PRESERVATION OF PANEER BY ANTIFUNGAL SUBSTANCES OF LACTOBACILLI AND ANTIMICROBIAL MILK PROTEINS

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

MASTER OF SCIENCE
IN
DAIRYING
(DAIRY MICROBIOLOGY)

BY
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This is to certify that the thesis entitled, **"PRESERVATION OF PANEER BY ANTIFUNGAL SUBSTANCES OF LACTOBACILLI AND ANTIMICROBIAL MILK PROTIENS "** submitted by **Ms. DESHMUKH PRANALI VILASRAO** towards the partial fulfilment of the award of the degree of **Master of Dairy Science** in Dairying **(DAIRY MICROBIOLOGY)** of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Shilpa 13/6/07

Dated: 13th June, 2007

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MAJOR ADVISOR & CHAIRPERSON

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ABSTRACT

Contamination of dairy products with undesirable yeasts and molds is a serious problem in the dairy industry. They can grow even at low pH value existing in fermented products such as cheese and yoghurt and cause spoilage of these products. In this context the antimicrobial potential of lactobacilli other than bacteriocins is currently being exploited due to their broad spectrum of activity. The preservation ability of lactobacilli is also being used since ancient times in food and feed due to synthesis of antimicrobial compounds such as organic acids, bacteriocin like inhibitory substances and antifungal substances. Though there are many reports on the use of LAB for bio-preservation of dairy and non dairy foods, the reports on the use of antifungal substances of lactobacilli in bio-preservation of foods are scanty. Three lactobacillus cultures *Lactobacillus casei* spp*. casei* NCDC 17, *L. acidophilus* NCDC 195, *Lb. collinoides* NCDC 02 were screened for antifungal activity in MRS broth, skim milk and whey supplemented with different ingredients like nitrogen sources (peptone and yeast extract), carbon sources (glucose, maltose, mannose) and Tween 80 at varying concentrations. *L. casei* spp. *casei* NCDC 17, *Lb. acidophilus* NCDC 195 showed maximum antifungal substance (AFS) production in skim milk and whey medium. *L. casei* spp *casei* NCDC 17 showed antifungal activity against yeast as well as mold cultures used in this study and were therefore used for further studies. The skim milk medium was prepared by supplemented with maltose (0.5%), peptone (1%) and Tween 80 (0.1 %) for AFS production by *Lb. casei* spp. *casei* NCDC 17. It *was* found to produce AFS having maximum antifungal activity against many fungi and also antibacterial activity against Gm+ve and Gm -ve bacteria. The antifungal substance synthesized in skim milk medium was applied for the bio preservation of paneer along with lactoferrin and pediocin. Among different combination treatments used, Treatment III (Pediocin along potassium sorbate and sodium citrate) and VI (Pediocin + Lactoferrin+ antifungal substance) gave relatively better results in preservation of paneer for more than 28 days at refrigeration temperature. On the basis of microbiological analysis and sensory evaluation of paneer, we can say that chemical anti fungal preservatives may be replaced by antifungal substances produced by *Lactobacilli.*

सारांश

डेरी उद्योग में दुग्ध उत्पादों का अवान्दित फंफूद से संदुषण एक विकट समस्या है। यह किण्वित पदार्थों जैसे चीज तथा योगर्ट में बहुत कम अम्लता पर भी प्रभावी रहते है तथा खाद्य पदार्थ को खराब करते हैं इस संदर्भ में आजकल बेक्टीरियोसिन के अतिरिक्त, विस्तृत वर्णक्रम क्रियाशीलता वाले जीवाणु विरोधी सामर्थ्य वाले लैक्टोबेसिलाई का दोहन किया जा रहा है। लैक्टोबेसिलाई के अम्ल, बेक्टीरियोसिन जेसे पदार्थ तथा फंफूद विरोधक पदार्थों के प्रजनन क्षमता के आधार पर खाद्य तथा चारा पदार्थों के परिरक्षण के लिए प्राचयीन समय से उपयोग हो रहा है। यद्यपि डेरी तथा अन्य खाद्य पदार्थों के परिरक्षण में लैक्टिक अम्ल जीवाणु के उपयोग की प्रचुर मात्रा में शोद्य अभिलेख पाए गए है किंतु लेक्टिक अम्ल जीवाणुओं के फंफूद विरोधक पदार्थों की भोजन परिरक्षण क्षमता संबन्धित अल्प आलेख है। तीन लैक्टोबेसिलाई जीवाणु समुह (लैक्टोबेसिलस केसाई उउपजाति केसाई NCDC 17, लैक्टोबेसिलस एसिडोफिलस NCDC 195, लैक्टोबेसिलस कोलिनोईडिस NCDC 02,) का चयन किया गया जिनकी फंफूद विरोधक पदार्थों के उत्पनादन क्षमता का विश्लेषण निम्न लिखित माध्यमों में किया गया था। MRS , वसा रहित दुग्ध तथा व्हे जिसे विभिन्न पदार्थों जैसे नाइट्रोजन स्त्रोत (पेपटोन, इस्ट एक्स्ट्रेकट) कारबन स्रेत (ग्लूकोस, मालटोस, मेनोजस) तथा ट्वीन 80 के विभिन्न मात्राओं से शेषपूरित किया गया। NCDC 195, ने वसा रहित दुग्ध तथा व्हे में फंफूद विरोधक पदार्थों के प्रजनन क्षमता में प्रमुखता दिखई। NCDC 17, ने उच्चतम फंफूद तथा ईरूट विरोधी कार्यशीलता दिखाई अतः आगे शोधों के लिए इस जीवाणु का चयन किया गया। फंफूद विरोधी पदार्थों की अधिकतम उत्पादन के लिए वसा रहित दुग्ध माध्यम को विभिन्न पदार्थों जैसे मालटोस 0.5 % , पेपटोन 1% तथा ट!वीन 80 0.1 % से शेषपूरित किया गया । NCDC 17 द्वारा उत्पादित फंफूद विरोधी पदार्थों को विभिन्न फंफूदों के विरूद्ध तथा जीवाणु विरोधी पदार्थों की विभिन्न जीवाणुओं के विरूद्ध कार्यशीलता पाई गई। फंफूद विरोधी पदार्थ का पीडियोसिन तथा लैक्टोफेरिन के साथ पनीर का परिरक्षण क्षमता के लिए जाँचा गया। विभिन्न मिश्रणों के उपचारों में से पीडियोसिन + लैक्टोफेरिन +फंफूद विरोधी पदार्थ ने पनीर का 4.0 Cपर 28 दिनों के लिए परिरक्षण के उच्चतर परिणाम दिखाए।

पनीर के सूक्ष्मजैविक तथा संवेदिक जाँच के आधार पर यह निष्कर्षित किया जा सकता है कि लैक्टोबेसिलाई द्वारा उत्पादित फंफूद विरोधी पदार्थों का उपयोग, रसायनिक परिरक्षण पदार्थों के सीन पर किया जा सकता है।

CHAPTER – 1

Introduction

1. INTRODUCTION

Recently, there has been a growing interest in bio-preservation, i.e., the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods due to the changing consumer requirements for fresh and nutritious food and to avoid the use of chemical preservatives. No single method is effective in providing protection against several types of spoilage microorganisms. Lactic acid bacteria (LAB) are the organisms of particular interest in bio-preservation. The preserving effect of LAB (*Lactococcus, Lactobacillus, Leuconostoc, Pediococcus*) mainly relates to the formation of organic acids such as lactic acid, acetic acid, hydrogen peroxide; diacetyl and the production of bacteriocins and bacteriocins like inhibitory substances (BLIS). The bacteriocins from LAB are bioactive peptides, derived from ribosomally synthesized precursors and with a bactericidal effect on a number of different gram-positive bacteria. The preserving ability of LAB has been used since ancient times in food and animal feed such as sauerkraut and silage.

The antibacterial effect of *Lactobacillus* is mainly due to the formation of various organic acids, H_2O_2, diacetyl, carbon dioxide, bacteriocin and antibiotic production. Bacteriocins are proteinaceous compound produced by bacteria that exhibit a bactericidal mode of action against related as well as unrelated organisms, Whereas, BLIS, the antagonistic substances, are having broader spectrum of activity than bacteriocins.

Molds and yeasts are significant spoilage organisms in different food and feed systems. Contamination of dairy products with undesirable yeasts and molds is a serious problem in the dairy industry. The commonly found fungi in dairy are *Geotrichicum, Aspergillus, Fusarium, Mucor, Penicillium, Candida, Rhodotorula, Kluyveromyces*, other filamentous yeasts and molds. Yeasts and moulds can grow even at low pH values, existing in fermented products such as cheese and yoghurt. Some species are able to grow in conditions not suitable for most microorganisms such as low a_W (as low as 0.62) and at pH values between 1.5 and 10.5. They can cause spoilage of fermented products and public health hazards due to the production of toxic and carcinogenic mycotoxins. Besides this problem, fungal spoilage leads to a significant

economic loss to the dairy industry. Worldwide, about 5-10 % of the food production is estimated to be spoiled by these organisms.

There are many reports on the production of antibacterial compounds by *Lactobacillus spp*, but reports on inhibition of yeasts and molds by these compounds are relatively very few. However, there has been some recent reports on the production of a proteinaceous antifungal substances by a *Lb. coryniformis*, other antifungal compounds like phenyllactic acid and 4-hydroxyphenyllactic acid, short-chain fatty acids, benzoic acid, methylhydantoin, mevalonolactone and cyclo- di-peptides (Gly-L-Leu) and a fungistatic bacteriocin-like substance, pentocin TV35b, produced by a strain of *Lactobacillus pentosus.* Antifungal proteins and polypeptides have been isolated from diverse group of organisms including bacteria. The mechanisms of these proteins include fungal cell wall polymer degradation, membrane channel and pore formation, damage of cellular ribosome etc.

Extensive studies have been done on the antibacterial effect of bacteriocin produced by *Lactobacillus* spp*.* and their use in bio-preservation along with some antifungal chemical preservatives. The current need of bio-preservation has renewed the interest in the search for antifungal substances produced by these food grade organisms. Therefore, looking into the potential of this important genus as the producer of antifungal compounds, the present work has been taken up to study the production of antifungal proteinaceous compounds by *Lactobacillus* spp. This might further help in preparation of a bio-preservative active against both bacteria and fungi.

There have been studies regarding antimicrobial milk proteins and peptides, and their biological role and biochemical mechanisms. Activated lactoferrin (ALF) is a new form of a naturally occurring protein from milk that acts as a powerful deterrent to pathogenic bacteria that may be present on a meat surface. Considered generally recognized as safe (GRAS) by the Food and Drug Administration and recently approved by U.S. Department of Agriculture for use on fresh beef ALF can be sprayed onto carcasses to help prevent bacterial contamination during processing or can be applied to a sub-primal or finished beef surface prior to final packaging to inhibit bacterial growth and extend shelf-life.

ALF has demonstrated microbial blocking activity against a variety of food borne pathogens including *E. coli* 0157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp., *Vibrio* spp., *Aeromonas hydrophila* and *Staphylococcus aureus* as well as food spoilage microorganisms; *Bacillus* spp., *Pseudomonas* spp. *and Klebsiella* spp. ALF also inhibits yeast and molds as well as viruses (Naidu ., 2002).

Paneer is of very high nutritive value and is rich in protein (Kanawjia and Singh. 2000). It is highly perishable product and its shelf life is low mainly due to microbial and physico-chemical change. So, many preservation techniques like brining, irradiation, freezing, use of chemical additives and preservatives have been tried by various workers to slow down or inhibit these deteriorative changes for extending its shelf life, But, on the other hand, recent trend is towards natural preservation techniques than preservation by chemical or other techniques. So bio-preservation of paneer is one such method.

In view of the above, the research project is being proposed with the following objectives:

1. To standardize the media composition for the production of antifungal substances using selected strains of lactobacilli.
2. To study the effect of antifungal substances of LAB in combination with antimicrobial milk proteins and peptides on the preservation of paneer.

CHAPTER – 2

Review of Literature

2. REVIEW OF LITERATURE

India has emerged as the largest milk producer (88 million tonnes per annum) in the world (IDF, 2003). Out of the total milk produced, approximately 52- 55% is utilized for the manufacture of indigenous dairy products (Patil, 2002). Approximately 5% of milk produced in India is converted into paneer, which is well-known acid coagulated indigenous milk product (ICMR, 2000). Annual paneer production in India is approximately 300,000 tonnes, which values to about Rs. 1,050 crores (Mathur, 1998). Majority of paneer manufacture is confined to the unorganised sector of dairy industry (Mathur, 1991). Paneer making practice was mainly confined to the northwest frontier regions of India, it is presently gaining popularity in other parts of country (Ghodekar, 1989). Paneer is of great value in diet because it is a rich source of high quality proteins, fat, minerals and vitamins. It is also highly perishable milk product and its spoilage occurs due to its microbial load and physico-chemical nature. Therefore, the shelf life of paneer is 7 days at refrigeration temperature.

2.1 Spoilage of paneer

The predominant spoilage and pathogenic microflora of paneer include bacterial species like *Staphylococcus, Campylobacter jejuni, Acinetobacter, Klebsiella, Streptococcus* etc. Molds like *Aspergillus flavus, A.fumigatus, A.niger, Fusarium, Rhizopus stolonifer, Mucor, Penicillium* (Vaishnavi *et al.*, 2001) and Yeasts like *Kluyveromyces marxianus*, *Debaryomyces* spp., *Candida* spp. During refrigerated storage of goat milk paneer at 4^0C, initial standard plate counts 3.94 log cfu/ gm, proteolytic bacteria 3.34 log cfu/gm, Staphylococcus species 2,9 log cfu/ gm, psychrotrophic bacteria(2.22 log cfu/gm) and yeast and mold count 1.0 log cfu/ gm were detected in paneer. However on 7^{th} day of storage microbial counts reached level of 6.08, 5.26, 4.9, 4.26, 2.6 log cfu/ gm respectively. The results indicate that good quality paneer from goat milk (Barbari) can be prepared and stored safely only for 3 days under refrigeration temperature. (Agnihotri and Pal., 1996).

2.2 Preservation of Paneer

Studies conducted by Bhattacharya *et al.* (1971) revealed that paneer could be stored for 6 days at 10^0C without much deterioration in quality but the freshness of product is lost after 3 days. Pal (1998) studied various

microbiological and biochemical changes in paneer during storage at 8± 2° C for 15 days of storage. The mesophilic counts, yeast and mould counts as well as coliforms increased. They also reported an increase in thiobarburitic value, titratable acidity and tyrosine value but residual pH and lactose decreased. Arora and Gupta (1980) reported that the storage of paneer at subzero temperature i.e. –13°C and –32°C for 120 days did not affect its flavour and appearance but the body and texture of the product was adversely affected and it became crumbly and fluffy after thawing. It was observed that the decrease in moisture was more at -13°C while an increase in NPN was more at -32^0C. On storage of paneer at 10°C for 6 days, there was no appreciable physico-chemical change up to 6 days, but putrid flavour was developed on 7^{th} day. Thermal sterilization of paneer was attempted to enhance the shelf life of paneer at ambient temp (Sachdeva, 1983). Paneer cubes packed in tins and sterilized by autoclaving at 15 psi for 15 min kept well over a period of 50 days at room temperature with slight browning and cooked flavour. Paneer cubes fried prior to sterilization, however, spoiled earlier due to development of oxidised flavour after 40 days of storage.

2.3 Shelf life of paneer

Generally surface spoilage limits the shelf life of paneer. Thus varieties of antifungal chemical agents have been used for controlling surface spoilage. Shukla *et al*. (1984) indicated a shelf life of 6 days at room temperature when raw paneer was dipped in 18% salt solution for 30 min. Rao *et al.* (1992) reported that incorporation of 0.1% potassium sorbate enhanced the shelf life of paneer from 6 days to 18 days at 5°C. Singh *et al.* (1988) reported that shelf life of paneer could be extended up to 16 days by dipping in brine (5%) overnight followed by packaging and storing at refrigeration temperature but this method was not found suitable since continuous dipping resulted in a very soft, fragile body and dull yellow appearance of the product towards the end of 10 days of storage period. Sachdeva and Singh (1990a) reported that the fungicide (delvocid) when used in combination with a germicide (H_2O_2) gave excellent results and paneer thus treated kept good for a period of 32 days at 8-10^0C. A putrefactive odour accompanied with slight bitterness developed after 32 days rendering the product unacceptable (Sachdeva and Singh 1990b). Singh *et al.* (1989) reported a shelf life of 36 days at room temperature by adding sorbic acid to milk (0.15%) and subsequent wrapping of paneer in sorbic acid coated paper. Singh and Kanawjia (1996) increased the shelf life of recombined milk paneer up to 50 days by dipping paneer blocks in 0.2% H_2O_2

along with 0.5% delvocid at 8^0C for 2 hrs followed by vacuum packaging. On the low fat paneer paraffining improved shelf life by more than 10 days. There was increase in TBA, tyrosine and titratable acidity and decrease in moisture and pH (Pal *et al*., 1993). Application of LP system and lysozyme to enhance the shelf life of paneer was studied in which enhanced shelf life observed was upto 24 days with LP system (control showed only 8 days) and combination of LP system and lysozyme increased the shelf life of paneer upto 28 days(Agrawal and Rattan Chand, 2001).

2.4 Antimicrobial activities of lactic acid bacteria

Lactic acid bacteria of genera Lactobacillus may be involved in the production of fermented foods as starter cultures .Lactic acid bacteria can produce many substances including organic acid(lactic ,acetic), Hydrogen peroxide, diacetyl secondary reaction products , bacteriocins and bacteriocin like inhibitory substances(BLIS) (Durlu–ozkaya *et al*., 2005). Bacteria can protect food from microbial spoilage by competitive growth by production of antagonistic metabolic product or by formation of other antimicrobial compounds (Schillinger *et al.,*1996).

2.4.1 Antibacterial activities

Inhibitory compounds produced by lactic acid bacteria and their mechanism of action are shown below:

Inhibitory compound	Mechanisms of action
Lactic and other volatile acids	Disruption of cellular metabolism
Hydrogen peroxide	Inactivation of essential biomolecules by superoxide anion chain reaction, activation of lactoperoxidase system
Carbon dioxide	Anaerobic environment and/or inhibition of enzyme decarboxylation and/or disruption of the cell membrane
Diacetyl	Interference with arginine utilization
Bacteriocin (secondary metabolites)	disruption of cytoplasmic membrane (in the case of nisin)

The antibacterial effect of *Lactobacillus* is mainly due to the formation of various organic acids, H_2O_2, diacetyl, carbon dioxide, bacteriocin and antibiotic

production (Hutten *et al*., 1995; Sanders *et al*., 1991). The bactericidal effect of H_2O_2 has been attributed to its strong oxidizing effect on the bacterial cell wall, SH-groups of cell proteins and memebrane lipid oxidation.(Lingren and Dobrogosz., 1990).

L. paracasei subsp. *paracasei* in coculture with *S. thermophilus* was inhibitory against microbial contaminants in fresh cream cheese with or without the addition of inulin, indicating the potential use of this combination in a probiotic and synbiotic product.(Buriti *et al*.,2007). *Lactobacillus plantarum* F1 and *L. brevis* OG1 isolated from Nigerian fermented foods, produced bacteriocins that had broad spectrum of inhibition against both pathogenic and food spoilage organisms and various lactic acid bacteria. The test organisms exhibited activities of 6400 and 3200 AU/ml respectively against *Escherichia coli* NCTC10418 and *Enterococcus faecalis* EF1, but did not inhibit *Candida albicans* ATCC10231 and *Klebsiella* sp. UCH15.(Ogunbanwo *et al*.,2003).

Eight strains of lactic acid bacteria producing bacteriocin were isolated from Burkina Faso fermented milk samples. These strains were identified as *Lactobacillus fermentum*, *Pediococcus spp*., *Leuconostoc mesenteroides subsp. meseteroides, Lactococcus.* The bacteriocin exhibited antibacterial activity against *Enterococcus faecalis* 103907 CIP, *Bacillus cereus* 13569 LMG, *Staphylococcus aureus* ATCC 25293, *Escherichia coli* 105182 CIP (Savadogo *et al*., 2004).

2.4.2 Antifungal activities of lactic acid bacteria

El-gendy and Marth (1981) conducted the first study on the antifungal activity of lactobacillus spp. They studied the growth of aflatoxigenic or non-toxigenic strains of *Aspergillus flavus*, *A.parasiticus*, *A.ochraceus* and twelve other spp of *Penicillium* in presence of some lactic culture. They found that the growth of *Aspergillus* spp was arrested even after two weeks at 15°C on addition of *Lactococcus lactis* subsp *lactis* and *Lactobacillus casei*. The appreciable inhibition of growth of 8 of the 12 *Penicillia* by the LAB was also recorded.

Karunaratne *et al.* (1990) also investigated the inhibition of mold growth in the presence of *lactobacillus*, namely, *L.acidophilus*, *L.bulgaricus*, and *L.plantarum* individually and along with a commercial silage inoculant, containing three different strain of the same species on growth and aflatoxin

production of *Aspergillus flavus* subsp*. parasiticus* NRRL 2999*.* Among all the *Lactobacillus* studied, *Lactobacillus acodophilus* was found to be the strongest inhibitor in terms of retarding growth and aflatoxin production by *Aspergillus flavus*.

Leuconostoc mesenterioeds ATCC 8293 and *L.s casei* subsp *casei* and *L. casei* subsp *rhamnosus* from cheese starter designated as AFT (Antifungal trio) suppressed the growth of mold mycelium and formation of conidiospores in milk agar medium *L. plantarum* VTT-E-78076 also produced an antifungal substance, active against *Fusarium* spp. All strains of *L. plantarum* isolated from Gouda and Edam cheese were also able to suppress the mold growth. In a similar study Haikara *et al.* (1995) also reported that *L. plantarum* (*L. plantarum* 601) had antifungal capabilities against *Fusarium* spp. and *Aspergillus niger*. The activity was further enhanced by cellulolytic and pectinolytic enzymes and by siderophores. Further, Dicks (1994) reported a *Lactobacillus* sp., which released a low molecular weight (smaller than 10kDa), non proteolytic antifungal substance in the supernatant effective against *Monilia* and probably acted by permeabilising the cell membrane.

Similarly, De Muynck *et al.*, 2004 assessed the potential of 17 lactic acid bacteria to inhibit the outgrowth of some common food-spoilage fungi. Thirteen strains showed antifungal activity of which five strains were very promising: *L. acidophilus* LMG 9433, *L. amylovorus* DSM 20532, *L. brevis* LMG 6906, *L. coryniformis* subsp*. coryniformis* LMG 9196 and *L. plantarum* LMG 6907.

Recently, Schwenninger *et al.,* (2005) isolated *Lactobacillus* spp from different food and feed samples such as raw milk, cheese, yoghurt, olives, sour dough, as well as corn and grass silage. Out of 1424 isolate tested 82 were shown to be inhibitory to different yeasts and mold cultures (*Candida spp, Zygosaccharomyces bailii*, and a *Penicillium* spp). These fungal cultures were isolated from spoiled yoghurt and fruits.

In a similar study , Durlu-Ozkaya *et al* ., (2005) isolated and identified yeasts from cheeses and reported the antifungal activities of some *Lactobacillus* spp. against the isolated yeast belonging to *Saccharomyces cerevisiae* (10 of 17) and one each of *Candida pseudotropicalis, C. krusei, C. lipolytica, C. lusitaniae, C. ciferrii, Torulopsis glabrata and Rhodotorula rubra*. Of all the test culture, *L. plantarum* Lp 21 had the maximum inhibitory effect

against all the *S. cerevisiae* strains. (Yang and Clausen., 2005) reported that the cell-free supernatants from *Lactobacillus casei* subsp. *rhamnosus* and *Lactobacillus acidophilus* grown in deMan Rogosa Sharpe (MRS) broth inhibited 95–100% growth of three mould fungi. Antifungal activity was attributed to one or more unknown heat and pH stable metabolites.

2.5 Antifungal substances of *Lactobacillus* spp.

Lactic acid bacteria produce a variety of compounds with antimicrobial activity. Lactic and acetic acids are produced as end products during lactic acid bacterial fermentation causing a reduction in pH, but other substances such as hydrogen peroxide, formic acid, propionic acid, acetoin and diacetyl, are also produced (Lindgren and Dobroqosz, 1990).

The precise mechanism of antimicrobial action can often not be defined because of a complex interaction between different compounds. Synergistic effects are often seen between the compounds involved in the antimicrobial action (Corsetti *et al*., 1998; Niku-Paavola *et al*., 1999). Much research has been directed towards identifying different antimicrobial substances, primarily antibacterial, in simple *in vitro* systems, but little is known about the overall mechanisms of complex preservation systems within food and feed environments (Earnshaw, 1992).

The inhibitory compounds are mainly fermentation products, proteinaceous compounds and low molecular weight inhibitory compounds. Microbial growth can be inhibited by the fermentation products like weak organic acids. Since at a certain concentration; they lower the pH level where many microorganisms cannot grow. The reports regarding the role of organic acids such as lactic acids, propionic acids, acetic acids in suppressing fungal growth have remained quiet insufficient.

Reiss (1976) observed that 0.75% lactic acid (approx. 80 mM) reduced the growth of *Aspergillus parasiticus*. Propionic acid negatively influence fungal growth, especially at lower pH (Woolford, 1984), and affect fungal membranes at pH values below 4.5 (Hunter and Segel, 1973). Moon (1983) found that mixtures of high concentrations of lactic, acetic and propionic acid inhibited yeast species that normally grow well in relatively high concentrations (100 mM) of the individual acids, except for propionic acid. The combination of lactic acid produced during LAB growth and the sodium acetate of de Man, Rogosa,

Sharpe (MRS) substrate a standard growth medium for LAB, has synergistic antifungal effects (Stiles *et al*., 2002).

Hydrogen peroxide accumulates in the environment since LAB does not produces catalase (Condon, 1987). The antimicrobial effect of hydrogen peroxide is well documented (Davidson *et al*., 1983). Fitzsimmons and Berry (1994) reported the inhibitory effect of this system against *Candida albicans*.

Diacetyl (2,3-butanedione) is the molecule responsible for the characteristic aroma associated with butter. It is produced by strains of all genera of lactic acid bacteria during citrate fermentation (Earnshaw, 1992). The antimicrobial effect of diacetyl is well documented, especially at pH below 7.0 (Jay, 1982). However, the amount of diacetyl needed to exert antimicrobial activity (close to 200 mM) will dramatically alter the taste and aroma of the product (Piard and Desmazeaud, 1991).

LAB produces proteinaceous compounds which are antibacterial, ribosomally synthesised, peptides, generally termed bacteriocins (Nes *et al*., 1996). A large number of bacteriocins have been characterized from lactic acid bacteria in recent years. The bacteriocins from lactic acid bacteria are commonly divided into three groups: class I – the lantibiotics; class II – the heat stable unmodified bacteriocins; class III the larger heat stable bacteriocins (Nes *et al*., 1996; Nes and Holo, 2000). These compounds are generally only active against closely related bacterial species and there is no evidence that bacteriocins have any effect on growth of yeast or moulds. In contrast, there are only few reports on the production on antifungal peptides produced by lactic acid bacteria. Several authors have reported that the antifungal activity of LAB is lost after treatment with proteolytic enzymes.

Batish *et al*., (1989) claimed that the antifungal substance produced by a lactic acid bacterium was of proteinaceous nature since it was degraded by proteinases. In a similar study by Roy *et al*., (1996) reported a proteinaceous AFS from *Lactococcus lactis* subsp. *lactis* with antagonistic activity against several filamentous fungi. After enzymatic treatment with chymotrypsin, trypsin and protienase E, the antifungal activity disappeared, indicating a proteinaceous nature of the antifungal substance.

Gourama and Bullerman (1995, 1997) showed that a commercially available silage inoculant with a combination of *Lactobacillus* species (*L.*

plantarum, L bulgaricus and *L. acidophilus*) exerted antifungal and anti-aflatoxin activity against *A. flavus*. Guorama and Bullerman (1995) found that *Lactobacillus casei* subsp. *pseudoplantarum* was responsible for the inhibitory activity. The activity was sensitive to treatments with the proteolytic enzymes trypsin and α-chymotrypsin, the activity was due to the production of a small peptide of less than 1 kDa. The antifungal proteinaceous compounds from *Lactobacillus* spp. has also been purified.

Okkers *et al*., (1999) purified and characterized a medium length bacteriocin like peptide TV35b from *Lactobacillus pentosus* with fungistatic effect against *Candida albicans* and bactericidal effect against many bacteria. The molecular size of pentocin TV35b was estimated to be between 2·35 and 3·4 kDa.

A proteinaceous compound produced by *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 had antifungal effect against several moulds and yeasts cultures like *Debaromyces hansenii* and *Kluyveromyces marxianus* (Magnusson and Schnu., 2001). The partially purified AFS was a small peptide (approx. 3kDa), heat stable (activity remains even after autoclaving), active in the pH range 3-6 and totally inactivated by proteinase K or trypsin.

Atanassova *et al*., 2003 reported that Lactobacillus paracasei subsp paracasei M3 – a starter strain in Bulgarian yellow cheese showed antiyeast activity against *Candida albicans*, *Candida blankii*, *Candida pseudointermedia* and *Saccharomyces cerevisiae*. The activity was due to the production of proteinaceous compounds of molecular weight 43 KDa. The AFS is heat stable (100 °C / 3 minute) and maximum activity detected at pH 6. The activity was found to be stable at pH 8. The maximum production of AFS occurs at late exponential LAB growth phase. The active compound is highly hydrophobic. They also reported that the gene coding for the active compound located most probably on the bacterial chromosome.

Several authors have reported the detection of antifungal low molecular weight compounds, but the number of purified and chemically characterized compounds is still low. Reuterin, a broad-spectrum antimicrobial substance originally described from *Lactobacillus reuteri*, is one of the most intensively studied low molecular weight inhibitory compounds (Talarico *et al*., 1988; Chung *et al*., 1989; Nakanishi, 2002). Reuterin is active against several

different types of microorganisms including gram-positive and gram-negative bacteria, yeast and fungi. Antifungal activity was shown against species of *Candida, Torulopsis, Saccharomyces, Aspergillus* and *Fusarium* (Chung *et al*., 1989).

Sjogren *et al.* (2003) identified and characterized four fatty acids which acts as an antifungal substances namely 3-(*R*)-hydroxydecanoic acid, 3-hydroxy-5-*cis*-dodecenoic acid, 3-(*R*)-hydroxydodecanoic acid and 3-(*R*)-hydroxytetradecanoic acid, from *Lactobacillus plantarum* MiLAB 14 .They found that yeasts were generally more sensitive to the hydroxylated fatty acids.

Magnusson *et al.* (2001) had found that there are low concentrations of several cyclic dipeptides present in MRS (broth and agar) and other complex media for growth of lactic acid bacteria. However, these amounts are not themselves high enough to exert antimicrobial activity in the applied bioassays. The cyclic dipeptides have antifungal activity at mg per ml concentrations, and hence are much less effective than the hydroxylated fatty acids. Niku-Paavola *et al*. (1999) discovered new types of antimicrobial compounds from the culture filtrate of *Lactobacillus plantarum* VTT E-78076. The active fraction included benzoic acid, 5-methyl-2,4-imidazolidinedione methylhydantoine), cyclo(glycyl-L-leucyl) , and tetrahydro-4-hydroxy- methyl-2H-pyran-2-one (mevalonolactone).

Lavermicocca *et al*. (2000) reported the production of phenyllactic acid and 4-hydroxyphenyllactic acid from *L. plantarum* 21b, which has antifungal activity against several species of filamentous fungi. Phenyllactic acid has also been identified from culture supernatants of *Lactobacillus plantarum* MiLAB 393 Ström *et al*., 2002, and from *Lactobacillus coryniformis* strain Si3.

Falguni and Vij (2006) recently reported the production of protienaceous antifungal substance by standard *Lactobacillus* cultures viz. *L. acidophilus, L. casei* and *L. collinodeis* in MRS broth. The AFS was heat stable at 100°C for 15 minutes, pH stable and storage stable for 6 days at refrigeration temperature. The active compound was hydrophobic in nature and was a small peptide (between 1 to 5 kDa).

2.6 Effect of Media and Additives

2.6.1 Effect of Growth media

Several workers have recorded the variations in the production of antimicrobial substances by *Lactobacillus* spp. In different growth medium, it was also noticed that the organism of same species and strains produce different amount of antimicrobial substances in different growth medium.

Lal (1987) used eight different medium (Elliker's broth,MRS broth, TYD broth, Trypticase soy broth, nutrient broth, yeast glucose broth, modified Chalmers medium and reconstituted skim milk 11%) for determining the effect of growth medium on the production of antifungal substance by Lactococcal spp. Maximum production of AFS occurred in Ellikers broth as compared to other media tested. Similar results were shown by Roy (1996).

2.6.2 Effect of Additives

Maximum bacteriocin production could be obtained by supplementing a culture medium with growth limiting factors, such as sugars, vitamins and nitrogen sources, by regulating ph or by choosing the best –adapted culture medium (*Vignolo et al*.,1995).

Ogunbanwo *et al.* (2003) obtained maximum bacteriocin production by supplementing 1 % glucose and 3 % yeast extract, 1-2 % NaCl, 0.5 % Tween 80 to normal MRS media. Addition of yeast extract (0.3-1.0% w/v) to milk medium enhanced both growth and bacteriocin production for all strains of Lactobacillus by Avonts et al(2004). Bacteriocin production was clearly observed in yeast extract supplemented milk medium for *L. acidophilus* IBB 801, L. *Johnsonii Lal, and L. gasseri* K7. *L. acidophilus* IBB 801, the only strain of dairy origin, displayed the best growth (10.5 log CFU/ ml) and bacteriocin production (3200 AU/ ml).

Optimization of bacteriocin production by *lactobacillus plantarum* ST13BR, a strain isolated from barley beer has been done in which Tween 80 in MRS broth increased bacteriocin production by more than 50 % whereas, meat extract or beef extract as a sole nitrogen source or a combination of the two (1:1) in MRS broth, stimulated bacteriocin production (6400 AU/ml) and the maximum bacteriocin production was recorded in presence of 2 % (W/V) maltose (Todorov *et al*., 2004).

Production of the anti-listerial bacteriocin, pediocin, by lactic acid bacteria (LAB) transformed with the cloning vector pPC418 (Ped^+, 9.1 kb) was influenced by composition of media. The amount of pediocin produced by *S. thermophilus* in skim milk and cheese whey supplemented with 0.5% yeast extract was estimated as 51 000 units/ ml and 25 000 units/ ml, respectively. Pediocin production remained essentially unchanged in reconstituted skim milk or whey media diluted up to 10-fold. The results demonstrate the capacity of recombinant strains of LAB to produce pediocin in a variety of growth media including skim milk and inexpensive cheese whey-based media, requiring minimum nutritional supplementation. (Somkuti and Steinberg, 2004).

Effect of Growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a Strain Isolated from Boza has been studied. Optimal production (12 800 AU/ml) was recorded in the presence of tryptone (20 g/L), a combination of tryptone and meat extract (1:0.6), or tryptone and yeast extract (1:0.6). Growth of strain ST194BZ in the presence of 10 or 20 g/L of D-mannose yielded bacteriocin levels of 12 800 AU/ml. In the presence of 30 or 40 g/L of mannose the activity levels doubled to 25 600 AU/ml.(Todorov *et al*., 2005) Effect of different carbon and nitrogen source on bacteriocin production by LAB isolated from agro-based waste studied. In which increase in the bacteriocin production was observed in MRS supplemented with 3% lactose and 2% peptone. Lactose was found to be better source of carbon than glucose (Lade *et al.,* 2006).

2.7 Biopreservation

Since Centuries, foods have been preserved by chilling, drying, acidification. Oxygen removal, fermentation, adding various preservatives etc. and these methods are applied in combination more recently. A new area of food stabilization called bio-preservation based on antagonistic displayed by microorganisms towards another organism is gaining interest. Bio-preservation refers to the storage life and enhanced safety of food using natural or controlled micro flora associated antibacterial products. In this context, LAB plays an important role in food stabilization. Due to the GRAS status of LAB the interest in using them for bio preservation has increased during recent years. Many studies have assessed the antibacterial effect of lactic acid bacteria but few reports are available on specific antifungal compounds of Lactobacilli.

Therefore this led to explore the antifungal substance production by the strains of Lactobacilli.

2.7.1 Biopreservation using bacteriocin / BLIS

Nisin, the most widely used bacteriocin has found application in biopreservation of various foods like dairy, meat, wine and canned foods (Triona and Hill, 1999). Plantaricin D, a bacteriocin produced by the *lactobacillus plantarum* strain was one of a few selected LAB that were isolated from vegetables foods and were considered for application in biopreservation. (Franz et *al.*, 1998). Lavermicocca *et al*. (2000) identified phenylactic acid from a *L. plantarum* strain originally isolated from sour dough They also demonstrated an improved shelf life of bread by addition of this strain as a starter in the sour dough .Similarly, Laitila. *et al*. (2002) indicated that *Lactobacillus plantarum* strains with known and selected characteristics could be used as a natural, food grade bio-control agent for management of problems caused by *Fusarium* fungi during germination of cereals. Mundticin, the bacteriocin produced by *E.. mundtii* was found to have potential as a bio-preservation agent for MA-stored mungbean sprouts when used in a washing step or a coating procedure (Bennik *et al.,* 1999).

The live cells of bacteriocin producing *Leuconostoc carnosum* 4010 was the most efficient method as it inhibited the growth of *L. monocytogenes* in cooked, sliced and gas packed saveloy stored at 5 and 10 c for four weeks (Jacobsen *et al*., 2003). *Lactobacillus sake* and *Lactobacillus curvatus* producing BLIS were inoculated individually or in combination on slices of beef has lower spoilage microbial counts substantially (Katikou *et al*.,2005). Another study, investigated the usefulness of two selected LAB, *Lb. sakei* ssp. *carnosus* and lactocin S producing *L. sake* 148 to extend the shelf life of cooked ham (Vermeiren *et al.,* 2006).

2.7.2 Biopreservation using milk proteins and peptides

2.7.2.1 Lactoferrin

The economic impact of food borne pathogen outbreaks and less than desired shelf life of vacuum packaging and refrigerated products and consumer demand for all natural food products have necessitated the development of effective natural antimicrobial preservation systems for the dairy industry. Lactoferrin (LF) is the main iron-glycoprotein present in the milk of various

mammals and it exerts an antimicrobial effect against a wide range of Gram-negative and Gram-positive bacteria, fungi and parasites. Many studies have indicated that LF has the potential to be used as a natural antimicrobial preservative in the food industry (Holley, 2003).

Bacteriostatic effect of lactoferrin has been reported against *L. monocytogenes* in UHT pasteurized milk. Fungistatic effect of lactoferrin in combination with antifungal drugs was analyzed against *Candida albicans.* (Kuipers *et al.,* 1999). Inhibition of *Penicillium commune* was reported by edible whey protein films incorporating lactoferrin and lactoferrin hydolysate(Seacheol and Krochta., 2005). Lactoferrin activity has been studied against *Salmonella typhimurium* and *Campylobacter jejuni* on poultry broiler skin and also *Listeria monocytogenes* in ready- to- eat foods (Naidu, 2002). Antifungal properties of lactoferricin B was analyzed against Yeasts like *Candida albicans, Cryptococcus curvatus, C.albidus and* Molds like *Aspergillus niger, A. fumigatus, Rhizopus oryzae* (Bellamy *et al.,* 1993).

Activated lactoferrin (ALF) is a new form of a naturally occurring protein from milk that acts as a powerful deterrent to pathogenic bacteria that may be present on a meat surface. ALF has demonstrated microbial blocking activity against a variety of food borns pathogens including *E. coli* 0157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp., *Vibrio* spp., *Aeromonas hydrophila* and *Staphylococcus aureus* as well as food spoilage microorganisms; *Bacillus* spp., *Pseudomonas* spp. *and Klebsiella* spp. ALF also inhibits yeast and molds as well as DNA and RNA viruses (Naidu, 2002).

2.7.2.2 Lactoferrin hydrolysate

The action of pepsin on lactoferrin produces peptides that have enhanced antimicrobial action as compared to lactoferrin (Tomita *et al.*, 1998). A peptide with 25 residues (17-41) from N-terminal of lactoferrin, named lactoferricin B has much stronger antibacterial activity than lactoferrin (Jones *et al.*, 1996). Multiple peptides have been isolated from pepsin hydrolysates of lactoferrin with varying microbial activities (Dionysius and Milme., 1997) isolated 3 peptides with different structure and activities. Lactoferricin B has been shown to be effective at concentrations as low as 3 µg/ l against a number of different strains of yeast and filamentous fungi (Bellamy *et al.*, 1994).

2.7.2.3 Other peptides

Three peptides produced by a *Lactobacillus acidophilus* DPC6026 fermentation of sodium caseinate and showing antibacterial activity against pathogenic strains *Enterobacter sakazakii* and *E. coli* were characterized by Hayes *et al*. (2006).These peptides may have bio-protective applicability and potential use in milk-based formula which has been linked to *E*. *sakazakii* infection in neonates. Three peptides αs1- CN f(21-29),α s1- CN f(30-37),α s1-CN f(195-208) produced by *Lactobacillus acidophilus* fermentation using sodium caseinate as substrate. Cationic peptides from α -s1 & α -s2 casein exhibited antibacterial activity against *Micrococcus luteus, Escherichia coli and Bacillus cereus.* The antibacterial activity of cationic peptides derived from α_{s1-} casein was greater than that of α_{s2-} casein, against both Gram positive and Gram negative organisms. (Bajaj *et al* ., 2005). The small antimicrobial peptide PAF26 (Ac-RKKWFW-NH2) has been identified by a combinatorial approach and shows preferential activity toward filamentous fungi like *Penicillium digitatum*. (Munoz *et al*., 2006).

CHAPTER – 3

Material and Methods

3. MATERIALS AND METHODS

The present study was carried out in four phases. In first phase screening of *Lactobacillus* culture was done. In second phase, supplementation of different ingredients in whey and milk based media for maximum antifungal activity was done for selected three *lactobacillus* cultures. In third phase, different preservation treatments with varying combinations of preservatives was given to paneer sample .In fourth phase storage studies of paneer was done at refrigeration (7^0C) temperature and the samples were subjected to microbiological and chemical analysis and also for sensory evaluation.

3.1 Chemicals

All the chemicals used in the present study were of Himedia, Qualigens, CDH, Sigma Companies, and were of analytical grade. Pediocin was procured from microbial metabolites laboratory, Dairy Microbiology Division. Packaging material was procured from packaging laboratory of Dairy Technology Division, NDRI, Karnal.

3.2 Microbial cultures

The details of the *Lactobacillus* cultures and yeast and mold cultures used in this study are given below:

3.2.1 *Lactobacillus* cultures

Three standard *Lactobacillus* cultures used in this study were procured from the National Collection of Dairy Culture (NCDC), NDRI, Karnal included.

- *Lactobacillus collinoides* (NCDC 02)
- *Lactobacillus casei* spp. *casei* (NCDC 17)
- *Lactobacillus acidophilus* (NCDC 195)

3.2.2 Indicator organisms

Two yeast and two mold cultures used in this study as indicator organisms were procured from the National Collection of Dairy Culture (NCDC), NDRI, Karnal also the bacterial cultures used were procured from the Institute Of Microbial Technology (IM.Tech.) Chandigardh. These are:

3.2.2.1 Yeast cultures

- *Kluyveromyces marxianus* (NCDC 41)
- *Candida guillermondii* (NCDC 44)
- *Torulopsis candida* (NCDC 43)
- *Saccharomyces cerevisiae* (NCDC 47)

3.2.2.2 Mold cultures

- *Aspergillus niger* (NCDC 267)
- *Rhizopus oryzae* (NCDC 52)
- *Penicillium roqueforti* (NCDC 170)
- Penicillium camemberti *(NCDC 56)*

3.2.2.3 Bacterial cultures

- *Staphylococcus aureus* (MTCC 1144)
- *Bacillus Cereus* (MTCC 1272)
- *Escherichia coli* (MTCC 739)
- *Pseudomonas aeroginosa* (MTCC 741)

3.3 Maintenance of cultures

3.3.1 Maintenance of *Lactobacillus* cultures

All the *Lactobacillus* cultures were maintained in litmus milk, and stored in refrigerator until use. These were periodically sub-cultured in the same medium once in a week. Each culture was activated by sub-culturing before use.

3.3.2 Maintenance of Fungal cultures

All the mold and yeast cultures were maintained on Yeast peptone dextrose agar (YPD – Hi-Media) at 30 °C. Yeast cultures were sub-cultured once in 15 days and mold cultures at 30 days intervals. The YPD agar slants of the molds and yeasts were stored in refrigerator until further use.

3.3.3 Maintenance of Bacterial cultures

All the bacterial cultures were maintained on Brain Heart Infusion agar at 37°C. The Pathogenic bacterial cultures were sub-cultured once in 15 days interval. BHI slants were stored in refrigeration temperature until further use. For prolonged storage 50% glycerol stock was used.

3.4 Purity of microbial cultures

The purity of microbial cultures was tested by microscopic examination using Gram staining, simple staining and fungal staining (Lactophenol cotton Blue) for bacterial, yeast and mold cultures, respectively

3.5 Screening of *Lactobacillus* cultures for antifungal activity

Three standard *Lactobacillus* cultures, *Lactobacillus casei* spp. *casei* NCDC 17, *Lactobacillus collinoides* (NCDC 02), *Lactobacillus acidophilus* (NCDC 195) were used in this study were tested for their antifungal activity against different yeast and mold cultures by agar spot and agar well diffusion methods.

3.5.1 Preparation of spore suspension of mold culture

All mold cultures were cultivated on YPD agar slant. After 3 – 5 days incubation at 30 °C, 10 ml of sterile saline water (0.8 % NaCl having 0.1 % Tween80) was added to slant culture and the mold spores were then scrapped from the slants with a sterile platinum loop. The spore suspension was vigorously agitated to disperse the spore clumps. The spore count was adjusted to 10^4 spore / ml of saline by adjusting optical density at 530 nm. Yeast cell count was also adjusted to 10^4 cfu / ml by using four hours old growth in YPD broth.

3.5.2 Agar spot assay

The overlay method was performed using MRS agar plates on which *Lactobacilli* were inoculated as two 2 cm long lines and incubated at 37°C for 48 hrs. The plates were then overlayed with 7 ml of soft agar (0.75 % agar) containing 10^4 yeast cells or fungal spores per ml. The plates were then incubated at 30°C for 48 hrs and examined for clear zones of inhibition around the bacterial streak (Magnusson *et al.,* 2001).

3.5.3 Preparation of cell free supernatant

The 24-hour fresh *Lactobacillus* culture was inoculated into MRS (De-Mann Rogosa Sharpe) broth and Skim milk @1% and incubated at 37°C for 48 hours. After incubation, the inoculated medium was centrifuged at 10,000 rpm at 10°C for 10 minutes. The supernatant was collected, filter sterilized (0.45 μm membrane filter) and concentrated to 10 fold. This concentrated supernatant was used for assessing antifungal activity by agar well diffusion assay.

3.5.4 Agar well diffusion assay

For the agar well diffusion assay, agar plates containing 10^4 spores of mold or yeast cells per ml agar were prepared. Wells, with a diameter of 4 mm, were then cut in the agar using a sterile cork-borer. A droplet of agar was added to each well in order to seal it to avoid leakage. Then, 100-µl of 10 fold concentrated samples was added to the wells and allowed to diffuse into the agar during a 5-h preincubation period at room temperature, followed by aerobic incubation at 30°C for 48 hours. The plates were then examined for the formation of clear zones around the wells filled with respective culture supernatants. The zones of inhibition surrounding the well (including well diameter) were measured with the help of a Calliper in terms of millimeters (Magnusson *et al.,* 2001).

3.6 Isolation of Lactoferrin

Lactoferrin was isolated from cow colostrums by the method of Law and Reiter (1977).

3.6.1 Preparation of Acid Whey

Colostrum samples of the first day of calving were collected from the Cattle Yard at National Dairy Research Institute, Karnal. Colostrum was diluted three-fold with distilled water and warmed to 40°C. Fat was removed using Alpha-Laval cream separator. Skimmed colostrums was acidified to pH 4.6 using 2 N HCl. The precipitated casein was removed and the clear whey was obtained by filtration through Whatman No.1 filter paper.

3.6.2 Cation-exchange chromatography

Weakly acidic cation exchanger CM-Sephadex C-50 was added to the acid whey @ 1 g/ litre and allowed to stir over magnetic stirrer for 1 h and thereafter allowed to settle for half an hour. The sample was then decanted taking care to prevent the loss of resin. The sample resin mixture was transferred over Buchnner funnel, layered with Whatman No.1 filter paper and fitted to the vacuum flask. Under gentle vacuum, the resin was washed with (about 2 litres) distilled water and 50 mm Tris-HCl buffer (pH 8.4) (about 2 litres) till the absorbance of eluate was <0.02 at 280 nm. The resin was packed in glass column (4 x 50 cm) and washed with one column volume Tris-HCl buffer (50 mm, pH 8.4), followed by two columns volume Tris-HCl (50 mm, ph 8.4) buffer containing 0.2 M NaCl. Finally lactoferrin was eluted with 50 mM Tris-Hcl buffer (pH 8.4) containing 0.5 M Nacl. The pink coloured crude lactoferrin was subjected to dialysis overnight against 50 mm Tris-HCl buffer (pH 8.4) with three changes. Crude Lactoferrin was further freeze dried and used for bio-preservation.

3.7 Supplementation of ingredients in milk and whey for AFS production

Different ingredients with varying concentrations given below were added in skim milk and whey to increase the antifungal activity of selected *Lactobacillus* cultures in milk and whey media. 50 ml of milk and whey supplemented with different ingredients were dispensed in flask and autoclaved. All the three previously selected cultures viz. *Lactobacillus collinoides* (NCDC 02), *Lactobacillus casei* spp *casei* (NCDC 17), *Lactobacillus acidophilus* (NCDC 195) were inoculated @1% in both the media and incubated at 37°C for 48 h.

3.7.1 *Effect of nitrogen source*

3.7.1.1 Effect of yeast extract

Yeast extract was incorporated in the skim milk at the concentration of 0.5.1.0, 1.5, 2.0, 2.5 and 3.0 percent before sterilization followed by culture inoculation, incubation then culture filtrate was tested for production of antifungal substance.

3.7.1.2 Effect of peptone

Peptone at the level of 0.5.1.0, 1.5, 2.0, 2.5 and 3.0 percent was added in skim milk before testing the culture filtrate for antifungal activity.

3.7.2 *Effect of carbon source*

3.7.2.1 Effect of glucose

Glucose was added to the skim milk at the level of 0.25, 0.5, 0.75, and 1.0 percent before sterilization.

3.7.2.2 Effect of maltose

Different concentration of maltose including 0.25, 0.5, 0.75, and 1.0 percent was supplemented to the skim milk followed by autoclaving.

3.7.2.3 Effect of mannose

Supplementation of skim milk was done by mannose at the level of 0.25, 0.5, 0.75, and 1.0 percent before sterilization followed by culture inoculation, incubation then culture filtrate was tested for production of antifungal activity.

3.7.3 Effect of Tween 80

Skim milk was supplemented with Tween 80 at the concentration of 0.1, 0.2, 0.3, 0.4, 0.5 percent, before inoculation with *lactobacillus* culture.

3.7.4 Effect of different ingredients in combination

Different nitrogen sources (peptone @ 1 % ,yeast extract @ 2 %) and carbon sources (glucose ,maltose, mannose @0.5 %) along-with Tween 80 @

0.1 % were used for maximum antifungal activity of *L.casei* spp. *casei* NCDC 17 in skim milk medium as given below:-

Sr. No.	Ingredients
1	Yeast extract + Glucose+ Tween 80
2	Yeast extract + Maltose + Tween 80
3	Yeast extract +Mannose + Tween 80
4	Peptone + Glucose + Tween 80
5	Peptone + Maltose + Tween 80
6	Peptone + Mannose + Tween 80

3.8 Production of Antifungal Substance

Antifungal substance which were obtained by selection and optimization of basal medium. The most promising *L.casei* spp *casei* NCDC 17 lactobacillus culture was used. The media selected was skim milk supplemented with peptone @1 % + maltose@ 0.5% +Tween 80 @ 0.1%. The antifungal substance produced was filter sterilized ,concentrated to 10 fold concentration by using distilled water and used for preservation of paneer.

3.9 Spectrum of antimicrobial activity of *L. casei* spp. *casei* NCDC 17

3.9.1 Antifungal activity

It was done as given in section 3.5 against the yeast and mold cultures

Yeast cultures: *Kluyveromyces marxianus* NCDC 41, *Candida guillermondii* NCDC 44, *Torulopsis candida* NCDC 43, *Saccharomyces cerevisiae* NCDC 47.

Mold cultures: *Aspergillus niger* NCDC 267, *Rhizopus oryzae* NCDC 52, *Penicillium roqueforti* NCDC 170, *Penicillium camemberti* NCDC 56.

3.9.2 Antibacterial activity of *Lactobacillus* cultures

The antibacterial activity in the culture supernatants was determined according to the agar well diffusion method of Schillinger and Lucke (1998) with slight modifications.

To the nutrient agar medium Tween 80 (0.1%) was added and the sterile medium was poured in to petriplates and the agar plates were dried overnight at room temperature .On the dried agar surface 100 µl of fresh overnight culture of bacteria ,the indicator strain (O.D. 0.35) was overlayed with soft agar (0.75 %) .wells (4mm) were punched out of the agar .100 µl of the culture supernatant of *Lb. casei* spp. *casei* NCDC 17 was placed into each well and incubated for 24 hours at 37 C .The plates were examined for zone of inhibition.

3.10 Preservation of paneer

Fresh paneer samples were procured from the Experimental Dairy of National Dairy Research Institute, Karnal. Paneer immediately after pressing when hot was collected aseptically in plastic bags and given different treatments of antifungal substances alongwith pediocin and lactoferrin.

3.10.1 Treatment of paneer with various antifungal agents.

3.9101.1 Procedure

1. Fresh Paneer blocks were cut into (1x1.5x2 inch) size weighing about 25 gms each. whole lot of paneer sample was divided into 7 lots and given the following treatments
2. The dipping solution was prepared (500 ml) by adding different preservatives in distilled water as follows:
    - Lactoferrin @150 µg/ml
    - Pediocin@ 4000 AU/ml
    - Sodium Citrate @ 0.6%

- Potassium Sorbate @ 0.1%
- Antifungal Substance @ 10 Fold Concentration

Different combinations of preservatives were added to sterilized distilled water according to the treatments required. The Ingredients were added in various combinations. Lactoferrin solution was Filter sterilized (0.22 µm) and added to dipping solution.

Sample	**Treatments**
C	Control
T-1	Pediocin + sodium citrate
T-2	Pediocin + sodium citrate + potassium sorbate
T-3	Pediocin + sodium citrate +lactoferrin
T-4	Pediocin + sodium citrate + antifungal substance
T-5	Pediocin + antifungal substance+ lactoferrin
T-6	Lactoferrin

(a) Control: First lot was dipped in the boiled and cooled distilled water for two hours which act as a control.

(b) Treatment 1: Second lot was dipped in the boiled and cooled distilled water for two hours in which pediocin and sodium citrate was added. It acts as a control for antifungal agent.

(***c) Treatment 2:*** Third lot was dipped in the boiled and cooled distilled water for two hours in which pediocin, sodium citrate and potassium sorbate were added which acts as a chemical antifungal agent.

(d) Treatment 3: Fourth lot was dipped in the boiled and cooled distilled water for two hours in which pediocin, sodium citrate and filter sterilized Lactoferrin was added which is antimicrobial milk protein.

(e) Treatment 4: Fifth lot was dipped in the boiled and cooled distilled water for two hours in which pediocin, sodium citrate was added and then each block was treated with 10 fold concentrated antifungal substance.

(f) Treatment 5: Sixth lot was dipped in the boiled and cooled distilled water for two hours in which pediocin and filter sterilized lactoferrin was added and then treated with 10 fold concentrated antifungal substance. This combination acts as a bio-preservative.

(g) Treatment 6: Seventh lot was dipped in the boiled and cooled distilled water for two hours in which only filter sterilized lactoferrin was added. This is also a natural preservative

Paneer blocks after dipping into the solutions for two hours were vacuum packaged in cryovac pouches.

3.11 Storage studies

All seven lots of paneer samples including control were kept at 7^0C (refrigeration) for storage study for 28 days. Analysis of samples was done on 0 day, 3 day, 7 day, 10 day, 15 day, 21 day and 28 day for the following parameters:

3.11.1 Microbiological parameters:

The stored samples of paneer were opened aseptically in an by UV irradiated inoculation chamber. 11 gm of paneer was aseptically weighed and transferred to a sterile mortar and a paste was prepared by adding 20 ml of sterile 2% sodium citrate solution from 99 ml of the solution. The sample was

dissolved in total 99 ml of 2% sodium citrate solution and then total plate counts and yeast and mould counts were carried out using this sample.

3.11.1.1 Total plate counts

It was carried out by preparing serial dilutions of the paneer sample as described in IS: 1479 (part III, 1977). Plate count agar was used and plates were incubated at 37^0C for 24 to 48 hours.

3.11.1.2 Yeast and mould counts

It was carried out by using potato dextrose agar and plates were incubated at 30^0C for 3 to 5 days.

3.11.2 Chemical parameters

Paneer samples were subjected to chemical analysis during storage. Paneer samples were grated and mixed well and subsequently used for analysis. Parameter studied were as follows

3.11.2.1 Titratable acidity

The titratable acidity of paneer was determined by method recommended by the Association Of Analytical Chemist (AOAC, 1984) for cheese. To 10 gm prepared paneer sample ,water at 40^0C was added to make up to 105 ml. It was mixed vigorously and then filtered. 25 ml of this filtrate, representing 2.5 gm sample was titrated against 0.1 N NaOH using phenolphthalein as indicator .results were expressed as percent lactic acid.

3.11.2.2 Moisture content

The moisture content of paneer was determined by method recommended by the Association of Analytical Chemist (AOAC, 1984) for cheese with some modification. 2 to 3 gm of prepared paneer sample was weighed in a moisture dish with tight fit cover. Then the sample was shredded by shredder equally distributed over the Petri-plate and kept in forced draft oven at 130 $\pm$1 ^{0}C . Sample was dried for 1.25 hr (with cover entirely off), then covered tightly, before

removing from oven, cooled and weighed .the loss in weight was expressed as moisture content.

3.11.3 Sensory evaluation of paneer

The sensory evaluation of paneer was done by a panel after definite interval of storage as mentioned above. The five point hedonic scale was used as per the score card given below.

SENSORY EVALUATION CARD FOR PANEER

Five point hedonic scale:

Poor	1
Fair	2
Good	3
Very good	4
Excellent	5

No.	**Attributes**	C	T-1	T-2	T-3	T-4	T-5	T-6
1	Appearance							
2	Flavor							
3	Body and texture							
4	Overall acceptability							

CHAPTER – 4

Results and Discussion

4. RESULTS AND DISCUSSION

This chapter includes the result on the screening of three standard *Lactobacillus* cultures for antifungal substance production in different growth media, their production in milk and whey medium supplemented with various ingredients to enhance the antifungal activity and the application of the antifungal substances (AFS) produced in preservation of paneer along with milk proteins. The results obtained in the present investigation have been presented in Tables, Figures, Histogram and Photographs.

4.1 Screening of Lactobacilli for antifungal activity

Three standard *Lactobacillus* cultures, *Lactobacillus collinoides* NCDC 02, *L. casei* spp *casei* NCDC 17 and *L. acidophilus* NCDC 195 showing antifungal activity in previous studies (Falguni and Vij , 2006) have been examined for antifungal activity against the test cultures by spot assay method (Plate1). It is quite evident that all the standard *Lactobacillus* cultures exhibited distinct antifungal activity against *R. oryzae* NCDC 52, A. *niger* NCDC 267, *K*. *marxianus* NCDC 41 and *C. gullerimondii* NCDC 44 fungal cultures by spot assay method.

4.2 Antifungal activity of *Lactobacillus* cultures in different growth media

Different growth media namely MRS, skim milk and whey were used for AFS production by three selected *Lactobacillus* cultures viz., *L. collinoides* NCDC 02, *L. casei* spp. *casei* NCDC 17, *Lb. acidophilus* NCDC 195. The results pertaining to the effect of different growth medium on the production of antifungal substances (AFS) has been presented in Table 1.

From the data therein, it is clear that maximum production of AFS by test culture was achieved in MRS medium. However, *L. acidophilus* NCDC 195 and *L. casei* spp. *casei* NCDC 17 also showed antifungal activity in skim milk and whey, whereas *L. collinoides* did not show any antifungal activity in skim milk and whey. Therefore, *L. casei* NCDC 17 and *L. acidophilus* NCDC

195 were selected for further studies for the production of AFS in skim milk and whey medium in order to use their AFS for preservation of paneer.

Tabel 1: Screening of *Lactobacillus* culture for anti-fungal activity.

Media	Lactobacillus cultures											
	02				17				195			
	Fungal cultures											
	41	44	52	267	41	44	52	267	41	44	52	267
	Zone of inhibition (mm diameter)											
MRS	7	9	20	20	12	12	21	14	14	11	12	13
Skim milk	-	-	-	-	-	13	12	8	-	12	11	17
Whey	-	-	-	-	-	12	10	6	-	11	10	9

***Lactobacillus* cultures:** *Lactobacillus collinoides* NCDC 02; *Lactobacillus casei* NCDC 17 and ,*Lactobacillus acidophilus* NCDC 195

Fungal cultures*:* *K. marxianaus* NCDC 41, *Candida guillermondii* NCDC 44; *Rhizopus oryzae* NCDC 52 and *Aspergillus niger* NCDC 267

Corsetti *et al.* (2004) also showed that maximum production of bacteriocin like substance (BLIS) by Lactobacilli occurs in MRS broth. Batish *et al*. (1990) reported negligible AFS production in MRS broth by Lactococci and they also reported that skim milk was better medium for the production of AFS by these culture.

However, the information regarding on the growth medium for AFS production are scanty in case of *Lactobacillus*. Most of the work has been reported by using MRS broth for production of AFS by *Lactobacillus* (Okkers

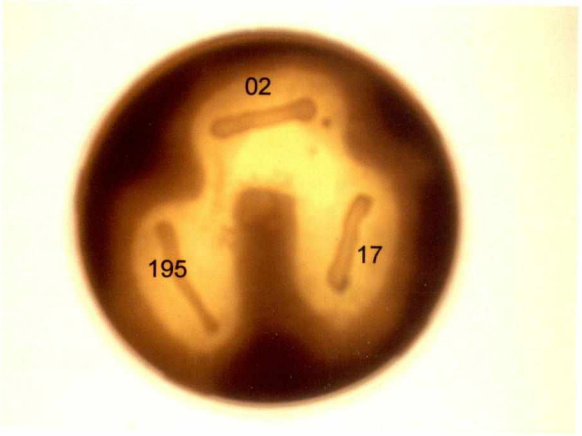

Plate1: Antifungal Activity of *Lactobacillus* Cultures Against *Rhizopus oryzae* NCDC 52 by agar spot assay

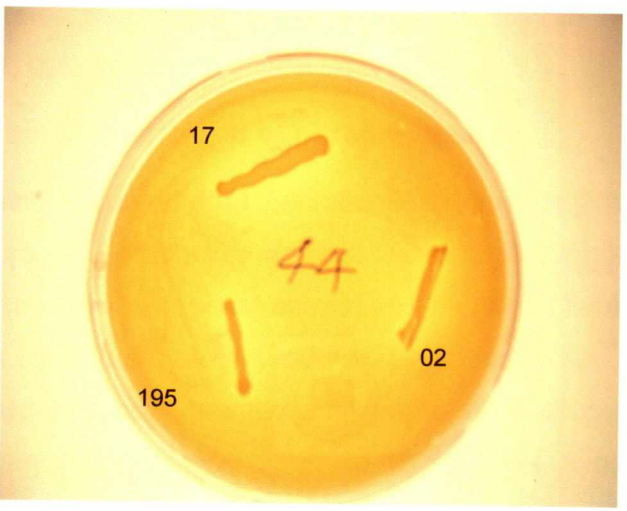

Plate 2 :Antiyeast activity of *Lactobacillus cultures* Against *Candida gullerimondii* NCDC 44 by agar spot assay

et al., 1999, Magnusson *et al*., 2001, Laitila *et al*., 2002, Aanassova *et al*., 2003 and Schwenniger *et al*., 2005, Falguni and Vij 2006).

From the results it is quite evident that as compared to MRS broth milk and whey do not appear to be an ideal medium for the optimum synthesis of AFS as it might lack some undefined constituents essential for the formulation of antifungal substance. Hence, appropriate supplementation of skim milk with suitable growth promoters and other desirable nutrients could be pre-requisite for the optimal production of AFS in skim milk and whey.

4.3 Effect of supplementation of ingredients

For achieving maximum antifungal activity by AFS production, two standard strains of *Lactobacillus* were tested. The skim milk and whey were used as basal medium supplemented with different nitrogen and carbon sources and Tween 80. The results are presented below.

In this investigation, different growth medium viz. skim milk and whey were supplemented with various ingredients and compared to maximum production of antifungal activity by the test cultures *L. casei* spp. *casei* NCDC 17 and *L. acidophilus* NCDC 195 against two yeasts (*K. marxianus* NCDC 41 and *C. guillermondii* NCDC 44) and two mold (*R. oryzae* NCDC 52 and *A. niger* NCDC 267) cultures.

4.3.1 Effect of supplementation of nitrogen source

It has been observed that *Lactobacillus casei* spp. *casei* NCDC 17 showed maximum AFS production against *Candida guillermondii* NCDC 44 in skim milk supplemented with yeast extract and peptone (Table 2). Clear zone of inhibition was observed by AFS produced by *Lb. casei* spp. *casei* NCDC 17 in skim milk medium supplemented with peptone at all concentrations but maximum antifungal activity was observed at 1% level of peptone whereas, addition of yeast extract at a concentration of 1.5-2.0% yielded maximum activity against *Candida guillermondii* NCDC44 and *Rhizopus oryzae* NCDC 52. Addition of nitrogen source in skim milk showed antifungal activity against *Rhizopus oryzae* NCDC 52. However, supplementation of yeast extract in skim milk as well as whey for AFS production by *Lb. casei* spp. *casei* NCDC 17 did not show any zone of inhibition against *A. niger* NCDC 267. Further, it

Table 2: Effect of supplementation of nitrogen sources in basal growth medium on antifungal activity* of *Lactobacillus casei* spp. *casei* NCDC 17.

Basal medium	Supplementary ingredients	Level of supplementation (%)	Diameter of zone of inhibition (mm) with well diameter 4 mm			
			NCDC 41	NCDC 44	NCDC 52	NCDC 267
Skim milk	Yeast Extract	0.50	-	16	14	-
		1.00	-	18	14	-
		1.50	-	20	16	-
		2.00	-	22	22	-
		2.50	-	16	18	-
		3.00	-	15	16	-
	Peptone	0.50	-	18	12	15
		1.00	-	22	12	17
		1.50	-	17	13	17
		2.00	-	18	15	19
		2.50	-	20	13	18
		3.00	-	22	12	15
Whey	Yeast Extract	0.50	-	15	-	-
		1.00	-	15	-	-
		1.50	-	14	-	-
		2.00	-	12	-	-
		2.50	-	13	-	-
		3.00	-	10	-	-
	Peptone	0.50	-	16	-	-
		1.00	-	15	-	-
		1.50	-	15	-	-
		2.00	-	13	-	-
		2.50	-	12	-	-
		3.00	-	12	-	-

NCDC 41:*Kluyveromyces marxianus*

NCDC 44: *Candida guillermondii*

NCDC 52: *Rhizopus oryzae*

NCDC 267: *Aspergillus niger*

*Mean of values of two independent experiments.

- No zone of inhibition was recorded.

was observed that AFS production was increased in skim milk supplemented with peptone 1.0-1.5% and zones of inhibition i.e. 22 mm and 15 mm were observed against *C. guillermondii* NCDC 44 and *R. oryzae* NCDC 52, respectively. Addition of peptone (@ 2 %) in skim milk showed antifungal activity against all the test cultures except *K. marxianus* NCDC 41. However, on further addition of peptone in basal media didn't show any increase in the antifungal activity. Supplementation of Yeast extract and peptone in whey for AFS production by NCDC 17 showed antifungal activity only against *C. guillermondii* NCDC 44. It has been observed that supplementation with yeast extract as well as peptone (@1%) in whey medium yielded maximum AFS production by *L. casei* spp. *casei* NCDC 17, though the zone of inhibition was small (10-16 mm.) as compared to milk medium.

Almost similar trend in AFS production by *L. acidophilus* NCDC 195 was observed in whey medium as well as in skim milk (Table 3). Addition of yeast extract (1.5-2.0%) showed maximum antifungal activity against both *Candida guillermondii* NCDC 44 and *Rhizopus oryzae* NCDC 52.

The largest diameter of zone of inhibition was 17 and 23 mm, respectively. But no significant inhibition was observed in case of *Kluyveromyces marxianus* NCDC 41 and *Aspergillus niger* NCDC 267 even with addition of nitrogen source in skim milk as well as in whey. While peptone supplementation (1.5-2.5%) in skim milk gave antifungal activity only against *C. guillermondii* NCDC 44 (zone diameter ranges from 22 to 23 mm). The other three fungal cultures such as *K. marxianus* NCDC 41, *R. oryzae* NCDC 52 and *A. niger* NCDC 267 were not significantly inhibited by *L. acidophilus* NCDC 195 in skim milk supplemented with peptone at all the concentration.

In whey, addition of yeast extract as well as peptone (@1.5%) each showed inhibitory activity against *C. guillermondii* NCDC 44 and *R. oryzae* NCDC 52. Whereas *K. marxianus* NCDC 41 and *A. niger* NCDC 267 were not inhibited by both types of supplementation in whey as well as skim milk.

Table 3: Effect of supplementation of different levels of nitrogen sources in basal growth medium on antifungal activity* of *Lactobacillus acidophilus* NCDC 195.

Basal Medium	Supplementary Ingredients	Level of Supplementation (%)	Diameter of zone of inhibition (mm) With well diameter 4 mm			
			NCDC 41	NCDC 44	NCDC 52	NCDC 267
Skim milk	Yeast Extract	0.50	-	15	18	-
		1.00	-	16	23	-
		1.50	-	16	20	-
		2.00	-	17	19	-
		2.50	-	16	17	-
		3.00	-	14	19	-
	Peptone	0.50	-	18	-	-
		1.00	-	18	-	-
		1.50	-	22	-	-
		2.00	-	22	-	-
		2.50	-	23	-	-
		3.00	-	18	-	-
Whey	Yeast Extract	0.50	-	15	16	-
		1.00	-	16	20	-
		1.50	-	16	18	-
		2.00	-	14	16	-
		2.50	-	14	16	-
		3.00	-	13	15	-
	Peptone	0.50	-	20	16	-
		1.00	-	20	16	-
		1.50	-	22	18	-
		2.00	-	20	19	-
		2.50	-	18	22	-
		3.00	-	16	20	-

NCDC 41:*Kluyveromyces marxianus* , NCDC 44: *Candida guillermondii*

NCDC 52: *Rhizopus oryzae* , NCDC 267: *Aspergillus niger*

*Mean of values of two independent experiments.

- No zone of inhibition was recorded.

Antifungal activity of AFS produced by *L. acidophilus* NCDC 195 was less having zone of inhibition 13 to 16 mm in case of yeast extract supplementation against *C. guillermondii* NCDC 44 than *R. oryzae* NCDC 52 (Zone of inhibition ranged between 15 to 20 mm) in both skim milk and whey. The influence of peptone incorporation in the whey on the production of AFS by *L. acidophilus* NCDC 195 was better against *C. guillermondii* NCDC 44 and *Rhizopus oryzae* NCDC 52 showing inhibitory zone diameter between 16-22 mm (Table 3).

4.3.2 Effect of Supplementation of Sugars

Maximum AFS production can be obtained by supplementation of growth medium. In order to exploit the beneficial effect of AFS, growth limiting factors such as sugars, glucose, maltose and mannose, were studied. The results pertaining to the effect of incorporation of different concentrations of sugars in skim milk and whey on the production of anti fungal substances by *L. casei* spp. *casei* NCDC 17 and *L. acidophilus* NCDC 195 have been presented in Table 4 and Table 5.

From the data presented therein, it is clear that glucose, maltose and mannose enhanced the antifungal activity of *L. casei* spp. *casei* NCDC 17 as well as *L. acidophilus* NCDC 195.

The antifungal activity in whey supplemented with different sugars by *Lactobacillus casei* spp. *casei* NCDC 17 was less as compared to skim milk. Among three sugars tested antifungal activity on glucose supplementation in whey was found to be more (22 mm) as compared to maltose (18mm) and mannose (15 mm), as diameter of zone of inhibition, against *Candida guillermondii* NCDC 44. However, the anti fungal substances (AFS) produced by *L. casei* spp. *casei* NCDC 17 in skim milk and whey supplemented with carbon sources did not show inhibitory effect against *K. marxianus* NCDC 41, *R. oryzae* NCDC 52 and *A. niger* NCDC 267.Whereas, addition of sugars in skim milk showed little antifungal activity against *R. oryzae* NCDC 52.

Table 4: Effect of supplementation of different levels of carbon sources in basal growth medium antifungal activity* of *Lactobacillus casei* spp*. casei* NCDC 17.

Basal medium	Supplementary ingredients	Level of supplementation (%)	Diameter of zone of inhibition (mm) with well diameter 4 mm			
			NCDC 41	NCDC 44	NCDC 52	NCDC 267
Skim milk	Glucose	0.25	-	18	16	-
		0.50	-	22	20	-
		0.75	-	20	16	-
		1.00	-	18	14	-
	Maltose	0.25	-	20	14	-
		0.50	-	24	16	-
		0.75	-	20	14	-
		1.00	-	18	13	-
	Mannose	0.25	-	20	14	-
		0.50	-	22	16	-
		0.75	-	20	12	-
		1.00	-	18	11	-
Whey	Glucose	0.25	-	15	-	-
		0.50	-	15	-	-
		0.75	-	20	-	-
		1.00	-	22	-	-
	Maltose	0.25	-	16	-	-
		0.50	-	17	-	-
		0.75	-	18	-	-
		1.00	-	17	-	-
	Mannose	0.25	-	14	-	-
		0.50	-	15	-	-
		0.75	-	14	-	-
		1.00	-	13	-	-

NCDC 41:*Kluyveromyces marxianus* , NCDC 44: *Candida guillermondii*

NCDC 52: *Rhizopus oryzae* , NCDC 267: *Aspergillus niger*

*Mean of values of two independent experiments.

- No zone of inhibition was recorded.

Table 5: Effect of supplementation of carbon sources in basal growth medium on antifungal activity* of *Lactobacillus acidophilus* NCDC 195.

Basal medium	Supplementary ingredients	Level of supplementation (%)	Diameter of zone of inhibition (mm) with well diameter 4 mm			
			NCDC 41	NCDC 44	NCDC 52	NCDC 267
Skim milk	Glucose	0.25	-	20	-	-
		0.50	-	22	-	-
		0.75	-	24	-	-
		1.00	-	22	-	-
	Maltose	0.25	-	19	-	-
		0.50	-	20	-	-
		0.75	-	16	-	-
		1.00	-	17	-	-
	Mannose	0.25	-	17	-	-
		0.50	-	19	-	-
		0.75	-	16	-	-
		1.00	-	16	-	-
Whey	Glucose	0.25	-	18	16	18
		0.50	-	20	22	16
		0.75	-	21	18	16
		1.00	-	22	15	15
	Maltose	0.25	-	15	18	15
		0.50	-	18	18	14
		0.75	-	20	18	14
		1.00	-	20	16	13
	Mannose	0.25	-	15	-	21
		0.50	-	16	-	22
		0.75	-	17	-	16
		1.00	-	17	-	20

NCDC 41:*Kluyveromyces marxianus,* NCDC 44: *Candida guillermondii*
NCDC 52: *Rhizopus oryzae* , NCDC 267: *Aspergillus niger*
*Mean of values of two independent experiments.
- No zone of inhibition was recorded.

Similarly, the effect of incorporation of different concentrations of sugars to skim milk and whey on the production of antifungal substance by *L. acidophilus* NCDC 195 is shown in Table 5. *L. acidophillus* NCDC 195 synthesized higher antifungal substance against test fungi. It was recorded that glucose (0.5%) supplemented in skim milk gave bigger zone of inhibition ranging from 22 to 24 mm against *C. guillermondii* NCDC 44.

Contrary to this, maltose supplementation exhibited smaller zone of inhibition i.e. 16 to 20 mm followed by mannose supplementation in skim milk (14 to 19mm). However, whey supplemented with glucose (0.5-1.0%) synthesized AFS having maximum antifungal activity (20 to 22 mm zone of inhibition). While maltose enriched whey exhibited lesser activity (18 to 20 mm) followed by mannose supplementation (16 to 17 mm) at same concentration against most sensitive yeast *Candida guillermondii* NCDC 44. Glucose and maltose supplemented whey was found to be effective for the production of AFS against *Rhizopus oryzae* NCDC 52 as well as *A. niger* NCDC 267 showing zone of inhibition. The antifungal activity ranging between 15 to 22 mm and 15-18 mm, respectively. Similarly, maltose enriched media at a concentration. 0.5% to 0.75% also registered zone of inhibition of 18 mm. against *Rhizopus oryzae* NCDC 52. Whereas, mannose supplementation in whey produced AFS having maximum inhibitory activity against *Aspergillus niger* NCDC 267 ranging from 20 to 24 mm. It is clear from the data in Table 5 that among different sugars tested addition of glucose, maltose and mannose enhanced AFS production at different concentration against *A. niger* NCDC 267.

4.3.3 Effect of supplementation of Tween 80

The data pertaining to the effect of incorporation of Tween 80 a surfactant, in the basal media on the production of AFS by *Lactobacillus casei* spp. *casei* NCDC 17 and *L. acidophilus* NCDC 195 has been presented in Table 6.

In case of *Lactobacillus casei* spp. *casei* NCDC 17, incorporation of Tween 80 at even lowest concentration (0.1%) in skim milk showed anti fungal activity against *C. guillermondii* NCDC 44 .having maximum zone of

inhibition of 22 mm. whereas, in whey it showed activity against *C. guillermondii* NCDC44 only. However, other test cultures such as *A. niger* NCDC267 were not inhibited.

Table 6: Effect of supplementation of surfactant in basal growth medium on antifungal activity* of *Lactobacillus casei spp. casei* NCDC 17 and *L.acidophilus* NCDC 195.

Basal medium	Culture	Supplementary ingredients	Level of supplementation (%)	Diameter of zone of inhibition (mm) with well diameter 4 mm			
				NCDC 41	NCDC 44	NCDC 52	NCDC 267
Skim milk	NCDC17	Tween 80	0.10	-	22	20	-
			0.20	-	20	18	-
			0.30	-	18	16	-
			0.40	-	17	16	-
			0.50	-	16	14	-
	NCDC 195	Tween 80	0.10	-	16	-	-
			0.20	-	15	-	-
			0.30	-	14	-	-
			0.40	-	14	-	-
			0.50	-	13	-	-
Whey	NCDC17	Tween 80	0.10	-	17	-	-
			0.20	-	15	-	-
			0.30	-	16	-	-
			0.40	-	14	-	-
			0.50	-	15	-	-
	NCDC 195	Tween 80	0.10	-	21	20	-
			0.20	-	20	20	-
			0.30	-	17	18	-
			0.40	-	16	18	-
			0.50	-	15	16	-

NCDC 41:*Kluyveromyces marxianus* , NCDC 44: *Candida guillermondii*
NCDC 52: *Rhizopus oryzae* , NCDC 267: *Aspergillus niger*
*Mean of values of two independent experiments.
- No zone of inhibition was recorded.

L. acidophilus NCDC 195 synthesized more AFS in whey supplemented with Tween 80 and showed anti fungal activity against *C.*

guillermondii NCDC 44 (21mm zone) and *R. oryzae* NCDC 52 (20 mm zone) whereas, in skim milk supplemented with Tween 80, *L. acidophilus* NCDC 195 gave maximum antifungal activity as zone of inhibition of 16 mm only against *Candida guilalermondii* NCDC 44 only. Also the antagonistic activity of Lb. acidophilus NCDC 195 was seen against *A. niger* NCDC 267 when 0.1- 0.2%) of concentration of Tween 80 was supplemented in whey.

It was observed that among all the fungal culture tested *C. guillermondii* NCDC 44 was found to be the most sensitive. Therefore, for comparison for antifungal activity on media standardization *C. guillermondii* NCDC 44 was selected.

4.3.4 Effect of supplementation of ingredients on *C. guillermondii* NCDC 44

The data recorded in Table 7 and Table 8 reviews the influence all the ingredients such as yeast extract, peptone, glucose, maltose, mannose and Tween 80 at different concentration on production of anti fungal substance by the *L.casei* spp. *casei* NCDC 17 and *L. acidophilus* NCDC 195 against *C. guillermondii* NCDC 44. The addition of yeast extract (1.5-2.0%) in skim milk showed more stimulatory effect on the AFS production by *L. casei* spp. *casei* NCDC 17 (15 to 22 mm. zones of inhibition). Whereas, in whey addition of yeast extract at all levels showed less antifungal activity (10 to 15 dia zone). Peptone supplementation in skim milk at a concentration of 1.0-1.5% showed maximum anti-fungal activity in the range of 20 to 23 mm. zone of inhibition, whereas, in case of whey antagonistic effect was very less (12 to 16 mm. zone of inhibition). The influence of production of antifungal substance by *L. casei* spp. *casei* NCDC 17 in skim milk supplemented with different sugars reflected the following results. Glucose (0.5%) supplementation in skim milk showed maximum anti-fungal activity, whereas, in whey, addition of glucose (0.75-1.0 %) level yielded similar zone of inhibition. Maltose supplementation (0.5%) in skim milk exhibited better anti fungal activity with largest zone of inhibition of 24 mm. However, in case of whey maltose and mannose supplementation up to 1.0% gave a lower inhibitory activity i.e. maximum 18 mm, whereas, mannose supplementation in skim milk showed maximum zone of 22 mm at 0.5% level. Similarly, the influence of Tween 80 on production of

Table 7: Effect of supplementation of different levels of nitrogen sources, carbon sources and Tween 80 in two basal growth media on antifungal activity* of *L. casei* NCDC 17 against *Candida guillermondii* NCDC 44.

Supplementary ingredients	Level of supplementation (%)	Diameter of zone of inhibition (mm) with well diameter 4 mm	
		Skim milk	Whey
Yeast Extract	0.05	16	15
	1.00	18	15
	1.50	20	14
	2.00	22	13
	2.50	16	13
	3.00	15	10
Peptone	0.50	18	16
	1.00	23	15
	1.50	20	15
	2.00	18	13
	2.50	18	12
	3.00	17	12
Glucose	0.25	18	15
	0.50	22	15
	0.75	20	20
	1.00	18	22
Maltose	0.25	20	16
	0.50	24	17
	0.75	20	18
	1.00	18	17
Mannose	0.25	20	14
	0.50	22	15
	0.75	20	14
	1.00	18	13
Tween 80	0.10	22	21
	0.20	20	20
	0.30	18	17
	0.40	17	16
	0.50	16	15

*Mean of values of two independent experiments.

AFS by *L. casei* spp. *casei* NCDC 17 in skim milk and whey showed that maximum effect on antifungal activity was at 0.1-0.2% addition of Tween 80. However, more effect was observed in skim milk.

Table 8 depicts the trend observed in skim milk and whey supplemented with various nutrients on the production of AFS by *L. acidophilus* NCDC 195. AFS production by *L. acidophilus* NCDC 195 was more in skim milk medium supplemented with yeast extract as compared to whey. It is evident from the Table 8 that *L. acidophilus* NCDC 195 showed less anti fungal activity in skim milk and whey on addition of yeast extract. On the other hand, it was observed that supplementation of peptone (1.5 %) in milk and whey substantially enhanced the antifungal activity.

It is evident from the Table 8 that glucose supplementation in skim milk at 0.75% showed maximum inhibitory action against *C. guillermondii* NCDC 44 (zone of inhibition ranges from 22 to 23 mm), whereas, maltose supplementation in skim milk and whey exhibited same activity (19 to 20 mm. zone of inhibition.) at a level of 0.25-0.50%. However, mannose addition in skim milk and whey gave almost similar antifungal activity in case of *L. acidophilus* NCDC.195 but less as compared to glucose and maltose. Similarly, addition of 0.01-0.02% of surfactant, Tween 80 showed maximum antifungal activity in both skim milk and whey.

Table 8: Effect of supplementation of different levels of nitrogen sources, carbon sources and Tween 80 in two basal growth media on antifungal activity* of *L. acidophilus* 195 against *Candida guillermondii* NCDC 44.

Supplementary ingredients	Level of supplementation (%)	Diameter of zone of inhibition (mm) with well diameter 4 mm	
		Skim milk	Whey
Yeast Extract	0.05	15	15
	1.00	16	16
	1.50	16	14
	2.00	17	13
	2.50	16	14
	3.00	14	13
Peptone	0.50	18	20
	1.00	18	20
	1.50	22	22
	2.00	22	20
	2.50	23	18
	3.00	18	16
Glucose	0.25	20	18
	0.50	22	20
	0.75	23	21
	1.00	22	22
Maltose	0.25	19	15
	0.50	20	18
	0.75	16	20
	1.00	17	20
Mannose	0.25	17	15
	0.50	19	16
	0.75	16	17
	1.00	16	17
Tween 80	0.10	16	21
	0.20	15	20
	0.30	14	17
	0.40	14	16
	0.50	13	15

*Mean of values of two independent experiments.

4.3.5 Effect of selected supplements in combination

The selected nitrogen and carbon source at optimal concentration was used for maximum AFS production by *L. casei* spp. *casei* NCDC 17 and *L. acidophilus* NCDC 195. The data recorded in Table 9 reveals that the effect of selected nitrogen and carbon source in combination along with 0.1 % Tween 80 in skim milk as in whey basal medium. It can be observed from the Table 9 that maximum zone of inhibition (25 mm) was observed on addition of peptone, maltose and Tween 80, followed by peptone, glucose and Tween 80 (23 mm). Whereas, in case of addition of yeast extract, glucose and Tween 80 and peptone, mannose and Tween 80 similar zone of inhibition (22 mm) was seen. From the Table 9 it is clear that the combination having peptone (1.0%), maltose (0.5%) and Tween 80 (0.1%) had synthesized maximum AFS in skim milk media by *L. casei* spp *casei* NCDC 17, the same combination was selected for further studies.

Table 9: Antifungal activity of AFS produced by *L. case* spp *casei* NCDC 17 in skim milk medium indifferent combinations of ingredients.

Ingredients	Zone Of Inhibition (mm)
Yeast extract + Glucose+ Tween 80	22
Yeast extract + Maltose + Tween 80	20
Yeast extract + Mannose + Tween 80	20
Peptone + Glucose + Tween 80	23
Peptone + Maltose + Tween 80	25
Peptone + Mannose + tween 80	22

The ability of AFS production in skim milk and whey supplemented with various concentration of yeast extract and peptone was studied. AFS production gradually increased with increasing the concentration of milk with nitrogen source. Addition of yeast extract and peptone in skim milk and whey improved AFS production as these nitrogen sources supply amino acids and small peptides. Yeast extracts containing both small peptide and vitamins improved AFS production.

When compared with the whey, the AFS production by *L. casei* spp. *Casei* NCDC 17 was more in milk then in whey, whereas, *L. acidophilus*

NCDC 195 showed the AFS production almost comparable in both the medium. But AFS production in skim milk was inhibitory to only yeast cultures, whereas, AFS produced by *L. acidophilus* NCDC 195 in whey was more inhibitory to both yeast and mold cultures.

Anonts *et al*. (2004) studied the bacteriocin production by seven *Lactobacillus* strains in MRS and milk medium, they reported that addition of 1% yeast extract to skim milk medium enhance bacteriocin production for all the strains used in milk. The best results were obtained by Lactobacillus cultures. Our finding also showed that Lactobacillus acidophilus as well as *L. casei* spp *casei* are producing more AFS in milk medium when supplemented with maltose (0.5%), peptone (1.0%) and Tween 80 (0.1%). Ogunbanwa *et al*., 2003 reported that large amount of bacteriocin was synthesized by *Lb. brevis* only when the medium was supplemented with glucose (1%), Tween 80 (0.5%), Yeast extract (2-3%) and NaCl (1-2%). Thus variation in the concentration of supplementation of cultivation media might have an influence on the amount of bacteriocin produce by microorganisms. Similar observations have been made previously by Daba *et al*., (1993) for mesenterosins.

Sanni *et al*., (1999) also reported that maximum bacteriocin production was obtained when glucose and peptone were varied to 0.25-0.5% in MRS broth, while bacteriocin activity was not detected at 2% glucose and peptone each.

Modification of carbon source of cultivation media should be considered for maximum production of bacteriocin that has potential use as food bio preservative. In another study, supplementation of 3% lactose and 2% peptone in MRS medium has increased bacteriocin production by LAB (Lade *et al*., 2006). They found that lactose was better source of carbon than glucose. Todorov *et al*. (2004) reported that addition of 3-4% mannose in growth media has doubled the production of bacteriocin. Our study also revealed that addition of peptone and maltose in the presence of surfactant Tween 80 has yielded maximum AFS production in skim milk based medium by *L. casei* spp. *casei* NCDC 17.

4.4 Spectrum of Antimicrobial activity of *Lactobacillus casei* spp. *casei* NCDC 17.

L. casei spp. *casei* NCDC 17.has been selected for AFS production in skim milk for preservation of paneer. Since paneer is a perishable dairy product due to its high microbial counts. The micro-flora of paneer contains both bacterial and fungal contaminants which are the primary factor of low shelf life of paneer. An attempt was made to test the antibacterial as well as antifungal activity of AFS produced by this potent culture against both spoilage and pathogenic microorganisms. Table 10 and 11 represents the spectrum of activity of AFS against pathogenic bacteria and spoilage fungi.

It is quite evident from the Table 10 that the AFS produced has maximum antifungal activity against *Penicillium camemberti* NCDC 56 and *P. roqueforti* NCDC 170 having a large zone of inhibition i.e. 40 mm diameter (Plate 2). Whereas, it showed comparatively much smaller zone of inhibition 20 mm and 22 mm in case of *A .niger* NCDC 267 and *R. oryzae* NCDC 52 ,respectively. However, yeasts were found to be more resistant. Among all the yeasts tested only *C. guillermondii* NCDC 44 was inhibited (zone of inhibition 25mm). Whereas, other three yeasts namely *Kluyveromyces marxianus* NCDC 44, *Sachharomyces cerevisiae* NCDC 47 and *Torulopsis candida* NCDC 43 were resistant to the AFS of *L. casei* spp *casei* NCDC17.

Table 10 : Spectrum of Antifungal Activity Of AFS Produced By *Lb casei* spp. *casei* NCDC 17 In Skim Milk Medium

Fungal Cultures	Zone of inhibition (mm)
Penicillium camemberti NCDC 56	40
Penicillium roquefortii NCDC 170	40
Rhizopus oryzae NCDC 52	22
Aspergillus niger NCDC 267	20
Candida guillermondii NCDC 44	25
Kluyveromyces marxianus NCDC 41	-
Saccharomyces cerevisiae NCDC 47	-
Torulopsis candida NCDC 43	-
- No zone of inhibition was recorded.	

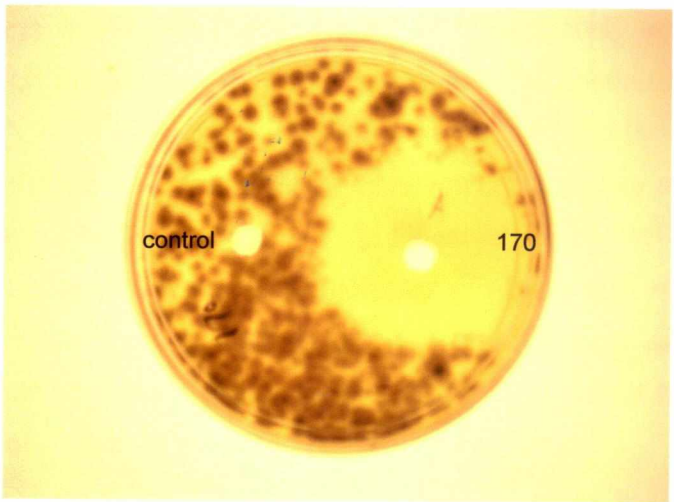

Plate 3 :Antifungal activity of *Lb. casei* spp.*casei* NCDC 17 Against *Penicillium roqueforti* NCDC 170 by agar well diffusion assay

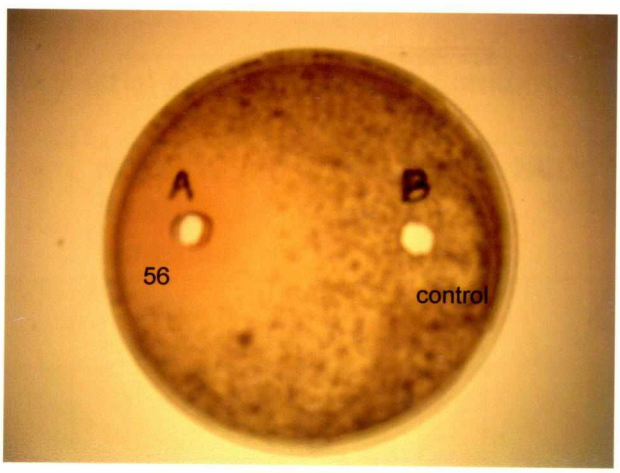

Plate 4 :Antifungal activity of *Lb. casei* spp.*casei* NCDC 17 Against *Penicillium camemberti* NCDC 56 by agar well diffusion assay

Data presented in Table 11 showed the inhibitory activity of AFS produced by *L. casei* spp *casei* NCDC 17 against four pathogenic bacterial cultures. It was found that maximum antibacterial activity was exhibited by AFS production in modified skim milk against *P. aeruginosa* MTCC 741 and *S. aureus* MTCC 1144 with a maximum zone of inhibition (30 mm) as shown in Plate 3 and Plate 4. A clear zone of inhibition (25mm) was also observed in case of *B. cereus* MTCC 1272 (Plate 5). However, *E. coli* MTCC 739 did not show any zone of inhibition by the AFS tested. It is quite evident from the results that the AFS produced by *L. casei* spp. *casei* NCDC 17 is having broad spectrum of activity. This potential of the culture was exploited for the preservation of paneer in the next section of investigation.

In the present investigation, *L. casei* spp. *casei* NCDC 17 showed broad spectrum of antimicrobial activity against many microorganisms. It inhibited molds like *Penicillium roquefortii*, *P. camembertii*, *A. niger* and *R. oryzae* and yeast such as *C. guillermondii*. Other Yeasts like *S. cerevisiae*, *T. candida* and *K. marxianus* were not inhibited. The AFS produced thereby also showed antibacterial activity against pathogenic strains of *B. cereus*, *P. aeruginosa* and *S. aureus* but did not give any inhibition against *E. coli*.

Table 11: Antibacterial Activity of AFS of Standard *Lactobacillus* Cultures

Bacterial cultures	**NCDC 17 A SKIM MILK (modified)**	**NCDC 17 B MRS**	**NCDC 195 MRS**	**NCDC 02 MRS**
S.aureus MTCC1144	30	15	15	12
B.cereus MTCC 1272	25	15	13	08
P.aeruginosa MTCC 741	30	23	20	18
E.coli MTCC 739	-	-	-	-

- No zone of inhibition was recorded.

Lactobacillus plantarum F1 and *L. brevis* OG1 produced bacteriocins that had broad spectrum of inhibition against both pathogenic and food spoilage organisms and various lactic acid bacteria. The activity was reported against *E. coli* NCTC10418 and *Enterococcus faecalis* EF1, but not inhibited *Candida albicans* ATCC10231 and *Klebsiella* sp. UCH15. (Ogunbanwo *et al*., 2003).

Savadogo *et al*., (2004) isolated and identified eight strains of lactic acid bacteria producing bacteriocin from Burkina Faso fermented milk samples. These strains were as *Lactobacillus fermentum*, *Pediococcus* spp., *Leuconostoc mesenteroides* subsp. *meseteroides*, *Lactococcus* spp.. The bacteriocin exhibited antibacterial activity against *Enterococcus faecalis* 103907 CIP, *Bacillus cereus* 13569 LMG, *Staphylococcus aureus* ATCC 25293, *Escherichia coli* 105182 CIP.

In a similar study, Durlu-Ozkaya *et al*., (2005) isolated and identified yeasts from cheeses and reported the antifungal activities of some *Lactobacillus* spp. against the isolated yeast belonging to *Saccharomyces cerevisiae* (10 of 17) and one each of *Candida pseudotropicalis*, *C.krusei, C.lipolytica, C.lusitaniae, C.ciferrii, Torulopsis glabrata* and *Rhodotorula rubra*. Of all the test culture, *L.plantarum* Lp 21 had the maximum inhibitory effect against all the *S.cerevisiae* strains.

Yang et al. (2005) reported that the cell-free supernatants from *Lactobacillus casei* subsp. *rhamnosus* and *Lactobacillus acidophilus* grown in deMan Rogosa Sharpe (MRS) broth inhibited 95–100% growth of three mould fungi. Antifungal activity was attributed to one or more unknown heat and pH stable metabolites.

L. paracasei subsp. *paracasei* in coculture with *S. thermophilus* was inhibitory against microbial contaminants in fresh cream cheese with or without the addition of inulin, indicating the potential use of this combination in a probiotic and synbiotic product (Buriti *et al*., 2007).

4.5 Preservation of paneer

Lactobacillus with antifungal effect could have potential as biopreservative, preventing growth of spoilage moulds, and yeast in food and

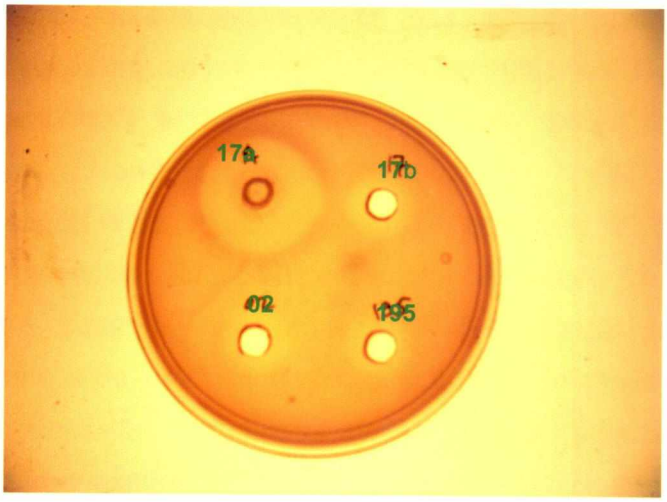

Plate 5 :Antibacterial Activity of *Lactobacillus* Cultures Against *S.aureus* MTCC 1144 *by agar well diffusion assay*

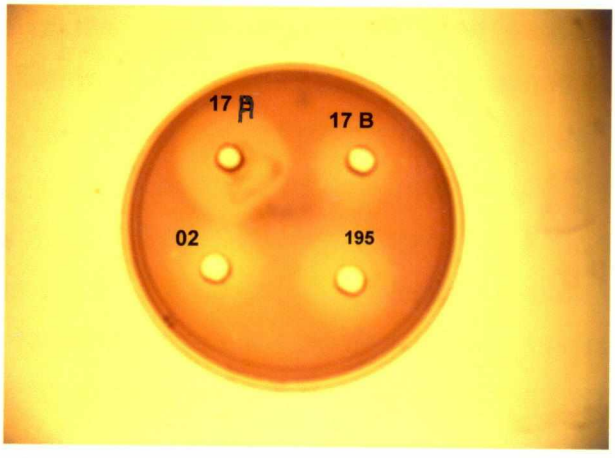

Plate 6 : Antibacterial Activity of *Lactobacillus* Cultures Against *Ps.aeruginosa* MTCC 741 by agar well diffusion assay

dairy products. In this study we report the antifungal properties of lactobacillus and their role in biopreservation of paneer. Paneer is perishable milk product, as it contains lot of microorganisms which may multiply rapidly and render it unsuitable for processing and unfit for human consumption. The antimicrobial potential of Lactobacilli and Lactoferrin has been extensively studied by several workers. However, influence regarding their application in preservation of food is scanty. Bacteriocin has bee used for preservation of dairy products especially for spoilage bacteria. Although, few reports have been published regarding the production of antifungal substances by Lactobacilli. There is no information on use of AFS as bio-preservative in dairy products. This necessitates studies of this line with the selection of potential Lactobacillus culture exhibiting high AFS production in milk and whey medium and their application in preservation of paneer alone and along with Lactoferrin. The results pertaining to this are presented in Tables, Graphs and Histograms.

4.5.1 Changes In Total Plate Counts

It is evident from the Table 12a that the total plate counts of all the samples of paneer increased during its storage. The rate of increase was faster in case of the samples dipped in boiled distilled water (control) as compared to other treatments. The initial count of all the samples ranged from 3.0 to 3.25 log cfu/ gm. The total plate counts of all the samples increased steadily during storage period. The total counts of control paneer sample increased sharply from 3.05 to 8.8 logs cfu/gm during the storage period up to 28 days. While the total plate count of treated samples with combination of different preservatives increased slow and steadily maximum of 7.4 cfu/ gm on 28^{th} day for the treatment I, which contains pediocin and sodium citrate but not any antifungal agent. Whereas, other five treatments total counts ranges from 6 to 6.9 cfu/ gm on 28th day (Fig. 1).

Table 12a: Changes of standard plate counts of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	3.05	3.2	3	3.16	3.3	3.26	3.14
3	4.38	4.2	4.18	3.5	4	4.08	4.4
7	5.6	5	4.9	4	5	5.17	5.2
10	6.5	6.1	5.7	5	5.5	5.34	5.1
15	7.3	6.5	6.3	5.4	6.3	5.5	5.7
21	7.5	6.6	6.7	5.6	6.5	5.8	5.9
28	8.8	7.4	6.9	5.9	7	6.1	6

P = Pediocin, SC = Sodium citrate
LF = Lactoferrin, PS = Potasium sorbate
AFS = Antifungal substance

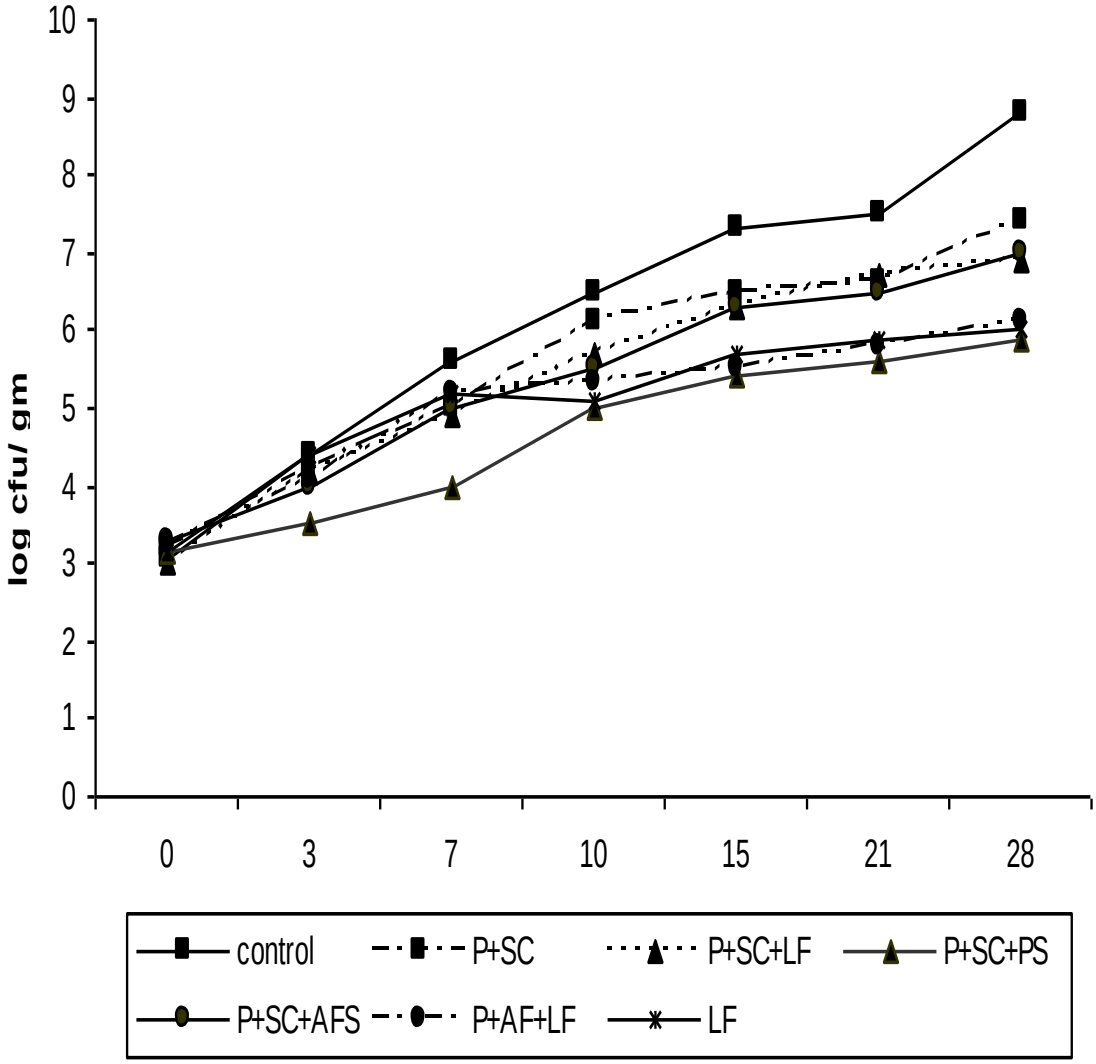

Fig. 1: Changes of total plate counts of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

The increase in the log cfu/ gm of control sample is very high i.e. up to 5.5 log cfu/gm as compared to the other treated samples which ranged from 2.8 to 4.2 log cfu/gm over the storage of 28 days. An increase of only 2.7 log cfu/gm was observed in treatment (III) followed by 2.8 log cfu/gm increased in treatment V (P+AF+LF) and treatment VI (LF) which was found to be comparable. Control sample registered sharp increase in total counts even after 10 days, whereas other samples showed moderate increase in the count after 21 days of storage. The TPC of paneer samples differs with different treatment during storage significantly ($P<0.05$) (Table 12b).

Table 12b: ANOVA table for total plate count during storage of panner sample

Analysis of Variance					
Source	Sum-of-Squares	DF	Mean-Square	F-Ratio	P
STORAGE	213.403	6	35.567	242.238	0.000
TREATMENTS	28.520	6	4.753	32.373	0.000
Error	19.675	134	0.147		

4.5.2 Changes In Yeast And Mold Counts

The initial yeast and mould counts of the samples dipped in boiled distilled water along with other treatments from I to VI varied over a range of 1.7 to 1.9 log cfu/ gm (Table 13a). The rate of increase of yeast and mold count was higher in case of control which leads to flavour defects on 15th day of storage, while other samples showed slow and steady increase in yeast and mold counts, which was controlled to 4.0 to 4.9 log cfu/ gm for treatment II to VI over the storage period. Treatment 1 having pediocin and sodium citrate but not any antifungal agent to prevent fungal growth has shown an increase in yeast and mold counts significantly high by 4 log cfu/ gm as compared to other treatments. In control sample there is sharp increase in yeast and mold count from 1.9 to 6.4 log cfu/ gm i.e. increased significantly by 4.5 log cfu/ gm as compared to other treated samples over the storage period of 28 days. Whereas, in treated samples, treatment V (P+AFS+LF) showed

better result in which yeast and mold counts increased by 2.1 log cycles only followed by treatment- VI having LF alone also showed increased log cfu/ gm by 2.4 only. In Treatment IV, III and II an increase in log cfu/ gm by 2.7, 2.8 and 2.9 cycles respectively was observed. (Fig. 2). The yeast and mold counts of paneer samples differs with different treatment during storage significantly ($P<0.05$) (Table 13b).

Table 13a: Changes of yeast and mold counts of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	1.9	1.95	2.0	1.8	1.9	1.9	1.7
3	2.6	2.7	2.3	2.3	2.5	2.3	2.4
7	4.2	3.8	3.6	3.8	3.9	3.1	3.1
10	4.9	4.7	4.4	4.2	4.2	4.3	4.3
15	5.3	5	4.6	4.3	4.4	4.5	4.5
21	5.8	5.5	4.7	4.4	4. 5	4.2	4.3
28	6.4	6.1	4.9	4.6	4.6	4.0	4.1

P = Pediocin, SC = Sodium citrate

LF = Lactoferrin, PS = Potasium sorbate

AFS = Antifungal substance

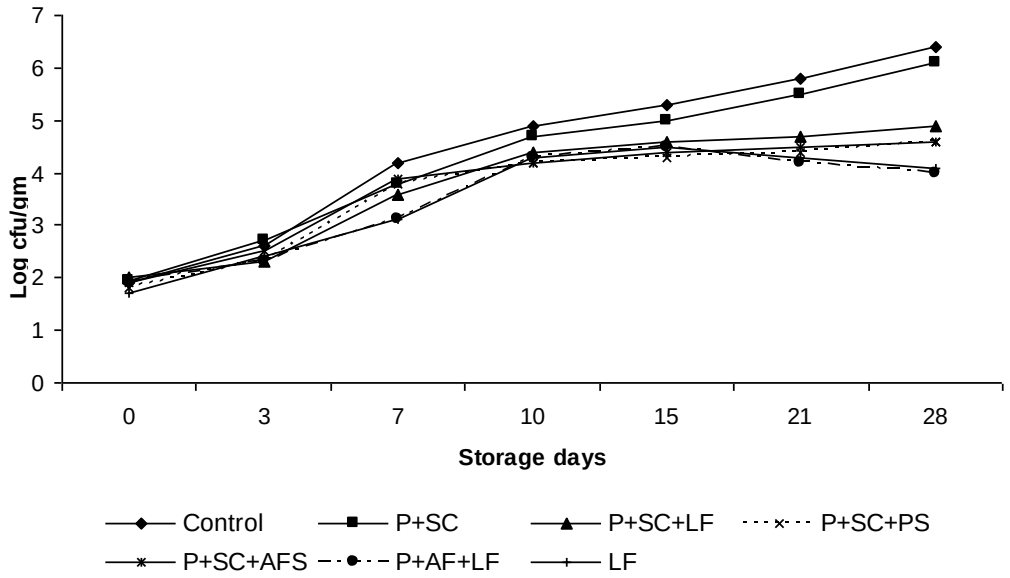

Fig. 2: Changes of yeast and mold counts of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Table 13b: ANOVA table for yeast and mold counts during storage of Panner sample

Analysis of Variance					
Source	Sum-of-Squares	DF	Mean-Square	F-Ratio	P
STORAGE	156.977	6	26.163	222.626	0.000
TREATMENTS	15.144	6	2.524	21.478	0.000
Error	15.748	134	0.118		

Data presented in Table 13 clearly reflects that the treatment V was better for controlling yeast and mold spoilage which is the most prominent cause of pander spoilage.

4.5.3 Changes In Moisture Content

The changes in the moisture content of the samples under refrigerated storage have been presented in Table 14, Fig. 3. In all the samples including control no significant change in moisture content was noticed over entire period of storage. Initial moisture content of paneer samples ranged from 52.45 to 52.86% which hardly reduced to one percent over entire storage period of paneer. The final moisture ranged from 51.20 to 51.60%.

Table 14: Changes of moisture content (%) of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	52.75	52.67	52.56	52.45	52.77	52.86	52.65
3	52.65	52.63	52.45	52.78	52.64	52.72	52.61
7	52.57	52.60	52.30	52.62	52.54	52.66	52.56
10	52.50	52.56	52.20	52.43	52.32	52.45	52.47
15	52.30	52.30	52.12	52.31	52.11	52.20	52.20
21	51.90	51.70	51.80	51.94	51.94	51.82	51.95
28	51.50	51.30	51.35	51.33	51.60	51.20	51.40

P = Pediocin , SC = Sodium citrate

LF = Lactoferrin, PS = Potasium sorbate

AFS = Anti-fungal substance

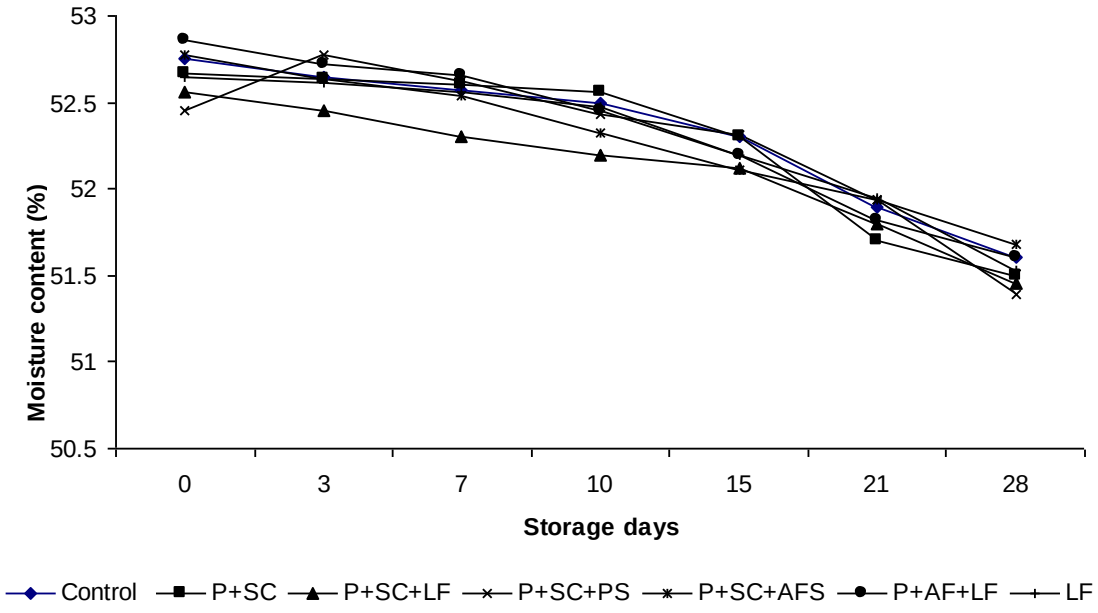

Fig. 3: Changes of moisture content (%) of Paneer samples with different preservation treatments during refrigerated storage (7±1°C)

4.5.4 Changes In The Titratable Acidity

As it evident from the data present in Table 15a and Fig. 4 that there was a slow but steady increase in acidity of all the samples of paneer during early stages of storage, followed by a sharp increase towards the end of storage period. The initial acidity of fresh paneer samples was 0.18% L.A. including control. On third day the acidity did not increase in treated samples except in case of control it increased to 0.21%. In control samples there is a linear increase in acidity up to 10 days by 0.02%. Acidity increases to 0.20% on 15th day. From 15th day onwards there is a sharp increase in the acidity up to 28th day, which reached up to 0.36% maximum. While in other treated samples from treatment II and VI, acidity remained in the range of 0.24 to 0.26% as Lactic acid up to 28 day. Whereas, in treatment II, it is 0.28% which is more as compared to the other treatments which may be due the increase in total counts and Yeast and mould counts of this sample due to the absence of antifungal preservative in this sample. The titratable acidity value clearly

indicates that the positive effect of different treatments on the preservation of paneer. All treatments are quite effective in inhibiting acid producing organisms, which is one of the main important reasons in paneer spoilage (Table 15 b).

Table 15a: Changes of acidity (% lactic acid) in Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	0.18	0.18	0.18	0.18	0.18	0.18	0.18
3	0.21	0.18	0.18	0.18	0.18	0.18	0.18
7	0.23	0.21	0.2	0.2	0.21	0.21	0.21
10	0.25	0.23	0.21	0.21	0.23	0.22	0.21
15	0.29	0.25	0.23	0.21	0.24	0.24	0.23
21	0.32	0.26	0.24	0.22	0.25	0.25	0.24
28	0.36	0.28	0.26	0.24	0.26	0.27	0.25

P = Pediocin, SC = Sodium citrate

LF = Lactoferrin, PS = Potasium sorbate

AFS = Anti-fungal substance

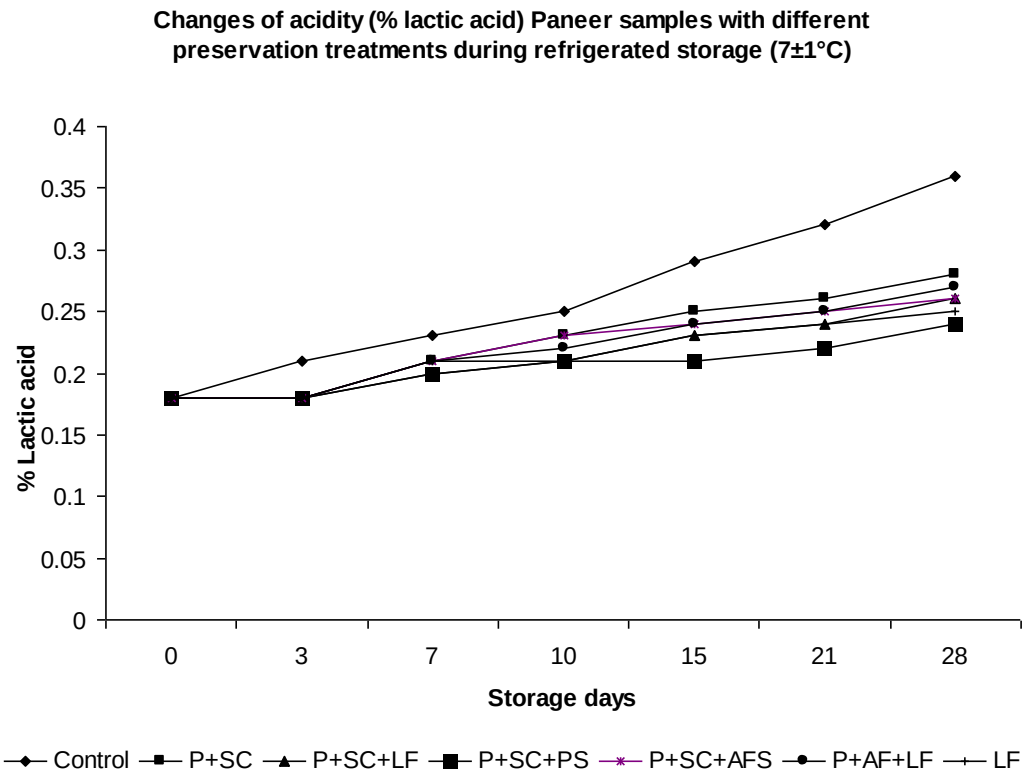

Fig. 4: Changes of acidity (% lactic acid) Paneer samples with different preservation treatments during refrigerated storage (7±1°C)

Table 15b: ANOVA table for acidity during storage of panner sample					
Analysis of Variance					
Source	Sum-of-Squares	DF	Mean-Square	F-Ratio	P
STORAGE	0.150	6	0.025	115.390	0.000
TREATMENTS	0.034	6	0.006	26.123	0.000
Error	0.029	134	0.000		

4.5.5 Sensory Analysis

Five point hedonic scale was used for the sensory evaluation of paneer during definite storage intervals .The results pertaining to sensory evaluation are present therein.

Table 16: Changes of colour and appearance scores* of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	4.93±0.07	4.80±0.06	4.87±0.03	4.97±0.03	4.90±0.06	4.90±0.05	4.63±0.31
3	4.57±0.29	4.77±0.15	4.70±0.12	4.97±0.03	4.90±0.06	4.63±0.32	4.93±0.03
7	4.40±0.26	4.27±0.32	4.50±0.29	4.67±0.17	4.63±0.20	4.40±0.20	4.50±0.28
10	3.80±0.15	4.00±0.06	4.67±0.17	4.17±0.17	3.67±0.34	3.73±0.27	4.37±0.33
15	4.23±0.37	4.10±0.21	3.90±0.10	4.17±0.16	4.50±0.28	4.33±0.16	4.17±0.33
21	3.33±0.09	3.50±0.06	3.70±0.06	4.00±0.06	3.53±0.09	3.63±0.03	3.53±0.09
28	3.30±0.06	3.40±0.06	3.50±0.06	3.43±0.29	3.23±0.15	3.30±0.06	3.30±0.17

P = Pediocin
SC = Sodium citrate
LF = Lactoferrin
PS = Potasium sorbate
AFS = Anti-fungal substance
*Mean±S.E., n = 5.

The scores of color and appearance were ranged from 4.63 to 4.97 for the paneer samples preserved with different treatments from treatment I to VI including control on 0^{th} day. During storage the scores were significantly decreased for all the paneer samples ($P<0.05$) .for control samples the score was decreased to 3.30 after 28 days. Similarly, in case of treated paneer samples scores after 28 days were in the range of 3.30 to 3.40. However, the preservation treatments did not affect the scores significantly for all the storage intervals. Color and appearance scores during storage decreased significantly ($P<0.05$) as shown in Table 16.

There is a significant different between the treated sample scores over the storage period of 28 days as compared to control sample (Table 17) The flavor scores were ranging between 4.87 to 4.93 on 0 day for the paneer samples. Up-to 10 day there is no significant change in flavor scores for all samples including control. But after 10^{th} day there was sharp decrease in the flavor scores of control sample. Whereas in other treated samples flavor scores decreases gradually. On 28^{th} day the control sample having flavor scores of 2.47±0.03 while in other treated samples it ranges from 3.03±0.06 to

3.17±0.07. Maximum scores were observed in treatment III (P+SC+PS) on 28th day. Flavor scores with different preservative treatments differs significantly (P<0.05) during storage.

Table17: Changes of flavour scores* of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	4.80±0.15	4.87±0.09	4.93±0.07	4.87±0.09	4.90±0.05	4.90±0.04	4.90±0.06
3	4.83±0.07	4.80±0.05	4.80±0.06	4.73±0.03	4.77±0.09	4.83±0.07	4.80±0.06
7	4.80±0.06	4.73±0.03	4.83±0.067	4.80±0.08	4.80±0.06	4.77±0.07	4.47±0.28
10	3.50±0.29	3.80±0.15	3.47±0.26	3.83±0.44	4.07±0.12	4.43±0.07	4.40±0.21
15	3.43±0.03	3.50±0.06	3.53±0.07	4.00±0.29	4.33±0.21	4.50±0.12	4.00±0.06
21	2.77±0.09	3.03±0.03	3.10±0.06	3.50±0.29	3.17±0.17	3.10±0.06	3.33±0.17
28	2.47±0.03	3.03±0.03	3.00±0.06	3.37±0.09	3.17±0.07	3.10±0.06	3.17±0.09

P = Pediocin
SC = Sodium citrate
LF = Lactoferrin
PS = Potasium sorbate
AFS = Anti-fungal substance
*Mean±S.E., n = 5.

The body and texture scores for all paneer samples ranges from 4.90±0.06 to 4.8±0.6 on 0th day and decreased significantly to 4.5 for all paneer samples. The body and texture scores of paneer samples with different preservative treatments and control did not differs significantly (Table18).

Table 18: Changes of body and texture scores* of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	4.90±0.06	4.80±0.06	4.93±0.07	4.90±0.06	4.90±0.06	4.97±0.03	4.90±0.06
3	4.80±0.05	4.80±0.05	4.80±0.08	4.86±0.03	4.90±0.08	4.77±0.03	4.83±0.03
7	4.73±0.03	4.80±0.08	4.83±0.03	4.80±0.08	4.80±0.05	4.87±0.03	4.77±0.03
10	4.66±0.03	4.70±0.10	4.67±0.03	4.70±0.02	4.71±0.02	4.74±0.02	4.66±0.02
15	4.68±0.02	4.68±0.02	4.67±0.02	4.64±0.02	4.66±0.02	4.68±0.02	4.60±0.02
21	4.63±0.02	4.64±0.02	4.61±0.02	4.67±0.02	4.59±0.02	4.51±0.02	4.53±0.02
28	4.57±0.02	4.57±0.02	4.59±0.02	4.51±0.02	4.53±0.02	4.55±0.02	4.57±0.02

P = Pediocin
SC = Sodium citrate
LF = Lactoferrin
PS = Potasium sorbate
AFS = Anti-fungal substance
*Mean±S.E., n = 5.

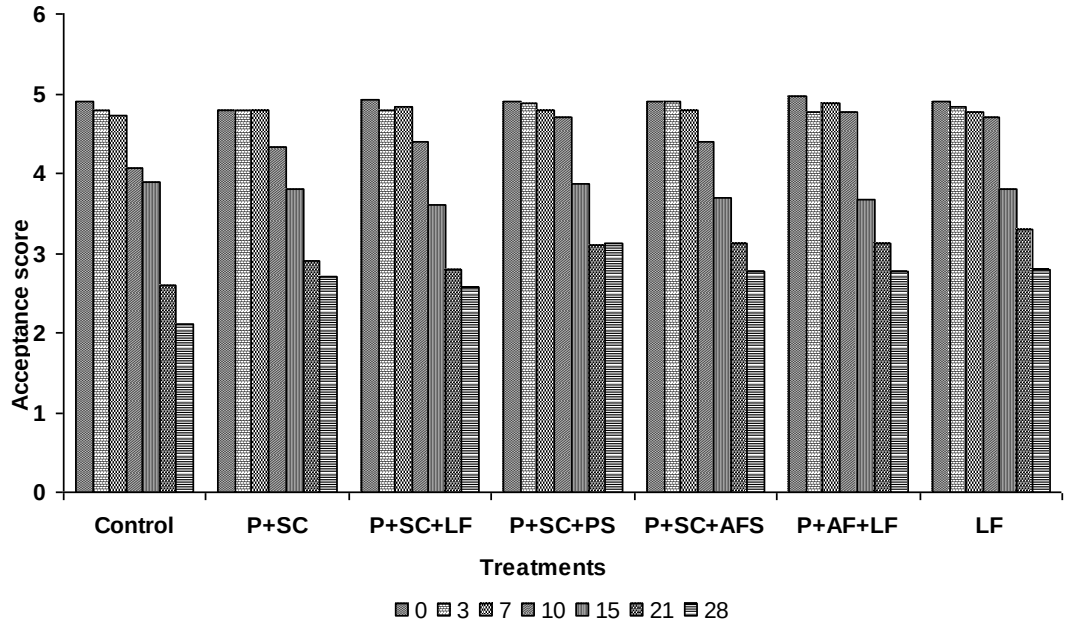

Fig. 5: Changes of overall acceptance scores* of Paneer samples with different preservation treatments during refrigerated storage (7±1°C)

Table 19 a and b, and Fig. 5 depicts that the overall acceptability of paneer sample. The initial scores for all samples was ranging from 4.8 to 4.9 which decreased significantly with the increasing storage duration ($P<0.05$). The overall acceptance scores of paneer samples preserved with (P+SC+PS) was (3.13) highest followed by treatment V, which was 2.87 on 28th day followed by treatment VI and V. Data presented in Fig. 5 clearly depicts that overall acceptability of samples treated with pediocin sodium citrate and potassium sorbate was more followed by treatments VI, V, and IV. Treatment V and VI are comparable. From these results we can say that the chemical fungal agents can be replaced with natural preservatives such as pediocin, AFS and lactoferrin. For paneer preservation treatment III and treatment V were found to be of good quality paneer after 28 days of storage while other treatment gave fair quality paneer while score for control sample indicates its poor quality from the 5 point hedonic scale.

Table 19a: Changes of overall acceptance scores* of Paneer samples with different preservation treatments during refrigerated storage (7±1°C).

Storage period (day)	control	Treatments					
		P+SC	P+SC+LF	P+SC+PS	P+SC+AFS	P+AF+LF	LF
0	4.90±0.06	4.80±0.06	4.93±0.07	4.90±0.06	4.90±0.06	4.97±0.03	4.90±0.06
3	4.80±0.06	4.80±0.06	4.80±0.06	4.87±0.03	4.90±0.06	4.77±0.03	4.83±0.03
7	4.73±0.03	4.80±0.06	4.83±0.03	4.80±0.06	4.80±0.06	4.87±0.03	4.77±0.03
10	4.07±0.09	4.33±0.09	4.40±0.06	4.70±0.06	4.40±0.06	4.77±0.03	4.70±0.12
15	3.90±0.17	3.80±0.06	3.60±0.06	3.87±0.03	3.70±0.06	3.67±0.07	3.80±0.06
21	2.60±0.12	2.90±0.06	2.80±0.056	3.10±0.06	3.13±0.09	3.13±0.15	3.30±0.06
28	2.10±0.10	2.70±0.06	2.57±0.03	3.13±0.12	2.77±0.12	2.87±0.09	2.80±0.06

P = Pediocin
SC = Sodium citrate
LF = Lactoferrin
PS = Potasium sorbate
AFS = Anti-fungal substance
*Mean±S.E., n = 5

Table 19b: ANOVA table for overall acceptability during storage of Paneer sample					
Analysis of Variance					
Source	Sum-of-Squares	DF	Mean-Square	F-Ratio	P
STORAGE	107.348	6	17.891	571.062	0.000
TREATMENTS	1.589	6	0.265	8.455	0.000
Error	4.198	134	0.031		

Food preservation is the process of treating and handling food in such a way as to stop or greatly slow down spoilage to prevent food borne illness while maintaining nutritional value density, texture and flavor of food. Similarly, in the present studies on preservation of paneer using different anti-fungal and antibacterial natural preservatives, the growth of spoilage organisms is slowed down while maintaining the sensory quality of paneer upto 28 day of storage at refrigeration temperature.

Generally surface spoilage limits the shelf life of paneer. Therfore, different types of antifungal chemical agents have been used for controlling surface spoilage. Shukla *et al*. (1984) indicated a shelf life of 6 days at room temperature when raw paneer was dipped in 18% salt solution for 30 min. In a similar study, Rao *et al.* (1992) reviewed that incorporation of 0.1% potassium sorbate enhanced the shelf life of paneer from 6 days to 18 days at 5^0C. Singh *et al*. (1988) reported that shelf life of paneer could be extended up to 16 days by dipping in brine (5%) overnight followed by packaging and storing at refrigeration temperature. dipping resulted in a very soft, fragile body and dull yellow appearance of the product towards the end of 10 days of storage period. in another study.

Sachdeva and Singh (1990a) reported that the (delvocid) when used in combination with a germicide (H_2O_2) gave fungicide excellent results and paneer thus treated kept good for a period of 32 days at 8-10^{0}C.

Pal (1998) studied various microbiological and biochemical changes in paneer during storage at 8±2°C for 15 days of storage. The mesophilic counts, yeast and mould counts as well as coliforms increased. They also reported an increase in thiobarburitic value, titratable acidity and tyrosine value but residual pH and lactose decreased.

In another study, the usefulness of two selected LAB, *Lb.sakei* ssp *carnosus* and laconic S producing Lusaka 148 to extend the shelf life of cooked ham was reported (Vermeiren *et al*., 2006).

Application of LP system and lysozyme to enhance the shelf life of paneer was studied in which enhanced shelf life observed was up to 24 days with LP system (control showed only 8 days) and combination of LP system and lysozyme increased the shelf life of paneer up to 28 days (Agrawal and Rattan Chand, 2001).

Many studies have indicated that lactoferrin has the potential to be used as a natural antimicrobial preservative in the food industry (Holley 2003). Inhibition of *Penicillium commune* was reported by edible whey protein films incorporating lactoferrin and lactoferrin hydolysate (Seacheol and Krochta*.,* 2005). Lactoferrin activity has been studied against *Salmonella typhimurium* and *Campylobacter jejuni* on poultry broiler skin and also *Listeria monocytogenes* in ready-to-eat foods (Naidu *et al*., 2003). Similar observations were found in the present studies while using lactoferrin alone as well as in combination with other natural preservatives for preservation of paneer.

CHAPTER – 5

Summary and Conclusion

5. SUMMARY AND CONCLUSION

The present investigation was undertaken to enhance the antifungal activity of the AFS producing strains of *Lactobacillus* cultures in skim milk and whey media by addition of various growth factor and to explore their potential for preservation of paneer. The salient findings of the present work are summarized below:

1. All the three *Lactobacillus* cultures viz. *L. collinoides* NCDC 02, *L. casei* spp. *casei* NCDC 17, *Lactobacillus acidophilus* NCDC 195 exhibited antifungal activity against all the indicator fungal cultures *Kluyveromyces marxianus* NCDC 41, *Candida guillermondii* NCDC 44, *Aspergillus niger* NCDC 267, *Rhizopus oryzae* NCDC 52 by spot assay method.
2. All the three *Lactobacillus* cultures exhibited antifungal activity in MRS broth by well assay method. *L. casei spp*. *casei* NCDC 17, *L. acidophilus* NCDC 195 also showed activity in milk and whey, whereas, *L. collinoides* NCDC 02 didn't show antifungal activity in milk and whey.
3. Addition of yeast extract (1.5-2.0%) and peptone (1.0-2.0%) in skim milk as well as whey enhanced antifungal activity against *C. guillermondii* NCDC 44 only by. *L. casei* spp. *casei* NCDC 17, whereas, peptone addition to milk enhanced antifungal activity against *C. guillermondii* NCDC 44, *A. niger* NCDC 267, *R. oryzae* NCDC 52 as well.
4. Addition of yeast extrat in skim milk and whey exhibited activity against *C. guillermondii* NCDC 44 and *R. oryzae* NCDC 52 by *L. acidophilus* NCDC 195,whereas, addition of peptone in whey showed activity against yeast and mold cultures, but addition of peptone in skim milk showed activity against yeast only.
5. In case of *L. casei spp*. *casei* NCDC 17, glucose and maltose (0.5%) supplementation in skim milk exhibited activity against both yeast and mold cultures whereas glucose and maltose in whey and mannose in both the media showed activity against *C. guillermondii* NCDC 44. *K. marxianus* NCDC 41 and *A. niger* NCDC 267 were not inhibited at all.

6. In case of *L. acidophilus* NCDC 195, addition of glucose and maltose in whey exhibited activity against all the test fungal culture except *K. marxianus* NCDC 41,whereas in skim milk the same supplementation showed activity against *C. guillermondii* NCDC 44.
7. Addition of Tween 80 in milk showed activity against both yeast and mold cultures by *L. casei* spp. *casei* NCDC 17 whereas, in whey ,activity was only against yeast. In case of *L. acidophilus* NCDC 195, the trend was reverse.
8. *C. guillermondii* NCDC 44 was the most sensitive fungal culture among all the test cultures followed by *R. oryzae* NCDC 52. *K. marxianus* NCDC 41 was the most resistant culture.
9. Addition of peptone (1.0%), maltose (0.5%) and Tween 80(0.1%) in skim milk was the best combination for maximum antifungal activity for *L. casei* spp. *casei* NCDC 17.
10. AFS produced in skim milk supplemented with the selected ingredients had broad spectrum of activity against bacterial and fungal cultures. *Staphylococcus aureus* (MTCC 1144), *Bacillus Cereus* (MTCC 1272), *Pseudomonas aeruginosa* (MTCC 741 were inhibited whereas, *Escherichia coli* (MTCC739) was not inhibited among pathogenic cultures tested. *C. guillermondii* NCDC 44, *A. niger* NCDC 267, *R. oryzae* NCDC 52.*P.roqueforti* NCDC 170, *P.camemberti* 56 were inhibited, whereas, *K. marxianus* NCDC 41, *S.cerevisiae* NCDC 47 and *T. candida* 43 were not inhibited.
11. On preservation of paneer at refrigerated temperature with the treatment of various chemicals (sodium citrate (SC), potassium sorbate (PS), and natural preservatives (pediocin(P), lactoferrin (LF) and AFS showed that natural preservatives and combination thereof with chemical preservative exhibited similar acceptance of paneer.
12. Total bacterial counts increased by 5.5 log cycles in case of untreated samples, whereas, in treated samples with P+SC+PS, P+AFS+LF and

Lactoferrin alone, there was increase of 2.7, 2.84 and 2.86 log cycle respectively.

13. In controlled sample, there was increase in 4.5 log cfu/gm of yeast and mold count, whereas, in P+SC+PS, P+AFS+LF and LF alone, there was an increase in yeast and mold counts by 2.6, 2.1 and 2.4 log cycle respectively.
14. Moisture content remained almost same in all samples during storage, whereas, acidity increased in control sample from 0.18 to 0.36%, while in treated sample acidity increased from 0.18 to 0.28% maximum.
15. Sensory evaluation of all the samples showed that there is no significant difference in appearance, body and texture attributes for different treatments and control samples, while flavour and overall acceptability scores differ significantly.

Conclusion

From the foregoing sections, it can be concluded that AFS produced by *Lactobacillus casei spp. casei* NCDC 17 has antifungal potential. The promising. AFS produced by this potential strain of *Lactobacillus* was having broad spectrum antimicrobial activity against many fungal and bacterial cultures, which is a unique characteristic of this culture. By judicious selection of ingredients for addition in skim milk, the production of AFS can be enhanced .The problem of yeasts and molds as well as bacteria in dairy and other foods can be minimized considerably as the AFS produced by the potential lactobacillus cultures has been successfully used in the preservation of paneer to retard /slow down the growth of yeast and mold as well as bacteria in the sample while retaining its overall quality.

However, further studies may be carried out on the production of natural preservatives in large scale for preparation of its powder form and its application in biopreservatoin of dairy and other foods.

Bibliography

BIBLIOGRAPHY

Avonts,L.,Erika, V.V.and Luc, D.V (2004). Cell growth and bacteriocin production of probiotic lactobacillus strains in different media. *International Dairy Journal,* 14(11): 947-955.

Agarwal,S.K. and Ratanchand, (2001). Effect of indigenous antimicrobial factors on the shelf life of paneer. MSc. Thesis. National Dairy Research institute, Karnal, India.

Agnihotri, M.K. and Pal, U. K. (1996). Quality and shelf life of goat milk paneer in refrigerated storage.*Small Ruminant research*, 20(1): 75-81

Arora, V.K. and Gupta, S.K. (1980). Effect of low temperature storage on paneer. Indian *J. Dairy Science*, 33: 374-380

AOAC. Official Methods Of Analysis. (1984). 14th Edn. AOAC, Arlington, VA, Section.

Atanassova, M., Choiset, Y., Dalgalarrondo. M., Chobert, J.-M., Dousset, X., Ivanova, I. and Haertle, T. (2003). Isolation and partial biochemical chracterization of a proteinaceous anti-bacteria and anti-yeast compound produced by L. paracasei subsp paracasei stain M3. *Int. Journal Of Food Microbiology,* 87, 63-73.

Bajaj,R.K., Narasimha, K., Mann, B., Sangwan, R.B. and Vij, S. (2005).Isolation of cationic peptides from buffalo α_{s1} – and α_{s2} –casein and their antibacterial activity. *Indian Journal Of Dairy Science*, 58(6), 387-391.

Batish, V.K., Grover, S. and Lal, R. (1989). Screening lactic starter cultures for antifungal activity. *Cultured Dairy Products Journal,* 24: 21-25.

Bellamy, W., Wakabayash, H., Takase, M., Kawase, K., Shimamura, S. and Tomita, M. (1993). Killing of *Candida albicans* by lactoferricin B, a potent

antimicrobial peptide derived from the N-terminal region of bovine lactoferrin. *Medi. Microb. Immunol. (Berl.)*, 182: 97-105.

Bellamy, W., Yamauchi, K., Wakabayashi, H., Takakura, N., Shimamura, S. and Tomita, M. (1994). Antifungal properties of lactoferricin B, a peptide derived from the N-terminal region of lactoferrin. *Letters Appl. Microbiol*., **18**: 230-233.

Bennik, M.H.J., Van, O.W., Smid, E.J. and Gorris, L.G.M. (1999). Biopreservation in modified atmosphere stored mungbean sprouts: the use of vegetable associated bacteriocinogenic Lactic acid bacteria to control the growth of Listeria monocytogenes. *Letters In Applied Microbiology,* 28: 226-232

Bhattacharya, D.C., Mathur, O.N., Srinivasan, M.R. and Samlik O. (1971). Studies on the methods of production and shelf life of paneer. *J. Food Science And Technology*, 8:117-120

Buriti, Flavia, C.A.,Cardarelli, Haissa R.,Saad., Susana, M.I (2007). Biopreservation by Lactobacillus paracasei in coculture with S.thermophilus in potentially probiotic and symbiotic fresh cream cheeses. *Journal Of Food Protection, 70*: 228-235

Chung, T.C., Axelsson, L., Lindgren, S.E. and Dobrogosz, W.J. (1989). In vitro studies on reuterin synthesis by Lactobacillus reuteri. *Microbial Ecology In Health And Disease,* 2: 137-144.

Condon, S. (1987). Responses of lactic acid bacteria to oxygen. *FEMS Microbiology Reviews ,* 46: 269-280.

Corsetti, A., Gobebetti, M., Rossi, J. & Damiani, P. (1998). Antimould activity of sourdough lactic acid bacteria: Identification of a mixture of organic acids produced by Lactobacillus sanfrancisco CB1. *Applied Microbiology And Biotechnology,* 50: 253-256.

Daba H, Lacroix C, Hang J, Simard R.E. (1993). nfluence of growth conditions on production and activity of mesenteric 5 by a strain of Leuconostoc mesenteries. *Appl. Microbial.Biotechnol.,* 39: 166-173.

Davidson, P.M., Post, L.S., Branen, A.L. & McCurdy, A.R. (1983). Naturally occurring and miscellaneous food antimicrobials. In Antimicrobials in foods. . Marcel Dekker Inc. New York, USA. *Edited by P.M. Davidson & A.L. Branen.* 385-392

De Muynck C, Leroy AI, De Maeseneire S, Arnaut F, Soetaert W, Vandamme EJ. (2004).Potential of selected lactic acid bacteria to produce food compatible antifungal metabolites. *Microbiol Res.*, 159(4):339-46.

Dicks, L.M.T. (1994). The control of post-harvest disease of fresh fruit by an antifungal compound produced by a *Lactobacillus* spp. International Symposium on lactic acid bacteria, Caen, Normandy, France,

Dionysius, D.A. and Milme, J.M. (1997). Antibacterial peptides of bovine lactoferrin : Purification and characterization. *J. Dairy Sci.*, 80: 667-674.

Durlu-Ozkaya F., Karabicak N., Kayali R. and Esen B. (2005). Inhibition of yeasts from traditional Turkish cheeses by *Lactobacillus* spp. *Int. Journal Of Dairy Tech*., 58: 111-114.

Earnshaw, R.G. (1992). The antimicrobial action of lactic acid bacteria: Natural food preservation systems. In The lactic acid bacteria in health and disease. Edited by B.J.B. Wood. 211-232. Elsevier. London, UK.

El-Gendy, S.M. & Marth, E.H. (1981). Growth and aflatoxin production by *Aspergillus parasiticus* in the presence of *Lactobacillus casei*. *Journal Of Food Protection, 44*: 211-212.

Falguni and Vij (2006). Production and characterization of proteinaceous antifungal substances (AFS) from *Lactobacillus* spp. MSc. thesis. National Dairy Research Institute, Karnal.

Fitzsimmons, N. & Berry, D.R. (1994). Inhibition of Candida albicans by Lactobacillus acidophilus: Evidence for the involvement of a peroxidase system. *Microbios* 80: 125-133.

Fronz, C.M.A.P., Toit,M.D., Olasupo, N.A., Schillinger, U. and Holzapfel, W.H. (1998). Pltaricin D, a bacteriocin produced by Lactobacillus plantarum BFE 905 from ready-to-eat salad: L*etters in Applied Microbiology*, 26:231-235.

Ghodekar D.R. (1989). Factors affecting quality of paneer- A Review, *Indian Dairyman,* 41(3): 161-164

Gourama, H. & Bullerman, L.B. (1995). Inhibition of growth and aflatoxin production of Aspergillus flavus by Lactobacillus Species. *Journal Of Food Protection*, 58:1249-1256.

Gourama, H. & Bullerman, L.B. (1997). Anti-aflatoxigenic activity of *Lactobacillus casei pseudoplantarum*. *International Journal Of Food Microbiology ,* 34: 131-143.

Haikara, A., and Laitila, A., (1995). Influence of lactic acid starter cultures on the quality of malt and beer. *Proceedings Of The European Brewery Convention Congress, Brussels, IRL Press: Oxford,* 249–256.

Hayes ,M., Ross, R.P.,Fitzgerald , G.F., Hill, C. and Stanton, C. (2006).Casein-derived antimicrobial peptides generated by *L. acidophilus* DPC6026.*Applied and environmental Microbiology*, 72(3):2260-2264

Hill, J.E. (1989). Method and inoculant for preserving agricultural products for animal feed. US Patent Application, 4:842-871

Holley,R.A.(2003).Use of lactoferrin and lactoferricin to inhibit growth of food pathogens and spoilage microorganisms in meat *ARDI PROJECT,* Canada.

Hunter, D.R. & Segel, I.H. (1973). Effect of weak acids on amino acid transport by Penicillium chrysogenum: Evidence for a proton or charge gradient as the driving force. *Journal of Bacteriology*, 113: 1184-1192.

Hutten, E., Noro, K., Yang, Z.,(1995). Purificaton and identification of antimicrobial substances produced by two *Lactobacillus casei* strains*. Int. Dairy J*., 5: 503 – 513.

ICMR (2000). Application of hazard analysis and critical control point for improvement of quality of processed foods*, ICMR Bulletin*, 30: 5.

IDF (2003). World dairy situation, *IDF bulletin* no.384: 39

Jacobsen,T., Budde,B.B., Koch,A.G., (2003). Application of Leuconostoc carnosum for biopreservation of cooked meat products. *Journal Of Applied Microbiology,* 95:242-249.

Jay, J.M. (1982). Antimicrobial properties of diacetyl. *Applied and Environmental Microbiology,* 44: 525-532.

Jones, E.M., Smart, A., Bloomberg, G., Burgess, L. and Millar, M.R. (1994). Lactoferrin, a new antimicrobial peptide. *J. Appl. Bacteriol.*, 77: 208-214.

Kanawjia, S. K. and Singh, S. (2000). Technologiacal Advances in paneer making, *Indian Dairyman,* 52(10): 45-50

Karunaratne, A., Wezenberg, E. and Bullerman, L.B. (1990). Inhibition of mould growth and aflatoxin production by Lactobacillus spp. *Journal Of Food Protection,* 53: 30–236.

Katikou, P., Ambrosiadis,I., Georgantelis, D., Koidis,P., Georgakis,S.A., (2005). Effect of Lactobacillus –protective cultures with bacteriocin-like inhibitory substancse producing ability on microbiological,chemical and sensory changes during storage of refrigerated vaccum-packaged sliced beef. *Journal Of Applied Microbiology,* 99(6): 1303-1313

Kuipers M. E., de Vries H. G., Eikelboom M. C., Meijer D. K. F. and Swart P. J. (1999). Synergistic Fungistatic Effects of Lactoferrin in Combination with Antifungal Drugs against Clinical Candida Isolates*. Antimicrobial Agents and Chemotherapy,* **43**(11): 2635-2641

Lade, H.S., Chitanand, M.P., Gyananath, G., Kadam, T.A. (2006). Studies on some peoperties of bacteriocins produced by Lactobacillus species isolated from Agro-Based waste. *The internet Journal Of Microbiology*, 2(2): 1-6.

Laitila, A., Alakomi, H.L., Raaska, L., Mattila-Sandholm, T. and Haikara, A. (2002). Antifungal activities of two Lactobacillus plantarum strains against Fusarium moulds in vitro and in malting of barley. *Journal of Applied Microbiology*, 93: 566-576.

Lal, R. (1987). Antifungal Potential of Lactic acid Bacteria, M.Sc thesis, Kuruksetra University, Kurukshetra.

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. & Gobetti, M. (2000). Purification and characterization of Novel antifungal compounds from the sourdough Lactobacillus plantarum strain 21B. *Applied and Environmental Microbiology,* 66: 4084- 4090.

Law, B.A. and Reiter, B. (1977). Isolation and bacteriostatic properties of lactoferrin from bovine milk whey. *J. Dairy Res.*, **44**: 595.

Lindgren, S.E., and Dobrogosz, W.J. (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews, 87*: 149-164.

Magnusson, J. and Schnu¨ rer, J. (2001). Lactobacillus coryniformis subsp. coryniformis strain Si3 produces a broad-spectrum proteinacaeous compound. *Applied and environmental Microbiology,* 67: 1–5.

Mathur, B. N. (1991). Indigenous milk products of India: the related research and technological requirements, *Indian Dairyman*, 42(2): 61-74

Mathur, B.N. (1998). Indigenous dairy product: technological aspects , Abstract Integration of Modern and traditional technology in the manufacture of high quality indigenous processed foods, held on 4th april,1998 at IISC, Bangalore, 27-31

Moon, N.J. (1983). Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate, and their synergistic mixtures. *Journal of Applied Bacteriology, 55*: 453-460.

Munoz, A., Lopez-Garcia, B. and Marcos, J.F. (2006). Studies on the mode of action of the antifungal hexapeptide PAF 26.Antimicrobial agents and chemotherapy, 50(11):3847-3855

Naidu, A.S. (2002). Activated lactoferrin – A new approach to meat safely. *J. Food Tech.*, 56(3): 40-45.

Nakanishi, K., Tokuda, H., Ando, T., Yajima, M., Nakajima, T., Tanaka, O. & Ohmomo, S. (2002). Screening of lactic acid bacteria having the ability to produce reuterin. *Japanese Journal of Lactic Acid Bacteria*, 13: 37-45.

Nes, I.F. & Holo, H. (2000). Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers*, 55: 50-61.

Nes, I.F., Diep, D.B., Håvarstein, L.S., Brurberg, M.B., Eijsink, V. & Holo, H. (1996). Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek,* 70: 113-128.

Niku-Paavola, M.L., Laitila, A., Mattila-Sandholm, T. & Haikara, A. (1999). New types of antimicrobial compounds produced by Lactobacillus plantarum. *Journal of Applied Microbiology,* 86: 29-35.

Ogunbanwo, S.T., Sanni, A.I. and Onilude, A. A. (2003). Characterization of bacteriocin produced by Lactobacillus plantarum F1 and Lactobacillus brevis OG1. *African Journal of Biotechnology*, 2 (8): 219-227.

Okkers, D.J., Dicks, L.M.T., Silvester, M., Joubert, J.J. & Odendaal, H.J. (1999).Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from Lactobacillus pentosus with a fungistatic effect on Candida albicans. *Journal Of Applied Microbiology,* 87:726-734.

Pal, M. A. (1998). Microbiologically related biochemical transformations in paneer during storage. *Indian J. Microbiology*, 38(3):21-23.

Pal, M. A., Yadav P. L. and Sanyal M. K. (1993). Effect of paraffining on the physico-chemical, microbilogical and sensory characteristics of low fat paneer at low temperature storage, *Indian J. dairy Science*, 46: 519-524

Patil, G.R. (2002). Present status of traditional dairy products, *Indian Dairyman*, 54 (2): 35- 46.

Piard, J.C. and Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Lait,* 71: 525-541.

Rao, K.V.S.S.; Zanjad, P.N. and Mathur, B.N. (1992). Paneer technology- A Review, *Indian J. Dairy Science*, 45:281-290.

Reiss, J. (1976). Prevention of the formation of mycotoxins in whole wheat bread by citric acid and lactic acid. (Mycotoxins in foodstuffs. IX). *Experientia,* 32: 168-169.

Roy, U., Batish, V.K., Grover, S. & Neelakantan, S. (1996). Production of antifungal substance by Lactococcus lactis subsp. lactis CHD-28.3. International *Journal Food Microbiology,* 32: 27-34.

Sachdeva, S. (1983). Production, packaging and preservation of paneer, Ph. D. thesis, Kurukshetra University, Kurukshetra.

Sachdeva, S. and Singh, S. (1990a). Shelf life of paneer as affected by antimicrobial agents- Part I Effect on sensory characteristics*, Indian J. Dairy Science*, 43 : 60-63.

Sachdeva, S. and Singh, S. (1990b). Shelf life of paneer as affected by antimicrobial agents- Part II Effect on microbiological characteristics, Indian J. Dairy Science, 43: 64-67.

Sanders, M.E., Kondo, J.K., Willrett, D.L., (1991). Application of LAB. In: Goldberg, I., Williams, R. (Eds.), Biotechnology and food ingredients. Van Nostrand Reinhold, New York, pp. 433 – 459.

Sanni A.I., Onilude A.A., Ogunbanwo S.T., Smith S.I., (1999). Antagonistic activity of bacteriocin produced by *Lactobacillus* species from ogi, an indigenous fermented food*. Journal Of Basic Microbial*, 39:189-195.

Savadogo, Aly ., Cheik, A.T., Ouattara, Imae, H.N., Bassole, Alfred S. Traore. (2004). Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Burkina faso fermented milk .*Pakistan Journal Of Nutrition*, 3(3):174-179.

Schillinger,U. (1996). Bacteriocins of Lactic acid bacteria.Biotechnology and food safety. D.Bills and S.Kung (eds). Butterworths, London, 55.

Schwenninger, S.M., Ur,A.H., Nielerer,B., Teuber,M. and Meile, L.(2005). Detection of antifungal properties in Lactobacillus paracasei spp. Paracasei SM20, SM29 and SM63 and molecular typing of the strains. *J. Food Protection*, 68:111-119.

Seacheol, M. and Krochta, J.M. (2005). Inhibition of pencillium commune by edible whey protein films incorporating lactoferrin, lactoferrin hydrosylate and lacto peroxidase. *Journal Of Food Sciene, 70*(2): M87.

Shukla, F.C., Madhu, B. and Sekhon, K.S. (1984). Studies on the technological aspects of processing and preservation of paneer, I.C.A.R Rep., New Delhi.

Singh, L., Mohan, M .S., Puttalingamma, V. and Sankaran, R. (1989). Preservation of paneer by sorbic acid*, J. Food Science And Technology*, 26(3): 129-132

Singh, S. and Kanawjia, S.K. (1996). Effect of hydrogen peroxide and delvocid on the enhancement of shelf life of recombined milk paneer, *Brief Communication of International Dairy Congress, Montreal*; 2: 537

Singh, S., Kanawjia, S.K. and Sachdeva, S. (1988). Extension of shelf life of paneer, *Asian J. Dairy Research*, 7: 147-151

Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J. & Kenne, L. (2003). Antifungal 3- hydroxy fatty acids from Lactobacillus plantarum MiLAB 14. *Applied and Environmental Microbiology*, 69: 7554-7557.

Somkuti and Steinberg (2004). Pediocin production by recombinant Lactic acid bacteria .*Biotechnol. Lett*. 25:473-477

Stiles, J., Penkar, S., Plockova, N., Chumchalova, J. and Bullerman, L.B., (2002) Antifungal activity of sodium acetate and Lactobacillus rhamnosus. *J. Food Prot*., 65: 1188–1191.

Ström, K., Sjögren, J., Broberg, A. & Schnürer, J. (2002). Lactobacillus plantarum MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and phenyl lactic acid. *Applied And Environmental Microbiology,* **68**: 4322-4327.

Suzuki, I., Nomura, M. and Morichi, T. (1991). Isolation of lactic acid bacteria which suppress mold growth and show antifungal action. *Milchwissenschaft,* 46 (10): 635-639.

Talarico, T.L., Casas, I.A., Chung, T.C. & Dobrogosz, W.J. (1988). Production and isolation of reuterin, a growth inhibitor produced by Lactobacillus reuteri. *Antimicrobial Agents and Chemotherapy,* 32: 1854-1858.

Todorov, S.D., Reenen, C.A., Dicks, L.M.T. (2004). Optimization of bacteriocin by lactobacillus plantarum ST13BR, a strain isolated from barley beer. *Food Technol. Biotechnol, 43(2): 165-173.*

Tomita, S., Shirasaki, N., Hayashizaki, H., Matsuyama, J., Benno, V. and Kiyosawa, I. (1998). Binding characteristics of bovine lactoferrin to the cell surface of clostridium species and identification of the lactoferrin binding protein. *Biosci. Biotech. Biochem.*, **62**(8): 1476-1782.

Triona, K., Hill, C. (1999). Potenitial in food preservation. Copyright@1999 Academic press.

Vaishnavi ,C.,Singh, S.,Grover,R.,(2001).Bacteriological study of indian cheese (paneer) sold in Chandigarh. *Indian Journal Of Medical Microbiology,* 19(4): 224-226

Vermeiren, L., Devlieghore, F., Debevere, J. (2005). Co-Culture experiments demonstrate the usefulness of Lactobacillus sakei 10A to prolong the shelf-life of a model cooked ham. *International Journal Of Food Microbiology,* 108: 68-77.

Vignolo, G.M., de Kairuz, M.N., de Ruiz Holgado AAP. and Oliver, G. (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. *J. Appl. Bacteriol,* 78: 5-10.

Woolford, M.K. (1984). The antimicrobial spectra of organic compounds with respect to their potential as hay preservatives. *Grass And Forage Science,* 39: 75-79.

Yang, W.V. and Clausen, C. A. (2005). Determining the Suitability of Lactobacilli Antifungal Metabolites for Inhibiting Mould Growth. *World Journal of Microbiology and Biotechnology*, 21: 977-981