PREPARATION OF PROTEIN-RICH FERMENTED WHEY BEVERAGE USING COLOSTRUM WHEY

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

DAIRY MICROBIOLOGY

BY

PRASHANTH S

B. Tech. (Dairy Technology)

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ICAR-NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

Karnal – 132 001 (Haryana), India

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Approved by

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CERTIFICATE

This is to certify that the thesis entitled "**PREPARATION OF PROTEIN-RICH FERMENTED WHEY BEVERAGE USING COLOSTRUM WHEY"** submitted by **MR. PRASHANTH S** in partial fulfilment of the requirement for award of the degree of **MASTER OF TECHNOLOGY** in **DAIRY MICROBIOLOGY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, **KARNAL (HARYANA)** is a bonafide research work carried out by her under my supervision and guidance. The work embodied in this thesis is original and no part has been submitted in part or full for the award of any diploma or degree of this or any other university.

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Affectionately Dedicated

To My

Beloved Parents,
Nephew Ashu

&

Respected Guide

ACKNOWLEDGEMENT

"Gratitude is the memory of the heart"

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Karnal

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(Prashanth. S.)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

NCDC	National Collection of Dairy Culture
CFU	Colony Forming Unit
CAGR	Compound Annual Growth Rate
ACE	Angiotensin Converting Enzyme
GI	Gastro Intestine
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
GMP	Glyco Macro Peptides
IGF	Insulin like Growth Factor
TGF	Transforming Growth Factor
WPC	Whey Protein Concentrate
LAB	Lactic Acid Bacteria
ATCC	American Type Culture Collection
MTCC	Microbial Type Culture Collection
NCTC	National Collection Type Culture
IMTECH	Institute of Microbial Technology
UHT	Ultra-High Temperature
HHP	High Hydrostatic Pressure
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
Ssp	Sub species
BP	Boiling Point
BSA	Bovine Serum Albumin
MRS	De Mann, Rogosa and Sharpe
VRBA	Violet Red Bile Agar
PET	Poly Ethylene Terephthalate
NDRI	National Dairy Research Institute
LRC	Livestock Research Centre
PBS	Phosphate Buffer Saline

TEAC	Trolox Equivalent Antioxidative Capacity
ANOVA	Analysis Of Variance
HPLC	High Performance Liquid Chromatography
SD	Standard Deviation
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
m/v	Mass by volume
L	Litre
µl	Micro litre
µMol/L	Micro Molar per Litre
µg	Microgram
kg	Kilogram
mg	Milligram
mm	Millimetre
cm	Centimetre
ml	Millilitre
min	Minute
nm	Nanometre
α-la	α-lactalbumin
°C	Degree Celsius
°	Degree
US$	US Dollar
OPA	O-pthaldehyde

"Preparation of protein-rich fermented whey beverage using colostrum whey"

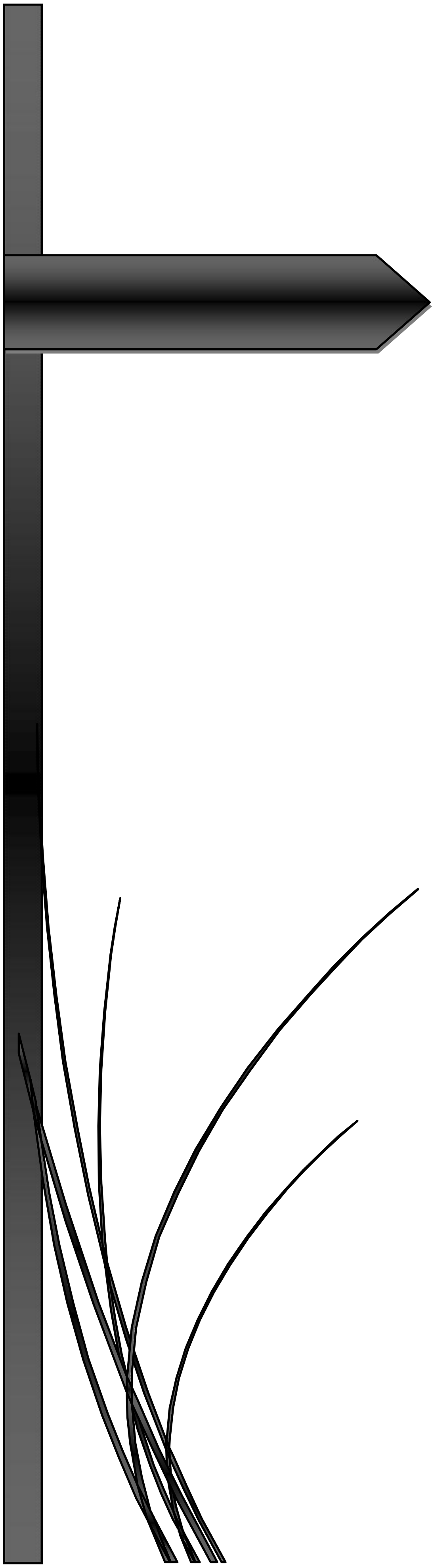
Abstract

Recently, the demand and popularity of healthy protein beverages has spiked across the globe. In this investigation, our main aim was to optimize the method for preparation of protein-rich fermented beverage using colostrum whey and to evaluate their bio-functional properties. Colostrum whey with high protein (>6%) was prepared by diluting colostrum with 0.5% water. Proteolytic NCDC cultures *L. acidophilus* NCDC 195, *L. rhamnosus* NCDC 24 and *L. rhamnosus* C25 (cheese isolate) were used for high protein whey fermentation. NCDC 195 alone and, in combination (195+25 & 195+24) were found to be the best for optimization of processing conditions for preparation of fermented whey beverage. The conditions for preparation of protein rich fermented whey beverage were optimized as 37^0C for 24h with 3% inoculum on the basis desirable pH (3.80-4.0), titratable acidity (0.9-1.10% L.A) and total *Lactobacillus* count (10^8 CFU/ml). The fermented whey beverages named as Drink 1, Drink 2 and Drink 3 were having protein (> 6%), peptides (>5mg/ml), pH of 3.9 and lactic count of > 8 log CFU/ml. Sensory evaluation of all the three drinks showed the overall acceptability above 8. The fermented whey beverages were having bio-functional properties and showed high antimicrobial activity against Gram-negative and Gram-positive pathogens. Drink 2 had highest antioxidant activity of 1991.47±7.01 µMol/L which corresponds to 85.62±0.301 % inhibition, followed by Drink 3 and Drink 1 with antioxidant activity of 1972±0.77 and 1908.62±0.001 µMol/L which corresponds to 84.82±0.033 & 82.043±0.001 % inhibition, respectively. ACE-inhibitory activity of Drinks was between 53.16 to 55.01%. The bio-functional properties of drinks during storage at 4^0C for 7 days showed no significant change. RP-HPLC chromatogram of fermented drinks revealed the generation of different peptides. Drink-1 showed 10 peaks of peptides, Drink-2 showed 15 peaks and Drink-3 showed 13 peaks of peptides. The protein rich fermented whey drinks may be used as functional food to improve the overall health of the consumers.

"कोलोस्ट्रम मट्ठा का उपयोग करके प्रोटीन युक्त किण्वित मट्ठा पेय तैयार करना"

सारांश

हाल ही में, दुनिया भर में स्वस्थ प्रोटीन पेय पदार्थों की मांग और लोकप्रियता बढ़ी है। इस जांच में, हमारा मुख्य उद्देश्य कोलोस्ट्रम मट्ठा का उपयोग करके प्रोटीन युक्त किण्वित पेय तैयार करने की विधि का अनुकूलन करना और उनके जैव-कार्यात्मक गुणों का मूल्यांकन करना था। उच्च प्रोटीन (>6%) के साथ कोलोस्ट्रम मट्ठा 0.5% पानी के साथ कोलोस्ट्रम को पतला करके तैयार किया गया था। प्रोटियोलिटिक एनसीडीसी कल्चर एल. एसिडोफिलस एनसीडीसी 195, एल रम्नोसस एनसीडीसी 24 और एल रम्नोसस सी25 (चीज आइसोलेट) का उपयोग उच्च प्रोटीन व्हे किण्वन के लिए किया गया था। अकेले एनसीडीसी 195 और संयोजन में (195+25 और 195+24) किण्वित मट्ठा पेय की तैयारी के लिए प्रसंस्करण की स्थिति के अनुकूलन के लिए सर्वोत्तम पाए गए। वांछित पीएच (3.80-4.0), अनुमापनीय अम्लता (0.9-1.10% एलए) और कुल लैक्टोबैसिलस गणना (108 सीएफयू / एमएल) के आधार पर 3% इनोकुलम के साथ प्रोटीन युक्त किण्वित मट्ठा पेय की तैयारी के लिए शर्तों को 24 घंटे के लिए 37^0C के रूप में अनुकूलित किया गया था। ड्रिंक 1, ड्रिंक 2 और ड्रिंक 3 नामक किण्वित व्हे पेय में प्रोटीन (> 6%), पेप्टाइड्स (> 5mg/ml), pH 3.9 और लैक्टिक काउंट> 8 लॉग CFU/ml थे। तीनों पेय के संवेदी मूल्यांकन ने समग्र स्वीकार्यता को 8 से ऊपर दिखाया। किण्वित मट्ठा पेय में जैव-कार्यात्मक गुण होते थे और ग्राम-नकारात्मक और ग्राम-पॉजिटिव रोगजनकों के खिलाफ उच्च रोगाणुरोधी गतिविधि दिखाते थे। ड्रिंक 2 में 1991.47 ± 7.01 μMol/L की उच्चतम एंटीऑक्सीडेंट गतिविधि थी जो 85.62 ± 0.301% निषेध के अनुरूप है, इसके बाद ड्रिंक 3 और ड्रिंक 1 की एंटीऑक्सीडेंट गतिविधि 1972 ± 0.77 और 1908.62 ± 0.001 μMol/L है जो 84.82±0.033 के अनुरूप है। 82.043 ± 0.001% निषेध क्रमश:। पेय की एसीई-निरोधात्मक गतिविधि 53.16 से 55.01% के बीच थी। 7 दिनों के लिए 40C पर भंडारण के दौरान पेय के जैव-कार्यात्मक गुणों ने कोई महत्वपूर्ण परिवर्तन नहीं दिखाया। किण्वित पेय के आरपी-एचपीएलसी क्रोमैटोग्राम ने विभिन्न पेप्टाइड्स की पीढ़ी का खुलासा किया। ड्रिंक -1 ने पेप्टाइड्स के 10 शिखर दिखाए, ड्रिंक -2 ने 15 चोटियों को दिखाया और ड्रिंक -3 ने पेप्टाइड्स के 13 शिखर दिखाए। उपभोक्ताओं के समग्र स्वास्थ्य में सुधार के लिए प्रोटीन युक्त किण्वित मट्ठा पेय का उपयोग कार्यात्मक भोजन के रूप में किया जा सकता है।



Chapter - 1

Introduction

INTRODUCTION

Demand for healthy protein beverages is growing all over the world and the global whey protein size was valued at US$ 7.4 Billion in 2018 and it is expected to register CAGR of 8.1% from 2019-2025 (Anon, 2019). Whey is a by-product derived from paneer or cheese preparation, because of its nutritional composition and larger volumes produced, whey is extensively used in production of food ingredients. The perfect way to reuse the liquid whey is by production of whey-based beverages due to its low capital, bland flavor, sensory value and nutritional value. Whey has been converted for the preparation of fermented whey beverage by utilizing LAB as a culture, because of its bio-functional properties of proteins and peptides (Abdulalim et al., 2018). The main constituents of whey as total solids (lactose) and also possess some important components which exhibit certain functional and nutritional properties like lactoferrin, Lactoperoxidase, immunoglobulins, lysozyme, anti-microbial and some growth factors. The main aims of utilization of huge volumes whey for preparation of whey beverage are to reduce disposal problems and production of healthy beverages which possess health benefits. The use of whey for production of beverage is considered has one of the best examples for value addition (Sabokbar and Khodaiyan, 2015). Whey proteins and their hydrolysates are easily digestible and can be used in production of healthy beverages. Whey can be used as raw material for production of high valued products like probiotic beverages because it contains nutritional components like minerals, proteins and milk sugar (lactose) and acts as a food matrix for the growth of probiotic microorganisms (Buriti et al., 2014; Castro et al., 2013). The functionality of beverages can be enhanced by addition of dietary fibres, probiotics, prebiotics, minerals and vitamins (Smithers, 2015).

The functional fermented whey beverages can be prepared by fermentation of whey with microorganisms to yield bioactive peptides. The bioactive peptides may be defined as specific protein fragments containing 3-20 amino acids that exert positive influence for normal functioning of body and promoting health (Kitts and Weiler, 2003). High protein beverages may enhance the health benefits like improving the muscle gain and their impairment. They may avoid loss of muscle and increase in the muscle mass after weight management.

Colostrum is the initial milk produced from mammals after parturition and its composition differs from the milk which is produced in later stage of lactation. Colostrum is rich in whey proteins and therefore, is a potential source of various bioactive peptides. The whey proteins such as lactoferrin, α-lactalbumin, Lactoperoxidase, lysozyme, β-lactoglobulin and their peptides may exhibit bio-functional activities like antimicrobial, ACE-inhibitory, antioxidative, opioid, antithrombotic, immunomodulatory, mineral binding as shown in **Fig.1.1** (Buttar et al., 2017).

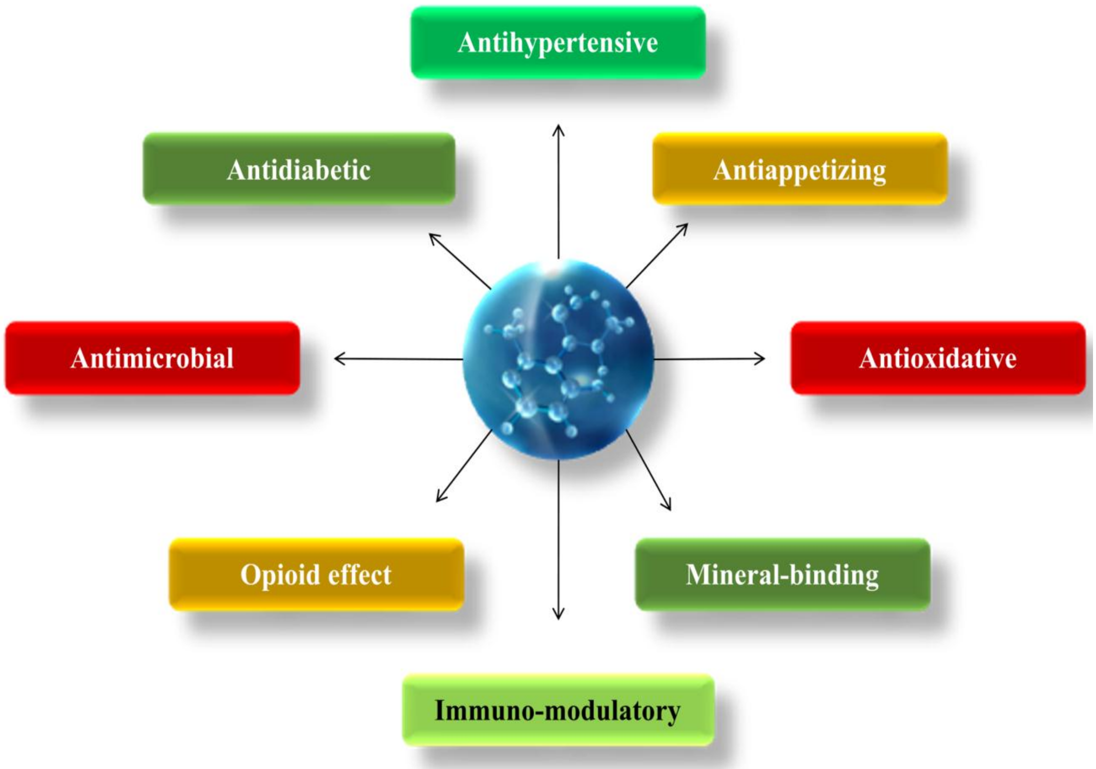


Figure 1.1 Therapeutic Effects of whey derived bioactive peptides

Colostrum contains several types of immunoglobulins, growth factors, cytokines, antibodies, lipids, lactoferrin, lysozymes, vitamins, and minerals, all of which help in the development of passive immunity, maintaining GI tract and growth of vital organs in the infant. Mostly, antimicrobial activity of colostrum is due to immunoglobulins, in addition to this colostrum also contains other antimicrobial factors like lactoferrin, lysozyme and Lactoperoxidase. In colostrum, lactoferrin and Lactoperoxidase are the most predominant non-specific antimicrobial components and many experiments have presented their antimicrobial activity against all kinds of microorganisms (Ayar et al., 2016).

Bovine colostrum has higher protein content than mature milk. The protein content in bovine colostrum ranges from 41-150 g/L and in mature milk is in the range of 30-35 g/L. The casein: whey protein ratio in normal milk is 80:20 with concentration <10 g/L of whey proteins. While, casein: whey protein ratio in bovine colostrum is 30:70 with concentration of 26 g/L of casein and 124 g/L of whey protein. The lactoferrin content in colostrum is 10.6 mg/ml, whereas lactoferrin is only <0.1 mg/ml in mature milk. Colostrum whey is considered to be an important source of whey protein compared to whey and consist of higher concentration of various proteins like lactoferrin, Lactoperoxidase, lysozyme, serum albumins, immunoglobulins, α-lactalbumin and β-lactoglobulin than mature milk (Pereira, 2014). α-lactalbumin is the major whey protein (40%) present in colostrum which helps in antimicrobial activity, absorption of calcium and other minerals, whereas β-lactoglobulin is present in lower concentrations The lactoferrin is around 10.6 mg/ml in colostrum whereas, lactoferrin is only <0.1 mg/ml. (Chatterton et al., 2006; Svensson et al., 2000).

Consumers are attracted towards utilization of bovine colostrum because of its immune-boosting properties. One of the novel trends in bovine colostrum market is incorporation of the bovine colostrum in dairy products. Besides this, bovine colostrum is also driving attention in the production of dietary supplements and infant formulae. The global bovine colostrum markets value stood at US$ 2.6 Billion in 2019 and to be estimated at US$ 4.3 Billion in 2025 @ CAGR of 6.4%. Asia Pacific excluding Japan leads the global market followed by North America and Europe and in that tablet & powder form are two prominent segments.

Although, reports are available on fermented whey drinks but there is a lack of information regarding the preparation of protein-rich fermented whey drinks using colostrum whey with bio-functional activities such as ACE-inhibitory, antimicrobial and antioxidative activity. Therefore, the present project has been conceptualized to exploit the higher concentration of colostrum whey for the preparation of bio-functional protein-rich fermented whey drinks with the following objectives:

1. Preparation of colostrum-whey supplemented protein-rich fermented whey-beverage
2. Evaluation of bio-functional properties of protein-rich fermented whey beverage
3. Characterization of bioactive peptides produced during preparation of protein-rich functional drink.

CHAPTER - 2

Review of Literature

2.1 Colostrum

The global bovine colostrum market value stood at US$ 2.6 Billion in 2019 and to be estimated at US$ 4.3 Billion in 2025 @ CAGR of 6.4%. Consumers are attracting towards bovine colostrum because of its immune-boosting properties. One of the novel trends in bovine colostrum market is incorporation of the bovine colostrum in dairy products, nutrition bars and functional beverages. Besides this, bovine colostrum is also driving attention in the production of dietary supplements and infant formulae. The third highest revenue segment in bovine colostrum market is dietary supplement. Thus, manufacturers are advancing their efforts in supplementation of adequate number of immunoglobulins in dietary supplements that are favorable in improving human gut. Asia Pacific excluding Japan leads the global market followed by North America and Europe and in that tablet & powder form are two prominent segments (Anon, 2020).

Milk and colostrum are considered as important source to enhance healthiness in children and adults. The readily availability of large quantities of immunoglobulins and lactoferrin in colostrum and different types of milk (Buffalo, Cow, Goat etc) make these secretions an important source to boost immunity in human beings (Hurley and Theil, 2011; Giansanti *et al*., 2016). Studies have revealed that bovine colostrum is 100-1000 times more effective than that of human colostrum. Therefore, bovine colostrum can be used as dietary supplement to promote well-being of humans (Elfstrand *et al*., 2002; Sarker *et al.,* 1998).

Generally, solid form of colostrum is more stable than liquid form because liquid form consists of high amount of moisture content that increases the chances of microbial spoilage (Jenny *et al.,* 1984). Proteins are the vital component present in colostrum. These proteins are rich source of amino acids and energy, which helps in maintenance of growth and health. However, utilization of colostrum is avoided due to its problems related to technical and hygienic aspects. Antimicrobial activity of colostrum reduces the possibilities of fermentation. High temperature processing of colostrum is not suitable because, proteins tend to denature at high temperatures (Korhonen *et al.,* 1998). Thus, colostrum processing is carried under low-temperature pasteurization and indirect steam drying (Godden *et al*., 2006; Seth and Das, 2011). Colostrum free from fats and lactose can be utilized for obese and lactose intolerant individuals (Elfstrand *et al.,* 2002).

2.2 Colostrum Composition and its Significance

Bovine colostrum is the initial milk produced by cows after calving, which is different from mature milk, by means of its nutrient profile and immunological composition. Colostrum comprises of macronutrients (carbohydrates, protein, fats, and oligosaccharides), micronutrients (minerals and vitamins), growth factors, immunological and anti-microbial compounds are not present in milk or present in minute concentrations. So, both bovine and human colostrum provide full diet to neonates with all essential nutrients during early phase of life. Particularly in ruminants, exchange of immune factors thru utero is not possible, so ingestion of colostrum provides protection with higher immunoglobulin content, without this survivability of ruminant is less (Godhia and Patel, 2013).

Table 2.1 Nutritional composition of bovine milk and colostrum

Composition	Milk	Colostrum
Dry matter, g/kg	122 g	153-245 g
Crude protein	34 g	41-140 g
Lactose	46 g	27-46 g
Fat	37 g	39-44 g
Ash	7 g	5-20 g
Lysozyme	0.07-0.6 mg/l	0.14-0.7 mg/l
Lactoperoxidase	20 mg/l	30 mg/l
Lysine	4.9	43.4 mg/ml
IgG	2	496 mg/ml
Lactoferrin	<0.1	10.6 mg/ml
IGF-I	33 ng/g	2500 ng/g
IGF-II	12 ng/g	25 ng/g

(Ayar *et al.,* 2016)

2.3 Bioactive Compounds of Colostrum

Colostrum is considered to be gift of nature to neonates which provides passive immunity. It also helps in synthesis of proteins with its desired functions. Colostrum is rich in bioactive compounds like Lactoferrin, Lactoperoxidase, Lysozyme Immunoglobulins and insulin like growth factor I and II these factors increase activity against pathogens (Tripathi and Vashista, 2006; Playford and Weiser, 2021).

2.3.1 Antimicrobial factors

2.3.1.1 Lactoferrin

Lactoferrin is an iron-binding glycoprotein with Molecular Weight of 80 kDa which is predominantly present in colostrum and lesser amounts in milk and some exocrine fluids like tears. The important role of lactoferrin is by chelating iron and activates immune and phagocytic functions in intestine. The concentration of lactoferrin in bovine colostrum is 1.5-5mg/ml, whereas in mature milk 0.1mg/ml (Marnila, 2005). Neutral pH and presence of bicarbonate ions are responsible for activity of lactoferrin. Since, the secretion of bicarbonate takes place in lumen of intestine, which favors the antimicrobial activity of lactoferrin. Lactoferrin binds to lipid-A which causes the release of LPS from bacteria cell wall. Lactoferrin changes permeability of membranes by binding to porin molecules of *Escherichia coli* and *Salmonella typhimurium.* Antiviral effects of lactoferrin showed against HSV-I and HIV-I (Tripathi and Vashista, 2006).

2.3.1.2. Lysozyme

Lysozyme is lytic and antimicrobial enzymes that have activity against Gram positive bacteria. This enzyme is most commonly found in mammalian body fluids like tears, saliva and colostrum. Major sources of lysozyme include egg albumen and human milk. Antimicrobial activity of lysozyme is due to cell wall degradation i.e., peptidoglycan layer leads to lysis of cell hydrophobic and cationic properties (Pellegrini *et al.*, 1999; Wheeler *et al.*, 2007). The activity of lysozyme is enhanced in the presence of Lactoferrin against *E. coli* (Pakkanen and Aalto, 1997). The concentration of lysozyme in bovine colostrum is 0.14-0.7 mg/l, whereas in normal milk is 0.07-0.6 mg/l (Singh, 2019). Milk lysozyme is effective against most Gram-positive and some Gram-negative microorganisms.

Antimicrobial activity of Lysozyme against *Escherichia coli* can be enhanced in presence of Lactoferrin and also found that lactoferrin damages the outer membrane of Gram-negative bacteria. Lysozyme encoding genes are found in cow and the purified enzyme from cow stomach and kidney, which is different from milk lysozyme. Amino acid content of milk lysozyme is strictly different from human and egg white lysozyme (Tripathi and Vashista, 2006).

2.3.1.3 Lactoperoxidase

Lactoperoxidase is a heme-group with Fe^{3+} containing chain glycoprotein and it is the most common antimicrobial enzyme present in colostrum. Major role of Lactoperoxidase is, in the presence of Hydrogen peroxide, the enzyme oxidizes the thiocyanate ions (SCN^-) to intermediate compounds that shows antimicrobial activity by oxidation of sulfhydryl groups in proteins that inhibits bacterial metabolism (Magacz *et al*., 2019). Lactoperoxidase system is effective against Gram-positive and some Gram-negative like *Staphylococcus aureus* (Kamau *et al.,* 1990), *Pseudomonas aeruginosa, Streptococcus mutans*, *Listeria monocytogenes* (Gaya *et al.,* 1991) and also protects from mammary gland infections. Antiviral effects of Lactoperoxidase showed against polio virus and HIV-I (Yamaguchi *et al.,* 1993).

The concentration of Lactoperoxidase in bovine colostrum is 11-45 mg/l, whereas in normal milk is 13-30 mg/l. Epithelial cells of mammary gland are expressed by Lactoperoxidase encoding genes, that helps to secrete Lactoperoxidase in milk. Interaction of Lactoperoxidase with Lysozyme forms a complex and this relation found to be specific and interaction of Lactoperoxidase and Lactoferrin found not synergistic, but additive and showed antimicrobial effect against *Streptococcus mutans* (Tripathi and Vashista, 2006).

Immunoglobulins

Bovine colostrum is considered to be good source for immunoglobulins and ingestion of this improves the immunity to calves after birth and also protects them from infectious intestinal and respiratory diseases, which are considered to be major reasons for death of calves. High serum IgG concentration in calves have low mortality rate compared to calves with serum IgG (<10 g/L). Immunoglobulin's concentrations in colostrum is of 100 times more compared to normal milk. The principal immunoglobulin present in colostrum is IgG_1;

however, IgG_2, IgA and IgM are present significantly in low amounts. Transfer of immunoglobulins from cow to its calf involves two processes i.e., initially, maternal immunoglobulins are concentrated in colostrum by absorbing from circulation then the transfer of colostrum immunoglobulins from intestinal lumen into the circulation of neonate. Sufficient amount of Immunoglobulins are transferred to calves by natural suckling. If, intake natural colostrum is not possible, in such cases immunoglobulins can be administered externally. The factors influencing passive transfer of Immunoglobulins includes number of antibodies utilized and age of the calf. The average requirement of colostrum Immunoglobulins is 80-100g and the Immunoglobulins concentration should be minimum concentration of 20g/L. Commercially colostral supplements are available, but concentration of Immunoglobulins is low compared to the natural colostrum (Tripathi and Vashista, 2006).

Table 2.2. The concentration of Immunoglobulins bovine milk and colostrum

Immunoglobulin	Bovine milk (g/L)	Colostrum (g/L)
IgG_1	0.31-0.40	52-87
IgM	0.03-0.06	3.7-6.1
IgA	0.04-0.06	3.2-6.2
IgG_2	0.03-0.08	1.6-2.1

(Singh, 2019)

2.3.2 Growth Factors

Several growth factors are present in both colostrum and normal milk which promotes growth and differentiation of cell. Several reports available on bovine colostrum and its growth factors that promote the growth of mammalian cells invitro and due to low amounts of growth factors in bovine milk promote less stimulation of cell (Yan and Charles, *2018)*. Insulin like Growth Factors (IGF-1 and IGF-2) belongs to the family of Insulin like growth factors, which contains insulin. Both IGF-1 and IGF-2 are acid and heat stable and helps in promoting cell development, differentiation and growth. The concentration of IGF-I and IGF-II in bovine colostrum is 50-2000 µg/l and 200-600 µg/l, whereas in normal milk is <10 µg/l and <10 µg/l respectively (McGuire *et al.*, 1991). IGFs are synthesized in colostrum by circulation, not from synthesis in mammary tissues. TGF-β is a very exciting growth factor present in bovine colostrum with several types of function which includes cell division, embryo development, helps in formation of cartilage bone, repair of tissues, controls immune system and it acts as growth inhibitor in connective tissues such as epithelial cells and lymphocytes. TGF-βs plays an important role in immunomodulatory activity against pathogenic microorganisms (Tripathi and Vashista, 2006).

Immune Factors

The external environment consists of wide range of pathogenic microorganisms includes Viruses, Fungi and Bacteria. If proper care is not taken, leads the microorganisms to multiply and may leads to death. In healthy animals these kinds of infections are short term because host develops a defense system against pathogenic species. The Immunoglobulins play important role in host's defense mechanism by developing antibodies. IgG is a predominant Immunoglobulin present in animals. Bovine milk consists of some specific antibodies are considered to be effective against enterogenic and enteropathogenic organisms, in human trials. Bovine colostrum is having ability for the prevention and treatment of various bacterial infections, by enhancing the host immunity (Jones *et al.,* 2019*)*

2.4 Therapeutic Applications of Colostrum

Colostrum is commonly served as a dietary supplement because of its antimicrobial, growth and immunomodulating factors which have positive impact on health of adults and children. Bovine colostrum can be utilized in production of nutraceuticals for humans due to its availability in large quantities. Colostrum doesn't cause any serious health issues to human beings and it is well accepted by adults, neonates and children.

Colostrum consists of Proline-rich peptides that control the performance of thymus gland. It also helps in relieving pain, swelling and inflammation caused by overproduction of T-cells and lymphocytes, due to hypersensitivity and allergy. Colostrum-based products enhance the function of thymus gland, which in turn regulates allergy and auto-immune disorders (Buttar *et al*., 2017). Lactoferrin helps in reduction of neurobehavioral dysfunctions facilitated by the pro-inflammatory cytokines (Choi *et al*., 2010; Undale *et al*., 2012). Uptake of colostrum lowers the partial amnesia through neuron cell death and cerebral ischemia by reducing the oxidative stress (Kim *et al*., 2012).

Immune sensitization or altered immunity is the major cause for coronary heart diseases and arterial atherosclerosis. Colostrum consists of Proline-rich peptides that help in control of cardiovascular diseases. The presence of insulin like growth factor (IGF-1) in colostrum may reduce the level of low-density lipoprotein (LDL)-cholesterol and enhances the level of high-density lipoprotein (HDL), and thus it avoids the formation arterial plagues (Buttar *et al.,* 2017). Leptin is a hormone chiefly made of adipose cells and enterocytes in intestine which controls hunger or desire to eat less. Leptin and IGF-I are present in colostrum, these reduces triglyceride content and cholesterol in lipidemic patients (Kim *et al*., 2009). It favors reduction of fat in body and increase lean body weight, thus helps an individual to lose weight.

Bovine k-casein glyco-macro-peptides (GMP) gained global attention due to its immunomodulatory effects, suppressing gastric secretion, inhibition of viral and bacterial adhesion and also favor the growth of *Bifidobacterium.* Bovine colostrum consists of lactoferrin that favors the growth and development of *Bifidobacterium.* GMP found to have ability to attach Cholera toxins and *Escherichia coli* (Brody, 2000). Collagen granules and colostrum powder comprises of growth factors, anti-inflammatory and antimicrobial factors and these factors promote the rapid healing of wounds by creating an interface between wound and outside environment (Singh *et al*., 2011). Bovine colostrum is having ability for the treatment of gastro-intestinal diseases and different kinds of cancer (Bagwe-Parab *et al,* 2020)

2.5 Whey Based Beverages

Whey is no more considered to be waste in dairy industry; instead, it is used as a raw material in preparation functional products like probiotic whey beverages. It is rich in components like milk sugar (lactose), milk proteins and minerals and these favors the growth and development of probiotic microorganisms (Castro *et al.,* 2013; Buriti *et al.,* 2014; Pescuma *et al.,* 2010). Past few decades, studies on supplemented whey beverage formulations and probiotic whey based have increased tremendously. Whey beverages supplemented with probiotics and prebiotics conferred the host by reducing blood pressure (Fluegel *et al*., 2010).

Whey can be supplemented directly, or in addition to dairy based powders like skim milk or butter milk powder etc., or added to beverages at different proportions. A probiotic whey beverage fermented with *L. acidophilus La-5 and B. animalis Bb-12* was prepared from acid whey enhanced with sweet whey powder or butter milk powder (5%). Storage studies were carried for 21days and throughout the period the counts were above 8 logs CFU/ml (Skryplonek and Jasińska, 2015).

Whey based beverage was formulated using chhana whey (75-78%) supplemented with watermelon juice (15%), sugar (7%) and varied proportions of Betel leaves concentrate (0, 1, 2, 3%). The ready beverage possesses red color and good taste. The beverage acceptability was increased with increase in betel leaves concentrate up to 2% and further increase in concentration (3%) affects the mouthfeel of beverage. Final product was subjected to in-bottle sterilization, cooling and stored in refrigerated condition (Naik *et al.*, 2009). Soft beverage was prepared using paneer whey (65%) added with guava pulp (25%) and sugar (10%). This beverage was subjected to various pasteurization temperature (60-70^0C) and timings (15-35 minutes) and checked for its physio-chemical and organoleptic properties for every 15days until 45days. Whey-guava beverage subjected to pasteurization i.e. (70^0C for 35 minutes) was found to be best in terms of sensory and organoleptic properties after 45days (Divya and Kumari, 2009).

Silva e Alves *et al*., (2018) developed a probiotic functional carbonated whey beverage from cheese whey fermented with probiotic species *Bifidobacterium animalis ssp. lactis.* The preparation of this beverage and incorporation of probiotic found to be quite easy. The final product had showed good probiotic counts and slight sedimentation during storage and the beverage microbiological quality satisfied the Brazilian standards.

Abdulalim *et al.,* (2018), developed and evaluated the functional product- mulberry whey beverage in cold storage for 21 days. Five different concentrations of product are prepared using sweet whey and black mulberry juice fermented with *L. rhamnosus and B. animalis.* Acidity was increased whereas, pH and phenolic contents decreased during storage. The antioxidant activity is increased by increasing in mulberry juice and decreased during cold storage. Sensory properties were improved with addition of mulberry juice. Results showed that the activity of *L. rhamnosus* GG and *B. animalis* ssp. *lactis* Bb-12 was high up to 14 days and gradually declined. All samples were accepted in terms of sensory properties, whereas sample SWBM3 (25% whey with 75% black mulberry juice) have showed high organoleptic scores.

Sabokhar and Khodaiyan, (2016) developed a novel beverage prepared from whey and pomegranate juice fermented with kefir grains @ 5-8% have showed good sensory properties and high antioxidant activity. Alcohol production was observed, when cheese whey is fermented with kefir grains (Koutinas *et al.,* 2009).

Whey beverage was developed with the addition of different concentration of tomato juice (5, 10 and 15%) and fermented with probiotic species like *L. plantarum and L. acidophilus* for 18 hours. The highest antioxidant activity of 9.073% was found in beverage with tomato juice concentration (15%). The beverage with tomato juice concentration (5%) have attained good sensory scores and found to be best formulation (Nuriwi *et al*., 2017).

Whey beverage was prepared from whey, skim milk and carrot juice fermented with yoghurt starters *(S. thermophilus, L. bulgaricus),* probiotic bacteria like *L. acidophilus and B. bifidum.* The final product showed 90.5 % antioxidant activity, viable cell counts 8.7 log CFU/ml, pH 4.6 and overall sensory score of 8.97. Implementation of this beverage production during cheese manufacture increases overall profit of industry and improves the product quality (Arsic *et al.,* 2018).

Singh *et al.,* (2016) prepared whey beverage supplemented with soy extract and curcumin and fermented with *L. acidophilus* NCDC 195 (LA 195) and *S. thermophilus* NCDC 323 (ST323). The product showed acidity of 1.38- 1.41% LA and total viable count of 9.09- 9.39 log CFU/ml after fermentation at 37^0C for 24h. Whey beverage made from two cultures have showed antimicrobial activity against *E. coli*, *B. cereus*, *S. aureus*, *L. monocytogenes, S. dysentriae* and *S. Typhi* having zone of inhibition (16 to 23 mm) and beverage made from combination of culture (LA195+ST323) supplemented with curcumin showed high antioxidant activity of 989.70 TEAC (µM/L).

Kumari et *al.,* (2015) reported on preparation of probiotic based fermented whey drink with *L. acidophilus* (16, 195) and yoghurt cultures (144) at 39^0C for 18h. The product showed $1x10^8$ CFU/ml counts, acidity of 1.10- 1.20% L.A and pH of 3.90- 4.20. Whey drink showed antimicrobial activity against Gram-positive and Gram-negative pathogens, antioxidative activity (3.23- 8.82 µmol/ml TEAC), ACE- inhibitory activity of 54.25- 70.86% and showed good sensory scores. There was no significant change in bio-functional properties even after storage of whey drink at refrigeration condition for 10 days. Similarly, whey fermented with *lactobacilli* showed good antimicrobial activity against antibiotic resistant pathogenic strains i.e., *E. faecalis* MTCC 439, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 15303, *S. aureus*, *Pseudomonas sp*., B. *cereus* ATCC 13061, *S. Typhi* NCTC 6017 (Yumlembam *et al.,* 2014).

Priyanka *et al.,* (2015) reported that the cheese isolate *L. rhamnosus* C6 was having proteolytic activity of 509.12 µg serine/ ml. *L. rhamnosus* C6 showed good probiotic attributes, as it survived at high bile concentration (up to 2%), low pH (pH 1.0) and cell surface hydrophobicity for n- hexadecane (28.53 ± 0.37%). The culture showed good growth in whey (9.19 log CFU/ml) and soy milk (8.88 log CFU/ml) after 48h of incubation and the fermentate of whey and soy milk fermented with *L. rhamnosus* C6 showed antimicrobial activity against *E. coli* 0157:H7 ATCC 35150, *S. dysenteriae* NCDC 107, *S. typhi* NCTC 6017, *L. monocytogenes* ATCC 15303, *B. cereus* ATCC 13061 and *S. aureus* MTCC 1144.

Whey is a good source for the production of non- probiotic soft beverages. Many reports are available on development of whey, whey hydrolysate or whey protein-based beverages especially, fruit-based whey beverages found to have high commercial values. Some examples of fruit-based whey beverages are whey supplemented with pomegranate juice (Aghda *et al.,* 2017), flaxseed and acerola pulp (da Silva *et al.,* 2015), orange juice (Sady *et al.,* 2013), grape juice (Amaral *et al.,* 2018), lemon (Singh *et al.,* 2014), araticum pulp (Costa de Lima *et al.,* 2016), pineapple (Baba *et al.,* 2016), apple juice (Goudarzi, 2015).

2.6 Colostrum Based Products

Setyawardani *et al.,* (2020) reported on kefir made of milk and colostrum-its physical and microstructural characteristics. Kefir was prepared by addition of kefir grains to varied proportions of milk and colostrum and kept for fermentation under room temperature for 24h. Result found to be kefir produced from mixture of milk-colostrum possess yellowish color whereas, kefir prepared from 100% colostrum showed slightly greenish in color. Strong, tight and compact microstructure of protein tissues was found in microstructure of kefir prepared from mixture of 40% milk and 60% colostrum.

Ayar *et al.,* (2016) investigated on the effect of bovine colostrum on the lactic microbial populations of yoghurt and kefir. Result found to be, effect of addition of colostrum to yoghurt, their effect on total mesophilic bacteria is negligible, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* counts were higher with addition of colostrum in yoghurt and in kefir, lactic *streptococci* and *Lactobacillus* counts were increased. However, addition of colostrum to yoghurt and kefir increased the functionality of beverage.

Fiorida *et al*., (2016) developed a probiotic kefir like beverage prepared from colostrum, honey, cow's milk and soyabean extract. Fermented product comprising honey with kefir grains of Mexican origin and sensory properties were increased than normal kefir beverage. Honey can be used as alternative substrate in preparation of bio-functional kefir beverage because of its important role on microbial DNA damage repair.

Study was conducted on supplementation of whey or colostrum to > 35 years old individuals to demonstrate its health claims. Colostrum supplemented individuals showed with increase in mineral density in bone, muscle strength, body composition, biochemical markers like lipids and blood glucose (Blair *et al*., 2020).

2.7 Proteins-Rich Beverages

High protein and energy density beverage was prepared from mixing the blends of malted white sorghum, yellow maize and soyabeans. Initially, cereal grains are malted for 48 and 72 hours, kilned and powdered to flour. Soya milk and malt extract mixed in 2:1 ratio with the addition of food additives like color, sweetening agents like sugar and glucose syrup to improve sensory attributes of beverage. The final product had protein content (12.3-17.8%), fat (2.63-10.13%), ash (1.25-5.30%) and energy value (135.40-166.52 kcal) and the beverage fermented for 72 hours was most preferred due to high sensory scores (Oluwole *et al.,* 2012).

Cho *et al.,* (2015) developed a functional high protein fermented beverage with the addition of whey protein concentrate (WPC 80), skim milk powder, sucrose and fermented with single starter i.e., *L. plantarum* DK211 isolated from Korean traditional fermented food named kimchi or used in combination with commercially available culture (*Lactococcus* *lactis* R704). Functional protein beverage was high protein (9%) and low-fat content (0.2%) product. Acid development was faster with combination of cultures than single culture. Final product had cell count of 10^9 CFU/ml after fermentation for 10h. The product exhibited antimicrobial, and antioxidant activity. The sensory attributes were acceptable and it was similar in most of properties, when compared to commercial protein drinks, except sourness. Thus, whey protein concentrates and LAB were used in preparation of a protein-rich fermented whey beverage with increased organoleptic and functional properties like anti-obesity and hypolipidemic activity.

2.8 Sensory and Storage

Probiotic beverage was developed from de-protenized whey fermented with *L. rhamnosus* NCDC 243, *B. bifidum* NCDC 2715 and *Propionibacterium freudenreichii subsp. shermanii* have showed probiotic counts over 8 log CFU/mL and high organoleptic properties after 10 days of storage (Maity *et al.*, 2008).

Probiotic whey beverage prepared from different proportions of whey (0, 20, 35, 50, 65 and 80%) cultured with yoghurt starters, *L. acidophilus and B. animalis Bb-12*. The beverage prepared from whey concentration (49%) showed high sensory properties. The concentration of whey in beverage does not affect viability and growth of probiotic microorganisms (Castro *et al.*, 2013).

Shukla *et al*., (2013) developed a probiotic whey beverage using cheese whey and pineapple juice fermented with *L. acidophilus* (1%). The mixture of cheese whey: pineapple juice i.e. (65: 35) fermented for 5hr have showed high overall acceptability in terms of sensory scores. The total counts in beverage were more than $1x10^6$ CFU/mL however, the counts decreased after 28 days.

A probiotic whey beverage fermented with *L. acidophilus La-5 and B. animalis Bb-12* was prepared from acid whey enhanced with sweet whey powder or butter milk powder (5%). The product was stable for 21days and the counts were above 8 log CFU/ml (Skryplonek and Jasińska, 2015).

A whey-based beverage was prepared with the supplementation of milk and fermented with *L. rhamnosus* ATCC 7469 culture. Throughout storage of 21 days the final product had showed good rheological and sensory properties and the probiotic counts of 7 log CFU/mL

(Bulatović *et al*., 2014). Besides this, the decrease in physical stability was found in probiotic whey-beverage prepared from cow's milk and reconstituted whey added with *L. acidophilus, B. animalis subsp. lactis* and *Streptococcus thermophilus* with the increase in proportion of reconstituted whey (Akpinar *et al.,* 2015).

Utilization of ultrafiltered whey permeate (concentrated by reverse osmosis) or retentate (rich in whey proteins) in preparation of probiotic whey beverage fermented with *L. acidophilus, L. casei and L. rhamnosus* @ 0.3% have showed viable cell counts over 7 log CFU/mL and acceptable sensory properties after 14 days of cold storage (Pereira *et al.*, 2015). Chocolate flavored probiotic whey beverage prepared from goat cheese whey, UHT goat milk in addition to prebiotics like oligofructose or inulin fermented with *B. lactis*. The beverage made from goat cheese whey (45%) and oligofructose (6%) with high probiotic counts after 28 days of cold storage (Da Silveira *et al*., 2015).

Nowadays consumers are attracting towards minimally processed foods so, technologies like non-thermal or emerging technologies are becoming popular. Some of non- thermal or emerging technologies used industrially include cold plasma (CP), supercritical carbon dioxide (SC-CO2), pulsed electric field (PEF), High hydrostatic pressure (HHP), ultrasonics (US), microwave heating, radiofrequency heating, ohmic heating (OH) (Amaral *et al.*, 2017; Cappato *et al.,* 2017; Coutinho *et al*., 2018; Monteiro *et al*., 2018).

SC-CO_2 treated grape juice-based whey drink at 14–16 or 18 MPa at 35 °C for 10 min. An increase in ACE inhibitory activity was observed with increased pressure and SC-CO_2 treated grape juice-based whey drink have showed high sensory properties than heat treated beverages (Amaral *et al.,* 2018). Shelf-life of fermented whey beverage can be extended up to 45days by the application of HHP at 400 MPa for 1 min. Besides this, the textural and flavor properties of whey beverage were preserved under cold storage for 45 days (Pega *et al.,* 2018).

2.9 Bio-Functional Properties of Whey Beverages

Fermentation of colostrum whey by proteolytic lactobacilli may release mixture of different bioactive peptides. These bioactive peptides may have various bio-functional properties including antimicrobial, antioxidant, anti-hypertensive as well as immunomodulatory properties and many more.

2.9.1 Antioxidative Activity

Sadat *et al.*, (2011) reported that antioxidant peptides (INYW, LDQW) were generated from bovine alpha-lactalbumin using thermolysin at 70^0C. Four antioxidant peptides (VGINYWLAHK, VLVLDTDYK, IDALNEK, KTKIPAVF) were identified in whey protein concentrate by hydrolyzing with alcalase, flavourzyme and corolase PP (Mann *et al.*, 2015). Production of Whey protein hydrolysates from cheese whey using alcalase enzyme and the antioxidant peptides like (VLDTDYK, VRTPEVDDE) were identified (Athira *et al.*, 2015).

Arsic *et al.*, (2018) prepared a functional fermented whey carrot beverage fermented with yoghurt cultures (*L. bulgaricus, S. thermophilus*) and probiotics like *L. acidophilus, B. bifidum* showed 90.5% antioxidant activity. Functional product- mulberry whey beverage was developed and evaluated during cold storage for 21 days. Five different concentrations of product were prepared using sweet whey and black mulberry juice fermented with *L. rhamnosus* and *B. animalis.* Acidity was increased whereas, pH and phenolic contents decreased during storage. The antioxidant activity of beverage and sensory properties were increased with the increase of mulberry juice and gradually decreased during cold storage (Abdulalim *et al.*, 2018).

Lopez *et al.*, (2020) reported the preparation of milk whey based fermented beverage with varied proportions of hydrolyzed collagen (0.3%, 0.5%, 0.75% & 1%) fermented with *L. rhamnosus, L. bulgaricus, and S. thermophilus.* The beverage with 1% collagen has showed radical inhibition for ABTS (48.30%), DPPH (30.06%), high protein content (9.75 g/L) and also observed with the increase in collagen concentration, the nutrition value, antioxidant activity and bioavailability of beverage increased.

The bioactive peptides derived from colostrum by fermentation with kefir grains enriched with selected yeasts have showed increase in antioxidant activity, after fermentation for 48hrs (Cotarlet *et al.*, 2019). Fajardo-Espinoza *et al.*, (2019) reported that peptides derived from bovine colostrum by enzymatic hydrolysis (pancreatin and pepsin). The peptides are subjected to ultrafiltration to obtain <10, 10-30, >30 kDa fractions and the highest antioxidant values and high iron binding capacity obtained in fractions less than 10kda and >30 kDa fractions, respectively.

Gaspar- Pintiliescu *et al.,* (2020) reported that bovine colostrum fermented with *Candida lipolytica* co-cultured with kefir grains have produced bioactive peptides. These peptides are subjected to ultracentrifugation to derive less than 10kDa peptides have shown high scavenging activity against ABTS radical's 63-92% inhibition.

2.9.2 Antihypertensive Activity

Fluegel *et al.,* (2010) reported that there was reduction in blood pressure in pre-hypertensive and hypertensive young men and women by the consumption of whey beverages. Results found to be helpful in treating pre-hypertension and stage-1 hypertension by using dietary treatment and also observed reduction in total and LDL cholesterol concentrations. Peptides from whey protein concentrate are derived by treating with flavourzyme at 50^0C for 30 min and these peptides are incorporated to *lassi*. Enriched *Lassi* showed ACE inhibitory activity of 79.67 % whereas, control sample showed 42.17 % of activity (Paul and Gosh, 2017).

ACE-inhibitory peptides (CMNESA, VLDTDYK, VAGTW, VFK, LAMA, YGL, LDAQSAPLR) derived from bovine whey proteins by proteolysis using different digestive enzymes (Leppala. 2000). Two novel ACE-inhibitory peptides IIAEK and IPAVFK were identified from tryptic digest of Beta-lactoglobulin through ultrafiltration (Power *et al.,* 2014).

Bartkiene *et al.,* (2018) reported on production of antimicrobial gummy candies using bovine colostrum and essential oil (*Thymus vulgaris or Eugenia caryophyllata*) fermented with probiotics (*Lactobacillus paracasei* LUHS244) have showed antimicrobial activity against *Proteus mirablis, Escherichia coli, Salmonella enterica, Staphylococcus aureus, Enterococcus faecalis, Streptococcus mutans* except *Pseudomonas aeruginosa.* Gummy candies made from bovine colostrum (3%), essential oil (0.2%) had good sensory properties and antimicrobial activity.

Villadoniga *et al.,* (2019) reported that novel ACE-inhibitory peptides (TTFHTSGY, GYDTQAIVQ) were generated from alpha-lactalbumin, a whey protein, by hydrolyzing with *Bromelia antiacantha* peptidases. Cotarlet *et al.,* (2019) reported that the bioactive derived from colostrum obtained by fermentation with kefir grains enriched with selected yeasts had ACE inhibitory activity.

Fajardo-Espinoza *et al.,* (2019) reported that bioactive peptides were generated from bovine colostrum whey by enzymatic hydrolysis (enzymes like pepsin and pancreatin) having ACE-inhibitory activity. The bioactive peptides derived from bovine colostrum fermented with *Candida lipolytica* co-cultured with kefir grains. These peptides are subjected to ultracentrifugation to derive <10kDa peptide fractions showed better modulation in ACE inhibition property (Gaspar-Pintiliescu *et al.,* 2020).

Recently, Manzano *et al.,* (2020) reported on cheese whey fermentation with native microbiota to release bioactive peptides with ACE-inhibitory activity. Additionally, the ACE-inhibitory activity increased from 22% in unfermented whey to 60-70% after 120h fermentation. Rodríguez-Hernández *et al*., (2020) reported on effect of probiotic cultures on ACE-inhibitory activity of whey based fermented beverage using Chris Hansen cultures like BCT-1 and ABT-4 (A-*L. acidophilous*, B-*Bifidobacterium animalis ssp lactis*, c-*L. paracasei*, T-*S. thermophilus*). During shelf-life, almost all whey based fermented beverage with the presence of commercial culture have showed ACE-inhibitory activity (22-100%). Whereas, whey beverage without probiotics showed highest ACE-inhibitory activity (100%) and whey beverage with ABT-4 showed ACE-inhibitory activity (80-100%).

2.9.3 Antimicrobial activity

Birkemo *et al*., (2009) found that peptides naturally found in bovine colostrum [i.e., casecidin 15(YQEPVLGPVRGPFPIIV), casecidin 17(YQEPVLGPVRGPFP)] had antimicrobial activity. The purified peptides showed antimicrobial activity against *Enterobacter sakazakii*, *E. coli* DPC6053.

Ali *et al.,* (2019) have done profiling of peptides from whey protein isolate medium fermented with *Lactobacillus helveticus* LH-2 and *L. acidophilus* La-5 using mass spectrometry. Ultra-filtrated 3kDa peptide fractions have shown anti-virulence activity against *Salmonella enterica ssp. enterica serovar Typhimurium*.

Demers-Mathieu *et al.,* 2013 reported on peptides extracted from tryptic hydrolysate of whey protein through ultrafiltration. Three peptides (IDALNENK (84-91), TPEVDDEALEK (125-135) & (84-91)] are derived from Beta-lactoglobulin. Peptide (125-135) has shown more inhibitory than peptide (84-91) against *Listeria monocytogenes* and *Staphylococcus aureus* whereas, peptide (36-42) was not inhibitory. Bovine whey proteins hydrolyzed with selected *Lactobacillus* strains (*L. plantarum, L. brevis*) have shown antimicrobial activity against Gram-positive, Gram-negative and some pathogenic and spoilage microorganisms (Messaoui *et al*., 2020).

Windayani *et al.*, (2020) reported kefir produced from colostrum fermented with kefir grains for 24-48h had antimicrobial activity. Colostrum kefir has antibacterial properties against both Gram-positive and Gram-negative food borne pathogenic bacteria. Cotarlet *et al.*, (2019) reported that the bioactive peptides derived from colostrum obtained by fermentation with kefir grains enriched with selected yeasts have shown strongest antimicrobial spectrum against *Bacillus subtilis* MIUG B1. In actual fact, the literature highlights the antimicrobial activity of fermented colostrum is linked to titratable acidity of samples, which means organic acids produced by lactic acid bacteria are the key components responsible to inhibit the growth of spoilage microorganisms. Recently, Ashok, (2020) reported that the peptides released from colostrum whey by microbial fermentation showed antimicrobial activity against Gram-positive and Gram- negative bacteria.

CHAPTER - 3

Materials and Methods

3.1. Prologue

This chapter deals with the materials, procedures, techniques and experimental methodologies that will be employed during the present investigation relating to the preparation of whey from colostrum, fermentation of colostrum whey by lactobacillus, optimization of preparation of protein rich fermented whey beverage, sensory evaluation and storage studies of whey beverage, followed by evaluation of bio-functional properties (antimicrobial, anti-oxidative, anti-hypertensive and immune-modulatory) of fermented whey beverage.

3.2. Materials

Chemicals, reagents and media, used in this study are purchased from Sigma Chemicals Industry, Navi Mumbai and Hi-Media Pvt. Ltd. Mumbai. Complete list of reagents and chemicals used in this study are cited in Appendix 1.

3.2.1 Culture Collection

Three cultures are used in this investigation, in which two cultures are obtained from National Collection of Dairy Culture, DM Division, Karnal and one culture from cheese isolated in our lab (Functional Fermented Foods and Bioactive Peptides Lab (FFFBAP). The cultures used in this study, growth medium and sources are as follows:

Table 3.1 Lactic Acid Bacteria used in manufacture of product

Strain type	Growth Medium	Origin
L. acidophilus NCDC195	MRS/Skim milk	Silage
L. rhamnosus NCDC24	MRS/Skim milk	Cheese
L. rhamnosus C25 KF806538	MRS/Skim milk	Cheddar cheese

3.2.2 Indicator Organisms

In this study, five pathogenic bacterial cultures are used as test organisms and they were procured from Microbial Type Culture Collection (MTCC), American Type Culture Collection (ATCC), National Collection Type Culture (NCTC) UK and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh. These are as follows:

Table 3.2 Pathogenic organisms used for antimicrobial activity

Pathogens	**Source**
Escherichia coli	ATCC 35150
Salmonella enterica ssp. *enterica* sero type *Abony*	NCTC 6017
Staphylococcus aureus	MTCC 1144
Bacillus cereus	ATCC 13061
Enterococcus faecalis	NCDC 115

3.3 Purity and Conformation of Cultures

3.3.1 Microscopic Examination

Microscopic examination of the *L. acidophilus* NCDC195, *L. rhamnosus* NCDC24, *L. rhamnosus* C25 as well as the test bacteria was subjected to Grams staining and simple staining to check the purity of the cultures.

3.3.2 Catalase test

The catalase test was performed by the slide method, using an inoculating needle; culture from well isolated colony was placed on clean glass slide. A drop of 3% hydrogen peroxide was added on this culture and observed effervescence, indicating positive for test and vice-versa.

3.3.3 Maintenance, Preservation and Propagation of cultures

Lactobacillus cultures and test organisms were propagated in broth media. *Lactobacillus* culture was maintained in litmus milk and stored in refrigerator until use. It was periodically sub-cultured in the same medium once in a week. Culture was activated by sub-culturing before use. All the test bacteria were maintained in nutrient broth/ Brain heart infusion broth (BHI) at 37°C. The test organisms were sub cultured once in 15 days interval. Nutrient agar slants were stored in refrigeration temperature until further used.

3.3.4 Proteolytic activity of *Lactobacillus* species

Proteolytic activity of *Lactobacillus* culture such as *L. acidophilus* NCDC195, *L. rhamnosus* NCDC24 and *Lactobacillus rhamnosus* C25 was measured by qualitatively method. The skim milk agar was prepared with 10 mL of skim milk and 90 mL of nutrient agar, autoclaved at pressure (10 psi for 15 min) separately to avoid coagulation and charring of milk and later mixed aseptically. Twenty ml of culture medium was then poured per plate and allowed to solidify. The cell free culture filtrates obtained are streaked on skim milk agar plates and incubated at 37°C for 24 h. Proteolytic activity was verified by a clear zone in the medium surrounding the wells.

3.4 Collection of colostrum

Colostrum was collected from Livestock Research Centre, NDRI Karnal. Initially skimming was done by centrifugation at 6000 rpm for 10 minutes.

3.4.1 Separation of whey from colostrum

Whey was separated from colostrum by rennet treatment. Colostrum was diluted (1:0.5) and slowly heated to 40-45°C and added with sufficient quantity of calf rennet (1: 4500) to give setting time of 20-30 min. Later, Whey was separated and filter sterilized using 0.45µm filter.

3.4.2 Physico-chemical properties of Colostrum-Whey

3.4.2.1 pH

pH is the negative logarithm of H^+ ions. pH was measured using pH meter (SDFCL, India).

3.4.2.2 Titratable Acidity

Acidity was determined by titration method. 10 ml of colostrum whey was taken into a conical flask. Two to three drops of phenolphthalein indicator were added and titrated against 0.1 Normality NaOH. Initial and final rundown values of NaOH were noted. Titrated three times to obtain an average rundown value. Titratable acidity is calculated using the formula:

Titratable Acidity = $\frac{9 * Normality\ of\ NaOH * Volume\ rundown\ of\ NaOH}{weight\ of\ Sample}$ % Lactic Acid.

(IS 1166 – 1973 Specification for condensed milk. Bureau of Indian Standards, New Delhi)

3.4.2.3 Estimation of Fat

The fat content of colostrum whey was determined by Mojonnier extraction method (AOAC, 2000). 10 g of sample was accurately weighed and transferred to the extraction tube. 1.25 ml of ammonia solution (sp. gr. 0.8974) was added to the sample and mixed well. Then 10 ml of ethyl alcohol (95 percent v/v) was added to the tube and shaken for two minutes. A 25 ml of diethyl ether (sp. gr. 0.72; peroxide free) was added to the tube and contents were shaken for one minute followed by addition of 25 ml petroleum ether (40-60°C BP) and the contents were properly mixed by vigorously shaking the tube for one minute. The tube was left untouched to separate the ethereal layer from the aqueous layer and the supernatant fat portion was decanted in clean, dry, previously weighed aluminum dish (alternatively, low rpm Mojonnier centrifuge can be used for fat layer separation). Extractions were repeated twice using 15 ml of diethyl ether and petroleum ether each time. The solvents were evaporated by heating the dish in the hot air oven at 102±2°C for 2h, cooled and weighed. Heating and weighing were repeated until weights did not show a difference more than 1mg. After this the dish was washed with petroleum ether to wash out fat, carefully leaving behind any insoluble residue in the dish. The dish was again dried and reweighed. The difference in weights represented the weight of fat extracted from the milk. Further, the correct weight of extracted fat was determined by blank determination. If reagent blank was found to be more than 0.5mg, it meant the need for purification or change of reagents. The fat percentage was calculated using the following formula:

Fat percentage (W/W) = $\frac{weight\ of\ fat\ (g)}{Weight\ of\ product\ (g)}$

3.4.2.4 Estimation of Protein

To prepare stranded curve dilutions of different concentration of bovine serum albumin (BSA) was prepared as ranged between 0.05 to 1mg/ml by mixing stock BSA solution

(1mg/ml) with distilled water. Pipetted put 0.2ml protein solution from these dilutions to different test tubes and added 2ml of alkaline copper sulphate reagent (analytical reagent). The solution was then mixed well and incubated at room temperature for 10min. then 0.2ml of Folin's-Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 min. zero the colorimeter with blank and the optical density at 660nm was taken. The absorbance against protein concentration was plotted to get a standard calibration curve. The absorbance of unknown sample was checked and determined concentration of the unknown sample using the standard curve (Appendix III).

3.4.2.5 Total Solids

Total solids percentage is a measure of the suspended and dissolved solids in water. Suspended solids are those that can be retained on a water filter. Dissolved solids are those that pass through a water filter. Initial weight of the empty, dry and clean aluminum dish was noted. Sample of around 3gm was weighed. After spreading the sample evenly, the dish was kept undisturbed for 24 to 36 h in hot air oven at 65 to 70˚C. The final weight of the sample was weighed after drying along with the dish. Again, dish was kept for drying for 1 hour and weighed. The above step was continued until the difference between the successive weightings was not more than 0.05. Total solids in the sample were measured using the formula given below:

$$\%\ \text{Total Solids} = \frac{W3-W1}{W2-W1} * 100$$

3.4.2.6 Lactose

Add 15ml of test solution to the 10ml or 25ml of the Fehling solution and heat to boiling over wire gauge. Boil for about 15 sec and add rapidly further quantities of the solution until only faintest perceptible blue colour remains. Then add 2 to 5 drops of methylene blue and complete the titration by adding the test solution dropwise. Find the corresponding titer from the factors table and apply correction factor.

$$\%\ \text{Lactose} = \frac{\text{Factor}}{\text{Titre}} * 100$$

3.4.3 Microbiological analysis

3.4.3.1 Total Lactic counts

Total lactic count resembles the total starter culture present in the final product. The media used was MRS agar. MRS media being a selective media, it only allows the growth and multiplication of fastidious *Lactobacillus* species. Serial dilutions were made, pour plated on MRS agar and plates were incubated at 37˚C in the inverted position for 24 to 48 h.

3.4.3.2 Coliform count

For the estimation of total coliform count in the final product, violet red bile agar media was used. As coliforms are facultative anaerobic in nature, such an environment was provided by plating double layer of the media. Serial dilutions were made, pour plated on Violet Red Bile Agar (VRBA) and plates were incubated at 37˚C in the inverted position for 24 to 48 h.

3.4.3.3 Yeast and Mold count

Yeast and mold in a product resemble hygiene practices involved in the product. For assessing fungal count in the final product, potato dextrose agar supplemented at the time of pouring agar with sterile 10% tartaric acid to maintain pH was used for favouring the growth of yeast and mold. Serial dilutions were made, pour plated on potato dextrose agar and plates were incubated at 25˚C for three to five days.

3.5 Growth behavior of *Lactobacillus* cultures in colostrum whey

Growth of *Lactobacillus* cultures (*L. acidophilus* NCDC195, *L. rhamnosus* NCDC24 and *Lactobacillus rhamnosus* C25) in colostrum whey were analyzed on the bases of total plate count, pH, titratable acidity of the samples after incubation period 24hr. The viable count of bacteria CFU/mL was determined by the standard method on MRS agar plates (Bio life, Milano, Italy) at 37 °C. Titratable acidity and pH were determined as per method describe in section 3.4.2.1 and 3.4.2.2.

3.6 Standardization of Processing Condition for Production of Protein-Rich Fermented Whey Beverage

3.6.1 Selection of culture

Two strains of *L. rhamnosus* (NCDC-24 and C-25) and one *L. acidophilus* (NCDC 195) were taken and standardized for different parameters such as pH, inoculums level, time and temp of incubation and sugar concentration.

3.6.2 Effect of inoculum level

To study the effect of inoculation level in the samples, filter sterilized colostrum whey was mixed with the cultures at different inoculation level @ 1%, 2% and 3%. After 0, 12, 24, 36 and 48 h incubation period at 37°C, samples were analyzed for various Physico-chemical and microbiological analysis for the selection of culture.

3.6.3 Effect of incubation period

To study the effect of incubation period in the samples, the filter sterilized colostrum whey with inoculated culture was incubated at different incubation time as 12, 24, 36 h and 48h. After incubation period, samples were analyzed for chemical and microbiological analysis.

3.6.4 Effect of incubation temperature

In filter sterilized colostrum whey, the starter cultures were added to bring out the fermentation at 37^0C.

3.7 Optimization of sugar level

To optimize the level of sugar in fermented whey beverage, beverage was supplemented with sugar @ 8, 10 and 12%. Then sugar level was selected on the basis of sensory evaluation and physico-chemical analysis.

3.8 Preparation of Protein Rich Fermented Whey Beverage

Fermented whey beverage was prepared as follows, colostrum is treated with rennet and subjected to filtration to yield colostrum whey, passed through filter (0.45µm), inoculated with 3% NCDC 195, 2% NCDC-195+ 1% C-25 & 2% NCDC-195+ 1% NDC-24 cultures, incubated at 37°C for 24h, added with flavor and sugar, transferred to PET bottles, and then stored at 4°C.

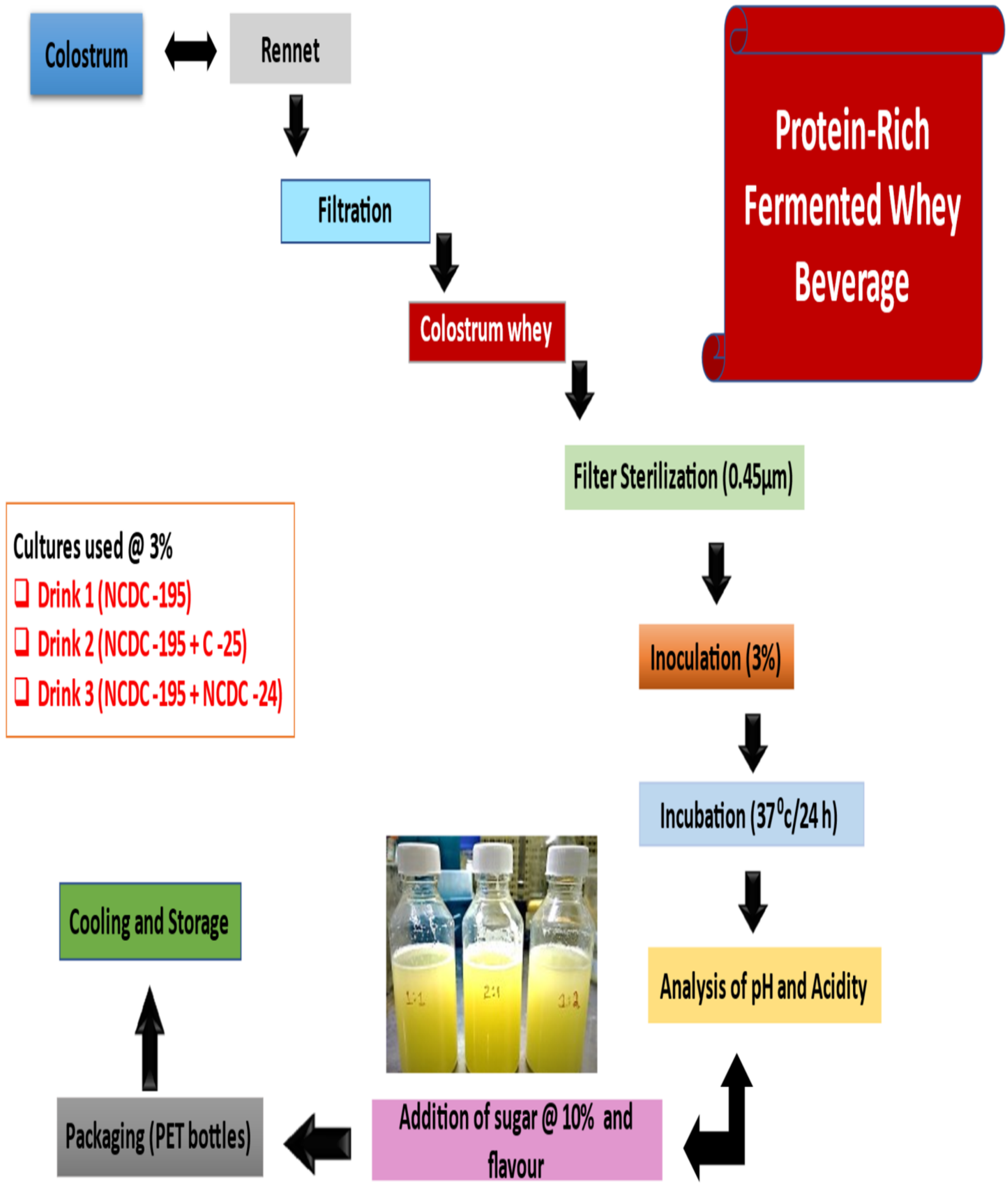


Fig. 3.3 Flowchart for Preparation of Protein-Rich Fermented Whey Beverage

3.9 Analysis of Protein Rich Fermented Whey Beverage

3.9.1 Physico-chemical analysis of Fermented Whey Beverage

Freshly prepared fermented whey beverage samples were analysed for physicochemical parameters- pH, titratable acidity (% lactic acid), total solids (%), fat percent (%), protein (%) and peptides.

3.9.1.1 pH

pH was determined as described earlier in section 3.4.2.1.

3.9.1.2 Acidity

Acidity was determined as described earlier in section 3.4.2.2.

3.9.1.3 Protein content

Protein content was determined as described earlier in section 3.4.2.4.

3.9.1.4 Estimation of peptides by OPA method

150µl of different concentrations of L-Leucine (0.1mg, 0.15mg, 0.2……0.5mg/ml) were taken in different small test tubes. 3ml of freshly prepared OPA solution was added and incubated it for 20 min at room temperature then absorbance was taken at 340nm.The experiment was performed in triplicates. All the reagents used in this part of the study are mentioned in Appendix IV.

3.9.2 Microbiological analysis of fermented whey beverage

Fermented whey beverage was analysed for a total of three parameters i.e., total lactic count, Coliform count and Yeast and Mold.

For microbiological analysis, 11ml of the sample was taken out from fermented whey beverage and diluted with 99 ml of 0.85% sodium chloride diluent and mixed uniformly. Serial dilutions were prepared and appropriate dilutions were plated by pour plate method on different growth media for enumeration of different microorganisms.

3.9.2.1 Total Lactic count

Total Lactic count was determined as described earlier in section 3.4.2.8.

3.9.2.2 Coliform count

Coliform count was determined as described earlier in section 3.4.2.9.

3.9.2.3 Yeast and Mold count

Yeast and Mold was determined as described earlier in section 3.4.2.10.

3.10 Sensory Evaluation of Protein Rich Fermented Whey Beverage

The sensory properties of fermented whey beverage were evaluated by untrained panel of five assessors recruited from students and staff members of NDRI, Karnal. A test form comprising 4 sensory attributes namely color and appearance, sedimentation, flavor/aroma; overall acceptability was given to each assessor. Sensory analysis for the freshly prepared fermented whey beverage was analysed by using nine-point hedonic score card and overall acceptability was noted.

3.11 Bio-Functional Properties of Protein Rich Fermented Whey Beverage

3.11.1 Antimicrobial activity of Protein rich fermented whey beverage

In order to find the maximum inhibitory activity of fermented whey beverage against different test pathogens, antimicrobial activity was checked by agar well diffusion method assay as per the method of Schillinger (1989) with some modifications as mentioned in section 3.6.3. A clear zone of inhibition of more than 1mm was considered positive inhibition.

3.11.2 Antioxidative activity of Protein rich fermented whey beverage

The antioxidant activity of fermented whey beverage was assayed according to the method described by Re et al., (1999). 2,2′-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration in 10 mL of water) and keeping the mixture in the dark at room temperature for 12-16 h before use at continuous agitation. The solution was diluted in 0.1 M phosphate-buffered saline (PBS, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm after equilibration at 30°C. An aliquot of 20µL of sample will be taken in a cuvette and to this 980µL in PBS solution will be added and are mixed for 10 sec. Decrease in absorbance at 734nm is recorded over 10 min at 10 sec interval using Spectrophotometer. Results were expressed as Trolox equivalent antioxidant capacity (TEAC) values. The experiment was performed in triplicates. All the reagents used in this part of the study are mentioned in Appendix V.

3.11.2.1 Standard curve of Trolox for ABTS$^+$ method

180 µl of ABTS working solution and 20 µl PBS was added to a well of 96 well micro plate and initial absorbance was recorded at 734 nm using microplate reader (Model: Infinite F200 Pro, Tecan, Austria). Calibration curve of Trolox, concentration ranging from 0-1000 µM was prepared by appropriate dilution of Trolox solution. 20 µl of standards were added to 180 µl of ABTS working solution.

The contents were mixed for 5 seconds and change in absorbance at 734 nm was recorded after 10 minutes. The standard curve was prepared by plotting concentration (µM) of Trolox (X-axis) v/s %inhibition (Y-axis) is mentioned in the Appendix V.

3.11.3 Determination of Angiotensin-I Converting Enzyme (ACE) inhibitory activity

The Hip-his-leu was dissolved in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. Then, 110 µl of 5 mM Hip-his-leu solution was mixed with 100µl 0.1 M sodium borate buffer (pH 8.3) then, 20 µl of fermented whey sample was added. The reaction was initiated by the addition of 20 µl (4 mU in 250 µl) of ACE enzyme and the mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 250µl of 1N HCI. The hippuric acid liberated by the ACE was extracted with 1.5ml ethyl acetate (by centrifugation at 3000g for 10 min). An aliquot of 750µl of the upper organic layer was collected and evaporated completely by heating at 95°C for 20 min. The sediment was dissolved in 1 ml of distilled water and absorbance was measured spectrophotometrically at 228 nm.

3.12 Storage Study of Fermented Whey Beverage

Storage study of fermented whey beverage was evaluated at refrigeration condition (4^0C) for 7 days and checked for its physicochemical analysis (pH and acidity), microbiological analysis (total lactic count, coliform count and yeast and mold count) and bio-functional activities (antimicrobial and antioxidant activity).

3.13 Reverse Phase High Performance Liquid Chromatography (RP-HPLC) of Fermented Whey Beverages

The fermented whey beverages are analysed through HPLC by the method given by Najafian and Babji (2014) with some modifications. Product sample (20µl) was injected into

HPLC system (Shimadzu, Japan) with the help of manual injector. The mobile phases were (A) 0.1% (v/v) tri-fluroacetic acid (TFA) in deionized water, and (B) 0.1% (v/v) TFA in 100% acetonitrile. A linear gradient of 100% eluent A for 5 min and with the following increase in eluent B: 0-5 min, 0% eluent B, 5-10 min, 0-50min eluent B, 10-30 min, 50-65% eluent B, 30-34 min, 65-100% eluent B, 35-40 min, 100% eluent B, 40-45 min 100-0% eluent B was performed for peptide separation. The flow rate was 0.6ml/min and the UV absorbance of the eluent was monitored at 214nm.

3.14 STATSTICAL ANALYSIS

Data was expressed as mean values with standard errors. In experiments, wherever required, two-way analysis of variance (ANOVA) was applied for comparison to test for any significant differences in the mean values. The differences among treatments were compared at 5 % level of significance.

CHAPTER - 4

Results and Discussion

RESULTS AND DISCUSSIONS

This chapter includes the results on the optimization on conditions for preparation of protein-rich fermented whey beverage using colostrum whey and bio-functional properties. The selected NCDC cultures i.e., *Lactobacillus acidophilus 195*, *Lactobacillus rhamnosus 24* and *Lactobacillus rhamnosus* C25 were used for preparation of fermented whey beverage. Evaluation of acceptability of fermented whey beverage after storage at refrigeration condition. Evaluation of antimicrobial, antioxidant and ACE-inhibitory activity of whey beverage was done. The results of the present study are represented in **Tables and Figures**. The culture *Lactobacillus acidophilus* 195 used in this study is probiotic strain and it is suitable in manufacture of fermented whey beverage and the cultures *Lactobacillus rhamnosus* 24 and *Lactobacillus rhamnosus* C25 were highly proteolytic and helps in production of bioactive peptides.

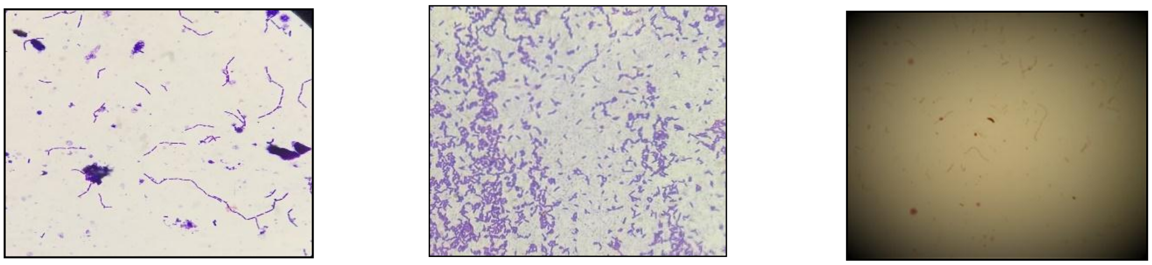
4.1 Purity of cultures

Purity and cell morphology of both test organisms and *Lactobacillus* cultures were examined using Gram's staining. The *Lactobacillus acidophilus* 195 was Gram-positive, rods arranged in chains whereas, *Lactobacillus rhamnosus* 24 and *Lactobacillus rhamnosus* C25 was Gram-positive, short rods. The test organisms were both Gram-positive and Gram-negative and in different arrangement and shape as shown in **Table 4.1 and Fig 4.1**. The *Lactobacillus* species were catalase negative and all the test organisms were catalase positive.

Table 4.1 Microscopic examination of *Lactobacillus* cultures and test organisms

Test organism	Gram's Reaction	Shape and Arrangement
Lactobacillus acidophilus NCDC 195	Positive	Rod
Lactobacillus rhamnosus C25 KF806538	Positive	Rod
Lactobacillus rhamnosus NCDC 24	Positive	Rod
Staphylococcus aureus MTCC 1144	Positive	Round

Bacillus cereus ATCC 14459	Positive	Rod
Escherichia coli ATCC *25922*	Negative	Rod
Enterococcus faecalis NCDC 115	Positive	Cocci
Salmonella enterica NCTC 6017	Negative	Rod



(i) (ii) (iii)

Fig 4.1 Morphology of *Lactobacillus* cultures: i) *Lactobacillus acidophilus* NCDC195 ii) *Lactobacillus rhamnosus* C25 iii) *Lactobacillus rhamnosus* NCDC24

4.2 Proteolytic activity of *Lactobacillus* cultures

Lactobacillus cultures were (screened for protease production on skim milk agar plate by indicating clear zone around the streaked colony and protocol mentioned in 3.3.4. Proteinases and peptidases are the enzymes responsible for proteolysis and these enzymes cleave large protein complexes into simple and smaller peptides.

Lactobacillus acidophilus NCDC195, *Lactobacillus rhamnosus* NCDC24 and *Lactobacillus rhamnosus* C25 were proteolytic in nature and showed a clear zone on skim milk agar around streaked area (**Table 4.2 and Plate 4.1**). Proteolytic system of Lactic Acid Bacteria consists of extracellular or cell envelope proteinases which initially cleave complex protein molecules to larger peptides. The intracellular peptidases, which further break the larger peptides to smaller peptides and amino acids and these, are transported through cytoplasmic membrane using specific transport proteins (Griffiths and Tellez, 2013). Gandhi and Shah, (2014) reported that *Lactobacillus acidophilus*, *L. helveticus*, *L. bulgaricus* and *S. thermophilus* have high proteolytic activity.

Table 4.2 Proteolytic activity of *Lactobacillus* cultures on skim milk agar

Sl.no	Culture	Zone of clearance
1.	*Lactobacillus acidophilus* NCDC195	++
2.	*Lactobacillus rhamnosus* C25	++
3.	*Lactobacillus rhamnosus* NCDC24	++

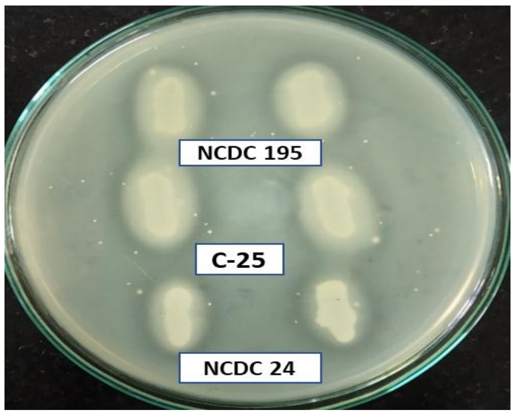


Plate 4.1 Proteolytic activity of *Lactobacillus* cultures

4.3 Physico-chemical analysis of colostrum whey

Colostrum samples were procured from Livestock Research Centre (LRC) of National Dairy Research Institute, Karnal from Gir and Sahiwal indigenous breeds and pooled. Whey was separated from colostrum by rennet treatment. Colostrum was diluted (1:0.5) and slowly heated to 40-45°C and added with sufficient quantity of calf rennet (1: 4500) to give setting time of 20-30 min. Later, Whey was separated and filter sterilized using 0.45µm filter. Initially Physico-chemical analysis of colostrum whey is evaluated which includes pH, acidity, fat, protein and lactose. The results related to these parameters are presented in **Table 4.3**.

The mean protein content of colostrum whey was 6.42±0.05 %, fat content was 0.206±0.01 %, lactose content was 2.46±0.10 %, and pH and acidity were 6.32±0.016 and 0.182±0.02 % L.A respectively (**Table 4.3**).

Table 4.3 Physico-chemical analysis of colostrum whey

Components	Percentage %
Fat	0.206±0.01
Protein	6.42±0.05
Lactose	2.46±0.10
pH	6.32±0.016
Acidity (%L. A)	0.182±0.02

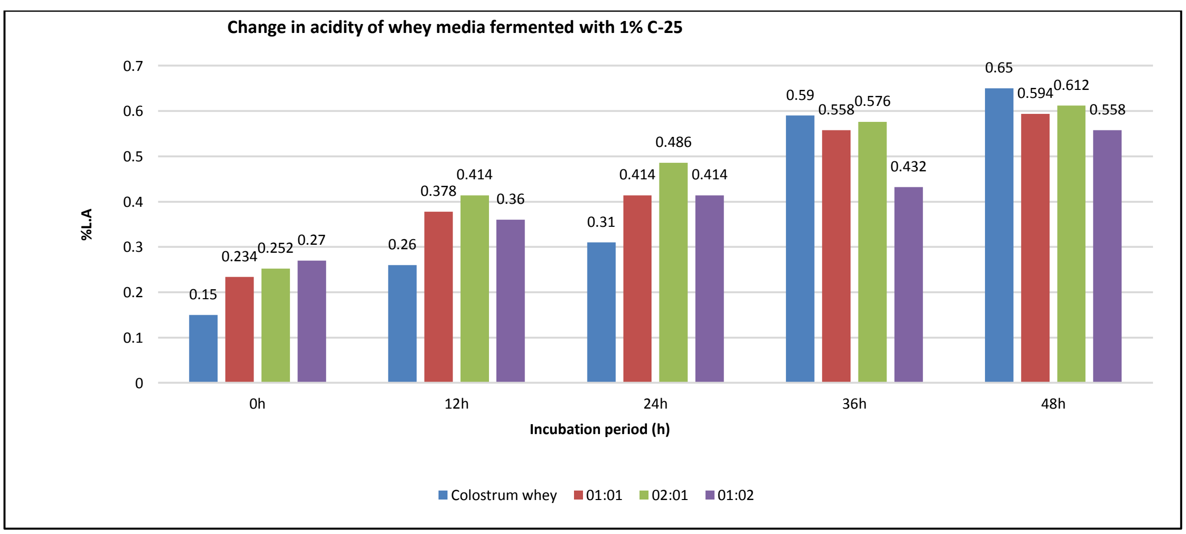
Data is presented as Mean ± SD (n=3)

Ayar et al., (2016) stated that average composition of colostrum serum had protein 15.3-25.4%, fat 3.9-4.4%, lactose 2.7-4.6%.

4.4 Selection of whey media

Whey media was prepared by mixing different proportions of colostrum whey: cheese whey (1:1, 2:1 & 1:2) and colostrum whey (1:0.5% water). Selection of whey media is evaluated by analyzing pH and acidity of different whey media fermented with *Lactobacillus rhamnosus* C25 culture at 1% and 2% concentration for 48h.

There was no significant difference in pH and acidity of different whey media fermented for 48h (F**ig 4.2 and 4.3**).



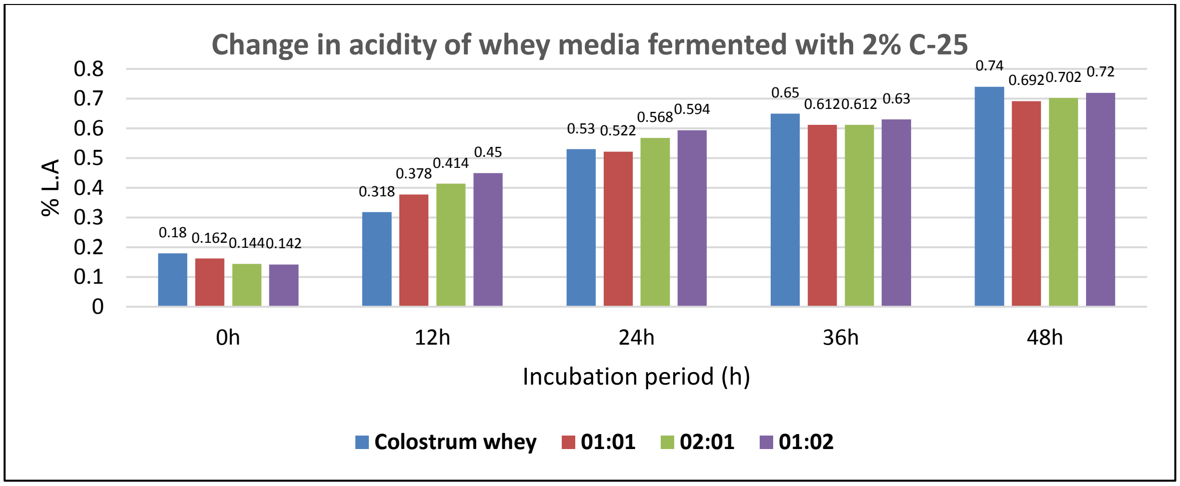


Fig 4.2 Change in acidity of whey media fermented with C25 culture @ 1% and 2%.

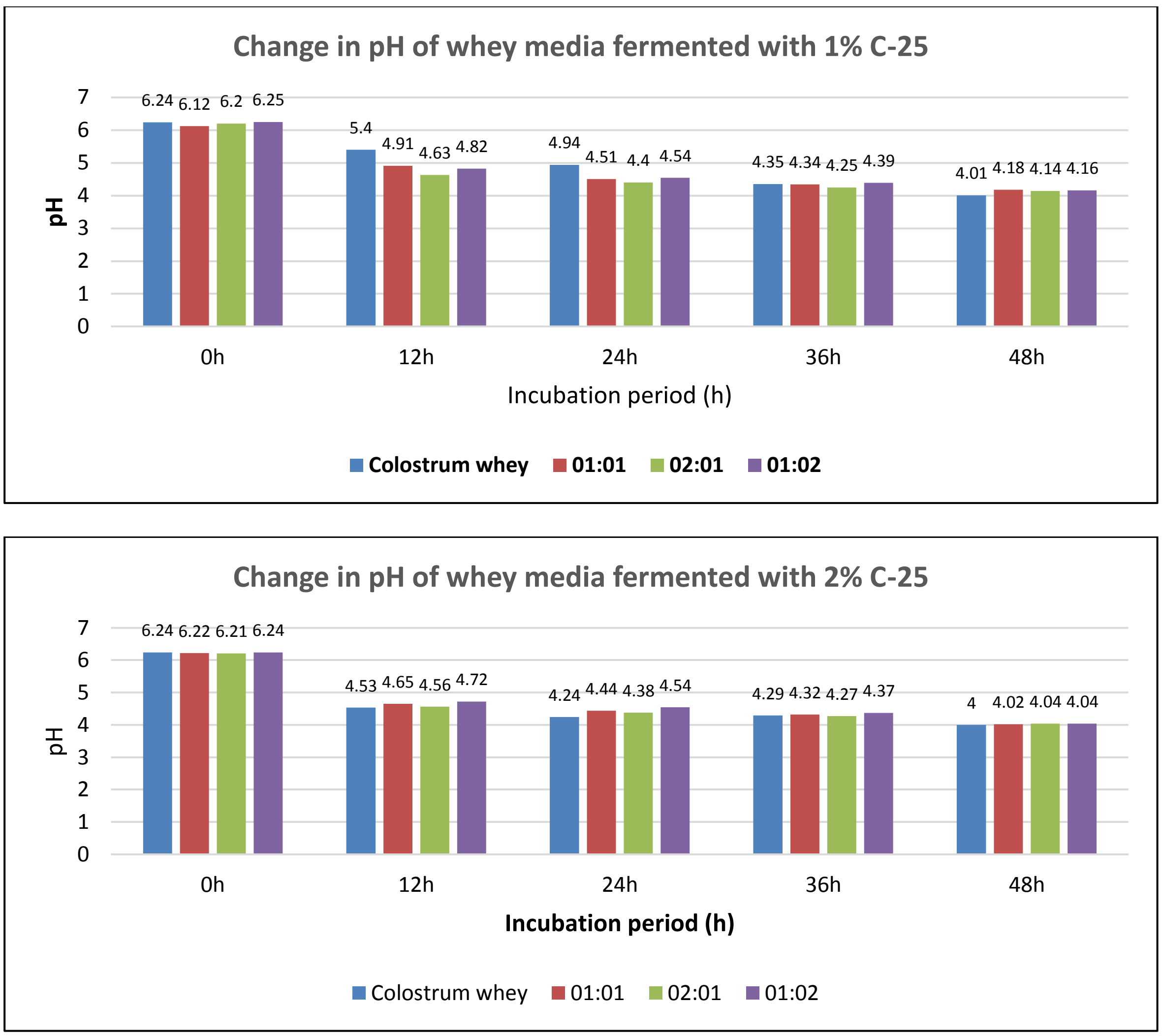


Fig 4.3 Change in pH of whey media fermented with C25 culture @ 1% and 2%.

Whey media was analysed for its protein content and found to be the combination of whey media (1:1, 2:1 & 1:2) have showed protein content of 2.3-4.3%, whereas, colostrum whey (1:0.5% water) showed 6.3-6.5% of protein content (**Table 4.4**). Hence, colostrum whey (1:0.5% water) was selected for further study for preparation of protein rich fermented whey beverage.

Functional high protein fermented beverage was prepared with the addition of whey protein concentrate (WPC 80), skim milk powder, sucrose and fermented with single starter i.e., *Lactobacillus plantarum* DK211 and *Lactococcus lactis* R704. The functional protein beverage had high protein (9%), low-fat content (0.2%) (Cho et al., 2015).

Table 4.4 Protein content (%) of whey media

Whey media	Protein content (%)	
	Before fermentation	After fermentation
1:1	3.5	3.2
2:1	4.25	4.04
1:2	2.57	2.44
Colostrum whey (1:0.5%)	**6.52**	**6.19**

4.5 Growth kinetics of different *Lactobacillus* cultures in colostrum whey

Proteolytic NCDC cultures *Lactobacillus acidophilus* NCDC 195; *L. rhamnosus* NCDC 24 and *L. rhamnosus* C25 (cheese isolate) were used for high protein whey fermentation. Growth kinetics of these selected *Lactobacillus* cultures was studied in order to distinguish their ability to ferment the colostrum whey with desired levels of pH, acidity and log count (CFU/mL). The *Lactobacillus* isolates were inoculated individually (195, 25, 24) and in combination (195+25, 195+24) in colostrum whey @ 1% in 37^{0}C for 48h.

4.5.1 Change in pH with the fermentation colostrum whey

pH of selected three single *Lactobacillus* cultures and two combinations with the fermentation colostrum whey were observed. pH values of selected *Lactobacillus* cultures @ 1% culture concentration are presented in **Table 4.5** and **Fig 4.4.**

The cultures 195, 195+ 25 and 195+ 24 showed pH in the range of 4.63-4.44, 4.34-4.27 and 4.19-4.12 with 24h, 36h and 48h incubation period respectively. Whereas, 25 and 24 cultures showed pH of 4.94, 4.6 and 4.34 with 24h, 36h and 48h incubation period respectively. From the above results obtained, cultures 25 and 24 are taking more time to show growth in colostrum whey, however cultures 195, 195+ 25 and 195+ 24 tend to adopt easily and showed maximum growth in colostrum whey by gradual decrease in pH with incubation period.

Table 4.5 pH values of colostrum whey fermented with *Lactobacillus* isolates in single and in combination

Incubation period / Cultures	0h	12h	24h	36h	48h
195	6.13±0.02[a]	5.11±0.04[b]	4.63±0.16[b]	4.34±0.09[a]	4.19±0.09[a]
25	6.24±0.01[b]	5.4±0.08[c]	4.94±0.04[c]	4.60±0.07[b]	4.32±0.08[b]
24	6.3±0.02[c]	5.6±0.07[d]	5±0.02[d]	4.64±0.04[c]	4.34±0.02[b]
195+25	6.22±0.02[b]	5.05±0.04[a]	4.44±0.02[a]	4.27±0.06[a]	4.12±0.04[a]
195+24	6.15±0.03[a]	5.03±0.02[a]	4.45±0.02[a]	4.30±0.02[a]	4.15±0.03[a]

Data is presented as Mean ± SD (n= 3)
a, b, c, d Means within row with different lowercase superscript letters are significantly different ($p < 0.05$) from each other.

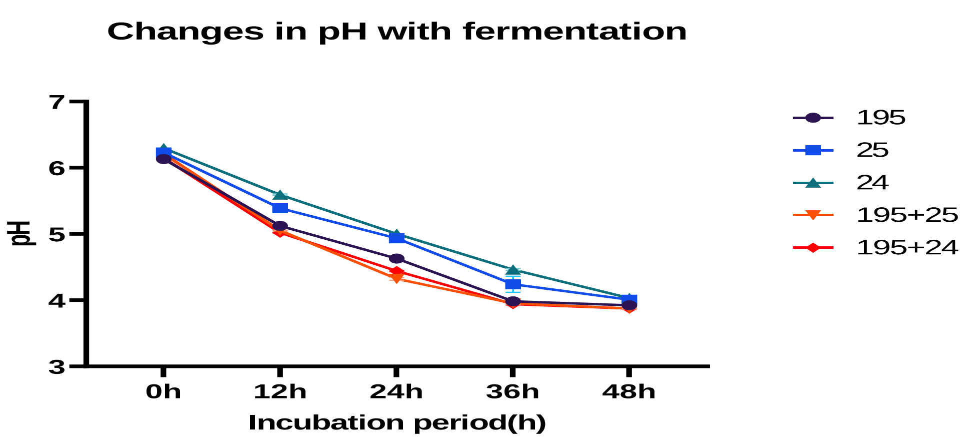


Fig 4.4 pH values of colostrum whey fermented with *Lactobacillus* isolates in single and in combination. These values are expressed in mean± S.D, n=3, p>0.05

4.5.2 Change in acidity with the fermentation of colostrum whey

Acidity of selected *Lactobacillus* cultures individually and in combinations with the fermentation colostrum whey was observed. Acidity values of selected *Lactobacillus* cultures @ 1% culture concentration are presented in **Table 4.6** and **fig 4.5.**

Similar to pH, the cultures 25 and 24 showed acidity of 0.3%, 0.59% and 0.65% L. A, whereas, 195, 195+ 25 and 195+ 24 cultures showed 0.58-0.62%, 0.65-0.69% and 0.76-0.80% with 24h, 36h and 48h incubation period respectively. This data represents that, cultures 195, 195+ 25 and 195+ 24 take less time to adopt in colostrum whey and achieved maximum growth in by gradual increase in acidity with incubation period.

Table 4.6 Acidity values of colostrum whey fermented with *Lactobacillus* isolates in single and in combination

Incubation period / Cultures	0h	12h	24h	36h	48h
195	$0.18{\pm}0.04^{a}$	$0.30{\pm}0.08^{b}$	$0.58{\pm}0.11^{b}$	$0.65{\pm}0.01^{c}$	$0.78{\pm}0.07^{b}$
25	$0.15{\pm}0.02^{b}$	$0.26{\pm}0.03^{c}$	$0.31{\pm}0.03^{c}$	$0.59{\pm}0.01^{d}$	$0.65{\pm}0.02^{c}$
24	$0.15{\pm}0.02^{b}$	$0.25{\pm}0.02^{c}$	$0.30{\pm}0.01^{c}$	$0.56{\pm}0.07^{e}$	$0.63{\pm}0.04^{c}$
195+25	$0.15{\pm}0.04^{b}$	$0.34{\pm}0.03^{a}$	$0.62{\pm}0.03^{a}$	$0.69{\pm}0.02^{a}$	$0.80{\pm}0.03^{a}$
195+24	$0.16{\pm}0.04^{ab}$	$0.32{\pm}0.03^{b}$	$0.60{\pm}0.02^{b}$	$0.67{\pm}0.02^{b}$	$0.76{\pm}0.03^{b}$

Data is presented as Mean ± SD (n= 3)
a, b, c, d Means within row with different lowercase superscript letters are significantly different ($p < 0.05$) from each other.

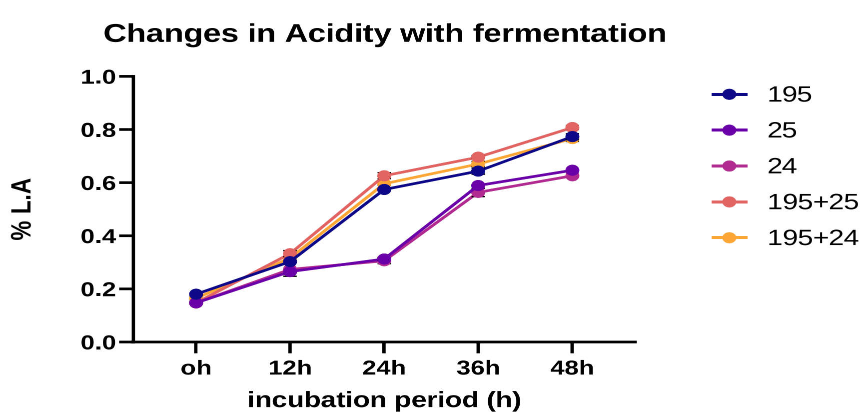


Fig 4.5 Acidity values of colostrum whey fermented with *Lactobacillus* isolates in single and in combination. These values are expressed in mean± S.D, n=3, p>0.05

Whey beverage was prepared from whey, skim milk and carrot juice fermented with yoghurt starters *(Streptococcus thermophilus, L. bulgaricus),* probiotic bacteria like *L. acidophilus and Bifidobacterium bifidum.* The final product showed 90.5 % antioxidant activity, viable cell counts 8.7 log CFU/mL, pH 4.6 and overall sensory score of 8.97. Implementation of this beverage production during cheese manufacture increases overall profit of industry and improves the product quality (Arsic et al., 2018).

4.5.3 Change in *Lactobacillus* count (log CFU/mL) with the fermentation colostrum whey

Log counts (CFU/mL) of selected *Lactobacillus* cultures individually and in combinations were evaluated and it was found that after fermentation, the cultures 195, 195+25 & 195+24 achieved 8 log counts/mL in 24h, whereas, the cultures C25 & NCDC 24 took 48h to achieve 8 log counts/mL. Log counts of selected *Lactobacillus* cultures @ 1% culture concentration are presented in **Table 4.7** and **Fig 4.6.**

Table 4.7 *Lactobacillus* counts of colostrum whey fermented with *Lactobacillus* cultures in single and in combination

Incubation period / **Cultures**	**0h**	**12h**	**24h**	**36h**	**48h**
195	6.40±0.02^{c}	7.14±0.11^{c}	8.07±0.31^{b}	8.25±0.04^{b}	8.27±0.53^{b}
25	6.21±0.06^{d}	6.95±0.32^{d}	7.69±0.32^{c}	7.9±0.06^{c}	8.16±0.21^{c}
24	6.18±0.02^{d}	6.42±0.24^{e}	7.52±0.22^{d}	7.84±0.31^{d}	8.07±0.52^{d}
195+25	6.52±0.04^{b}	7.38±0.21^{a}	8.16±0.02^{a}	8.32±0.22^{a}	8.31±0.11^{a}
195+24	6.47±0.22^{a}	7.23±0.31^{b}	8.1±0.24^{a}	8.23±0.06^{b}	8.223±0.21^{b}

Data is presented as Mean ± SD (n= 3)
a, b, c, d Means within row with different lowercase superscript letters are significantly different ($p< 0.05$) from each other.

After fermentation to achieve 8 log counts the cultures 195, 195+25 & 195+24 took 24h whereas, the cultures 25 & 24 took 48h to achieve 8 log counts. From the above results, it signifies those 25 and 24 cultures have longer lag phase and takes more time to show growth in colostrum whey, however cultures 195, 195+25 & 195+24 tends to adopt easily in colostrum whey and showed maximum counts of 8.22-8.31 log counts (CFU/mL) in 48h. So, based on pH, acidity and *Lactobacillus* count best combination was selected for further optimization of the product. The best combinations were found to be **195, 195+25 and 195+24**.

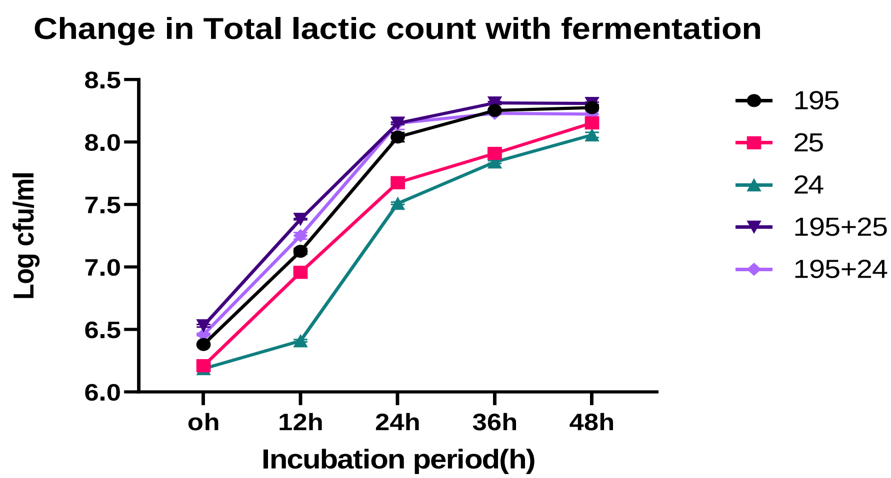


Fig 4.6 *Lactobacillus* counts (log CFU/mL) of colostrum whey fermented with *Lactobacillus* isolates in single and in combination. These values are expressed in mean± S.D, n=3, p>0.05

Probiotic beverage was developed from de-protenized whey fermented with *L. rhamnosus* NCDO 243, *B. bifidum* NCDO 2715 and *Propionibacterium freudenreichii subsp. Shermanii* have showed probiotic counts over 8 log CFU/mL and high organoleptic properties after 10 days of storage (Maity et al., 2008).

Pereira et al., (2015) reported that on utilization of ultrafiltered whey permeate (later concentrated by reverse osmosis) or retentate (rich in whey proteins) in preparation of probiotic whey beverage fermented with *L. acidophilus, L. casei* and *L. rhamnosus* @ 0.3% have showed viable cell counts over 7 log CFU/mL and acceptable sensory properties after 14 days of cold storage.

4.6 Optimization of processing conditions for preparation of protein-rich fermented whey beverage

Based on the results obtained from preliminary study of screening of cultures, 195 (*L. acidophilus*), 195+25 (*L. acidophilus+ L. rhamnosus*) and 195+24 (*L. acidophilus+ L. rhamnosus*) cultures were selected for further studies. Processing conditions for preparation of protein rich fermented whey beverage using 195, 195+25 and 195+24 cultures were optimized with decrease in desired pH, increase in desired acidity and total *Lactobacillus* count. Here, the optimization of fermented whey beverage is done for different inoculum level (i.e., 1%, 2% & 3%) and incubation period (12h, 24h, 36h, 48h) at incubation temperature (37^0C).

4.6.1 Change in pH with different levels of inoculum and incubation period

Inoculum level is a significant factor and plays an important role in obtaining maximum growth of cultures. Variation in levels of inoculum might change the growth of culture, pH, acidity, and production of antimicrobial components.

From the data presented in **Fig 4.7,** it was observed that at 37^0C, there was significant decrease in pH between 1%, 2% and 3% inoculum levels ($p>0.05$) and 12h, 24h, 36h, 48h incubation period ($p>0.05$) among all the *Lactobacilli* cultures. At 3% inoculum level and incubation period of 24h the cultures have shown desired pH of 3.8-4.0 and found no significant difference in pH between 24h, 36h and 48h incubation period of cultures.

Kumari et al., (2015) observed the considerable decrease in pH during fermentation of paneer whey at 37^0C, 39^0C and 42^0C. Decrease in pH in the range of 3.98-4.12 was observed at 1% inoculum level for all the cultures and at 2% inoculum level observed the pH in range of 3.65-3.82 for LAB isolates, whereas 3.68-3.74 for combination of LAB and yoghurt cultures. The maximum decrease in pH of 4.14 is observed in LAB and 4.13 pH observed in combination LAB and yoghurt cultures at 1.5% inoculum level at 42^0C. From these results it shows that change in pH is varied with different inoculum levels.

Whey beverage was prepared from whey, skim milk and carrot juice fermented with yoghurt starters *(S. thermophilus, L. bulgaricus)*, probiotic bacteria like *L. acidophilus and B. bifidum.* The final product showed 90.5 % antioxidant activity, viable cell counts 8.7 log

CFU/mL, pH 4.6 and overall sensory score of 8.97. Implementation of this beverage production during cheese manufacture increases overall profit of industry and improves the product quality (Arsic et al., 2018).

Table 4.8 pH values with different levels of inoculum and incubation period

Cultures / **Incubation period**	**Inoculum level**	**195**	**195+25**	**195+24**
0h	**1%**	6.13 ± 0.03^{Ba}	6.15 ± 0.06^{Bb}	6.13 ± 0.03^{Ba}
	2%	6.13 ± 0.05^{Ba}	6.12 ± 0.03^{Ba}	6.12 ± 0.05^{Ba}
	3%	6.10 ± 0.02^{Aa}	6.08 ± 0.02^{Aa}	6.08 ± 0.02^{Aa}
12h	**1%**	5.11 ± 0.04^{Ba}	5.05 ± 0.04^{Ca}	5.03 ± 0.02^{Ba}
	2%	4.89 ± 0.08^{Ab}	4.76 ± 0.09^{Ba}	4.8 ± 0.01^{Aa}
	3%	4.82 ± 0.03^{Ac}	4.69 ± 0.07^{Aa}	4.75 ± 0.01^{Ab}
24h	**1%**	4.63 ± 0.16^{Cb}	4.44 ± 0.02^{Ca}	4.45 ± 0.02^{Ca}
	2%	4.24 ± 0.12^{Ba}	4.2 ± 0.07^{Ba}	4.22 ± 0.03^{Ba}
	3%	3.98 ± 0.06^{Aa}	3.96 ± 0.13^{Aa}	3.96 ± 0.02^{Aa}
36h	**1%**	4.34 ± 0.09^{Ba}	4.27 ± 0.06^{Ca}	4.30 ± 0.02^{Ba}
	2%	4.04 ± 0.04^{Aab}	4.0 ± 0.09^{Bb}	4.02 ± 0.04^{Aa}
	3%	3.96 ± 0.01^{Ab}	3.92 ± 0.09^{Aa}	3.94 ± 0.06^{Aa}
48h	**1%**	4.19 ± 0.09^{Ca}	4.12 ± 0.04^{Ca}	4.15 ± 0.03^{Ca}
	2%	3.98 ± 0.09^{Ba}	3.96 ± 0.07^{Ba}	3.98 ± 0.12^{Ba}
	3%	3.95 ± 0.07^{Ab}	3.9 ± 0.13^{Aa}	3.91 ± 0.02^{Aa}

Data is presented as Mean ± SD (n= 3)

A, B, C, a, b, c, d, e Mean ± SD bearing different superscripts column wise (capital alphabets) and row wise (small alphabet) differ significantly ($p < 0.05$).

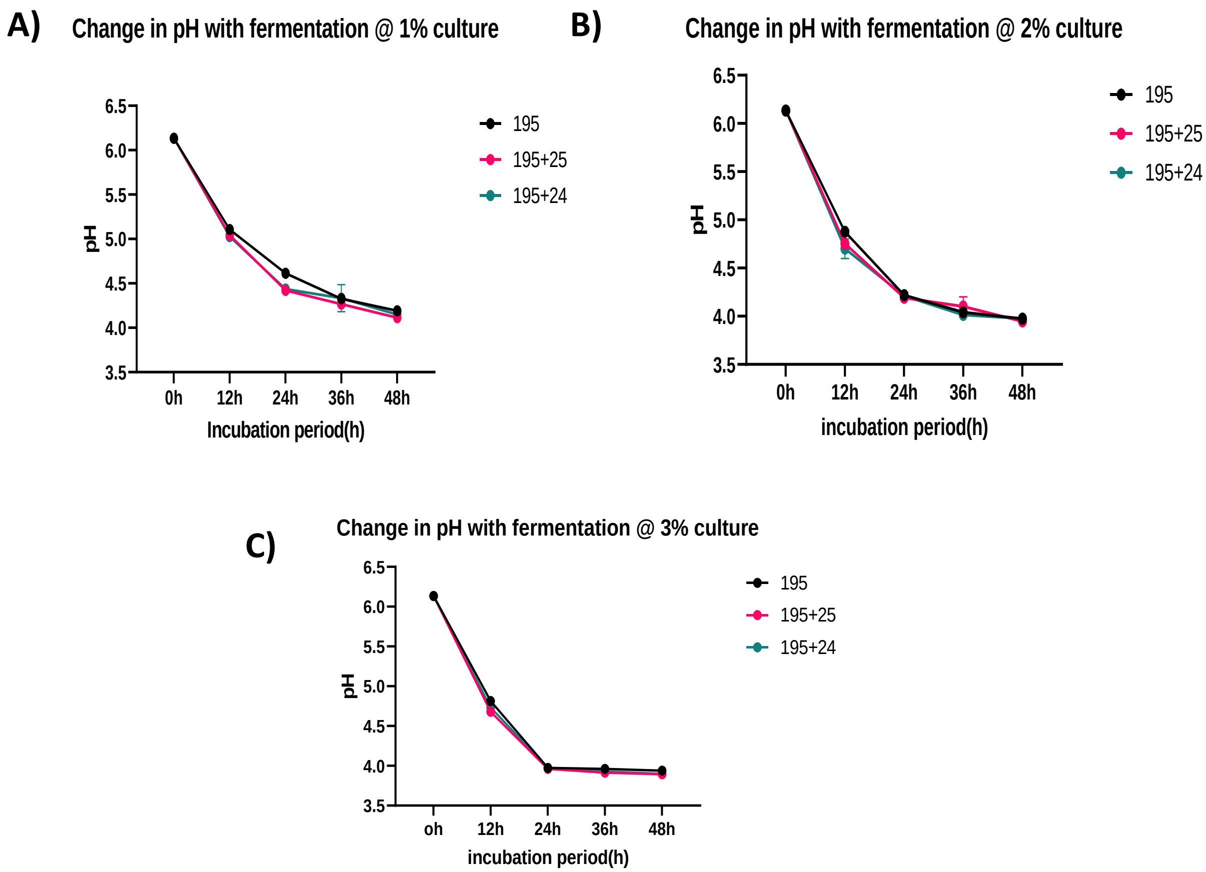


Fig 4.7 pH values with different levels of inoculum and incubation period (A) 1%, (B) 2% and (C) 3%. These values are expressed in mean± S.D, n=3, p>0.05

4.6.2 Change in acidity with different levels of inoculum and incubation period

Temperature and incubation period have shown the important effect on the cultures viability because each microorganism will have an optimum growth temperature and specific log period phase, where the culture shows its maximum growth and produce antimicrobial components. So, the cultures i.e., 195, 195+25 and 195+24 are used for fermentation of colostrum whey at different incubation period of 12h, 24h, 36h and 48h.

From the data presented in **Table 4.9 & Fig 4.8,** it was observed that at 37^0C there was significant increase in acidity between 1%, 2% and 3% inoculum levels (p>0.05) and 12h, 24h, 36h, 48h incubation period (p>0.05) among all the isolates. At 3% inoculum level and incubation period of 24h the cultures have showed desired acidity of 0.95-1.05% L.A and found no significant difference in acidity between 24h, 36h and 48h incubation period of all three cultures.

Increase in acidity in the range of 0.68-0.89% L. A was observed at 1% inoculum level for all the cultures, at 1.5% inoculum level cultures have showed acidity of 0.92-1.13% L. A. The maximum increase in acidity of 1.14-1.28% L.A is observed at 2% inoculum level at 42^0C. From these results it shows that increase in acidity is varied with different inoculum levels and incubation period.

Table 4.9 Acidity values with different levels of inoculum and incubation period

Cultures / **Incubation period**	**Inoculum level**	**195**	**195+25**	**195+24**
0h	**1%**	$0.18±0.02^{Ba}$	$0.18±0.02^{Ba}$	$0.18±0.02^{Ba}$
	2%	$0.20±0.04^{Aa}$	$0.21±0.02^{Aa}$	$0.20±0.04^{Aa}$
	3%	$0.20±0.04^{Aa}$	$0.21±0.02^{Aa}$	$0.20±0.04^{Aa}$
12h	**1%**	$0.30±0.08^{Cb}$	$0.34±0.03^{Ca}$	$0.32±0.03^{Ca}$
	2%	$0.35±0.10^{Bb}$	$0.38±0.04^{Ba}$	$0.36±0.11^{Bb}$
	3%	$0.4±0.04^{Ab}$	$0.47±0.04^{Aa}$	$0.42±0.04^{Ab}$
24h	**1%**	$0.58±0.11^{Cb}$	$0.62±0.03^{Ca}$	$0.60±0.02^{Ca}$
	2%	$0.66±0.02^{Bc}$	$0.75±0.05^{Ba}$	$0.72±0.04^{Bb}$
	3%	$0.95±0.13^{Ac}$	$1.05±0.01^{Aa}$	$0.98±0.03^{Ab}$
36h	**1%**	$0.65±0.01^{Cb}$	$0.69±0.02^{Ca}$	$0.67±0.02^{Cb}$
	2%	$0.87±0.11^{Ba}$	$0.89±0.03^{Ba}$	$0.888±0.02^{Ba}$
	3%	$0.98±0.02^{Ac}$	$1.13±0.01^{Aa}$	$1.05±0.03^{Ab}$
48h	**1%**	$0.78±0.07^{Cb}$	$0.80±0.03^{Ca}$	$0.76±0.03^{Cb}$
	2%	$0.89±0.12^{Bb}$	$0.90±0.01^{Bab}$	$0.92±0.02^{Ba}$
	3%	$1.06±0.03^{Ac}$	$1.15±0.08^{Aa}$	$1.1±0.02^{Ab}$

Data is presented as Mean ± SD (n= 3)

A, B, C, a, b, c, d, e Mean ± SD bearing different superscripts column wise (capital alphabets) and row wise (small alphabet) differ significantly ($p < 0.05$).

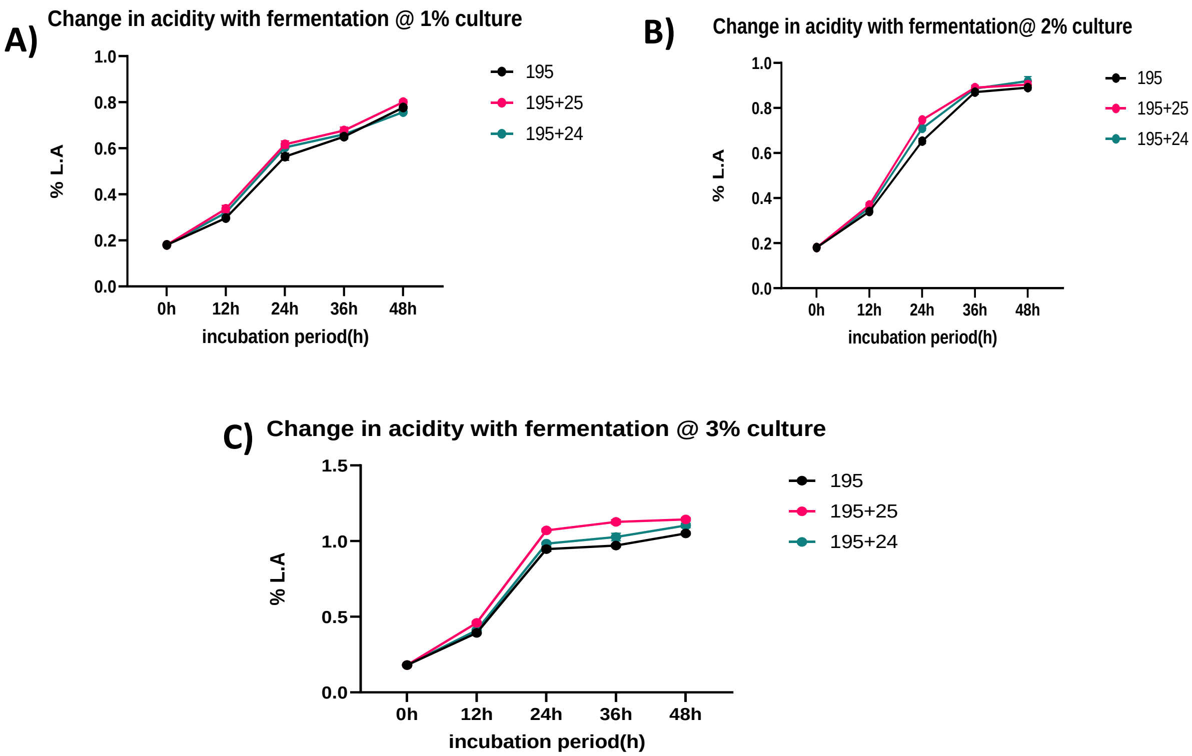


Fig 4.8 Acidity values with different levels inoculum and incubation period (A) 1%, (B) 2% and (C) 3%. These values are expressed in mean± S.D, n=3, p>0.05

Kumari et al., (2015) observed the considerable increase in acidity during fermentation of paneer whey at 37^{0}c, 39^{0}C and 42^{0}c. The maximum increase in acidity was found in case alone LAB (0.81% L.A) whereas with the combination of yoghurt cultures have shown 0.61% L.A at different durations of fermentation. And postulated the reason for slow acid production i.e., the incubation temperature 37^{0}C was not favorable for the growth of yoghurt cultures than LAB.

Singh et al., (2016) investigated on development of whey beverage supplemented with soy extract and curcumin fermented with *L. acidophilus* NCDC 195 (LA 195) and *S. thermophilus* NCDC 323. Results found to be, final product have showed acidity of 1.38-1.41% LA and total viable count were 9.09- 9.39 log CFU/mL after fermentation at 37^{0}C for 24h

4.6.3 Change in total *Lactobacillus* count with different levels of inoculum and incubation period

Total *Lactobacillus* counts were evaluated by plating on MRS agar for colostrum whey fermented at different inoculum levels and incubation period. From the data presented in **Fig 4.9,** it was observed that at 37^0C there was significant increase in *Lactobacillus* between 1%, 2% and 3% inoculum levels ($p>0.05$) and 12h, 24h, 36h, 48h incubation period ($p>0.05$) among all the isolates. At 3% inoculum level and incubation period of 24h the cultures have showed high *Lactobacillus* counts (Log CFU/mL) of 8.6-8.8 and found no significant difference in *Lactobacillus* counts between 24h and 36h.

At 48h incubation period the cultures have showed decrease in *Lactobacillus* counts and found significant difference in counts between 24h, 36h and 48h. This signifies that the cultures have attained decline phase and showed the decrease in *Lactobacillus* counts at 48h **(Table 4.10).**

From the results obtained, the conditions for preparation of protein rich fermented whey beverage were optimized as 37^0C for 24h with 3% inoculum size on the basis of desirable pH (3.80-4.0), titratable acidity (0.9-1.10% L.A) and total *Lactobacillus* count (10^8 CFU/mL).

In a similar study, Kumari, (2015) reported that the optimized conditions for preparation of probiotic whey beverage were 1.5% inoculum size at 39^0C for 18h incubation period, which showed maximum growth of probiotic cultures alone and in combination. These conditions were optimized based on attaining desired acidity of (1.10-1.20%) L.A, pH of (4.20-3.90) and total lactic count (10^8 CFU/mL)

Shukla et al., (2013) developed a probiotic whey beverage using cheese whey and pineapple juice fermented with *Lactobacillus acidophilus* (1%). The mixture of cheese whey: pineapple juice i.e. (65: 35) fermented for 5hr have showed high overall acceptability in terms of sensory scores. The total counts in beverage were more than 10^6 CFU/mL but the counts decreased after 28 days.

A whey-based beverage was prepared with the supplementation of milk and fermented with *Lb. rhamnosus* ATCC 7469 culture. Throughout storage of 21 days the final product had showed good rheological and sensory properties and the probiotic counts of *7 log* CFU/mL (Bulatović et al., 2014).

Table 4.10 Total *Lactobacillus* count CFU with different levels of inoculum and incubation period

Cultures / Incubation period	Inoculum level	195	195+25	195+24
0h	1%	6.21 ± 0.06^{Aa}	6.21 ± 0.06^{Ba}	6.21 ± 0.06^{Ba}
	2%	6.22 ± 0.04^{Ab}	6.21 ± 0.06^{Bb}	6.24 ± 0.05^{Aa}
	3%	6.22 ± 0.04^{Aa}	6.24 ± 0.05^{Aa}	6.24 ± 0.05^{Aa}
12h	1%	7.14 ± 0.11^{Cc}	7.38 ± 0.21^{Ca}	7.23 ± 0.31^{Cb}
	2%	7.25 ± 0.06^{Bc}	7.43 ± 0.09^{Ba}	7.31 ± 0.08^{Bb}
	3%	7.58 ± 0.03^{Ab}	7.67 ± 0.08^{Aa}	7.66 ± 0.03^{Aa}
24h	1%	8.07 ± 0.31^{Cb}	8.16 ± 0.02^{Ca}	8.1 ± 0.24^{Cb}
	2%	8.33 ± 0.10^{Bb}	8.47 ± 0.12^{Ba}	8.42 ± 0.06^{Ba}
	3%	8.66 ± 0.07^{Ab}	8.79 ± 0.01^{Aa}	8.70 ± 0.18^{Ab}
36h	1%	8.25 ± 0.04^{Cb}	8.32 ± 0.22^{Ca}	8.23 ± 0.06^{Cb}
	2%	8.41 ± 0.09^{Bb}	8.58 ± 0.04^{Ba}	8.55 ± 0.05^{Ba}
	3%	8.69 ± 0.04^{Ab}	8.83 ± 0.05^{Aa}	8.74 ± 0.02^{Ab}
48h	1%	8.21 ± 0.53^{Cb}	8.32 ± 0.11^{Ca}	8.20 ± 0.21^{Cb}
	2%	8.42 ± 0.03^{Bb}	8.48 ± 0.11^{Ba}	8.49 ± 0.05^{Ba}
	3%	8.56 ± 0.02^{Aa}	8.54 ± 0.14^{Aa}	8.57 ± 0.12^{Aa}

Data is presented as Mean ± SD (n= 3)

A, B, C, a, b, c, d, e Mean ± SD bearing different superscripts column wise (capital alphabets) and row wise (small alphabet) differ significantly ($p < 0.05$).

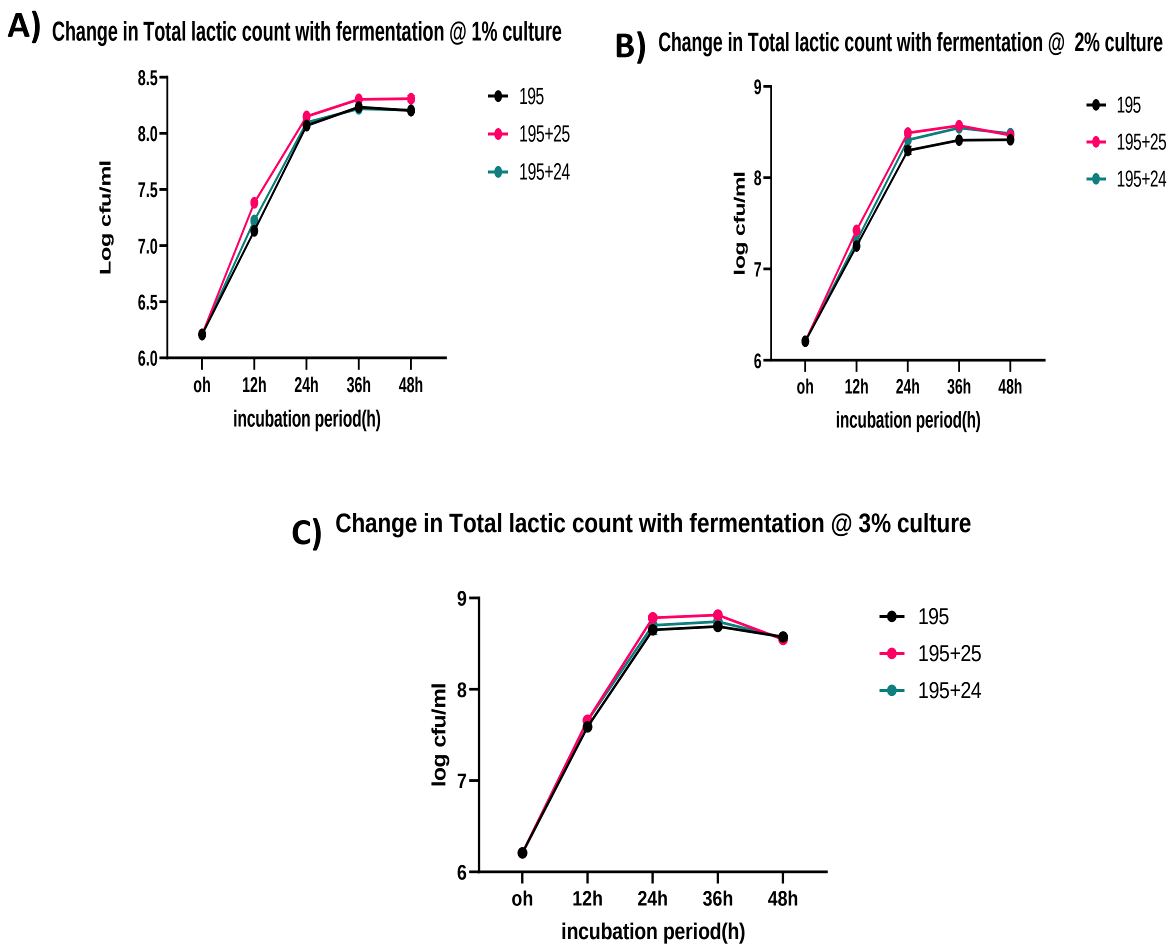


Fig 4.9 Total *Lactobacillus* count with different levels inoculum (A) 1%, (B) 2% and (C) 3%. These values are expressed in mean± S.D, n=3, p>0.05

Cheese isolate *L. rhamnosus* C6 found to be proteolytic bacteria with proteolytic activity of 509.12 µg serine/ mL. This isolate showed the good probiotic attributes as it survived at high bile concentration (upto 2%), low pH (pH 1.0) and cell surface hydrophobicity for nhexadecane (28.53 ± 0.37%). The culture has showed good growth in whey (9.19 logs CFU/mL) and soy milk (8.88 logs CFU/mL) after 48h of incubation (Priyanka et al., 2015).

4.6.4 Optimization of sugar level

Sugar is optimized at three different concentrations of 50% sugar syrup such as 8%, 10% and 12% were added in fermented whey beverage, which is prepared under optimized conditions and evaluated by sensory values. Based on the sensory score obtained, it was found that 10% of sugar in whey beverage has showed maximum sensory values.

4.7 Preparation of protein-rich fermented whey beverage

Based on the results obtained from preliminary study of screening of cultures and optimized conditions were selected for preparation of protein-rich fermented whey beverage using colostrum whey.

Whey was separated from colostrum by rennet treatment. Colostrum was diluted (1:0.5) and slowly heated to 40-45°C and added with sufficient quantity of calf rennet (1: 4500) to give setting time of 20-30 min. Later, Whey was separated and filter sterilized using 0.45µm filter. Filtered whey was inoculated with 3% of 195(Drink 1), 195+25(Drink 2) and 195+24(Drink 3) cultures, incubated at 37°C for 24h, analysed for pH and acidity, added with flavor and sugar, transferred to PET bottles, and then stored at 4°C.

4.8 Physico-chemical and microbiological analysis of fermented whey beverage

The parameters like pH, acidity, protein and peptides were evaluated under physico-chemical analysis of fermented whey beverage. Drink 1 had pH of 3.97±0.02, acidity of 0.96±0.06% L.A, protein of 6.23±0.11% and peptides of 5.1±0.02 mg/mL. Similarly, pH of 3.92±0.08, acidity of 1.05±0.04% L.A, protein of 6.19±0.04% and peptides of 5.34±0.02 mg/mL were observed for Drink 2. Whereas, Drink 3 had pH of 3.94±0.03, acidity of 0.95±0.01% L.A, protein of 6.20±0.01% and peptides of 5.22±0.02 mg/mL **(Table 4.11)**.

Table 4.11 Physico-chemical analysis of fermented whey beverage

Drinks / **Components**	**Drink 1**	**Drink 2**	**Drink 3**
pH	3.97±0.02	3.92±0.08	3.94±0.03
Acidity (% L.A)	0.96±0.06	1.05±0.04	0.95±0.01
Protein (%)	6.23±0.11	6.19±0.04	6.20±0.01
Peptides (mg/mL)	5.1±0.02	5.34±0.02	5.22±0.02

Data is presented as Mean ± SD (n=3)

Ayar et al., (2016) reported on the average composition of colostrum added yoghurt and kefir samples includes pH of 4.67- 4.73, protein of 3.21- 3.47%, fat of 2.80- 3.20%, viscosity of 10.2- 18.5 P and non-fat dry matter of 8.27-8.60%. Setyawardani et al., (2020) reported the average pH of kefir made of colostrum at different concentrations were 3.98-3.91.

The total *Lactobacillus* counts of Drink 1, Drink 2, and Drink 3 were 8.59±0.21, 8.64±0.12, 8.62±0.08 *Log* CFU/mL, respectively. Coliform counts and, yeast and mold count of fermented whey drinks were nil **(Table 4.12)**. Absence of coliform counts signifies the good hygienic and sanitary processing in manufacture of product. Similarly, absence of yeast and mold count signifies stringent sanitary management during product preparation.

Table 4.12 Microbiological analysis of fermented whey beverage

Drinks / **Parameters**	**Drink 1**	**Drink 2**	**Drink 3**
Total Lactobacillus count (Log CFU/mL)	8.59±0.21	8.64±0.12	8.62±0.08
Coliform count	Nil	Nil	Nil
Yeast and Mold count	Nil	Nil	Nil

Data is presented as Mean ± SD (n=3)

Ayar et al., (2016) reported the total mesophilic aerobic bacteria was 6.06 and 6.24 log CFU/g, *Streptococcus thermophilus* showed 7.54 and 7.57 log CFU/g, *L. bulgaricus* showed 7.15 and 7.44 log CFU /g.

Cho et al., (2015) reported production of functional high protein fermented beverage with the addition of whey protein concentrate (WPC 80), skim milk powder, sucrose and fermented with single starter i.e., *Lactobacillus plantarum* DK211 and *Lactococcus lactis* R704. The functional protein beverage had high protein (9%), low-fat content (0.2%) and the product has reached a cell count of 10^9 CFU/mL in 10h.

Silva e Alves et al., (2018) developed a probiotic functional carbonated whey beverage from cheese whey fermented with probiotic species *Bifidobacterium animalis ssp. lactis.* The preparation of this beverage and incorporation of probiotic found to be quite easy. The final product had showed good probiotic counts and slight sedimentation during storage and the beverage microbiological quality satisfied the Brazilian standards.

4.9 Sensory evaluation of protein-rich fermented whey beverage

Sensory evaluation is an important aspect for any newly prepared product. This part of study helps the producer to evaluate their products in view of, whether the newly prepared product is accepted by the consumer or not. In this present study, the developed product was analyzed for sensory characteristics like its color and appearance, sedimentation, flavor, acidity, and overall acceptability. Sensory aspects of newly developed fermented whey beverage Drink 1, Drink 2 and Drink 3 were evaluated by a group of 10 members using 9-point hedonic scale. Sensory analysis of fermented whey beverage is presented in **Table 4.13** and **Fig 4.10.**

The sensory scores were given for color and appearance, sedimentation, flavor and acidity of fermented whey beverage (Drink 1) were 8.2±0.2, 7.4±0.2, 7.2±0.2 and 7.4±0.2 respectively whereas, for fermented whey beverage (Drink 2) was given scores 8.4±0.2, 7.7±0.4, 7.4±0.1 and 7.6±0.1 respectively and for fermented whey beverage (Drink 3) were 8.0±0.3, 7.3±0.2, 7.2±0.1 and 7.6±0.2 respectively. However, overall acceptability of freshly prepared fermented whey beverage Drink 1, Drink 2 and Drink 3 were 8.1±0.3, 8.2±0.2 and 8.0±0.2 out of 10, respectively. No significant ($p>0.05$) difference was observed in acidity and overall acceptability of drinks and found the significant ($p>0.05$) difference in color and appearance, sedimentation, and flavor attributes between the drinks. From these results it may be inferred that all the three fermented whey drinks had good sensory values and overall acceptability.

Probiotic whey beverage prepared from different proportions of whey (0, 20, 35, 50, 65 and 80%) cultured with yoghurt starters, *L. acidophilus and B. animalis Bb-12*. It was reported that the beverage prepared from whey concentration (49%) had high sensory properties based on Weibull distribution data. The concentration of whey in beverage had not affected the viability and growth of probiotic microorganisms (Castro et al., 2013).

Table 4.13 Sensory evaluation of fermented whey beverage

Drinks / Parameters	Drink 1	Drink 2	Drink 3
Colour and Appearance	8.2 ± 0.2^{b}	8.4 ± 0.2^{a}	8.0 ± 0.3^{c}
Sedimentation	7.4 ± 0.2^{b}	7.7 ± 0.4^{a}	7.3 ± 0.2^{c}
Flavour	7.2 ± 0.2^{b}	7.4 ± 0.1^{a}	7.2 ± 0.1^{c}
Acidity	7.4 ± 0.2^{a}	7.6 ± 0.1^{a}	7.6 ± 0.2^{a}
Overall acceptability	8.1 ± 0.3^{a}	8.2 ± 0.2^{a}	8.0 ± 0.2^{a}

Data is presented as Mean ± SD (n= 3)

a, b, c, d Means within columns with different lowercase superscript letters are significantly different ($p < 0.05$) from each other.

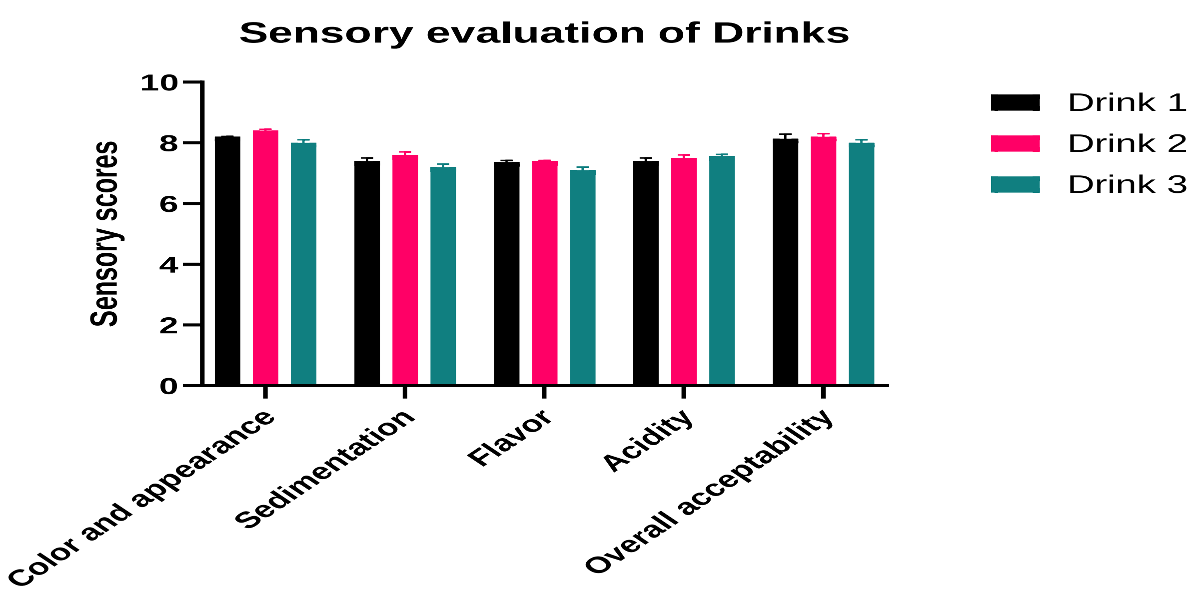


Fig 4.10 Sensory evaluation of fermented whey drinks

Shukla et al., (2013) developed a probiotic whey beverage using cheese whey and pineapple juice fermented with *Lactobacillus acidophilus* (1%). The mixture of cheese whey: pineapple juice i.e. (65: 35) fermented for 5hr have showed high overall acceptability in terms of sensory scores.

Whey beverage was prepared from whey, skim milk and carrot juice fermented with yoghurt starters *(Streptococcus thermophilus, Lactobacillus bulgaricus)*, probiotic bacteria like *Lactobacillus acidophilus and Bifidobacterium bifidum.* The final product showed 90.5 % antioxidant activity, viable cell counts 8.7 log CFU/mL, pH 4.6 and overall sensory score of 8.97. Implementation of this beverage production during cheese manufacture increases overall profit of industry and improves the product quality (Arsic et al., 2018).

Sabokhar & Khodaiyan, (2016) developed a novel beverage prepared from whey and pomegranate juice fermented with kefir grains @ 5-8% has showed good sensory properties and high antioxidant activity.

4.10 Bio-functional properties of fermented whey beverage

4.10.1 Anti-microbial activity of fermented whey beverage

Antimicrobial activity of fermented whey beverage was evaluated by using agar well diffusion assay method. Colostrum whey was fermented with 195, 195+25 and 195+24 isolates. The fermented whey beverage was subjected to centrifugation at 6000rpm for 15min to remove microbial cells. The obtained supernatant was used for determination of antimicrobial activity against pathogens. Zone of inhibition of fermented whey beverages i.e., Drink 1, Drink 2, and Drink 3 with 195, 195+25 and 195+24 isolates against different pathogens is presented in **Table 4.14**, **Fig 4.11, and Plate 4.2**.

Fermented whey beverage (Drink 1) prepared using NCDC195 exhibited maximum antimicrobial activity against *Staphylococcus aureus* (12.33±0.33), *Bacillus cereus* (13±0.58), *Escherichia coli* (12±0.577), *Enterococcus faecalis* (11±0.577) and *Salmonella enterica* (13±0.58). Fermented whey beverage (Drink 2) by 195+25 exhibited maximum antimicrobial activity against *Staphylococcus aureus* (12±0.577), *Bacillus cereus* (13±0.577), *Escherichia coli* (13±0.58), *Enterococcus faecalis* (13.33±0.33) and *Salmonella enterica* (17.66±0.33). Fermented whey beverage (Drink 3) by 195+24 isolates exhibited maximum antimicrobial activity against *Staphylococcus aureus* (11±0.58), *Bacillus cereus* (13±0.58), *Escherichia coli* (13.6±0.33), *Enterococcus faecalis* (12±0.58) and *Salmonella enterica* (13±0.577). No significant difference ($p>0.05$) in antimicrobial activity was observed between Drink 1, Drink 2, and Drink 3. Production of bioactive peptides and antimicrobial substances during fermentation may be the reasons for antimicrobial activity of fermented whey beverage...

Table 4.14 Antimicrobial activity of fermented whey beverage

Drinks	Drink 1	Drink 2	Drink 3
Test bacterium	Zone of inhibition in mm (including well dia 6mm)		
Staphylococcus aureus	12.33±0.33[a]	12±0.577[a]	11±0.58[b]
Bacillus cereus	13±0.58[a]	13±0.577[a]	13±0.58[a]
Escherichia coli	12±0.577[b]	13±0.58[a]	13.6±0.33[a]
Enterococcus faecalis	11±0.577[c]	13.33±0.33[a]	12±0.58[b]
Salmonella enterica	13±0.58[b]	17.66±0.33[a]	13±0.577[b]

Data is presented as Mean ± SD (n= 3)

a, b, c, d Means within rows with different lowercase superscript letters are significantly different ($p < 0.05$) from each other.

Windayani et al., (2020) reported antimicrobial activity of kefir produced from colostrum fermented with kefir grains for 24-48h. The colostrum kefir has antibacterial properties against both Gram-positive and Gram-negative food borne pathogenic bacteria. After 48 hours fermentation of kefir, there was increase in antibacterial activity and titratable acidity whereas, decrease in pH value. Ali et al., (2019) reported on profiling of peptides from whey protein isolate medium fermented with *Lactobacillus helveticus* LH-2 and *Lactobacillus acidophilus* La-5 using mass spectrometry. Ultra-filtrated 3kDa peptide fractions had anti-virulence activity against *Salmonella enterica ssp. enterica serovar Typhimurium*.

Bartkiene et al., 2018 reported that production of antimicrobial gummy candies using bovine colostrum and essential oil (*Thymus vulgaris or Eugenia caryophyllata*) fermented with probiotics (*Lactobacillus paracasei* LUHS244) had antimicrobial activity against *Proteus mirablis, Escherichia coli, Salmonella enterica, Staphylococcus aureus, Enterococcus faecalis, Streptococcus mutans* except *Pseudomonas aeruginosa.*

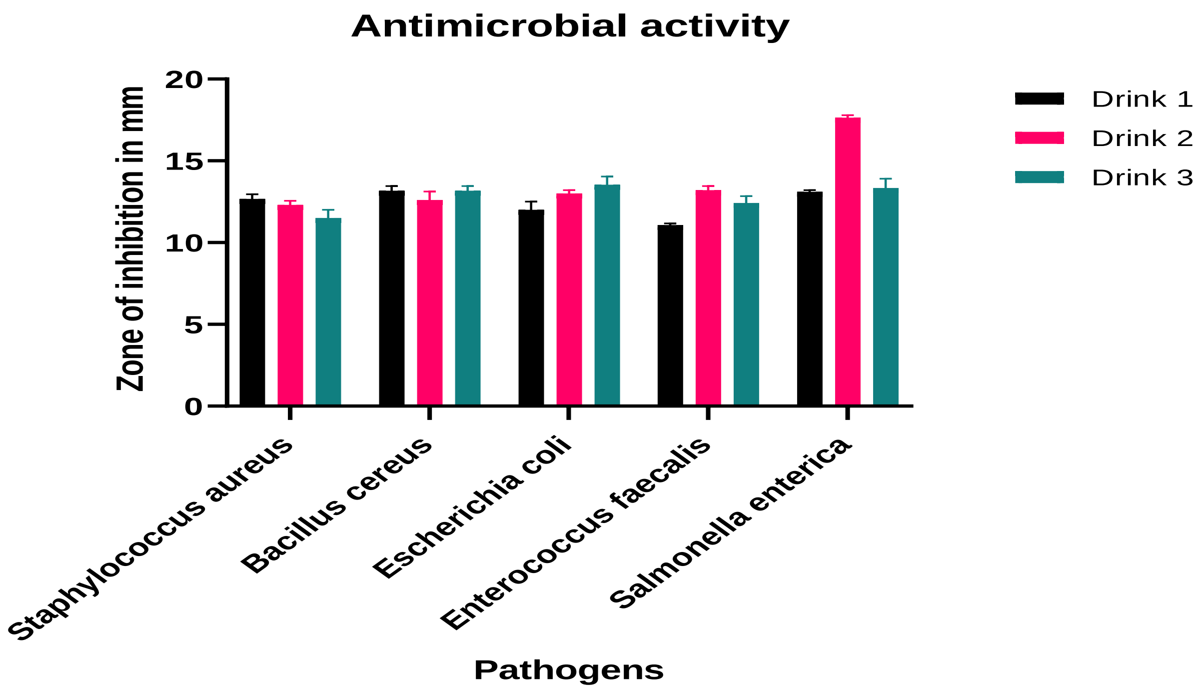
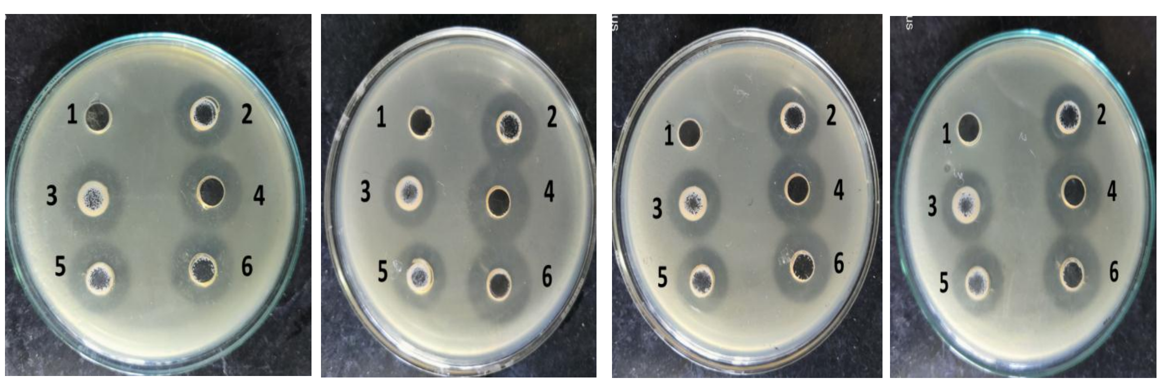


Fig 4.11 Anti-microbial activity of fermented whey beverage. These values are expressed in mean± S.D, n=3, p>0.05



S. aureus ***B. cereus*** ***E. coli*** ***S. enterica***

Plate 4.2 Antimicrobial activity of fermented whey beverage. (1) Control, (2) Drink-1, (3 & 4) Drink 2, (5 & 6) Drink 3

Cotarlet et al., 2019 reported that the bioactive peptides derived from colostrum obtained by fermentation with kefir grains enriched with selected yeasts have shown strongest antimicrobial activity against *Bacillus subtilis* MIUG B1. The antimicrobial activity of fermented colostrum is linked to titratable acidity of samples, which means organic acids produced by lactic acid bacteria are the key components responsible to inhibit the growth of spoilage microorganisms.

4.10.2 Antioxidative activity of fermented whey beverage

Anti-oxidative activity of fermented whey beverage was evaluated by using $ABTS^{+}$ assay method using Trolox as standard, which is Vitamin-E analogue, known to have maximum capacity to scavenge free radicals and expressed as Trolox Equivalent Antioxidative capacity (TEAC). In the present investigation, antioxidative activity was found in fermented whey beverage by different combination of *Lactobacillus* isolates. Antioxidative activity of fermented whey beverages Drink 1, Drink 2 and Drink 3 was evaluated. Data pertaining to this is presented in **Table 4.15 and Fig 4.12**. All the three Drinks had good antioxidant activity than control. However, Drink 2 showed highest antioxidant activity of 1991.47 TEAC µMol/L which corresponds to 85.62±0.301% inhibition followed by Drink-3 with antioxidant activity of 1972.88 TEAC µMol/L which corresponds to 84.82±0.033% inhibition. Similarly, Drink-1 showed antioxidant activity of 1908.62TEAC µMol/L which corresponded to 82.043±0.001% inhibition. However, a significant difference (p>0.05) in anti-oxidative activity was observed between Drink 1, Drink 2 and Drink 3.

Table 4.15 Antioxidative activity of fermented whey beverage

Parameters	**% Inhibition**	**TEAC (µMOL/L)**
Drinks		
Control	41.83 ± 0.33^{d}	970.77±7.92
Drink 1	82.043 ± 0.001^{c}	1908.62±0.001
Drink 2	85.62 ± 0.301^{a}	1991.47±7.01
Drink 3	84.82 ± 0.033^{b}	1972.88±0.77

Data is presented as Mean ± SD (n= 3)

a, b, c, d Means within columns with different lowercase superscript letters are significantly different (p< 0.05) from each other.

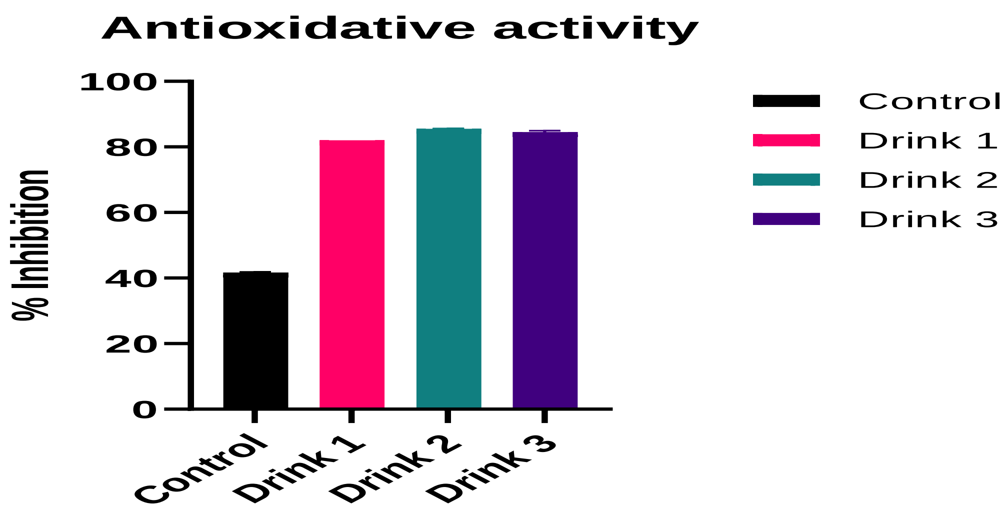


Fig 4.12 Antioxidative activity of fermented whey beverage. These values are expressed in mean± S.D, n=3, p>0.05

Anti-oxidative activity of these drinks may be due to production of bioactive peptides during fermentation. These peptides are responsible for antioxidative activity and various peptides are released from colostrum whey by LAB fermentation. The bioactive peptides derived from colostrum by fermentation with kefir grains enriched with selected yeasts have showed increase in antioxidant activity, after fermentation for 48h (Cotarlet et al., 2019). Fajardo-Espinoza et al., *(*2019) reported that antioxidative properties of peptides derived from bovine colostrum by enzymatic hydrolysis (pancreatin and pepsin). The peptides are subjected to ultrafiltration to obtain <10, 10-30, >30 kDa fractions and the highest antioxidant values and high iron binding capacity obtained in fractions less than 10kDa and >30 kDa fractions, respectively.

León-López et al., (2020) reported that milk whey based fermented beverage with varied proportions of hydrolyzed collagen (0.3%, 0.5%, 0.75% & 1%) fermented with *Lactobacillus rhamnosus, L. bulgaricus, Streptococcus thermophilus* had antioxidative activity. The beverage with 1% collagen had radical inhibition for ABTS (48.30%), DPPH (30.06%), high protein content (9.75 g/L) and observed with the increase in collagen concentration, the nutrition value, antioxidant activity and bioavailability of beverage increased. Arsic et al., (2018) also reported that functional fermented whey carrot beverage prepared by fermentation with yoghurt cultures (*L. bulgaricus, S. thermophilus*) and probiotics like *L. acidophilus; Bifidobacteria bifidum* had 90.5% antioxidant activity.

Gaspar-Pintiliescu et al., (2020) reported that bovine colostrum fermented with *Candida lipolytica* co-cultured with kefir grains have produced bioactive peptides. These peptides are subjected to ultracentrifugation to derive less than 10kDa peptides have shown high scavenging activity against ABTS radical's 63-92% inhibition.

4.10.3 ACE-inhibitory activity of fermented whey beverage

ACE-inhibitory activity of fermented whey beverage was evaluated by using method of Hernandez et al., 2007. In the present investigation, ACE-inhibitory activity was seen in fermented whey beverage by different combination of *Lactobacillus* cultures. ACE-inhibitory activity of fermented whey beverages Drink 1, Drink 2 and Drink 3 was evaluated. Three Drinks have shown good ACE-inhibitory activity than control. Amongst three drinks, Drink-2 had high ACE-inhibition **(55.01%),** followed by Drink-1**(54.25%)** and Drink-3**(53.16%).** Significant difference (p>0.05) in ACE-inhibitory activity was observed between Drink 1, Drink 2 and Drink 3**. (Table. 4.16 and Fig. 4.13)**

Rodríguez-Hernández et al., (2020) reported on effect of probiotic cultures on ACE-inhibitory activity of whey based fermented beverage using Chris Hansen cultures like BCT-1 and ABT-4 (A-*Lactobacillus acidophilus*, B-*Bifidobacterium animalis ssp lactis*, c-*Lactobacillus paracasei*, T-*Streptococcus thermophilus*). During shelf-life, almost all whey based fermented beverage with the presence of commercial culture had shown ACE-inhibitory activity (22-100%). and whey beverage with ABT-4 had shown ACE-inhibitory activity (80-100%).

Table 4.16 ACE-inhibitory activity of fermented whey beverage

Parameters	**% ACE- Inhibition**
Drinks	
Control	31.03^{d}
Drink 1	54.25^{b}
Drink 2	55.01^{a}
Drink 3	53.16^{c}

Data is presented as Mean ± SD (n= 3)
a, b, c, d Means within columns with different lowercase superscript letters are significantly different (p< 0.05) from each other.

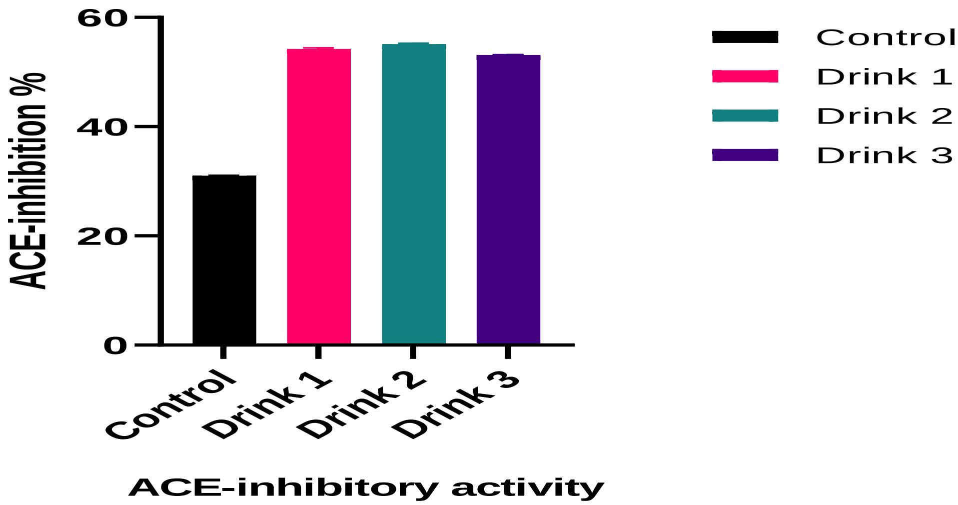


Fig 4.13 ACE-inhibitory activity of fermented whey beverage. These values are expressed in mean± S.D, n=3, p>0.05

ACE inhibition property of these drinks is due to production of bioactive peptides during fermentation. Various number of ACE-inhibition peptides are released from colostrum whey by LAB fermentation. Cotarlet et al., (2019) reported on the bioactive peptides derived from colostrum by fermentation with kefir grains enriched with selected yeasts have showed ACE inhibitory activity. In the present investigation also, these whey-derived peptides exhibited bio-functional properties like antimicrobial, antioxidative and ACE-inhibitory activity.

Fajardo-Espinoza et al., (2019) investigated the production of bioactive peptides from bovine colostrum whey by enzymatic hydrolysis (enzymes like pepsin and pancreatin) having ACE-inhibitory activity. The bioactive peptides derived from bovine colostrum fermented with *Candida lipolytica* co-cultured with kefir grains. <10kDa peptide fractions showed better modulation in ACE inhibition property (Pintiliescu et al., 2020). Peptides from whey protein concentrate are derived by treating with Flavorzyme at 50^0C for 30 min and these peptides are incorporated to lassi. Enriched Lassi had ACE inhibitory activity of 79.67 % whereas, control sample showed 42.17 % (Paul and Gosh. 2017).

Mazorra-Manzano et al., (2020) reported that cheese whey fermentation with native microbiota to release bioactive peptides with ACE-inhibitory activity. Additionally, the ACE-inhibitory activity increased from 22% in unfermented whey to 60-70% after 120h fermentation.

4.11 Shelf-life study of fermented whey beverage

Shelf-life study of fermented whey beverage was evaluated at refrigeration condition (4^0C) for 7 days. Samples were drawn and evaluated for physicochemical, microbiological analysis and bio-functional activities at 0^{th}, 3^{rd}, $5^{th,}$ and 7^{th} day intervals of time.

4.11.1 pH of fermented whey beverage during storage at 4^0C for 7days

Decrease in pH was observed in fermented whey drinks during course of storage at 4^0C for 7days.Drink 1 have showed decrease in pH from 0^{th} day (3.98±0.02) to 7^{th} day (3.91±0.06), Drink 2 have showed decrease in pH from 0^{th} day (3.92±0.08) to 7^{th} day (3.86±0.03) and Drink 3 showed decrease in pH from 0^{th} day (3.94±0.03) to 7^{th} day (3.88±0.02). And found significant difference (p>0.05) in pH from 0^{th} to 7^{th} day of storage (**Table 4.17 & Fig 4.14**).

Table 4.17 pH of fermented whey beverage during storage at 4^0C for 7days

pH	**Storage period**			
Drinks	**0^{th} Day**	**3^{rd} Day**	**5^{th} Day**	**7^{th} Day**
Drink 1	3.98 ± 0.02^{b}	3.96 ± 0.04^{b}	3.93 ± 0.07^{a}	3.91 ± 0.06^{a}
Drink 2	3.92 ± 0.08^{c}	3.90 ± 0.04^{bc}	3.89 ± 0.02^{ab}	3.86 ± 0.03^{a}
Drink 3	3.94 ± 0.03^{b}	3.93 ± 0.11^{b}	3.90 ± 0.01^{a}	3.88 ± 0.02^{a}

Data is presented as Mean ± SD (n= 3)

a, b, c, d Means within rows with different lowercase superscript letters are significantly different (p< 0.05) from each other.

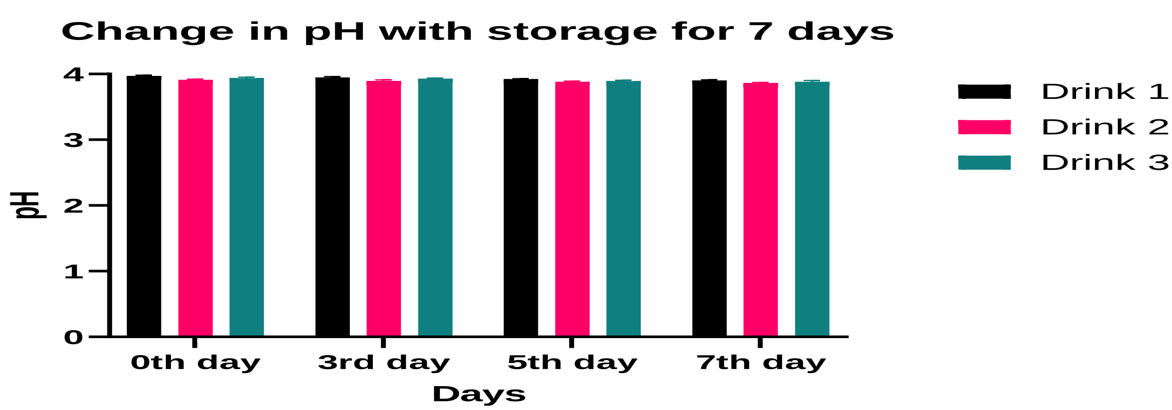


Fig 4.14 pH of fermented whey beverage during refrigeration condition

4.11.2 Acidity of fermented whey beverage during storage at 4^0C for 7days

Increase in acidity was observed in fermented whey drinks during course of storage at 4^0C for 7days. An increase in acidity was in Drink 1 from 0^{th} day (0.970±0.08) to 7^{th} day (1.030±0.12), in Drink 2 (1.053±0.04) to 1.117±0.02) and in Drink 3 (0.98±0.01 to 1.030±0.06). A significant difference (p>0.05) in acidity from 0^{th} to 7^{th} day of storage was observed (**Table 4.18 & Fig 4.15**).

Table 4.18 Acidity of fermented whey beverage during storage at 4^0C for 7days

Acidity	Storage period			
Drinks	**0^{th} Day**	**3^{rd} Day**	**5^{th} Day**	**7^{th} Day**
Drink 1	$0.970±0.08^{c}$	$0.990±0.03^{bc}$	$1.020±0.06^{ab}$	$1.030±0.12^{a}$
Drink 2	$1.053±0.04^{b}$	$1.053±0.04^{b}$	$1.090±0.01^{a}$	$1.117±0.02^{a}$
Drink 3	$0.98±0.01^{b}$	$0.983±0.02^{b}$	$1.013±0.03^{ab}$	$1.030±0.06^{a}$

Data is presented as Mean ± SD (n= 3)

a, b, c, d Means within rows with different lowercase superscript letters are significantly different (p< 0.05) from each other.

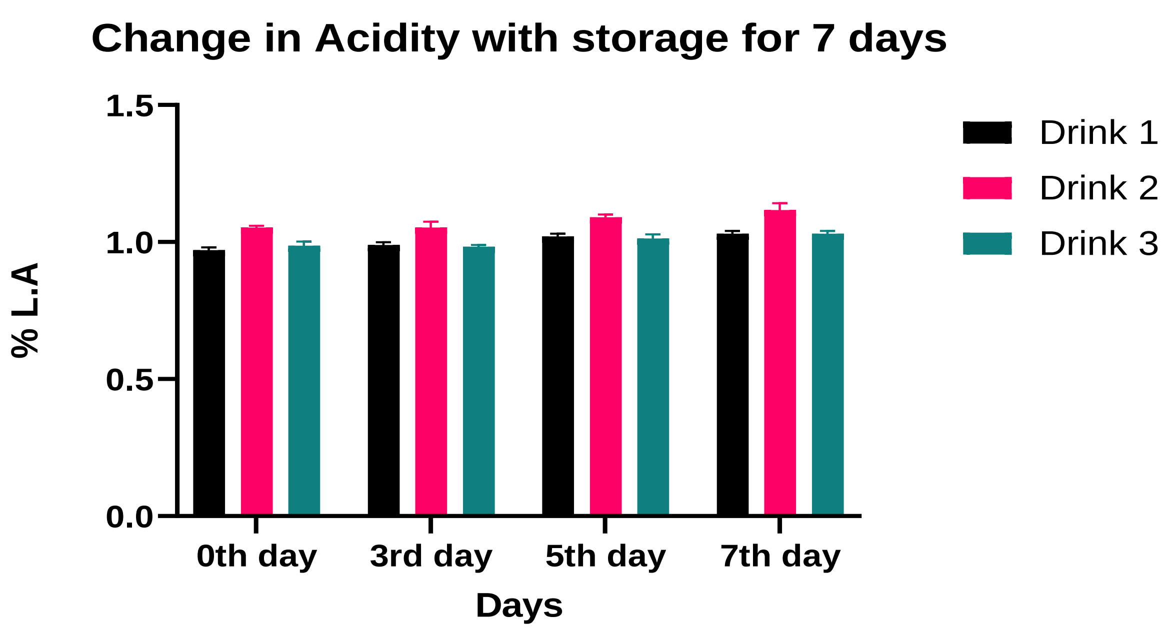


Fig 4.15 Acidity of fermented whey beverage during refrigeration condition

4.11.3 Total *Lactobacillus* counts of fermented whey beverage during storage at 4^{0}C

Total *Lactobacillus* count was found stable in fermented whey drinks during course of storage at 4^{0}C for 7days. Total *Lactobacillus* count in Drink 1, Drink 2 and Drink 3 remained same (i.e., 8.59-8.62, 8.56-8.70 and 8.58-8.64 log CFU/mL respectively) even after storage at 4^{0}C for 7days. No significant difference ($p>0.05$) was observed in total *Lactobacillus* count from 0th to 7th day of storage of drinks (**Table 4.19 & Fig 4.16**).

Table 4.19 Total *Lactobacillus* count of fermented whey beverage during storage at 4^{0}C for 7days

Total Lactobacillus count	Storage period			
Drinks	**0th Day**	**3rd Day**	**5th Day**	**7th Day**
Drink 1	8.59±0.21^{ab}	8.60±0.08^{a}	8.62±0.12^{a}	8.58±0.15^{b}
Drink 2	8.66±0.12^{a}	8.68±0.13^{a}	8.70±0.07^{a}	8.56±0.04^{b}
Drink 3	8.62±0.08^{ab}	8.62±0.04^{a}	8.64±0.05^{a}	8.58±0.02^{a}

Data is presented as Mean ± SD (n= 3)
a, b, c, d Means within rows with different lowercase superscript letters are significantly different ($p< 0.05$) from each other.

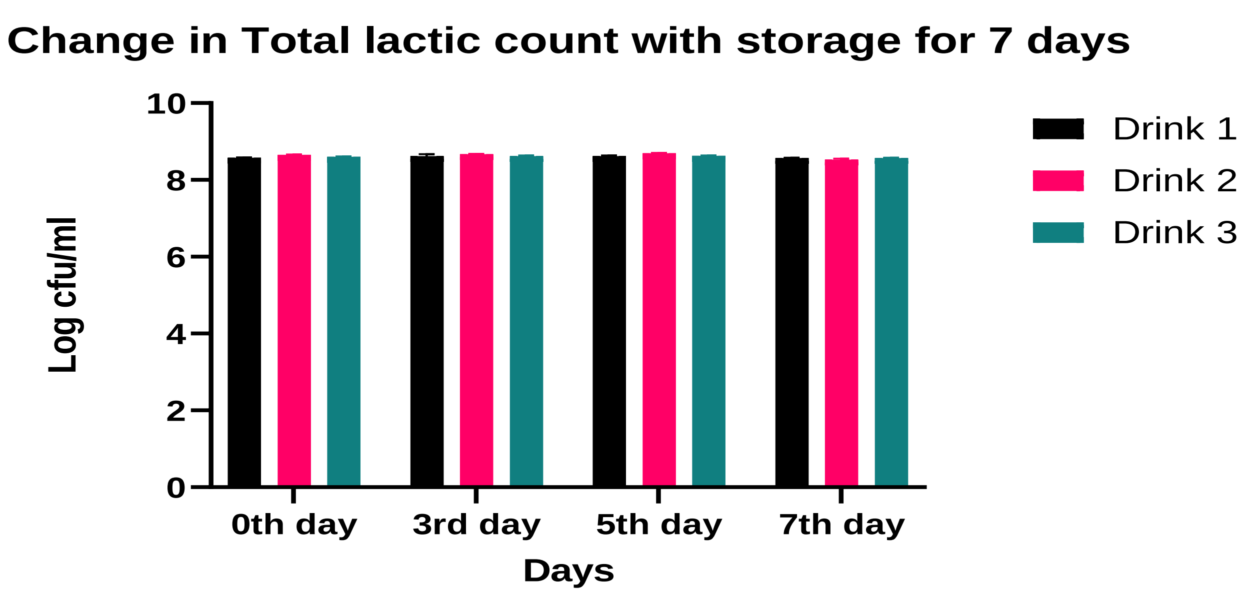


Fig 4.16 Total *Lactobacillus* count of fermented whey beverage during refrigeration condition

Probiotic beverage was developed from de-protenized whey fermented with *L. rhamnosus* NCDO 243, *B. bifidum* NCDO 2715 and *Propionibacterium freudenreichii subsp. shermanii* have showed probiotic counts over 8 logs CFU/mL and high organoleptic properties after 10 days of storage (Maity et al., 2008).

Shukla et al., (2013) developed a probiotic whey beverage using cheese whey and pineapple juice fermented with *Lactobacillus acidophilus* (1%). The mixture of cheese whey: pineapple juice i.e. (65: 35) fermented for 5h have showed high overall acceptability in terms of sensory scores. The total count in beverage was more than 10^6 CFU/mL but the counts decreased after 28 days.

A probiotic whey beverage fermented with *L. acidophilus La-5 and B. animalis Bb-12* was prepared from acid whey enhanced with sweet whey powder or butter milk powder (5%). Storage studies were carried for 21days and throughout the period the counts were above 8 logs CFU/mL (Skryplonek and Jasińska, 2015). Chocolate flavored probiotic whey beverage prepared from goat cheese whey, UHT goat milk in addition to prebiotics like oligofructose or inulin fermented with *B. lactis*. The beverage made from goat cheese whey (45%) and oligofructose (6%) have showed high probiotic counts after 28 days of cold storage (Da Silveira et al., 2015).

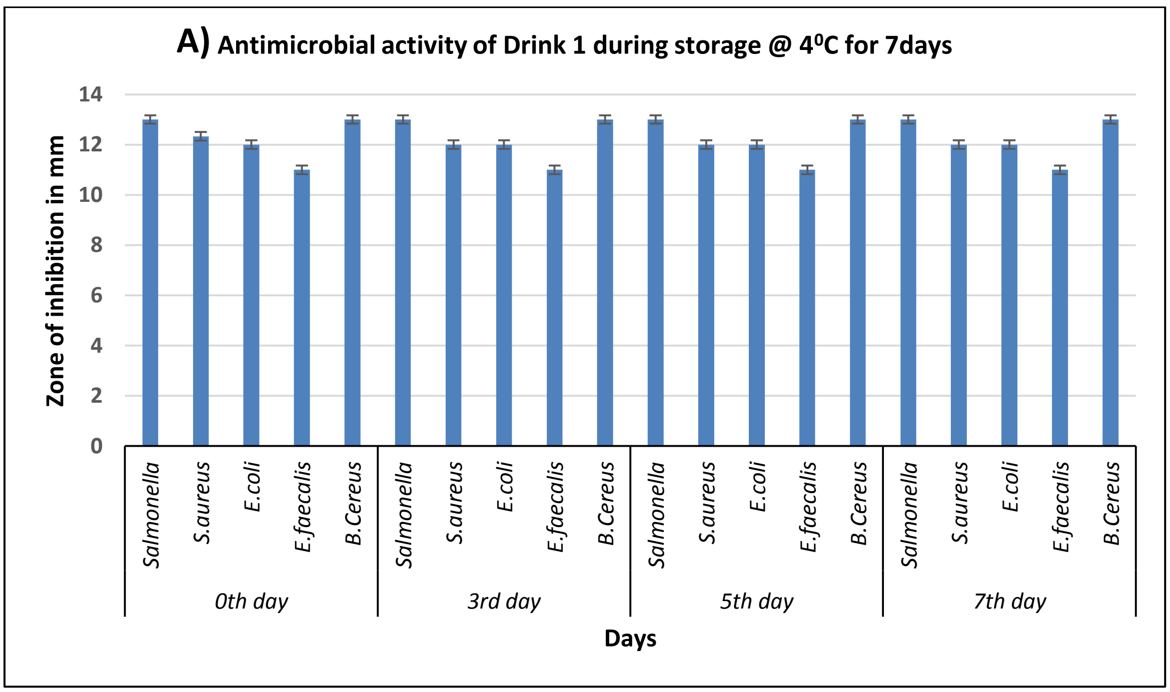
4.11.4 Anti-microbial activity of fermented whey beverage during storage at 4^0C

Anti-microbial activity was found stable in fermented whey drinks during course of storage at 4^0C for 7days. All the drinks showed antimicrobial activity after preparation called as 0^{th} day.

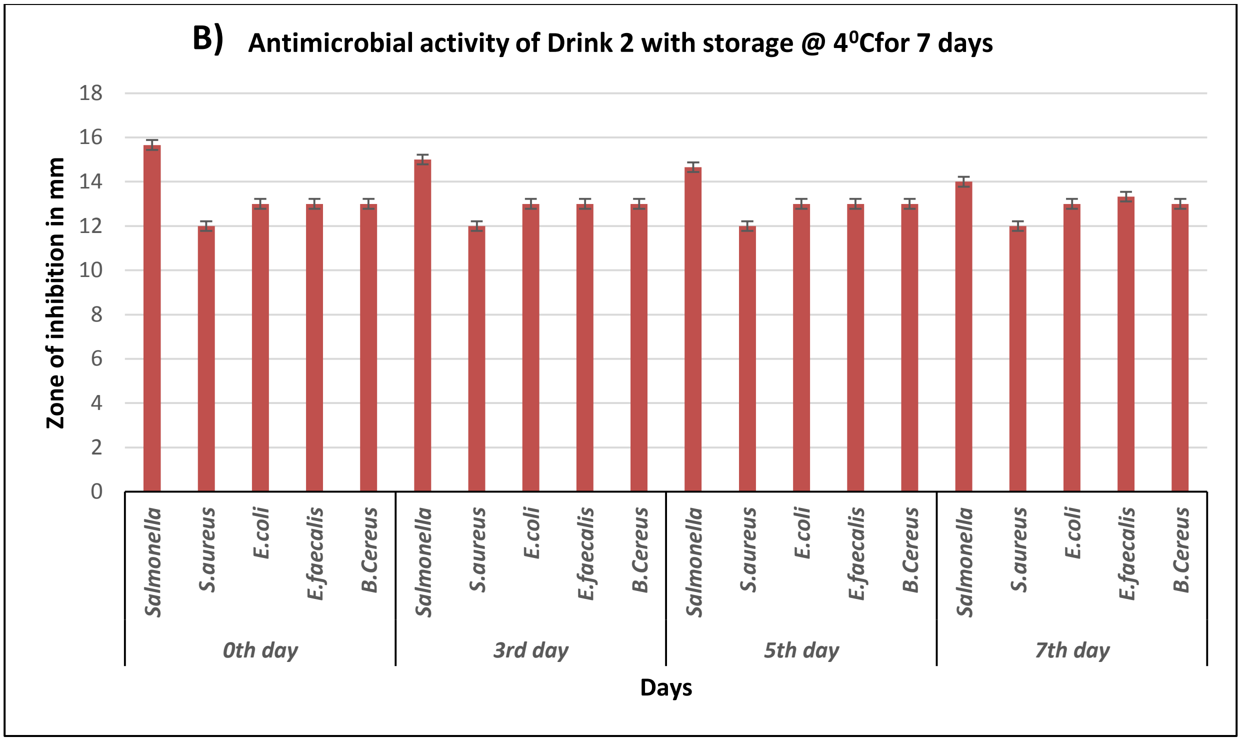
Drink 1 exhibited activity against *Staphylococcus aureus* (12.33±0.33), *Bacillus cereus* (13±0.58), *Escherichia coli* (12±0.577), *Enterococcus faecalis* (11±0.577) and *Salmonella enterica* (13±0.58). The antimicrobial activity of drinks on 7^{th} day was found stable in all the drinks. **Drink 1** showed antimicrobial activity against *Staphylococcus aureus* (12.33±0.20), *Bacillus cereus* (13±0.24), *Escherichia coli* (12±0.05), *Enterococcus faecalis* (11±0.02) and *Salmonella enterica* (13±0.68)]. Similar results were observed in Drink 2 and Drink 3 also.

Drink 2 exhibited antimicrobial activity against all the test pathogens like *Staphylococcus aureus* (12±0.577), *Bacillus cereus* (13±0.577), *Escherichia coli* (13±0.58), *Enterococcus* *faecalis* (13.33±0.33) and *Salmonella enterica* (17.66±0.33) on 0^{th} day. The antimicrobial property of the drink 2 was stable after 7 days storage with zone of inhibition against test bacteria like *Staphylococcus aureus* (12±0.564), *Bacillus cereus* (13±0.24), *Escherichia coli* (13±0.44), *Enterococcus faecalis* (13±0.04) and *Salmonella enterica* (15±0.57)].

Drink 3 also showed similar trend of antimicrobial activity. The zone of inhibition against all the pathogens was observed as *Staphylococcus aureus* (11±0.58), *Bacillus cereus* (13±0.58), *Escherichia coli* (13.6±0.33), *Enterococcus faecalis* (12±0.58) and *Salmonella enterica* (13±0.577) on the day of preparation. The antimicrobial activity was stable after storage of 7 days. The antimicrobial activity for **Drink 3** against all the test organism was observed as *Staphylococcus aureus* (11±0.38), *Bacillus cereus* (13±0.58), *Escherichia coli* (13±0.33), *Enterococcus faecalis* (12±0.22) and *Salmonella enterica* (13±0.64). No significant difference ($p>0.05$) was observed in anti-microbial activity (Zone of inhibition in mm) from 0^{th} to 7^{th} day of *storage of* drinks (**Fig 4.17 A-C**).



Studies on utilization of ultrafiltered whey permeate (later concentrated by reverse osmosis) or retentate (rich in whey proteins) in preparation of probiotic whey beverage fermented with *L. acidophilus, L. casei and L. rhamnosus* @ 0.3% have showed viable cell counts over 7 log CFU/mL, antimicrobial and acceptable sensory properties after 14 days of cold storage (Pereira et al., 2015).



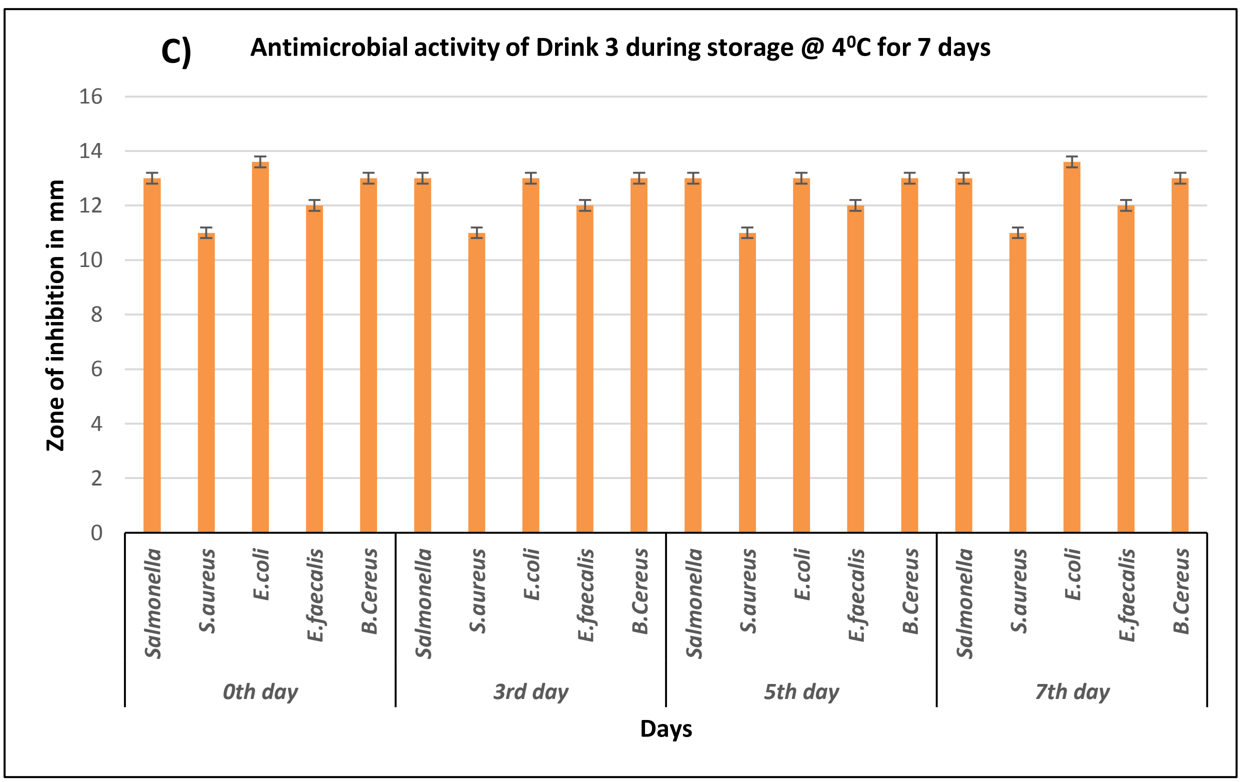


Fig 4.17 Anti-microbial activity of fermented whey beverage [A) Drink 1 B) Drink 2 C) Drink 3] during refrigeration condition

Bartkiene et al., 2018 reported on production of antimicrobial gummy candies using bovine colostrum and essential oil (*Thymus vulgaris or Eugenia caryophyllata*) fermented with probiotics (*Lactobacillus paracasei* LUHS244) have showed antimicrobial activity against *Proteus mirablis, Escherichia coli, Salmonella enterica, Staphylococcus aureus, Enterococcus faecalis, Streptococcus mutans* except *Pseudomonas aeruginosa.* Gummy candies made from bovine colostrum (3%), Essential oil (0.2%) have showed good sensory properties and antimicrobial activity, therefore these candies can be preferred to consumers.

Ali et al., (2019) have done profiling of peptides from whey protein isolate medium fermented with *Lactobacillus helveticus* LH-2 and *Lactobacillus acidophilus* La-5 using mass spectrometry. Ultra-filtrated 3kDa peptide fractions have shown anti-virulence activity against *Salmonella enterica ssp. enterica serovar Typhimurium.*

4.11.5 Anti-oxidative activity of fermented whey beverage during storage at 4^0C for 7days

Anti-oxidative activity was found stable in fermented whey drinks during course of storage at 4^0C for 7days. Anti-oxidative activity of drinks on 0^{th} day (82.06- 85.6% inhibition) and anti-oxidative activity of drinks on 7^{th} day (81.6- 85.4% inhibition). No significant difference ($p>0.05$) was observed in anti-oxidative activity (% inhibition) from 0^{th} in to 7^{th} day of storage of drinks (**Fig 4.18**).

Fluegel et al., (2010) reported on reduction in blood pressure in prehypertensive and hypertensive young men and women by the consumption of whey beverages. Results found to be helpful in treating prehypertension and stage 1 hypertension by using dietary treatment and also observed reduction in total and LDL cholesterol concentrations.

SC-CO_2 treated grape juice-based whey drink at 14–16 or 18 MPa at 35 °C for 10 min. An increase in ACE inhibitory activity with increase in application pressure was reported.SC-CO_2 treated grape juice-based whey drink have showed high sensory properties than heat treated beverages (Amaral et al., 2018).

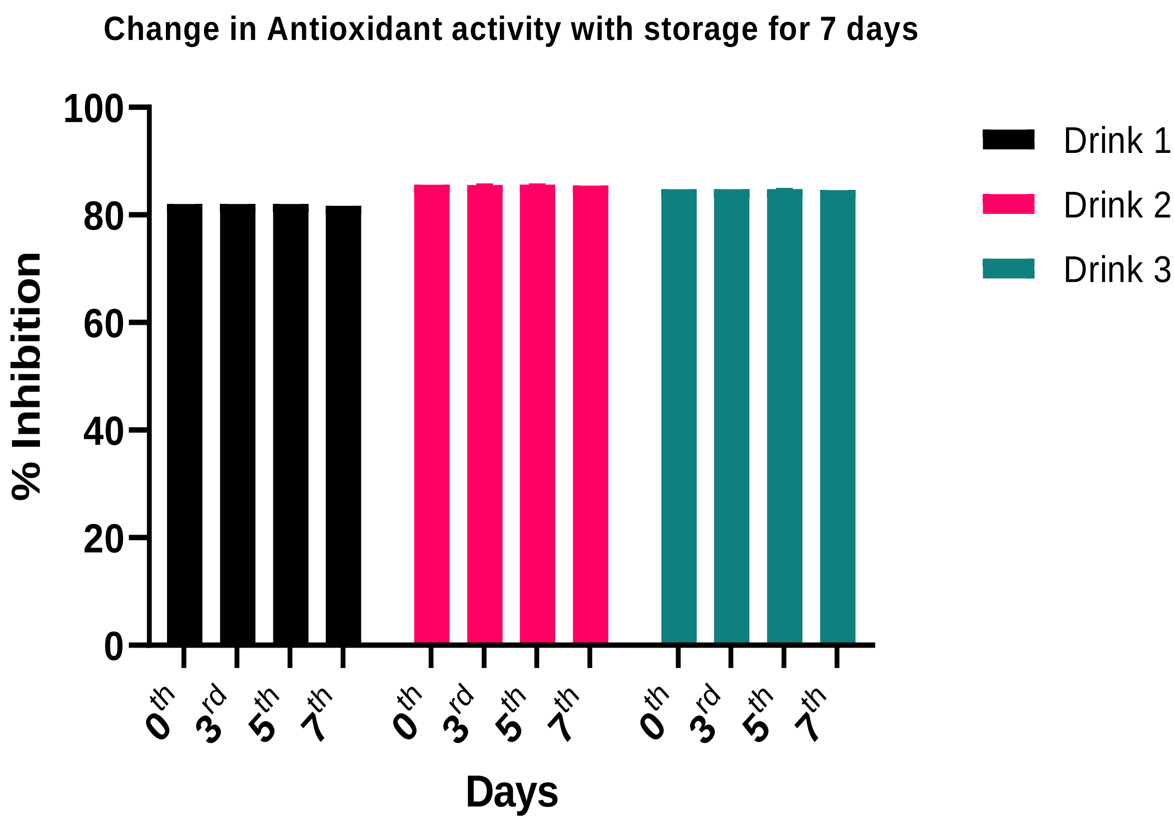
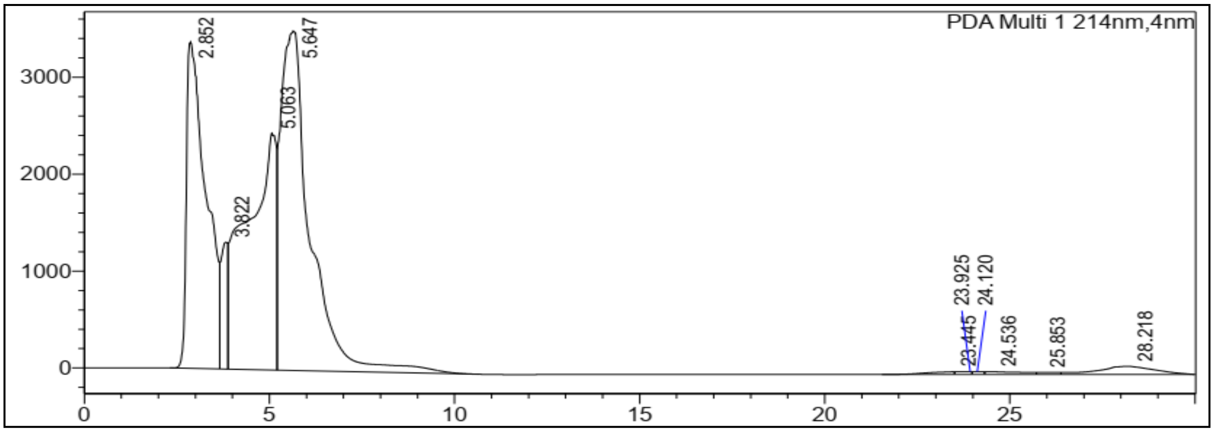


Fig 4.18 Anti-oxidative activity of fermented whey beverage during refrigeration condition

4.12 RP-HPLC chromatogram of fermented whey beverages

RP-HPLC chromatogram of fermented whey beverages reveals the generation of different bioactive peptides. These peptides are analysed through HPLC using the method given by Najafian and Babji (2014). With the help of manual sample injector, the fermented whey sample (20µL) was injected into HPLC system (Shimadzu, Japan) and run for 30min.

Sample of Drink 1 was subjected to HPLC, the chromatogram showed total of 10 peaks of peptides with different retention time that lasted up to 28min. 4 peptides showed retention time of 2.852-5.647 min, 5 peptides showed 23.445-25.883 min and 1 peptide showed retention time of 28.218 minutes **(Fig 4.19).** Peptides in Peak **1, 3 and 4** showed higher concentration.



<Peak Table>

PDA Ch1 214nm

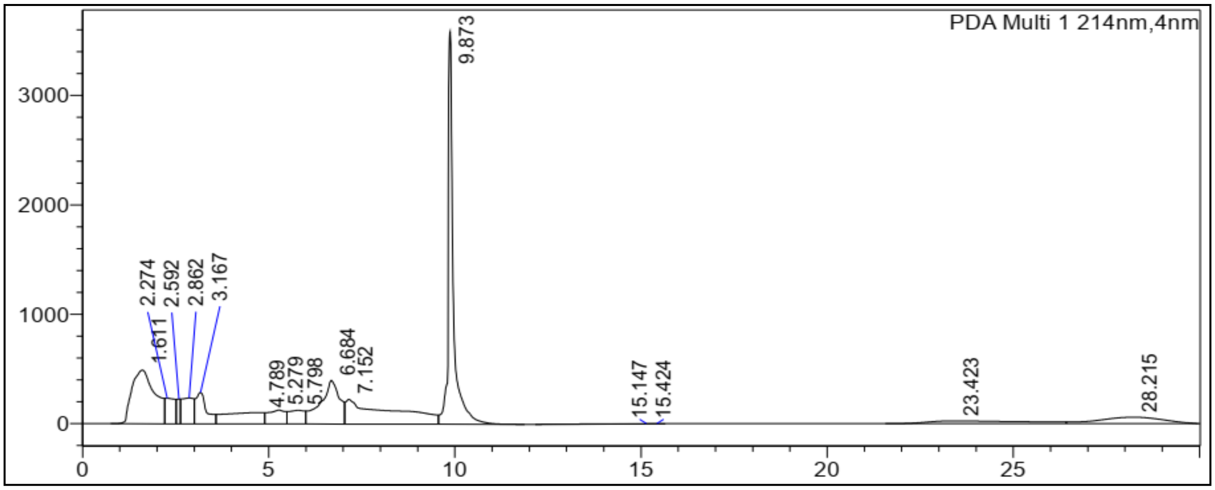
Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	2.852	123902121	3356380	25.463		
2	3.822	16711993	1309580	3.434		V
3	5.063	136144208	2444464	27.979		V
4	5.647	195376880	3503551	40.151		V
5	23.445	1400077	27397	0.288		
6	23.925	777774	27849	0.160		V
7	24.120	569033	28028	0.117		V
8	24.536	2021412	29570	0.415		V
9	25.853	809233	21033	0.166		V
10	28.218	8888415	82841	1.827		V
Total		486601147	10830694			

Fig 4.19 RP-HPLC chromatogram and peak table of drink 1

Birkemo et al., (2009) found that peptides naturally found in bovine colostrum [i.e., casecidin 15(YQEPVLGPVRGPFPIIV), casecidin 17(YQEPVLGPVRGPFP)] had antimicrobial activity. The purified peptides showed antimicrobial activity against *Enterobacter sakazakii* and *Escherichia coli* DPC6053.

Bovine whey proteins hydrolyzed with selected *Lactobacillus* strains (*Lactobacillus plantarum*, *Lactobacillus brevis*) have shown antimicrobial activity against Gram-positive, Gram-negative and some pathogenic and spoilage microorganisms (Messaoui et al., 2020).

The chromatogram of Drink 2 has shown total of 15 peaks of peptides with different retention time that lasted up to 28min. Out of which, 9 peptides showed retention time of 1.611-6.684 min, 4 peptides showed 7.152-15.424 min and 2 peptide showed retention time of 23.423 and 28.215 min. **(Fig 4.20).**



<Peak Table>
PDA Ch1 214nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	1.611	21143787	489731	15.715		
2	2.274	4250005	233029	3.159		V
3	2.592	1589772	226645	1.182		V
4	2.862	5189268	235603	3.857		V
5	3.167	5834931	286727	4.337		V
6	4.789	7598050	103578	5.647		V
7	5.279	4083366	126930	3.035		V
8	5.798	3601469	124794	2.677		V
9	6.684	14277629	398456	10.612		V
10	7.152	19508124	226398	14.500		V
11	9.873	35463629	3586323	26.359		V
12	15.147	190589	851	0.142		
13	15.424	5613	502	0.004		V
14	23.423	4769885	25295	3.545		
15	28.215	7034865	58852	5.229		V
Total		134540983	6123713			

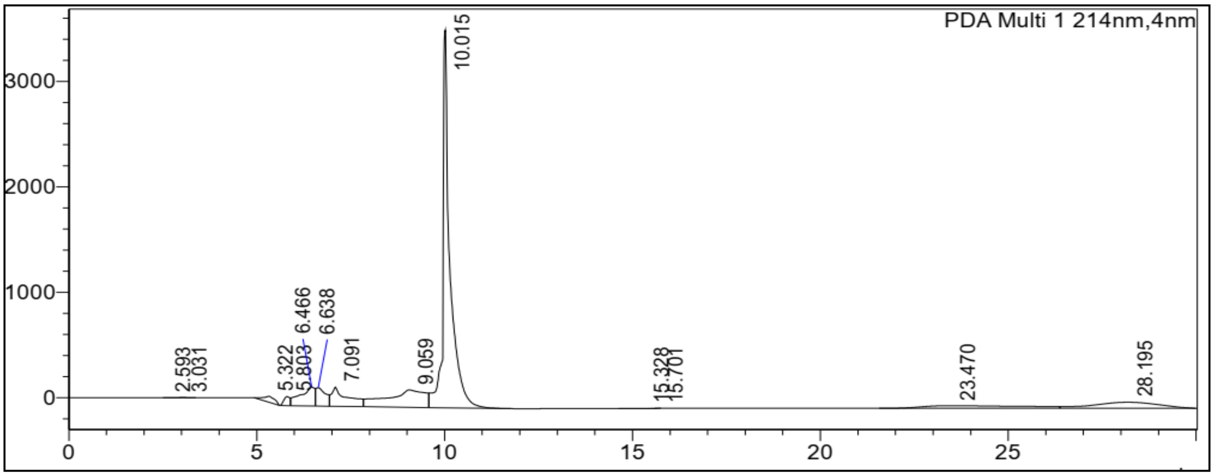
Fig 4.20 RP-HPLC chromatogram and peak table of drink 2

Fajardo-Espinoza et al., (2019) reported that bioactive peptides were generated from bovine colostrum whey by enzymatic hydrolysis (enzymes like pepsin and pancreatin) having ACE-inhibitory activity. The bioactive peptides derived from bovine colostrum fermented with *candida lipolytica* co-cultured with kefir grains. These peptides were subjected to ultracentrifugation to derive <10kDa peptide fractions showed better modulation in ACE inhibition property (Gaspar-Pintiliescu et al., 2020).

Cotarlet et al., (2019) reported that the bioactive derived from colostrum obtained by fermentation with kefir grains enriched with selected yeasts had showed ACE inhibitory activity.

A total of 15 peaks of peptides were observed in the chromatogram of Drink 3 with different retention time of 28min. Out of which 9 peptides showed retention time of 1.611-

6.684 min, 4 peptides showed 7.152-15.424 min and 2 peptide showed retention time of 23.423 and 28.215 min. **(Fig 4.21).** The concentration of peptide in **peak 9** was very high.



<Peak Table>

PDA Ch1 214nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark
1	2.593	1279	230	0.001		V
2	3.031	33575	1999	0.039		V
3	5.322	1218808	57308	1.412		
4	5.803	976196	86826	1.131		
5	6.466	4814491	178822	5.579		V
6	6.638	3168352	174189	3.671		V
7	7.091	5399947	180820	6.257		V
8	9.059	11576740	164910	13.415		V
9	10.015	47261985	3580800	54.767		V
10	15.328	5196	155	0.006		
11	15.701	1654	53	0.002		V
12	23.470	4837448	25148	5.606		
13	28.195	7001101	58016	8.113		V
Total		86296773	4509275			

Fig 4.21 RP-HPLC chromatogram and peak table of drink 3

Sadat et al., (2011) reported that antioxidant peptides (INYW, LDQW) were generated from bovine alpha-lactalbumin using thermolysin at 70^0C.

Four antioxidant peptides (VGINYWLAHK, VLVLDTDYK, IDALNEK, KTKIPAVF) were identified in whey protein concentrate by hydrolyzing with alcalase, flavourzyme and corolasePP (Mann et al., 2015).

Production of whey protein hydrolysates from cheese whey using alcalase enzyme and the antioxidant peptides like (VLDTDYK, VRTPEVDDE) were identified (Athira et al., *2015).*

CHAPTER - 5

Summary and Conclusions

SUMMARY AND CONCLUSION

- Colostrum samples of first three days were procured from (LRC) Livestock Research Centre of National Dairy Research Institute, Karnal from Sahiwal cow.
- Colostrum whey was separated by rennet treatment of pooled colostrum. Colostrum was diluted (1:0.5) and slowly heated to 40-45°C and added with sufficient quantity of calf rennet (1: 4500) to give setting time of 20-30 min. Later, Whey was separated and filter sterilized using 0.45µm filter.
- Physico-chemical analysis of Colostrum whey was evaluated. The mean protein content of colostrum whey was 6.42±0.05 %, fat content was 0.206±0.01 %, lactose content was 2.46±0.10 %, pH and acidity were 6.32±0.016 and 0.182±0.02 % L.A respectively.
- Proteolytic NCDC cultures *Lactobacillus acidophilus* NCDC 195, *L. rhamnosus* NCDC 24 and *L. rhamnosus* C25 (cheese isolate) were used for high protein whey fermentation.
- Growth kinetics of different *Lactobacillus* cultures i.e., alone (195, 25 & 24) and combination (195+25 & 195+24) were grown in colostrum whey. Out of which 195, 195+25 and 195+24 were selected on the basis of their growth.
- The conditions for preparation of protein rich fermented whey beverage were optimized as 37^0C for 24h with 3% inoculum on the basis desirable pH (3.80-4.0), titratable acidity (0.9-1.10% L.A) and total *Lactobacillus* count (10^8 CFU/ml).
- The parameters like pH, acidity, protein and peptides were evaluated under Physico-chemical analysis of fermented whey beverage. Drink 1 had pH of 3.97±0.02, acidity of 0.96±0.06% L.A, protein of 6.23±0.11% and peptides of 5.1±0.02 mg/ml, Drink 2 had pH of 3.92±0.08, acidity of 1.05±0.04% L.A, protein of 6.19±0.04% and peptides of 5.34±0.02 mg/ml whereas, Drink 3 had pH of 3.94±0.03, acidity of 0.95±0.01% L.A, protein of 6.20±0.01% and peptides of 5.22±0.02 mg/ml.
- The total *Lactobacillus* counts of Drink 1, Drink 2, Drink 3 were 8.59±0.21, 8.64±0.12, 8.62±0.08 *log* CFU/ml, respectively. Coliform and yeast and mold

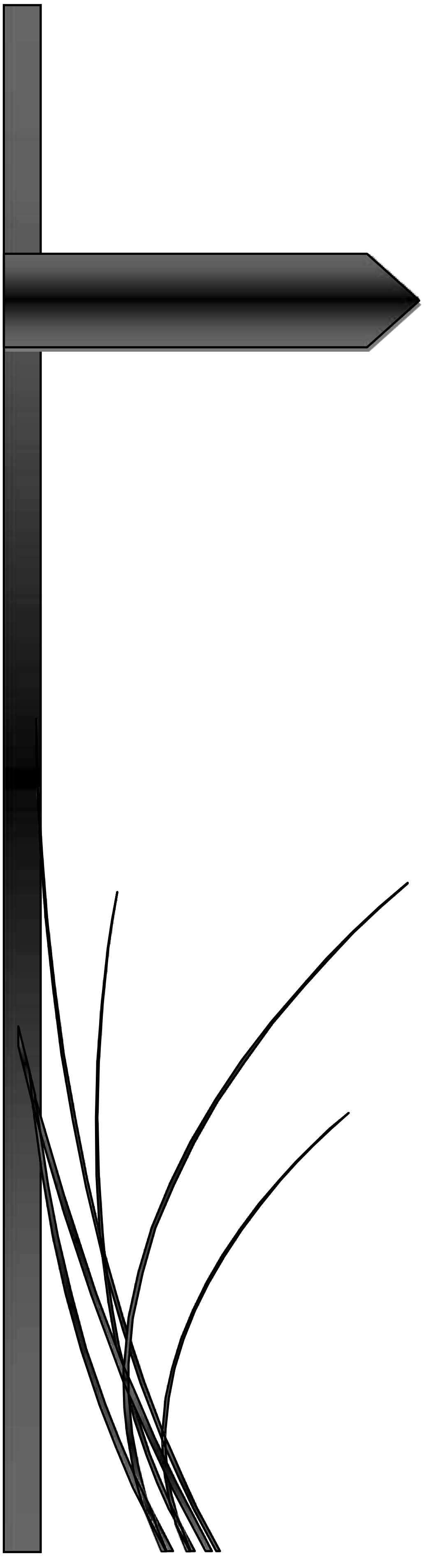
were absent in fermented whey drinks.

- ❖ All the three drinks (Drink 1, Drink 2 & Drink 3) exhibited good sensory values and overall acceptability above 8.
- ❖ Fermented whey beverages (Drink 1, 2 and 3) had maximum antimicrobial activity against *Staphylococcus aureus* (12.33±0.33, 12±0.577, 11±0.58), *Bacillus cereus* (13±0.58, 13±0.577 and 13±0.58), *Escherichia coli* (12±0.577, 13±0.58 and 13.6±0.33), *Enterococcus faecalis* (11±0.577, 13.33±0.33&12±0.58) and *Salmonella enterica* (13±0.58, 17.66±0.33 and 13±0.577), respectively No significant difference ($p>0.05$) in antimicrobial activity was observed between Drink 1, Drink 2 and Drink 3.
- ❖ Three Drinks exhibited good antioxidant activity than control. Amongst three drinks, Drink 2 showed highest antioxidant activity (1991.47 TEAC µMol/L) followed by Drink-3 (1972.88 TEAC µMol/L) and Drink-1 (1908.62TEAC µMol/L). Significant difference ($p>0.05$) in anti-oxidative activity was observed between Drink 1, Drink 2 and Drink 3.
- ❖ All the three Drinks also had good ACE-inhibitory activity than control. However, among all the drinks, Drink-2 showed higher ACE-inhibition (55.01%), followed by Drink-1(54.25%) and Drink-3(53.16%). A significant difference ($p>0.05$) in ACE-inhibitory activity was observed between Drink 1, Drink 2 and Drink 3.
- ❖ Decrease in pH and increase in acidity was observed storage of 7days at 4^0C. A significant difference ($p>0.05$) in pH and acidity from 0^{th} to 7^{th} day of storage.
- ❖ Total *Lactobacillus* counts (CFU/ml), anti-microbial and anti-oxidant activity of drinks was found to be stable even after storage for 7 days at 4^0C. No significant difference ($p>0.05$) was observed from 0^{th} to 7^{th} day of storage of drinks
- ❖ RP-HPLC chromatogram of Drinks showed different number of peaks in the Drinks. Drink-1 showed 10 peaks of peptides, Drink-2 showed 15 peaks and Drink-3 showed 13 peaks of peptides.

Conclusion

On the basis of present investigation protein rich fermented whey beverages were developed by using proteolytic lactic acid bacteria NCDC195 (*L. acidophilus*), C25 (*L.*

rhamnosus) and NCDC 24 (*L. rhamnosus*). All the drinks were accepted based on sensory scores and have antioxidative, antimicrobial and ACE-inhibitory activities. The fermented whey drinks possess potential health benefits because of production of bioactive peptides by the action of proteolytic bacteria *L. acidophilus*, *L. rhamnosus* and *L. rhamnosus* and presence of bioactive components in colostrum whey. Shelf life of beverages was stable at 4^0C for 7 days. Hence, from the results it may be concluded that protein rich fermented whey beverage with proteolytic lactic acid bacteria can be used as bio-functional whey beverage and bioactive proteins or peptides derived from product may be used to increase functionality of beverages.



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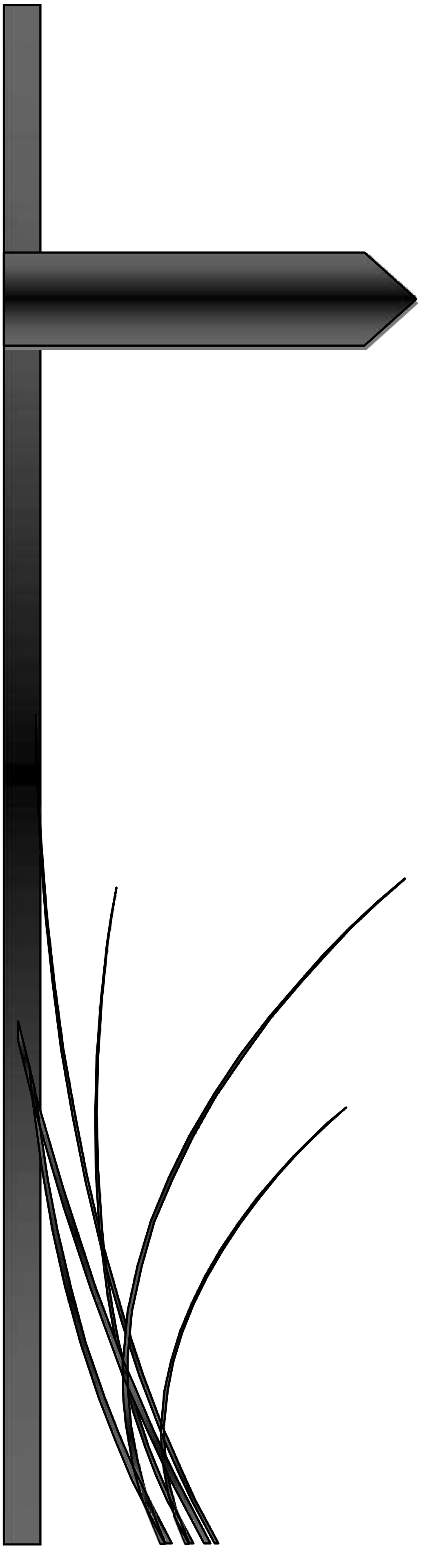

Appendices

REAGENTS FOR ANALYSIS OF FERMENTED WHEY BEVERAGE

Phenolphthalein indicator

Here, 0.5 g of Phenolphthalein indicator was dissolved in 50 ml of 95% ethanol and volume was made to 100 ml by distilled water. It was filtered and kept for 2 days at room temperature for stabilization.

Sodium hydroxide (0.1 N)

Calculation:

$$\text{Normality} = \frac{\text{weight} * 1000}{\text{Eq. wt.} * \text{Vol. (ml)}}$$

Where; Eq. wt. = equivalent weight

Vol. = volume

Here, 4g of NaOH (molecular weight – 40 g) is added to 1000 ml of distilled water and standardized with 0.1 N of oxalic acid.

Hydrochloric acid (0.1 N)

Calculation:

$$\text{Normality} = \frac{\text{weight} * 1000}{\text{Eq. wt.} * \text{Vol. (ml)}}$$

Where; Eq. wt. = equivalent weight

Vol. = volume

Here, 3.66 g of HCl (molecular weight – 36.5 g) is added to 1000 ml of distilled water.

APPENDIX II

MEDIA USED FOR CULTURE ISOLATION, MAINTENANCE AND MICROBIOLOGICAL ANALYSIS

Composition of different types of media used in the study, are given below. Sterilization of media and reagents were performed at 121^{0} C for 15 min under 15 psi pressures.

1. M 17 BROTH

Ingredients	Quantity (Gms / Litre)
Peptic digest of animal tissue	2.50
Casein enzymic hydrolysate	2.50
Papaic digest of soyabean meal	5.00
Yeast extract	2.50
Beef extract	5.00
Lactose	5.00
Ascorbic acid	0.50
Disodium - ß - glycerophosphate	19.00
Magnesium sulphate	0.25
Final pH (at 25°C)	7.1±0.1

For preparation of M 17 agar media, agar powder was added at the rate of 1.5 % in MRS broth.

2. De MANN ROGOSA SHARPE BROTH OR MRS BROTH

Ingredients	Quantity (Gms / Litre)
Proteose peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Dextrose	20.00

Polysorbate 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.00
Final pH (at 25°C)	6.5±0.2

For preparation of MRS agar media, agar powder was added at the rate of 1.5 % in MRS broth.

3. **MRS AGAR PH 5.2**

MRS broth was prepared (previously mentioned above). To obtain pH-modified MRS agars,1.0 M HC1 was used to adjust the pH of the medium to 5.2 or 5.8.

4. GLYCEROL STOCK (50 %)

Ingredients	**Quantity**
Glycerol	50.00 ml
Distilled water	50.00 ml

Sterilized by autoclave. Glycerol stock added in equal amount to the culture in each cryovial, at the of preservation.

5. BHI BROTH

Composition	**Ingredients (Gms / Litre)**
Calf brain infusion	12.50
BHI powder	5.00
Proteose peptone	10.00
Dextrose (Glucose)	2.00
Sodium chloride	5.00
Disodium hydrogen phosphate	2.50
Final pH (at 25°C)	7.4±0.2

For preparation of BHI agar media, agar powder was added at the rate of 1.5 % in BHI broth.

6. NUTRIENT AGAR (NA)

Ingredients	Quantity (Gms / Litre)
Peptone	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	15.00
Final pH (at 25°C)	7.4±0.2

7. SOFT AGAR

Similar to the Nutrient agar (above mentioned), agar is added at the rate of 0.8 % in soft agar instead of 1.5 % in nutrient agar.

8. SALINE SOLUTION (pH 7.0)

Ingredients	Quantity
NaCl	8.00 g
Distilled water	1000 ml

9. VIOLET RED BILE AGAR (VRBA)

Ingredients	Quantity (Gms / Litre)
Peptone	7.00
Yeast extract	3.00
Sodium chloride	5.00
Bile salts mixture	1.50
Lactose	10.00
Neutral red	0.03
Crystal violet	0.002
Agar	15.00
Final pH (at 25°C)	7.4±0.2

10.POTATO DEXTROSE AGAR

Ingredients	Quantity (Gms / Litre)
Potatoes, infusion from	220.00
Dextrose	20.00
Agar	15.00
Final pH (at 25°C)	5.6±0.2

11. SKIM MILK AGAR

Ingredients	Quantity (Gms / Litre)
Skim milk powder	28.00
Casein enzymic hydrolysate	5.00
Yeast extract	2.50
Dextrose	1.00
Agar	15.00

APPENDIX III

REAGENTS USED FOR PROTEIN ESTIMATION

A. Copper sulphate solution (1%w/v)

1g of copper sulphate was dissolved in distilled water and volume is made up to 100ml.

B. Sodium potassium tartrate solution (2% w/v)

2.0 g of sodium potassium tartrate solution was dissolved in distilled water and volume is made up to 100 ml.

C. Sodium hydroxide solution (0.2M)

8g of sodium hydroxide pellet was dissolved in distilled water and volume made up to 1L.

D. Sodium carbonate solution (4% w/v)

4g of sodium carbonate was dissolved in distilled water and volume made up to 100ml.

E. Alkaline reagents

To 49ml of reagent ‘C’, 49ml reagent ‘D’ was added and then 1ml of reagent ‘A’ was added followed by 1ml of reagent ‘B.Ths. reagent was prepared fresh as and when required.

F. Folin’s reagent (1N):

To 5ml of folin’s and ciocalteau’s phenol reagent, 5ml of distilled water was added. This dilution was done just before use.

APPENDIX IV

REAGENTS USED FOR PEPTIDES QUANTIFICATION (OPA METHOD)

1. 0.1 M Borax

3.81 g of borax (molecular weight 381 g) was added to 60 mL of distilled water taken in a glass beaker and was dissolved by keeping it on magnetic stirrer for 15-20 min and then its volume was made 100 ml. it was stored at 4^0 C.

2. 20 % Sodium Dodecyl Sulphate or SDS

20 g of SDS was dissolved in 80 mL of distilled water and volume was made 100 mL. It was stored at room temperature.

3. β- Mercaptoethanol

4. Methanol

Preparation of OPA Reagent:

- It should be prepared fresh for every sample testing
- 80mg of OPA dissolve in 2ml of methanol was added
- To this add 50ml borax solution and 5ml of 20% SDS solution was added
- Then add β- mercaptan ethanol 200µl
- Then make final volume up to 100ml with distilled water 42.8ml

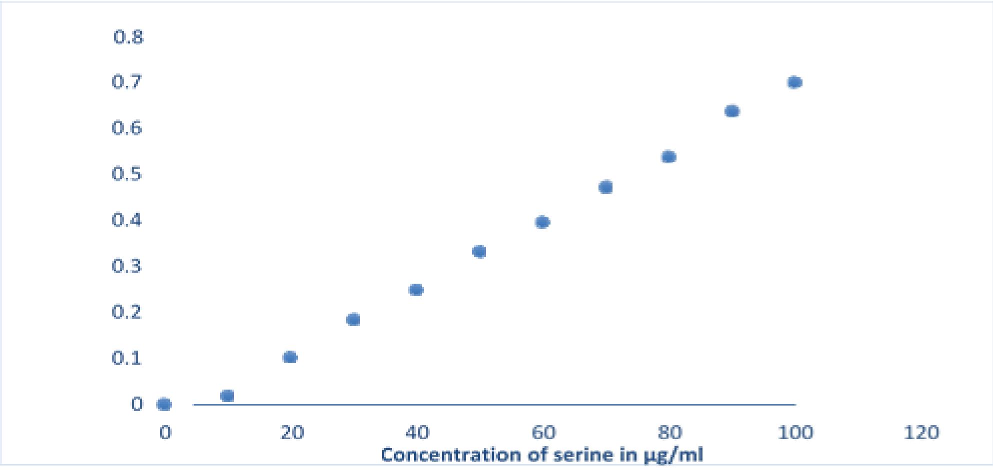

Figure 7.1 Standard curve of serine for proteolytic activity

APPENDIX V

REAGENTS USED FOR ANTIOXIDANT ACTIVITY

1.Potassium persulfate solution (140 mM)

1.892 gm of potassium persulfate was dissolved in double distilled water and the volume was made up to 50 ml freshly prepared before use.

2. ABTS stock solution (7mM)

[2,2'-Azinobis (3-ethylbenzo-thiazoline)-6-sulfonic acid]diammonium salt]

19.2 mg of ABTS was dissolved in 5 ml of double distilled water and was added with 88 µl of 140 mM potassium persulfate solution (2.45 mM final concentration). The mixture was stirred in an amber color bottle in dark for 12-16 h for production of sufficient free radicals.

3.Phosphate buffered saline (PBS, pH 7.4)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl. 1.44 g of Na2HPO4 and 0.24 g of KH2PO4 in 800 ml distilled water, adjusted Ph to 7.4 with 1 N HCl and made the volume up to 1 liter with distilled water and filtered through 0.45µ filter.

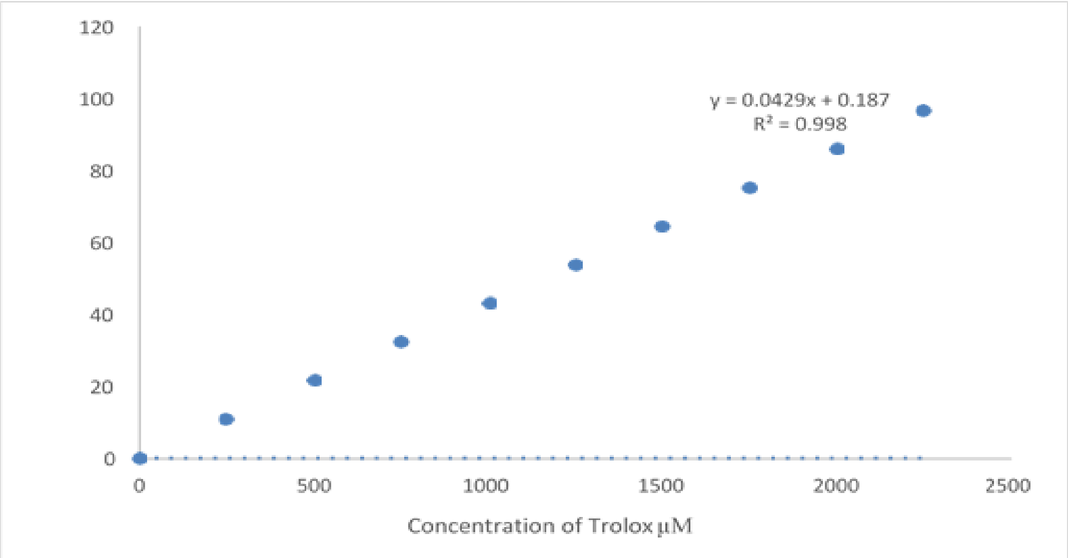

Figure 7.2 Standard curve of Trolox for ABTS method

APPENDIX VI

REAGENTS USED FOR ACE-INHIBITORY ACTIVITY

1. **Hippuryl-L-histidyl-L-leucine (HHL):**

   10.74 mg of HHL (Sigma, FW 429.5) was dissolved in 5 ml of 0.1 M sodium borate buffer (pH 8.3) with 0.3 M NaCI, pH 8.3.

2. **Sodium Borate buffer (0.1 M, pH 8.3) containing 0.3 M NaCI:**

   Sodium tetra borate :3.81 g

   NaCI: 1.75 g

   Dissolved in 80ml of distilled water, pH was adjusted to 8.3 and finally volume was made to 100ml with distilled water.

3. **Angiotensin converting enzyme (ACE):**

   ACE from rabbit lung (Sigma CAT. No. A6778), 1 unit was dissolved in 5ml of distilled water and stored at -20°C.

   ACE-inhibitory activity was expressed as percentage. Percent inhibition was calculated as follows:

   Inhibition activity (%) = (B-A)/ (B-C) x 100

   Where,

   A = the absorbance in the presence of ACE and ACE-inhibitory component (sample)

   B = the absorbance without ACE-inhibitory component

   C = the absorbance without ACE

   Inhibition was expressed as the concentration of component that inhibits 50% of ACE activity (IC 50) and 1 unit of ACE-inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions.

APPENDIX VII

REAGENTS USED FOR LANE-ENYON METHOD

1. Fehling solution- It is prepared by mixing equal volumes of Fehling solution A and B.

Fehling solution-A Dissolve 34.639g of copper sulphate inn water and few drops of 0.1N H_2SO_4 to prevent turbidity. Dilute it to 500ml and filter through prepared asbestos.

Fehling solution-B Dissolve 173g of Rochelle salt of sodium hydroxide in water. Dilute to 500ml with water. Allow to stand for 2days and filter through prepared asbestos.

2. Acetic acid solution: 10% (W/V; aqueous) solution.

3. Methylene blue indicator: 1% (W/V; aqueous) solution.