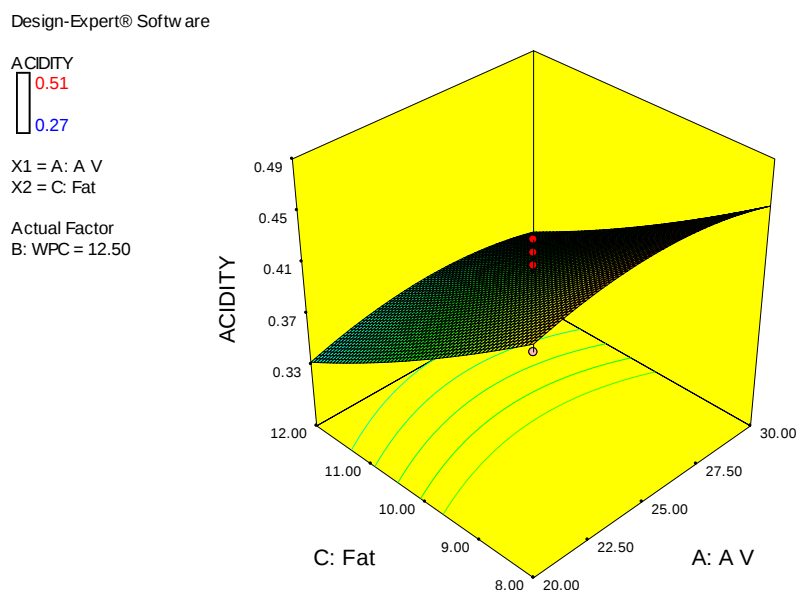


Fig.5.3.7(a) Response surface plot relating to acidity as influenced by the levels of fat and *Aloe vera* juice

The effect of *Aloe vera* juice at linear level was not significant but the term being positive showed that with the increase in *Aloe vera* juice content, acidity of the ASIC mix increased. This could be attributed to the natural acidity of the *Aloe vera* juice which on addition into the ice cream mix increased the acidity. However, at the quadratic level, the term for *Aloe vera* juice was negative which indicated that with increasing *Aloe vera* juice level, acidity of the ASIC mix

decreased. These results are in accordance with the findings of Singh *et al.* (2012) who developed a *lassi* added with *Aloe vera* juice and observed that the titrable acidity (% lactic acid) of the *lassi* increased significantly ($p \leq 0.05$) with the increasing level of *Aloe vera* juice in milk from 0 to 15%. But further increase in the level of *Aloe vera* juice in milk from 15 to 30% resulted in decreased acidity. This could be attributed to the buffering capacity of *Aloe vera* solids at higher levels.

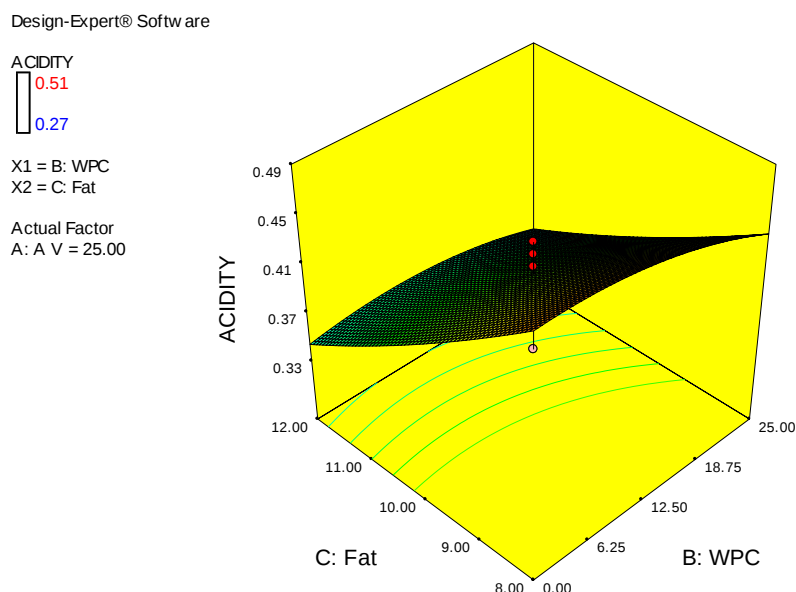


Fig.5.3.7(b) Response surface plot relating to acidity as influenced by the levels of fat and WPC

Reddy *et al.*, (1987) used liquid channa whey as a substitute for milk solids not fat in ice cream mixes and observed that with the increase in the level of liquid channa whey, titrable acidity of ice cream mix increased. Hofi *et al.* (1993) replaced SNF with WPC in the ice cream and found that with the increase in WPC: SNF ratio from 0:100 to 100:0, acidity of the mix increased. However, in the present study, at linear level of WPC, a not significant negative value was observed for acidity. The reason for this could be that in the above mentioned studies the level of WPC addition was more whereas, in the present study only a small amount of SMP was substituted with WPC. This small addition of the WPC

was not sufficient enough to bring about the significant change in the acidity and might have worked as a buffering agent that resulted in minor and not significant decrease in the acidity of the mix.

The coefficient of determination (R^2) was 0.84 and the adequate precision was found to be 9.49 which was considerably higher than the minimum desirable i.e. 4 (for high prediction ability).

The acidity of ASIC mix could be predicted by the equation (for actual values of the variables) given below:

$$\text{Acidity} = 0.69328 + 0.021471 * AV - 0.012112 * WPC - 0.065736 * Fat + 4.00000E-004 * AV * WPC + 2.50000E-004 * A V * Fat + 3.00000E-004 * WPC * Fat - 5.71704E-004 * AV^2 - 8.01589E-005 * WPC^2 + 1.28821E-003 * Fat^2$$

5.3.3.2 Effect of process variables on pH

The pH of an ice cream mix can be used as an indicator of ice cream mix quality. The normal pH of ice cream mix is about 6.3 (Marshall and Arbuckle, 1996). The MSNF content of the ice cream mix has a remarkable effect on the pH of the ice cream mix. As the MSNF portion of a mix increases, the normal acidity increases and pH decreases (Arbuckle, 1986).

The pH value of the ASIC mix ranged between 6.14 and 6.62. The lowest pH value was recorded in the ice cream mix added with 30% *Aloe vera* juice, 25% WPC and 8% fat. Whereas, the maximum pH value was noted in the product having 20% *Aloe vera* juice, 25% WPC and 12% fat (Table 5.3.7).

The coefficient of estimates of the pH model showed that *Aloe vera* juice had a significant effect on pH at all the three levels viz., linear, quadratic and interaction (Table 5.3.8). The linear term of the model for *Aloe vera* juice being negative ($p \leq 0.05$) indicated that the pH of ice cream mix decreased with increasing *Aloe vera* juice level. This can be related to the low pH of the *Aloe vera* juice. The pH of normal ice cream mix is 6.3 and the addition of an ingredient like *Aloe vera* juice with a pH value of 4.5 can be the reason for the decrease in pH of the ASIC mix. However, at the quadratic level, the term for

Aloe vera juice was positive which indicated that with increasing *Aloe vera* juice level, pH of the ASIC mix increased. These results are in agreement with the observations of Singh *et al.* (2012) who developed a *lassi* with *Aloe vera* juice and observed a significant ($p \leq 0.05$) decrease in pH of the *lassi* when *Aloe vera* juice level was increased from 0 to 15%. But further increase in the level of *Aloe vera* juice showed no significant decrease in the pH of *lassi*. This could be attributed to the buffering capacity of *Aloe vera* solids at higher levels.

WPC did not show any significant effect on the pH at linear level (Table 5.3.8). But the quadratic term of WPC was significant ($p \leq 0.05$) resulting in the response curve being concave upward (Fig.5.3.8 a). The interaction term of *Aloe vera* juice and WPC was negative indicating that with simultaneous increase in the concentration of *Aloe vera* juice and WPC, the pH of ASIC mix decreased significantly ($p \leq 0.05$) (Fig.5.3.8a).

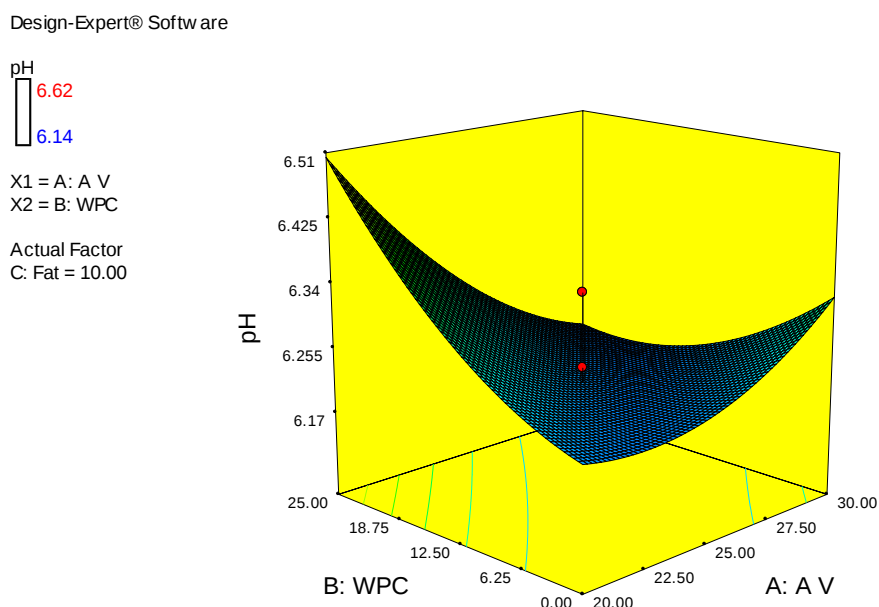


Fig.5.3.8(a) Response surface plot relating to pH as influenced by the levels of WPC and *Aloe vera* juice

The regression analysis of data presented in Table 5.3.8 indicated that the coefficient of determination (R^2) was 0.79 and the adequate precision was found

to be 6.88 which were higher than the minimum desirable (4) required for high prediction ability.

The pH of ASIC mix could be predicted by the equation (for actual values of the variables) given below:

$$\text{pH} = 7.05031 - 0.091352 * \text{AV} + 0.041673 * \text{WPC} + 9.06694\text{E-}003 * \text{Fat} - 1.66000\text{E-}003 * \text{AV} * \text{WPC} + 3.75000\text{E-}004 * \text{AV} * \text{Fat} - 4.50000\text{E-}004 * \text{WPC} * \text{Fat} + 1.96987\text{E-}003 * \text{AV}^2 + 3.03866\text{E-}004 * \text{WPC}^2 + 3.79285\text{E-}004 * \text{Fat}^2$$

5.3.3.3 Effect of process variables on specific gravity

According to Marshall *et al.* (2003), specific gravity of ice cream mix normally varies from 1.0544 to 1.1232. During the present study, specific gravity of the ASIC mix recorded was between 1.032 to 1.122 (Table 5.3.7). Lowest specific gravity was obtained for formulation prepared from 25% *Aloe vera* juice, 12.5% WPC and 10% fat. Formulation made with 25% *Aloe vera* juice, 12.5% WPC and 10% fat showed maximum specific gravity which means there was no significant difference in the specific gravity of the ASIC mix. The regression coefficient model estimates showed that none of the ingredients had significant impact on the specific gravity of the ASIC mix (Table 5.3.8).

5.3.4 Effect of *Aloe vera* juice, WPC and fat on viscosity of ASIC mix

Viscosity is an important property of the ice cream mix and a definite level of it is essential for proper whipping and retention of air. Among the various ingredients, milk fat and stabilizer influence the viscosity more. Ice cream mix has both apparent and true viscosity. The true viscosity of the ice cream mix may range from 50 to 300 cP. The higher the viscosity of ice cream mix, the greater is the power required to freeze the mix (Marshall and Arbuckle, 1996). The viscosity of the ASIC mix ranged from 46.5 cp to 276.2 cp (Table 5.3.9). The ASIC prepared with 30% *Aloe vera* juice, 25% WPC and 12% fat was least viscous, whereas, the ASIC prepared with 20% *Aloe vera* juice, 0% WPC and 8% fat showed maximum viscosity (Table 5.3.9). Among the ingredients, *Aloe vera* juice and fat had significant effect on the viscosity of the ASIC mix (Table 5.3.10).

The partial regression coefficients indicated that *Aloe vera* juice had a significantly ($p \leq 0.01$) negative effect on the viscosity of ASIC mix at linear level (Table 5.3.10). Thus, with the increasing *Aloe vera* juice level (20% to 30%), viscosity decreased proportionally irrespective of the levels of the other two ingredients (Fig.5.3.9a). In one of the related studies, Hussain (2013) developed *Aloe vera* supplemented probiotic lassi and reported reduction in viscosity of the product with increase in *Aloe vera* juice. In a similar study, Singh *et al.* (2012) developed a *lassi* with *Aloe vera* juice and showed that the viscosity of both medium fat and low fat *lassi* samples decreased significantly ($p \leq 0.05$) with increase in addition of *Aloe vera* juice level from 0 to 15%. The authors reported that high moisture content of *Aloe vera* juice could be the reason for decrease in viscosity of *lassi*.

Design-Expert® Software

VISCOSITY

276.2

46.5

X1 = A: A V

X2 = B: WPC

Actual Factor

C: Fat = 10.00

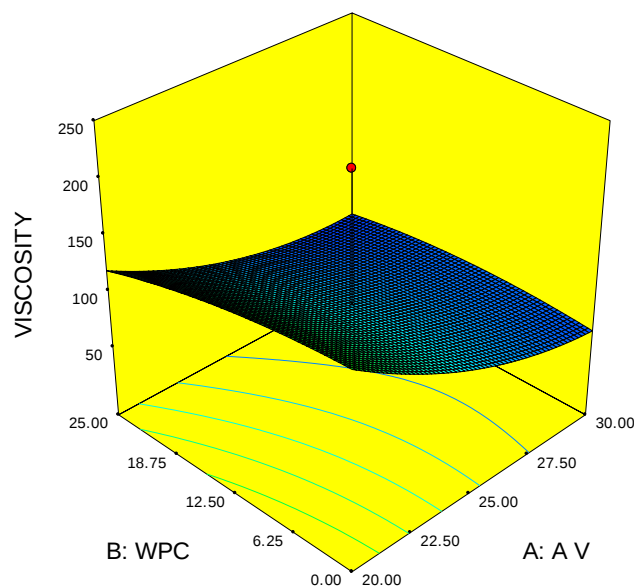


Fig.5.3.9(a) Response surface plot relating to viscosity as influenced by the levels of WPC and *Aloe vera* juice

At linear level, fat content also showed a negative significant ($p \leq 0.05$) effect on the viscosity of ASIC mix (Table 5.3.10), which indicated that with the increase in fat from 8% to 12%, there was a decrease in viscosity at all the levels

of *Aloe vera* juice and WPC (Fig.5.3.9b). These observations are also in agreement with the findings of Singh (2007) who noted that with the increase in milk fat level in low fat fibre enriched fruit ice cream mix, there was a decrease in viscosity. On the contrary, Marshall *et al.* (2003) observed an increase in viscosity with the increase in the levels of fat in ice cream mix.

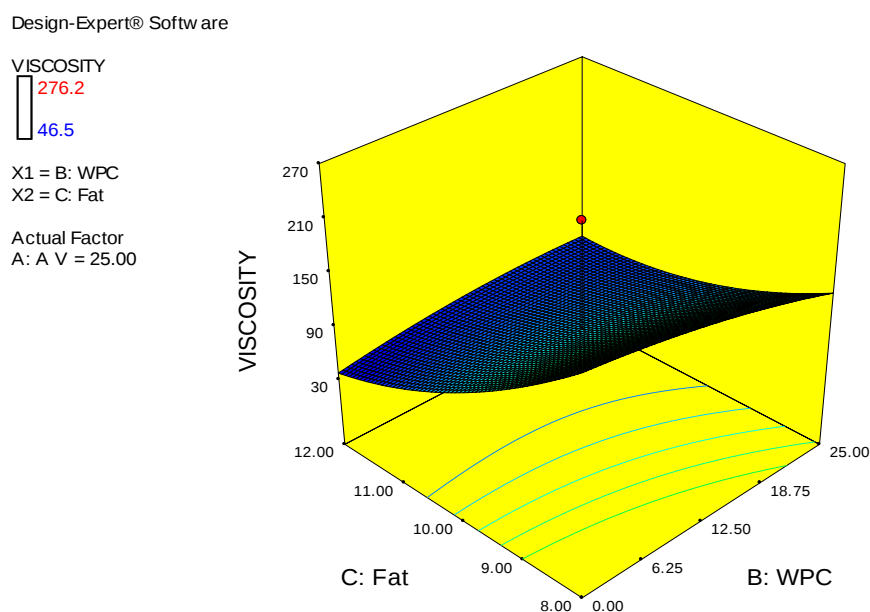


Fig.5.3.9(b) Response surface plot relating to viscosity as influenced by the levels of fat and WPC

At linear level, WPC showed a negative effect although not at significant level, which implied that with the increase in WPC there was a decrease in viscosity. These findings are in agreement with the findings of Lee and White (1991), which used whey protein concentrate to replace different levels of solids not fat in vanilla ice cream at 25, 50, 75 or 100 percent, respectively and observed that viscosity of the ice cream mixes decreased as the percentage of substitution of whey protein concentrate increased.

The coefficient of determination (R^2) was 0.78 and the adequate precision value at 7.4 was considerably higher than the minimum desirable i.e. 4 (for high prediction ability). The viscosity of ASIC mix could be predicted by the equation (for actual values of the variables) given below:

$$\text{Viscosity} = 2083.79176 - 59.33632 * \text{AV} - 12.06878 * \text{WPC} - 185.15900 * \text{Fat} + 0.14426 * \text{AV} * \text{WPC} + 1.39413 * \text{AV} * \text{Fat} + 0.87835 * \text{WPC} * \text{Fat} + 0.71438 * \text{AV}^2 - 0.049749 * \text{WPC}^2 + 5.51667 * \text{Fat}^2$$

Table 5.3.9 Effect of *Aloe vera* juice, WPC and fat on viscosity of ASIC mix

Run	AV	WPC	FAT	Viscosity (cP)
1	20.00	0.00	8.00	276.2
2	25.00	12.50	10.00	66.8
3	20.00	0.00	12.00	70.1
4	30.00	0.00	12.00	48.2
5	20.00	25.00	12.00	76.3
6	25.00	12.50	10.00	210.2
7	25.00	12.50	6.64	266.5
8	25.00	12.50	10.00	66.2
9	30.00	0.00	8.00	154.6
10	16.59	12.50	10.00	248.7
11	25.00	-8.52	10.00	77.5
12	25.00	12.50	13.36	62.8
13	25.00	12.50	10.00	68.5
14	25.00	33.52	10.00	83.0
15	30.00	25.00	8.00	109.0
16	25.00	12.50	10.00	69.2
17	30.00	25.00	12.00	46.5
18	25.00	12.50	10.00	65.8
19	20.00	25.00	8.00	150.6
20	33.41	12.50	10.00	56.8

Table 5.3.10 Regression coefficients and ANOVA of quadratic model for viscosity of ASIC mix as influenced by *Aloe vera* juice, WPC and fat levels

Factor	VISCOSITY
Intercept	91.72
A-A V	-39.37 * *
B-WPC	-11.53
C-Fat	-57.99*
AB	9.02
AC	13.94
BC	21.96
A ²	17.86
B ²	-7.77
C ²	22.07
R-Squared	0.7857
Adeq Precision	7.431
Model F value	4.07 *
Lack of fit	ns

** Highly Significant ($p \leq 0.01$); * Significant ($p \leq 0.05$); ^{ns} Non-significant

5.3.5 Effect of *Aloe vera* juice, WPC and fat on overrun and melting quality and firmness of ASIC

The quadratic models for overrun, melting quality and firmness in ASIC preparation were obtained through successive regression analysis. The values of their responses and partial coefficients of regression of the three factors are presented in Tables 5.3.11 and Table 5.3.12, respectively.

Table 5.3.11 Effect of *Aloe vera* juice, WPC and fat on overrun and melting and firmness of ASIC

Run	AV	WPC	FAT	Overrun	% Melt/h	Firmness (N)
1	20.00	0.00	8.00	85.49	71.40	81.29
2	25.00	12.50	10.00	64.28	64.60	41.19
3	20.00	0.00	12.00	68.00	55.07	58.25
4	30.00	0.00	12.00	72.08	28.11	65.90
5	20.00	25.00	12.00	77.92	64.48	70.66
6	25.00	12.50	10.00	66.88	60.32	41.85
7	25.00	12.50	6.64	63.14	82.48	34.46
8	25.00	12.50	10.00	61.30	82.31	41.80
9	30.00	0.00	8.00	75.89	83.67	44.04
10	16.59	12.50	10.00	81.60	81.60	92.47
11	25.00	-8.52	10.00	64.41	72.06	77.76
12	25.00	12.50	13.36	72.36	6.61	48.00
13	25.00	12.50	10.00	65.20	64.10	36.36
14	25.00	33.52	10.00	53.30	60.69	42.07
15	30.00	25.00	8.00	60.50	68.64	18.41
16	25.00	12.50	10.00	66.30	65.30	39.02
17	30.00	25.00	12.00	66.66	45.39	46.89
18	25.00	12.50	10.00	68.80	65.00	45.85
19	20.00	25.00	8.00	60.51	80.00	71.04
20	33.41	12.50	10.00	72.20	28.25	45.24

Table 5.3.12 Regression coefficients and ANOVA of fitted quadratic model for overrun, % melt/h and firmness of ASIC as influenced by *Aloe vera* juice, WPC and fat

Factor	OVERRUN	% MELT/HR	FIRMNESS (N)
Intercept	65.37	66.70	41.03
A-A V	-2.39	-9.88**	-13.58**
B-WPC	-3.99**	0.083	-7.51**
C-Fat	1.30	-17.45**	3.64**
AB	-0.72	-1.97	-5.85**
AC	0.30	-5.87	9.22**
BC	5.61**	4.14	3.66 *
A ²	4.66	-2.66	9.73**
B ²	-1.72**	1.39	6.57**
C ²	1.42	-6.33	-0.040
R-Squared	0.8567	0.8343	0.9725
Adeq Precision	10.066	7.781	25.838
Model F value	6.64 **	5.60 **	39.37 **
Lack of fit	ns	ns	Ns

** Highly Significant ($p \leq 0.01$) * Significant ($p \leq 0.05$) ^{ns} Non-significant

5.3.5.1 Effect of process variables on overrun

Overrun in ice cream means the increase in the volume of ice cream over the volume of mix used, which is achieved by incorporation of air. The overrun in ice cream is important because it influences the quality and is responsible for profitability. In the present study, the overrun of ASIC ranged from 53.3% to 85.49%. Lowest overrun was observed in the ASIC formulation with 25% *Aloe*

vera juice, 33.52% WPC and 10% fat while the maximum overrun was recorded for the product with 20% *Aloe vera* juice, 0% WPC and 8% fat (Table 5.3.11). The coefficient of determination (R^2) was 0.86 and the adequate precision was found to be 10.07 which was appreciably higher than the minimum desirable i.e. 4 (for high prediction ability).

Among all the three ingredients, WPC had a significant ($p \leq 0.01$) effect on the overrun of ASIC at linear and quadratic levels besides during its interaction with *Aloe vera* juice (Table 5.3.12). At linear level, overrun of the product decreased significantly ($p \leq 0.01$) with the increasing WPC levels (Fig.5.3.10a). However, interaction of WPC with fat had a significantly ($p \leq 0.01$) positive effect on the overrun of ASIC. At quadratic level, WPC had a significantly ($p \leq 0.01$) negative impact on the overrun of ASIC (Table 5.3.12) and the response surface was concave upward (Fig.5.3.10b). Similar findings have been reported by Asadinejad *et al.*, (2013) who studied the effect of whey protein concentrate on properties of ice cream and observed a decrease in overrun as the level of WPC replacement increased. Similarly, Thompson *et al.*, (1983), who substituted whey protein concentrate (WPC) for dried skim milk (DSM) in ice cream observed a reduction in overrun of ice cream with increased levels of WPC addition.

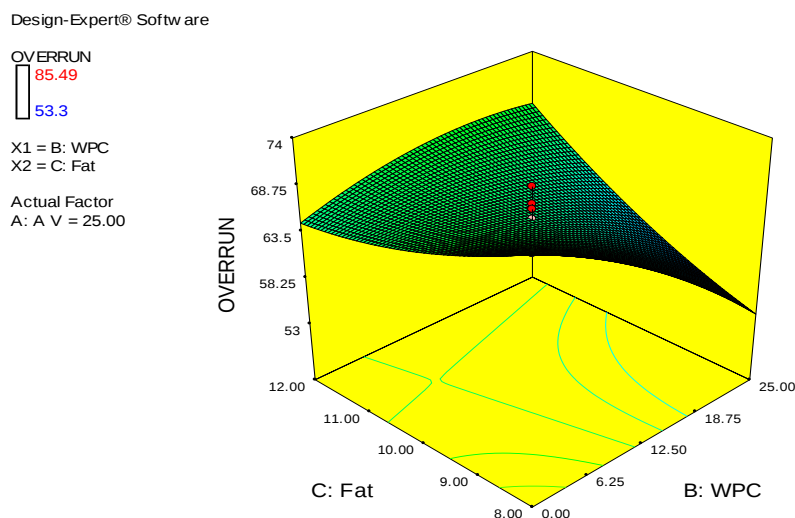


Fig.5.3.10(a) Response surface plot relating to overrun as influenced by the levels of fat and WPC

The partial regression coefficients for overrun indicated that at linear level, fat had a not significant but positive effect on overrun indicating that with the increase in fat content of the mix, overrun of the product increased. This observation was in unison with the findings reported by Marshall *et al.* (2003), Verma (2002) and Singh (2007). Dleuzewski *et al.*, (1981) also revealed that overrun increased with the increase in fat content and decreased with the increase in SNF content.

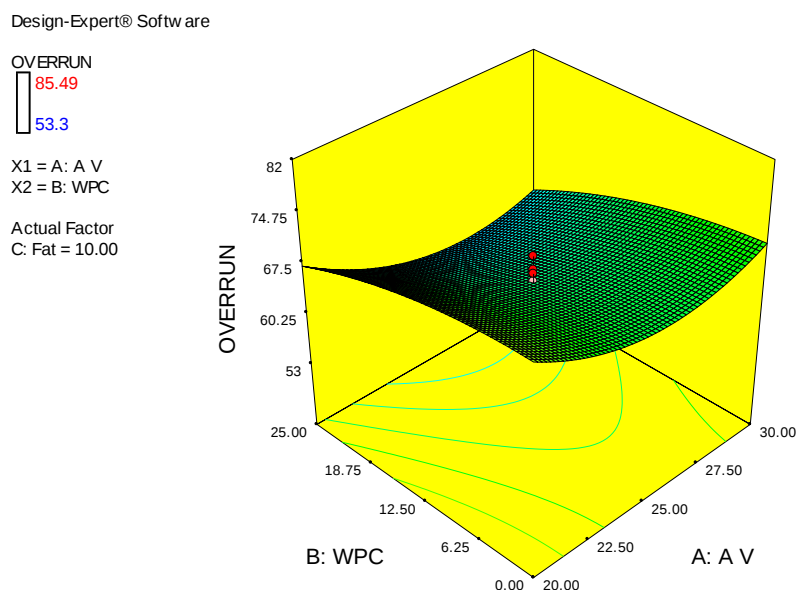


Fig.5.3.10(b) Response surface plot relating to overrun as influenced by the levels of WPC and A/oe vera juice

The overrun of ASIC could be predicted by the equation (for actual values of the variables) given below:

$$\text{Overrun} = 257.08749 - 9.95156 * A V - 1.99993 * WPC - 10.02327 * Fat - 0.011500 * A V * WPC + 0.030375 * A V * Fat + 0.22435 * WPC * Fat + 0.18628 * A V^2 - 0.011026 * WPC^2 + 0.3555 * Fat^2$$

5.3.5.2 Effect of process variables on melting quality

The melt-down rate of ice cream is affected by many factors, including the amount of air incorporated, the nature of the ice crystals, and the network of fat globules formed during freezing. How the ice cream melts down (melt-down) is one of the factor affecting appearance of the product, either adversely as a curdled, wheyed-off melted product, or favorably as an especially smooth, creamy, rich-appearing melted product.

In addition to sensorily evaluating the melting quality of ice cream, melting quality of ASIC was also quantitatively determined in terms of percentage melt per hour. The % melt/h of the ice cream varied from 6.61 to 83.67 %. The lowest % melt/h was obtained in the ice cream with 25% *Aloe vera* juice, 12.5% WPC and 13.36% fat and the ice cream with 30% *Aloe vera* juice, 0% WPC and 8% fat showed the maximum % melt/h (Table 5.3.11), implying that formulation with the highest fat content melted at the slowest rate, providing ice cream a good melting quality.

The partial regression coefficient indicated that at linear level fat had a significant effect ($p \leq 0.01$) with a negative sign, implying that the % melt/h decreased with the increasing fat level. It can be seen from Fig. 5.3.11(a) that as the fat level increased from 8% to 12%, the % melt/h decreased. This observation is in accordance with the findings of Roland *et al.* (1999) who observed that increase in the fat content of ice cream mix improved melting property of the final product. Ohmes *et al.*, (1998) also reported that ice creams containing fat would be expected to melt more slowly than non fat ice creams containing similar amounts of total solids and stabilizers / emulsifiers.

At linear level, *Aloe vera* juice had a significant negative effect ($p \leq 0.01$) on % melt/h of the ice cream. This implied that with the increasing *Aloe vera* juice content, % melt/h decreased. It is shown in the Fig.5.3.11(b) that as the level of *Aloe vera* juice increased from 20% to 30%, the % melt/h decreased gradually.

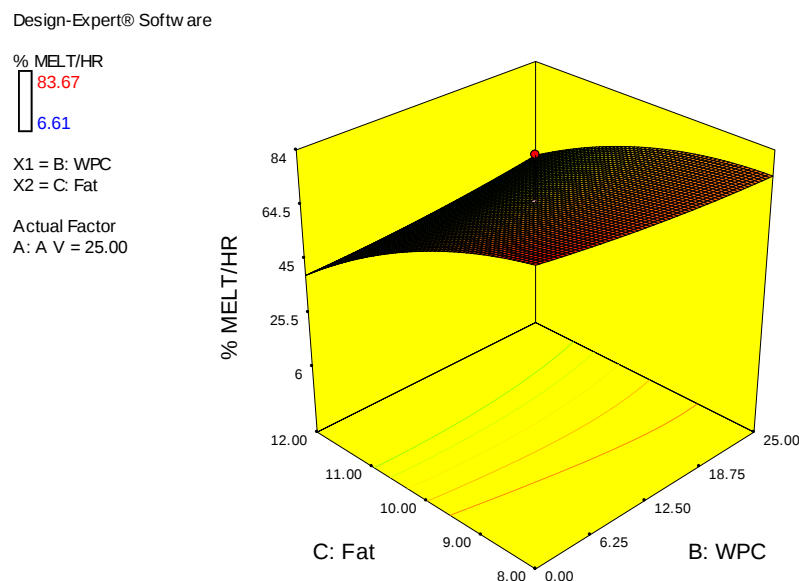


Fig.5.3.11(a) Response surface plot relating to % melt/h as influenced by the levels of fat and WPC

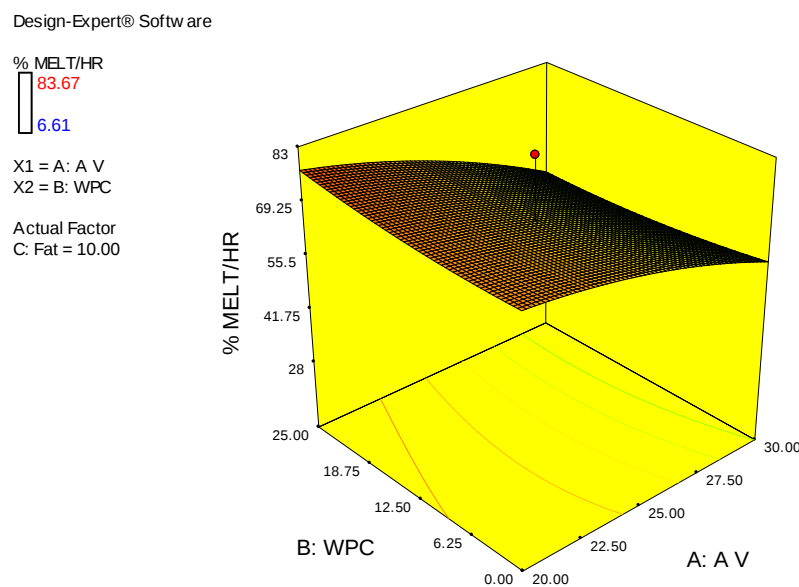


Fig.5.3.11(b) Response surface plot relating to % melt/h as influenced by the levels of WPC and Aloe vera juice

Increase in the WPC level resulted in an increase in melting rate (% melt/h) of ASIC. However, the effect of WPC was not significant. Tirumalesha and Jayaprakasha (1998) studied the effect of an admixture of spray dried WPC and buttermilk powder on physico-chemical and sensory characteristics of ice cream and observed that the melting resistance decreased with the increasing level of the admixture into the mix.

The coefficient of determination (R^2) was 0.83 and the adequate precision was found to be 7.78. The not-significant “lack of fit test” suggested that the respective quadratic model could be used to predict the effect of the ingredients level on melting rate of ice cream (Table 5.3.12).

The melting rate of ASIC could be predicted by the equation (for actual values of the variables) given below:

$$\text{Melting rate (\% Melt/h)} = -156.05941 + 9.60969 * AV - 1.08339 * WPC + 35.53323 * Fat - 0.031520 * AV * WPC - 0.58700 * AV * Fat + 0.16560 * WPC * Fat - 0.10641 * AV^2 + 8.88207E-003 * WPC^2 - 1.58256 * Fat^2$$

5.3.5.3 Effect of process variables on firmness

The firmness of ice cream is related to its structure. The air cells of the ice cream structure are essentially spherical although there is some distortion due to fat and ice crystal formation. The material surrounding these air cells is a non-Newtonian fluid containing small ice crystals and clumps of fat (up to 80%). In ice creams with low fat content, the rheology of the composite fluid surrounding the air cells will be altered due to the reduction in the fat clumps which predominate the composite fluid of conventional ice cream structure. The firmness of ice cream is affected by such factors as the overrun, ice crystal size, ice phase volume, and the extent of fat destabilization.

The firmness of ice cream was determined by Texture Analyser using Warner blade attachment. The firmness of ASIC ranged from 18.41 to 92.47 N. The regression analysis of the data presented in Table 5.3.12 showed that the coefficient of determination (R^2) was 0.97 and the “lack of fit test” was not

significant, indicating that the model was sufficiently accurate for predicting the firmness of ASIC with any combination of the factors level within the range evaluated. The adequate precision value at 25.84 was appreciably higher than the minimum desirable i.e. 4 (for high prediction ability). Lowest firmness value was recorded in the ice cream with 30% *Aloe vera* juice, 25% WPC and 8% fat and the maximum firmness value was obtained for the product with 16.59% *Aloe vera* juice, 12.5% WPC and 10% fat (Table 5.3.11).

The partial co-efficient of regression model indicated that at the linear level all the three ingredients had a significant ($p \leq 0.01$) effect on the firmness of ASIC. *Aloe vera* and WPC had negative ($p \leq 0.01$) whereas fat had positive ($p \leq 0.01$) effect on firmness of ice cream. The firmness of the ASIC increased gradually with the increase in fat (Fig.5.3.12a,b). This observation is in agreement with the findings of Aime *et al.* (2001) who used a knife attachment of Texture Analyser to measure the firmness of ice cream with fat level increasing from 0.4% to 10% and observed that the fat free (0.4% fat) ice cream sample had a lower firmness value than the regular fat (10% fat) ice cream.

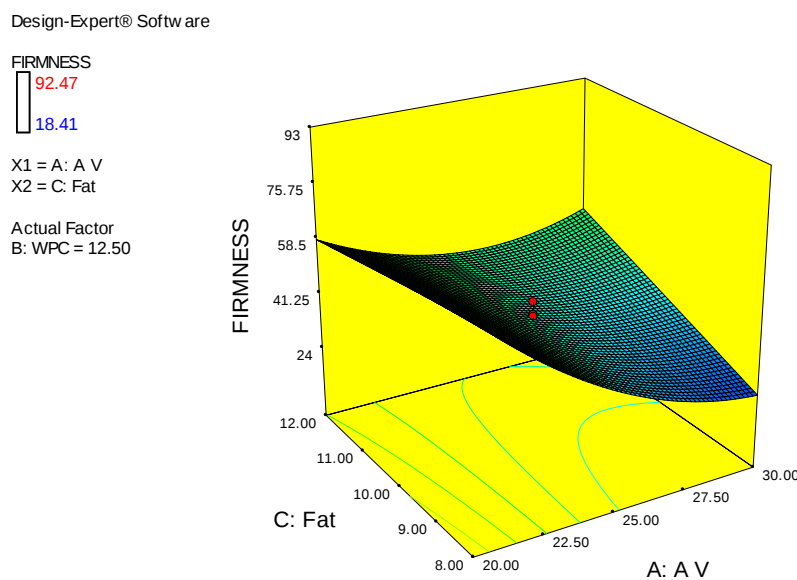


Fig.5.3.12(a) Response surface plot relating to firmness as influenced by the levels of fat and *Aloe vera* juice

However, with increased levels of WPC incorporation the firmness of ASIC decreased significantly (Fig.5.3.12b). Verma (2002) also reported that with the increase in WPC content, firmness of ice cream decreased. Saritha *et al.* (1998) used spray dried whey powder as a substitute for SMP at levels ranging from 10 to 100% in the preparation of ice cream and observed that hardness of ice cream reduced upto 40% substitution. Tirumalesha and Jayaprakasha (1998) studied the effect of an admixture (50:50) of spray dried WPC and buttermilk powder on physico-chemical and sensory characteristics of ice cream and observed that the hardness of ice cream decreased with increasing level of the admixture in to the ice cream.

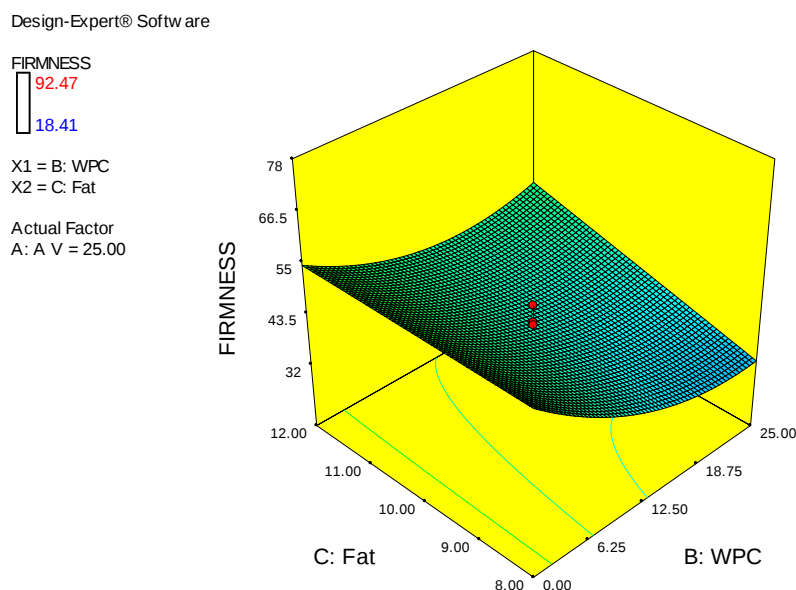


Fig.5.3.12(b) Response surface plot relating to firmness as influenced by the levels of fat and WPC

It can be seen from Fig.5.3.12(a) that as the *Aloe vera* juice level increased from 20 to 25 percent, the firmness of ASIC decreased rapidly but further increase in *Aloe vera* juice level caused the firmness to plateau. At quadratic level, both *Aloe vera* juice and WPC had a positive significant effect ($p \leq 0.01$) on the firmness of the ice cream (Table 5.3.12), the response surface being concave upward (Fig.5.3.12c).

Table 5.3.12 revealed that all the three factors significantly affected the firmness of ASIC at interaction level. The response-factor relationship between *Aloe vera* juice and fat ($p \leq 0.01$) and interaction between WPC and fat were positive.

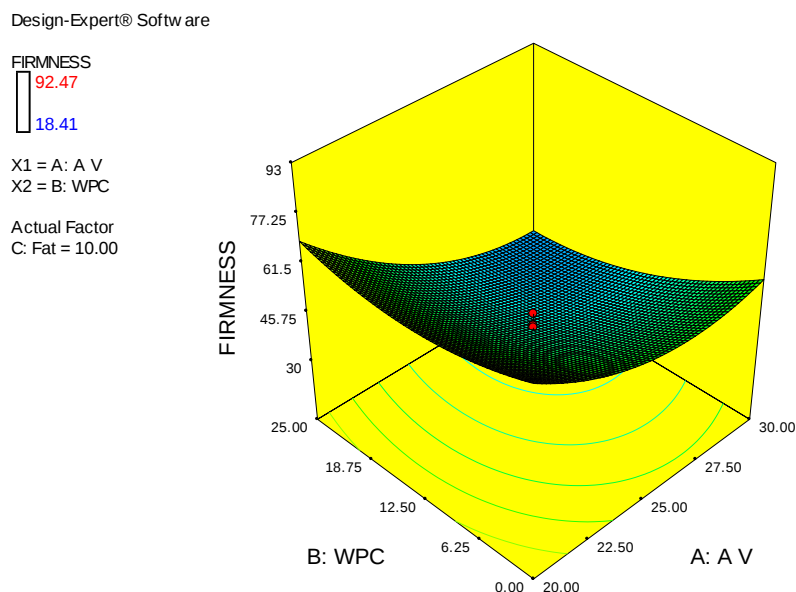


Fig.5.3.12(c) Response surface plot relating to firmness as influenced by the levels of WPC and *Aloe vera* juice

The firmness score of ASIC could be predicted by the equation (for actual values of the variables) given below:

$$\text{Firmness} = 566.51049 - 30.21900 * AV - 0.77500 * WPC - 22.85971 * Fat - 0.093600 * AV * WPC + 0.92200 * AV * Fat + 0.14640 * WPC * Fat + 0.38907 * AV^2 + 0.042022 * WPC^2 - 0.010050 * Fat^2$$

5.3.6 Optimized ASIC formulation

The Response Surface Design expert (version 7.5.1) was used in order to arrive at the best combination of the ingredient-based variables. Sensory attributes, physico-chemical and other properties were taken as the criteria for optimization, because they determine the acceptability of the product. Table

5.3.13 shows the upper and lower limits of the goal set for constraints in optimization of the ASIC formulation.

Table 5.3.13 Goal set for constraints in optimization of ASIC

Name	Goal	Lower Limit	Upper Limit
A V	is in range	20	30
WPC	is in range	0	25
Fat	minimize	8	12
B & T	maximize	7	7.92
SWEETNESS	maximize	7.5	7.81
FLAVOR	maximize	7.16	7.73
CREAMINESS	maximize	6.8	7.8
MELTING Q	maximize	7.2	7.71
O.A.	maximize	7.2	7.93
OVERRUN	is in range	53.3	85.49
% MELT/HR	is in range	6.61	83.67
ACIDITY	is in range	0.27	0.51
pH	is in range	6.14	6.62
VISCOSITY	is in range	46.5	276.2
SP. GRAVITY	is in range	1.032	1.122
FIRMNESS	is in range	18.41	92.47

Four suggested solutions for ASIC were obtained using Design Expert as shown in Table 5.3.14. It was observed that solution no. 1 had higher desirability value of 0.84. The predicted values of different responses for solution 1 were

compared with the experimental values obtained for the product actually prepared using the optimized formulation for validation.

Table 5.3.14 Suggested solutions for the major ingredients of ASIC

S.No.	Suggested formulation			Desirability
	<i>Aloe vera</i> juice (%)	WPC (%)	Fat (%)	
1	20	25	8	0.84
2	20.79	25	8	0.83
3	21.70	25	8	0.82
4	22.80	25	8	0.80

5.3.6.1 Validation of the optimized formulation

ASIC was prepared by using the optimized combination having maximum desirability of 0.84. It was subjected to evaluation for sensory, physico-chemical and rheological attributes. The data obtained for various attributes are presented in Table 5.3.15. The t-test (assuming equal variance) was used for comparing the actual and predicted values. The t-test indicated that there was no significant difference between the predicted and observed values except for body & texture, overall acceptability and firmness. These differences may be ascribed to the experimental error.

Table 5.3.15 Verification of the predicted values for the optimization parameters of the optimized product

Parameter	Predicted value	Observed value ^a	t _{0.05}
Body and texture score	7.84	7.96	-1.5 *
Sweetness score	7.71	7.84	-2.12 ^{ns}
Flavor score	7.59	7.68	-2.06 ^{ns}

Creaminess score	7.73	7.85	-2.15 ^{ns}
Melting quality	7.71	7.75	-1.31 ^{ns}
Overall acceptability	7.72	7.89	-3.27*
Overrun	62.22	63.66	-1.63 ^{ns}
% Melt/h	78.45	80.33	-2.13 ^{ns}
Acidity (% la)	0.4	0.41	-1.69 ^{ns}
Viscosity	192.66	185.16	2.23 ^{ns}
pH	6.47	6.45	2.23 ^{ns}
Specific gravity	1.09	1.082	0.44 ^{ns}
Firmness	71.13	68.97	2.93*

^aMeans from triplicate experiments

5.4 SELECTION OF PROBIOTIC BACTERIA

The large mucosal surface area of the adult human makes it the largest body area interacting with the environment (Collins *et al.*, 1998). Although sterile at birth, we acquire commensal enteric microflora resulting in the development of a complex ecosystem in the gastrointestinal tract. Over the last 10 years, there has been a steady increase in the consumption of dairy products containing bacterial strains claimed to promote well being (Morelli, 2007). Lactic acid bacteria (LAB) are the common microorganism in foods constituting the natural intestinal microflora of humans (Tannock, 1995). There is a large scientific consensus that, in order to assess the properties of probiotic bacterial strains, it is mandatory to perform a preliminary *in vitro* assessment (FAO/WHO, 2001, 2002). Strains belonging to species normally inhabiting the human gut have been shown to behave better when assayed for their *in vitro* resistance to low pH or to simulated gastric juice (Conway, 1987). There is an ambiguous situation among the enteric species of lactobacilli, strains with a documented ability to colonize the human gut showed poor performance in the *in vitro* assay (Charteris *et al.*,

1998; Mishra and Prasad, 2005) but the same strains show excellent results when analysed *in vivo* (Fonden *et al.*, 2000). This disagreement between *in vitro* and *in vivo* results points out towards the need to refine these kinds of tests. Several papers (Acharya & Shah, 2002; Collado and Sanz, 2006; Mishra and Prasad, 2005) suggest that a probiotic bacterial strain should be analyzed according to the following or similar scheme: (i) it must survive during gastric transit, (ii) it should tolerate bile salts, (iii) it must adhere to gut epithelial tissue, (iv) it needs to possess antimicrobial activity and antibiotic susceptibility.

5.4.1 Probiotic activity verification of the cultures

In the present study, four probiotic LAB strains viz., NCDC-624 (*L.plantarum*), NCDC-625 (*L.plantarum*), NCDC-626 (*L.rhamnosus*) and NCDC-627 (*L.paracasei ssp. paracasei*) were evaluated to validate their probiotic attributes. The results of the study are being described hereunder.

5.4.1.1 Acid Tolerance

The pH of gastric acid varies between 1.5-4.5 over a period of 2h, depending on the entering time and the type of gastric contents (Verdenelli *et al.*, 2009). Most of the microorganisms ingested get destroyed by the gastric juice (pH 2.0) in the stomach (Charteris *et al.*, 1998). Minimum 90 min incubation time in acidic broth is essential because the time from entrance to release from the stomach is 90 min. However, the passage time is increased by further digestive processes (Cebeci and Gurakan., 2003). Therefore, resistance to human gastric transit is an important selection criterion for probiotic organisms. Recent developments in new delivery systems and use of specific foods, clearly demonstrate that acid sensitive strains can be buffered through the stomach. It is, therefore, expected that the relatively much acid tolerant strains of *Lactobacillus* would show better gastric survival, if consumed along with the fermented milk products or within a food matrix.

The effect of different pH conditions on the viability of probiotic strains is presented in Table 5.4.1a,b. In general, all four strains showed lower viability at pH 2.0 than at pH 3.0 and pH 4.0. At pH 2.0, after an incubation

time of 180 min, NCDC-626 and NCDC-627 showed a 4-5 log reduction in viability whereas NCDC-624 and NCDC-625 showed 6 log reduction and 5 log reduction, respectively after 180 min of exposure. At pH 3.0, viability of cells of NCDC-624 and NCDC-626 decreased by less than one log cycle after 180 min of exposure while NCDC-625 and NCDC-627 showed slightly more than one log cycle reduction under identical conditions of incubation. When the pH was raised to 4.0, the viability improved and all the strains registered less than one log cycle reduction after 180 min of incubation time. At pH 7.0, however, all the four strains showed an increase in count at the end of incubation. The study thus revealed that though all the strains evaluated were able to tolerate the acidity of gastric environment, they were unable to show any growth due to the acidic stress.

Table 5.4.1(a) Effect of different pH on the viability of probiotic strains

	pH 7.0					pH 4.0				
	0 min	30 min	60 min	120 min	180 min	0 min	30 min	60 min	120 min	180 min
NCDC-624	9.00±0.2	9.05± 0.5	9.03± 0.3	9.22± 0.1	9.17± 0.3	9.09± 0.4	9.04± 0.2	8.69± 0.4	8.98± 0.3	8.88± 0.2
NCDC-625	9.20±0.4	9.36± 0.5	9.27± 0.2	9.41± 0.3	9.37± 0.5	9.00± 0.2	9.17± 0.2	8.74± 0.4	8.87± 0.5	8.67± 0.7
NCDC-626	9.37±0.4	9.41± 0.3	9.29± 0.6	9.46± 0.5	9.49± 0.8	9.30± 0.5	9.24± 0.1	9.11± 0.4	9.06± 0.4	9.12± 0.2
NCDC-627	9.27±0.7	9.12± 0.2	9.19± 0.3	9.06± 0.6	9.13± 0.5	9.12± 0.5	8.99± 0.3	8.95± 6.0	8.67± 0.3	8.95± 0.9

Viable cell count (log cfu mL⁻¹) after exposure to low pH(4.0,3.0,2.0) with control (pH 7.0) at different time intervals (min) at 37 °C

Table 5.4.1(b) Effect of different pH on the viability of probiotic strains

	pH 3.0					pH 2.0				
	0 min	30 min	60 min	120 min	180 min	0 min	30 min	60 min	120 min	180 min
NCDC-624	9.01±0.3	8.67±0.5	8.64± 0.8	8.24± 0.3	8.10± 0.2	9.08± 0.4	8.91± 0.1	5.93± 0.7	4.97± 0.6	3.04± 0.2
NCDC-625	9.33±0.9	9.23±0.6	8.62± 0.5	8.23± 0.4	8.12± 0.7	9.21± 0.3	8.68± 0.1	6.79± 0.5	5.01± 0.6	4.29± 0.9
NCDC-626	9.38±0.9	9.01±0.7	8.69± 0.4	8.24± 0.3	8.41± 0.6	9.29± 0.1	8.63± 0.6	6.72± 0.1	5.01± 0.8	4.87± 0.7
NCDC-627	9.08±0.8	8.76±0.7	8.20± 0.6	7.87± 0.4	7.82± 0.2	9.26± 0.5	8.54± 0.5	6.19± 0.4	5.02± 0.0	4.91± 0.9

Viable cell count (log cfu mL⁻¹) after exposure to low pH(4.0,3.0,2.0) with control (pH 7.0) at different time intervals (min) at 37 °C

Our results are in agreement with the observations recorded in a study conducted by Mishra and Prasad (2005) who reported higher survival of probiotic strains at higher pH (pH 3.0) than at lower pH (pH 2.0). Mourad and Nour-Eddine (2006) and Cebeci and Gurakan (2003) also studied acid tolerance of *Lactobacilli* strains and observed decrease in viability of probiotic organisms at low pH after similar incubation periods.

5.4.1.2 Bile Tolerance

The bile salt tolerance pattern of the strains is presented in Table 5.4.2a,b. All the selected strains were able to tolerate 0.5%, 1% and 2% bile concentrations. During 3h of incubation at 0.5% bile salt concentration, all the four strains showed only minor reduction in counts except NCDC-625 which showed one log reduction. When the bile concentration was raised to 1%, the survivability was adversely affected and all the strains showed 1-2 log cycle reduction barring NCDC-627 which decreased by less than one log cycle. At still higher bile concentration (2%), all the four probiotic strains showed more than 2 log reductions after 3h. Among all strains, NCDC- 627 appeared to be more tolerant at higher bile concentration. This implies that this strain of probiotic may survive the bile concentration of 0.2-2.0% in the small intestine (Gunn,2000). Bile entering the duodenal section of the small intestine is reported to reduce the survival of bacteria by targeting lipids and fatty acids of cell membrane.

Studies carried out in the past have revealed that different strains of probiotic bacteria including *L. paracasei* and *L. plantarum* exhibit varying levels of tolerance to bile salts (Gilliland and Walker 1990; Mishra and Prasad, 2005; Sieladie *et al.*, 2011). Our results are also consistent with their observations.

Table 5.4.2(a) Bile salt tolerance pattern of the strains

	0% bile			0.5% bile		
	0h	1h	3h	0h	1h	3h
NCDC-624	9.19±0.6	9.06±1.0	9.22±1.8	9.07±0.6	8.87±0.4	9.16±0.9
NCDC-625	8.91±0.7	9.17±2.0	9.11±1.2	9.19±1.1	9.11±1.1	8.18±0.5
NCDC-626	8.90±0.4	8.85±1.9	8.95±1.4	9.20±0.9	9.19±0.4	9.31±0.6
NCDC-627	9.02±0.6	8.73±0.9	8.97±1.1	9.02±0.8	9.14±0.9	8.82±1.0

Viable cell count (log cfu mL⁻¹) after exposure to different bile concentration (0.5%,1%,2%) with control (0%) at different time intervals (h) at 37 °C

Table 5.4.2(b) Bile salt tolerance pattern of the strains

	1% bile			2% bile		
	0h	1h	3h	0h	1h	3h
NCDC-624	9.00± 0.6	8.86± 0.7	7.85± 1.8	9.05± 0.9	8.01± 0.6	6.77± 0.5
NCDC-625	8.73± 0.6	8.62± 0.4	7.56± 0.8	9.01± 0.3	8.04± 0.5	6.79± 0.2
NCDC-626	9.08± 0.6	8.21± 0.7	7.01± 1.8	8.87± 0.3	7.08± 0.4	6.64± 0.7
NCDC-627	9.19± 0.9	8.92± 0.9	8.62± 0.6	9.00± 0.7	7.60± 1.1	6.81± 0.9

Viable cell count (log cfu mL⁻¹) after exposure to different bile concentration (0.5%,1%,2%) with control (0%) at different time intervals (h) at 37 °C

5.4.1.3 Cell Surface Hydrophobicity (CSH)

In the present study, all the four strains namely NCDC-624 (*L.plantarum*), NCDC-625 (*L.plantarum*), NCDC-626 (*L.rhamnosus*) and NCDC-627 (*L.paracasei* ssp. *paracasei*) were evaluated for their cell surface hydrophobicity towards three hydrocarbons i.e. n-hexadecane, n-octane and xylene, which may reflect the colonization potential of the organism to intestinal lumen. The results pertaining to the surface hydrophobicity of the strains are given in Table 5.4.3. As evident from the table, NCDC- 627 has relatively more affinity towards all the three hydrocarbons as compared to the other strains. The percent cell surface hydrophobicity values of NCDC- 627 observed for the three hydrocarbons i.e, n-hexadecane, n-octane and xylene were 52%, 62% and 65%, respectively.

The variation in hydrophobicity of probiotic bacteria explains the fact that adhesion depends upon the origin of strains as well as surface properties (Ambrosini *et al.*, 1998). The large differences in the cell surface hydrophobicity could be due to the expression of cell surface proteins among the strains of a species as well as due to environmental conditions which could affect the expression of surface proteins. Morelli (2007) and Mishra and Prasad (2005) however showed that adhesion index is a strain –specific feature, but there was no apparent relationship with the original environment from which the strain was isolated.

Verdenelli *et al.* (2009) also studied *in vitro* adherence to human HT29 cell line and noted that *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from human faeces performed better than the commercial *Lactobacillus* strains belonging to the same species. This adhesion ability of good bacteria to intestinal epithelial cells gives competition to the coming intruders inside host cell. Higher CSH may favor the colonization of mucosal surfaces and play a role in the adhesion of bacteria to epithelial cells by preventing wash-out especially in the small intestine where flow rates are relatively higher (Schillinger *et al.*, 2005). The ability to adhere can give information about the possibility of probiotics to colonize and may modulate the host immune system (Klayaung *et al.*, 2008).

Table 5.4.3 Cell surface hydrophobicity with different hydrocarbons

Hydrocarbon	NCDC-624	NCDC-625	NCDC-626	NCDC-627
n- hexadecane	31 ± 1.73	38 ± 0.97	13 ± 1.78	52 ± 0.87
n- octane	34 ± 1.08	41 ± 1.51	12 ± 0.57	62 ± 1.04
Xylene	58 ± 0.75	41 ± 1.29	52 ± 0.98	65 ± 0.75

Mean ± S.D [Hydrophobicity (%) = $(O.D_{600} \text{ before mixing} - O.D_{600} \text{ after mixing}) / (O.D_{600} \text{ before mixing}) \times 100$]

5.4.1.4 Antibiotic Susceptibility

One of the crucial criteria for evaluating the safety of potential probiotics is its antibiotic susceptibility, since bacteria used as probiotics may serve as host of antibiotic resistant genes, which can be transferred to pathogenic bacteria. (Klayaung et al., 2008). As per the European Union (EU) Scientific Committee on Animal Nutrition (SCAN) guidelines, bacteria used in feeds should not contain any acquired antibiotic resistances (SCAN, 2002). Antibiotics are commonly classified based on their mechanisms of action, chemical structure, or spectrum of activity. Most of the antibiotics target bacterial functions or growth processes. Out of the 14 antibiotics used in the present study, vancomycin, penicillins, ampicillin, gentamicin, cephazolin, cephuroxime and cephalothin function by disrupting the bacterial cell wall synthesis. Antibiotics like amikacin, clindamycin, erythromycin chloramphenicol and tetracycline leave the bacterium unable to synthesize proteins vital to its growth. Whereas, antibiotic ofloxacin and co-trimoxazole inhibit synthesis of nucleic acids and folate, respectively.

During the present investigation, all the four strains of probiotic bacteria were exposed to 14 different antibiotics. The results of the study are presented in Table 5.4.4. All the four strains were found to be either sensitive or moderately sensitive to amikacin, ampicillin, chloramphenicol, cefazolin, cephalothin, clindamycin, erythromycin, gentamicin, penicillin and tetracycline. NCDC- 626

was resistant to co-trimoxazole, cefuroxime, ofloxacin and vancomycin while both NCDC- 624 and NCDC- 625 were resistant to ofloxacin and vancomycin. However, of all the four probiotic strains studied, NCDC- 627 was either sensitive or moderately sensitive to all the antibiotics.

Table 5.4.4 Antibiotic susceptibility of different probiotic strains.

Antibiotic	NCDC-624	NCDC-625	NCDC-626	NCDC-627
Co-Trimoxazole (25mcg)	S	S	R	S
Tetracycline (30mcg)	S	S	S	S
Gentamicin (10mcg)	S	S	S	S
Amikacin (30mcg)	S	S	M	S
Chloramphenicol (30 mcg)	S	S	S	S
Ampicillin (10mcg)	S	S	M	M
Cephazolin (30mcg)	S	S	S	M
Cephuroxime (30mcg)	S	S	R	S
Ofloxacin (1mcg)	R	R	R	S
Erythromycin (15mcg)	S	S	S	S
Clindamycin (2mcg)	S	S	S	S
Vancomycin (30mcg)	R	R	R	S
Penicillin (10 units)	S	S	S	S
Cephalothin (30mcg)	S	S	S	S

S: sensitive i.e inhibition >50%; M: moderately sensitive i.e inhibition 10-30 %; R: resistant i.e no inhibition

Vancomycin is a glycopeptide antibiotic and has been reserved as a drug of “last resort” in the past, as it was used only after treatment with other antibiotics had failed. However, vancomycin resistance is now a widespread phenomenon among Lactobacilli. Some LAB strains like *L. casei*, *L. plantarum*, *Enterococcus* spp., *Pediococcus* spp. and *Leuconostoc* spp. have been reported

to be resistant to vancomycin. (Mishra and Prasad, 2005). Davis *et al.*, (2007) reported that the resistance of heterofermentative and facultative heterofermentative lactobacilli to vancomycin is intrinsic due to the presence of D-Ala-D-lactate in their peptidoglycan. Out of the 13 *L.plantarum* strains studied by Cebeci and Gurakan (2003) for antibiotic susceptibility, all the strains were susceptible to erythromycin, clindamycin and cephalosporin and four cultures showed resistance to penicillins. However, in the present study, both the *L. plantarum* strains were susceptible to penicillin along with erythromycin, clindamycin and cephalosporin. This variation can be attributed to the activities of specific strains against the antibiotics.

Verdenelli *et al.* (2009) carried out antibiotic susceptibility test of two probiotic cultures viz., *Lactobacillus rhamnosus* and *Lactobacillus paracasei* and observed that both the strains were resistant to vancomycin and gentamicin. Whereas in the present study, *L.paracasei ssp. paracasei* was found susceptible to vancomycin which added to the probiotic potential of our culture.

Since, NCDC 627 was found susceptible to all the tested antibiotics belonging to the major classes of antibiotics used in human clinical therapy it can be considered as its positive trait for use in probiotic food.

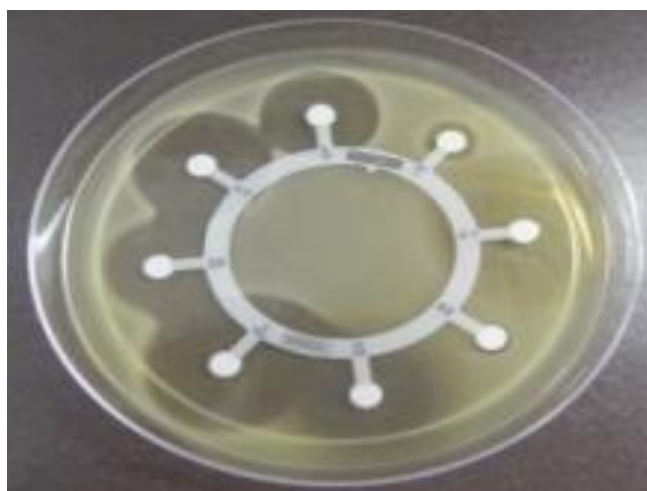


Plate 1. Inhibitory zones of *Lactobacillus* strains formed due to their susceptibility for the antibiotics

5.4.1.5 Antimicrobial Activity

Antimicrobial activity is thought to be an important means for probiotic bacteria to competitively exclude or inhibit activities of harmful or pathogenic intestinal microbes. Inhibition of the growth of pathogen can be through production of antimicrobial components such as organic acid-Lactic acid, hydrogen peroxide and bacteriocins (Jin *et. al.*, 1996). Lactobacilli are natural components of human intestinal microbial flora and these fermentative organisms produce organic acids such as acetic and lactic acids, which tend to lower the intestinal pH and inhibit the multiplication of harmful or pathogenic microorganisms.

The antagonistic activity exhibited by different lactobacilli strains used in this study against most common enteric organisms was determined by well diffusion method by measuring the diameter of the zone of inhibition as presented in Table 5.4.5. The results indicated that all the *Lactobacillus* species were active against the tested indicator strains. The zones of inhibition of indicator organism tested ranged from 7.2 to 10.4 mm in diameter. NCDC- 627 showed maximum inhibition activity against *Escherichia coli*, *Salmonella enteritidis* and *Shigella spp.* with diameter of zones of inhibition ranging from 8.1 to 10.3mm; 9.0 to 10.2mm and 8.9 to 10.3mm respectively. While for *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhi*, NCDC- 626 showed maximum inhibition activity with diameter of zones of inhibition ranging from 7.2-10.4mm; 8.5-9.7mm and 9.1-10.0mm respectively. For the same pathogenic micro-organisms, NCDC- 627 showed inhibition activity very close to NCDC-626, that ranged from 9.3mm to 10.3mm. For pathogenic microorganism *Escherichia faecalis* and *Micrococcus luteus*, NCDC- 626 showed maximum inhibition activity. NCDC-625 showed maximum inhibition activity against *Pseudomonas aeruginosa* with inhibition zone diameter of 9.2 to 9.9mm.

Table 5.4.5 Antimicrobial activity of different probiotic strains.

Organism	NCDC-624	NCDC-625	NCDC-627	NCDC-626
<i>Escherichia coli</i> , ATCC 25922	8.1 ± 0.4	9.4 ± 0.5	10.3 ± 0.5	10.0 ± 0.6
<i>Enterococcus faecalis</i> , MTCC 439	8.0 ± 0.3	9.2 ± 0.4	8.7 ± 0.4	10.2 ± 0.5
<i>Listeria monocytogenes</i> , ATCC 15303	7.2 ± 0.3	9.1 ± 0.4	10.3 ± 0.6	10.4 ± 0.6
<i>Micrococcus luteus</i> , NCDC131	7.4 ± 0.4	8.2 ± 0.4	7.9 ± 0.3	9.0 ± 0.4
<i>Pseudomonas spp</i> (Clinical isolate of AIIMS)	9.2 ± 0.4	9.9 ± 0.6	9.4 ± 0.3	9.3 ± 0.5
<i>Staphylococcus aureus</i> , (Clinical isolate of AIIMS)	8.5 ± 0.4	8.6 ± 0.4	9.3 ± 0.4	9.7 ± 0.4
<i>Salmonella enteritidis</i> , MTCC 3291	9.0 ± 0.5	9.2 ± 0.5	10.2 ± 0.6	9.2 ± 0.4
<i>Shigella dysenteriae</i> , NCDC 107	10.1 ± 0.6	8.9 ± 0.4	10.3 ± 0.6	10.2 ± 0.6
<i>Salmonella typhi</i> , NCDC 113	9.4 ± 0.4	9.1 ± 0.3	9.9 ± 0.5	10.0 ± 0.6

Mean ± S.D. Zone diameter >6 represents strong inhibitory action against potential pathogen

Our results are in agreement with the findings of Jamaly *et al.* (2011) who isolated *L. paracasei* and *L. plantarum* strains from Moroccan fermented dairy foods and evaluated their antimicrobial activity. They observed that both the probiotic cultures had strong inhibitory effect against some common food borne pathogens. Several other workers have also reported inhibitory activity of lactobacilli against common pathogens like *Escherichia coli*, *Salmonella*, *Shigella*, *S. aureus*, *E. faecalis* and *B. cereus* etc in the past (Oyetayo, 2004; Reddy *et al.* 2006; Savadogo *et al.* 2004). Besides, Gaudana *et al.* (2010) observed strong inhibitory activity of *L. rhamnosus* CS25 strain (isolated from faeces of human child) against both Gram-positive and Gram-negative bacteria.

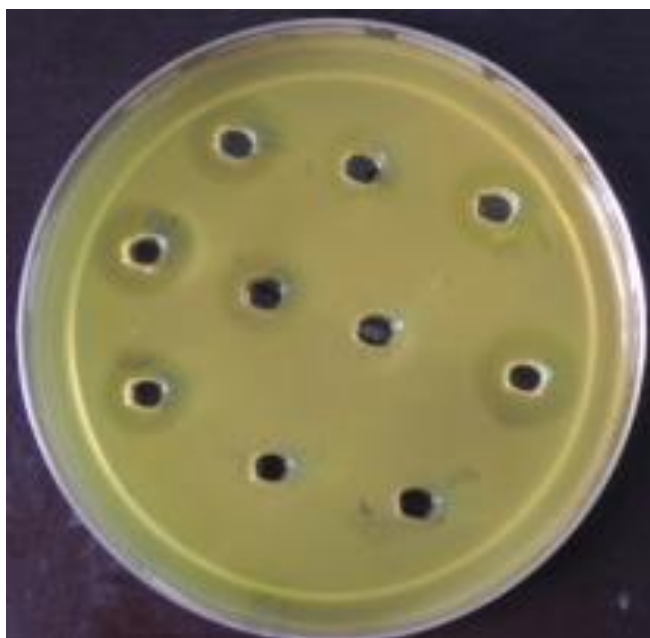


Plate 2. Antagonistic effect of probiotic lactobacilli culture against pathogens

5.4.2 Survivability of probiotics with *Aloe vera* at low temperature ($-20 \pm 2^{\circ}\text{C}$)

All the four probiotic cultures (NCDC-624, NCDC-625, NCDC-626 and NCDC-627) were added @ 1% to reconstituted skim milk (10% TS) samples containing *Aloe vera* juice (previously pasteurized) added @ 20% and incubated at 37°C for 48h. After 48h of incubation period, samples were placed in deep freezer maintained at $-20 \pm 2^{\circ}\text{C}$ for 40 days. After 40 days, the stored samples

were evaluated for the low temperature tolerance of the selected strains by enumeration of probiotic count.

Table 5.4.6 Viable cell count * (Log_{10} cfu/ml) of probiotic cultures

Days	Viable cell count (Log_{10} cfu/ml)			
	NCDC-627	NCDC-625	NCDC-626	NCDC-624
0	9.08 ± 0.04	8.77 ± 0.06	9.63 ± 0.12	8.95 ± 0.04
40	8.97 ± 0.07	8.59 ± 0.11	9.13 ± 0.02	8.74 ± 0.04

* Mean $\pm$ SE from three replicates

Table 5.4.6 presents the data showing effect of low temperature ($-20 \pm 2^{\circ}\text{C}$) on the survival rate of the four probiotic bacteria after 40 days. Results showed that low temperature had significant ($p < 0.05$) effect on survival rate of these bacteria. NCDC-626 had the highest viable count at $-20 \pm 2^{\circ}\text{C}$ after 40 days of storage followed by NCDC-627, NCDC-624 and NCDC-625. However, there was 0.5 log reduction in the counts of NCDC-626. NCDC-627 showed 0.11 log reduction in the count, which was least among the four probiotic cultures.

Our results corroborate well with the observations of Homayouni *et al.* (2008) who studied the effect of low temperature (4 and -20°C) on the survival rate of four probiotic bacteria after 1, 2 and 3 months in MRS broth medium and noted better resistance of *Lactobacillus* genera at low temperatures. Haynes and Playne (2002) also observed good low temperature resistance of *Lactobacillus paracasei* subsp. *paracasei* (LCS1) in low fat ice cream.

5.4.3 Selection of Best Probiotic Strain

Probiotic attributes and low temperature tolerance of four probiotic LAB strains viz., NCDC-624 (*L.plantarum*), NCDC-625 (*L.plantarum*), NCDC-626 (*L.rhamnosus*) and NCDC-627 (*L.paracasei* ssp. *paracasei*) were studied to select the best strain for incorporation into ASIC mix to prepare *Aloe vera* supplemented probiotic ice cream (ASPIC). Out of the four probiotic cultures

evaluated, NCDC – 627 was selected, as it possessed better acid tolerance, antimicrobial activity and antibiotic susceptibility, highest % cell surface hydrophobicity and better low temperature tolerance.

5.5 INCORPORATION OF SELECTED PROBIOTIC BACTERIA IN ASIC

ASPIC was prepared by adding milk fermented with selected probiotic strain to the ASIC mix, followed by freezing. A part of the total calculated quantity of milk (4% and 8%) meant for the ASIC formulation was used for preparation of fermented milk, by inoculating sterilized milk (4% and 8% of total milk separately) with NCDC 627 @ 2% rate and incubating it at 37 °C for 16h. The viable probiotic bacteria was enumerated in the ASPIC samples immediately after freezing.

Table 5.4.7 shows the effect of level of addition of fermented milk on the viable count of probiotic bacteria after freezing. The ice cream prepared with 8% fermented milk resulted in statistically higher viable cell counts (8.19 ± 0.02) as compared to the ice cream prepared with 4% fermented milk. Therefore, the rate of addition of fermented milk to ASIC was selected as 8% for the preparation of *Aloe vera* supplemented probiotic ice cream.

Table 5.4.7 Viable cell count* (Log_{10} cfu/g) of probiotic bacteria @ 4% and 8% fermented milk addition

4% Fermented milk addition	8% Fermented milk addition
6.88 ± 0.03^b	8.19 ± 0.02^a

*Mean $\pm$ SE from three replicates

5.5.1 Incorporation Of Commercial DVS Probiotic Bacteria In ASIC

Based on the results of studies conducted to select the level of incorporation of NCDC culture in the ASIC mix, the commercial La5 DVS culture was also added to 8% of the total milk and incubated at 37 °C for 2h to obtain fermented milk for further incorporation into the ASIC mix. The procedure for preparation of ice cream remained as described in section 5.5.

5.6 PROXIMATE COMPOSITION AND CALORIFIC VALUE OF ASPIC

5.6.1 Proximate Composition

ASPIC samples were prepared and analyzed for their proximate composition. The proximate composition of the final formulation of ASPIC is given in Table 5.6.1.

Table 5.6.1 Proximate composition of ASPIC

Constituents	Formulated Ice cream (NCDC culture)	Formulated Ice cream (DVS culture)
Total Solids (%)	39.80 ± 0.40	39.50 ± 0.23
Fat (%)	8.10 ± 0.20	8.00 ± 0.10
Protein (%)	4.88 ± 0.20	4.90 ± 0.22
Ash (%)	1.20 ± 0.04	1.30 ± 0.06
Carbohydrates (%)	25.60*	25.40*
Energy (Kcal/100ml)	194.80	193.20

* Calculated by difference. Values are mean ± standard error (from three determinations)

As can be seen from Table 5.6.1, both the ice cream formulations contained fat more than 2.5 percent but less than 10.0 percent, and milk protein more than the prescribed minimum protein content of 3.5 percent, as suggested under FSSAI regulations for medium fat ice cream. Therefore, both the developed products can be categorized as medium fat ice cream.

5.6.2 Calorific Value

The energy value of ASPIC was calculated by taking the energy value for fat, protein and carbohydrate as 9.0, 4.0 and 4.0 kcal/g respectively. The energy

value was computed to be 194.8 kcal per 100ml for ASPIC with NCDC and 193.2 kcal per 100ml for ASPIC with DVS culture.

5.7 STORAGE STUDY

During prolonged storage, ice cream is likely to undergo physical and biochemical changes which might affect not only sensory acceptability of the product but may alter many other desirable properties. This section presents results of changes in sensory and microbiological quality of the ASPIC with NCDC probiotic culture during storage at -20 ± 2 °C for 3 months.

5.7.1 Organoleptic Changes

New product development is a challenging task, as consumers expect a product that is tasty and healthy simultaneously. Consumers cannot accept a functional probiotic ice cream with added ingredients that contribute unpleasant flavors to the product even if it is beneficial for health. For functional ingredient like probiotics, consumers must be made aware of health benefits of probiotics to convince that the functional probiotic ice cream is better than the traditional one. The sensory properties of the products are not modified when the probiotic cultures are added to them intensely (Champagne *et al.*, 2005). Previous studies that are based on sensory evaluation also suggest no clear differences between the tastes of products containing probiotic strain and the control prepared using support culture (Saxelin *et al.*, 1999). Probiotic ice creams which are not fermented do not present problems owing to microbial metabolism as they are stored at low temperature that minimize the biochemical reactions of probiotic microorganisms (Cruz *et al.*, 2009). The addition of probiotic cultures has also extended the shelf life of milk based products (O' Riordan and Fitzgerald, 1998) by inhibiting the development of undesirable psychrotrophic flora. The production of a probiotic ice cream with high sensory acceptance therefore requires technical knowledge for successful development of the product with good sensory performance in comparison with the conventional ice cream (Favaro-Trindade *et al.*, 2006). However, development of probiotic ice cream with good sensorial quality is possible (Vardar & Oksuz, 2007).

There are several studies addressing sensory properties in probiotic foods and many of them show no change in acceptability when adding probiotics to dairy products. Nousia *et al.*, (2010) developed a probiotic ice cream by incorporating *Lactobacillus acidophilus* LMGP-21381 in a standard ice cream mix and assessed the developed product for the sensory characteristics during 45 weeks of storage. The sensory parameters viz. aroma, taste and texture obtained high scores in the sensory evaluation during storage period. In another study, efficiency of a nonfermented ice cream for delivering *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* to consumers was evaluated by Abghari *et al.*, (2011). The authors reported that the addition of the microorganisms had no significant effect on the sensory properties of the finished product. Heenan *et al.*, (2004) developed a nonfermented, vegetarian frozen probiotic soy dessert and carried out a study to detect sensory differences between products containing probiotics (*L. acidophilus* MJLA1, *S. boulardii* 74012) and an uninoculated control. The samples of frozen desserts were stored for 0, 4 and 7 months and compared using triangle tests. Product inoculated with *L. acidophilus* MJLA1 could not be distinguished from the control sample.

Recently, ice creams with *Aloe vera* as one of the constituent have also been prepared and subjected to sensory evaluation for consumer acceptance. Manoharan *et al.*, (2012) incorporated *Aloe vera* juice at different levels (5, 10, 15, 20, 25, 30, 35, 40 and 45 %) in ice cream mix. The authors concluded that the inclusion of *Aloe vera* juice in the ice cream significantly altered the organoleptic scores of the ice cream samples. *Aloe vera* juice at 20 % inclusion level, among the different inclusion levels (5-45%), had the maximum scores, without much affecting its acceptability. Srisukh *et al.*, (2006) also prepared pandan-flavored, coconut-flavored and orange-flavored *Aloe* ice creams using fresh *Aloe vera* pulp and carried out sensory evaluation of the developed *Aloe* ice creams. Sensory evaluation of the *Aloe* ice cream showed highest average score for pandan-flavored *Aloe* ice cream followed by Coconut-flavored and orange-flavored *Aloe* ice creams.

Table 5.7.1 Changes in sensory scores* of ASPIC during storage

Attributes	Storage period in days						
	0	15	30	45	60	75	90
Color & Appearance ^{ns}	8.00±0.20	8.00±0.20	7.78±0.07	7.75±0.00	7.80±0.05	7.65±0.25	7.60±0.20
Body & Texture ^{ns}	8.00±0.00	8.00±0.00	7.76±0.20	7.70±0.00	7.65±0.25	7.50±0.33	7.35±0.15
Creaminess ^{ns}	7.85±0.05	7.80±0.05	7.75±0.00	7.73±0.07	7.85±0.04	7.60±0.20	7.59±0.15
Sweetness ^{ns}	7.90±0.00	7.88±0.02	7.80±0.00	7.85±0.05	7.80±0.10	7.70±0.20	7.64±0.14
Flavor ^{ns}	7.95±0.05	7.85±0.05	7.85±0.00	7.80±0.00	7.80±0.00	7.75±0.25	7.65±0.21
Melting Quality ^{ns}	7.75±0.00	7.74±0.01	7.70±0.20	7.75±0.04	7.70±0.10	7.67±0.16	7.55±0.05
Overall Acceptability ^{ns}	7.85±0.05	7.85±0.05	7.80±0.00	7.85±0.02	7.81±0.15	7.75±0.25	7.60±0.05

* Mean ± SE from three replicates

^{ns} Non-significant difference (p>0.05)

Organoleptic properties of the ASPIC were assessed by a panel of judges using nine-point hedonic scale with 1 as “dislike extremely” and 9 as “like extremely”. The organoleptic quality of the product was evaluated in terms of various important sensory attributes, viz., color and appearance, body and texture, creaminess, sweetness, flavor, melting quality and overall acceptability. The results are delineated in Table 5.7.1. The probiotic and fermented taste was not found particularly noticeable in the product throughout the storage period. High pH value of the resultant ice cream could be the reason for this (Salen *et al.*, 2005). All ASPIC samples supplemented with the NCDC probiotic strain were found acceptable with no marked herb flavor due to the presence of *Aloe vera* juice. Generally, the mean sensory scores for all sensory attributes decreased slightly during storage, the decreases being statistically not significant. There was no significant ($p>0.05$) difference among all the organoleptic properties of ASPIC throughout the storage period of 90 days. Among all the sensory parameters, color & appearance and body & texture attributes of the ice cream obtained highest mean sensory scores of 8.00 on zero day of storage. Whereas, after 90 days of storage flavor of ASPIC received highest mean sensory score of 7.65.

5.7.1.1 Changes in sensory color and appearance score

The average sensory score for color and appearance decreased from the initial value of 8.00 to 7.60 at the end of 3 months of storage (Fig. 5.7.1). The change in sensory score for color and appearance over a period of 3 months was not significant ($P>0.05$) and remained stable during the storage period. Erratic storage conditions such as temperature fluctuations, surface evaporation due to improper packaging and quality of the coloring matter added are the reasons generally responsible for change in color and appearance during storage (Verma, 2002). Kumar (2009) also showed that the color and appearance of probiotic and synbiotic ice cream did not show any significant variations during storage period of 120 days.

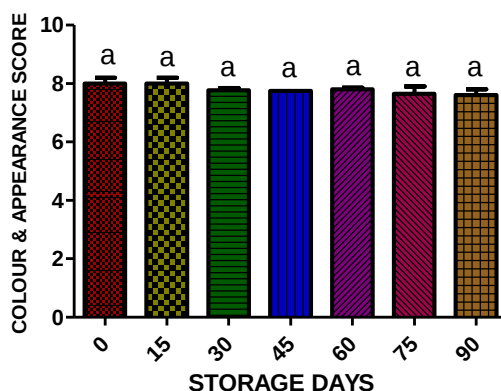


Fig. 5.7.1 Changes in color and appearance scores of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.1.2 Changes in sensory body and texture score

For body and texture, the mean sensory score during the storage period of three months varied between 8.00 and 7.35 (Fig.5.7.2). Generally, two main types of texture defects viz., sandiness and iciness are encountered in ice cream during storage leading to lower sensory scores. But, during the present study, decrease in body and texture score was not significant ($P > 0.05$) even after 3 months of storage. Similar results were obtained by Singh (2007) for low fat, fibre enriched fruit ice cream during storage for 35 days. Kumar (2009) also noted statistically not significant variation in mean body & texture score after 60 days of storage of probiotic ice cream.

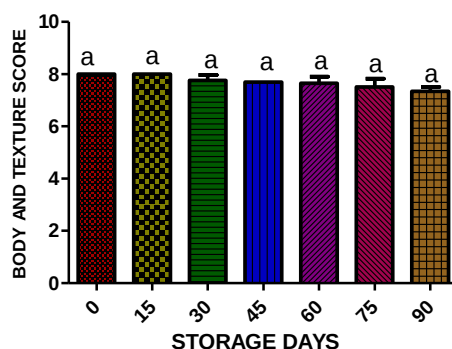


Fig. 5.7.2 Changes in body and texture scores of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.1.3 Changes in sensory creaminess score

Creaminess of ice cream is a critical characteristic that determines the acceptability of the product. The average value for creaminess was 7.85 at the beginning of storage and it decreased to 7.59 after 3 months (Fig. 5.7.3). The change in creaminess score over a period of 3 months was not significant ($P > 0.05$). Singh (2007) also reported no significant changes in creaminess score of low fat, fibre enriched fruit ice cream after 35 days of storage.

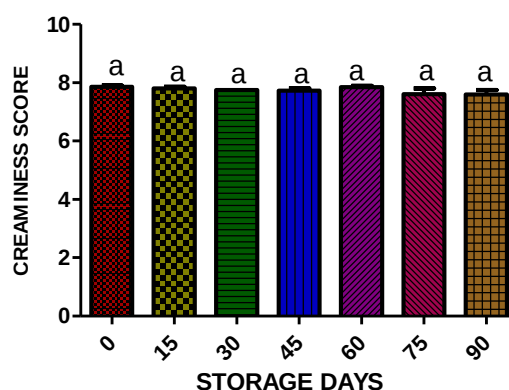


Fig. 5.7.3 Changes in creaminess scores of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.1.4 Changes in sensory sweetness score

The variation in sweetness scores of ASPIC during storage is presented in Table 5.7.1. The average values of sensory score for sweetness varied from 7.90 to 7.64. It is evident from the table that sweetness did not decrease significantly ($P > 0.05$) during the storage period.

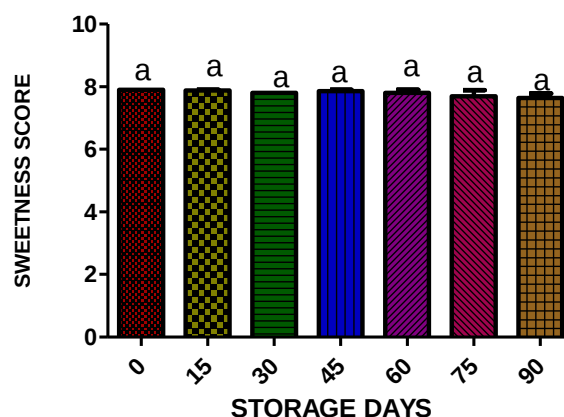


Fig. 5.7.4 Changes in color and appearance scores of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.1.5 Changes in sensory flavor score

According to Homayouni *et al.*, (2012), with respect to choice of food, flavor is the primary indicator that is followed by health benefit considerations. The flavor profiles created by probiotic ice cream can be different from that of conventional ice cream. The mean flavor scores for ASPIC presented in Table 5.7.1, revealed a not- significant ($P > 0.05$) decrease in flavor scores with the advancement of storage. At the start of the storage, i.e., on zero day of storage, average flavor score was 7.95, which dropped to 7.65 after 3 months. Most of the panelists commented that they did not notice the flavor *Aloe vera*, although the samples had been prepared with 20% of *Aloe vera* juice in the formulation. A mild flavor like vanilla was sufficient to give acceptable flavor to the product as no pronounced *Aloe vera* or probiotic flavor was observed in any of the ASPIC sample. Thus, it can be inferred that the flavor of the developed ice cream remained acceptable upto 3 months of storage duration. These findings are in line with those of Salen *et al.* (2005) who prepared probiotic ice cream with five different probiotic cultures and reported that all ice cream samples were acceptable without any marked off-flavor after 2 weeks of storage. Supplementation with probiotic bacteria has been found to have little effect on

flavor, texture or other sensory characteristics of ice cream (Mohammadi *et al.*, 2011).

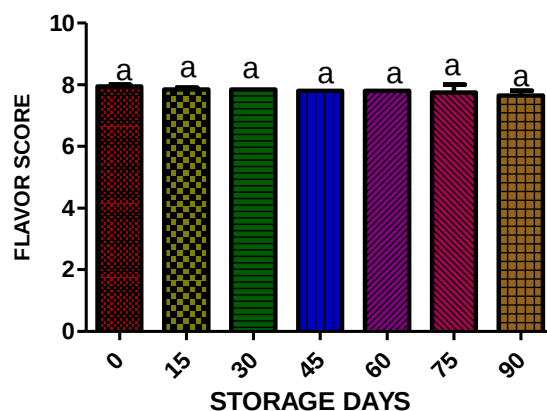


Fig. 5.7.5 Changes in flavor scores of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.1.6 Changes in sensory melting quality score

The sensory melting quality scores dropped from initial 7.75 to 7.55 after 3 months of storage. However, the decrease in the melting quality score was not significant ($P>0.05$) over the entire storage duration (Fig.5.7.6). The results are in agreement with the findings of Specter and Sester (1994) and Verma (2002), who reported a statistically not-significant ($P>0.05$) change in melting quality of frozen dessert during the storage period of 140 and 90 days, respectively.

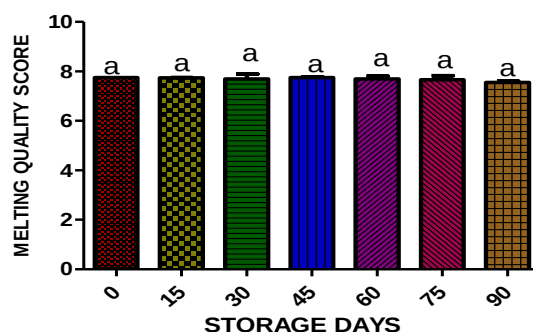


Fig. 5.7.6 Changes in melting quality scores of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.1.7 Changes in sensory overall acceptability score

The overall acceptability of the non-fermented probiotic ice cream and the conventional ice cream is similar, however, it is the low pH of the fermented probiotic ice cream that has a negative effect on sensory acceptance of the product as ice cream is not characterized as acidic food product. (Homayouni *et al.*, 2012) It is evident from Table 5.7.1 that mean sensory score for overall acceptability decreased, although not significantly ($P>0.05$), over the storage period of 3 months. The average scores varied from 7.85 to 7.60. No significant effect on overall acceptability attribute of the frozen dessert has been reported by Specter and Sester (1994) during 140 days of storage.

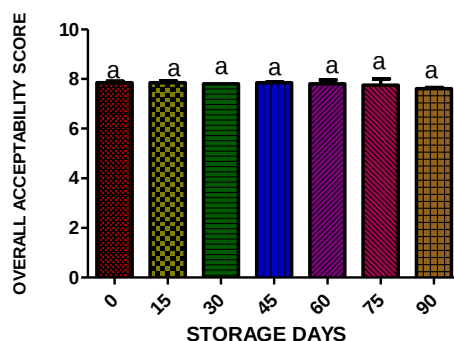


Fig. 5.7.7 Changes in overall acceptability scores of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.2 Microbiological Changes

Ice cream is an excellent matrix for microbial proliferation as it has all the essential nutrients viz., sugar, proteins and moisture, oxygen as well as suitable pH for the growth of microorganisms. Ice cream is generally stored at sub-zero, i.e., -20 ± 2 °C temperature which is not likely to permit microbial growth during storage. However, microbial quality of the product was evaluated, as it is an essential part of product development and also survivability of the added probiotic microorganism during storage is critical for the success of probiotic ice cream. Microbiological quality of ASPIC was evaluated in terms of *Lactobacillus* count, standard plate count (SPC), coliform count and yeast and mold count. The analysis was done at an interval of 15 days over a storage period of 3 month at -20 ± 2 °C. The results are presented in Table 5.7.2.

Table. 5.7.2 Changes in microbiological counts* of ASPIC during storage

Attributes	Storage period in days						
	0	15	30	45	60	75	90
<i>Lactobacilli</i> count (log cfu/g)	8.22±0.06 ^a	8.16±0.05 ^a	7.99±0.09 ^b	7.88±0.03 ^c	7.57±0.03 ^d	7.53±0.04 ^d	7.34±0.04 ^e
SPC count (log cfu/g)	5.30±0.06 ^a	5.23±0.04 ^a	5.14±0.05 ^{ba}	5.05±0.02 ^{bc}	4.95±0.05 ^c	4.18±0.06 ^d	3.81±0.06 ^e
Yeast & mold count (per g)	28.33±4.4 ^a	13.00±1.52 ^b	9.33±0.67 ^{cb}	7.33±0.33 ^{cbd}	5.67±0.33 ^{cd}	2.67±0.67 ^d	1.67±0.67 ^d
Coliforms count (per g)	--	--	--	--	--	--	--

* Mean ± SE from three replicates

**Mean with same superscripts in a row (a,b,c,d) do not differ significantly (p<0.01)

5.7.2.1 *Lactobacillus* count

The change in *Lactobacilli* count in ice cream samples during storage is presented in Table 5.7.2 and Fig.5.7.8. Initially, the viable probiotic count was 8.22 ± 0.06 log cfu/g which subsequently decreased to 7.34 ± 0.04 log cfu/g after 3 months of storage at -20 ± 2 °C. There was no significant ($p>0.05$) reduction in bacterial counts till 15th day of storage. However, the counts decreased significantly ($p<0.05$) after 15th day till 60th day of storage. The counts again showed no significant ($p>0.05$) reductions between 60th and 75th day of storage but after that the counts again decreased significantly ($p<0.05$) till 90th day. The overall decrease in the *Lactobacilli* count was 0.88 log cfu/g during a storage period of 3 months. Our results showed that ASPIC had number of viable probiotic organisms higher than the recommended minimum 10^6 cfu/g throughout 3 months of storage. In a similar study, Hekmat and McMohan (1992) reported 2 log cycle reduction in the *L. acidophilus* count in ice cream at the end of 17 weeks of storage at -29 °C. Salen *et al.*, (2005) manufactured probiotic ice cream by mixing fortified milk fermented with probiotic strains with an ice cream mix and evaluated the survival of cultures during 12 weeks of frozen storage at - 26 °C. The authors observed a decrease in viable counts by 2.23, 1.68, 1.54, 1.23 and 1.77 log cfu/g for *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri*, *Lactobacillus gasseri* and *Lactobacillus rhamnosus*, respectively, during the storage period. Akalin and Erisir (2008) studied the effects of inulin and oligofructose on the rheological characteristics and probiotic culture survival in low-fat probiotic ice cream during storage for 90 days. The authors observed that the counts decreased (0.3 to 0.9log cfu/g) significantly ($p<0.05$) throughout the storage. Abghari *et al.*, (2011) evaluated the efficiency of a non fermented ice cream for delivering *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* to consumers and observed that both the microorganisms survived and the counts were higher than 10^7 CFU g⁻¹ during 12 weeks of storage at -19 °C. During freezing and storage of ice cream, reduction in the survival of probiotic bacteria has been also reported by several other workers in the past (Christiansen *et al.*, 1996, Hagen

and Narvhus, 1999). However, there are reports suggesting no significant decrease in viable bacterial count in ice cream throughout 180 days of storage at $-20 \pm 1^{\circ}\text{C}$ (Basygjit *et al.*, 2006). Turgut and Cakmakci (2009) also investigated the possible use of probiotics in ice cream manufacture and observed that the viable *L.acidophilus* and *B.bifidum* counts did not decrease significantly throughout 90 days of storage. Nousia *et al.*, (2010) developed a probiotic ice cream by incorporating *Lactobacillus acidophilus* LMGP-21381 in a standard ice cream mix and assessed the survivability of the probiotic strain during 45 weeks of storage at -15°C and -25°C . The authors observed no significant change in the viable counts of *L. acidophilus* during frozen storage. In the present study, the viable *Lactobacilli* count dropped during storage but remained above the recommended minimum limits of 10^6 cfu/g implying that although the frozen storage of the product adversely affected the probiotic culture survival, the bacterial cells remained at levels sufficient to offer the suggested therapeutic effects.

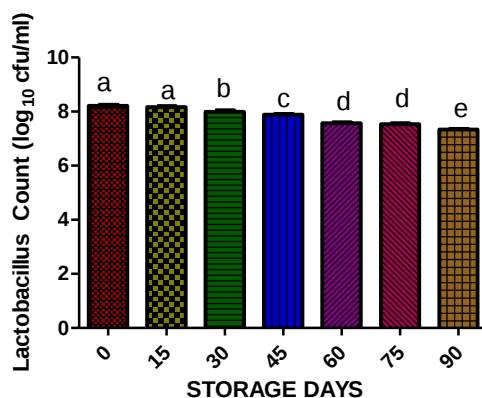


Fig. 5.7.8 Changes in *Lactobacillus* count of ASPIC during storage of 90 days at $-20 \pm 2^{\circ}\text{C}$

5.7.2.2 Standard Plate Count

The changes in standard plate count (SPC) of the ASPIC during storage are given in Fig. 5.7.9. The SPC count decreased significantly ($p < 0.05$) from

initial 5.30 ± 0.06 log cfu/g to 3.81 ± 0.06 log cfu/g after 3 months of storage (Table 5.7.2). The decline in the SPC was not significant ($p > 0.05$) for the first 15 days but the count decreased significantly ($p < 0.05$) afterwards till 90th day. This could be attributed to sub-zero (-20 ± 2 °C) storage temperature, which does not permit the growth of microbial population during storage. Kambamanoli Dimou (1993) also reported that deep freezing stabilizes the microbial count of ice cream and microorganisms present in ice cream do not proliferate. Some sensitive species (Gram-negative) die during storage and their population reduce. The SPC of the ASPIC was within the limit as specified by FSSAI regulations.

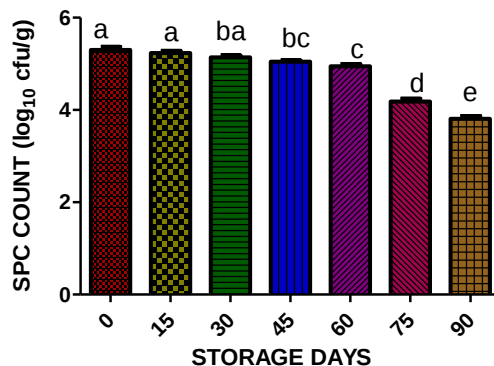


Fig. 5.7.9 Changes SPC count of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.2.3 Yeast and mold count

The results of studies involving status of yeast & mold during storage of ASPIC are illustrated in Fig. 5.7.10. The yeast & mold count of the fresh ice cream samples was 28.33 ± 4.41 log cfu/g which decreased to 1.67 ± 0.67 log cfu/g after 3 months of storage, the decrease being significant at 5% level of significance throughout storage. This reduction in yeast & mold count can be attributed to the sub-zero (-20 ± 2 °C) temperature of storage of ASPIC which hindered the growth of yeast & mold and caused reduction in their population during storage. Singh (2007) and Verma (2002) have also separately reported a gradual decrease in average count during storage of ice cream at -20 °C.

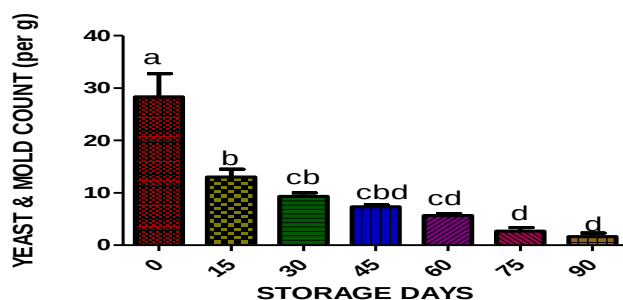


Fig. 5.7.10 Changes in color yeast & mold count of ASPIC during storage of 90 days at -20 ± 2 °C

5.7.2.4 Coliform count

Coliforms are indicators of hygienic practices employed during product manufacture. They enter the product through wash water, clothes and utensils or equipments that are not sanitized properly before use. It was found that coliforms were absent in 1 g sample of ASPIC throughout the storage period. Absence of coliforms could be due to high heat treatment given during the pasteurization of ice cream mix and subsequent aseptic handling during processing.

5.8 COST ESTIMATION OF ASPIC

After optimizing the levels of ingredients and the process parameters for the manufacture of ASPIC, the cost of production was estimated. In order to work out the cost of production of the ice cream, the following assumptions were made:

- 1) Plant capacity to be 500 kg ice cream mix per day.
- 2) Plant to be operated in single shift for 300 working days in a year.
- 3) Milk and cream to be procured through a contractor and to be delivered at the factory site in chilled condition.
- 4) *Aloe vera* juice to be procured in ready-made form in bulk.
- 5) The finished product to be packaged in ice cream cups and 1 litre ice cream bricks.
- 6) Losses were assumed to be 0.5%.

5.8.1 Raw Material Requirement

Although, the overrun observed for the optimized formulation was less, the quantity of raw materials required per day was estimated according to the composition of the ice cream assuming 100% overrun because for our project we used a batch freezer (also the model was very old) but in industries continuous freezers are used that can give higher overrun. The quantities of raw materials taken for the manufacture of 500 kg ice cream mix per day were 222.95 kg milk (6.5% fat), 36.35 kg cream (67% fat), 47.76 kg SMP, 15.92 kg WPC-35, 75.00 kg sugar, 1.00 kg stabilizer, 1.00 kg emulsifier, 100.00 kg *Aloe vera* juice, 0.75 kg flavor and probiotic culture (Table 5.8.1).

Table 5.8.1 Assumptions regarding quantity of raw materials required/day

Ingredient	Requirement/day	Output (100% overrun)
Milk (fat- 6.5%)	222.95 kg	995 litre (Including 0.5% losses)
Cream (fat- 67%)	36.35 kg	
SMP	47.76 kg	
WPC- 35	15.92 kg	
Sugar	75.00 kg	
Stabilizer(Alginate-D2)	1.00 kg	
Emulsifier (GMS)	1.00 kg	
<i>Aloe vera</i> juice	100.00 kg	
Flavour (vanilla)	0.75 kg	
Culture (NCDC)	800.00 ml	
Culture (DVS)	800.00 g	

5.8.2 Capital requirement

The built-up area and the plot of land area taken were 750 sq.mtrs. and 950 sq.mtrs, respectively. The price of plot of land was taken as Rs. 2,000 per

sq.mtrs. and the cost of built-up area as Rs. 4,000 per sq. mtrs. Thus, the calculated cost of land was considered as Rs. 19.00 lakh and building cost as Rs. 30.00 lakh. The total cost of land and building including land development cost was Rs. 49,50,000.00. The depreciation cost on building was calculated (@ 5%) to be Rs. 2,47,500.00 (Table 5.8.2).

A comprehensive list of the major processing equipments used for manufacturing 995 litre ice cream daily, corresponding to 2,98,500 litre ice cream per annum is given in Table 5.8.3. The total depreciation cost for these items was calculated as Rs. 7,90,000.00, the details of which are given in Table 5.8.3. The costs shown are location specific for the estimation. The same format, however, could be adopted for costing of ice cream prepared under any given situation.

Table 5.8.2 Cost of land and building and depreciation on building

Particular	Size (Sq. mtrs.)	Rate/ sq.mtrs. (Rs.)	Estimated cost (Rs.)	Rate of depreciation (%)	Annual depreciation (Rs.)
Land	950	2000	19,00,000.00		
Land development cost			50,000.00		
Building	750	4000	30,00,000.00	5.00	2,47,500.00
Total			49,50,000.00		2,47,500.00

Table 5.8.3 Equipment and vehicle cost

Item	Capacity	No.	Rate (Rs.)	Estimated cost (Rs.)	Rate of depreciation (%)	Annual depreciation (Rs.)
Batch pasteurizer	250 L/hr	1	5 lakh	5 lakh	10	50,00
Ageing Vat	250 L	2	3 lakh	6 lakh	10	60,000
Homogenizer	250 L/hr	1	3.5 lakh	3.5 lakh	10	35,000
Continuous freezing machine	200 L/hr	1	5 lakh	5 lakh	10	50,000
Packaging machine		1	2 lakh	2 lakh	10	20,000
Plate heat exchanger	300 L/hr	1	1 lakh	1 lakh	10	10,000
Friama grinder	500 L/hr	1	50,000	50,000	10	5,000
Cold room	10 X 15 ft	1	4.5 lakh	4.5 lakh	10	45,000
Crates		100	500	50,000	10	5,000
Steam Boiler	100kg/hr	1	1.7 lakh	1.7 lakh	15	25,500
Power generator	100 KVA	1	25 lakh	25 lakh	15	3,75,000
Water and steam line fittings			50,000	50,000	7	3,500
Laboratory equipment			50,000	50,000	10	5000
Weighing scale	100 kg	2	5000	10,000	10	1000
Refrigerated vehicle		1	10 lakh	10 lakh	10	1,00,00
Total				65.8 lakh		7,90,000

5.8.3 Direct Costs

5.8.3.1 Cost of raw materials

For calculating the cost of various ingredients used in the manufacture of ASPIC, the current (2013-14) market rates were considered (Table 5.8.4). The various raw materials required for manufacturing 2,98,500 litre ice cream per annum were worked out. It may be observed from Table 5.8.4 that the total cost of raw materials works out to be Rs. 1,42,30,575.00 (with NCDC culture) and Rs. 1,97,50,575.00 (with DVS culture) per annum.

Table 5.8.4 Cost of raw materials

Sr. No.	Item	Requirement (kg)		Rate /kg (Rs.)	Annual cost (Rs.)
		Daily	Annual		
1	Buffalo milk	222.95	66,885	35	23,40,975
2	Cream	36.35	10,905	200	21,81,000
3	SMP	47.76	14,328	200	28,65,600
4	WPC-35	15.92	4,776	500	23,88,000
5	Flavor	0.75	225	300	67,500
6	Stabilizer	1.00	300	100	30,000
7	Emulsifier	1.00	300	100	30,000
8	Sugar	75.00	22,500	35	7,87,500
9	<i>Aloe vera</i> juice	100.00	30,000	110	33,00,000
10	Culture (NCDC)	800.00 ml	240.00 Ltr	1000	2,40,000
11	Culture (DVS)	800.00 g	240.00 kg	24,000	57,60,000
Total	(with NCDC culture)				1,42,30,575
Total	(with DVS culture)				1,97,50,575

5.8.3.2 Labour and supervision

In accordance with the manufacturing operations to be performed in single operating shifts, the requirements of the personnel needed for the manufacture of 2,98,500 litre ice cream per annum were computed. The persons directly involved in the production were plant operators and manufacturing labour. The total direct cost for labour and supervision was estimated at Rs. 7,50,000.00 per annum (Table 5.8.5).

Labour and supervision charges are specific for the situations considered and are proportional to the volume of production. Since these charges are likely to depend on the complexity of plant, extent of automation and manufacturing practices, these need to be calculated for the specific situations.

Table 5.8.5 Direct cost of personnel

Sr.No.	Staff	Number	Monthly salary (Rs.)	Monthly cost (Rs.)	Annual cost (Rs.)
1	Labourers	4	4000	16,000	1,92,000
2	Skilled workers	02	5000	10,000	1,20,000
3	Mechanic/ Electrician	01	7,500	7,500	90,000
4	Boiler attendants	01	7,500	7,500	90,000
5	Lab analyst	01	7,500	7,500	90,000
6	Lab attendant	01	4000	4000	48,000
7	Technical Supervisors	01	10,000	10,000	1,20,000
				62,500	7,50,000

5.8.3.3 Packaging

Proper and attractive packaging not only helps in retaining the quality attributes, but also promotes sale of the product. In this costing exercise, 100 ml ice cream cups (for packing 70% of the ice cream produced) and 1 litre ice cream bricks (for 30% of the ice cream produced) were envisaged for retail selling of the product. The total cost of packaging for 2,98,500 litre ice cream was reckoned to be Rs. 45,37,800.00 (Table 5.8.6).

Table 5.8.6 Cost of packaging materials

Sr. No.	Type of packaging materials	Requirement		Rate / piece (Rs.)	Annual cost (Rs.)
		Daily	Annual		
1	Ice cream cups (100ml)	6,965.00	20,89,500.00	2.00	41,79,000
2	Ice cream bricks (1 litre)	299.00	89,700.00	4.00	3,58,800
Total					45,37,800

Since the cost of packaging depends on the size and cost of packaging materials, the total cost incurred in the packaging has to be assessed separately for a given size of packaging in a given type of system.

5.8.3.4 Utilities

To estimate the total direct cost, the apportioned costs for various utility services viz. electricity, steam and water were considered. The estimate of electricity requirement is given in Table 5.8.8. Individual items of utility services were determined and itemized in Table 5.8.7. It may be seen that the total cost of utilities worked out to be Rs. 2, 89,500.00 per annum.

Table 5.8.7 Electricity requirements

S.No.	Particulars	kWh	Total working hours	kWh (units/ day)	kWh/ Annum
1	Batch pasteurizer	12	3	36	10,800
2	Homogenizer	3	3	9	2,700
3	Ageing Vat	2	15	30	9,000
4	Freezer	3	3	9	2,700
5	Plate Chiller	4	2	8	2,400
6	Friama grinder	1	1	1	300
7	Packaging machine	2	3	6	1,800
8	Fan, A/C, Tube light, etc.			50	15000
9	Lab equipment			20	6000
	Total			169	50,700

The extent of various utility services depends upon the efficiency of the plant. The cost of utilities, therefore, varies from plant to plant and thus needs to be figured out for a given situation.

Table 5.8.8 Charges on power and utilities

S.No.	Particulars	Requirements		Rate (Rs.)	Annual cost (Rs.)
		Daily	Annual		
1	Electricity	169 kWh	50,700 kWh	5 / kWh	2,53,500
2	Water	2000 Ltr.	600000 Ltr.	10/ 1000 Ltr.	6000
3	Fuel				30,000
	Total				2,89,500

5.8.4 Indirect Costs

The indirect costs are situation specific and are proportional to the volume of production. Therefore, depending upon the existing facilities, the indirect costs need to be computed individually for a particular situation.

5.8.4.1 Detergents and chemicals/glassware

Under this category, the expenditure incurred on common detergents such as caustic soda, teepol etc. were considered. The estimate for detergents is presented in Table 5.8.9.

Table 5.8.9 Cost of detergents and chemicals

S. No.	Item	Annual cost (Rs.)
1	Caustic soda, teepol, etc.	2,500.00
2	Glass wares/chemicals	3000.00
Total		5,500.00

5.8.4.2 Manpower (Adm.)

The total indirect cost for administrative personnel is given in Table 5.8.10.

Table 5.8.10 Indirect cost for administrative staff

S. No.	Staff	Number	Monthly salary (Rs.)	Monthly cost (Rs.)	Annual cost (Rs.)
1	Factory Manager	1	25,000	25,000	3,00,000
2	Assistant Manager	1	15,000	15,000	1,80,000
3	Clerks	1	5000	5000	60,000
4	Store keeper	1	7,500	7,500	90,000
5	Accountant	1	7,500	7,500	90,000
6	Attendants	1	4000	4000	48,000
7	Security staff	1	4000	4000	48,000
8	Driver	1	4000	4000	48,000
	Total			72,000	8,64,000

5.8.5 Fixed Costs

Under the fixed costs, the elements included were interest on total capital investment, maintenance cost and depreciation cost (vide Table 5.8.2 and 5.8.3).

5.8.5.1 Interest on capital investment

The total capital investment comprises of interest on fixed capital and working capital. The working capital was calculated on the basis of cost of raw materials for one month of production and one month salary of the staff involved in the production of ASPIC. The total fixed and working costs and their interests at the rate of 12 per cent are given in Table 5.8.11. The total interest on these capitals was computed to be Rs. 15,90,537.00 per annum.

Table 5.8.11 Interest on capital

Sr. No.	Particulars	Amount (Rs.)	Annual interest @ 12% (Rs.)
1	Fixed capital (land, building, plant and machinery)	1,15,30,000.00	13,83,600.00
2	Working capital (being the value of raw materials and salaries and utilities for one month)	17,24,481.25	2,06,937.80
	Total		15,90,537.00

5.8.5.2 Maintenance

The estimate for maintenance of equipment, building and other expenses is shown in Table 5.8.12

5.8.6 Total Cost

The various direct, indirect and fixed costs involved in the production of ASPIC per annum are elucidated in the preceding sections. The total of direct, indirect and fixed costs is shown in Table 5.8.12.

Table 5.8.12 Total cost of developed ice cream

S. No.	Component	Annual cost (Rs.)
A. Direct costs		
1	Raw materials	
	(with NCDC culture)	1,42,30,575.00
	(with DVS culture)	1,97,50,575.00
2	Packaging	45,37,800.00
3	Manpower (labour and supervision)	7,50,000.00
4	Utilities	2,89,500.00
	Sub-total	
	(with NCDC culture)	1,98,07,875.00
	(with DVS culture)	2,53,27,875.00

B. Indirect costs		
1	Manpower (Administrative)	8,64,000.00
2	Estimated expenditure on detergents, glass wares and chemicals for quality control	5,500.00
	Sub-total	8,69,500.00
C. Fixed costs		
1	Depreciation	10,37,500.00
2	Interest	15,90,537.00
3	Maintenance (equipment, building, etc.)	50,000.00
	Sub-total	26,78,037.00
Total (A+B+C)		
(with NCDC culture)		2,33,55,412.00
(with DVS culture)		2,88,75,412.00

5.8.7 Net Manufacturing Cost

The total manufacturing cost was calculated to be Rs. 2,33,55,412.00 (with NCDC culture) and Rs. 2,88,75,412.00 (with DVS culture) per annum and the total production 2,98,500.00 litre per annum including 0.5% losses. (Table 5.8.13).

Table 5.8.13 Net manufacturing cost

Item	Particular
Total manufacturing costs	
(with NCDC culture)	2,33,55,412.00
(with DVS culture)	2,88,75,412.00
Total production (Litre)	2,98,500.00
Estimated cost of production of Aloe ice cream per litre	
(with NCDC culture)	Rs. 78.24
(with DVS culture)	Rs. 96.74

In conclusion, the cost of production of the ASPIC was estimated as **Rs. 78.24** (with NCDC culture) and **Rs. 96.74** (with DVS culture) per liter. Under commercial conditions at higher scale of handling, the cost of production is expected to be lower.

5.9 CONSUMER ACCEPTANCE STUDIES

Consumer acceptance studies are very important for ascertaining the acceptability and marketing prospects of a newly developed product. The ASPIC developed with both the probiotic cultures (NCDC and commercial DVS culture) in the study were presented to 150 respondents representing all income and age groups and including both males and females.

5.9.1 ASPIC with commercial DVS probiotic culture

For the ASPIC with DVS culture, the consumer panel consisted of 54.67% males and 45.33% females. The division of the respondents according to the gender and age group is represented in Table 5.9.1. The consumers' preference for conventional ice cream is presented in Table 5.9.2 and consumers' frequency of consumption of ice cream is shown in Table 5.9.3. In the survey, it was found that none of 150 respondents disliked eating ice cream. The respondents' liking for the ice cream ranged between 'Moderate' to 'Extreme' (Table 5.9.2). The majority of the consumers ate ice cream either 'weekly' or 'for-nightly', some ate it 'occasionally' and a few 'rarely' (Table 5.9.3).

Table 5.9.1 Division of respondents according to the gender and age groups

Age groups	No. of respondents		
	Male	Female	Total
< 20	9.00 (6.00)	10.00 (6.70)	19.00 (12.67)
20-29	27.00 (18.00)	51.00 (34.00)	78.00 (52.00)
30-39	9.00 (6.00)	1.00 (0.67)	10.00 (6.70)
40-49	20.00 (13.33)	4.00 (2.66)	24.00 (16.00)
50-59	14.00 (9.33)	1.00 (0.67)	15.00 (10.00)
>60	3.00 (2.00)	1.00 (0.67)	4.00 (2.67)
Total	82.00 (54.67)	68.00 (45.33)	150.00 (100.00)

*Figures in parentheses indicate percentage

Table 5.9.2 Consumers' preference for conventional ice cream

Preference	No. of respondents		
	Males	Females	Total
Like extremely	8.00 (5.33)	17.00 (11.33)	25.00 (16.67)
Like very much	48.00 (32.00)	38.00 (25.33)	86.00 (57.33)
Like moderately	26.00 (17.33)	13.00 (8.67)	39.00 (26.00)
Total	82.00 (54.67)	68.00 (45.33)	150.00 (100.00)

*Figures in parentheses indicate percentage

Table 5.9.3 Frequency of consumption of conventional ice cream

Frequency	No. of respondents		
	Males	Females	Total
Weekly	48.00	40.00	88.00
Fortnightly	20.00	11.00	31.00
Occasionally	12.00	17.00	29.00
Rarely	2.00	0.00	2.00
Never	-	-	-
Total	82.00	68.00	150.00

Table 5.9.4 shows the consumers' rating of ASPIC. All the 150 respondents (i.e. 100%) 'liked' the product. However, 46.67% of the respondents rated the product as 'excellent', 40.67% as 'very good', 10.67% as 'good', and 2.00% as 'fair' (Table 5.9.4 and Fig. 5.9.1). No adverse remarks or criticism was received. Expected frequency of consumption of ASPIC by consumers is represented in Table 5.9.5.

Table 5.9.4 Consumer rating of ASPIC

Rating	No. of respondents		
	Males	Females	Total
Excellent	42.00 (28.00)	28.00 (18.67)	70.00 (46.67)
Very good	33.00 (22.00)	28.00 (18.67)	61.00 (40.67)
Good	4.00 (2.67)	12.00 (8.00)	16.00 (10.67)
Fair	3.00 (2.00)	0.00 (0.00)	3.00 (2.00)
Total	82.00 (54.67)	68.00 (45.33)	150.00 (100.00)

*Figures in parentheses indicate percentage

In the present survey, 98.00 percent of the respondents were 'willing' to buy the product even if priced 25% higher than that of regular ice cream in the market (Table 5.9.6 and Fig. 5.9.2).

Table 5.9.5 Expected frequency of consumption of ASPIC by consumers

Frequency	No. of respondents		
	Males	Females	Total
Weekly	52.00	42.00	94.00
Fortnightly	17.00	14.00	31.00
Occasionally	8.00	10.00	18.00
Rarely	5.00	2.00	7.00
Never	-	-	--
Total	82.00	68.00	150.00

Table 5.9.6 Consumers' willingness to buy the ASPIC at 25% higher price in comparison to conventional ice cream

Whether willing to buy at higher cost	No. of respondents		
	Males	Females	Total
Yes	80.00 (53.33)	67.00 (44.67)	147.00 (98.00)
No	2.00 (1.33)	1.00 (0.67)	3.00 (2.00)
Total	82.00 (54.67)	68.00 (45.33)	150.00 (100)

*Figures in parentheses indicate percentage

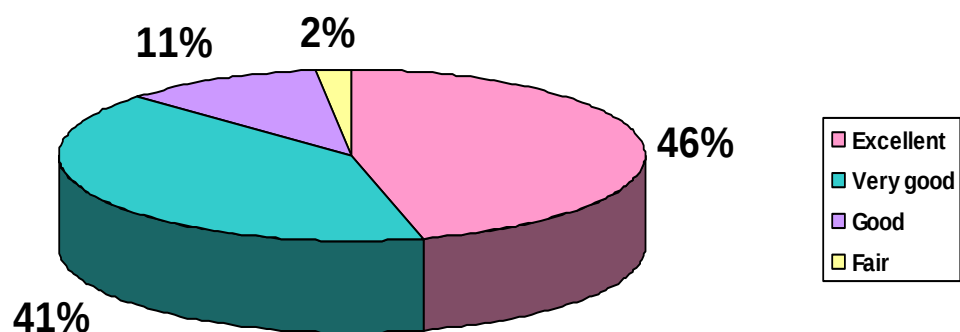


Fig. 5.9.1 Consumer rating of the ASPIC with commercial DVS culture

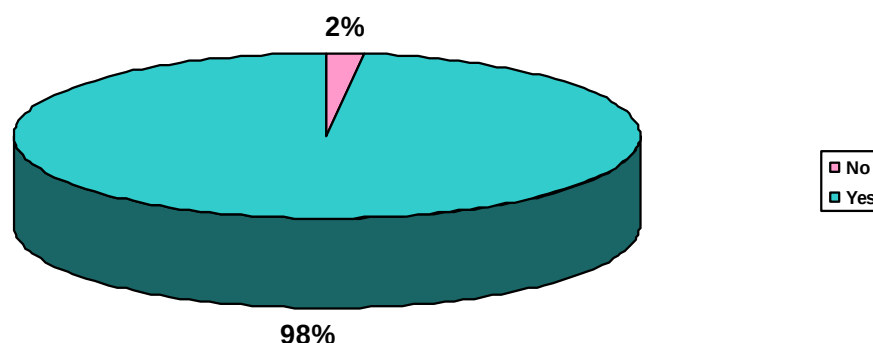


Fig. 5.9.2 Consumers' willingness to buy ASPIC with commercial DVS culture, even if priced 25% percent higher in comparison with conventional ice cream

5.9.2 ASPIC with NCDC probiotic culture

ASPIC with NCDC probiotic culture prepared using the process developed in the present study was presented to 150 respondents representing all income and age groups (Table 5.9.7) of potential consumers. This consumer panel consisted of 64.67% males and 35.33% females. The consumers' degree of liking and frequency of consumption of the conventional ice cream is presented in Table 5.9.8 and Table 5.9.9 respectively. None of the 150 respondents disliked eating ice cream.

Table 5.9.7 Division of respondents according to the gender and age groups

Age groups	No. of respondents		
	Male	Female	Total
< 20	19.00 (12.67)	11.00 (7.33)	30.00 (20.00)
20-29	35.00 (23.33)	30.00 (20.00)	65.00 (43.33)
30-39	7.00 (4.67)	2.00 (1.33)	9.00 (6.00)
40-49	17.00 (11.33)	8.00 (5.33)	25.00 (16.67)
50-59	16.00 (10.67)	2.00 (1.33)	18.00 (12.00)
>60	3.00 (2.00)	0.00 (0.00)	3.00 (2.00)
Total	97.00 (64.67)	53.00 (35.33)	150.00 (100.00)

*Figures in parentheses indicate percentage

Table 5.9.8 Consumer rating of conventional ice cream

Preference	No. of respondents		
	Males	Females	Total
Like extremely	34.00 (22.67)	17.00 (11.33)	51.00 (34.00)
Like very much	48.00 (32.00)	30.00 (20.00)	78.00 (52.00)
Like moderately	15.00 (10.00)	6.00 (4.00)	21.00 (14.00)
Total	97.00 (64.67)	53.00 (35.33)	150.00 (100.00)

*Figures in parentheses indicate percentage

Table 5.9.9 Frequency of consumption of conventional ice cream

Frequency	No. of respondents		
	Males	Females	Total
Weekly	57.00	34.00	91.00
Fortnightly	24.00	10.00	34.00
Occasionally	14.00	8.00	22.00
Rarely	1.00	0.00	1.00
Never	1.00	1.00	2.00
Total	97.00	53.00	150.00

As can be seen from Table 5.9.10, consumer's response to ASPIC with NCDC probiotic culture was extremely favorable. All the 150 respondents (i.e. 100%) 'liked' the product. Table 5.9.10 and Table 5.9.11 represents the consumers' degree of liking and expected frequency of consumption of the developed ice cream, respectively. Out of the 150 respondents, 97.33 percent were 'willing' to buy the product even if priced 25% higher than that of regular ice cream in the market (Table 5.9.12 and Fig. 5.9.4).

Table 5.9.10 Consumer rating of the ASPIC with NCDC probiotic culture

Rating	No. of respondents		
	Males	Females	Total
Excellent	45.00 (30.00)	23.00 (15.33)	68.00 (45.33)
Very good	40.00 (26.67)	27.00 (18.00)	67.00 (44.67)
Good	12.00 (8.00)	3.00 (2.00)	15.00 (10.00)
Total	97.00 (64.67)	53.00 (35.33)	150.00 (100)

*Figures in parentheses indicate percentage

Table 5.9.11 Consumers' expected frequency of consumption of ASPIC with NCDC probiotic culture

Frequency	No. of respondents		
	Males	Females	Total
Weekly	60.00	37.00	97.00
Fortnightly	27.00	11.00	38.00
Occasionally	9.00	5.00	14.00
Rarely	1.00	-	1.00
Never	-	-	-

Table 5.9.12 Consumers' willingness to buy the ice cream at 25% percent higher price in comparison with conventional ice cream

Whether willing to buy at higher cost	No. of respondents		
	Males	Females	Total
Yes	93.00 (62.00)	53.00 (35.33)	146.00 (97.33)
No	4.00 (2.66)	0.00 (0.00)	4.00 (2.67)
Total	97.00 (64.67)	53.00 (35.33)	150.00 (100.00)

*Figures in parentheses indicate percentage

So, it could be inferred that ASPIC with both the probiotic cultures (commercial DVS culture and NCDC culture) developed during the present study were acceptable enough to be launched at commercial scale. This observation is also substantiated by the fact that out of the 150 respondents, more than 97% respondents were willing to buy the product even if priced 25% higher. The consumers were appreciative of the fact that the product contained two neutraceuticals with potential health benefits.

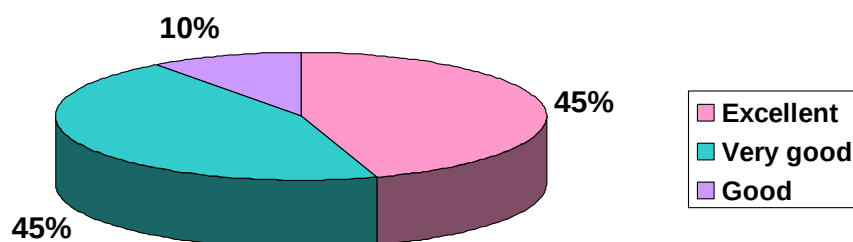


Fig. 5.9.3 Consumer rating of the ASPIC with NCDC culture

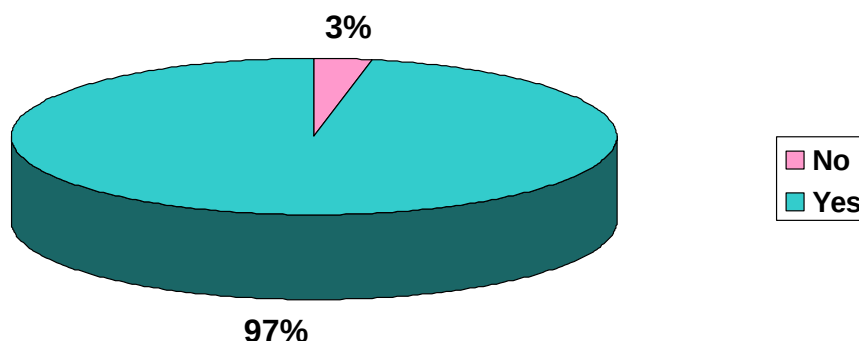


Fig. 5.9.4 Consumers' willingness to buy ASPIC with NCDC culture, even if priced 25% percent higher in comparison with conventional ice cream

5.10 COMPARISON OF SENSORY ATTRIBUTES OF ASPIC WITH MARKET SAMPLE

The sensory attributes of ASPIC prepared with both the probiotic cultures viz., NCDC-627 (AV- NCDC) and DVS culture (AV-DVS) were compared with the standard market samples by offering them to a panel of sensory judges. Out of the four market samples, two were popular brands having national presence viz., Kwality Walls (KW) and Amul (AM), whereas, the remaining two represented products popular in the local market of Karnal viz., NDRI ice cream (NIC) and Mohit ice cream (MIC).

As probiotic and *Aloe* ice creams are not available in the nearby market areas, normal vanilla flavored ice cream was considered for comparison of sensory attributes. Vanilla flavored market samples were chosen as ASPIC developed during the study were also flavored with vanilla.

Table 5.10.1 Comparison of sensory attributes of ASPIC with market sample

ATTRIBUTES	KW	AM	MIC	NIC	AV-DVS	AV-NCDC
Color & Appearance	7.85± 0.28	7.77± 0.24	7.21± 0.33	8.00* ± 0.00	7.41± 0.15	7.56± 0.18
Body & Texture	7.31± 0.41	7.53± 0.25	6.37± 0.32	7.77± 0.11	8.00*± 0.12	7.81± 0.13
Creaminess	7.39± 0.37	7.60± 0.32	5.96± 0.51	7.50± 0.26	7.75*± 0.25	7.69± 0.16
Sweetness	7.35± 0.34	7.67± 0.25	6.87± 0.20	7.33± 0.21	7.75± 0.17	7.94*± 0.06
Flavor	7.27± 0.31	7.78* ±0.19	5.83± 0.42	6.54± 0.58	7.33± 0.16	7.62± 0.18
Melting Quality	7.00± 0.46	7.13± 0.41	6.73± 0.46	7.42± 0.20	8.00*± 0.13	7.81± 0.13
Overall Acceptability	7.28± 0.32	7.46± 0.28	6.25± 0.32	6.90± 0.59	7.50± 0.13	7.66± 0.16

MEAN ± SE . * Indicates highest average score

Table 5.10.1 shows the comparison between sensory attributes of ASPIC and market samples. For color and appearance, highest score was obtained by NIC (8.00) followed by KW, AM, AV-NCDC, AV-DVS and MIC. Lower color scores for ASPIC can be attributed to the presence of *Aloe vera* juice, which was dull in appearance with a greenish tinge. Therefore, the color of ice cream with *Aloe vera* was comparatively intense and somewhat off white whereas, market samples of ice creams were bright white in color. However, the color of ice cream with *Aloe vera* when viewed individually was not objectionable and was quite acceptable.

The body and texture score was highest for ASPIC with DVS culture (8.00) followed by ASPIC with NCDC probiotic culture, NIC, AM, KW and MIC. The highest score of 7.75 was obtained by AV-DVS for creaminess followed by AV-NCDC, AM, NIC, KW and MIC. In case of sweetness, highest score was given to AV-NCDC followed by AV-DVS, AM, KW, NIC and MIC.

Amul is a well known ice cream brand and is quite popular across the country. AM received highest score for flavor followed by AV-NCDC, AV-DVS,

KW, NIC and MIC. For melting quality, highest score was obtained by AV-DVS followed by AV-NCDC, NIC, AM, KW and MIC. In case of overall acceptability, highest score was awarded to AV-NCDC followed by AV-DVS, AM, KW, NIC and MIC.

In the present study, both the ice cream with *Aloe vera* obtained good sensory scores and showed acceptable sensory characteristics. ASPIC with DVS culture or ASPIC with NCDC probiotic culture were rated better in terms of all sensory attributes except color & appearance and flavor. However, this fact should also be kept in mind that all the market sample were plain vanilla ice cream whereas both the developed ice cream were with two nutraceuticals viz., *Aloe vera* and probiotics. Out of the two nutraceuticals, *Aloe vera* juice possessed an off-taste and off-flavor. So, it can be concluded that frozen product like ice cream can serve as a carrier for both *Aloe vera* and probiotics without affecting the sensory attributes.

5.11 VALIDATION OF IMMUNOMODULATORY EFFECT OF THE DEVELOPED PRODUCT

Various bio-protective factors present in herbs like *Aloe vera* and probiotics provide protection against different pathogens (including bacteria, viruses, fungi etc.) and side effects of conventional chemotherapy by inducing para-immunity and non-specific immunomodulation. The notion of immunomodulation is related to the non-specific activation of the function and efficiency of lymphocytes, macrophages, natural killer cells, granulocytes and complement functions and also to the production of several effector molecules generated by activated cells (Vigila and Baskaran, 2008). These factors also play vital role in maintaining a favorable gut microflora that in turn suppresses the growth of microbes responsible for many infectious diseases. Ice creams with several functional ingredients are available in the market nowadays. However, the presently available ice creams in the country are devoid of such bioprotective factors especially a combination of *Aloe vera* and probiotics. Attempts were therefore made to develop a technology for the manufacture of *Aloe vera* supplemented

probiotic ice cream (ASPIC). In the present study, *in vivo* animal studies were conducted to investigate the immunomodulatory efficacy of the ASPIC using cyclophosphamide as an immunosuppressant.

5.11.1 Immunomodulatory Studies:

The immunomodulatory potential of ASPIC was validated through an *in vivo* trial conducted over a period of 14 days per group. The Swiss albino female mice were divided into two batches; each batch being further divided into four groups with 6 mice each. The mice groups were fed for 13 days and were sacrificed on the 14th day. In batch I, the intraperitoneal injection of immunosuppressant (cyclophosphamide (CP) @ 10 mg/kg body weight) was given for the first 3 days only whereas, in batch II, the intraperitoneal injection of immunosuppressant (cyclophosphamide @ 10 mg/kg body weight) was given daily for 13 days. Each batch was divided into the following four groups, according to the feed given for 13 days:

- Group-I (CD) : Control diet
- Group-II (CIC) : Control ice cream
- Group-III (DVS) : ASPIC with DVS probiotic culture
- Group- IV (NCDC) : ASPIC with NCDC probiotic culture

After 13 days of dietary regime, mice were sacrificed on the 14th day and peritoneal fluid, spleen, blood, intestine and organs (small intestine, liver and kidney) were collected. Peritoneal fluid was analyzed for macrophage count and % phagocytosis. Lymphocyte proliferation index was analyzed by splenocytes in spleen and immunoglobulin IgA was analyzed using small intestine. Blood samples were analyzed for complete blood count. Weight of the organs (liver, spleen, kidney, small intestine) was also checked for any significant difference. All the following parameters were analyzed for both the batches.

5.11.2 Macrophage count in peritoneal fluid

The immune response to microorganisms relies both on innate and acquired immunity. The non specific immune response is the first line of defense to protect the host against the foreign antigen. Immune responses are mediated by white blood cells (e.g. neutrophils and macrophages) and intestinal epithelial cells. Macrophages are versatile cells that play many roles. As scavengers, they rid the body of worn-out cells and other debris. As secretory cells, macrophages are vital to the regulation of immune responses and the development of inflammation.

Macrophages in peritoneal fluid were determined using the Neubauer ruling hemocytometer. The cell suspension was diluted with culture medium and cells were counted. The results pertaining to macrophage count are presented in Table 5.11.1 and Figures 5.11.1 and 5.11.2. In both the batches (I and II), with different doses of immunosuppressant i.e. cyclophosphamide, ASPIC having DVS culture (group III) showed significantly ($p < 0.01$) higher macrophage count than the ASPIC with NCDC probiotic culture (group IV).

The effect of dose levels of an immunosuppressant was highlighted by the comparison between the two batches which showed lower macrophage counts in all the groups of batch-II (cyclophosphamide injection given for 13 days) than batch-I (cyclophosphamide injection given for 3 days), implying that the dose of immunosuppressant for longer duration of 13 days in batch II led to fairly higher reduction in the macrophage count.

Table 5.11.1 Macrophage count* in peritoneal fluid

	Macrophage count			
	CD (I)	CIC (II)	DVS (III)	NCDC(IV)
Batch I	120.80 ± 5.51 ^c	170.80 ± 9.08 ^c	725.00 ± 27.84 ^a	236.30 ± 19.13 ^b
Batch II	106.30 ± 9.55 ^c	122.90 ± 5.51 ^{bc}	463.30 ± 15.90 ^a	150.00 ± 3.61 ^b

*Mean ± SE from 6 replicates

**Mean with same superscripts in a row (a,b,c,d) do not differ significantly ($p < 0.01$)

In a study to evaluate the effect of probiotic cultures on peritoneal macrophage count by Gill *et al.* (2000), the authors reported that the group of mice fed with diet supplemented with probiotic bacteria viz., *L. rhamnosus*, *L. acidophilus* or *B. lactis* showed significant increase in the count of peritoneal macrophages as compared to the control group.

Use of herbs for improving the resistance of body against common infections and pathogens has been a guiding principle of Ayurveda. *Aloe vera* is known for medicinal uses and has been investigated for different pharmacological properties in the past by several workers. A number of studies have indicated immunomodulating activities of the polysaccharides present in *Aloe vera* gel and suggested that this increase in immunomodulatory activities occur via activation of macrophage cells (Zhang and Tizard, 1996; Chow *et al.*, 2005; Im *et al.*, 2005). Our observations are in accordance with the results of the previous works on probiotics and *Aloe vera* individually. Thus, it can be inferred from the present study that a combination of *Aloe vera* and probiotics could enhance macrophage count which is an indicator of better immune response.

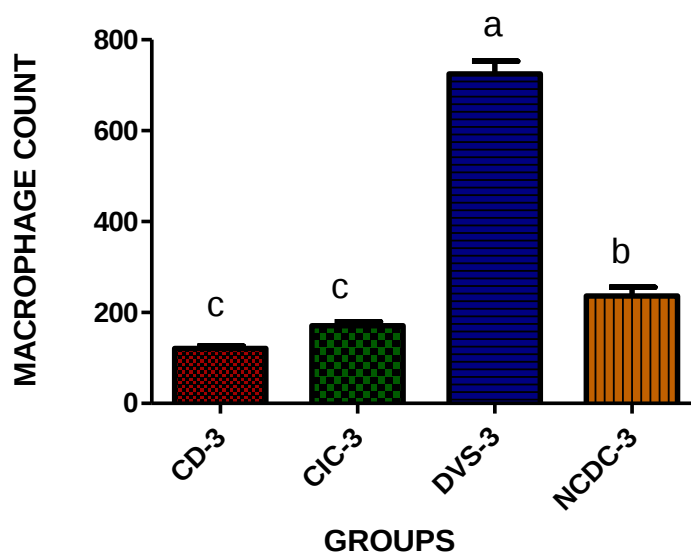


Fig. 5.11.1 Macrophage count of groups with CP dose for 3 days

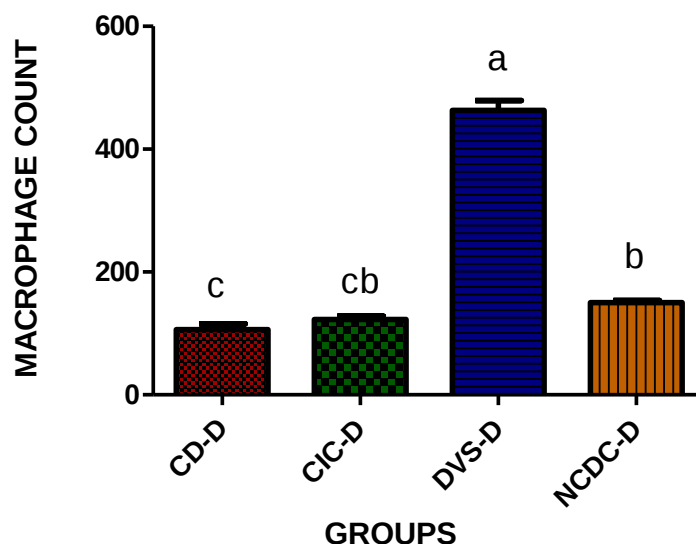


Fig. 5.11.2 Macrophage count of groups with daily CP dose

5.11.3 Lymphocyte count in spleen

Lymphocytes are a type of white blood cells in the vertebrate immune system and are the major cellular agents of adaptive immunity. Lymphocyte count in spleen was determined using the Neubauer ruling hemocytometer. The cell suspension was diluted with culture medium and cells were counted. The results for lymphocyte count are presented in Table 5.11.2 and Figures 5.11.3 and 5.11.4. It is apparent from Figure 5.11.3 (batch I) that the lymphocyte count of groups fed with ASPIC containing commercial DVS culture (group III), control ice cream (group II) and control diet (group I) were less than that of group in which mice were fed with ASPIC containing NCDC probiotic culture (group IV). However, in batch II, there was no significant difference in the lymphocyte counts of group III containing commercial DVS culture and group IV with NCDC probiotic culture (Figure 5.11.4) showing no significant difference due to different probiotic cultures. Whereas, the difference between probiotic cultures had a significant effect on lymphocyte count in batch I.

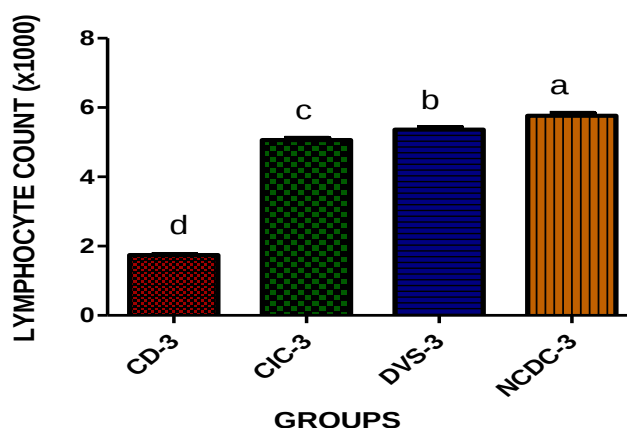
Table 5.11.2 Lymphocyte count* in spleen

	Lymphocyte count (x1000)			
	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
Batch I	1.74 ± 0.03 ^d	5.06 ± 0.06 ^c	5.36 ± 0.07 ^b	5.76 ± 0.07 ^a
Batch II	2.69 ± 0.06 ^c	4.42 ± 0.04 ^b	5.73 ± 0.04 ^a	5.85 ± 0.21 ^a

*Mean ± SE from 6 replicates

**Mean with same superscripts in a row (a,b,c,d) do not differ significantly (p<0.01)

Lactic acid bacteria (LAB) particularly the genus *Lactobacilli* has been correlated with a variety of health benefits including the enhancement of immune system. Gill *et al.*, (2000) and Kirjavainen *et al.*, (1999) reported that gram-positive commensal strains of probiotic *Lactobacilli* found in the human and mouse gastrointestinal tracts exert their beneficial health effects by several ways and one of the ways is through enhanced lymphocyte proliferation. A similar effect of *Lactobacilli* in enhancing lymphocyte count has been observed in the present study. According to Reynolds and Dweck (1999), in *Aloe vera* some components like acemannan are present that are associated with the stimulation of the antigenic response of human lymphocytes as well as the formation of all types of leucocytes from spleen and bone marrow as studied in irradiated mice.

**Fig. 5.11.3 Lymphocyte count (X1000) of groups with CP dose for 3 days**

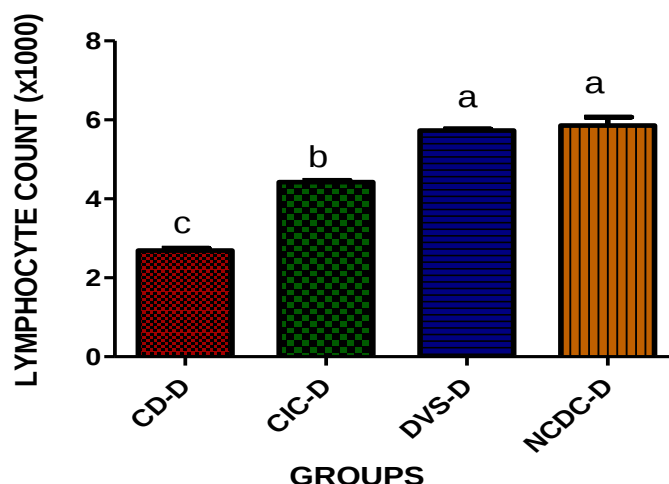


Fig. 5.11.4 Lymphocyte count (X1000) of groups with daily CP dose

5.11.4 %Phagocytosis of peritoneal macrophages

The activation of systemic immune response by feeding ASPIC was evaluated by measuring the phagocytic activity of peritoneal macrophages. One of the important functions of macrophages is to ingest and destroy inhaled particles by phagocytosis. The % phagocytosis (phagocytic activity) was expressed in terms of numbers of yeast cells ingested per 100 peritoneal macrophages.

In this study, an increase in percentage phagocytosis was observed for both the batches differing in immunosuppressant dose levels. This increased phagocytic activity could be attributed to increased production of phagocytosis cells as stimulated by the presence of probiotic and *Aloe vera* in the feed. It is apparent from Table 5.11.3 and Figures 5.11.5 and 5.11.6 that in both the batches (differing in dose levels of the immunosuppressant), the group fed with ASPIC having NCDC (group IV) showed significantly ($p < 0.01$) higher phagocytic activity followed by group III (mice fed with ASPIC having DVS probiotic culture). However, in both the batches (I and II), no significant difference in percentage

phagocytosis was observed in either the group fed with control ice cream (group II) or the group fed with control diet (group I) (Table 5.11.3).

Table 5.11.3 %Phagocytosis* of peritoneal macrophages

	% Phagocytosis			
	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
Batch I	13.15 ± 1.03 ^c	12.27 ± 0.57 ^c	18.65 ± 0.56 ^b	27.39 ± 0.98 ^a
Batch II	5.87 ± 0.62 ^c	6.09 ± 0.12 ^c	16.65 ± 0.69 ^b	28.33 ± 0.68 ^a

*Mean ± SE from 6 replicates

**Mean with same superscripts in a row (a,b,c,d) do not differ significantly (p<0.01)

Several workers in the past have reported species dependent variation in the efficacy of lactic acid bacteria in enhancing phagocytosis, as also observed in the present study. The structural differences in the cell wall composition of different bacterial strains are suggested to be responsible for the differences in the efficacy of different strains (Erickson and Hubbard, 2000). Perdigon and Alvarez (1992) and Schfirrin *et al.*, (1995) have concluded that the efficiency of stimulation of phagocytic cells depend on the ability of the strains to survive in the gastro intestinal tract, adherence to gut mucosa and their concentration above a critical level.

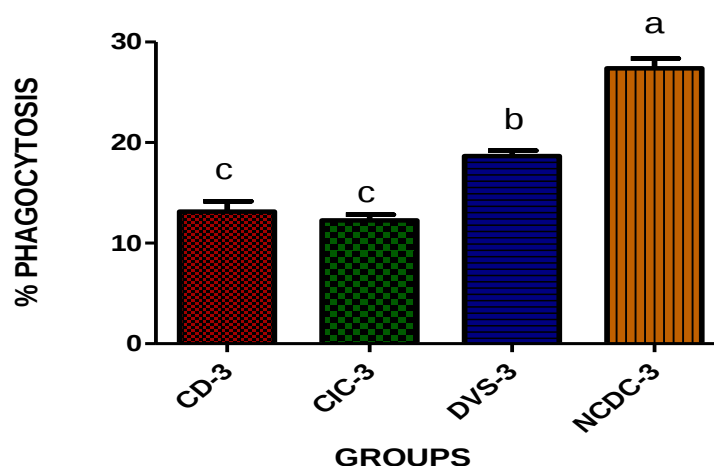


Fig. 5.11.5 % Phagocytosis of groups with CP dose for 3 days

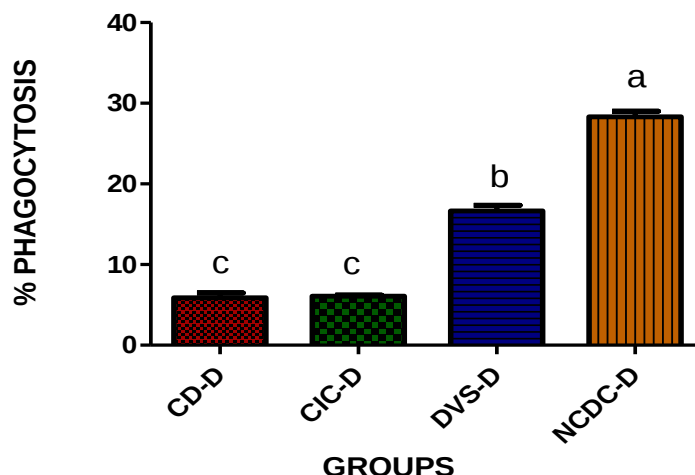


Fig. 5.11.6 % Phagocytosis of groups with daily CP dose

Increase in percentage phagocytosis by the administration of probiotics have been reported by several workers in the past. Schiffirin *et al.*, (1995) reported that *in vitro* phagocytosis of *E. coli* by human blood leucocytes was enhanced after administration with probiotics viz., *Lactobacillus acidophilus* LA1 and *Bifidobacterium bifidum* Bb 12. In a study by Gill *et al.*, (2000), supplementation with probiotics viz. *L. rhamnosus*, *L. acidophilus* or *B. lactis* resulted in a significant increase in the phagocytic activity of peritoneal macrophages as compared to the control mice group. Kapila, (2004) also demonstrated that the feeding of probiotic culture *Lactobacillus casei* 19 and milk fermented with *Lactobacillus casei* 19 activated the peritoneal macrophages by increasing phagocytic activity. Another worker, Meena *et al.* (2008) reported that lactic acid bacteria as well as immunoactive components released in milk had positive impact on phagocytic activity. Yadav (2012) and Salaria (2012) in independent studies also observed higher percentage of phagocytosis in mice fed with soy based probiotic yoghurt and probiotic infant food formulation, respectively.

Modulation of immune system, by several plant materials, for alleviation of diseases has been an interesting approach since ancient times. To evaluate the immunomodulatory property of *Aloe vera* gel, Madan *et al.* (2008) carried out an experiment in mice and the authors observed that *Aloe vera* gel consumption had stimulated phagocytic activity in mice.

Thus, our observations are in accordance with the previous works on probiotics and *Aloe vera* on stimulation of phagocytic cells. However, in the above discussed studies, the authors showed individual effect of probiotics and *Aloe vera* intake on phagocytic activity in animal models but our observations relate to the combined effect of both probiotics and *Aloe vera* on percentage phagocytosis.

5.11.5 Lymphocyte proliferation index

The lymphocyte proliferation in the animals was determined by 3-[4, 5-dimethyl thazol-2yl]-2, 5-diphenyl tetrazolium bromide/tetrazolium (MTT) assay. The colorimetric assay measures number and activity of living cells at the end of the assay. In the present study, the lymphocyte proliferative response with two different mitogens having different response to B lymphocytes (B cells) and T lymphocytes (T cells) viz., lipopolysaccharide (LPS) and Concanavalin A (Con A) was determined after 13 days of feeding trials, the results of which are presented in Tables 5.11.4 and 5.11.5. The proliferation index (PI) or stimulation index (SI) represents the ratio of absorbance with and without the addition of mitogen measured at 540nm.

Table 5.11.4 Lymphocyte proliferation index* using LPS as mitogen

	Lymphocyte Proliferation Index (Lipopolysaccharide as mitogen)			
	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
Batch I	0.86 ± 0.03 ^c	0.99 ± 0.00 ^b	1.08 ± 0.02 ^a	1.07 ± 0.04 ^{ba}
Batch II	0.94 ± 0.01 ^c	0.96 ± 0.00 ^c	1.23 ± 0.01 ^a	1.06 ± 0.01 ^b

*Mean ± SE from 6 replicates

**Mean with same superscripts in a row (a,b,c,d) do not differ significantly ($p < 0.01$)

The results pertaining to lymphocyte proliferation index with LPS as mitogen are depicted in Table 5.11.4 and Figures 5.11.7 and 5.11.8. It was observed that in both the batches (I and II), the mice groups fed with ASPIC having DVS culture (group III) showed significantly ($p < 0.01$) higher lymphocyte proliferation index than the other three groups. However, in batch I (immunosuppressant injection given only for 3 days) no significant difference in lymphocyte proliferation index was observed between different probiotic cultures i.e. there was no significant difference between group III and group IV whereas in batch II (immunosuppressant injection given for 13 days) the difference in lymphocyte proliferation index between groups fed with ASPIC having different probiotic cultures i.e., group III and group IV was significant ($p < 0.01$) (Table 5.11.4).

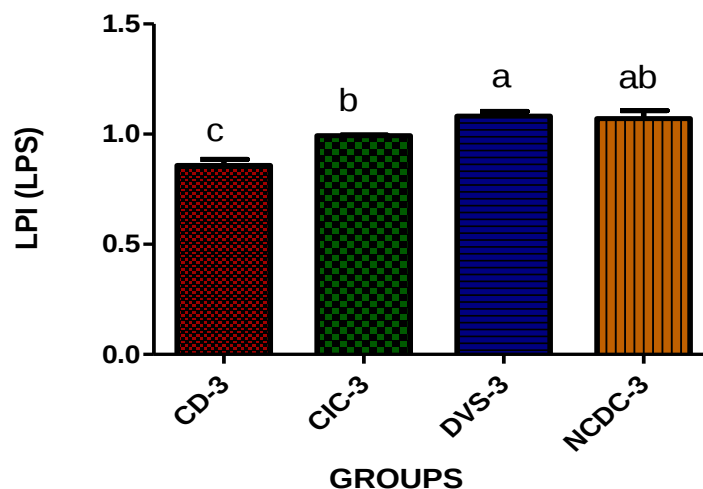


Fig. 5.11.7 Lymphocyte proliferation index with LPS as mitogen

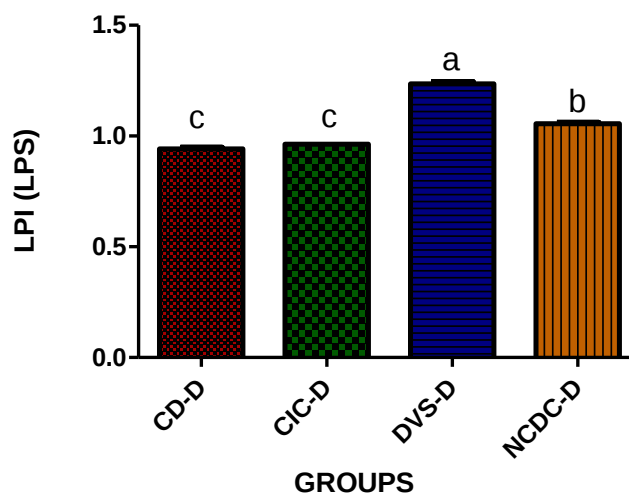


Fig. 5.11.8 Lymphocyte proliferation index with LPS as mitogen

The results pertaining to lymphocyte proliferation index with Con A as mitogen are expressed in Table 5.11.5. It is apparent from Figures 5.11.9 and 5.11.10 that for both the batches (I and II), group fed with ASPIC having NCDC probiotic culture showed higher lymphocyte proliferation index than the other three groups. Similar to lymphocyte proliferation with LPS as mitogen, in lymphocyte proliferation with Con A as mitogen, there was no significant difference in lymphocyte proliferation index of the different probiotic cultures i.e. there was no significant difference between group III and group IV whereas in batch II (immunosuppressant injection given for 13 days) the difference in lymphocyte proliferation index between groups fed with ASPIC having DVS culture and NCDC probiotic cultures i.e., group III and group IV was significant ($p < 0.01$)

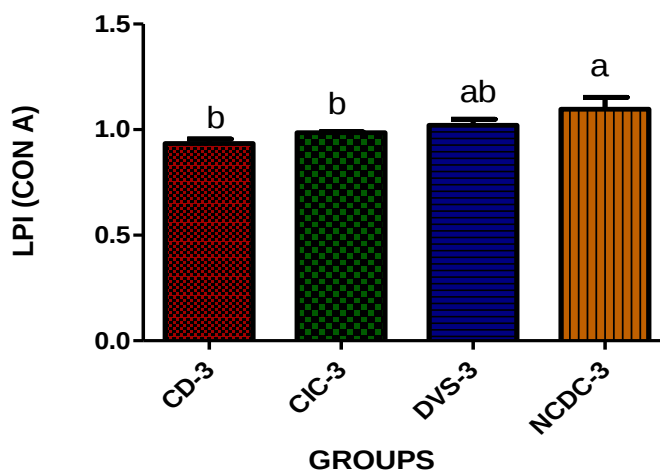
Table 5.11.5 Lymphocyte proliferation index* using Con A as mitogen

	Lymphocyte Proliferation Index with Concanavalin A as mitogen			
	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
Batch I	0.94 ± 0.02 ^b	0.98 ± 0.00 ^b	1.02 ± 0.03 ^{ab}	1.09 ± 0.06 ^a
Batch II	0.88 ± 0.00 ^c	0.94 ± 0.00 ^c	1.09 ± 0.04 ^b	1.22 ± 0.01 ^a

*Mean ± SE from 6 replicates

**Mean with same superscripts in a row (a,b,c) do not differ significantly (p<0.01)

The mitogens viz., LPS and Con A vary in their response to B lymphocytes (B cells) and T lymphocytes (T cells). Each B cell and T cell is specific for a particular antigen. The mitogen LPS shows the response of B-cells which are related to humoral immunity and the proliferative response of T-cells due to Con A indicates cell mediated autoimmunity. As the lymphocyte proliferation index with LPS as a mitogen was more for the mice group fed with ASPIC with DVS culture, it can be interpreted that the response of T cells as affected by DVS culture was less than that of the B cells. However, for group IV in which mice were fed with ASPIC having NCDC probiotic culture, lymphocyte proliferation index with Con A was more, showing that the response of T cells as affected by NCDC culture was higher than that of B cells.

**Fig. 5.11.9 Lymphocyte proliferation index with CON A as mitogen**

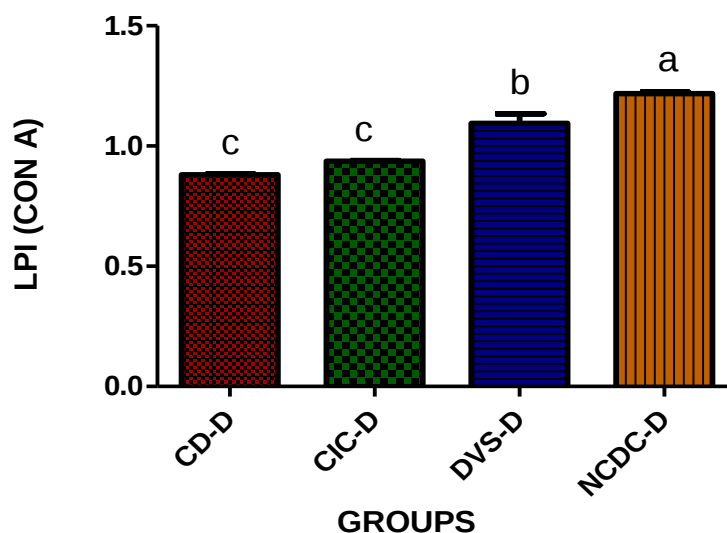


Fig. 5.11.10 Lymphocyte proliferation index with CON A as mitogen

In the past, studies have been conducted that indicate lymphocyte proliferation activity of probiotics in animal models. Shalini (2008) carried out a study and evaluated the efficiency of probiotics in enhancing lymphocyte proliferation by feeding mice with probiotic dahi for seven days. The author observed a significant ($p < 0.01$) increase in lymphocyte proliferation in mice group fed with probiotic than the control mice group. In another study, Paturi *et al.*, (2008) observed that the proliferative responses of splenocytes to concanavalin A (Con A) and lipopolysaccharide (LPS) were significantly higher in mice fed with diets incorporated with *L. acidophilus* LAFTI L10 after the 14-day feeding trial. Meena *et al.*, (2008) also reported that lactic acid bacteria had positive impact on lymphocyte proliferation. Salaria (2012) also observed enhanced lymphocyte proliferation in mice fed with probiotic infant food formulation than the infant food formulation without probiotics showing the effect of probiotics on lymphocyte proliferation.

Studies have also been conducted that indicate lymphocyte proliferation activity of *Aloe vera* in animal model. In *Aloe vera*, acemannan is an essential

component and its activity has been associated with stimulation of the antigenic response of human lymphocytes as well as the formation of all types of leucocytes from both spleen and bone marrow as studied in irradiated mice by Reynolds and Dweck (1999). Our results are in agreement with the above discussed studies.

5.11.6 Measurement of immunoglobulin (IgA) levels in Intestine

IgA is the predominant immunoglobulin of mucosal surface indicating mucosal immunity. It plays a significant role in the local immune system that deals with food antigens as well as harmful bacteria and viruses. Anti-inflammatory and immune-regulating activities are the important features of IgA. Considering beneficial effects of IgA, increase in the levels of this antibody after immunosuppression using cyclophosphamide and effect of different feed on the immunosuppressed mice in terms of change in IgA levels were determined by ELISA. The results of feeding different diets on the IgA level are shown in Table 5.11.6 and Fig. 5.11.11 and Fig. 5.11.12.

In batch I, IgA level in mice group fed with control diet and control ice cream were 279.2 µg and 334.7 µg respectively. It can be observed from Fig. 5.11.11 that there was a significant ($p < 0.01$) increase in the level of IgA in mice groups fed with ASPIC i.e., group III (455.2 µg) and group IV (437.3 µg) in comparison to control group. However, there was no significant difference in IgA levels among the ASPIC (group III) with DVS probiotic culture and ASPIC (group IV) with NCDC probiotic culture (Table 5.11.6).

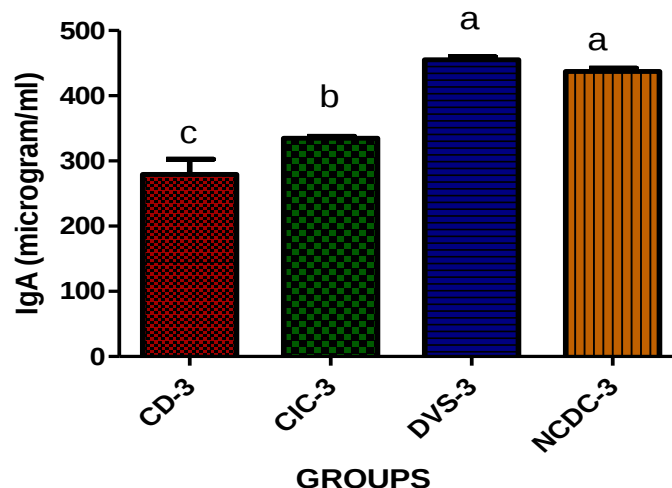


Fig. 5.11.11 IgA levels (microgram/ml) of groups with CP dose for 3 days

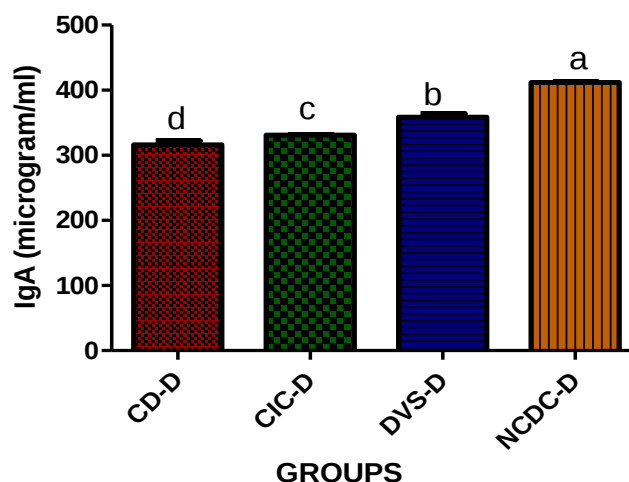


Fig. 5.11.12 IgA levels (microgram/ml) of groups with daily CP dose

The variations in the IgA concentrations of mice challenged with immunosuppressant for 13 days are mentioned in Table 5.11.6 and Figure 5.11.12. On studying the augmentation in IgA concentration, significant difference among all the four groups was observed. Group IV (ASPIC with NCDC probiotic culture) showed significantly ($p < 0.01$) higher IgA levels than the other three groups (Table 5.11.6).

Table 5.11.6 IgA levels(microgram/ml)* in mice intestine

	IgA (microgram/ml)			
	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
Batch I	279.20 ± 23.48 ^c	334.70 ± 3.12 ^b	455.20 ± 4.52 ^a	437.30 ± 4.82 ^a
Batch II	315.90 ± 6.50 ^d	331.10 ± 0.82 ^c	358.70 ± 5.16 ^b	411.80 ± 1.24 ^a

*Mean ± SE from 6 replicates

**Mean with same superscripts in a row (a,b,c,d) do not differ significantly (p<0.01)

Several workers in the past have studied food products with probiotics as a functional ingredient and reported that products with probiotic culture increase the IgA levels in mice irrespective of the type of food product and probiotic culture used in the study. Studies in the past have indicated definite role of bioactives in *Aloe vera* in immunomodulation. In one of the *in vivo* study by Kwon *et al.*, (2011) feeding of diet incorporated with *Aloe vera* peel extract resulted in elevated levels of IgG and IgA besides promotion of anti-inflammatory cytokines. In another relevant study, Malin *et al.* (1996) observed that yogurt with *Lactobacillus* GG or *Bifidobacterium lactis* increased IgA secreting cells in human model and the required dose of probiotic was 2x10¹⁰cfu/day. Shalini (2008) also observed that seven days feeding with probiotic dahi significantly increased secretory IgA antibodies in mice. Vandana (2011) reported an enhanced IgA levels in small animal group fed with probiotic whey drink. Gopalrao (2011) also studied the effect of *Lactobacillus reuteri* and observed an increased level of IgA in animal model. In recent studies, Yadav (2012) and Salaria (2012) reported a statistically significant increase in the level of IgA in soy based probiotic yoghurt and probiotic infant food formulation, respectively.

5.11.7 Blood parameters

Red blood cell (RBC) count, haemoglobin level, percentage neutrophils and platelet counts are some of the important haematological parameters which constitute the key components of the immune system. These blood components are known to recognize the foreign antigens and mounts an immune response. An increase or decrease in the concentration of these cells affect the health/immune constitution of the body (Jayathirtha and Mishra, 2004).

In the present study, immunomodulatory activity of the ASPIC was investigated by evaluating the effect of two nutraceuticals viz. *Aloe vera* and probiotics on various hematological parameters in swiss albino female mice. In batch I (cyclophosphamide injection given only for the initial three days) no significant ($p>0.01$) change in hematological parameters was observed for all the four groups (Table 5.11.7).

Table 5.11.7 Blood parameters* of batch with 3 day dose of CP

Blood parameters				
Parameters	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
% Neutrophils	64.00 $\pm$ 4.16 ^a	62.00 $\pm$ 0.58 ^a	74.00 $\pm$ 6.24 ^a	71.00 $\pm$ 3.51 ^a
RBC count (million/Cu mm)	5.40 $\pm$ 1.60 ^a	7.37 $\pm$ 0.89 ^a	7.97 $\pm$ 0.18 ^a	7.80 $\pm$ 0.18 ^a
Haemoglobin (gm %)	10.25 $\pm$ 0.02 ^a	10.97 $\pm$ 1.55 ^a	12.50 $\pm$ 0.23 ^a	11.75 $\pm$ 0.49 ^a
Platelet count (Lac/Cumm)	1.54 $\pm$ 0.61 ^a	3.08 $\pm$ 0.38 ^a	3.42 $\pm$ 0.70 ^a	3.26 $\pm$ 0.07 ^a

*Mean $\pm$ SE from 6 replicates

**Mean with same superscripts in a row (a) do not differ significantly ($p<0.01$)

In batch II, feeding ASPIC showed a significant ($p<0.01$) increase in % neutrophils, RBC count and haemoglobin level in mice groups. Table 5.11.8 and Figures 5.11.13 to 5.11.16 depict the results observed. The group IV i.e., the mice group fed with ASPIC having NCDC probiotic culture showed higher % neutrophils and RBC counts than the other three groups. However, there was no significant increase in the platelet counts among all the four groups.

Table 5.11.8 Blood parameters* of batch with daily dose of CP

Blood parameters				
Parameters	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
% Neutrophils	58.33 $\pm$ 1.45 ^b	57.33 $\pm$ 3.93 ^b	65.00 $\pm$ 1.53 ^b	74.67 $\pm$ 2.91 ^a
RBC count (million/Cu mm)	6.49 $\pm$ 0.10 ^b	6.51 $\pm$ 0.20 ^b	6.76 $\pm$ 0.33 ^b	7.85 $\pm$ 0.08 ^a
Haemoglobin (gm %)	9.80 $\pm$ 0.40 ^b	10.83 $\pm$ 0.73 ^{ba}	11.03 $\pm$ 0.59 ^{ba}	11.93 $\pm$ 0.09 ^a
Platelet count (Lac/Cumm)	2.45 $\pm$ 0.65 ^a	3.31 $\pm$ 0.04 ^a	3.53 $\pm$ 0.43 ^a	3.49 $\pm$ 0.20 ^a

*Mean $\pm$ SE from 6 replicates

**Mean with same superscripts in a row(a,b) do not differ significantly (p<0.01)

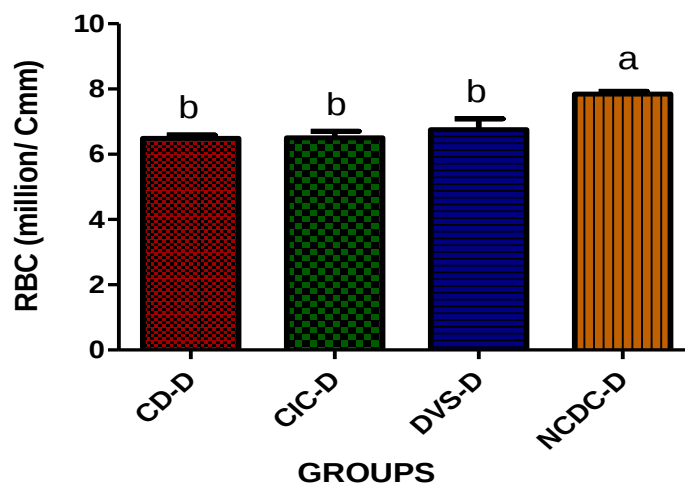


Fig. 5.11.13 RBC count (million/Cu mm) of groups with daily CP dose

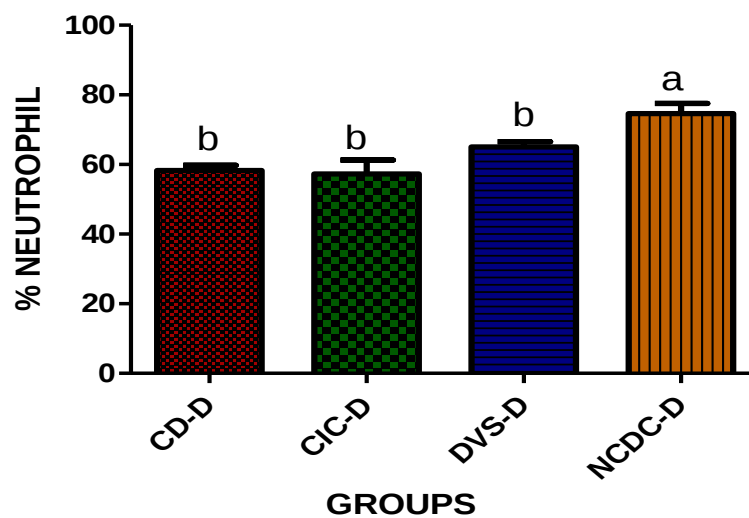


Fig. 5.11.14 % Neutrophil count of groups with daily CP dose

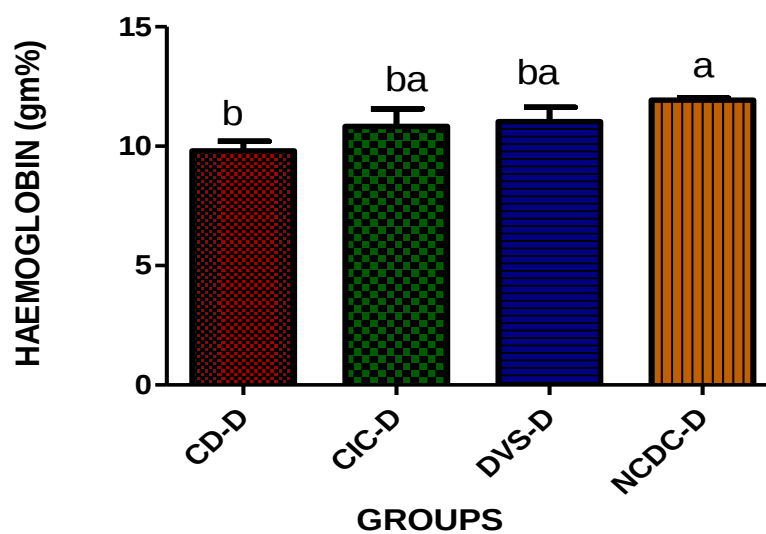


Fig. 5.11.15 Haemoglobin (gm %) of groups with daily CP dose

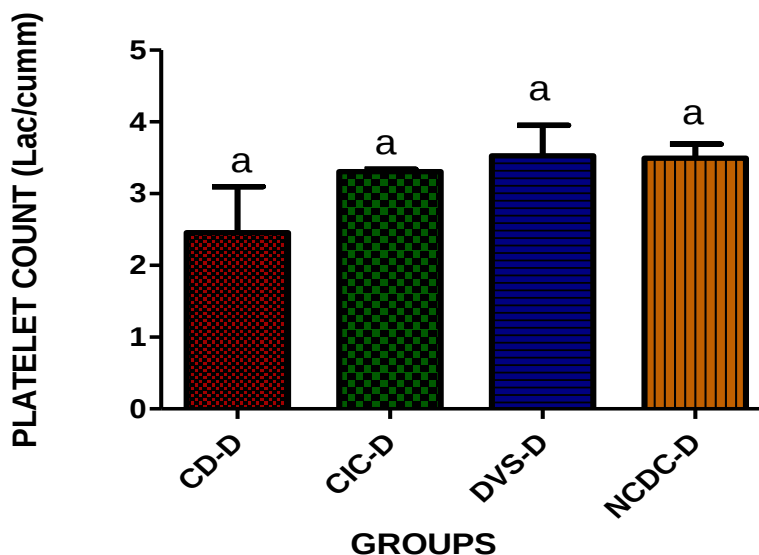


Fig. 5.11.16 Platelet count (Lac/cumm) of groups with daily CP dose

Previous studies using herb or selected plant material have shown a positive response on haematological parameters in animal model. Rahman *et al.*, 2011 and Gupta *et al.*, 2010 in separate studies to evaluate immunomodulatory activity of plant material investigated the activity of methanol extract of fruit of *Solanum xanthocarpum* (Solanaceae) and effect of *Moringa oleifera* Lam. (commonly known as drumstick) extract on cyclophosphamide induced toxicity in swiss albino mice, respectively. The authors concluded that the selected plant material possessed a definite positive response on immunomodulation. The results of the present study are in good agreement with the observations recorded in both the above mentioned studies.

5.11.8 Organ Weight

The changes in organs' weight are frequently connected with the effects of treatment and are widely accepted as criterion for evaluation of toxicities associated with the test article (Black 2002, Wooley 2003). In toxicological studies, the evaluation of organ weight plays a significant role in the assessment

of pharmaceuticals and chemicals (Sellers *et al.*, 2007). Although, organ weights indicate test article related effects, the detectable weight changes in organs may not necessarily be related to treatment or any adverse effect (Sellers *et al.*, 2007).

In the present study, difference in organs' weight among groups fed with different diets was observed. In batch I, no significant ($p>0.01$) difference in organs' weight was observed among all the four groups fed with different diets viz. control diet (group I), control ice cream (group II), ASPIC with DVS culture (group III) and ASPIC with NCDC culture (group IV) (Table 5.11.9).

Table 5.11.9 Organ's weight* of groups with 3 days dose of CP

Weight of organs (g)				
Organ	CD	CIC	DVS	NCDC
Kidney	0.41 ± 0.05^a	0.38 ± 0.04^a	0.43 ± 0.02^a	0.41 ± 0.06^a
Liver	1.43 ± 0.10^a	1.55 ± 0.12^a	1.45 ± 0.11^a	1.53 ± 0.11^a
Spleen	0.14 ± 0.05^a	0.15 ± 0.01^a	0.19 ± 0.09^a	0.15 ± 0.05^a
Small Intestine	1.26 ± 0.10^a	1.44 ± 0.17^a	1.16 ± 0.18^a	1.31 ± 0.02^a

*Mean $\pm$ SE from 6 replicates

**Mean with same superscripts in a row (a) do not differ significantly ($p<0.01$)

In batch II i.e., the batch in which cyclophosphamide injection was given for 13 days, significant increase in kidney weight was observed in group III and IV as compared to groups fed with control diet (group I) and control ice cream. (group II). However, there was no significant effect of the type of probiotic cultures on kidney weight as the difference between group III and group IV was not significant. The group fed with ASPIC with DVS culture showed significant increase ($p<0.01$) in liver weight than the other three groups. Whereas, no significant difference was observed in weight of spleen and small intestine among all the four groups.

Table 5.11.10 Organs' weight* of groups with daily dose of CP

Weight of organs (g)				
Organ	CD (I)	CIC (II)	DVS (III)	NCDC (IV)
Kidney	0.40 ± 0.01 ^b	0.42 ± 0.01 ^b	0.52 ± 0.03 ^a	0.53 ± 0.00 ^a
Liver	1.34 ± 0.02 ^b	1.43 ± 0.01 ^b	1.80 ± 0.01 ^a	1.56 ± 0.07 ^b
Spleen	0.10 ± 0.00 ^a	0.13 ± 0.01 ^a	0.14 ± 0.02 ^a	0.16 ± 0.02 ^a
Small Intestine	1.20 ± 0.01 ^b	1.22 ± 0.02 ^a	1.26 ± 0.08 ^a	1.22 ± 0.05 ^a

*Mean ± SE from 6 replicates

**Mean with same superscripts in a row (a,b) do not differ significantly (p<0.01)

Several studies are available on evaluation of effects of feeding fermented milks with probiotic cultures which indicate no significant effect on organs' weight like liver, kidney and spleen (Lee *et al.*, 2006; Takemura *et al.*, 2010 and An *et al.*, 2011). Rather S.A. (2012) carried out a study to evaluate the effect of Aloe vera and probiotic dahi on the progression of obesity and observed that the organs' weight (kidney, liver and spleen) didn't change significantly in experimental mice as compared to the control mice groups.

Various reports are also available on the evaluation of effects of plant material on the organs' weight. In a study by Gupta *et al.* (2010) to evaluate the immunomodulatory effect of *Moringa oleifera* Lam. extract on cyclophosphamide induced toxicity in mice. The authors observed a significant dose related increase in size and weight of thymus and spleen after the treatment in mice. The authors also observed that the administration of cyclophosphamide resulted in a significant reduction in weight of thymus and spleen. While in the animals treated with cyclophosphamide along with the plant extract, these parameters were found to be increased. However, no significant difference was observed in weight of liver and kidney after the treatment.

In the present study, the significant difference in liver and kidney weight in batch II could be attributed to the higher doses of cyclophosphamide.

Chapter 6

Summary and Conclusions

SUMMARY AND CONCLUSION

The present study entitled “Technology development for the manufacture of *Aloe vera* supplemented probiotic ice cream” was carried out and the results obtained during the current investigation are summarized as follows:

6.1 PRELIMINARY TRIALS

6.1.1 Out of the two forms of *Aloe vera* i.e., gel and juice, *Aloe vera* juice was selected @ 20-30% addition level for development of *Aloe vera* supplemented ice cream.

6.1.2 Sensory evaluation of mix with ten different flavors suggested vanilla flavor @ 0.15% addition for the preparation of *Aloe vera* supplemented ice cream.

6.1.3 Lymphocyte proliferation index showed non significant ($p > 0.05$) decrease in immunomodulatory activity of *Aloe vera* upon heating. However, to avoid any destruction of polysaccharides of *Aloe vera* juice at higher temperatures, heating treatment of 70°C/10 min was selected as a heat pretreatment of *Aloe vera* juice before addition into ice cream mix.

6.2 OPTIMIZATION OF INGREDIENTS FOR *Aloe vera* SUPPLEMENTED PROBIOTIC ICE CREAM

6.2.1 RSM experiment was framed incorporating three factors, namely, *Aloe vera* juice, fat and WPC. The three-factor combinations gave 20 experiments based on the Central Composite Rotatable Design (CCRD). After conducting RSM trials, following results were obtained.

6.2.2 The colour and appearance scores by sensory evaluation of the product were not significantly influenced by the ingredient mix.

6.2.3 Body & texture of ice cream generally decreased with increasing *Aloe vera* juice level. The interaction effect between *Aloe vera* juice and fat had a positive significant ($p < 0.05$) effect on the same. Milk fat had a significant

- effect ($p < 0.01$) at the quadratic level. Increasing the milk fat level in formulation improved the body & texture scores.
- 6.2.4 Addition of *Aloe vera* juice decreased the sweetness score. The linear term of the model for *Aloe vera* juice was negative ($p \leq 0.01$) indicating thereby that the sweetness of ice cream generally decreased with increasing *Aloe vera* juice level. The interaction effect between WPC and fat had a negative significant ($p < 0.05$) effect on the same. The quadratic term being negative ($p < 0.01$) with *Aloe vera* juice level displayed a convex-downward response.
- 6.2.5 Flavour of ice cream generally increased with increasing WPC level. The interaction effect between WPC and fat had a negative significant ($p < 0.01$) effect on the same. The quadratic term being negative ($p < 0.01$) with WPC level displayed a convex-downward response.
- 6.2.6 Creaminess score of ice cream generally increased with increasing fat level. The interaction effect between fat and *Aloe vera* juice had a positive significant effect ($p \leq 0.01$) and between fat and WPC, a net negative effect ($p \leq 0.01$) was observed on the same. The interaction effect between WPC and fat had a negative significant ($p < 0.01$) effect on the same. The quadratic term being positive ($p < 0.05$) with fat level displayed a convex-upward response.
- 6.2.7 The linear term of the model for *Aloe vera* juice level was negative ($p \leq 0.01$) indicating thereby that the overall acceptability of ice cream generally decreased with increasing *Aloe vera* juice level. The interaction effect between WPC and fat had a negative significant ($p < 0.05$) effect on the same. The quadratic term being negative ($p < 0.05$) with *Aloe vera* juice level and positive ($p < 0.01$) with fat level indicating that highest overall acceptability score was observed at intermediate levels of *Aloe vera*, while extreme levels of fat.
- 6.2.8 All three components (L^* , a^* and b^*) of the colour profile of the ice cream mix were not significantly influenced by the ingredients in the mix.

- 6.2.9 Acidity of ice cream mix generally decreased with increasing fat level, irrespective of level of other ingredients.
- 6.2.10 pH of ice cream mix generally decreased with increasing *Aloe vera* juice level. The interaction effect between *Aloe vera* juice and WPC had a negative significant ($p < 0.01$) effect on the same.
- 6.2.11 Specific gravity of the ice cream mix was not significantly influenced by the ingredients in mix.
- 6.2.12 Viscosity of ice cream mix generally decreased with increasing *Aloe vera* juice level and increasing fat level.
- 6.2.13 WPC was found to affect the overrun of the product at all the levels significantly.
- 6.2.14 Percentage melt/h generally decreased with increasing *Aloe vera* juice and fat level.
- 6.2.15 With the increasing level of fat, firmness increased, but decreasing *Aloe vera* juice and WPC levels, resulted in increase in firmness. The interaction of fat with *Aloe vera* juice ($p \leq 0.01$) or WPC ($p \leq 0.05$) was found to increase firmness of the product significantly. While interaction between *Aloe vera* and WPC had a net negative effect ($p \leq 0.01$) on the firmness of product.
- 6.2.16 The solution obtained with highest desirability of 0.84 (AV-20%, WPC-25% and Fat- 8%) was selected as the optimum one and was verified by using the suggested levels of *Aloe vera* juice, WPC and fat in the ice cream mix for the preparation of an *Aloe vera* supplemented ice cream and comparing the predicted values with observed values for the physico-chemical properties and sensory characteristics of the resulting ice cream.

6.3 TESTING OF *IN VITRO* PROBIOTIC POTENTIAL

6.3.1 Probiotic attributes of four probiotic LAB strains viz., NCDC-624 (*L.plantarum*), NCDC-625 (*L.plantarum*), NCDC-626 (*L.rhamnosus*) and NCDC-627 (*L.paracasei ssp. paracasei*) were studied to reconfirm their probiotic attributes.

6.3.2 All four probiotic strains showed a good survival in acidic condition. Though the strains were able to tolerate the acidity of gastric environment, they were unable to show any growth under the acidic stress.

6.3.3 All four probiotic strains showed more than 2 log reduction after 3h at 2% bile concentration. Among all the strains, NCDC- 627 was found to be more bile tolerant.

6.3.4 NCDC- 627 has relatively more affinity for all three hydrocarbons than rest. The percent cell surface hydrophobicity values of NCDC- 627 observed for three hydrocarbons i.e, n- hexadecane, n-octane and xylene were 52%, 62% and 65%, respectively.

6.3.5 Out of the four NCDC strains studied for antibiotic susceptibility NCDC- 627 was either sensitive or moderately sensitive to all the antibiotics.

6.3.6 *Lactobacillus* species were found active against the tested indicator strains. The zones of inhibition of indicator organism tested were ranging from 7.2 to 10.4 mm in diameter.

6.4 SELECTION OF PROBIOTIC CULTURE

6.4.1 Evaluation of low temperature tolerance showed that out of the four cultures, highest log₁₀ cfu/g count was obtained by NCDC-626, followed by NCDC-627, NCDC-624 and NCDC-625. However, there was statistically no significant difference in the counts of NCDC-626 and NCDC-627.

6.4.2 Out of the four probiotic cultures NCDC – 627 was selected, as it possessed better acid tolerance, better antimicrobial activity, better

antibiotic susceptibility, highest %CSH and better low temperature tolerance.

6.5 VALIDATION OF IMMUNOMODULATORY PROPERTIES

6.5.1 To validate immunomodulatory properties of the developed products animal trials were carried out. Mice were divided into two batches, each with four groups. One batch was given peritoneal injection (10mg/kg body weight) of cyclophosphamide (CP) for first 3 days and the other batch was given peritoneal injection of CP daily for 13 days. Each batch was then further divided into four groups, according to the feed Group-1 (CD): control diet, Group-2 (CIC): control ice cream, Group-3 (DVS): *Aloe vera* supplemented probiotic ice cream with DVS probiotic, and Group- IV (NCDC): *Aloe vera* supplemented probiotic ice cream with NCDC probiotic culture.

6.5.1.1 Macrophage count:

For batch 1, the macrophage count for group I was 120.8 ± 5.51 , for II was 170.8 ± 9.08 , for group III was 725 ± 27.84 and 236.3 ± 19.13 for IV. Statistical analysis showed significantly higher ($p < 0.01$) count in group III and group IV other two groups. Significant ($p < 0.01$) difference between two cultures was also observed.

For batch-2, the macrophage count for CD, CIC, DVS and NCDC were 106.3 ± 9.55 , 122.9 ± 5.51 , 463.3 ± 15.90 and 150 ± 3.61 respectively. The difference of which varied significantly ($p < 0.01$). Group IV showed higher counts than group I, II and III, however no significant difference in between group II and III.

6.5.1.2 Lymphocyte count:

For batch-1, the count ($\times 1000$) for I was 1.74 ± 0.03 , for II was 5.06 ± 0.06 , for group III was 5.36 ± 0.07 and 5.76 ± 0.07 for group IV. Significantly higher ($p < 0.01$) count in group IV than all other groups was observed. The count in all the groups varied significantly ($p < 0.01$).

For batch-2, the count (x1000) for CD, CIC, DVS and NCDC were 2.69 ± 0.06 , 4.42 ± 0.04 , 5.73 ± 0.04 and 5.85 ± 0.21 respectively. There was a non significant difference in counts of group III and IV. In both the batches, group IV showed higher counts than group I, II and III.

6.5.1.3 % Phagocytosis:

For batch-1, the % phagocytosis by group IV was significantly higher than group III ($p < 0.01$) and group II and I ($p < 0.01$). % Phagocytosis for group III was also significantly higher ($p < 0.01$) than II and I. A non significant decrease was observed in group II from group I.

For batch-2, % phagocytosis was highest for group IV followed by group III, II and I. Statistically significant difference ($p < 0.01$) was observed among all the groups except group II and group I.

6.5.1.4 Lymphocyte proliferation index (LPI):

In case of mitogen LPS, for batch-1, the LPI after 13 days was 0.86 for group I, 0.99 for group II, 1.08 for group III and 1.07 for group IV. There was a significant ($p < 0.01$) increase in LPI of group IV as compared to group I and II. For batch-2, highest LPI was observed for group III. There was a significant increase ($p < 0.01$) in LPI value in group III and IV and also from control and control ice cream group.

For the mitogen Con A, in batch-1, the LPI after 13 days was 0.94 for group I, 0.98 for group II, 1.02 for group III and 1.09 for group IV. There was no significant difference among group II & III and group III & IV. For batch-2, highest LPI was observed for group IV. Significant increase ($p < 0.01$) in LPI value in group IV as compared to I, II and III. Group IV was also significantly ($p < 0.05$) higher than group III.

6.5.1.5 IgA content:

For batch-1, the results indicated increase in the level of IgA in group III (455.2 μg) and group IV (437.3 μg) than that of control ice cream group (334.7 μg) and control group (279.2 μg). This increase was found to be

statistically significant ($p < 0.01$). However, there was no significant difference among the cultures i.e. group III and IV. For batch-2, level of IgA in group IV (411.8 μg) and group III (358.7 μg) was higher than that of control ice cream group (331.1 μg) and control group (315.9 μg).

6.5.1.6 Blood parameters:

For batch-1, the group with 3 day cyclophosphamide injection, all the parameters showed non significant difference in counts.

For batch-2, significant increase in % neutrophils, RBC and haemoglobin level was observed. No significant ($p > 0.01$) difference in platelet count was observed.

6.5.1.7 Organ Weight:

In batch-1, the difference in weight for all the organs was non significant.

In batch-2, there was a non significant increase in weight of spleen and small intestine. However, there was an increase in weight of liver of group fed with ASPIC containing DVS culture.

6.6 GROSS COMPOSITION OF ASPIC

6.6.1 The final formulation contained 8.0 percent fat, which is more than 2.5 percent but less than 10.0 percent, and milk protein 4.9 percent, which is more than prescribed minimum protein content of 3.5 percent, as suggested by FSSAI regulations for medium fat ice cream.

6.6.2 The energy value of *Aloe vera* supplemented probiotic ice cream was calculated by taking the energy value for fat, protein and carbohydrate as 9.0, 4.0 and 4.0 kcal/g respectively. The energy value computed to be 193.2 kcal per 100ml *Aloe vera* supplemented probiotic ice cream.

6.7 STORAGE STUDY OF ASPIC

6.7.1 During storage over the period of 3 months, no significant ($p > 0.05$) change in scores of sensory attributes was observed.

6.7.2 Statistical analysis indicated significant ($p < 0.05$) differences among the *Lactobacilli* counts made every 15 days during 3 month. Reduction of 0.88 $\log_{10}$ cfu/g during 3 month storage was observed. The survival rate was 89.29%, equivalent to a count of $7.34 \pm 0.04 \log_{10}$ cfu/g of ice cream.

6.7.3 A significant ($p < 0.05$) reduction in SPC count from 20.67×10^4 ($\log_{10}$ cfu/g : 5.30 ± 0.12) to 6.66×10^3 ($\log_{10}$ cfu/g : 3.81 ± 0.11) at the end of 3 months of storage was observed.

6.7.4 Significant ($p < 0.05$) reduction in yeast and mold count from 28.33 ± 7.64 to 1.67 ± 1.15 at the end of 3 months of storage was observed.

6.7.5 Coliforms were absent in 1 g sample throughout the storage period.

6.8 COMPARISON OF ASPIC WITH MARKET SAMPLES

6.8.1 A comparison of sensory attributes of *Aloe vera* supplemented ice cream developed with both the probiotic cultures viz., *Aloe vera* supplemented ice cream with NCDC (AV- NCDC) and *Aloe vera* supplemented ice cream with DVS culture (AV-DVS), with market samples showed acceptable sensory characteristics. Except, color & appearance and flavor either AV-NCDC or AV-DVS obtained highest mean sensory score.

6.9 CONSUMER RESPONSE STUDY OF ASPIC

6.9.1 *Aloe vera* supplemented probiotic ice creams developed through the present study was acceptable enough to be launched at commercial scale. In separate studies for both the ice creams, out of 150 respondents, 97.33 percent were 'willing' to buy the ASPIC (with NCDC-627 culture), and 98.00 percent for ASPIC (with DVS culture) even if priced 25% higher than that of regular ice cream in the market.

6.10 COST OF PRODUCTION OF ASPIC

6.10.1 The cost of production of the *Aloe vera* supplemented probiotic ice cream was estimated as Rs. 78.24 (with NCDC culture) and Rs. 96.74 (with DVS culture) per ltr. Under commercial conditions at higher scale of handling, the cost of production is expected to be lower.

6.11 CONCLUSIONS

6.11.1 Ice cream enriched through the addition of *Aloe vera* and probiotics constitutes a new product with notable immunomodulatory potential, even greater than the potential presented by the standard ice cream, a delicacy appreciated worldwide

6.11.2 *Aloe vera* juice could be supplemented at 20% into ice cream without hindering the consumer acceptance of the product.

6.11.3 Consumption of ASPIC could potentially deliver health benefits by the supply of natural immunomodulatory agents.

6.11.4 The incorporation of two nutraceuticals viz., *Aloe vera* and probiotics in the ice cream formulation promoted an increase in macrophage count, lymphocyte count, percentage phagocytosis, lymphocyte proliferation and IgA levels in mice.

6.11.5 *Aloe vera* supplemented probiotic ice cream with acceptable consumer quality and better immunoprotective effect could be manufactured at reasonable cost.

6.12 FUTURE RECOMMENDATIONS

6.12.1 A concentrated form of *Aloe vera* need to be used in place of *Aloe vera* juice to minimize its supplementation effects on sensory and physico-chemical parameters of ASPIC.

6.12.2 Chromatographic techniques may be used to know the bioactive molecules of *Aloe vera*.

6.12.3 Application of different preservation methodologies need to be conducted to improve the shelf-life of ASPIC

6.12.4 More immunoprotective parameters and long feeding procedures may be evaluated to get a clear picture about the immunomodulatory effects of *Aloe vera* and probiotic combination

6.12.5 All the above mentioned results show that ice cream can be utilized successfully as a carrier for combination of two novel nutraceuticals without affecting the sensory attributes, with a great consumer acceptance as shown by consumer acceptance survey. The developed products are validated to possess immunomodulatory properties in mice model. However, further clinical trials are required to establish the fact in humans.

Chapter 7

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Chapter 8

Appendices

Annexure I

i) PUM buffer

Contents	Quantity (g)
K ₂ HPO ₄	10
KH ₂ PO ₄	10
Urea	10
MgSO ₄ 7H ₂ O	0.05
All the ingredients were dissolved in a part of distilled water first and finally the volume was made up to 1000 ml with the same. Final pH was adjusted to 7.1.	

ii) PBS saline

Contents	Quantity (g)
NaCl	8
KCl	0.2
Na ₂ HPO ₄	1.15
KH ₂ PO ₄	0.2
All the ingredients were dissolved in a part of distilled water first and finally the volume was made up to 1000 ml with the same. Final pH was adjusted to 7.0.	

iii) Glycerol stock solution:

Glycerol stock solution was prepared by combining equal parts of glycerol and distilled water followed by autoclaving the mixture at 121 °C for 15 min. Glycerol stock added in equal amount to the culture in each cryovial, at the time of preservation.

ANNEXURE II

Division of Dairy Technology
NATIONAL DAIRY RESEARCH INSTITUTE
Karnal (Haryana)

Score Card for Sensory Evaluation of *Aloe vera* Ice cream

Product particulars: _____

Date: _____

Kindly evaluate the given samples of Ice cream for colour & appearance, body & texture, creaminess, sweetness, flavor, melting quality and overall acceptability using the following 9-point hedonic scale and enter the score for each sample in the space provided in the below table.

<u>Hedonic ratings</u>	<u>Score</u>
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				
Colour & Appearance					
Body & Texture					
Creaminess					
Sweetness					
Flavour					
Melting Quality					
Overall Acceptability					

Remarks (if any) : _____

Signature : _____

Name : _____

ANNEXURE III

CONSUMER SURVEY OF *ALOE VERA* SUPPLEMENTED PROBIOTIC ICE CREAM

Dear Consumer,

We have developed “*Aloe vera* Supplemented Probiotic Ice cream” prepared by incorporating *Aloe vera* juice, WPC and Probiotics. Kindly provide your honest opinion about the product.

Note: Please tick mark [✓] your preference or write in few words at appropriate places.

- 1. Name of consumer:**

- 2. Age:** < 20 [] 20-29 [] 30-39 [] 40-49 [] 50-59 []
 >60 []

- 3. Gender:** Male ☐ Female ☐

- #### 4. Occupation:

5. Do you like Ice cream? Yes ☐ No ☐

If yes, how much? **Moderately** ☐ **Very much** ☐ **Extremely** ☐

- 6. How often you take Ice cream?**

Weekly ☐ Fortnightly ☐ Occasionally ☐
Rarely ☐ Never ☐

7. Did you like this Ice cream? Yes ☐ No ☐

- 8. If yes, degree of liking?**

Excellent	☐	Very Good	☐
Good	☐	Fair	☐

- 9. Are you ready to buy this product knowing that it is beneficial for health and cost of the product is higher (say, 25% higher) than that of regular ice cream in the market?**

Yes ☐ No ☐

- 10. If yes, then possible frequency of consuming the product:**

Weekly ☐ **Fortnightly** ☐ **Occasionally** ☐
Rarely ☐ **Never** ☐

Date:

Place:

(Signature)

PHOTOGRAPHS RELATED TO MY PROJECT WORK



1. Experimental animals in small animal house during the study



2. Housing of small animals in cages



3. Powdered chalk diet



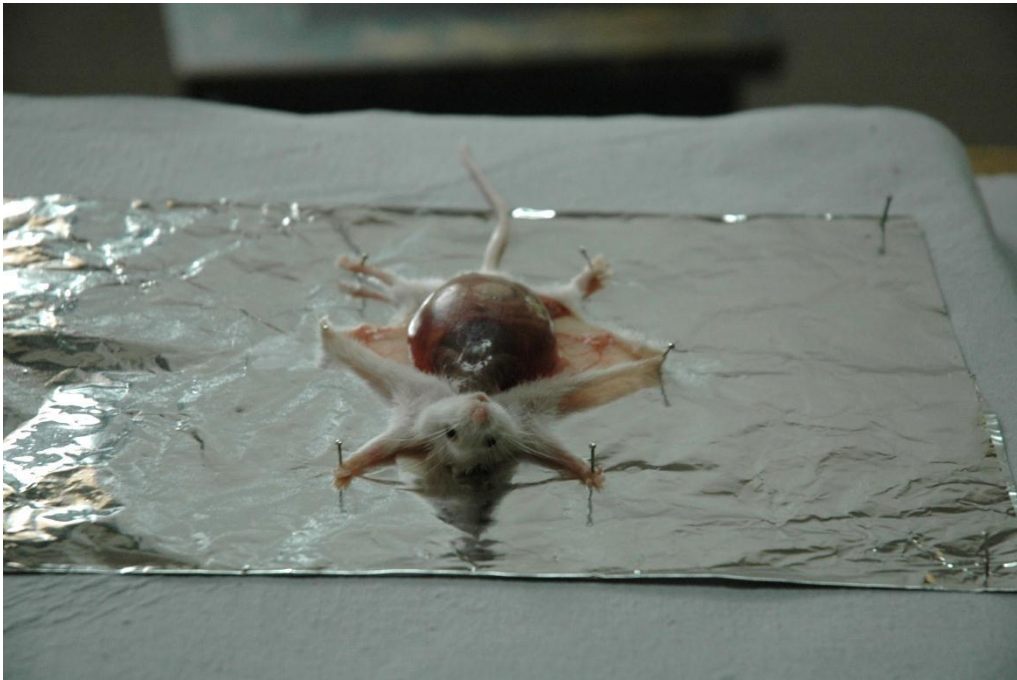
4. Chalk diet mixed with melted ice cream



5. Intraperitoneal cyclophosphamide injection to small animals



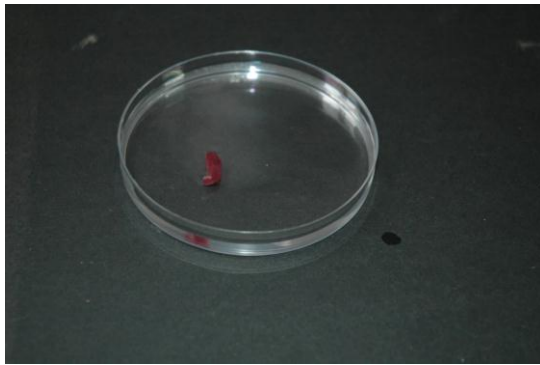
6. Dissection of small animals



7. Collection of peritoneal fluid



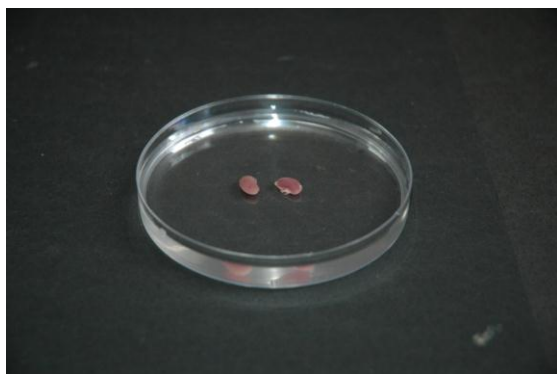
8. Collection of blood from the heart of animal for blood analysis



a.) Spleen



b.) Small Intestine

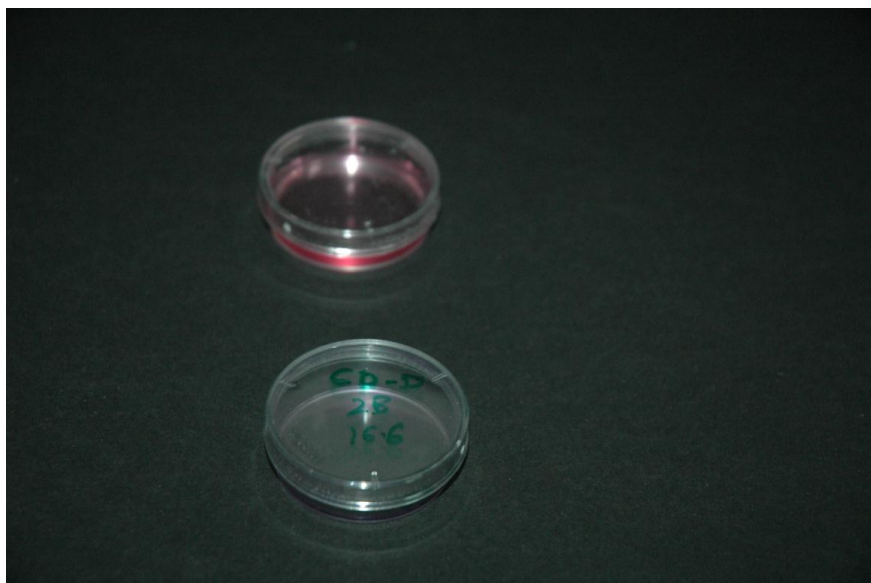


c.) Kidneys

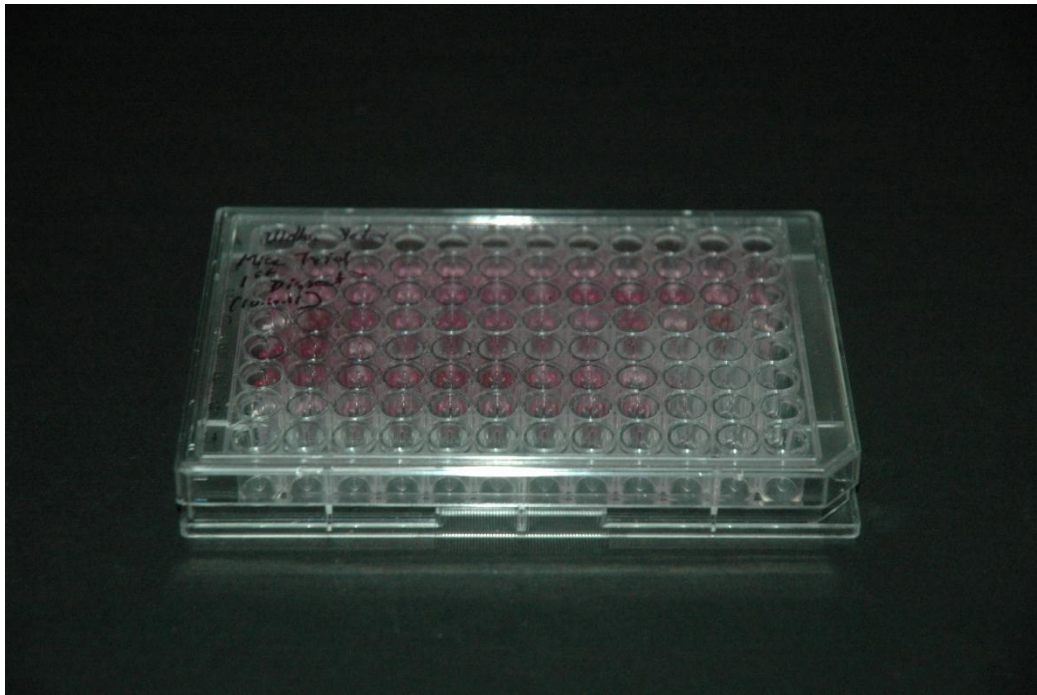


d.) Liver

9. Organs of small animal for weight measurement



10. Culture plate to analyse % phagocytosis



11. Tissue culture plate to analyse Lymphocyte Proliferation Index



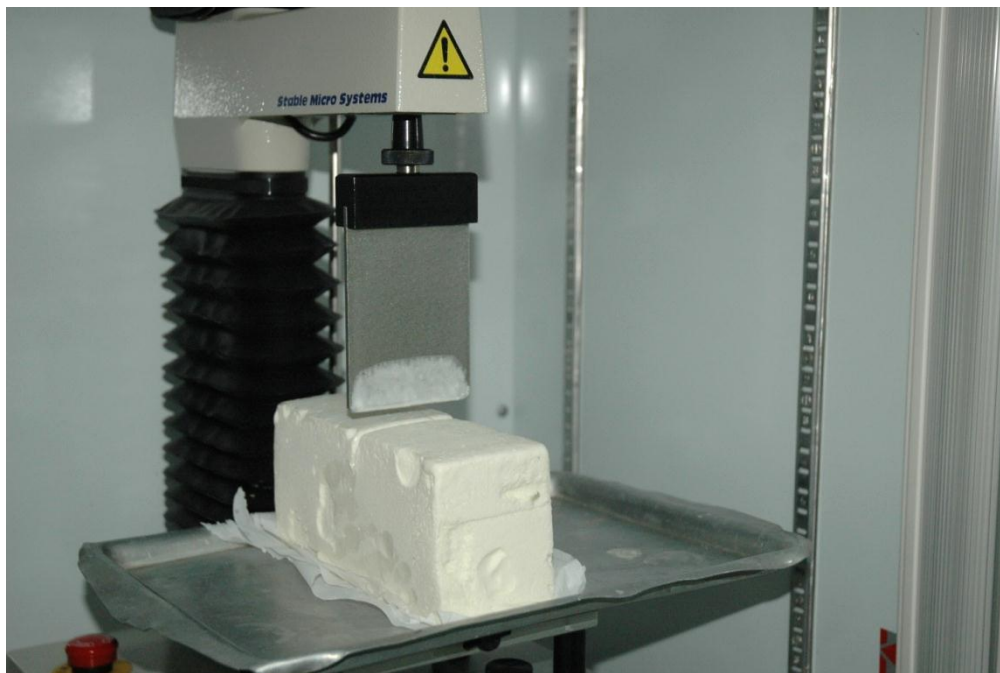
12. Viscometer for viscosity measurement



13. Aloe vera supplemented probiotic ice cream



14 (a). Texture analysis of ice cream with warner blade



14 (b). Texture analysis of ice cream with warner blade



15. Hunter color lab for instrumental color analysis