

**CHARACTERIZATION OF HUMAN STRAINS OF
LACTOBACILLUS ACIDOPHILUS ISOLATES
FOR THEIR SUITABILITY IN PREPARATION OF
MILK BEVERAGE AND THEIR ANTIBACTERIAL
CUM THERAPEUTIC ABILITY**

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ABSTRACT

A B S T R A C T

CHARACTERIZATION OF HUMAN STRAINS OF LACTOBACILLUS ACIDOPHILUS ISOLATES FOR THEIR SUITABILITY IN PREPARATION OF MILK BEVERAGE AND THEIR ANTIBACTERIAL CUM THERAPEUTIC ABILITY

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The present study was planned and conducted to examine the characteristics of Lactobacillus acidophilus strains isolated from the human intestinal and urino-genital tracts for their suitability in preparation of acceptable acidophilus milk and for their antibacterial as well as therapeutic abilities. The strains of L.acidophilus LBKV₃ and LBKI₄ were isolated from the vaginal mucosa of human female and faeces of bottle-fed infant respectively and were identified by morphological, physiological and bio-chemical tests. From among a total of 280 isolates obtained from the above stated sources the two strains of L.acidophilus LBKV₃ and LBKI₄ were selected for further characterization on the basis of sensory properties of curd and resistance to phenol, bile salt and low pH.

One strain each of mastitic Escherichia coli, Pseudomonas aeruginosa and Salmonella typhosa were obtained from Veterinary College, Gujarat Agricultural University, Anand Campus, Anand, and one strain of Lactobacillus bulgaricus (LBW) was procured from N.D.R.I. Karnal. One mixed strain lactic culture LF-40 and Streptococcus thermophilus (CH₁) was imported from Christen Hansen's Laboratory, Denmark. One strain each of Bacillus cereus and enterotoxigenic Staphylococcus aureus was obtained from Department of Dairy Microbiology, Dairy Science College, Anand. The strains of L.acidophilus were maintained in deMan, Rogosa and Sharpe (MRS) broth. While the strains of E.coli, Staph. aureus, Ps.aeruginosa, B.cereus and Salm.typhosa were maintained on nutrient agar slants. Sterilised skim milk was used for preserving the mixed lactic culture, LF-40. While L.bulgaricus and Str.thermophilus were maintained on MRS and lactose purple agar ^{stabs} ~~slants~~ respectively.

Growth rates in terms of log viable counts and titratable acidity of selected isolates of L.acidophilus strains LBKV₃ and LBKI₄ were determined in skim milk at 37°C. Acidophilus milks were organoleptically evaluated by a panel of judges.

The strains were also tested for their antibacterial activity against E.coli (Mastitic), B.cereus, Ps.aeruginosa, Salm.typhosa and enterotoxigenic Staph.aureus. The activated strains of L.acidophilus were inoculated in skim milk at 2.5 per cent level and incubated at 37°C for 12 to 72 h. The cell

free-culture filtrate (CFC-filtrates) were obtained by centrifuging the curd at 3000 rpm for 40 min. The CFC-filtrates were assayed for antibacterial activity against above mentioned food spoilage and pathogenic organisms by cup-assay technique.

Antibacterial activity of two strains of L.acidophilus against mastitic E.coli and enterotoxigenic Staph.aureus during production and storage of acidophilus milk was compared.

Simultaneously mixed lactic culture LF-40, and yoghurt culture (Str.thermophilus CH₁, and L.bulgaricus LBW) were inoculated in skim milk and their antibacterial activity was also assessed at 37 C and 15 C storage temperatures after 12 h incubation at 37 C. Inhibitory activity was expressed in terms of reduction in viable counts of the pathogenic organisms at selected time intervals i.e. 12, 16, 20, 24, 36, 48, 60 and 72 h of storage at 37 C and 4, 8, 12, 24, 36, 48 and 60 h of storage at 15 C for all the four cultures including two L.acidophilus strains.

To determine the implantation properties of the isolates, the fresh acidophilus milks prepared by using L.acidophilus LBKV₃ and that prepared by using L.acidophilus LBKI₄ containing viable cells of L.acidophilus LBKV₃ and LBKI₄ respectively were fed to two groups of human test subjects having six healthy and one adult having gastro-intestinal discomforts in each of the two groups. Acidophilus milk was fed at the rate of 200 g per day for a period of one week. The faecal samples of the test subjects were tested for content of

lactobacilli and coliforms, one day before starting feeding and at the interval of 2 days and one week during and one and two weeks after stopping the feeding.

Six commercially available therapeutic preparations intended to supply viable lactobacilli viz., Vizylac, Sporolac (Tablet), Zyspore, Lactisyn, Lactobacil and Sporolac (powder) were analysed to detect the actual viable cells of lactobacilli and contaminants other than lactobacilli, if any present.

To determine the antibacterial and therapeutic properties in vivo acidophilus milk at the rate of 200 g/day containing 2×10^7 viable cells of L.acidophilus was fed to 11 patients suffering from gastro-intestinal and related complaints like constipation, diarrhoea, ulcerative inflammations etc., for a period of 9 days. Clinical confirmations were obtained from the physician.

The results of the study have revealed that the L.acidophilus strain LBKV₃ isolated from vaginal surface was superior for all desirable characteristics with respect to product quality, implantation ability and antibacterial activity to that of gastro-intestinal isolate, LBKI₄.

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C E R T I F I C A T E

This is to certify that the thesis entitled "CHARACTERIZATION OF HUMAN STRAINS OF LACTOBACILLUS ACIDOPHILUS ISOLATES FOR THEIR SUITABILITY IN PREPARATION OF MILK BEVERAGE AND THEIR ANTIBACTERIAL CUM THERAPEUTIC ABILITY" submitted by Shri Chandraprakash Dattarao Khedekar in partial fulfilment of the requirements for the degree of Master of Science in Dairy Microbiology of the Gujarat Agricultural University is a record of bonafide research work carried out by him under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Anand

Date: 15 July 1988



(J. M. DAVE)
MAJOR ADVISOR

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INTRODUCTION

CHAPTER - 1

I N T R O D U C T I O N

In the recent years increasing number of research publications are seen on the health benefits of consuming live Lactobacillus acidophilus cells. Consequent to this there is a strong need felt to obtain promising strains of this organism for use in preparation of milk products having added therapeutic and related benefits for consumers.

A strong scientific opinion is building up in favour of such products and occasional expressions have indicated the future trends in this regard (Driessen and Puhon, 1987).

Lactobacillus acidophilus therapy in the form of fermented milk such as acidophilus milk, Yakult etc., has been successfully used in prevention and control of enteric disorders as well as different inflammatory processes.

Keeping the above said guide lines in view the present study was planned to investigate the following aspects.

- i) Isolation, identification and selection of human strains of L. acidophilus from different body sources.
- ii) To characterize the isolates with a view to establish their antagonistic effects against selected pathogenic and spoilage types of organisms.

- iii) To study patterns of inhibition of pathogenic organisms during production and storage of acidophilus milk.
- iv) To assess the acceptability of acidophilus milk based on organoleptic attributes.
- v) To examine the ability of these strains to implant in the human intestines.
- vi) To assess the therapeutic properties in vivo by treating human patients suffering from a variety of gastro-intestinal and related discomforts.

REVIEW OF LITERATURE

CHAPTER - II

REVIEW OF LITERATURE

The lactic acid bacteria consist of rod and spherical shaped, gram-positive, asporogenous organisms. They are widely distributed in nature and are easily isolated from mucous surfaces of mammals, green plants, milk and fermented foods. In man, the lactobacilli are found in the mouth, lower intestine and vagina. Because lactic acid is ^{the} principal end product of their carbohydrate fermentation, they have been exploited by man for thousands of years in food preservation. This was first done unknowingly as fermentations were allowed to proceed under the total influence of the natural food flora. More recently, man has learned to select certain species of these bacteria and use them under controlled conditions to produce the desired quality and stability in food products. Today these bacteria are indispensable in the manufacture of many products such as silage, dairy products, meat products, poultry products, vegetables, fruits, and plant seed products (Smith and Palumbo, 1981).

Lactobacilli constitute the predominant bacterial flora of the adult vagina. Lactobacillus acidophilus, L. casei, L. fermentum and L. cellobiosus occur in vagina and are collectively known as 'DODERLIEN'S BACILLI'. They ferment the glycogen deposited in the vaginal epithelial cells forming lactic acid

which accounts for highly acidio pH (about 4.0-4.5) of the vagina (Hurley et al., 1974⁴ and Anonymous, 1988).

The lactic acid starters are used in manufacture of yoghurt, acidophilus milk, cultured butter milk, sour cream, kefir, koumiss and ~~varieties~~^{varieties} of cheeses. In preparation of the plant seed products too, like fermented soy milk, peanut milk, sufu, shoyu, mahewu, ogi, fermented dough or sour dough bread they are indispensable.

Primitive man was constantly exposed to various lactic acid bacteria from the moment of birth and these microorganisms established themselves to become important members of the indigenous flora. With the onset of technological revolution in the food industry, however, types of organisms to which modern man is exposed^{to} by eating has changed drastically.

Since last few decades alongwith other lactic acid bacteria a prominent candidature is of Lactobacillus acidophilus. L. acidophilus is one of the predominant lactobacilli present in human and animal intestinal tract (Draser and Hill, 1974; Sandine et al., 1972⁵). Taxonomically it is placed in the family Lactobacillaceae under part 16 of the Bergey's manual of Determinative Bacteriology (Buchanan and Gibbons, 1974⁶). Further, as classified by Orla-Jenson (1919, 1943⁷) L. acidophilus is a member of thermobacterium group and is homofermentative.

In recent years, increasing emphasis is being placed on products cultured with specific micro-organisms to which special

nutritional, dietetic and therapeutic properties have been attributed. Among these, L.acidophilus has received the most extensive attention, especially for therapeutic and dietary purposes.

More than 200 species of bacteria have been isolated from man, and there might be more, especially the strict anaerobes (IDF, 1983), for considerable changes in the intestinal microflora occur from the day a baby is born until he or she becomes adult. Thus, in breast-fed infants, bifidobacteria represented 99 per cent of their faecal flora (IDF, 1983 and Rasic and Kurmann, 1983). But supplementation of their diet with a cow's milk formula resulted in large increases of bacteroides, in relation to bifidobacteria, in their faeces. This change suggests that breast milk could contain antibacterial substances which inhibit pathogens but not bifidobacteria. So that consequently, breast-fed infants are more resistant to infections than bottle-fed infants (IDF, 1983).

It appears that a large number of organisms are present in the stomach and small intestine of rats, whereas a much lower number of bacteria is present in human. However, in the large intestine and faeces, a more complex flora is present. Although lactobacilli, in general, represent a smaller percentage of the total intestinal flora, their metabolic functions make them more important than the others (Hawksworth et al., 1971).

Many factors affect the ecology of the alimentary tract, these include ~~the~~ pH, oxygen availability, level of specific substrates, presence of secretions and bacterial interactions (IDF, 1983).

2.1 CULTURAL AND BIOCHEMICAL CHARACTERISTICS USED IN IDENTIFICATION OF LACTOBACILLI

Much of the present day classification of lactobacilli is based on the outstanding work of Orla-Jenson (1919⁵, 1943). He divided the genus Lactobacillus into three main groups: Thermobacterium, Streptobacterium and Betabacterium, according to their growth temperatures and end products of carbohydrate fermentation. These groups were further subdivided into a number of species.

Further studies of the heterofermentative lactobacilli were made by Federson (1938)⁶, who separated five groups by means of growth temperatures and sugar fermentation. This work was later extended by Shinwell (1948)⁷ to differentiate brewery lactobacilli. Rogosa et al. (1947)⁸ suggested a number of biochemical tests which would give a key to the genus Lactobacillus. Rogosa et al. (1953)⁹ amplified this work and applied it to classification of oral lactobacilli, which they were able to classify into many different species. Briggs (1953)¹⁰ divided the lactobacilli into eight groups by means of physiological tests. deMan (1956^{a, b})¹¹ characterized species belonging to the thermobacteria and

streptobacteria. Rogosa and Sharpe (1959)¹ have made a comprehensive study of the classification of the lactobacilli using different methods.

The MRS medium of deMan, Rogosa and Sharpe (1960) was used for general cultivation of strains, determination of growth temperatures and in a modified form as the basal medium for biochemical tests.

The homofermentative lactobacilli can be distinguished by two tests. (i) the ability of heterofermentative strains to produce CO_2 from glucose using Gibson and Abd-el-Malek's (1945) method, (ii) the ability with some exceptions to produce NH_3 from arginine (Briggs, 1953)². The amount of acid formed in the milk is useful for differentiating some species of thermobacteria (Rogosa et al., 1953; deMan, 1956^{a, b})³.

The ability to split aesculin can be demonstrated by the formation of black precipitate in the presence of ferric ammonium citrate in medium (Davis, 1955⁴; Naylor and Sharpe, 1958)⁵ or by quenching of the fluorescence of aesculin. Rogosa and Sharpe (1959)⁶ and Wheater (1955)⁷ found that growth of an organism in the presence of bile salts was a characteristic of certain species of lactobacilli.

2.2 DESIRABLE CHARACTERISTICS IN LACTOBACILLUS ACIDOPHILUS STRAINS FOR THEIR THERAPEUTIC USE

Seven species of lactobacilli are frequently isolated from the intestinal tract; L.acidophilus, L.casei, L.cellobiosus, L.fermentum, L.plantarum, L.salivarius and Bifidobacterium bifidum (L.bifidus) (Gilliland, 1979)%. Of these microorganisms, the most commonly suggested as dietary adjuncts are L.acidophilus, B.bifidus and L.casei.

Utilization of L.acidophilus strains in preparation of a variety of food products is based on certain important physiological and biochemical abilities of these strains. An ideal culture of L.acidophilus to be used as dietary adjunct having therapeutic properties should have several desirable characteristics:

2.2.1 Tolerance to Gastric Acidity

It is desirable that the organism be a normal inhabitant of the intestinal tract of the healthy human host. If it has to function in the intestinal tract, it must survive the upper digestive tract to reach the intestines. There are a number of factors contributing to the control of bacteria in the digestive tract (Gilliland, 1979)%. Gastric acid is known to possess antibacterial activity in the human stomach and intestine and the Yakult Honsha Co.Ltd. (1971)% compared different species of lactobacilli for their relative resistance to gastric acidity. Their study indicated that there were differences among the

species tested. L.acidophilus, L.casei, and L.plantarum survived about 3 h exposure, but L.bulgaricus did not survive the first hour. Recently, Petterson et al.(1983) analysed the ileostomy contents of four volunteers, all with terminal or distal ileostomy. Acidophilus milk (500 ml) was consumed the day before the examination and on the day of sampling together with a normal diet. They observed that L.acidophilus NCDO 1748 survived passage through the stomach and intestine of all subjects, whereas L.bulgaricus - from yoghurt - survived only in one case. In addition, Gilliland (1979) has reported that some Lactobacilli exhibited resistance to lysozyme, which might indicate their ability to overcome this enzyme in the digestive system. Sandine (1979) reported that in spite of gastric juice and bile, fatty acids, organic acids, lysozyme, lysolecithin and antibiotics are the other important inhibitory substances present in gastro-intestinal tract. Beneficial effects of consuming live lactobacilli cells can only be derived if the same can maintain viability and activity in the carrier food before consumption and can reach the intestine in active condition.

Another such in vitro study, where human gastric juice, in proportions normally secreted in the stomach during digestion was added to homogenized breakfast mixture (orange juice, cheese sandwich and Coffee). To portions of this, butter milk, yoghurt and acidophilus milk were added. After 3 h of digestion it was

found that only L.acidophilus survived in appreciable numbers as compared with other lactic acid bacteria (Alm and Petterson, 1980)'.
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2.2.2 Oxygen Requirement

This characteristic is very important with respect to both culture production and maintenance of viability. B.bifidum is an anaerobic microorganism, and problems would, therefore, be expected during production and maintenance of the viability of the organism before consumption. Both L.acidophilus and L.casei are facultative anaerobes so that the difficulties of maintaining anaerobic conditions during their production and storage do not exist (Gilliland, 1979)'.
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2.2.3 Tolerance to Surface Tension

Surface tension is another factor influencing the establishment and growth of bacteria in the intestinal tract. Albus (1921)' studied the resistance of lactobacilli to different levels of surface tension, i.e. 40.4, 42.6, and 45.6 dynes. The depression of the surface tension of the medium was made by sodium ricinoleate. None of the L.bulgaricus strains grew at 40.4 dynes at 38 C for 7 days. However, all strains of L.acidophilus and the single strain of B.bifidum tested grew rapidly at the lowest surface tension: L.casei strains grew at 42.6 dynes.
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Bile acids may play a role in controlling the growth of bacteria in the gut. They are synthesised by the liver and normal human bile contains two primary acids which are cholic and chenodeoxy cholic acids, and a secondary bile acid, deoxycholic acid. In bile, they are coupled by an amide linkage to the amino acids glycine and taurine (White et al., 1973).

The bile salts play a role in fat absorption and any free bile acids may be absorbed by passive diffusion from the small or large intestine. The absorbed bile acids then return to the liver where they are reconstituted and resecreted in the bile (Draser and Hill, 1974)^c. Although, deconjugation is one of the main microbial reactions which alter the bile acids, the deconjugated form is more inhibitory to bacteria than the conjugated acid (Sandine, 1979)^c.

In one study, six strains of L. acidophilus and thirteen strains of L. casei of human origin were tested for their ability to deconjugate glycocholate and taurocholate. All strains of L. acidophilus deconjugated taurocholate and only one strain deconjugated glycocholate, while none of the L. casei isolates deconjugated taurocholate and only nine strains deconjugated glycocholate. Some strains of L. acidophilus and L. casei can liberate free bile acid, which might enhance their antagonistic actions towards intestinal pathogens. If this is so, this action could help in maintaining a favourable balance among species present in the intestinal tract (Gilliland and Speck, 1977^b)^c.

Another study has indicated that neither L.bulgaricus nor L.lactis could occur normally in the intestinal tract, because of their inhibition by oxgall in lactobacillus selective agar (Gilliland and Speck, 1977^a). Nevertheless, some authors reported that strains of L.bulgaricus can survive passage through the intestinal tract of both adults and children (IDF, 1983)^c, there is less doubt about the capacity of L.acidophilus to survive passage through the intestinal tract (IDF, 1983)^c.

L.acidophilus strains have been reported to resist presence of 2 per cent and 4 per cent bile salts (Wheater, 1955^c; Rogosa and Sharpe, 1959^c; Muelhens, 1967)^c.

Marmur (1961)^c had shown the resistance of lactobacilli to lysozyme which is one of the enzymes having deleterious effect on microorganisms.

Recently Gilliland et al. (1984)^c reported that in a feeding trial involving newborn, bile-resistant strains of L.acidophilus caused greater increases in numbers of facultative lactobacilli in the upper and small intestines than did the strain exhibiting lower resistance to bile.

2.2.4 Phenol Tolerance

Phenol is another such chemical compound which may influence the survival of L.acidophilus in the intestine. Since phenol is the product of the putrefactive processes in the large intestine, the resistance to this and other similar

compounds like indole, skatol, etc. may be used as an indication of the intestinal surviving ability of bacterial cultures. In this, L.acidophilus is able to grow in presence of upto 0.5 per cent (Rasic and Kurmann, 1978). Resistance to even 0.6 per cent phenol in milk by a strain of L.acidophilus NK 10 has also been reported (Koroleva, 1977).

2.2.5 Adhesion to Intestinal Surface/Mucosa

To establish successfully in the intestinal tract, bacterial strains also must be able to adhere physically and multiply on these surfaces (Klaenhammer, 1982). Kleeman and Klaenhammer (1982) studied these mechanisms of adhesion and suggested that in vitro adherence combined with subsequent in vivo studies, may provide a basis for screening candidate organisms for use in microbiotic supplements. Recently, Hood and Zottola (1987) studied the adherence properties of L.acidophilus electron microscopically and concluded that adherence of this organism specifically to human foetal intestinal cells was related to presence of polysaccharides in the cell wall.

In another such study Conway et al. (1987) reported that adhesion of lactic acid bacteria to human and pig intestinal cells and human ileal cells was increased in presence of 1 per cent skim milk.

2.2.6 Other Desirable Functions

Among other desirable functions carried out by lactobacilli including L.acidophilus in the intestinal tract is production of antimicrobial substances like organic acids, antibiotics, hydrogen peroxide etc. and aid in maintaining lower oxidation-reduction potential. These altogether can exert the antagonistic effect towards undesirable micro-organisms in the intestinal tract (Gilliland, 1979).

If the candidate organism possesses drug resistance, it will be an additional advantage, as it will have higher chances of survival even when the host is under antibiotic treatment. In this regards RodriguezFerri et al.(1979) initiated some work to develop drug resistance in the cultures of L.acidophilus.

In order to select cultures supposed to have relatively higher resistance to different conditions used in manufacture of acidophilus products survivor strains are screened through successive processing stresses (Pien, 1977; Cerna, 1981).

Combinations of different strains of L.acidophilus with special abilities can also be grown in association to improve the performance of each other. An USSR Patented Starter is prepared by 3 specially selected strains of L.acidophilus of which 2 possess high antagonistic properties towards pathogens and 1 has high proteolytic activity (Koroleva et al., 1983).

The last but important parameter in selection of lactobacillus culture for dietary use is formulation of growth medium and incubation conditions to yield high populations that are adapted to enzymatic or physical states required for cell stability during frozen or dried storage and have proper biological activity (Klaenhammer, 1982).

2.3 ACIDOPHILUS MILK PRODUCTS

During the last 15-20 years, there has been a truly dramatic rise in the numbers and varieties of different cultured milk product available which has contributed to the increase in consumption of these products in many countries. In particular, increasing emphasis is being placed on products cultured with specific micro-organisms to which special nutritional, dietetic and therapeutic properties have been attributed. Among these L.acidophilus has received most extensive attention, and as a result there are many acidophilus products available in different forms in different parts of the world.

Many types of foods have been suggested as carriers of L.acidophilus. These have included confectionary products, sherbet and jellies, but because the viability of the organism could not be maintained properly these foods were rated as 'unsuccessfull' (Speck, 1980). However, milk is the most precious food material known to mankind and the diversity of cultured products makes it an essential component in the diet

of many people. It is essential that individuals receive a diet with a high milk or lactose supplement, or that growth factors for this bacteria are incorporated in the preparation (Hawley *et al.*, 1959). Therefore, milk or cultured products would appear to be ideal vehicles for L.acidophilus (Nahaisi, 1986).

Traditionally the acidophilus milk (sour) is made by culturing L.acidophilus in severely heat treated milk, incubated for about 18-24 h at 37 C till acidity reached upto 0.6-0.7 per cent lactic acid (Foster *et al.*, 1958).

Acidophilus milk (sour) was the first dietary product developed containing large number of viable L.acidophilus cells. Process variations have been reported from the USSR (Glazachev, 1961), Yugoslavia (Svigir-Varga, 1970), Czechoslovakia (Teply and Hylmer, 1974) and India (Gandhi and Nambudripad, 1979^a) in a manufacture of acidophilus milk. This product is seldom flavoured with carrot juice, tomato juice, vanillin or cocoa (Svigir-Varga, 1970). Addition of 5 per cent carrot or tomato juice can also be done (Foster *et al.*, 1958). Possibilities of shortening the fermentation time of acidophilus milk has been also investigated (Miller and Pahan, 1980).

However, this product has encountered many problems during its expansion in the market of which the major difficulties are:

2.3.1 Slow Growth of L.acidophilus in milk

L.acidophilus particularly human strains are very slow growing in milk (Nahaisi, 1986)'. Relating to this problem, the addition of 0.5 per cent yeast extract has been advocated to shorten the coagulation time from 72 h to only 3 h and still shorter fermentation times can be accomplished using 1-5 per cent dextrose or grape sugar (Canadian patent, 1979)'. Also, the addition of 1-5 per cent tomato juice, 500 ppm peptone or 0.5 ppm trypsin to the milk can shorten the fermentation time (Miller and Puhon, 1980)'.

At the NIRD Reading (UK), the growth of two strains of L.acidophilus of human origin was accelerated by the sulphur - containing amino acid, cysteine, whereas methionine and cystine were found not stimulatory; it was suggested that the release of free thiol (SH) groups reduced the oxidation-reduction potential and this resulted in the observed effect (Marshall et al., 1982)'.

Final acidity and storage temperature are indicated to be the two important factors responsible for viability of cells (Kulp, 1931)'. It is preferable to cease incubation when the acidity approaches 0.65 per cent lactic acid (Foster et al., 1958; Kulp, 1931 and Reed, 1982)' and the viability can be maintained for 7 days at 5 C storage. However, Kulp (1931)' has suggested temperatures of 12-16 C for more than one week storage.

2.3.2 Sour Taste and Unattractive Flavour

The poor or unattractive flavour is another problem usually associated with milk fermented with L.acidophilus, because flavour is a key factor in consumer acceptance and demand. Despite excellent therapeutic value of this product, it did not become much popular due to very sour taste and the intense cooked flavour imparted to the product due to initial high heat treatment of the milk. To overcome sour taste of the product and to improve the palatability of the product it has been presented in variety of other forms with addition of flavourings or with other aroma forming bacteria. These products include A-38, acidophilus yoghurts, sweet acidophilus milk, acidophilus-yeast milk, acidophilin, acidophilus ice-cream, acidophilus paste, acidophilus concentrates, drink containing acidophilus, acidophilus whey etc. L.acidophilus is also being incorporated in various infant formulae. Freeze dried, vacuum dried or even spray dried preparations of acidophilus are also available in powder, granules or tablet forms.

L.acidophilus is also being used in ripening of cream for butter making (Lazauskas, 1974,1976) in preparing fermented pea-nut milk or soy milk (Smith and Palumbo, 1981) and also to ferment colostrum for feeding to young calves (Ellinger et al., 1980).

Poor flavour of acidophilus milk could be corrected by an understanding of the biochemical pathways leading to acetaldehyde in fermented milk: L.acidophilus is known to produce only low levels of acetaldehyde. Threonine aldolase is one acetaldehyde-forming enzyme, and Marshall and Cole (1983) have demonstrated its activity in L.acidophilus.

The addition of 0.1 and 0.5 per cent threonine to milk fortified with whey proteins increased the acetaldehyde produced by L.acidophilus 1748 from 3.0 ppm (in control) to 21 and 41 ppm respectively (Marshall and Cole, 1983).

Another method of improving the flavour of acidophilus milk might be through genetic manipulation, but little work has been done in this area (Nahaisi, 1986).

Tremendous growth in yoghurt consumption has been reported in the literature over the past few years, and this product could provide an alternative route for solving the problem of the poor flavour associated with acidophilus milk.

Acidophilus yoghurt is manufactured by incubating the yoghurt culture and L.acidophilus together, but it can also be made by mixing a normal yoghurt with an acidophilus-fermented milk (Lockie, 1980). However, L.acidophilus, when added to yoghurt tends to decrease in numbers during storage at 4-5 C. Such antagonism was found to be due to L.bulgaricus and hydrogen peroxide appeared to be the main substance responsible for the loss in viability of L.acidophilus (Gilliland and

Speck, 1977^o and Schioppa et al., 1982). In contrast Manus (1979) stated that blending milk cultured separately with L.acidophilus and yoghurt or butter milk produced a product containing viable L.acidophilus with an acceptable flavour.

In another experiment, yoghurt containing the normal yoghurt microflora and 30 million L.acidophilus per ml was produced and it was claimed that L.acidophilus remained alive (Muelhens, 1970). In addition, L.acidophilus, together with a mixed yoghurt culture produced a firm yoghurt with an acceptable flavour (Labropoulos et al., 1982).

The development of acidophilus products has been along several primary lines. A popular one has been to take advantage of the increased use of yoghurt as a carrier for L.acidophilus. 'Biograde', a product developed in Germany, which contains L.acidophilus and bifidobacteria among others, such as S.thermophilus (Speck, 1980).

Acidophilus-yoghurt popular in Western countries and America is made by inoculating heat treated and homogenized milk with the yoghurt culture and L.acidophilus culture, the latter should be an intestinal strain. Both cultures are separately prepared and added together at the end of incubation (Rasic and Kurmann, 1978 and Vedamuthu, 1974). Preparation of acidophilus yoghurt replacing L.bulgaricus with L.acidophilus plain or fruit flavoured by vat method is also reported (Angevine, 1972).

'Aco yoghurt' is a Swiss made product. A milk culture of L.acidophilus is added to yoghurt before packaging.

'Bioghurt', culture contains L.bulgaricus and S.thermophilus in addition to L.acidophilus. Another cultures used for this product contains L.acidophilus and S.lactis (Speck, 1980);

'A-38', is a product developed in Denmark. It is manufactured by mixing milk inoculated with a buttermilk starter and milk cultured with L.acidophilus in a ratio of 9:1 or 8:2 and the latter does not affect the flavour of the original product (Lang and Lang, 1978). As early as 1931, Myers grew L.acidophilus in sterile medium, harvested the cells, and then added them to pasteurized milk which provided L.acidophilus population comparable to that in regular acidophilus milk. This was the first sweet acidophilus milk reported. Duggan et al.(1959) prepared a frozen concentrate of L.acidophilus for preparation of a palatable acidophilus milk. Speck (1975^a) developed a product using pasteurized low fat milk to which a concentrate of bile resistant human strain of L.acidophilus was added. Several million viable and bile resistant cells of L.acidophilus were present per ml and the population could be maintained for 2-3 weeks with proper refrigeration. Further the flavour of the milk remained unaltered by the culture (Speck, 1980). Feeding trials have demonstrated that humans showed a significant increase of

lactobacilli in faecal contents after consumption of sweet acidophilus milk (Gilliland et al., 1978). Survival of L.acidophilus in three brands of commercial sweet acidophilus milk was also investigated (Young and Nelson, 1978). Noticeable decline in viable count of L.acidophilus was found after storage for 23-24 day at 4 ± 2 C and notable off-flavours developed in 14-21 days.

Acidophilus yeast milk differs from acidophilus milk in that in addition to L.acidophilus, Yeast (lactose fermenting or brewers' or bakers' yeast which ferment sucrose but not lactose) are used for fermenting the milk. It is contented that yeasts and acidophilus bacteria mutually stimulate their viability and as a result of this a considerable amount of antibiotics accumulate (Lang and Lang, 1975).

Acidophilin differs from acidophilus milk in the composition of the microflora, flavour and therapeutic properties. It is made by fermenting milk by a mixed starter consisting of cultures of L.acidophilus, S.lactis and Kefir culture (Lang and Lang, 1975).

Acidophilus paste is a product obtained by partial elimination of whey from acidophilus milk, has a consistency of thick cream and contains upto 8 per cent fat and upto 80 per cent moisture. Acidophilus paste may be made with flavourings, such as sugar in form of 65 per cent sugar syrup, cocoa and candied fruit. This product is said to have an excellent

flavour, fine consistency, and is considered very suitable for feeding infants (Lang and Lang, 1975). Another paste like product containing L.acidophilus is also developed in Russia specially for feeding infants. It is prepared from concentrated or reconstituted skim milk having 30 per cent total solids, added with more than 30 per cent cream to have more than 12 per cent milk fat, maize oil supplemented with vitamin A and D₂, malt extract, sugar syrup and water soluble vitamins with glycerophosphate. Acidophilus concentrate can be prepared by centrifuging L.acidophilus cells grown in a specially developed culture medium. It results in a concentration of about a hundred fold and gives a product which will maintain a substantial portion of its viability for upto 3 months when stored below 5 C. In normal production lots, it contain atleast 50 million cells per millilitre. The liquid concentrate can be taken orally or can be readily mixed with cold food, liquid or juice (Gandhi and Nambudripad, 1979^a).

At NDRI Karnal, a product called sweet acidophilus ice-cream was developed by direct incorporation of a culture of L.acidophilus in form of a cell concentrate (Kaul, 1979). Duthie et al. (1981) also described the ice-cream as an ideal vehicle for L.acidophilus.

An interesting product patented in the USA is a microbiologically stable acidophilus pudding containing 25-45 per cent water, sugar (not specified), 3-15 per cent fat, as well as

alginate stabilizer, emulsifier and flavourings. It is semi-soft at freezer temperatures and has an extended shelf-life at room temperature. A quinine salt may be added to reduce sweetener and other additives including upto 50 per cent fruits like strawberries, raspberries or cherries. However, there is no mention regarding the number of viable cells in the finished product and during storage (Mann, 1984).

In Japan a very successful product has been merchandised by the Yakult Co., for a number of years. Starting materials are skim milk or non-fat milk solids and sweetening agents such as sucrose, glucose, liquid sugar and starch syrup. Final flavouring is accomplished by the addition of fruit juices and flavouring essences. The milk is cultured with an intestinal isolate, Shirota strain (L. casei or acidophilus) to produce the desired acidity. In addition to lactic acid, minor products are also formed which include citric, succinic, malic and acetic acids, acetaldehyde, diacetyl and acetoin. After fermentation the product, Yakult, is maintained under refrigeration (Speck, 1975^a).

Hylmar and Pokorana (1986) suggested the method of production of pure dairy cultures with improved dietetic and medicinal properties.

2.4 DRIED ACIDOPHILUS MILK PRODUCTS

The traditional acidophilus sour milk and other products such as acidophilus yeast milk, acidophilus paste and some other related products were not much appreciated by consumers because of their unpleasant flavour. But some of their special merits with respects to nutritional and therapeutic properties led to development of palatable preparations such as bioghurt, biograde, acidophilus-yoghurt, sweet acidophilus milk etc. There are continuous efforts being made to improve organoleptic properties of these products to have greater consumers' acceptance with retention of their excellent therapeutic value. Dried acidophilus products in the form of powder, capsules and tablets prepared using lyophilization, vacuum drying or spray drying and such other sophisticated techniques appear to be very attractive proposals. Dehydration of acidophilus milk products may offer advantages which are basic to other dried food products such as long term preservation in nutritionally and biologically stable forms. Convenience in handling, storage, marketing, consumption and maintenance of viable cell population during storage has been reported to be promising (Holdman, 1975; Brennan et al., 1983; Prajapati et al., 1987).

2.5 INHIBITION OF GROWTH OF PATHOGENIC ORGANISMS DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK

Park and Marth (1972) observed that Salmonella typhimurium could grow in an artificial medium acidified to pH 4.4 with

lactic acid but that the organism was inactivated in cultured skim milk at similar pH. They concluded that factors other than acid production were involved in the inhibition of Salm. typhimurium in yoghurt.

To determine factor(s)* responsible for bactericidal properties of yoghurt, Rubin and Vaughan (1979) indicated the factors augmented lactic acid inhibition of Salm. typhimurium were low pH and low oxidation-reduction potential. The die-off rate of Salm. typhimurium was more rapid in yoghurt whey than in whole yoghurt, indicating that the casein fraction was partially able to protect ~~protect~~ Salmonella.

It has been suggested that the antibacterial activity of lactic cultures, and particularly of L. acidophilus, may be, atleast partially, due to lactic acid (Shahani et al., 1977).

Spillmann et al. (1978) investigated the possibility of detection of antimicrobial activity in commercial yoghurts as well as from acidophilus milks and other fermented milk products. No pronounced antimicrobial activity was detected in commercial yoghurts and acidophilus milks. Since the fractionation of the crude extract showed that only fractions containing lactic acid had an inhibitory effect, they concluded that lactic acid was responsible for inhibition.

Rubin et al. (1982) determined how lactic acid inhibits growth of Salm. typhimurium in yoghurt. This inhibition was demonstrated by microscopic examination not to be due to

bacteriolysis. Neither growth nor metabolic activity could be initiated after cells were washed in phosphate buffer and exposed to 1.5 per cent lactic acid for 1 h at 37 C, indicating that lactic acid inhibition is irreversible. Thus they concluded that the inhibition of Salm.typhimurium in yoghurt was from the intracellular dissociated moiety of lactic acid.

The main factor of inhibition and preservation in foods involving lactic acid fermentations is the rapid pH drop. Thus the acid moiety is the primary cause of inhibition. However, studies directed at elucidating the inhibitory nature of lactic acid fermentations have found a substantial effect due to other metabolites such as hydrogen peroxide (Dahiya and Speck, 1968; Gilliland and Speck, 1975).

Skim milk fermented by various lactic cultures was tested for antimicrobial activity by agar diffusion technique, using Ps.aeruginosa, Ps.fluorescens and B.subtilis as test organisms. L.acidophilus, L.bulgaricus and Str.thermophilus strongly inhibited the growth of test organisms (Pulusani et al., 1979). Shahani et al. (1977) were able to obtain lactic acid free preparations of acidophilin, an antibiotic substance from an active sample of acidophilus milk, using the silica gel method. Thus combined effect of lactic acid and these metabolites plays an important role in the overall inhibition of spoilage and pathogenic organisms.

Contamination of cheese with Staph. aureus is a recurrent problem in many parts of the world and has been studied extensively by Ibrahim and Baldock (1981).

Lactobacillus species isolated from oysters produced a substance inhibitory to Pseudomonas, Bacillus and Proteus species, the most sensitive being Pseudomonas. The inhibitory substance accumulated in Lactobacillus culture media, reaching maximum concentrations in 4 to 5 days at 30 C. The active substance was dialyzable, heat labile, and inactivated by catalase. Inhibitor production paralleled H_2O_2 formation in Lactobacillus cultures led to conclude Price and Lee (1970) that the observed inhibition had resulted from H_2O_2 produced by lactobacilli.

The potential for contamination of milk with Staph. aureus and the subsequent hazard associated with growth and enterotoxin production in other fermented products like yoghurt and acidophilus milk is possible. The inhibitory factors produced in a lactic acid fermentation have been studied under conditions of long incubation or storage times (Shahani et al., 1976; Shahani et al., 1977; Dahiya and Speck, 1968). Recently, Attai et al. (1987) has reported the inhibitory effect of yoghurt and acidophilus yoghurt cultures on a pathogenic strain of Staph. aureus introduced under simulated production conditions. They indicated the involvement of lactic acid, hydrogen peroxide and possible bacteriocin production in the inhibitory phenomenon.

2.6 THERAPEUTIC PROPERTIES OF PRODUCTS CONTAINING LACTOBACILLUS ACIDOPHILUS CELLS.

The importance of fermented milks, like dahi and butter milk as therapeutic agents in the treatment of gastro-intestinal disorders had been recognised in the Ayurvedic system of Medicine in India from very early times (Laxminarayana, 1981). The use of fermented milks in Western countries for the above purpose became popular in the beginning of the 20th century following the observations of Metchnikoff (1908), when he published the book entitled 'Prolongation of Life'. During the intervening three quarters of this century many research reports have generally tended to confirm this concept that 'toxic' substances produced by harmful bacteria affecting man's health and longevity' could be combated by the ingestion of lactobacilli amenable to the environment of the intestinal tract (Speck, 1975^a). While some scientific literature did not support Metchnikoff's theory, it is now generally agreed that L.acidophilus, rather than L.bulgaricus, is the species of lactobacilli capable of residence in the intestinal tract.

The health claims of ingesting live L.acidophilus cells could be due to several possible mechanisms which may include, restoration of normal intestinal flora through competition with and antagonism to harmful flora and human pathogens, accumulation of their metabolites including acids in the intestine and enhancement in the normal functioning of digestive

tract (Kopeloff, 1926; Rettger, 1935; Kuemmerle, 1958; Yakult Honsha Co.Ltd., 1971; Speck, 1976; Sandine, 1979; Gilliland, 1979; Shahani and Chandan, 1979; Speck, 1980; Konecny, 1986; Prajapati et al., 1986; Andres, 1987; Gilliland, 1987; Gurr, 1987).

2.6.1 Antimicrobial Activity* of L.acidophilus

Antimicrobial effects of products containing L.acidophilus against several human pathogens, enteric organisms and food spoilage microorganisms have been verified by a number of research workers through in vitro and in vivo studies.

Vakil and Shahani (1965) observed in vitro inhibitory effect of L.acidophilus against several Gram-positive and Gram-negative organisms. Vincent et al. (1959) claimed that antibiotic 'lactocidin' from certain strains of intestinal L.acidophilus had a broad antibacterial spectrum exhibiting bactericidal action against several Gram-negative, Gram-positive and acid fast bacteria and molds. Neri (1961, 1973) observed bacteriostatic effects of the substances produced by L.acidophilus against B.subtilis, Staph.aureus, E.coli, Salm.typhi, Shigella dysenteriae and Brucella melitensis.

Tomio-Karvoio (1963) observed bactericidal effects of the antibiotic substances of L.acidophilus in the whey of acidophilus milk against several pathogens.

Singh and Laxminarayana (1973) reported higher susceptibility of spore-forming aerobes, coliform bacteria, Staphylococci including pathogenic strains of E.coli and Staph.aureus to the

inhibitory action of lactobacilli. Neutralised culture filtrates of 12 strains of L.acidophilus showed inhibitory activity towards B.subtilis, Micrococcus flavus and Staph.aureus but not against strain of E.coli (Mitic et al., 1973).

Findings of Mikolajcik and Hamdan (1975^b) have shown that antibacterial agents from L.acidophilus are active against such diverse bacterial species as Salmonella, Shigella, Staphylococcus, Klebsiella, Proteus, Bacillus, Pseudomonas, Escherichia and also against Vaccinia and Polio viruses.

Shahani et al.(1976) reported that cultures of L.acidophilus and L.bulgaricus exhibited moderate to very strong inhibitory activity towards pathogenic strains of Salmonella, Shigella, Pseudomonas, Staphylococcus, Klebsiella and Vibrio spp. Gilliland and Speck (1977^a) studied antagonistic action of L.acidophilus towards intestinal and food borne pathogens in associative cultures. Hosono and Tokita (1977) observed that bacterial cell extract of L.acidophilus ATCC-3205 showed inhibitory action against multiplication of E.coli K-12 strain over a wide range of pH.

Shahani et al.(1977) observed a broad spectrum antibacterial activity of acidophilin isolated from L.acidophilus DDS₁ against variety of enteric pathogens and food spoilage organisms. Spillmann et al.(1978) reported antibacterial activity of one strain of L.acidophilus against B.subtilis, Ps.fluorescens and Staph.aureus.

Products made with L.acidophilus-R have been reported to show higher antibacterial activity towards E.coli, M.flavus, Staph.aureus, Salm.waltevrenden and B.subtilis (Gandhi and Nambudripad, 1979^b).

Smith and Stiles (1980) reported the use of some oral and vaginal Streptococci in inhibition of normal and pathogenic vaginal bacteria.

Several isolates from the faeces of bottle-fed infants identified as L.acidophilus strains showed variable antibacterial activity against E.coli (Patel, 1982). Culture filtrates of L.acidophilus-R and L.acidophilus-860 showed inhibitory activity against M.flavus C-504 and Ps.fragi, but other four strains failed to give any antibacterial activity (Amin, 1982).

Kujawa and Roczniak (1982) reported 6 strains of L.acidophilus, isolated from calf faeces to kill E.coli strains in milk within 48-72 h at 37 C. Gritsenko et al. (1983) also observed antagonistic properties of 10 strains of L.acidophilus towards E.coli, Salmonella spp. and Staph.aureus. Singh (1983) reported the inhibition of E.coli, Pseudomonas fragi, M.flavus and Staph.aureus by culture filtrate of L.acidophilus grown in cow, buffalo or goat milk.

Gandhi and Nambudripad (1980) observed in vitro inhibitory effect of L.acidophilus strains by using four strains they had prepared acidophilus yoghurt (AY) and acidophilus yeast milk (ASM). The latter contained 1.5 per cent lactic acid and showed

greatest inhibitory activity against E.coli, B.subtilis, M.flavus and Staph.aureus. Optimum temperature for antibacterial activity was 40 C.

Prajapati et al. (1984) observed the antibacterial activity of L.acidophilus LB₁H₃^{*} against E.coli and concluded that inhibition was attributed not only to the lactic acid but also some other antimicrobial compounds like hydrogen peroxide and other inhibitory substances.

Findings of Kim (1984) have shown that L.acidophilus grown in tomato juice, produced an antimicrobial compound active against several test microorganisms. The inhibitory substance was extracted from the tomato juice medium with methanol and ethanol and purified by gel filtration. B.subtilis, E.coli, Staph.aureus, Ps.fluorescens, Proteus vulgaris and Shigella dysenteriae were inhibited by purified preparations.

Alm (1985) reported that daily consumption of more than 500 ml acidophilus milk shortened the duration of Salmonella carrier state in randomly selected human carriers.

Study of inhibition of some pathogenic and potentially pathogenic bacteria in cultured milk products concluded that the strains of Salm.typhimurium, Shigella flexneri, Y.enterocolitica, Ps.aeruginosa, B.cereus and Staph.aureus were most susceptible to the antibacterial activity of acidophilus milk as compared to yoghurt and soured milk (Konecny, 1986).

Prasad and Gandhi (1987) studied some factors affecting the production of antibacterial substances in L.acidophilus strain R against fourteen different types of microorganisms. Micrococcus flavus was the most sensitive organism to antibacterial activity and production of antimicrobial activity was highest in sterilised skim milk as compared to cow's whole milk, buffalo milk, goat milk and a whey-lactose-yeast extract medium.

Studies of Attaie et al.(1987) indicated that inhibition of Staph.aureus was highest during acidophilus-yoghurt production as compared to standard yoghurt and acidified yoghurt production.

Danielson et al.(1987) reported higher antimicrobial activity of L.acidophilus against B.subtilis than E.coli when grown in MRS broth supplemented with 0.2 or 0.4 per cent bile salts.

The inhibitory effect of lactobacilli against yeast and mold was recently investigated by Collins and Hardt (1980). They found that L.acidophilus was capable of suppressing the growth of Candida albicans.

Reports on antiviral effects of L.acidophilus are also available in the literature. Klebanoff and Belding (1974) reported antagonistic relationship between H_2O_2 generating bacteria (Str.mitis and L.acidophilus) and viruses (Poliovirus). Hamdan and Mikolajcik (1974) observed the adverse effect of

'acidophilin' produced by L.acidophilus against polio and vaccinia viruses. Uchida et al.(1978) reported that infective titres of Herpes Simplex Virus-1, Chikungunya and Influenza type-A were reduced to 0 after approximately 1 h incubation at 37 °C in presence of culture filtrate of L.acidophilus or Bifidobacterium bifidum or Str.faecalis. Gilbert et al.(1983) suggested that fermentation of edible food wastes with L.acidophilus for feeding to livestock offers a possible alternative to cooking the food waste to prevent spread of diseases as the organism was found to inhibit Newcastle diseases virus, infectious canine hepatitis virus, porcine picornavirus, frog virus and bovine diarrhoea virus. Foot and mouth virus treated by acidophilus skim milk for four hours at 38 °C was non virulent for suckling mice but it did not find protective against subsequent infection in guinea pigs (Gorskill et al., 1972).

2.6.2 Mechanism of Antimicrobial Action of L.acidophilus

Mechanism by which L.acidophilus inhibits microbial growth is still not fully understood. A number of factors may be responsible for the antimicrobial activity exhibited by these organisms, which can be summarised as under -

2.6.2.1 Antibiotic Production

A number of reports have been written concerning antibiotic production by L.acidophilus. Strains of lactobacilli vary

in their ability to produce these inhibitors, and in addition, cultural conditions affect the amount produced within strains (Sandine, 1979; Prasad and Gandhi, 1987).

L.acidophilus appears to produce multiple inhibitors, e.g. Acidophilin (Vakil and Shahani, 1965), Lactocidin (Vincent et al., 1959), Lactolin (Kodama, 1952) and Lactacin (Barefoot and Klaenhammer, 1985), but at this stage, it is not clear whether these inhibitors are the same or of different substances (Nahaisi, 1986). Shahani et al. (1976) found that the optimum temperature for L.acidophilus to produce antibiotic compounds is 37 C. In addition, this microorganism has to grow in milk in order to produce any inhibitory substances.

In one study L.acidophilus exhibited inhibitory activity against B.subtilis, Serratia marcescens, Proteus vulgaris, Ps.fluorescens, Ps.aeruginosa, E.coli, Sarcina lutea, Str.laotis and Staph.aureus. Such inhibition was attributed to an antibiotic substance produced by the L.acidophilus. Since its normal metabolites did not demonstrate any suppressive action against these pathogenic microorganisms (Shahani et al., 1976).

Skorodurnova (1959) claimed that an acidophilus yeast milk produced with cultures of acidophilus bacteria and a lactose fermenting yeast was antibiotically active against Mycobacterium tuberculosis and several intestinal diseases.

Thus antibacterial activity may be partially attributed to the heterogenous antibiotic like substances and not only to the presence of organic acids and hydrogen peroxide (Hamdan, and Mikolajc 1974; Hosona and Tokita, 1977; Sukhova et al., 1978; Mehta et al., 1983; Barefoot and Klaenhammer, 1985; Konecny, 1986; Prasad and Gandhi, 1987; Rao and Gandhi, 1988).

2.6.2.2 Acid Production

The production of lactic acid during fermentation lowers the pH of the medium and thus prevents the growth of pathogenic, gram-negative microorganisms. In addition, volatile acids have some antimicrobial activity under conditions of low oxidation-reduction potential (Rasic and Kurmann, 1978).

Due to fermentation of milk by acidophilus bacteria numerous metabolites are produced, important among them being the lactic acid, acetic acid, and other organic acids (Ritter, 1956; Kao and Frazier, 1966; Trammer, 1966; Amster and Jost, 1980; Mikolajcik and Hamdan, 1975^b; Gilliland and Speck, 1977^a; Patkul et al., 1977; Uchida et al., 1978; Alm, 1982^{a, b}; Amer and Lammerding, 1983; Kanbe, 1986).

2.6.2.3 Hydrogen Peroxide Production

Some species of the genus Lactobacillus produce hydrogen peroxide. Collins and Aramaki (1979) studied the production of hydrogen peroxide in 10 per cent reconstituted skim-milk and low-fat milk by four species of L. acidophilus. Strain differences

were observed in respect of production and in addition, continuous agitation enhanced production during growth at 37 C, or storage at 4° C.

Numerous reports are available showing the possible inhibitory effect of hydrogen peroxide produced by the cells of lactobacilli during their metabolism present in small quantity (Dahiya and Speck, 1968; Price and Lee, 1970; Gilliland and Speck, 1977^b; Klebanoff and Belding, 1974; Collins and Aramaki, 1979; Wheeler et al., 1982).

A few workers have reported the antimicrobial effect due to the combined effect of lactic acid and antibiotic substances produced by cells (Singh and Laxminarayana, 1973; Mikolajcik and Hamdan, 1975^{a, b}). Gilliland and Speck, (1977^a) and Gilliland, (1985) reported this antimicrobial activity probably due to the combination of factors including acids, hydrogen peroxide and other inhibitory substances.

2.6.3 Implantation Properties of Lactobacillus acidophilus in Human Intestine and Its Effect on the Intestinal Microflora

In making the proposition that the ingestion of live organisms in acidophilus products may enhance the beneficial effects of the indigenous flora, it has been implied that they must be capable of surviving the acidic conditions of the stomach and passing down the gastrointestinal tract in large numbers and filling an ecological niche.

L. acidophilus is known to constitute a substantial part of the normal intestinal microflora living in association with a number of other groups of microorganisms e.g. Colliform bacteria, Streptococci, Bacteroides and Clostridia and at the same time exerting a regulatory influence on the relative proportions of different species (Laxminarayana, 1981). Table 2.1 summarises the data on approximate numbers of Lactobacilli present in the intestinal tract. The lactobacilli range from not detectable in some individuals, except for the faeces to form a million to a billion per gram. Citing these findings Sandine (1979) observed that their absence in certain 'healthy' persons would suggest that while their function may be desirable, they may also be dispensible. Table 2.2 indicates the relative proportion of other microflora in the gastrointestinal tract.

TABLE 2.1: NUMBER OF LACTOBACILLI IN THE HUMAN STOMACH, INTESTINAL TRACT AND FAECES^a

Sample source	:Viable count :(range log 10/g)
Stomach	N ^b
Upper small intestine	N-6
Lower small intestine	N-8
Large intestine	N-9
Faeces	3.6-9.3

a: Adopted from Drasar (1974) and Drasar and Hill (1974) as cited by Prajapati (1984)-

b: N less than 10 per gram

TABLE 2.2: APPROXIMATE NUMBER OF VARIOUS BACTERIA IN THE HUMAN STOMACH, INTESTINE AND FAECES^a

Genus	Mean log ₁₀ viable count/g wet weight				
	Stomach	Upper small	Lower small	Large	Faeces
<u>Bacteroides</u>	N ^b	2.5	2.5	8.0	10.5
<u>Bifidobacterium</u>	N	2.0	4.0	7.0	10.5
<u>Clostridium</u>	N	N	N	N	3.5
Enterobacteria	N	N	3.3	7.0	6.0
Enterococci	N	N	2.3	7.0	3.5
<u>Lactobacillus</u>	N	1.0	N	6.5	4.0
Verilonella	N	N	N	3.0	3.0
Yeasts	N	1.0	2.3	N	1.0
% Lactobacilli	0	14	0	0.5	5x10 ⁻⁶

a: Adopted from Hawksworth et al. (1971) as cited by Sandine (1979)

b: N = less than 10 per gram

Any serious disturbance or destruction of the normal microbial population due to change of diet or drastic use of wide spectrum antibiotics will provide favourable conditions for the growth of pathogenic organisms leading to gastroenteritis and other intestinal diseases. Another serious consequence of this condition is the development of antibiotic resistant character (R-factor) to other gram-negative organisms in the

vicinity (Smith, 1967). L.acidophilus has been found to play an important role in inhibiting the growth of such pathogens and also in restoring the normal ecological balance of the microbial population in the intestine.

Diet may influence the microflora in a number of ways. Firstly, it might supply substrates for bacterial growth. Secondly, diet may influence various aspects of gut physiology such as acid and bile secretion and motility in ways that preferentially inhibit or encourage certain microbial populations. Thirdly, components of the diet may have antibacterial properties. Fourthly, organisms introduced into the diet in fermented products may exert an influence on the existing ecology encouraging beneficial populations to flourish and suppressing harmful ones. They may prevent colonization of a habitat by competing more effectively than an invading strain for essential nutrients or sites of adhesion or making local environment unfavourable for the growth of the invader by producing antibacterial substances. It is the last two aspects that we are mainly concerned with in considering the effect of cultured produce on the gut microflora (Gurr, 1987).

There is now sufficient experimental evidence to prove that oral administration of fermented milk or cultured preparations containing viable cells of L.acidophilus can be effective in overcoming the above effects and in restoring the normal balance of microbial population in the intestine (Speck, 1975^b;

Vanovsky, 1975; Ferreira and De, 1979; USA National Dairy Council, 1979; Ramanath et al., 1980; Garvie et al., 1984; Ratoliffe et al., 1986; Gilliland, 1987; Gurr, 1987). Rodriguez-Ferri et al. (1979) have suggested that the yoghurt, containing drug resistant strains of L.acidophilus, L.bulgaricus and Str.thermophilus, can be manufactured and used to restore the intestinal flora in presence who have undergone prolonged antibiotic treatment.

Smith (1924) observed that with the administration of acidophilus the number of intestinal E.coli got reduced substantially and the flora in the stools consisted almost entirely of L.acidophilus.

In one of the studies (Myers, 1931) a non-fermented milk containing high number ($\sim 3 \times 10^8$ /millilitre) of added cells of L.acidophilus, the test subjects consumed 0.95 litre per day. The laotobacilli count in faecal matter were found to increase and coliform counts were found to decrease.

Twinning-McMath (1959) suggested that L.acidophilus could be used to maintain a stable protective intestinal flora while pathogenic organisms were being eliminated.

L.acidophilus (Shirota-Strain) used in preparing Yakult in Japan, when orally administered at 5×10^9 cells in 180 ml cow milk resulted in increase of the L.acidophilus (Shirota-Strain) and in decrease of Enterococci in the intestinal contents (Yakult Honsha Co. Ltd., 1971).

Zychowicz et al. (1974) showed therapeutic and implantation properties of L. acidophilus by feeding acidophilus milk to the two groups of children suffering from ²Salmonellosis and Shigellosis. Cure was obtained in most cases.

Speck (1975^b) suggested that it would be desirable to have L. acidophilus bacteria regularly available for intestinal implantation by inclusion in the diet of humans.

Collins and Petric (1975) gave the beneficial activity of L. acidophilus in reducing undesirable microflora in fermented colostrum.

Gilliland and Speck (1977^b) studied inhibitory action of L. acidophilus towards intestinal and food-borne pathogens in laboratory.

Consumption of acidophilus milk by volunteers suffering from gastro-intestinal complaints resulted in an approximately 10 fold decrease in total and coliform counts and 10 fold increase in lactobacilli counts in the stools. Implantation of L. acidophilus was confirmed, and volunteers reported partial or good improvement in their condition (Gandhi and Nambudripad, 1978).

Gilliland et al. (1978) determined the influence of consuming non-fermented milk containing L. acidophilus on faecal flora of healthy males. Results showed increase in facultative lactobacilli in their faeces which was found to

decrease after discontinuation of feeding. Sukhova et al. (1978) tried to implant L.acidophilus in new-born babies for a period of 6 weeks and reported that the tolerance by the infants was good and beneficial effect on the intestinal microflora was indicated.

In the feeding trials of pasteurized milk containing L.acidophilus isolated from either calf or human intestines to new born calves by Gilliland et al. (1979), increase in number of lactobacilli and decrease in number of coliforms in faeces was observed. Further, the calf strain of L.acidophilus gave the greatest increase in lactobacilli, while the human strain gave the greatest decrease in coliforms.

In a review on importance of feeding L.acidophilus to calves has suggested that feeding of fermentable cereal carbohydrates together with lactose allows the L.acidophilus culture to become established and create a more balanced intestinal microflora, thus preventing disorders such as diarrhoea (Jensen, 1979). Ayebo et al. (1980) observed no significant effect of ingestion of normal milk or acidophilus milk on faecal total aerobic counts, but decrease in faecal coliforms and increase in lactobacilli count was observed, when acidophilus milk was consumed.

Speck (1980) described the use of lactobacilli especially L.acidophilus as dietary supplements with particular emphasis on selecting strain with required cell viability,

resistance to bile and capacity of becoming established in the intestinal tract. Pollmann *et al.* (1980^a) observed high L.acidophilus counts in the tissues from gastro-intestinal tract of pigs fed L.acidophilus DDS-1 strain. However, the faecal flora counts were not significantly correlated with tissue lactobacilli counts. In conventional piglets, given L.acidophilus by stomach tube, increase in lactobacillus population in the gastro-intestinal tract while decrease in E.coli counts were observed.

Pollmann *et al.* (1980^b) in a study with 36 crossbred pigs, fed a maize-soyabean meal diet with 10 per cent lactose and L.acidophilus culture, highest lactobacillus counts in the gastro-intestinal tract were observed. However, supplementation with either L.acidophilus or probios was not found effective in suppressing E.coli in faeces.

Ellinger *et al.* (1980) observed decrease in faecal coliform counts with time in a group of calves receiving whole milk treated with L.acidophilus (frozen concentrated culture) at 5×10^8 organisms/litre.

Sharp increase in faecal counts of lactobacilli, coliforms and clostridia were reported which had been drastically reduced as a result of treatment with tetracycline-hydrochloride in rats when viable cells of L.acidophilus were orally administered (Ramanath *et al.*, 1980).

Kleeman and Klaenhammer (1982) reported two mechanisms in an in vitro study of testing adherence of lactobacillus species to human fetal intestinal cells. One mechanism requiring calcium was found non specific and allowed all lactobacilli tested to adhere, while the other system, not requiring calcium was found in 4 human strains of L.acidophilus.

Mautone et al. (1982) reported beneficial effect of feeding Bifidobacterium bifidum + L.acidophilus to 22 healthy neonates, as it markedly decreased gram-positive cocci and increased gram-positive bacilli in faeces. Patel (1982) reported increase in lactobacilli and decrease in coliform and enterococci counts during feeding of L.acidophilus carried in different cereal and pulse based media in dried form to human volunteers. However, after stopping feeding, the behaviour of counts was reversed.

Germ-free rats on a standard chow diet did not become colonized with L.bulgaricus or Str.thermophilus after feeding a small amount of yoghurt (Garvie et al., 1984). They have indicated the yoghurt bacteria survive while feeding continued, but do not colonize and are rapidly eliminated.

In a feeding trial involving new born dairy calves, supplementation of the diet with the more bile resistant strain of L.acidophilus caused greater increase in numbers of facultative lactobacilli in the upper small intestines than did the strain exhibiting lower resistance to bile (Gilliland et al., 1984).

The vital importance of colonization of certain parts of the human body by microorganisms is discussed, with particular reference to the intestinal flora and immune defence systems in the body. The role of bifidobacteria and L.acidophilus is noted, and the importance of regular consumption of cultured milk products in maintaining desirable microflora is emphasized (Klupsch, 1985).

Prajapati et al. (1986) reported that daily feeding of 10 g AMBTS (Acidophilus milk + Banana paste + Tomato juice concentrate + Sugar) with 200 ml pasteurized milk to 6 adults for 15 days resulted in an increase in faecal lactobacilli from 41 million to 550 million per gram and a decrease in faecal coliforms from 7 million to 0.63 million per gram, indicating successful implantation of L.acidophilus LB₁H₃.

Inclusion of beneficial intestinal bacteria, such as L.acidophilus, in milk and milk products, functions of these bacteria in the human gut and their survival during gastrointestinal processes are considered (Andres, 1987).

In vivo studies with human volunteers by Conway et al. (1987) confirmed that L.acidophilus strain could survive in the stomach for at least 20-40 minutes. All four strains which they have studied could adhere to some extent to human and pig intestinal cells and adhesion to human ileal cells was increased in the presence of 1 per cent skim milk.

Electron microscopy studies of adherence properties of L.acidophilus revealed that all strains adhered to the glass coverslips but no attachment fibrils were observed. One of the strains, BG₂FO₄ was known to adhere specifically to human foetal intestinal cells. The ability to adhere was related to presence of polysaccharide (Hood and Zottola, 1987).

Characteristics of host specificity in the strains of L.acidophilus has been indicated (Speck, 1975^a; Shahani and Ayebo, 1980; Laxminarayana, 1981; Tannoek et al., 1982; Mayra-Makinen et al., 1983). Thus for successful implantation in the human intestine, a human strains of L.acidophilus has much higher merits.

Speck (1976) has stated in a conclusive manner that ingestion of 10^8 to 10^9 L.acidophilus cells daily gives satisfactory results in maintaining normal balance of intestinal flora. Similarly Hawley et al. (1959) reported this level to be the most satisfactory.

2.7 LACTOBACILLUS ACIDOPHILUS IN TREATMENT OF HUMAN DISEASES

One of the interesting stories associated with Lactobacillus dates back to the early days of Bacteriology and the work of the eminent Bacteriologist, Metchnikoff in 1899. He observed that people in the Balkan Countries lived to an exceptionally old age and attributed this to their custom of regularly consuming lactobacillus by way of fermented milk.

2.7.1 In Gastro-intestinal Disorders

Metchnikoff (1908) advanced the theory that lactic acid bacteria (*Laetobacillus*) would inhibit the growth of putrefactive types of bacteria in the intestine and thus prevent the formation and absorption of toxic products which cause diarrhoea, dyspepsia and constipation. He successfully cured many of his patients suffering from dysentery by oral administration of live lactobacilli.

Kuemmerle (1957) reported the use of a commercial live culture preparation of *L. acidophilus* in therapy of a number of different gastro-intestinal disorders. Children suffering from various intestinal disturbances were cured with the administration of a commercial *L. acidophilus* preparation called 'Acidophilus Zyma' (Anonymous, 1959).

Lachner and Bieler (1961) demonstrated therapeutic value of 'Acidophilus Zyma' in the treatment of different intestinal disorders including enteritis, dyspepsia and X-ray damage in young persons.

Eklsenina and Tarnopolskaya (1960) treated 61 patients of chronic colitis with acidophilus milk and found better tolerance than fresh milk with complete reduction of their severity. However, acidophilus lactose tablets showed low therapeutic effect among 14 patients. A fermented milk product rich in *L. acidophilus* organisms was found effective in treating children suffering from paratyphoid or colic infections (Siegenthaler *et al.*, 1960).

Aritaki and Ishikawa (1962) cured infantile diarrhoea by feeding 30 ml of L.acidophilus milk in 13 out of 17 cases.

Dyspeptic infants infected with E.coli made rapid recovery after treatment with L.acidophilus milk culture (Tomio-Karovic and Panjeck, 1962). In vitro and in vivo laboratory tests have shown that milk fermented with a pure culture of L.acidophilus contained antibiotic which proved effective in the treatment of not only various disorders in digestive tract, but also of different inflammatory processes. It proved its effectiveness in various parenteral inflammatory processes caused by microbes resistant to all the antibiotics of daily practice (Tomio-Karovic, 1963).

In the middle of this century several workers have reported that L.acidophilus and L.bulgaricus fermented milk products could be used successfully in the treatment of gastroenteritis, specific or non-specific diarrhoea, skin infections and herpetic and aphthous stomatitis (Gordon et al., 1957; Siver, 1961; Abbott, 1961; Gillespie and Dimmick, 1951).

Freeze dried cultures of L.acidophilus proved to be very good success in the treatment of gastro-intestinal complaints (Tomio-Karovic and Krivec, 1970).

In the field tests of feeding Yoghurt, Japanese workers have obtained remarkable success in control and prevention of several gastro-intestinal disorders of infants as well as adults (Yakult Honsha Co. Ltd., 1971). No beneficial effect

was observed by Pearce and Hamilton (1974) when encapsulated freeze dried preparation of L.acidophilus was administered in a carbohydrate containing clear fluid diet to children below three years with acute diarrhoea.

Use of cultured milk products for treatment of liver and intestinal disorders has been described in a review by Surazynski et al.(1973). Two more reports (Gandhi and Nambudripad, 1979^b; Anonymous, 1978), also stated that feeding fermented L.acidophilus milk helped controlling gastro-intestinal disorders in humans. Tomic-Karovic (1974) presented a report of cases of various diseases treated with acidophilus milk including rheumatism, furunculosis and other purulent conditions in diabetics, infectious hepatitis as well as wounds, ulcerations and acne. Zychowicz et al.(1974) obtained cure in most of the cases of salmonellosis and shigellosis in children by feeding acidophilus milk. Feeding of acidophilus-yeast milk in addition to specific modification proved much effective in contributing to improvement and cure of the clinical conditions in 51 children suffering from cholepathia and pancreas malfunction manifested by impaired enzyme secretion (Bukhgalter, 1974).

Diseases of gastro-intestinal tract including diarrhoea were controlled by feeding of L.acidophilus in farm animals including calves, pigs, lambs and poultry (Nikolic et al., 1973; Shilovskaya and Bastrykina, 1978; Jensen, 1979).

Alm (1985) showed that daily consumption of more than 500 ml acidophilus milk shortened the duration of the Salmonella carrier state.

2.7.2 In Constipation

Acidophilus milk was suggested as a means of increasing the efficiency of bowel transport to some degree, in cases of habitual constipation (Graf, 1983). Thus a weakness of the abdominal muscles can occur in hospitalised, elderly or bed-ridden patients, and strong laxatives can cause complete evacuation of the bowel, including essential electrolytes (Alm et al., 1983).

Daily consumption of about 200 ml of acidophilus milk by hospitalised constipated men and women aged between 54 and 92 years reduced the use of laxatives, and there appeared to be a tendency towards an increased number of defaecations (Alm et al., 1983). In ~~future~~^{the} study, a daily intake of 300-400 ml of 'filmjolk' by 50 patients for a period of 90 days resulted in a low defaecation frequency and a need for laxatives. The 'filmjolk' was prepared by mixing nine parts of cultured buttermilk with one part of acidophilus milk, the former being made with a mixed culture of Str.lactis, Str.cremoris, Str.lactis sub.sp.diacetylactis and Leuconostoc cremoris (Alm et al., 1983). This mixture was less effective than acidophilus milk alone.

Sterilised acidophilus milk was not successful in increasing the bowel transport of constipated patients (Rettger and Cheplin, 1983), and this indicates the need for viable L.acidophilus in such treatments. In order to explain this action, Graf (1983) suggested that L.acidophilus affects the microbiological balance/interactions at the surface of the bowel mucosa. Such an effect, an 'excitation' of the mucosa, which spreads to the muscle layers.

2.7.3 Anticancerous Effects

Approximately 80-90 per cent of human cancers are of environmental aetiology and conceivably preventable (Draser and Hill, 1974).

Nitrosamines may be produced in vivo from secondary amines and nitrite. The human stomach with its low pH favours the nitrosation of dimethylamine and the formation of dimethyl nitrosamine and other nitrosamines. Nitrosamines can be produced by bacteria (Rowland and Grasso, 1975).

Strains of Lactobacilli and E.coli were found more active in degrading nitrosamines than bifidobacteria or bacteroides: they degraded diphenyl nitrosamine more rapidly than dimethyl nitrosamine (Rowland and Grasso, 1975).

Feeding non-fermented acidophilus milk containing viable L.acidophilus to Sprague-Dawley rats for 30 days significantly lowered the activity of β -glucosidase and β -glucuronidase in

their faecal matter. These two enzymes have been reported to convert procarcinogens into carcinogens (Sinha et al., 1979). In another study, 21 subjects were fed milk containing viable L.acidophilus (2×10^7 per ml) to investigate their effect on faecal bacterial enzyme activity. A highly significant decrease (2-4 fold) in the activities of β -glucuronidase, nitroreductase and azoreductase were observed in all subjects, but only during the period of feeding of lactobacilli (Goldin and Gorbach, 1985). Another diet containing a large population of viable L.acidophilus significantly lowered the activities of faecal nitroreductase and β -glucuronidase in meat-eating individuals (Goldin and Gorbach, 1980).

Additional studies, using Ehrlich ascite tumour cells, indicated that extracts of L.acidophilus and yoghurt possessed anti-tumour activity (Shahani, 1983). However, any conclusions concerning the role of acidophilus products in cancer treatment is dependent on further research work, particularly in vivo.

2.7.4 Effect on Body Cholesterol Level

Hypercholesteremia is believed to be one of the major factors bringing about atherosclerotic heart disease (Shahani, 1983). Intestinal bacteria can degrade cholesterol during its transit through the bowel and while coprostanol and coprostanone are the major faecal metabolites of cholesterol, smaller amounts of cholestanone are also formed (Draser and Hill, 1974).

Feeding non-fermented acidophilus milk to experimental animals (rats significantly reduced the serum blood cholesterol and the level decreased from 141.8 mg/100 ml to 102.5 mg/100 ml (Sinha, 1978).

Feeding of L. acidophilus in the diet has been reported to reduce serum cholesterol level in infants (Harrison and Peal, 1975) and in rats (Grunewald, 1982), while in a study by Thompson et al. (1982) no significant effect was observed.

Recently Danielson et al. (1987) reported 30-80 per cent reduction in cholesterol levels by all three strains of L. acidophilus in an MRS broth supplemented with 0.2 or 0.4 per cent bile salts.

Thus, it appears that some strains of L. acidophilus could play a role in the metabolism of cholesterol.

2.7.5 In Post Oral Antibiotic Treatment Complications

Antibiotics and other chemotherapeutic agents are indispensable and important drugs of choice to combat majority of infectious diseases. Ideally, they should selectively destroy pathogens and ~~and~~ not the normal flora of human intestines along with disease causing bacteria.

But the experience is otherwise. The oral administration of antibiotics, chemotherapeutics and steroid drugs over long period of time adversely affect the normal flora, particularly lactobacillus and disturb the 'balance of nature' in the intestine.

The disturbance in normal flora results into serious infections due to drug resistant and putrefactive bacteria such as *Staphylococcus*, *Proteus*, *Pseudomonas* and *Candida* (*Monilia*) Yeasts causing an array of discomfort such as diarrhoea, dyspepsia, flatulence, constipation, oral thrush, B-complex deficiencies and vaginitis. The putrefactive bacteria produce ammonia which is absorbed by liver in sufficiently large quantity in patients having hepatic insufficiency and leads to Hepatic coma (Anonymous, 1988). If this is so, then resistant strains may cause serious therapeutic problems in the host and perhaps, spread resistance to other species (Nordbring, 1983).

There is considerable agreement among scientists and physicians that the regular intake of acidophilus milk ensures that the bowel is colonised with a desirable microflora, and that such organisms assist in the control of intestinal disorders. In addition, acidophilus yoghurt helps to restore the normal balance of the intestinal flora after antibiotic treatment (Vedamuthu, 1974; Keogh, 1978). Thus many types of antibiotics are more severe against gram positive bacteria, including lactobacilli, than against gram negative types, for example *E.coli* and this effect upsets the balance of the microflora (Davis, 1970; Vedamuthu, 1974). Therapy with *L.acidophilus* has been advocated for such symptoms.

2.7.6 In Lactose Intolerant People

Lactose metabolism is a problem affecting many people in different parts of the world. Lactose intolerance is nearly 100 per cent in some populations on the continents of Africa and Asia. In North Europe, the majority are highly tolerant. In the United States, approximately 70 per cent of the black race are intolerant to some degree, and about 15 per cent of Caucasians (Campbell and Marshall, 1975).

Lactose intolerance may result from either a congenital deficiency of the intestinal β -galactosidase enzyme, or a lack of lactose activity of varying duration resulting from a number of intestinal injuries, for example gastro-enteritis (IDF, 1983). If this exists, then the colonic microflora would ferment the dietary lactose, resulting in flatulence, abdominal discomfort and diarrhoea (Rasic and Kurmann, 1983). Partial fermentation of the lactose present in cultured milks makes available such foods to lactose intolerant persons without adverse symptoms, as about 20-50 per cent of the lactose present in milk may be fermented by the bacteria (Alm, 1982^b). The absence of lactose intolerance after consumption of fermented milk, such as yoghurt could be explained by a slower passage of the lactose into the duodenum, in combination with lactose breakdown by the yoghurt microorganisms or the enzyme β -galactosidase could be liberated by autolysis of the bacterial cells, and in turn, increase the benefit to a lactose intolerant person

(Hawksworth et al., 1971; Rasio and Kurmann, 1983). If this is so, the fact that the microflora used in the production of many fermented milks can not survive in the human intestinal tract may not be so important, although the use of human strains of lactobacilli such as L.acidophilus, might extent the benefit to many lactose malabsorbers.

Speck et al. (1983) have examined the effect of feeding pasteurised, low-fat milk containing 2×10^7 per ml L.acidophilus cells to six, adult, Caucasian females with a history of lactose intolerance. An increase in their blood glucose was observed, and it was suggested that the increased numbers of L.acidophilus in the intestine provided better lactose absorption. In another study Alm (1982^{a,b}) observed that the abdominal distress and diarrhoea in eight lactose intolerant persons-resulting from the consumption of 500 ml of low-fat milk - disappeared after the consumption of yoghurt or acidophilus milk. Cultured milks, in particular acidophilus milk or yoghurt, should be recommended in formulating diets for lactose intolerant individuals. Use of L.acidophilus in treatment of lactose malabsorption is reported by Kim and Gilliland (1983) and Gilliland and Kim (1984). But Payne et al. (1981) reported that L.acidophilus containing milk when fed to lactose malabsorbants it did not give significant advantage.

2.7.7 Other Diverse Benefits of Consuming L.acidophilus

Tomio-Karovic (1959) fed acidophilus milk (half a litre per day) to 9 patients with psoriasis and found complete disappearance of the disease after a month in 3, improvement in 5 and no improvement in 1. Tomio-Karovic (1962) indicated that the ingestion of acidophilus milk or whey by mice for 5 days reduced the number of deaths due to intraperitoneal injection of pneumococcus species isolated from pneumonia sputum.

Tomio-Karovic (1964) reported that when acidophilus milk was used for upto 2 months to treat 500 cases of serious urological infection by strains of E.coli, Proteus, Staph. aureus, Str.haemolyticus and Ps.pyocyanea gave very good results in 80 per cent, improvement in 15 per cent and unsuccessful in five per cent of the cases.

L.acidophilus in milk was successfully used to treat two patients with hepatic encephalopathy (Macbeth et al., 1965). Direct application of the freeze dried preparation of L.acidophilus to post-operative wounds and decubital sores also gave good results (Tomio-Karovic and Krivec, 1970).

Berger and Karovic (1961) studied that treatment of bacterial vaginitis by local application with freeze-dried L.acidophilus milk in 22 women had a beneficial effect on the vaginal flora in the majority of cases. Gillespie et al. (1963) treated 58 young women suffering from lesions of acne vulgaris

with 7×10^{10} viable cells of L.acidophilus in sweetened and flavoured acidophilus milk daily for five days per week. An improvement in skin conditions was observed and maintained throughout the six months of experiment in 47 cases.

Successful use is reported of acidophilus preparation, as a protective agent in post operative electron irradiation to breast cancer sites and in the treatment of post irradiation ulcers. Reference is also made of an experiment in which consumption of acidophilus milk prevented formation of lesions in guinea pigs subjected to X-rays, and generally had beneficial physiological effects (Tomic-Karovic and Rudez, 1975).

Selected strains of L.acidophilus and L.bulgaricus grow well on jojoba seed meal and were found to reduce levels of cyano toxicants by apparently modifying the cyano groups. Further, this treatment also resulted in increased palatability of the meal which is used in livestock feeds for cattle, sheep and poultry (Verbiscoar et al., 1981)

It has been stated by Gandhi and Nambudripad (1979^a) that acidophilus milk therapy not only relieves such conditions as uncomplicated gastro-intestinal disorders but also a feeling of general vigour and health and controbutes to longevity. Further the experiments have revealed that the body weight of a child, a man or an animal increases when fed with acidophilus milk preparation.

Possible indirect use of cultured milk products has also been cited in alleviation of anxiety and depressive symptoms (Ayebo and Shahani, 1980).

Recently Linn et al.(1986) patented a method for treatment and prevention of mastitis using L.acidophilus.

2.8 PREVALENCE OF VIABLE LACTOBACILLI IN COMMERCIALY AVAILABLE THERAPEUTIC PREPARATIONS

L.acidophilus and probably other bile resistant lactobacilli found in the digestive tract have several beneficial roles on the health and well-being of the gastro-intestinal tracts of human and animals (Sandine, 1979 and Sandine et al., 1972). Abnormal and abusive food habits, antibiotic intake and digestive disorders might affect the growth and multiplication of L.acidophilus and reduce the number of these beneficial bacteria. This in turn could facilitate growth of undesirable normal and transient microflora and produce different abnormalities of the digestive system (Sandine et al., 1972; Speck, 1976; 1978). Dietary supplementation of live L.acidophilus cells to improve or maintain intestinal health, to treat intestinal diseases and to overcome lactose intolerance, cancer, cholesterol and constipation treatment has been advocated (Gilliland et al., 1978; Sandine, 1979; Speck, 1978; Sinha et al., 1979; Shahani, 1983; Sinha, 1978; Graf, 1983).

A number of L.acidophilus and Lactobacillus - containing dried products are now available at retail stores for use as dietary adjuncts for humans and animals. Many manufacturers claim their products contain large numbers of viable lactobacilli even after long storage. Gilliland and Speck (1977^c) examined several liquid and dried acidophilus products and found that very few dried products had viable L.acidophilus cells even in relatively low numbers. L.acidophilus fed orally in the form of freeze-dried capsules to nine hospitalized adult males failed to appreciably alter the faecal aerobic lactobacilli counts. However, viability of lactobacilli in the freeze-dried preparations was not reported (Dharma and Hoskins, 1972), raising the question as to whether any viable cells were fed. Similar evidence was also obtained by Gilliland et al. (1978). A recent survey in USA on prevalence of viable L.acidophilus in commercial products brought out the fact that only one out of four brands from the health food group contained 3.3×10^3 viable L.acidophilus cells per gram against 9×10^7 claimed by the manufacturer. Other samples from the same group did not have any L.acidophilus cells. They contained other lactobacilli, coliforms and gram-negative, lactose negative, motile rods (Brennan et al., 1983). The establishment of the lactobacilli requires consumption of moderate numbers of L.acidophilus (Speck, 1978), and a daily intake of 10^8 - 10^9 viable L.acidophilus was reported to be the most satisfactory level (Hawley et al., 1959; Brennan et al., 1983 and Nahaisi, 1986).

MATERIALS & METHODS

CHAPTER - III

M A T E R I A L S A N D M E T H O D S

3.1 ISOLATION AND IDENTIFICATION OF CULTURES

A total of two hundred and eighty cultures were isolated from the faeces of 24 bottle-fed infants of 1 to 2 years age and vaginal surfaces of 6 females between 16 to 24 years age. The faecal samples and vaginal surface swabs collected were diluted in sterile distilled water and plated on deMan Rogosa and Sharpe (MRS) agar. Predominating colony types were isolated from MRS agar plates prepared from above samples. When sufficient colonies were present, well isolated ones were picked into tubes of sterile MRS broth from each plate. Isolates were incubated at 37 C for 24 to 48 h (until growth was evident). Purity was established by streaking the isolates on MRS agar. All the isolates were catalase negative and gram positive rods. They were identified using the scheme of Rogosa and Sharpe (1959). Selected distinguishing biochemical and physiological characteristics from among those listed in the ^{1st} ~~9th~~ edition of Bergey's Manual of Systematic Bacteriology were used to identify the isolates. From among the two hundred eighty isolates, from the above said two sources, sixteen were identified as *Lactobacillus* species.

3.2 SELECTION AND MAINTENANCE OF CULTURES

Out of sixteen isolates, *Lactobacillus* species, *L. acidophilus* strains LBKV₃, from vaginal mucosa and LBKI₄ from gastro-intestinal tract giving tolerance to bile salt (4 per cent), phenol (0.5 per cent), sodium chloride (4 per cent) and pH 2 were selected for the present study. Similarly some other basic characteristics were also compared.

The comparisons were made by inoculating loopfulls of active cultures of both the strains grown separately in MRS broth at 37 C for 24 h into each of the following media in duplicate tubes.

- i) MRS broth added with 2 per cent and 4 per cent bile salts.
- ii) MRS broth added with 2 per cent and 4 per cent sodium chloride.
- iii) MRS broth having pH 7, 5, 4, 3 and 2.
- iv) Skim milk added with 0.4 and 0.5 per cent phenol.

All the inoculated tubes were incubated at 37 C for 24 to 96 h and growth was observed as development of turbidity as compared to the control tube incubated simultaneously. In case of skim milk-phenol medium positive growth was judged by clotting of the milk. Observations were recorded every 24 h. To verify tolerance (ability to remain viable) to above mentioned factors, loopfulls from tubes showing no growth

after 48 h of incubation were inoculated into plain MRS broth tubes and incubated for 48 h. Cultures able to develop turbidity were taken as tolerant. One strain each of Escherichia coli (mastitic), Salmonella typhosa and Pseudomonas aeruginosa was procured from collection of pathogenic organisms, Department of Veterinary Microbiology, Veterinary College, Gujarat Agricultural University, Anand Campus, Anand. One strain each of Staphylococcus aureus and Bacillus cereus was obtained from the Department of Dairy Microbiology, Dairy Science College, Anand. Mixed strain culture, IF-40 and lactic culture Streptococcus thermophilus (CH₁) was imported from Christen Hansen Laboratory, Denmark. One strain of Lactobacillus bulgaricus (LBW) was procured from National Collection of Dairy Organisms, National Dairy Research Institute, Karnal.

All the above strains of L.acidophilus were maintained in sterile screw-cap glass tubes of MRS broth. The mixed strain culture, IF-40, Str.thermophilus (CH₁) and L.bulgaricus (LBW) were maintained in sterilised skim milk. Staph.aureus, E.coli, Ps.aeruginosa, B.cereus and Salm.typhosa were maintained on Nutrient agar slants. The cultures were stored at refrigeration temperature (5 C) between transfers, which were subcultured weekly to maintain their activity. The cultures were subcultured atleast three times in MRS broth before use in the experiment.

3.3 CULTURE MEDIA

The composition and methods for preparation of the media used in the study are given in Appendix-I.

3.4 GROWTH STUDIES ON *L.ACIDOPHILUS* LBKV₃ and LBKI₄

Growth characteristics of the strains LBKV₃ and LBKI₄ were determined according to the method given by Mikolajcik and Hamdan (1975^a).

Activated cultures were inoculated at 2.5 per cent rate in sterilised skim milk and incubated at 37 C. Viable cell counts and acidity were determined by withdrawing the aliquots samples at selected time intervals.

3.4.1 Viable Cell Count

At selected time periods, i.e. 0, 16, 20, 24, 36, 48, 72, 96 and 120 h of incubation, one millilitre sample was taken and appropriately diluted using 9 ml phosphate buffer, IS:1479, Part III (1962) dilution blanks. One millilitre from the suitable dilutions were poured in petridishes and then MRS agar was added and mixed. Number of colonies were counted after incubating the plates at 37 C for 48 h.

3.4.2 Titratable Acidity

Titratable acidity of the inoculated milk samples, as per cent lactic acid was determined at selected intervals as stated above according to the method specified in Part I of IS:1479 (1960) using 10.0 g sample.

3.5 DETERMINATION OF ANTIBACTERIAL ACTIVITY OF *L.ACIDOPHILUS* LBKV₃ AND LBKI₄ AGAINST SELECTED PATHOGENIC AND SPOILAGE TYPE OF MICROORGANISMS

Antibacterial activity of the milk inoculated with the strains LBKV₃ and LBKI₄ was determined after 12, 24, 48, 72 and 96 h of incubation by cup-well assay technique as described in BS:4020 (1974) against E.coli (mastitic), Salm. typhosa, Staph.aureus (enterotoxigenic), Ps.aeruginosa and B.cereus. The fermented milk medium was centrifuged at 3000 revolution per minute (rpm) for 40 min, and the clear supernatant was boiled in screw-cap tubes for 5 minutes after recording its pH. Cell-Free Culture filtrate (CFC-filtrate) so obtained was finally stored in the same screw-cap glass tubes.

The control was prepared by adding lactic acid in sterilised skim milk to achieve 2 per cent lactic acid. This was then centrifuged for 40 min at 3000 rpm and the pH of the supernatant was adjusted to the pH of CFC-filtrate by using 0.1 N hydrochloric acid (HCl).

3.5.1 Cup-Well Assay Technique

3.5.1.1 Preparation of Inoculum of Assay Organisms

Twenty-four hour old cultures of E.coli, Salm.typhosa, Ps.aeruginosa, Staph.aureus and B.cereus grown in Nutrient broth were centrifuged at 3000 rpm for 30 min in sterilised centrifuged tubes. The supernatant was decanted and cells

were suspended in sterile normal saline (0.9 per cent NaCl) solution, centrifuged and washed cells were resuspended in sterile normal saline solution. The percentage transmittance of the washed cell suspensions were suitably adjusted to 50 per cent at 550 nm (using Spectronic-20 Spectrophotometer) diluting it with sterile normal saline. The number of cells in cell suspensions were then determined by the pour plate method. The cell suspensions were adjusted to contain approximately 10^7 cells per millilitre. The readjusted cell suspension of E.coli and Salm.typhosa were spread individually on the surface of MacConkey's agar at the rate of 1 millilitre per plate. Similarly the readjusted cell suspensions of Staph.aureus was spread on the surface of Staphylococcus Agar-110 added with 10 per cent skim milk prior to pouring the sterilised plates and those of B.cereus and Ps.aeruginosa were spread individually on the surface of Nutrient agar plates at the rate of 1 millilitre per plate.

3.5.1.2 Assay Procedure

The procedure recommended in the British Standard Method (BS:4020, 1974) with slight modification was followed. The assay media (Nutrient agar, MacConkey's agar and Staphylococcus agar) were melted and tempered to 45 ± 2 °C and equal amounts of media were transferred to individual sterilized petridishes separately and allowed to solidify. The petridishes were transferred to a refrigerator (5 °C) for one hour to

facilitate the boring of wells in the inoculated medium. The plates were transferred to incubator at 37 C after spreading 1 millilitre of the cell suspension of the test organism on the agar surface uniformly to make the plates dry. Three wells were made at equal distance in the solidified agar medium in each petridish using a sterile hollow stainless steel gel cutter (7 mm diameter).

The CFC-filtrates of two cultures were transferred in 0.1 millilitre aliquotes into two wells individually using sterilized standard graduated 0.1 millilitre pipettes, and third well was filled with 0.1 millilitre of control filtrate. Petriplates were left undisturbed for one hour to facilitate the diffusion of the culture filtrates into the medium. The plates were incubated at 37 C for 24 hours and diameter of zones of inhibition was measured.

3.6 PREPARATION OF ACIDOPHILUS MILK

For preparation of acidophilus milk method recommended by Nahaisi (1986) with slight modifications was followed.

A schematic diagram showing processing steps for manufacture of acidophilus milk is given in Figure 3.1.

3.7 SENSORY EVALUATION

Acidophilus milks prepared by using L. acidophilus LBKV₃ and LBKI₄ were brought to about 20 C. These were organoleptically evaluated by 6 technical persons as per 9 point hedonic preference scale.

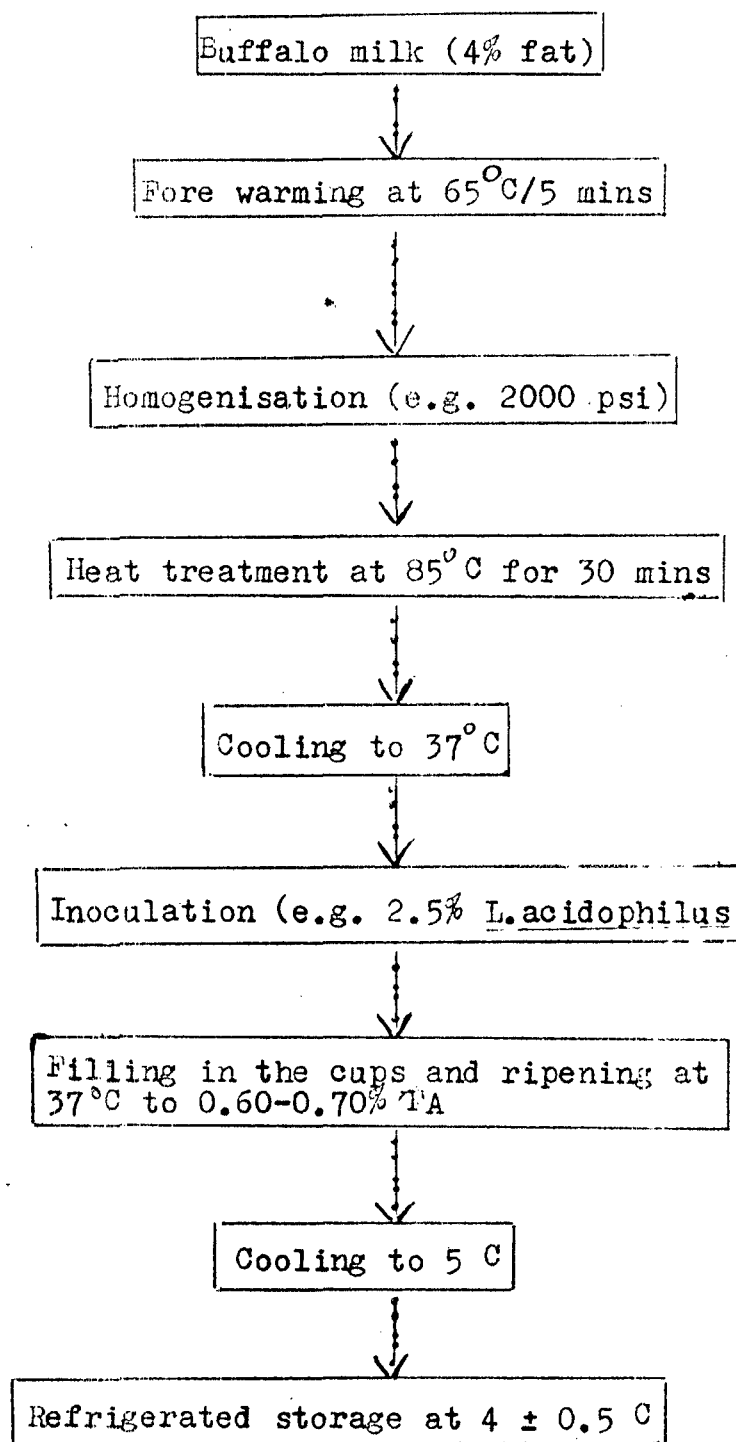


FIGURE 3.1: FLOW DIAGRAM SHOWING PROCESSING STEPS FOR MANUFACTURE OF ACIDOPHILUS MILK

3.8 STUDY OF IMPLANTATION CHARACTERISTICS OF L.ACIDOPHILUS LBKV₃ AND LBKI₄

Fresh acidophilus milk containing on an average 3×10^7 live cells of L.acidophilus LBKV₃ per gram was fed to a group of seven test subjects at the rate of 200 g per day for a period of a week. Another group of seven volunteers was fed with fresh acidophilus milk containing 3×10^7 live cells of L.acidophilus LBKI₄ per gram at the rate of 200 g per day for a period of 7 days to study its implantation characteristics.

The fourteen test subjects, selected for the study were all adults including 13 males and 1 female. One in each of the seven volunteers' groups was having complaints of gastro-intestinal discomforts. The faecal samples of all the volunteers were examined immediately after collection for lactobacilli and coliform counts before starting the feeding and at the intervals of 2 days and one week during feeding and one and two weeks after stopping the feeding.

3.8.1 Lactobacilli Count

Faecal samples were diluted in sterilised phosphate buffer solution, appropriate dilutions were plated on MRS agar. After solidification they were overlaid with uninoculated melted MRS agar and incubated at 37 C for 48 h. Counts were expressed as c.f.u. of lactobacilli per gram of the faecal sample.

3.8.2 Coliform Count

Faecal samples were diluted in sterilised phosphate buffer solution, appropriate dilutions were plated on MacConkey's agar. After solidification they were overlaid with uninoculated melted MacConkey's agar and incubated at 37 C for 24 h. Counts were expressed as c.f.u. of coliforms per gram of the faecal sample.

3.9 ANALYSIS OF COMMERCIALY AVAILABLE PREPARATIONS CONTAINING LACTOBACILLI

Six types of commercially available preparations containing lactobacilli, namely, Sporolac (Uni-Sankyo, Ltd., Hyderabad), Lactobacil (Inter-Care Ltd. Mehsana), Lactisyn (Griffon Lab., Bombay), Vizylac (Unichem Ltd., Bombay) and Zyspore (Key-West Ltd., Bombay) were collected from various pharmaceutical stores of Anand.

One gram of each of the preparations was appropriately dissolved in 9.0 millilitre of sterilized phosphate buffer. The preparations labelled to contain spores of L. sporogenes were subjected to heat treatment by holding the first dilution tubes in waterbath at 80 C for 5 min. followed by cooling in chilled water and plating in duplicate. Preparations containing L. sporogenes were plated on both MRS as well as sporogenes agar media, while those containing L. acidophilus were plated on MRS agar as well as Nutrient agar to detect presence of contaminants, if any. Plates were incubated at 37 C for 24-48 h.

The viable counts of preparations were expressed in c.f.u. per ampoule/capsule/sachet/tablet, depending upon the form of availability.

3.10 INHIBITION PATTERNS OF LBKV₃ AND LBKI₄ AGAINST SELECTED PATHOGENS IN MILK

3.10.1 Antibacterial Activity Against Staph.aureus

Antibacterial activity of L.acidophilus strains LBKV₃ and LBKI₄ was determined by using twenty four hours old cultures of these strains activated in sterilised skim milk. Similarly yoghurt culture (Str.thermophilus CH₁ and L.bulgaricus LBV) and mixed strain culture LP-40 were also activated. These cultures were inoculated into sterilised skim milk flasks containing 300 millilitre of milk at the rate of two per cent. Subsequently all the four flasks were inoculated with 0.1 ml quantity of twenty four hours old culture of Staph.aureus grown in nutrient broth. Initial count of Staph.aureus was recorded after incubation of plates for 24-36 h.

The contents of the flasks were thoroughly mixed by using Cyclomixer and aseptically distributed in presterilized test tubes in equal quantities of 15 ml per tube. After labelling the tubes one set of tubes for each of the four cultures mentioned above was incubated at 37 C. After twelve hours of incubation one set of the tubes for each of the cultures was transferred to B.O.D. incubator adjusted at 15 C while other set was incubated at 37 C.

3.10.1.1 Staphylococcal Count

At selected time intervals i.e. 0, 12, 16, 20, 24, 36, 48, 60 and 72 h of incubation at 37 C and 4, 8, 12, 24, 36, 48 and 60 hours of incubation at 15 C one millilitre quantity was taken from each of the four cultures individually and appropriately diluted using 9.0 ml phosphate buffer dilution blanks. Suitable dilutions were poured in petridishes and then melted Staphylococcus agar (S-110 Difco, 1977) was added and mixed. Number of colonies were counted after incubating the plates at 37 C for 24-48 h.

3.10.1.2 Titratable Acidity

Titratable acidity of the incubated milk samples as per cent lactic acid was determined at the above mentioned intervals according to the method specified in Part I of IS:1479 (1960) using 10.0 g sample.

3.10.2 Antibacterial Activity Against E.coli

Antibacterial activity of LBKV₃ and LBKI₄ strains was determined by using twenty four hours old cultures of these strains activated in sterilized skim milk. Similarly Yoghurt culture (Str.thermophilus CH₁ and L.bulgaricus LBW) and that of mixed strain culture LF-40 were also activated. These cultures were inoculated into sterilised skim milk flasks containing appropriately measured 300 millilitre of skim milk at the rate of two per cent. Subsequently all the four flasks

were inoculated with 0.1 ml quantity of twenty four hours old culture of E.coli grown in nutrient broth. Initial coliform count was recorded after incubation of plates for 24 h.

The contents of the flasks were thoroughly mixed by using Cyclomixer and aseptically distributed in presterilised test tubes in equal quantities of 15 millilitre per tube. The tubes were then incubated at 37 C for 12 h, after which fifty per cent tubes from each of the four cultures were transferred to BOD incubator adjusted at 15 C, while other set was incubated at 37 C.

3.10.2.1 Coliform Count

At selected time intervals, i.e. 0, 12, 16, 20, 24, 36, 48, 60 and 72 h of incubation at 37 C and 4, 8, 12, 24, 36, 48 and 60 h of incubation at 15 C, one millilitre quantity was taken from each of the four cultures individually and appropriately diluted using 9.0 ml phosphate buffer dilution blanks. Suitable dilutions were poured in petridishes and then MacConkey's agar was added and mixed. After solidification they were overlayered with uninoculated melted MacConkey's agar and c.f.u. were counted after 24 h incubation at 37 C.

3.10.2.2 Titratable Acidity

Titratable acidity of the inoculated milk samples as per cent lactic acid was determined at above mentioned intervals according to the method specified in Part I of IS:1479 (1960) using 10.0 g sample.

3.11 ACIDOPHILUS THERAPY TO PATIENTS SUFFERING FROM GASTRO-INTESTINAL DISCOMFORTS

Patients belonging to a heterogenous age group and suffering from a variety of gastro-intestinal and related disturbances were selected according to physicians recommendation.

Eleven patients were selected out of which three were suffering from chronic constipation, two each from diarrhoea and ulcerative stomatitis and one each from ulcerative colitis, leucocytis, glossitis with stomatitic gingivitis, and piles inflammation.

3.11.1 Preparation of Acidophilus Milk for Acidophilus Therapy

Heat treated (85 C/30 min) skim milk was inoculated at 3 per cent rate with 24 h old active cultures of L.acidophilus LBKV₃ and LBKI₄ separately. The inoculated milk was distributed in 100 ml quantity in presterilised plastic cups. These cups were incubated at 37 C for 24 h and then cooled to 15 C.

3.11.2 Dose of Acidophilus Milk

Acidophilus milk thus prepared contained about 2×10^7 viable cells of L.acidophilus LBKV₃ per millilitre with 1.17 per cent titratable acidity as lactic acid. Acidophilus milk prepared by using LBKI₄ strain contained 1.9×10^7 viable cells per millilitre having 1.01 per cent lactic acidity.

Such products prepared by using two Lactobacillus acidophilus strains were fed at the rate of 200 g per day to the patients over a period of 9 days. It was suggested to take 100-200 ml of milk half an hour before or after taking acidophilus cells. Clinical response was recorded after 3, 6 and 9 days of commencing the treatment.

RESULTS & DISCUSSION

CHAPTER - IV

R E S U L T S A N D D I S C U S S I O N

4.1 ISOLATION AND IDENTIFICATION OF LACTOBACILIUS ACIDOPHILUS STRAINS

Strains of L.acidophilus were isolated from faeces of bottle-fed infants and vaginal surfaces of healthy females between 16-25 years of age. The isolates were identified to species level by subjecting them to various morphological and physiological tests, the results of which are presented in Table 4.1 and Appendices II and III.

The strains of L.acidophilus LBKV₃ and LBKI₄ fermented cellobiose, fructose, galactose, glucose, lactose, maltose, melibiose, salicin, sucrose and trehalose but not arabinose, mannitol, rhamnose, ribose, sorbitol and xylose. Both the isolates were negative for gas production from sodium gluconate and glucose and were able to hydrolyse esculin. But none of them was able to produce ammonia from arginine (Table 4.2).

The morphological, physiological and biochemical characteristics of both the isolates presented in Table 4.1 and 4.2 respectively are comparable with those suggested for L.acidophilus by Buchanan and Gibbons (1974) and except in case of melibiose and trehalose where the present isolates fermented the sugars without delay unlike reported by Kandler and Norbert (1986).

TABLE 4.1: MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS
OF LACTOBACILLUS ACIDOPHILUS LBKV₃ AND LBKI₄

Characteristics	<u>Lactobacillus acidophilus</u> strains	
	LBKV ₃	LBKI ₄
1. Gram's reaction	+ve	+ve
2. Morphology (Grown in skim milk for 24 h)	Thin, short rods with rounded ends, occurring singly, in pairs and short chains	Thin, long, slender rods occurring singly, in pairs and in short chains
3. Spores	-ve	-ve
4. Catalase	-ve	-ve
5. Colony characte- ristics on MRS agar	Round, subsurface and chalky white colonies with varying size (dia. 1 to 8 mm), rough surfaces	Irregularly shaped (spindle, triangu- lar), round colonies with varying size (dia. 1 to 10 mm), rough surface, fuzzy with radiate projections
6. Growth at 15 °C	-ve	-ve
7. Growth at 45 °C	+ve	+ve
8. Growth at 50 °C	-ve	-ve

TABLE 4.2: BIOCHEMICAL CHARACTERISTICS OF LACTOBACILLUS
ACIDOPHILUS LBKV₃ AND LBKI₄

Characteristics	: <u>Lactobacillus acidophilus</u> strains		
	: 1*	: LBKV ₃	: LBKI ₄
Production of acid from			
Arabinose	-	-	-
Cellobiose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Melibiose	d	+	+
Mannitol	-	-	-
Rhamnose	-	-	-
Ribose	-	-	-
Salicin	+	+	+
Sorbitol	-	-	-
Sucrose	+	+	+
Trehalose	d	+	+
Xylose	-	-	-
Production of gas from			
Gluconate	-	-	-
Glucose	-	-	-
Production of NH ₃ from			
Arginine	-	-	-
Esculin hydrolysis	+	+	+

1* pattern of characteristics as reported by Kandler and
Norbert (1986) for L. acidophilus

Key: + Growth present within 24-48 hrs.

- No growth upto 96 hrs.

d Growth delayed upto 120 hrs.

TABLE 4.3: CHARACTERISTICS USED FOR SCREENING OF THE
ISOLATES FOR THEIR ABILITY TO WITHSTAND
GASTRO-INTESTINAL ENVIRONMENT

Characteristics	<u>Lactobacillus acidophilus strains</u>	
	LBKV ₃	LBKI ₄
1. Bile resistance	Grew in 2 per cent bile salt MRS medium and resistant to 4 per cent bile salt	Grew in 2 per cent bile salt MRS medium and resistant to 4 per cent bile salt.
2. Phenol resistance	Grew in 0.4 per cent phenol in milk and resistant to 0.5 per cent phenol in milk	Grew in 0.4 per cent phenol in milk and tolerate 0.5 per cent phenol in milk
3. Salt tolerance	Grew in 2 and 4 per cent sodium chloride containing MRS broth	Grew in 2 and 4 per cent sodium chloride containing MRS broth
4. pH tolerance	Grew in MRS broth having ^{a pH of} 5, 4 and 3 but not at 2.	Grew in MRS broth having ^{a pH of} 5, 4 and 3 but not at 2.

4.2 CRITERIA EMPLOYED IN CULTURE SELECTION

The basic consideration in selecting LBKV₃ and LBKI₄ for use in the study was their natural habitat, since both were selected from the human body flora, the former from the vaginal mucosa and the latter from the faeces of the bottle-fed infants, as has been suggested by Gilliland *et al.*, 1975; Moore and Holdeman, 1974 and Mutai *et al.*, 1971. The additional positive merits were their resistance to phenol, bile salts and low pH (Table 4.3) which enable their viable passage through the gastro-intestinal tract and allow them to establish and multiply there in the existing nutritional and ecological conditions (Wheater, 1955; Rogosa and Sharpe, 1959; Muelhens, 1967; Rasic and Kurmann, 1978; Gilliland, 1979 and Sarra and Dellaglic, 1984).

4.3 GROWTH STUDIES ON LACTOBACILLUS ACIDOPHILUS LBKV₃ AND LBKI₄

Patterns of growth of the culture LBKV₃ and LBKI₄ in terms of increase in viable counts and production of lactic acid in skim milk at 37 C are shown in Figure 4.1 and Table 4.4. The culture LBKV₃ remained in exponential phase upto 20 h at 37 C, giving 9.42×10^8 cells per millilitre and acidity value of 0.982 per cent lactic acid. However, the culture LBKI₄ remained in exponential phase upto 18 h at 37 C giving 8.4×10^8 cells per millilitre and acidity value of 0.80 per cent lactic acid. Thenafter both the cultures entered stationary phase of about 36 h. The acidity during this phase and thereafter increase progressively upto 120 h of incubation.

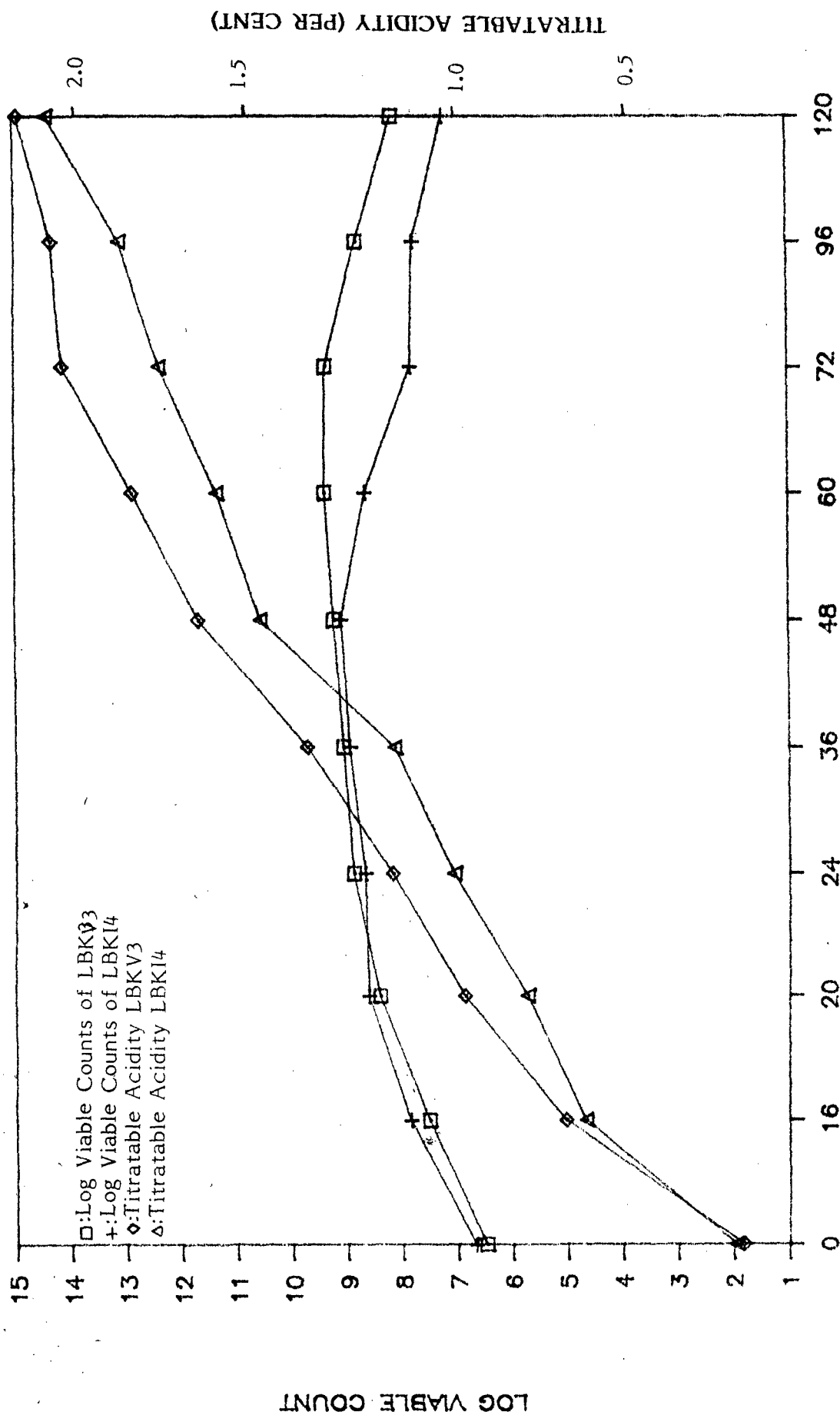


FIG.4.1: GROWTH PATTERNS OF LACTOBACILLUS ACIDOPHILUS LBKV3 AND LBKI4
IN SKIM MILK INCUBATED AT 37°C

TABLE 4.4: COMPARATIVE APPRAISAL OF GROWTH PATTERNS OF
LACTOBACILLUS ACIDOPHILUS LBKV₃ AND LBKI₄ IN
TERMS OF VIABLE CELL COUNTS AND TITRATABLE
ACIDITY IN SKIM MILK INCUBATED AT 37 C

Incubation period (hours)	Viable cell counts (log ₁₀ of counts/g)		Titratable acidity (as per cent lactic acid)	
	LBKV ₃	LBKI ₄	LBKV ₃	LBKI ₄
0	6.480	6.670	0.260	0.280
16	7.520	7.858	0.720	0.670
20	8.420	8.620	0.982	0.820
24	8.900	8.686	1.170	1.010
36	9.080	8.970	1.390	1.164
48	9.240	9.100	1.670	1.510
60	9.390	8.660	1.840	1.620
72	9.380	7.820	2.020	1.770
96	8.820	7.778	2.050	1.874
120	8.150	7.720	2.134	2.060

	Period	Treatment	Treatment x Period	Period	Treatment	Treatment x Period
SEM	0.03	0.0145	0.045	0.007	0.004	0.011
C.D at 5%	0.0844	0.0471	0.1266	0.013	0.013	0.036

Statistical analysis using split plot design with one main and one sub plot revealed that on an average the difference between the growth rates of two cultures was highly significant (Appendix IV and V). Culture LBKV₃ on an average gave significantly higher log viable count of 8.54 as compared to 7.94 for that of LBKI₄. Similarly on an average the difference between the rate of acid production of the two cultures was highly significant. Culture LBKV₃ on an average developed significantly higher acidity of 1.42 per cent as compared to 1.27 for that of LBKI₄.

However, the interaction between the culture and period was significantly different. This indicated that the viable count of the culture did not remain consistent during different time intervals.

The maximum cell count for LBKV₃ was observed at 60 h of incubation and that for LBKI₄ was observed at 48 h of incubation. This indicates that the former culture required more time to attain maximum population, but at 48 h of incubation it gave higher counts as compared to those given by LBKI₄. This indicated superiority of LBKV₃ over LBKI₄ as far as acid production and viable cell counts were concerned.

With respect to maximum acidity development, the results are comparable with those of Prajapati (1984).

With respect to maximum cell mass production strain LBKI₄ compared favourably with those of Patel (1982) and Prajapati (1984) who obtained maximum cell mass at 48 h of incubation. In LBKV₃ showed similar viable count at 60 h of incubation. Mikolajcik and Hamdan (1975^a) using L. acidophilus NCDO₃, cultured in skim milk incubated at 98.6 F, observed the end of log phase after 8 h of incubation giving about 4×10^8 cells/ml, and the viable count was slightly lower at the end of 24 h of incubation.

Continuous rise in acidity even after the population entered the stationary phase observed in the present study is in agreement with the results of Mutai et al. (1971); Rasic and Kurmann (1978); Prajapati (1984). Such rise in acidity could be attributed to the activity of the cells and their enzymes (Rasic and Kurmann, 1978; Gilliland, 1979).

One of the aims of this study was to decide the stage of fermentation at which there would be maximum number of viable cells present per unit volume of the curd so that the active cell population to be fed to the volunteers, minimum quantity would be required for feeding optimum cell numbers to the consumers for appropriate response as suggested by Hawley et al. (1959) and Speck (1978). Cultures at 36 h of growth were used for feeding.

4.4 ACCEPTABILITY OF THE ACIDOPHILUS MILKS

Acidophilus milks prepared under the present study were organoleptically evaluated to decide their acceptability.

Table 4.5 shows the preference score for acceptability of these two types of acidophilus milks, i.e. prepared by using two different L.acidophilus strains. The weighted score of 7.38 and 6.83 out of 9 for LBKV₃ and LBKI₄ acidophilus milks respectively indicated that the products were graded as 'liked moderately' to 'liked very much' by the judges.

TABLE 4.5: SCORING OF THE ACIDOPHILUS MILKS FOR ACCEPTABILITY ON NINE POINT HEDONIC SCALE

Acceptability of product	Maximum scope	LBKV ₃ Per cent consumer's response	LBKI ₄ Per cent consumer's response
Liked extremely	9	16.66	13.88
Liked very much	8	27.77	16.66
Liked moderately	7	36.11	33.33
Liked slightly	6	16.66	22.22
Neither liked nor disliked	5	2.77	2.77
Disliked slightly	4	0.0	11.11
Disliked moderately	3	0.0	0.0
Disliked very much	2	0.0	0.0
Disliked extremely	1	0.0	0.0
Weighted average		7.38	6.83
		SEM	C.D.
Cultures		0.034	0.095
Judges		0.165	-
Judges x Treatment		0.082	-

The statistical analysis was done to compare the acceptability of the two types of acidophilus milks by using Factorial CRD (Appendix VI), revealed that the average score obtained by LBKV₃ was significantly higher than that of LBKI₄. Further, the scores given by different judges were significantly different from each other. But the interaction effect of judges and two cultures was non-significant.

4.5 IMPLANTATION PROPERTIES OF LACTOBACILLUS ACIDOPHILUS LBKV₃ AND LBKI₄

Acidophilus milk containing on an average 3×10^7 cells of L. acidophilus LBKV₃ and LBKI₄ was fed at the rate of 200 g per day ^{per person} for 7 days to two groups having seven human test subjects in each. The faecal samples of the subjects were examined for lactobacilli and coliform counts before the start of feeding, during feeding and after completion of feeding.

The number of viable cells of L. acidophilus fed everyday to each individual was coming to about 3×10^9 which was about optimum suggested by Hawley et al. (1959) and Speck (1976) for successful implantation of the lactobacilli in the intestine and for maintaining the normal balance of intestinal flora.

4.5.1 Initial Faecal Flora of the Test Subjects Group A*

The microbiological analysis of the faecal samples collected from the test subjects prior to beginning of feeding

*To which L. acidophilus LBKV₃ was fed.

of acidophilus milk showed much variation in the counts of the bacterial groups examined (Table 4.6).

The log values of viable counts of faecal lactobacilli and coliforms per gram of sample ranged from 7.20 to 7.95 and 7.10 to 8.11 respectively. The average log values of the same bacteria were 7.54 and 7.37 per gram of sample respectively.

TABLE 4.6: VARIABILITY OF FAECAL FLORA FROM SEVEN TEST SUBJECTS PRIOR TO FEEDING ACIDOPHILUS MILK*

Type count	Medium	Log of counts/g	
		Range	Average
Lactobacilli	MRS agar	7.2-7.95	7.54
Coliforms	MacConkey's agar	7.1-8.11	7.37

*Acidophilus milk containing L. acidophilus LBKV₃

4.5.2 Initial Faecal Flora of the Test Subject Group B***

The microbiological analysis of the faecal samples collected from the test subjects prior to beginning of feeding of acidophilus milk showed much variation in the counts of the bacterial groups examined (Table 4.7).

The log values of viable counts of faecal lactobacilli and coliforms per gram of sample ranged from 7.27 to 8.44 and 6.88 to 7.65 respectively. The average log values of the same bacteria were 7.67 and 7.22 per gram of sample respectively.

***To which L. acidophilus LBKI₄ was fed

TABLE 4.7: VARIABILITY OF FAECAL FLORA FROM SEVEN TEST
SUBJECTS PRIOR TO FEEDING ACIDOPHILUS MILK**

Type count	Medium	Log of counts/g	
		Range	Average
Lactobacilli	MRS agar	7.27-8.44	7.67
Coliforms	MacConkey's agar	6.88-7.65	7.22

**Acidophilus milk containing L.acidophilus LBKI₄

Drasar (1974) and Darsar and Hill (1974) recorded a wide variation in the log counts of lactobacilli in the human faeces ranging from 3.6 to 9.3 per gram. Patel (1982) reported log counts of lactobacilli ranging from 6.23 to 8.74 per gram. Prajapati (1984) recorded the log counts of lactobacilli ranging from 6.99-8.09 per gram. The counts recorded in the present study were close to the counts observed by these authors. The range of coliform counts recorded by Patel (1982) and Prajapati (1984) was wider than observed in the present study which could be due to the variable nature of human flora with individuals. Further, the subjects tested by Prajapati (1984) were having some gastro-intestinal discomforts.

4.5.3 Effect of Feeding Lactobacillus acidophilus LBKV₃

The effect of feeding L.acidophilus LBKV₃ in milk on faecal lactobacilli and coliform counts in the test subjects are presented in Table 4.8 and 4.9 respectively.

TABLE 4.8: EFFECT OF FEEDING ^{MILK CULTURES OF} LACTOBACILLUS ACIDOPHILUS LBKV₃ ~~3~~ ON FAECAL LACTOBACILLI
COUNTS IN HUMAN SUBJECTS

Period	Individuals							Average
	1	2	3	4	5	6	7	
Before feeding	7.65 (4.5x10 ⁷)	7.34 (2.2x10 ⁷)	7.53 (3.4x10 ⁷)	7.95 (9x10 ⁷)	7.88 (7.6x10 ⁷)	7.20 (1.6x10 ⁷)	7.27 (1.9x10 ⁷)	7.54
During feeding	7.60 (4x10 ⁷)	7.3 (2x10 ⁷)	7.59 (3.9x10 ⁷)	8.23 (17x10 ⁷)	8.39 (24.7x10 ⁷)	7.25 (1.8x10 ⁷)	7.46 (2.9x10 ⁷)	7.68
	One week	9.27 (190x10 ⁷)	8.46 (29x10 ⁷)	9.75 (573x10 ⁷)	9.77 (588x10 ⁷)	9.01 (104x10 ⁷)	8.28 (19x10 ⁷)	9.11 (131x10 ⁷)
After stopping feeding	One week	8.52 (33.6x10 ⁷)	8.76 (58.6x10 ⁷)	9.57 (378x10 ⁷)	8.72 (25.7x10 ⁷)	9.60 (407x10 ⁷)	8.85 (71.6x10 ⁷)	8.27 (19x10 ⁷)
	Two weeks	8.31 (20.5x10 ⁷)	7.54 (3.5x10 ⁷)	8.29 (19.8x10 ⁷)	8.45 (28.7x10 ⁷)	8.80 (64.1x10 ⁷)	7.60 (4.10 ⁷)	7.77 (6x10 ⁷)
Average	8.27	7.88	8.55	8.62	8.73	7.84	7.98	
Figures in the parentheses are the mean viable counts								
			SEM	Period	Treatment	Treatment x Period		
				0.019	0.1707	0.0112		
			C.D. at 5%	-	0.224	0.318		

It could be observed from both these tables that the feeding of the acidophilus milk resulted in ~~an~~ increase in lactobacilli and decrease in coliforms in the faeces of all the subjects. However, the extent of increase or decrease as the case may be varied with the individuals.

The contents of lactobacilli in the faeces samples showed significant increase in all the test subjects during the first week of feeding (Table 4.8). The range of increase in lactobacilli count was observed to be from 12 to 173 times at the end of feeding as compared to the counts at the start of feeding. After stopping the feeding within one week, 5 out of 7 test subjects showed significant decline in lactobacilli counts, from what they had at the completion of feeding. However, 2 out of 7 test subjects showed significant increase in lactobacilli counts even one week after stopping the feeding. Further, the counts at this stage were still significantly higher than the initial population before starting of feeding. After 14 days of stopping the feeding the average counts of lactobacilli were significantly higher than the initial.

As could be observed from Table 4.9 the coliform counts in faeces decreased significantly in all the human test subjects at the end of feeding period as compared to initial counts. The range of decrease in coliform count was observed to be from 4 to 65 times at the end of second week of feeding

TABLE 4.9: EFFECT OF FEEDING ^{MILK CULTURES OF} LACTOBACILLUS ACIDOPHILUS LBKV ₃ ~~ON~~ ON FAECAL COLIFORM COUNTS IN HUMAN TEST SUBJECTS

Period	Individuals							Average
	1	2	3	4	5	6	7	
Before feeding	7.37 (23.7x10 ⁶)	8.11 (130x10 ⁶)	7.27 (18.7x10 ⁶)	7.16 (14.7x10 ⁶)	7.10 (12.4x10 ⁶)	7.32 (21x10 ⁶)	7.27 (18.9x10 ⁶)	7.37
During 48 hours feeding	7.30 (20x10 ⁶)	7.30 (20x10 ⁶)	7.12 (13.4x10 ⁶)	7.13 (13.7x10 ⁶)	6.97 (9.5x10 ⁶)	7.28 (19x10 ⁶)	7.27 (18.8x10 ⁶)	7.19
One week	6.56 (3.7x10 ⁶)	6.30 (2x10 ⁶)	6.43 (2.7x10 ⁶)	6.00 (1x10 ⁶)	5.58 (0.38x10 ⁷)	6.41 (2.6x10 ⁶)	6.68 (4.8x10 ⁶)	6.28
After One week stopping feeding	7.28 (19.3x10 ⁶)	6.17 (1.5x10 ⁶)	6.76 (5.8x10 ⁶)	6.00 (1x10 ⁶)	6.68 (4.8x10 ⁶)	6.60 (4x10 ⁶)	6.53 (3.4x10 ⁶)	6.57
Two weeks	7.14 (13.9x10 ⁶)	6.72 (5.3x10 ⁶)	7.36 (23x10 ⁶)	6.57 (3.8x10 ⁶)	7.02 (10.6x10 ⁶)	7.23 (17.1x10 ⁶)	6.53 (3.4x10 ⁶)	6.94
Average	7.13	6.92	6.99	6.57	6.67	6.97	6.85	

Figures in the parentheses are the mean viable counts

Period	Treatment	TreatmentxPeriod
SEM	0.0842	0.0947
C.D. at 5%	0.2386	-
	-	0.0119

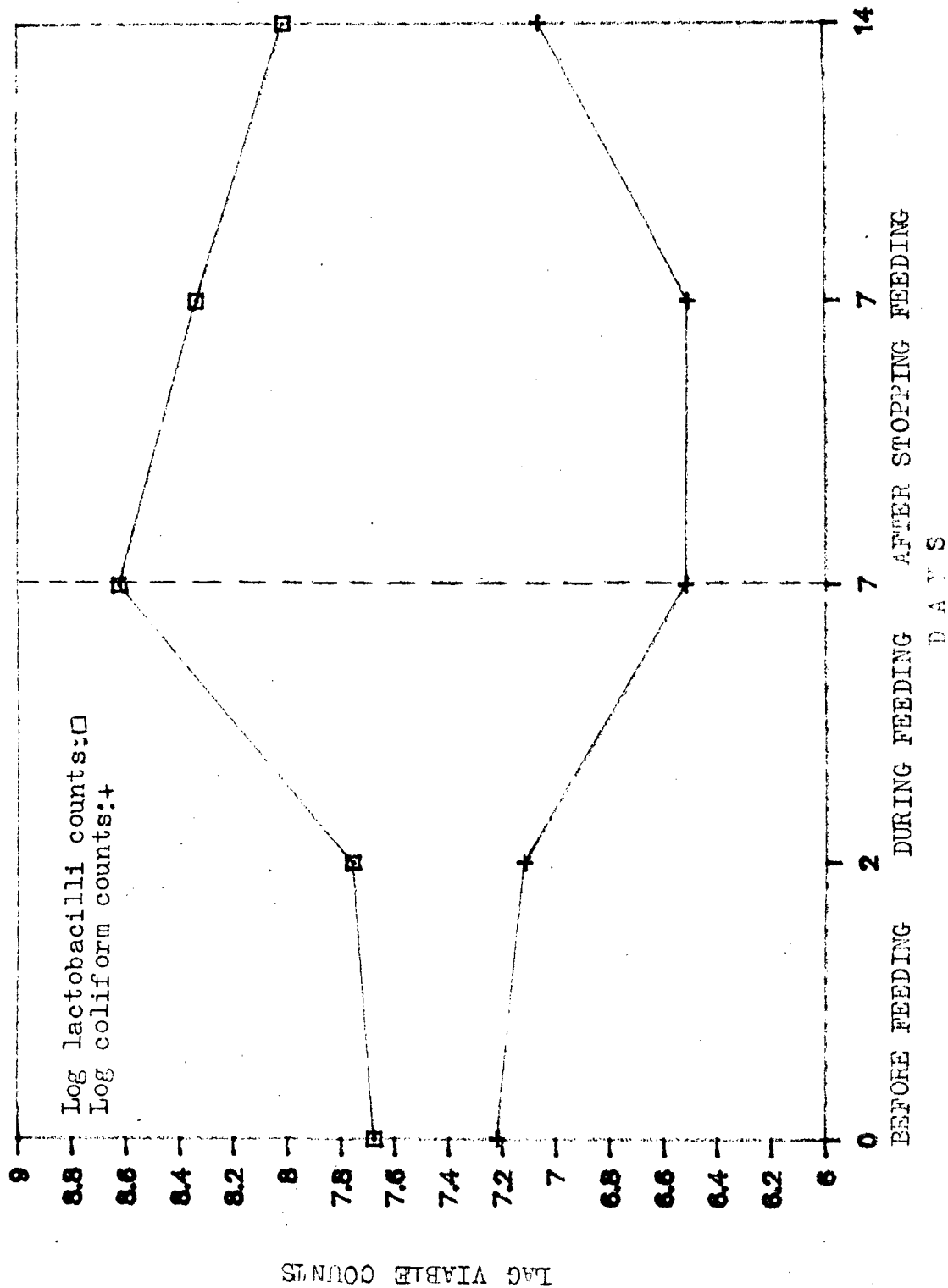


FIG 4.2 : EFFECT OF FEEDING MILK CULTURE OF LACTOBACILLUS ACIDOPHILUS LBV₃ ON FAECAL COLIFORMS AND LACTOBACILLI IN HUMAN SUBJECTS.

as compared to the counts before start of feeding. After stopping feeding the faecal coliform counts of 4 out of 7 test subjects started showing increase, while in the other two there was further decline and in one of them the coliform count was constant as compared to the count at the end of feeding.

However, the coliform counts remained significantly lower in 5 out of 7 test subjects and lower in 2 after stopping feeding as compared to initial count before commencement of feeding.

The relation between the behaviour of lactobacilli and coliform counts in the faeces of the test subjects before, during and after stopping feeding could be seen in Figure 4.2 and Table 4.10. It could be seen that as Lactobacilli increased in the faeces, the coliforms decreased, while the trend was reversed after stopping feeding. Further, the faeces examined after two weeks of stopping feeding revealed that lactobacilli remained significantly higher (8.11) than the initial count (7.54) and the coliforms remained significantly lower (6.94) than the initial count (7.37).

MILK CULTURES OF
 TABLE 4.10: EFFECT OF FEEDING LACTOBACILLUS ACIDOPHILUS
LBKV₃ ON THE LOG LACTOBACILLI AND COLIFORM
 COUNTS IN THE FAECES OF TEST SUBJECTS

Period	Lactobacilli counts	Coliform counts
Before feeding	7.54	7.37
During feeding) 2 days	7.68	7.19
During feeding) 7 days	9.09	6.28
After stopping) 7 days	8.89	6.57
feeding)14 days	8.11	6.94

4.5.4 Effect of Feeding Lactobacillus acidophilus LBKI₄

The effect of feeding L. acidophilus LBKI₄ in milk on faecal lactobacilli and coliform counts of the test subjects are presented in Table 4.11 and 4.12 respectively.

It could be observed from these tables that the feeding of the acidophilus milk resulted in ~~an~~ increase in lactobacilli and decrease in coliforms in the faeces of all the test subjects. However, the extent of increase or decrease as the case may be varied with the individuals.

The counts of lactobacilli in the faecal samples showed significant increase in all the subjects during the first week of feeding (Table 4.11). The range of increase in lactobacilli count was observed to be from 3 to 42 times at the end of feeding as compared to the counts at the start of feeding.

TABLE 4.11: EFFECT OF FEEDING LACTOBACILLUS ACIDOPHILUS LBK1₄ IN MILK ON FAECAL LACTOBACILLI COUNTS IN HUMAN TEST SUBJECTS

Period	I n d i v i d u a l							Average
	1	2	3	4	5	6	7	
Before feeding	7.36 (2.3x10 ⁷)	7.83 (6.8x10 ⁷)	7.27 (1.9x10 ⁷)	8.44 (27.6x10 ⁷)	7.55 (3.6x10 ⁷)	7.50 (3.2x10 ⁷)	7.80 (6.4x10 ⁷)	7.67
During feeding								
48 hours	7.46 (2.9x10 ⁷)	7.83 (6.8x10 ⁷)	7.49 (3.1x10 ⁷)	8.63 (43.3x10 ⁷)	7.76 (5.8x10 ⁷)	7.60 (4x10 ⁷)	7.56 (3.7x10 ⁷)	7.76
One week	8.76 (58x10 ⁷)	8.38 (24x10 ⁷)	7.77 (6x10 ⁷)	9.54 (351x10 ⁷)	9.18 (152x10 ⁷)	8.20 (15.9x10 ⁷)	8.63 (43x10 ⁷)	8.63
After stopping feeding								
One week	7.81 (6.5x10 ⁷)	8.44 (27.7x10 ⁷)	7.72 (5.3x10 ⁷)	9.04 (110x10 ⁷)	8.26 (18.4x10 ⁷)	8.74 (55.4x10 ⁷)	8.37 (23.6x10 ⁷)	8.34
Two weeks	7.47 (3x10 ⁷)	8.29 (19.6x10 ⁷)	7.63 (4.3x10 ⁷)	8.81 (64.6x10 ⁷)	8.01 (10x10 ⁷)	8.13 (13.5x10 ⁷)	7.78 (6.1x10 ⁷)	8.01
Average	7.77	8.15	7.57	8.89	8.15	8.03	8.03	

Figures in the parentheses are the mean viable counts

SEM	0.079	Treatment	0.1707	TreatmentxPeriod	0.0162
C.D at 5%	-		0.224		0.318

TABLE 4.12: EFFECT OF FEEDING LACTOBACILLUS ACIDOPHILUS LBK1₄ IN MILK ON FAECAL COLIFORM COUNTS
IN THE HUMAN TEST SUBJECTS

Period	I n d i v i d u a l s							Average
	1	2	3	4	5	6	7	
Before feeding	7.42 (26.9x10 ⁶)	6.88 (7.6x10 ⁶)	7.65 (44.9x10 ⁶)	6.94 (8.9x10 ⁶)	7.32 (21x10 ⁶)	7.38 (24x10 ⁶)	7.00 (10x10 ⁶)	7.22
During feeding	7.32 (21x10 ⁶)	6.62 (4.2x10 ⁶)	7.47 (29.9x10 ⁶)	6.93 (8.6x10 ⁶)	7.31 (20.6x10 ⁶)	7.36 (23x10 ⁶)	6.86 (7.4x10 ⁶)	7.12
One week	6.77 (5.9x10 ⁶)	6.00 (1x10 ⁶)	6.65 (4.5x10 ⁶)	5.99 (0.98x10 ⁶)	6.81 (6.5x10 ⁶)	7.00 (10.3x10 ⁶)	6.46 (2.9x10 ⁶)	6.52
After stopping feeding	6.57 (3.8x10 ⁶)	6.44 (2.8x10 ⁶)	6.69 (4.9x10 ⁶)	5.67 (0.47x10 ⁶)	5.79 (0.63x10 ⁶)	7.27 (19x10 ⁶)	7.16 (14.5x10 ⁶)	6.51
Two weeks	7.00 (10x10 ⁶)	7.50 (32.2x10 ⁶)	7.26 (17.8x10 ⁶)	6.30 (2x10 ⁶)	6.94 (8.8x10 ⁶)	7.25 (17.9x10 ⁶)	7.20 (15.9x10 ⁶)	7.06
Average	7.02	6.69	7.14	6.37	6.83	7.25	6.94	

Figures in the parentheses are the mean viable counts

SEM	0.0842	Treatment	0.0947	TreatmentxPeriod	0.0119
C.D. at 5%	0.2386	-	-	-	-

After stopping the feeding within one week 4 out of 7 test subjects showed significant decline in lactobacilli counts, from what they had at the completion of feeding. However, 1 out of 7 test subjects showed significant increase in lactobacilli counts even one week after stopping feeding. Two subjects out of 7 showed slight decline in lactobacilli count. After 14 days of stopping the feeding the average count of lactobacilli was significantly higher than the initial count, in 5 out of 7 subjects and it was higher in the rest two subjects.

As could be observed from Table 4.12 the coliform counts in faeces decreased significantly in all the human test subjects at the end of feeding period as compared to initial counts. The range of decrease in coliform counts was observed to be 2 to 10 times at the end of first week of feeding as compared to the counts before the start of feeding. After stopping feeding, the faecal coliform counts of 4 out of 7 test subjects showed increase and in three there was continuous decline in coliform counts as compared to the count at the end of feeding. However, the counts remained significantly lower in 4 and lower in 1 out of 7 test subjects, but in rest two of them the faecal coliform count was observed to be higher after two weeks of stopping the feeding as compared to initial population before the commencement of feeding.

The relation between the behaviour of lactobacilli and coliform counts in the faeces of the test subjects before,

during and after stopping feeding could be seen in Fig.4.3 and Table 4.13. It could be seen that as lactobacilli increased in the faeces, the coliforms decreased, while the trend was reversed after stopping feeding. Further, the faeces examined after two weeks of stopping feeding revealed that lactobacilli remained significantly higher (8.01) than the initial count (7.67) and coliforms remained significantly lower (7.06) than the initial count (7.22).

TABLE 4.13: EFFECT OF FEEDING ^{MILK CULTURE OF} LACTOBACILLUS ACIDOPHILUS LBK1₄ IN MILK ON THE LOG LACTOBACILLI AND COLIFORM COUNTS IN THE FAECES OF TEST SUBJECTS

Period		Lactobacilli counts	Coliforms counts
Before feeding		7.67	7.22
During feeding	2 days	7.76	7.12
	7 days	8.63	6.52
After stopping feeding	7 days	8.34	6.51
	14 days	8.01	7.06

The observed numerical relationship of the lactobacilli and the coliforms enumerated in the faeces samples agree with Gilliland et al. (1978,1979); Ayebo et al. (1980); Gandhi and Nambudripad (1978); Patel (1982) and Prajapati (1984). However, Sarra and Dellaglio (1984) observed an increase in not only

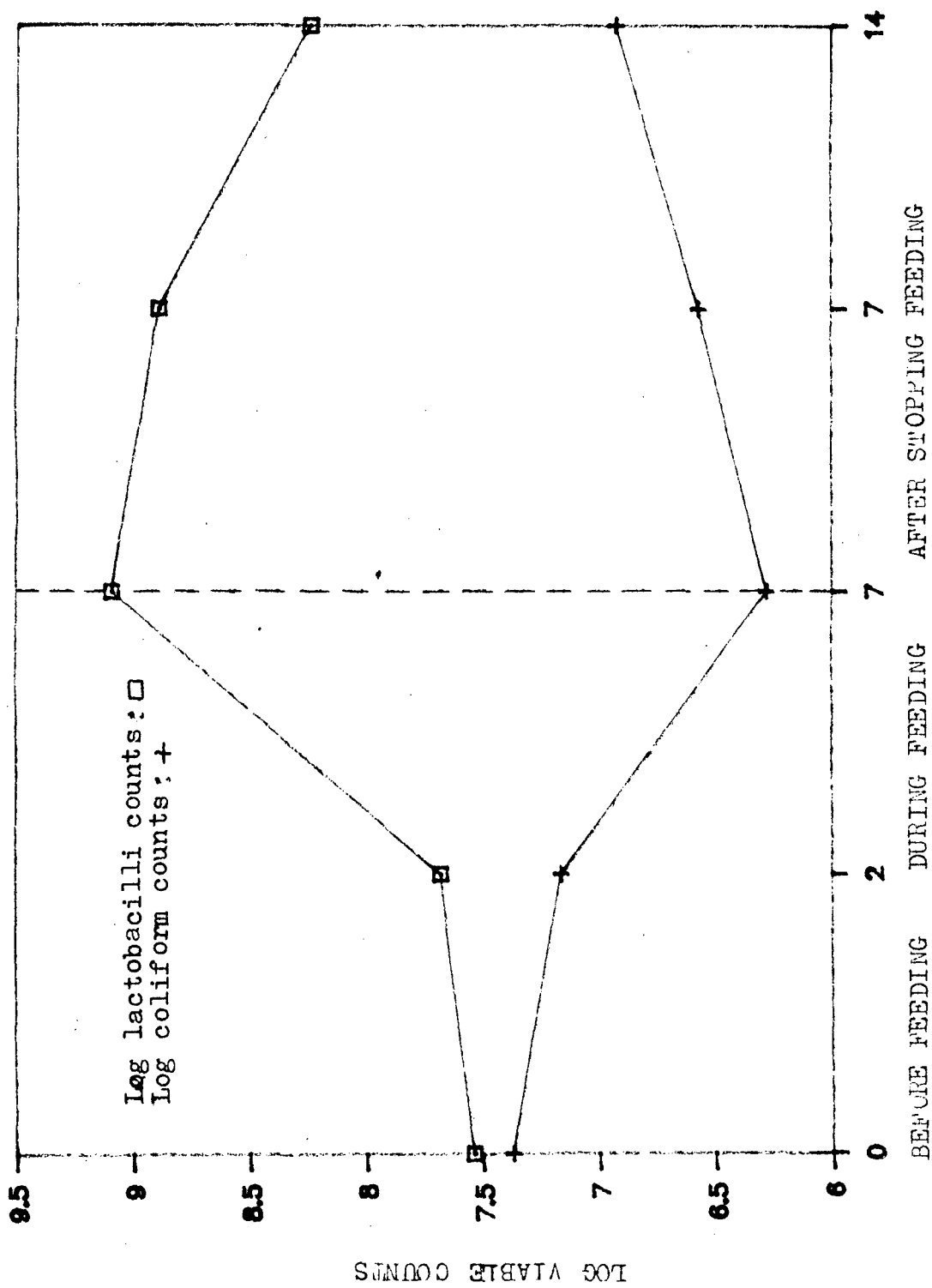


FIG 4.3 : EFFECT OF FEEDING MILK CULTURE OF LACTOBACILLUS ACIDOPHILUS LBK1₄ ON FAECAL COLIFORMS AND LACTOBACILLI IN HUMAN SUBJECTS.

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(7)

lactobacilli but also the coliform counts. Similar results were recorded for coliform counts in one test subject from group A and two from group B in the present study. These authors deduced that the lactobacillus cells supplied to the subjects were responsible for stimulation of coliforms and thus increase in the number of same after the end of feeding trial. Variability in the effect of feeding with the individuals observed in this study was similar to that reported by Gilliland et al. (1978), ~~and~~ (1977) and Prajapati (1984).

Mutai et al. (1971) justified the positive implantation of his strain of lactobacillus on the ground that the intestinal population of lactobacilli in the test subjects took 3 weeks to come to initial population. In the present study, two weeks after stopping feeding the lactobacilli count of both the subject groups remained at significantly higher levels than the initial counts.

From an oral enquiry of the test subjects it was found that they felt relief in their gastro-intestinal discomforts which they were experiencing before the start of the feeding.

4.5.5 Comparative Appraisal of Implantation Ability of

L. acidophilus LBKV₃ and LBKI₄

Statistical analysis applying Split-Plot design (Appendices VII and VIII) revealed that the difference in implantation ability of the two cultures was non-significant. But effect of

feeding two cultures on faecal lactobacilli counts and coliform counts of the two test subject groups at different periods was significantly different. Further, interaction between the periods and the cultures was significantly different for lactobacilli count but same was non-significant for coliform counts of the two test subject groups. From the view point of the implantation ability of the cultures LBKV₃ was observed to show superiority over LBKI₄.

4.6 ANTIBACTERIAL ACTIVITY OF LACTOBACILLUS ACIDOPHILUS

LBKV₃ AND LBKI₄

The observations on in vitro inhibitory effect of CFC-filtrates of the strains LBKV₃ and LBKI₄ grown in skim milk at 37 C against various food poisoning and spoilage types of organisms are presented in Table 4.14 and 4.15 respectively. It was seen that the antibacterial substances produced by both of the L.acidophilus strains were found to be active against all the five test organisms, namely B.cereus, Staph.aureus, Ps.aeruginosa, Salm.typhosa and E.coli. Antibacterial activity of 60 h old CFC-filtrates of L.acidophilus strains was most inhibitory towards B.cereus followed by E.coli, Staph.aureus and Salm.typhosa; Ps.aeruginosa was the most resistant among the test organisms (Plate I and II).

It is evident from Fig.4.4 and 4.5 that CFC-filtrates of the L.acidophilus strains exhibited inhibition of the test organisms from 12 h onwards. Further, it could be seen that



PLATE-I

INHIBITION OF SELECTED PATHOGENIC AND FOOD SPOILAGE
ORGANISMS BY LACTOBACILLUS ACIDOPHILUS STRAINS

A: Bacillus cereus

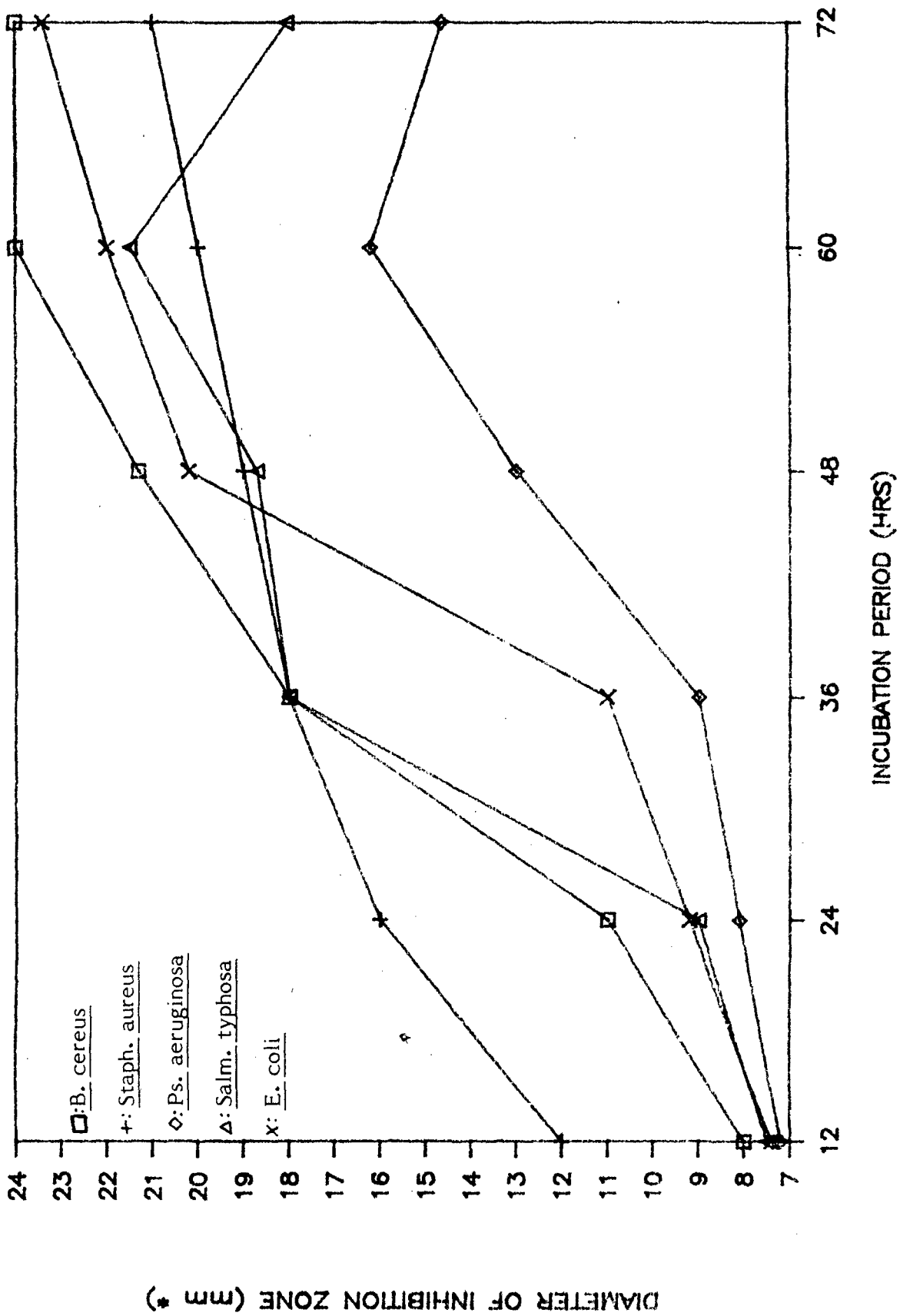
B: Staphylococcus aureus

C: Pseudomonas aeruginosa

1: CFC-Filtrate of LBKV3

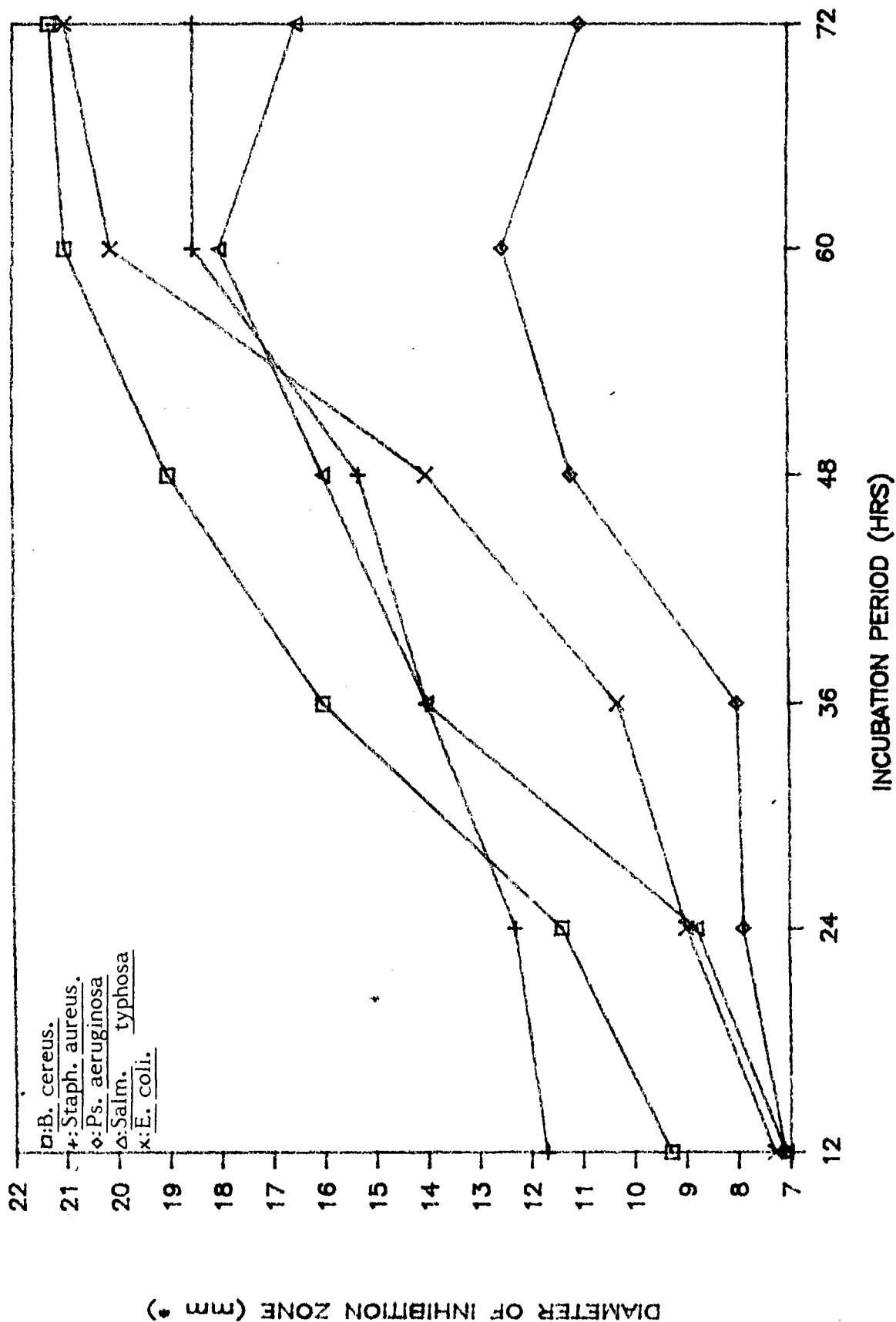
2: CFC-Filtrate of LBKI4

3: Control



* Including 7 mm bore diameter

FIG.4.4: ANTIBACTERIAL ACTIVITY OF LACTOBACILLUS LBKV3 AGAINST SELECTED FOOD SPOILAGE AND PATHOGENIC ORGANISMS



* Including 7 mm bore diameter ^{SCC FILTRATES OF}
FIG.4.5: ANTIBACTERIAL ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS LBK14* AGAINST SELECTED
FOOD SPOILAGE AND PATHOGENIC ORGANISMS



PLATE-II

INHIBITION OF PATHOGENIC ORGANISMS BY
LACTOBACILLUS ACIDOPHILUS STRAINS

D: Salmonella typhosa

E: Escherichia coli (Mastitic)

1: CFC-Filtrate of LBKV3

2: CFC-Filtrate of LBKI4

3: Control

maximum inhibitory effect of both the Lacidophilus strains against B.cereus, Staph.aureus and E.coli was observed at 72 h however, that for Ps.aeruginosa and Salm.typhosa it was maximum at 60 h and there was slight decline in inhibitory activity after 72 h.

The decreased activity in antibacterial activity observed with both strains of L.acidophilus could be attributed to lowering of pH and reduction in concentration of some of the antibacterial components. Rao and Gandhi (1988) had similar observations with their cultures.

Further, it may be noted that Ps.aeruginosa was not inhibited upto 36 h of CFC filtrates from both L.acidophilus strains.

TABLE 4.14: ANTIBACTERIAL ACTIVITY OF LACTOBACILLUS ACIDOPHILUS

LBKV₃

Test organisms	:Cell free filtrates of <u>L.acidophilus</u> LBKV ₃					
	: Period of incubation (h)					
	: 12	: 24	: 36	: 48	: 60	: 72
	: Inhibition zone diameter (mm*)					
<u>Bacillus cereus</u>	8.0	11.0	18.0	21.3	24.0	24.0
<u>Staphylococcus aureus</u>	12.0	16.0	18.2	19.2	20.0	21.0
<u>Pseudomonas aeruginosa</u>	7.2	8.1	9.0	13.0	16.0	14.6
<u>Salmonella typhosa</u>	7.5	9.0	18.0	18.7	21.5	18.0
<u>Escherichia coli</u>	7.4	9.2	11.0	20.2	22.0	23.4

*Including 7 mm bore diameter

TABLE 4.15: ANTIBACTERIAL ACTIVITY OF LACTOBACILLUS
ACIDOPHILUS LBKI₄

Test organisms	Cell-free filtrates of L.acidophilus LBKI ₄					
	Period of incubation (h)					
	12	24	36	48	60	72
	Inhibition zone diameter (mm*)					
<u>Bacillus cereus</u>	9.3	11.4	16.0	19.0	21.0	21.3
<u>Staphylococcus aureus</u>	11.7	12.3	14.0	15.3	18.5	18.5
<u>Pseudomonas aeruginosa</u>	7.1	7.9	8.0	11.2	12.5	11.0
<u>Salmonella typhosa</u>	7.1	8.8	14.0	16.0	18.0	16.5
<u>Escherichia coli</u>	7.3	9.0	10.3	14.0	20.1	21.0

*Including 7 mm bore diameter

TABLE 4.16: COMPARATIVE APPRAISAL OF ANTIBACTERIAL ACTIVITIES
OF L.ACIDOPHILUS LBKV₃ AND LBKI₄ 60 h CELL-FREE
FILTRATES

Sr. No.	Test organisms	Inhibition zone diameter (mm*)		
		LBKV ₃	LBKI ₄	Control
1	<u>Bacillus cereus</u>	24.0	21.0	15.0
2	<u>Staphylococcus aureus</u>	20.0	18.5	8.5
3	<u>Pseudomonas aeruginosa</u>	16.0	12.5	14.5
4	<u>Salmonella typhosa</u>	21.5	18.0	16.0
5	<u>Escherichia coli</u>	22.0	20.1	8.0

*Including 7 mm bore diameter

From Table 4.16 it could be seen that the CFC-filtrates of both the cultures at 60 h showed higher inhibitory activity as compared to control. Further, neutralization of CFC-filtrates partially reduced the inhibitory activity but antibacterial activity still persisted in the CFC-filtrates. This clearly suggested that a factor other than acidity was responsible for inhibitory activity. This observed behaviour is supported by the reports from several workers, who attributed the antimicrobial effect of L.acidophilus to the combined effect of lactic acid and antibiotic substances (Singh and Laxminarayana, 1973; Mikolajcik and Hamdan, 1975^{a,b}) or to the combination of factors including acids, hydrogen peroxide and other inhibitory substances Gilliland and Speck, 1977^a; Hylmar and Pokorna, 1986; Prasad and Gandhi, 1987). As far as the susceptibility of different test organisms to the inhibitory effect of L.acidophilus strains was concerned our results are in agreement with those reported by Danielson et al. (1987) who had shown more susceptibility of Bacillus as compared to Escherichia.

Unlike ours Rao and Gandhi (1988) and Gandhi and Nambudripad (1980) reported greatest inhibitory activity of L.acidophilus R strain against E.coli followed by Bacillus spp. and Staph.aureus.

Like ours, Mikolajcik and Hamdan (1975^a) did not observe inhibition of Pseudomonas and E.coli by L.acidophilus NCDO₃, upto 12 h of incubation.

It was also noted that points of increase in inhibitory activity coincided with the onset of stationary phase of the cultures. From this it could be assumed that above said stage of growth might be associated with either production or release of the inhibitory components. Presence of high numbers of L.acidophilus cells in the product exhibited maximum antibacterial activity.

CFC-filtrate of L.acidophilus LBKV₃ was highly inhibitory against all the five test organisms as compared to that of L.acidophilus LBKI₄.

4.7 INHIBITION OF PATHOGENIC MICRO-ORGANISMS DURING PRODUCTION AND STORAGE OF ACIDOPHILIC MILK

The observations on in vitro inhibitory effect of L.acidophilus strains, mixed lactic and yoghurt cultures against enterotoxigenic Staph.aureus during production and storage of acidophilus milks, curd and yoghurt respectively are shown in Fig.4.6.

4.7.1 Inhibition of Enterotoxigenic Staphylococcus aureus

It could be seen from Fig.4.6 and appendices IX and X that there was steady increase in Staphylococcal counts upto 16 h of incubation at 37 C in acidophilus milk containing LBKV₃ but after 20 h of incubation count started decreasing and after 60 h it became less than what that was initially present. In case of acidophilus milk containing L.acidophilus LBKI₄, there

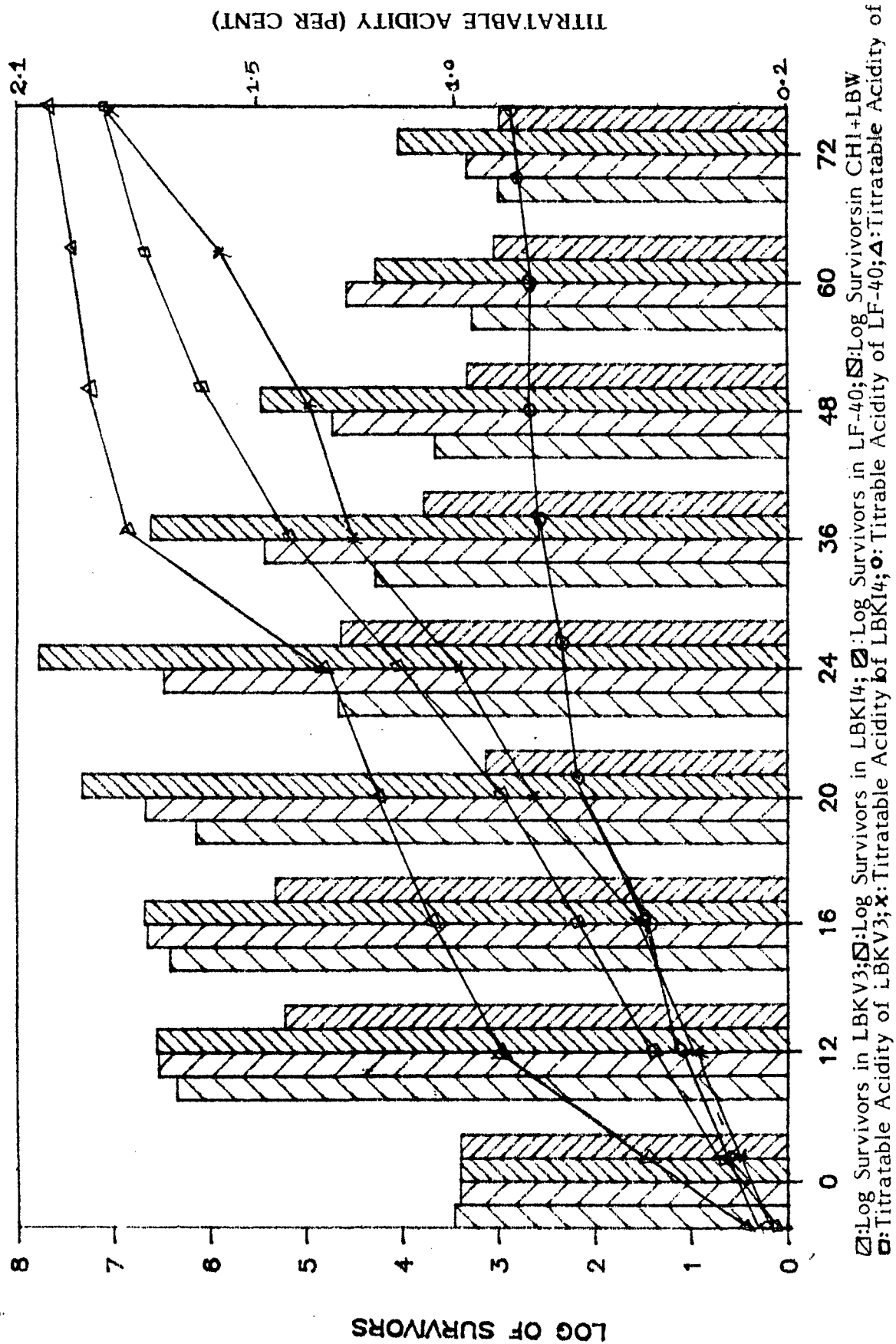


FIG.4.6 INHIBITION OF STAPH. AUREUS DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK STORED AT 37 C

was steady increase in staphylococcal counts upto 20 h of incubation but from 24 h onward there was rapid decline and after 72 h of incubation at 37 C the counts were lower than those present initially.

In case of acidophilus milk containing L.acidophilus LBKI₄, there was steady increase in staphylococcal counts upto 20 h of incubation but from 24 h onward there was rapid decline and after 72 h of incubation at 37 C the counts were lower than those present initially.

In skim milk inoculated with mixed strain lactic culture, LF-40, there was steady increase in staphylococcal counts upto 24 h but after 36 h the counts progressively decreased upto end of incubation at 37 C remaining higher than those initially inoculated.

In case of skim milk containing mixed yoghurt culture (CH₁+LBW) increase in staphylococcal counts was observed upto 16 h but after 20 h there was rapid decline which resulted into reduction in staphylococcal counts to lower than initial levels within 48 h of incubation at 37 C. Fig. 4.6 and Appendix X revealed that there was steady increase in acidity in all the cultures used.

It could be observed from Fig. 4.7 and appendix XI and XII that the acidophilus milk containing LBKV₃ had shown reduction in staphylococcal counts after 8 h of storage at 15 C but the counts steadily increased upto 24 h of incubation at

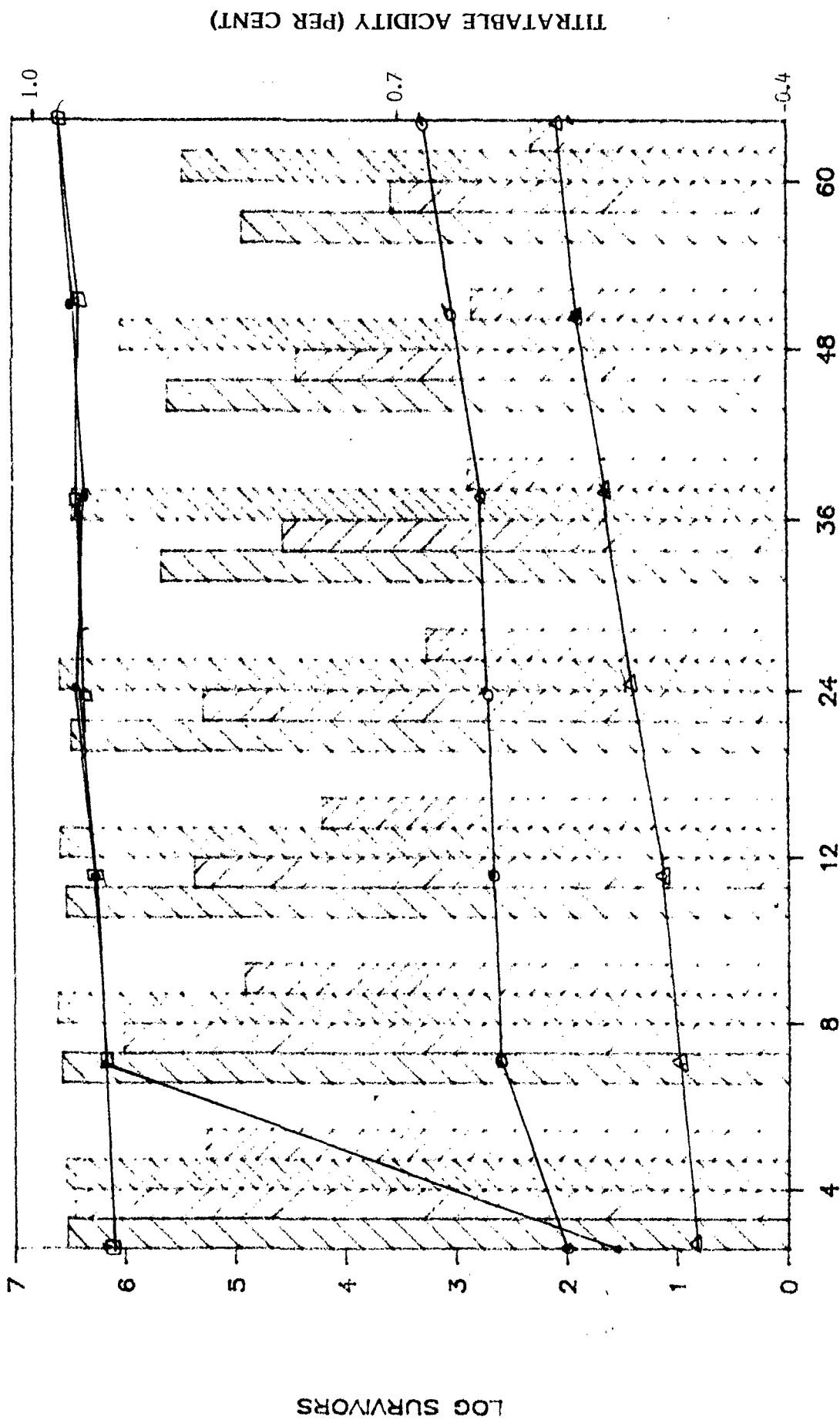


FIG. 4.7: INHIBITION OF STAPH. AUREUS DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK (AND OTHER FERMENTED MILKS)

15 C in acidophilus milk containing LBKI₄. In case of milk inoculated with mixed strain lactic culture, LP-40, the staphylococcal counts steadily increased upto 36 h while in milk inoculated with yoghurt culture the count started decreasing after a storage period of 8 h at 15 C. Fig. 4.7 and appendix XII, reveal that there was very slow increase in acidity values of all the test cultures upto 60 h at 15 C.

Inhibitory effect was observed after 4 h and 48 h of storage at 15 C by milk inoculated with yoghurt culture and mixed lactic culture respectively.

Further, it could be seen that the maximum inhibitory activity was observed ^{between} ~~after~~ 48 ~~to~~ 60 h of incubation in both the acidophilus milks. These results are parallel to those obtained for these cultures in in vitro inhibitory studies using cup-well assay technique.

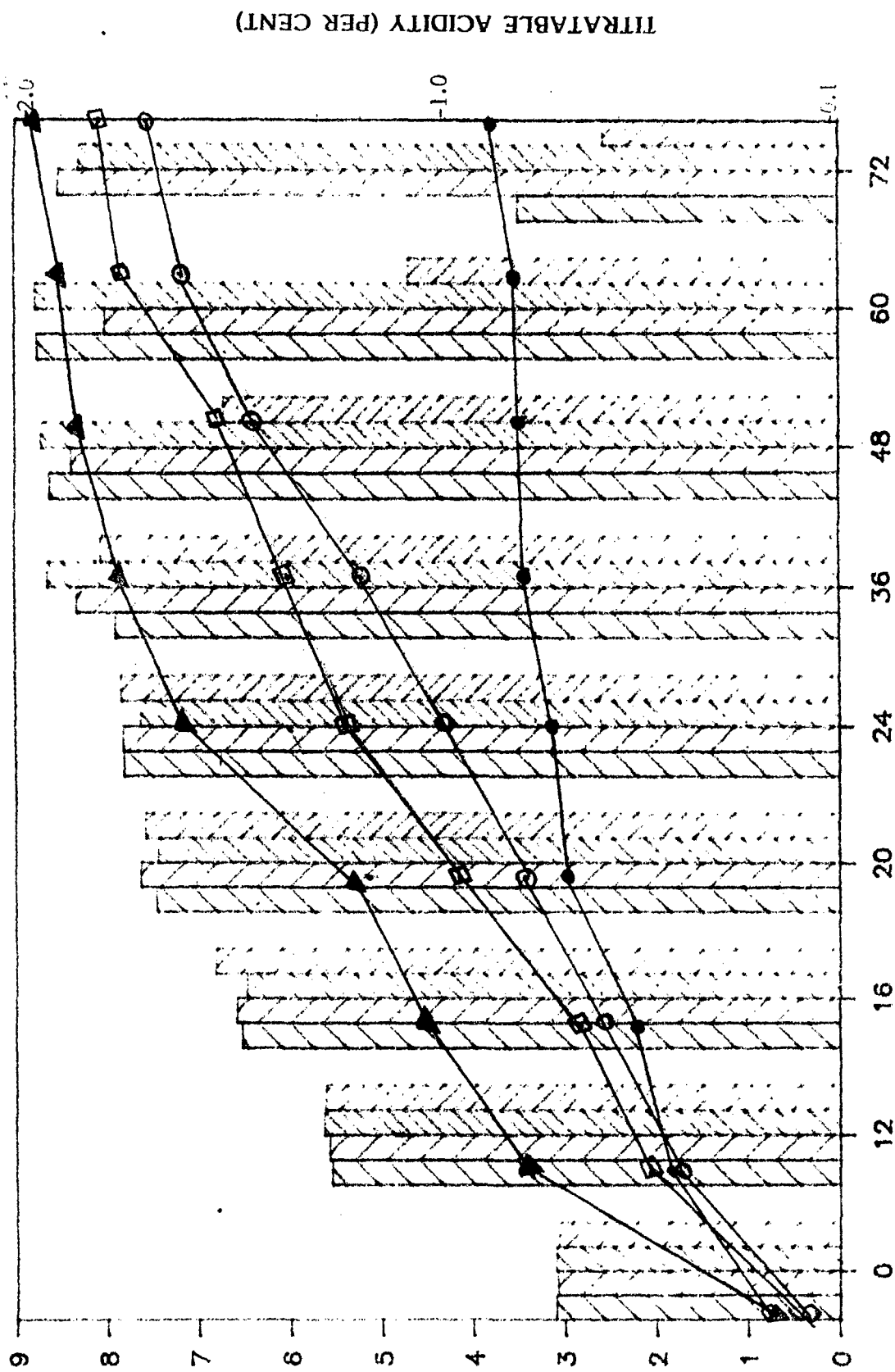
From Fig. 4.6 it could be seen that the inhibitory effect was not completely due to increase in acidity in case of acidophilus milk. Konecny (1986) made similar observations. For milk inoculated with yoghurt culture and mixed lactic culture there was better relationship between the rise in acidity and the decrease in the count of Staph. aureus.

In case of skim milks inoculated with L. acidophilus strains, LP-40 culture and yoghurt culture stored at 15 C after 12 h incubation at 37 C, the inhibitory activity of

acidophilus milk containing L.acidophilus IBKV₃ strain was observed from 3 h onwards and that for IBKI₄ after 24 h.

Statistical analysis using split-plot design with one main and one sub-plot (Appendices IX to XII) revealed that the differences between antibacterial activities of four cultures were non-significant at 37 C. However, the inhibitory activities of four cultures were significantly different at 15 C. At both the temperatures viz., 37 C and 15 C IBKV₃ had higher antibacterial activity against Staph.aureus. At both the storage temperatures the rate of acid production was significantly different.

The published literature has not shown any report of identical studies to make any comparison possible with the present data. However, the results obtained by Attaie et al. (1987) for acidophilus-yoghurt are comparable with those of present study for yoghurt culture. But highest inhibitory activity of L.acidophilus observed by Konecny (1986) among various lactic cultures tested is in agreement with the results of present study. Results obtained on effect of different storage temperatures on inhibitory activity are similar to those by Konecny (1986), who proposed that the higher storage temperature had a greater inhibitory effect than lower one.



INCUBATION PERIOD (HRS) AT 37 C

□:Log Survivors in LBKV3; ○:Log Survivors in LBKI4; ●:Log Survivors in LF-40; ▲:Log Survivors in CHI+LBW

□:Titratable Acidity of LBKV3; ○:Titratable Acidity of LBKI4; ●:Titratable Acidity of LF-40; ▲:Titratable Acidity of CHI+LBW

FIG.4.8: INHIBITION OF E.COLI DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK (STORED AT 37 C)

4.7.2 Inhibition of Mastitic Escherichia coli

The observations on in vitro inhibitory effect of L. acidophilus strains, mixed lactic and yoghurt cultures against mastitic E. coli during production and storage of acidophilus milks, curd and yoghurt respectively are shown in Fig. 4.8 and 4.9. It could be seen from Fig. 4.8 and Appendices XIII and XIV that there was steady increase in coliform counts upto ~~60~~ 60 h of incubation at 37 C in acidophilus milk containing L. acidophilus LBKV₃ but after that there was decline in counts upto 72 h till they reached the numbers inoculated initially.

In case of acidophilus milk containing L. acidophilus LBKI₄ counts steadily increased upto 72 h and the same was true for milk inoculated with IF-40. In case of milk inoculated with yoghurt culture (CH₁+LBW) coliform counts increased upto 36 h and there was sharp decline in counts.

It could be observed that inhibitory activity of L. acidophilus strains was not completely due to increase in acidity. Konecny (1986) had similar results using Acidophilus-Yoghurt cultures. In case of milk inoculated with yoghurt culture the inhibitory activity increased linearly with acidity.

It could be observed from Fig. 4.9 and Appendices XV and XVI, that the acidophilus milk containing L. acidophilus LBKV₃ showed inhibition of growth of E. coli after 60 h of storage at 15 C. None of the remaining cultures was able to show any inhibitory activity against E. coli upto 60 h of storage
yoghurt culture has shown inhibition of E. coli after 36h.

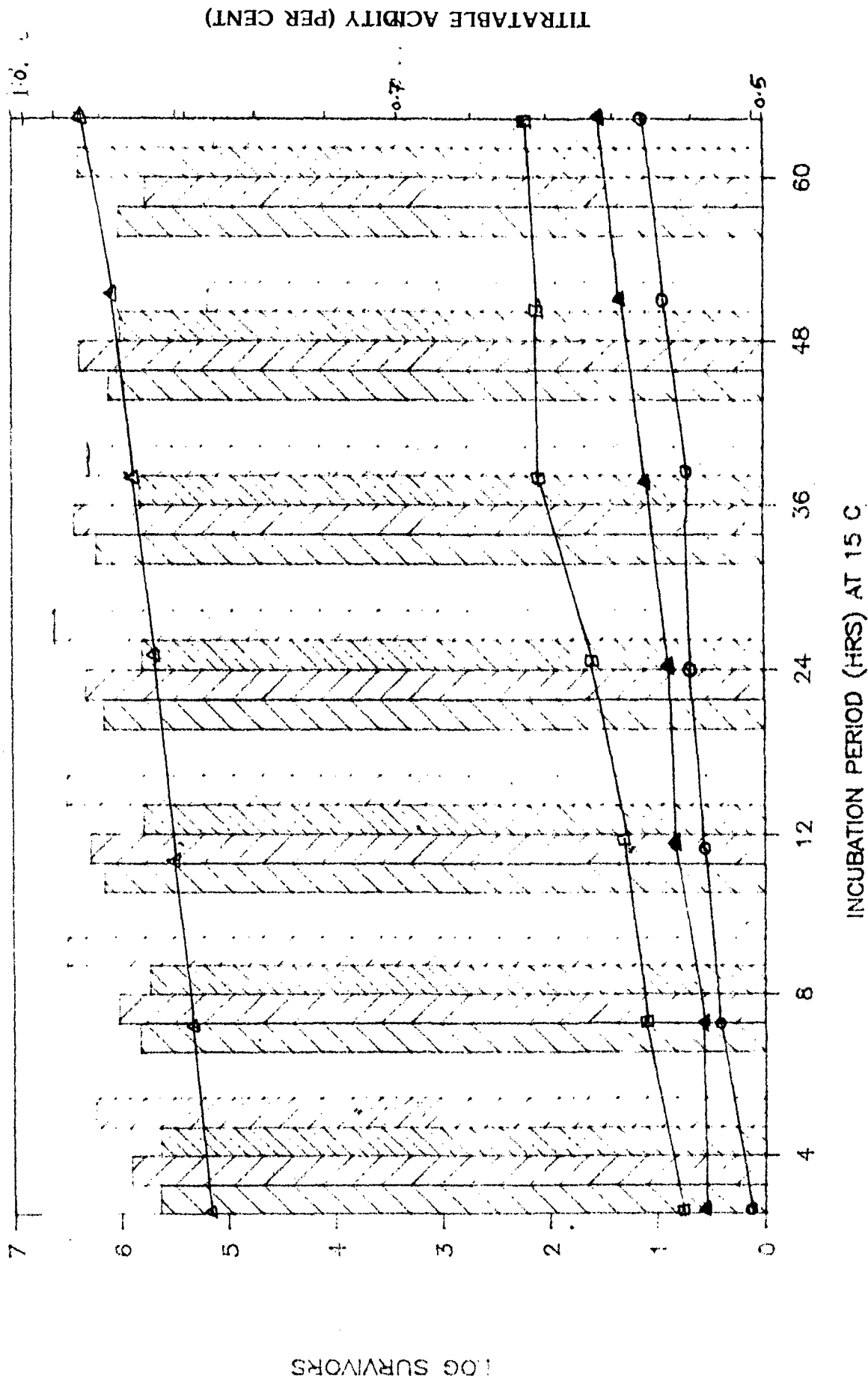


FIG 4.9: INHIBITION OF E. COLI DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK (STORED AT 15 C)

at 15 C. Results of inhibition of E.coli by the four cultures are in agreement with those obtained by Goel et al. (1971) for yoghurt cultures. Rapid inhibition at higher temperature as compared to lower one is supported by reports of Konecny (1986).

Statistical analysis using Split-Plot design with one main and one sub-plot (Appendices XIII to XVI) revealed that the differences in inhibitory activity of four cultures were highly significant and same was true for the rate of acid production. Further, the inhibitory activity of L.acidophilus IBKV₃ against E.coli was significantly higher than that of IBKI₄.

4.8 ACIDOPHILUS MILK IN TREATMENT OF HUMAN DISEASES

Acidophilus milk was prepared from skim milk by following the method given by Nahaisi (1986) with slight modifications.

4.8.1 For Patients Suffering From Constipation

Three patients, 30-35 years of age, suffering from chronic constipation and one of recent onset were earlier treated by the physician using chemotherapeutic agents like Erythromycin, Metronidizol and supplemented with B-complex (as shown in Table 4.17) but there was no positive response in any of the cases. Under these circumstances Acidophilus therapy was started by feeding 200 g of acidophilus milk per day containing 2×10^7 viable cells per gram for nine days. Response was recorded after 3, 6 and 9 days during feeding period.

It could be observed from the contents of table that there was improvement in the bowel motion within 3 days of feeding the acidophilus milk in case of patient having recent history of constipation and he was clinically cured within 6 days of feeding. The patients having chronic constipation showed slight improvement after therapy for 6 days followed by complete recovery after 9 days of treatment.

The results are in agreement with those reported by Graf (1983) who suggested acidophilus milk for increasing the efficiency of bowel transport. Similarly Alm *et al.* (1983) recommended acidophilus milk in treatment of hospitalised patients suffering from constipation. Sterilized acidophilus milk was not successful in increasing the bowel treatment of constipated patients (Rettger and Cheplin, 1983), and this indicated the need for viable L.acidophilus in such treatments. Graf (1983) offered possible mode of action of L.acidophilus which could^{have} affected the microbiological balance/interactions at the surface of the bowel mucosa. Such an effect caused an 'excitation' of mucosa which spread to the muscle layers.

4.3.2 For Patients Suffering From Ulcerations

Five patients between 27 to 38 years suffering from various ulcerative complaints like ulcerative colitis, inflammation of piles, buccal ulceration, ulcerative stomatitis and opthous ulcers were treated with chemotherapeutic agents such as Salazopyrin, Tinidazol and Metronidizol upto five days

without notable response. They were taken to 200 g of acidophilus milk (per day) containing 2×10^7 viable cells per gram for a period of nine days. Response was recorded after 3, 6 and 9 days during feeding as shown in Table 4.17.

It could be observed from the contents of the table that out of five patients one showed improvement in the oral ulcers after 3 days of feeding acidophilus milk, while two showed improvement after 6 days. All the patients were clinically cured from oral ulcers, rectal ulcers and inflammation of piles after 9 days of feeding.

Tomio-Karovic (1974) reported use of acidophilus milk for the treatment of various diseases including wounds and ulcerations.

Gordon et al. (1957); Siver (1961); Abbott (1961) and Gillespie and Dimmick (1951) reported the use of acidophilus milks in treatments of skin infection and stomatitis.

Such ulcerative complaints arise due to the deficiency of B-group of vitamins and hence it was assumed that the acidophilus organisms synthesized some of them or resulted in correction of intestinal flora which in turn caused the synthesis of deficient vitamins as reported by Nordbring (1983).

TABLE 4.17 CASE HISTORY OF PATIENTS SUFFERING FROM VARIOUS GASTRO-INTESTINAL AND RELATED DISCOMFORTS AND THEIR RESPONSE TO ACIDOPHILUS THERAPY

Sr. No.	Gender	Age	Complaints	Disease	Chemotherapy	Response of feeding Acidophilus Milk *			Clinical Report
						After 3 days	After 6 days	After 9 days	
A	B	C	D	E	F	G	H	I	J
1.	M	32	Abdominal discomfort, constipation, changes in bowel motion.	Chronic ulcerative colitis	Salazopyrin 300 mg for 3 days without response, from 4th day onwards Salazopyrin & acidophilus milk was given	Relief from abdominal discomfort	Improvement in constipation	Totally relieved from the ulcers and constipation	Salazopyrin followed by acidophilus milk clinically cured the patient from ulcerative colitis
2.	M	38	Oral ulcers Bleeding from rectum with mucus in stools	Inflammation of piles	Metronidazol 400 mg for 5 to 7 days resulted into no improvement and acidophilus milk was recommended	Mucous disappeared after 48 hours of feeding and improvement in oral ulcers	Improvement of rectal bleeding, oral ulcers got healed completely	Recovery from all ailments	Cured from the piles inflammation and stated discomforts
3.	M	27	Oral ulcers, Diarrhoea, burning pain at anal region.	Buccal Ulceration Gingival swelling and proctitis	Metronidazol 200 mg with B-complex and Erythromycin 100 mg upto 5 days without response & acidophilus milk was recommended	Diarrhoea cured within 48 hours of feeding acidophilus milk	Burning pains also disappeared after 5 days of acidophilus milk feeding	Recovered from oral and anal ulcers	Chemotherapy followed by acidophilus therapy resulted into immediate recovery of patient.
4.	M	26	Severe Diarrhoea & gas problems	Diarrhoea	No chemotherapy was given	Diarrhoea cured within 2 days of feeding acidophilus milk **	Gas problem disappeared	-	Recovered from diarrhoea within 48 hours
5.	M	32	Ulcerative Stomatitis	Ulcerative stomatitis <u>Entamoeba histolytica</u> Cysts were observed in stools	Tinidazol 600 mg twice/day along with B-complex No response upto 3 days Acidophilus milk was suggested	No response except slight improvement in loose motions	Loose motions reduced & ulcers got healed. Relieved from stomatitis	No complaint of gastro-intestinal disorders	Patient was clinically cured from ulcerative stomatitis.
6.	M	32	Chronic Constipation & gas problem	Chronic constipation & ophus ulcers	Metronidazol 400 mg for 3 days with B-complex without response. Acidophilus milk was given.	Improvement in gas problem within 48 hours of acidophilus milk feeding	Improvement in constipation & recovery from gas problem	Recovered from both the complaints	Ophus ulcers and chronic constipation was cured by chemotherapy followed by acidophilus milk

A	B	C	D	E	F	G	H	I	J
7.	M	24	Constipation, Gastrointestinal itching, loss of appetite	Glossitis with stomatitis & gingivitis	B-complex tablets 400 ~~mg~~ for 4 days then acidophilus milk	Improvement in appetite and itching in intestinal tract	Relief in abdominal pain	Recovered from all the stated discomforts	Clinically cured
8.	M	35	Chronic Constipation (from 3 months)	Chronic constipation & Stomatitis	B-complex tablet 400 ~~mg~~ from 15 days then acidophilus milk was given	Slight relief from stomach problems	Problem of constipation improved slightly	Recovered from constipation & stomach problem	Clinically cured from constipation and stomatitis
9.	F	6	Diarrhoea, Vomiting & loss of appetite	Leucocytes prominent in stools	Metronidazol 200 mg/day for 3 days followed by acidophilus milk	Vomiting & Diarrhoea cured	Improvement in appetite	Feeling completely normal	Recovered from all complaints
10.	M	32	Loose motions with foul smell	Diarrhoea, stomatitis & gingivitis	Antibiotics 200 mg twice a day for 4 days with B-complex 100 ~~mg~~ /day. Slight improvement and suggested to take acidophilus milk	Recovered completely from loose motions	No discomfort observed	-	Rapidly recovered from gingivitis, diarrhoea and stomatitis
11.	M	30	Buccal cavity ulcers, difficulty in swallowing, constipation	Constipation, pharyngitis, inflammation of salivary gland	Erythromycin 200 mg tablets & B-complex 100 ~~mg~~ for 5 days. Poor response observed. Acidophilus milk was suggested	Improvement in constipation problems	Oral ulcers healed partially and recovered from constipation	Recovered from constipation and ulceration	Clinically recovered from stated complaints

* Acidophilus milk 200g containing 2×10^7 viable cells / gram

** Acidophilus milk 300g containing 2×10^7 viable cells / gram

4.8.3 For Patients Suffering From Diarrhoea

Two patients including a girl of 6 years and an adult male of 26 years were taken to acidophilus therapy. Prior to this the girl was already treated with chemotherapeutic agents like Metronidazol (200 mg per day) for 3 days without any response. Acidophilus milk 200 g (per day) containing 2×10^7 viable cells of L. acidophilus was administered for six days and response was recorded after 3 and 6 days of feeding (Table 4.17).

It was observed that the patient who did not receive any drug got clinically cured within a period of two days of feeding acidophilus milk. The other patient who was previously treated with Metronidazol got clinically cured after three days of feeding acidophilus milk. Our results are in agreement with those reported by Aritaki and Ishikawa (1962) who cured infantile diarrhoea by feeding 30 ml of acidophilus milk in 13 out of 17 patients. Identical results were reported by Tomic-Karovic and Panjeck (1962) and Yakult Honsha Co., Ltd. (1979). Several workers have reported the use of acidophilus milk in treatment of gastro-enteritis and specific or non-specific diarrhoea (Gordon et al., 1957; Siver, 1961; Abbott, 1961; Gillespie and Dimmick, 1951).

Identical results were reported by Nikolic et al. (1973); Shilovskaya and Pactorykina (1978) in treatment of farm animals.

4.8.4 Acidophilus Therapy For A Patient Suffering From Glossitis, Stomatitis And Gingivitis

A patient of 24 years suffering from above mentioned complaints was treated with B-complex tablets (400 ug/day) for 4 days but without notable response. He was administered acidophilus milk (200 g/day) containing 2×10^7 viable cells of L.acidophilus for 9 days. The response is recorded in Table 4.17. It was observed that after 3 days of feeding of acidophilus milk there was an improvement in all the complaints and patient was clinically cured from all discomforts after 9 days of feeding of acidophilus milk. Similar results were reported by Gillespie and Dimmick (1951) and Anonymous (1978).

4.9 PREVALENCE OF LACTOBACILLI IN SELECTED COMMERCIAL THERAPEUTIC PREPARATIONS AVAILABLE IN INDIA

A total of 34 samples from the 'Sporolac' (Uni-Sankyo Ltd., Hydrabad), 'Lactobacil' (Intercare, Mehsana, Gujarat), 'Lactisyn' (Griffon Lab., Bombay), 'Zyspore' (Key-West, Bombay) and 'Vizylac' (Unichem Pvt.Ltd. Bombay) were enumerated with MRSA for lactobacilli and sporolactobacilli and Sporolac Agar (Sneath, 1986) for L.sporogenes spores. Nutrient agar was used for detecting contaminants present, if any only in case of preparations containing L.acidophilus (Appendix XVII).

TABLE 4.18: BACTERIAL COUNTS AND OTHER MICROORGANISMS IN
DRIED LACTOBACILLUS PREPARATIONS ANALYSED
FROM INDIAN MARKET

Sample	No. of batches tested	Colony forming units on		
		MRS agar	Sporogenes: agar	NA
Sporolac (Powder)	2	8.5×10^5	2.8×10^5	-
Sporolac (Tablet)	3	1.16×10^5	0.67×10^5	-
Lactobacil	2	0.77×10^5	-	2.05×10^2
Lactisyn	3	2.0×10^7	-	0.97×10^2
Vizylac	3	1.1×10^6	1.6×10^6	-
%spore	3	1.9×10^6	1.04×10^6	-

In 'Sporolac' group 8 samples from five batches were examined. Samples from this group have given relatively higher counts on MRS agar as compared to SA. But in both the cases counts were ten fold less than what ^{was} claimed by the manufacture.

In case of 'Lactobacil' from 2 batches three samples were analysed and average counts were only 77,000 lactobacilli per gram against 10 million L. acidophilus labeled on the sachet.

In case of 'Lactisyn' 8 samples from three batches were tested and 2×10^7 lactobacilli per ampoule were recorded as against 2x490 total lactobacilli claimed by manufactures. In this case contaminants were comparatively less than that of Lactobacil.

In case of 'Vizylac' and 'Zyspore', 15 samples from three batches of each product were analysed and 1.5×10^6 and 1.04×10^6 spores of L. sporogenes were recorded per capsule as against 40×10^6 and 60×10^6 respectively claimed by manufacturers (Table 4.18).

Most lactobacilli containing therapeutic preparations available in India included in this study had very small numbers of viable lactobacilli. These products were produced by different methods and none of the samples procured was stored under refrigerated conditions. These factors might have contributed to such low counts and wide variation in cell viability among products from the different groups.

A daily dose of 10^8 to 10^9 viable L. acidophilus cells is thought to be necessary for their beneficial role in humans (Gilliland et al., 1978 and Speck, 1978). At this recommended dose level none of the samples would be able to supply this number and thus could be effective as a dietary adjunct.

However, recent feeding trials with human subjects using dried commercial acidophilus products have failed to control the undesirable effects of enteropathogenic E. coli (Clements et al., 1981 and Pozo-Olaro et al., 1978). Small numbers of viable L. acidophilus in these products may be a reason for this failure.

The products containing L.acidophilus had organisms other than lactobacilli. Their presence could be due to poor sanitary conditions used in manufacture of these products. Possible health implications from the consumption of these products needs to be evaluated.

No one report is available in cited literature indicating the analysis of lactobacilli containing therapeutic preparations available in Indian market but our results agree with those of Gilliland and Speck (1977^c); Speck (1978) and Brennan et al. (1983).

SUMMARY & CONCLUSIONS

CHAPTER - V

S U M M A R Y A N D C O N C L U S I O N S

The investigation was carried out to determine the comparative account of product quality, implantation ability in human intestine and antibacterial activity in vivo and in vitro of Lactobacillus acidophilus strains isolated from two different human body sources.

For this study the strains of L. acidophilus were isolated from the faeces of 24 bottle fed infants of 1 to 2 years of age and vaginal mucosa of human females from 16 to 24 years of age. Their morphological, physiological and biochemical properties were confirmed. Two strains of L. acidophilus LBKV₃ and LBKI₄ isolated from vaginal surface and gastro-intestinal tract respectively, possessing resistance to phenol, bile salts, low pH and giving good organoleptic characteristics of curd were selected for further study.

Growth studies of the strains LBKV₃ and LBKI₄ determined in terms of increase in log viable counts and titratable acidity in skim milk at 37 C revealed that at about 36 h of incubation, which was the beginning of their maximum stationary phase, isolates could produce 123×10^7 and 92.5×10^7 cells/ml respectively. The acidity at this stage was 1.39 and 1.17 per cent lactic acid respectively for L. acidophilus LBKV₃ and LBKI₄, however the maximum acidity, 2.16 and 2.06 per cent lactic acid respectively, was produced at the end of 120 h of incubation.

Acidophilus milks were prepared utilizing the LBKV₃ and LBKI₄ strains and were organoleptically evaluated by a panel of judges using nine point hedonic scale. The weighted scores of 7.38 and 6.83 out of 9 awarded to acidophilus milks containing L.acidophilus LBKV₃ and LBKI₄ respectively indicated that the products were graded into "liked very much" to "liked moderately" categories respectively.

The antibacterial activity of the strains of L.acidophilus LBKV₃ and LBKI₄ was checked by cup assay method against pathogenic and spoilage types of organism viz., E.coli (Mastitic), B.cereus, Staph.aureus (enterotoxigenic), Ps.aeruginosa and Salm.typhosa. Strain LBKV₃ showed higher antibacterial activity between the two strains tested. B.cereus was found to be the most susceptible followed by Staph.aureus, Salm.typhosa and E.coli. Ps.aeruginosa was most resistant among the cultures tested.

The antibacterial activity was found to increase with increase in incubation period from 12 to 72 hrs. but for Salm.typhosa and Ps.aeruginosa the extent of antibacterial activity was found to decrease from 60 h onwards.

Inhibition of mastitic E.coli and enterotoxigenic Staph.aureus during production and storage of acidophilus milks prepared by using two L.acidophilus strains and stored at 15 C and 37 C revealed that Staph.aureus was inhibited from 20 h onwards and after 60 h of storage at 37 C the count was

sufficiently lower than what was initially inoculated in skim milk containing L.acidophilus LBKV₃. In case of milk containing LBKI₄ strain there was steady increase in staphylococcal counts upto 20 h but subsequently counts were observed to decrease and were sufficiently lower than originally inoculated after 72 h storage at 37 C. In case of skim milk inoculated with mixed lactic culture, LF-40, there was steady increase in staphylococcal counts upto 24 h and then the decline was observed but counts were sufficiently higher than initially inoculated. In case of skim milk inoculated with yoghurt culture, counts showed decline from 20 h onwards and were sufficiently lower than initial number after 48 h of storage at 37 C.

Milks inoculated with L.acidophilus strains LBKV₃ and LBKI₄, mixed lactic culture LF-40, and yoghurt culture (CH₁ + LBW) along with staphylococci and stored at 15 C after incubating at 37 C for 12 h showed decline in staphylococcal counts after 8 h of storage in milk inoculated with LBKV₃, milk inoculated with LBKI₄ showed decline after 12 h of storage, while decline in staphylococcal counts were observed after 24 and 8 h of storage respectively in milks inoculated with LF-40 and yoghurt culture.

Results of inhibition of E.coli during production and storage of milks inoculated with two acidophilus strains, one mixed lactic culture and one with yoghurt culture revealed that in all the cases the coliform counts steadily increased

upto 48 h of storage at 37 C and then there was decline in E.coli counts after 48 h onward in case of LBKV₃, 60 h onward in case of LBKI₄, and 36 h onward in case of yoghurt culture. But there was no reduction in coliform counts of milk inoculated with mixed lactic culture, LF-40, upto 60 h of storage at 37 C.

Skim milk inoculated with LBKI₄, LBKV₃ and yoghurt culture stored at 15 C after 12 h inoculation at 37 C showed decline in coliform counts after 48, 48 and 36 h incubation respectively but there was no decline in coliform counts in milk inoculated with LF-40.

The implantation properties of the individual isolates of L.acidophilus were studied by feeding the acidophilus milks to human test subjects for a period of one week. The effects of feeding on the intestinal content of lactobacilli and coliforms were determined by analysing the faecal samples of the test subjects before administration, during and after stopping the administration. Counts of lactobacilli were found to increase while coliforms decreased significantly during feeding, whereas the trend got reversed when the feeding was discontinued. However, the lactobacilli counts in the faecal samples were significantly higher even after two weeks of stopping feeding and that of coliforms were significantly lower than the initially present. This study conclusively indicated the implantation ability of both the L.acidophilus strains. However, the extent of implantation varied with the

individuals. Further L.acidophilus LBKV₃ was observed to be superior to LBKI₄ in terms of implantation ability.

Analysis of commercially available preparations intended to supply viable lactobacilli revealed that all six products tested had very small numbers of viable bacteria and all contained organisms other than lactobacilli.

The above findings indicated that the isolates of L.acidophilus strains varied in their growth characteristics and patterns of growth cycles. Both the strains gave organoleptically acceptable products.

Both the strains showed antagonistic action against selected pathogenic and spoilage types of bacteria, however, there was variation between the strains. The strain LBKV₃ exhibited relatively higher inhibitory activity against pathogens during production and storage of acidophilus milk.

Encouraging therapeutic benefits were observed while using the cultures for treatment of human patients suffering from a variety of gastro-intestinal and related discomforts.

Limited viability of lactobacilli in commercially available preparations was detected by analysing six types of selected therapeutic preparations available in Indian market.

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APPENDICES

APPENDIX - I

COMPOSITION AND METHODS FOR PREPARATION OF THE CULTURE MEDIA USED IN THE STUDY

1. Sterilised Skim Milk :

Freshly separated cow's skim milk was obtained from student's Training Dairy of the College. This was distributed in test tubes or flasks in required quantities and sterilised in an autoclave at 121°C (15 psi) for 20 min.

2. Skim milk-phenol :

Two lots of skim milk were added with 0.4 and 0.5 per cent (w/v) phenol and then sterilised in an autoclave at 121°C (15 psi) for 20 min.

3. MRS Broth (deMan, Rogosa and Sharpe, 1960) :

Peptone	10.0 g
Meat extract	10.0 g
Yeast extract	5.0 g
D-glucose	20.0 g
Sodium acetate	5.0 g
Tri-ammonium citrate	2.0 g
K_2HPO_4	2.0 g

MgSO ₄ · 7H ₂ O	0.2 g
MnSO ₄ · 5H ₂ O	0.05 g
Tween-80	1.0 ml
Distilled water	1000.0 ml

All the ingredients were dissolved in distilled water and final pH was adjusted to 6.4 - 6.6 and sterilised in an autoclave at 121 C (15 psi) for 20 min.

4. MRS Agar :

MRS agar was prepared by incorporating 1.5 per cent agar-agar powder (Bacteriological grade) in MRS broth after adjustment of the pH.

5. MRS Broth - Bile Salt :

MRS broth was fortified with 2 per cent and 4 per cent (w/v) bile salt (sodium taurocholate) before sterilisation.

6. MRS Broth - NaCl :

MRS broth was fortified with 2 per cent and 4 per cent (w/v) sodium chloride before sterilisation.

7. MRS Broth Having Different pH values :

The pH of different lots of MRS broth was adjusted to 7, 5, 4, 3 and 2 before sterilization with concentrated HCl.

8. Normal Saline (Isotonic Salt Solution) :

Sodium chloride	0.9	g
Distilled water	100.0	ml

Dissolved, distributed 10 ml each into test tubes, cotton plugged and sterilised at 121 C for 20 min.

9. Nutrient Broth : (Difco Manual, 1977) :

Peptone	5.0	g
Beef extract	3.0	g
NaCl	8.0	g
Distilled water	1000.0	ml

Final pH of medium was adjusted to 7.0 ± 0.1 and sterilised by autoclaving at 121 C for 20 min.

10. Nutrient Agar :

Nutrient agar was prepared by incorporating 1.5 per cent agar-agar powder (Bacteriological grade) in the nutrient broth.

11. Mac-Conkey's Agar (Difco Manual, 1977) :

Peptone	17.0 g
Proteose peptone	3.0 g
Sodium taurocholate	1.5 g
Lactose	10.0 g
Sodium chloride	5.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Distilled water	1000.0 ml
pH	7.2 $\pm$ 0.1

All the ingredients were dissolved in water, pH was adjusted & then the indicator was added. The medium ^{sub}is then filtered, distributed into 100 ml quantities in flasks and sterilised by autoclaving at 121 C for 20 min.

12. Phosphate Buffer For Preparing Dilutions :

(IS : 1479, Part-III, 1962)

Stock Solution of Phosphate buffer was prepared by dissolving 34.0 g of KH_2PO_4 in 500 ml distilled water. ^{ph}PH was adjusted to 7.2 with 0.1 N NaOH solution and the total volume was made upto 1 litre with distilled water.

For use as dilution water, 1.25 ml of the stock phosphate buffer solution was diluted to 1 litre with distilled water. It was filled in 99 ml or 9 ml aliquots in flasks or tubes and sterilised by autoclaving at 121 C for 20 min.

13. Carbohydrate Fermentation Media :

MRS medium ^{5.9}is used for general cultivation and also as a basal medium for biochemical characterisation of strains of lactobacilli. The method employed by Sharpe (1962) was used.

The MRS broth recommended as basal medium was devoid of glucose and meat extract and added with 0.0025 per cent (w/v) Bromocresol purple as an indicator.

To the above basal medium sugars were added at 1 per cent level separately. Cellobiose, galactose, lactose, mannitol, metibiose, salicin, sorbitol, sucrose and trehalose were added before sterilisation of basal medium, while maltose and esculin were added as seitz filtered solution to the basal medium after sterilization due to susceptibility of these carbohydrates to heat decomposition.

The sugar broth were inoculated with washed cell suspensions of the organisms at 0.1 millilitre per tube and after incubation the results were recorded.

14. Staphylococcus Medium - 110 :

Tryptone	10.0 g
Yeast extract	2.5 g
Gelatin	30.0 g
d-Mannitol	10.0 g
Lactose	20.0 g
Sodium chloride	75.0 g
Dipotassium phosphate	5.0 g
Agar	15.0 g
Distilled water	1000.0 g

All the ingredients were dissolved in distilled water by heating to boil and final pH was adjusted to 7.0 and sterilised in an autoclave at 121 C (15 psi) for 20 min.

15. Sporogenous Agar :

Yeast extract	5.0 g
Peptone	5.0 g
Glucose	20.0 g
Calcium carbonate	10.0 g
Agar	15.0 g

All the ingredients were dissolved in 1000ml. 7
distilled water and final pH was adjusted to 6.5
and sterilised in an autoclave at 121 C (15 psi)
for 20 min.

APPENDIX II

BIOCHEMICAL CHARACTERISTICS OF SELECTIVELY ISOLATED VAGINAL AND GASTROINTESTINAL LACTOBACILLI

Characteristics LBKV1 LBKV2 LBKV3 LBKV4 LBKV5 LBKV6 LBK11 LBK12 LBK13 LBK14 LBK15 LBK16 LBK17 LBK18 LBK19 LBK110

Production of

Acid From:

Arabinose	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	d	+	+	-	+	+	+	+	d	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	d	+	+	+	+	+	+	+	+
Melibiose	+	+	+	d	+	+	+	d	+	+	d	+	+	+	+	-
Mannitol	+	-	-	-	-	-	+	+	d	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	d
Ribose	-	-	-	-	+	-	+	+	-	-	-	-	d	+	-	d
Salicin	+	+	+	+	+	+	+	-	+	+	+	+	d	+	+	+
Sorbitol	-	-	-	d	-	-	-	-	-	-	-	-	+	+	+	d
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	-	+	+	+	+	+	-	-	+	+	+	-	d	+	+
Xylose	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

Production of

Gas From:

Gluconate	-	-	-	-	+	d	+	d	-	-	-	-	-	-	d	-
Glucose	-	-	-	-	+	-	+	+	-	-	-	-	+	d	-	-

Production of

NH From:

3

Arginine	-	-	-	-	d	-	-	d	-	-	-	-	-	-	-	+
Esculin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Key:

* All characteristics are similar to those given by Kandler and Norbert (1986) for *L. acidophilus*

+ Growth present within 24-48 hrs.

- No growth upto 96 hrs.

d Growth delayed upto 7 days.

LBKV--- Vaginal isolate

LBK1--- Gastrointestinal isolate

APPENDIX-III

PHYSIOLOGICAL CHARACTERISTICS OF SELECTIVELY ISOLATED VAGINAL AND GASTROINTESTINAL LACTOBACILLI

CHARACTERISTICS	LBKV1	LBKV2	LBKV3	LBKV4	LBKV5	LBKV6	LBKI1	LBKI2	LBKI3	LBKI4	LBKI5	LBKI6	LBKI7	LBKI8	LBKI9	LBKI10
Growth at 15 C	-*	d*	-*	-*	-*	-*	-*	-*	-*	-*	d*	++	-*	d*	d*	-*
Growth at 45 C	+	+	+	+	d	-	-	+	+	+	+	-	+	+	+	+
Growth at 50 C	d	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Bile Resistance																
2%	G	T	G	T	T	-	T	-	-	G	T	-	-	-	T	-
4%	T	T	T	-	-	-	-	-	-	T	-	-	-	-	-	-
Phenol Resistance																
0.4%	T	-	G	-	T	T	-	-	T	G	T	-	-	T	-	-
0.5%	-	-	T	-	-	-	-	-	-	T	-	-	-	-	-	-
Salt Tolerance																
2%	T	G	G	G	-	T	T	T	T	G	G	T	G	G	T	T
4%	-	-	G	T	-	-	T	-	-	G	T	T	T	T	-	-
pH Tolerance																
5	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
4	G	G	G	G	G	G	G	G	G	G	G	G	G	G	d	-
3	T	T	G	T	T	T	T	T	G	G	T	T	T	T	-	-
2	-	T	T	-	-	T	-	-	T	T	-	-	T	T	-	-

Key:

+ Growth present within 24-48 hrs.

- No growth upto 96 hrs.

-* No growth upto 10 days.

d* Growth delayed upto 12 days

d Growth delayed upto 7 days

T Tolerance

G Growth Present

LBKV--- Vaginal isolate

LBKI-- Gastrointestinal isolate.

APPENDIX - IV

COMPARATIVE APPRAISAL OF GROWTH RATES OF L. ACIDOPHILUS LBKV₃ AND LBKI₄ (MEAN OF LOG VIABLE COUNTS AT DIFFERENT PERIODS*)

Cultures: (T)	Incubation period (P) (Hrs)										Mean : T
	: 0	: 16	: 20	: 24	: 36	: 48	: 60	: 72	: 96	: 120	
LBKV ₃	6.4800	7.520	8.420	8.900	9.080	9.24	9.39	9.380	8.82	8.150	8.5380
LBKI ₄	6.6700	7.858	8.620	8.686	8.970	9.100	8.66	7.820	7.778	7.22	8.1382
Mean P	6.575	7.689	8.520	8.793	9.025	9.170	9.025	8.60	8.299	7.685	

* Average log viable counts of five replications.

ANOVA Table

Source	: D.F.	: S.S.	: M.S.	: Cal.F.	: Tab.F.	: Sem.	: C.D.
Treatment	1	3.995	3.995	380.5**	0.094	0.0145	0.0471
Error (A)	8	0.084	0.0105	-	-	-	-
Period	9	59.026	6.558	655.8**	0.032	0.03	0.0844
T x P	9	8.967	0.996	99.6**	0.045	0.045	0.1266
Error (B)	12	0.718	0.01	-	-	-	-
Total	99						

** Difference is highly significant at 5% level of significance.

APPENDIX - V

COMPARATIVE APPRAISAL OF GROWTH RATES OF *L. ACIDOPHILUS* LBKV₃ AND LBKI₄ (MEAN RATE OF ACID PRODUCTION *)

Cultures (T)	Incubation periods (P) (Hr)											Mean
	0	16	20	24	36	48	60	72	96	120	T	
LBKV ₃	0.26	0.72	0.982	1.17	1.39	1.67	1.84	2.02	2.05	2.134	1.4236	
LBKI ₄	0.28	0.67	0.82	1.01	1.164	1.51	1.62	1.77	1.874	2.06	1.2778	
Mean P	0.27	0.695	0.901	1.09	1.277	1.59	1.73	1.895	1.962	2.097		

* Average titratable acidity values of five replications

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F.	Tab.F.	Sem.	C.D.
Treatment	1	0.532	0.532	532**	7.710	0.004	0.013
Error (A)	8	0.009	0.001	-	-	-	-
Period	9	33.016	3.67	616.6**	2.010	0.007	0.023
T x P	9	0.166	0.184	306 **	2.010	0.011	0.036
Error (B)	72	0.046	0.0006	-	-	-	-
Total	99						

** The rate of acid production of two cultures is highly significant at 5% level of significance.

APPENDIX - VI

COMPARATIVE ACCOUNT OF ACCEPTABILITY OF ACIDOPHILUS MILKS PREPARED USING LACTOBACILLUS ACIDOPHILUS LBKV₃ AND LBKI₄

Cultures (T)	: Scoring for acceptability on nine point hedonic scale*						: Mean : T
	: (Response of judges)						
	J ₁	J ₂	J ₃	J ₄	J ₅	J ₆	
LBKV ₃	2.9714	2.5476	2.6739	2.6361	2.6762	2.7634	2.7114
LBKI ₄	2.9714	2.5032	2.4400	2.4727	2.6295	2.5752	2.5987
Mean J	2.9714	2.5254	2.5570	2.5544	2.6528	2.6693	

* Scores are average values of six replications.

ANOVA Table

Source	: D.F.	: S.S.	: M.S.	: Cal.F.	: Tab.F.	: SEM.	: C.D.
Treatment	1	0.229	0.229	5.618**	4.000	0.034	0.035
Judges	5	1.642	0.328	8.057**	2.370	0.058	0.165
T x J	5	0.134	0.027	0.658 ^{NS}	2.370	0.082	-
Error	60	2.446	0.041	-	-	-	-
Total	71						

** : Scores for two cultures are significantly different.

N.S. : Difference between scores allowed for individual cultures by different judges is non-significant.

APPENDIX - VII

COMPARATIVE ACCOUNT OF EFFECT OF FEEDING LACTOBACILLUS ACIDOPHILUS LBKV₃ AND LBKI₄ ON FAECAL LACTOBACILLI COUNTS OF HUMAN TEST SUBJECTS*

Cultures (T)	Log lactobacilli counts at different periods (P)						Mean T
	Before	During feeding		After stopping		14-days	
	feeding						
	2-days	7-days	7-days	7-days	14-days		
LBKV ₃	7.5457	7.6886	9.0929	8.8986	8.2514	8.2954	
LBKI ₄	7.6786	7.7614	8.6371	8.340	8.0171	8.0869	
Mean P	7.6121	7.725	8.865	8.619	8.1343		

* Log faecal lactobacilli counts of seven human test subjects.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F	Tab.F	Sem.	C.D.
Treatment	1	0.761	0.761	1.044 ^{NS}	4.75	0.1707	-
Error (A)	12	8.742	0.7285	-	-	-	-
Period	4	16.704	4.176	47.45**	2.56	0.079	0.224
T x P	4	1.330	0.335	3.784**	2.56	0.112	0.318
Error (B)	48	4.203	0.088	-	-	-	-
Total	69						

** Log viable count differences are highly significant.

NS Difference in effects of the two cultures on faecal Lactobacilli counts are non-significant.

APPENDIX - VIII

COMPARATIVE ACCOUNT OF EFFECT OF FEEDING LACTOBACILLUS ACIDOPHILUS LBKV₃ AND LBKI₄ ON FAECAL COLIFORM COUNTS OF HUMAN TEST SUBJECTS*

Culture (T)	Log coliform counts at different periods (P)						Mean T
	Before feeding :	During feeding		After stopping feeding			
	:	2 days	7 days	7 days	14 days	:	
	:	:	:	:	:	:	
LBKV ₃	7.3714	7.1657	6.2800	6.5743	6.9386		6.8720
LBKI ₄	7.2271	7.1243	6.5257	6.5129	7.0629		6.8906
Mean P	7.2993	7.1600	6.4029	6.5436	7.0007		

*Log faecal coliform counts of seven human test subjects

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F	Tab.F	Sem.	C.D.
Treatment	1	0.006	0.006	0.019 ^{NS}	4.75	0.0947	-
Error (A)	12	3.768	0.314	-	-	-	-
Period	4	8.535	2.134	21.490**	2.56	0.0842	0.2386
T x P	4	0.363	0.091	0.9164 ^{NS}	2.56	0.0119	0 -
Error (B)	48	4.767	0.0993	-	-	-	-
Total	69						

NS : Difference in effects of the two cultures on faecal coliform counts are non-significant.

** : Differences between log coliform counts are highly significant at 5% level of significance.

APPENDIX - IX

INHIBITION OF STAPHYLOCOCCUS AUREUS DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK AT 37 C (MEAN OF LOG SURVIVORS AT DIFFERENT INCUBATION PERIODS*)

Cultures (T)	Incubation periods (P) at 37 C (Hr)										Mean T
	0	12	16	20	24	36	48	60	72	:	
LBKV ₃	3.454	6.34	6.420	6.146	4.662	4.284	3.664	3.266	2.99		4.58
LBKI ₄	3.408	6.532	6.654	6.670	6.482	5.430	4.730	4.570	3.32		5.31
LF-40	3.40	6.552	6.680	7.32	7.770	6.620	5.470	4.272	4.03		5.79
LBW+CH ₁	3.39	5.220	5.320	5.132	4.644	3.780	3.320	3.040	2.98		4.9
Mean P	3.413	6.161	6.2685	6.067	5.889	5.0285	4.296	3.387	3.33		

* Average log survivor values of five replications.

ANOVA Table

Source	:	D.F.	:	S.S.	:	M.S.	:	Cal.F.	:	Tab.F.	:	Sem.	:	C.D.
Treatment		3		4099.075		1366.36		0.852 ^{NS}		3.24		5.97		-
Error (A)		16		25663.29		1603.9		-		-		-		-
Period		8		14952.93		1898.116		1.165 ^{NS}		2.01		0.111		-
P x T		24		39273.48		1636.39		1.020 ^{NS}		1.60		17.91		-
Error (B)		128		205322.20		1604.08		-		-		-		-
Total		179												

NS : Differences in log survivors are non significant.

APPENDIX - X

INHIBITION OF STAPHYLOCOCCUS AUREUS DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK AT 37 C. (MEAN TITRATABLE ACIDITY VALUES AT DIFFERENT INCUBATION PERIODS*)

Cultures (T)	Incubation periods (P) at 37 C (Hr)											Mean T
	0	12	16	20	24	36	48	60	72			
LBKV ₃	0.240	0.44	0.66	0.89	1.13	1.45	1.640	1.778	1.88			1.1231
LBKI ₄	0.254	0.42	0.56	0.83	1.004	1.29	1.41	1.590	1.88			1.0264
LF-40	0.200	0.49	0.55	0.694	0.72	0.81	0.83	0.850	0.87			0.668
CH ₁ +LBW	0.250	0.82	1.08	1.16	1.348	1.834	1.92	1.950	2.02			1.3689
Mean P	0.236	0.5425	0.697	0.8935	1.050	1.346	1.45	1.542	1.6625			

* Average titratable acidity values of five replications.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F.	Tab.F	Sem.	C.D.
Treatment	3	11.399	3.80	3800**	3.24	4.714	0.013
Error (A)	16	0.015	0.001	-	-	-	-
Period	8	38.68	4.835	4835**	2.01	7.07	0.0197
P x T	24	5.147	0.214	21.4**	1.6	0.014	0.039
Error (B)	128	0.188	0.001	-	-	-	-
Total	179						

** Rate of acid production of four cultures at different periods is highly significant at 5% level of significance.

APPENDIX - XI

INHIBITION OF STAPHYLOCOCCUS AUREUS DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK (MEAN OF LOG SURVIVORS AT DIFFERENT INCUBATION PERIODS AT 15 C*)

Cultures (T)	Incubation periods (P) at 15 C (Hr)								Mean T
	4	8	12	24	36	48	60		
LBKV ₃	6.462	6.012	5.372	5.292	4.552	4.426	3.568		6.042
LBKI ₄	6.53	6.57	6.534	6.48	5.662	5.602	4.922		5.0977
LF-40	6.542	6.61	6.584	6.58	6.460	6.030	5.458		6.3234
CH ₁ +LBW	5.272	4.922	4.214	3.266	2.886	2.840	2.308		3.6726
Mean P	6.2015	6.0285	5.676	5.4045	4.89	4.7245	4.06		

* Average values of five replications.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F.	Tab.F	Sem.	C.D.
Treatment	3	150.069	50.023	6252.1**	3.24	0.324	0.042
Error (A)	16	0.129	0.008	-	-	-	-
Period	6	70.419	11.736	978**	2.19	0.0245	0.07
T x P	18	16.138	0.897	74.79**	1.68	0.0489	0.136
Error (B)	96	1.160	0.012	-	-	-	-
Total	139						

** Differences in log survivors in four cultures at different intervals are highly significant at 5% level of significance.

APPENDIX - XII

INHIBITION OF STAPHYLOCOCCUS AUREUS DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK (MEAN OF TITRATABLE ACIDITY AT DIFFERENT INCUBATION PERIODS AT 15°C)

Cultures (T)	Incubation periods (p) at 15 C (Hr)								Mean T
	4	8	12	24	36	48	60	:	
LBKV ₃	0.57	0.624	0.63	0.636	0.638	0.666	0.6795		0.6348
LBKI ₄	0.484	0.484	0.498	0.528	0.540	0.562	0.556		0.5217
LF-40	0.530	0.530	0.536	0.548	0.558	0.564	0.570		0.548
CH ₁ +LBW	0.930	0.930	0.940	0.952	0.944	0.960	0.968		0.946
Mean P	0.6285	0.642	0.651	0.666	0.670	0.688	0.693		

* Average values of five replications.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F	Tab.F	Sem.	C.D.
Treatment	3	3.998	1.333	2.668**	3.24	0.003	0.01
Error (A)	16	0.008	0.0005	-	-	-	-
Period	6	0.068	0.0113	37.66**	2.10	0.011	0.03
T x P	18	0.016	0.0008	2.666**	1.68	0.0077	0.02
Error (B)	96	0.036	0.0003	-	-	-	-
Total	139						

** Acidity values of four cultures at different periods are significantly different.

APPENDIX - XIII

INHIBITION OF ESCHERICHIA COLI DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK AT 37 C (MEAN OF LOG SURVIVORS AT DIFFERENT INCUBATION PERIODS*)

Cultures (T)	Incubation periods (P) at 37 C (Hr)										Mean
	0	12	16	20	24	36	48	60	72	72	T
LBKV ₃	3.100	5.546	6.530	7.470	7.820	7.910	8.620	8.750	3.490	3.490	6.5818
LBKI ₄	3.080	5.580	6.590	7.640	7.830	8.330	8.390	8.020	3.520	3.520	6.5533
LF-40	3.100	5.620	6.482	7.450	7.640	8.640	8.710	8.770	8.300	8.300	7.1924
CH ₁ +LBW	3.070	5.620	6.820	7.590	7.850	8.076	6.720	4.690	2.560	2.560	5.8884
Mean P	3.0875	5.5965	6.6055	7.5375	7.785	8.239	8.110	7.557	4.4675	4.4675	

* Average log survivor values of five replications.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F	Tab.F.	Sem.	C.D.
Treatment	3	38.311	12.77	22803.37**	3.24	0.0034	0.01
Error (A)	16	0.009	0.00056	-	-	-	-
Period	8	520.797	65.099	203434.38**	2.01	0.0004	0.0011
T x P	24	134.547	5.606	17518.75**	1.60	0.008	0.022
Error (B)	118	0.042	0.00032	-	-	-	-
Total	179						

** Log survivor values are significantly different.

APPENDIX - XIV

INHIBITION OF ESCHERICHIA COLI DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK AT 37 C (MEAN TITRATABLE ACIDITY VALUES AT DIFFERENT INCUBATION PERIODS*)

Cultures (T)	Incubation periods (P) at 37 C (Hr)										Mean T
	0	12	16	20	24	36	48	60	72		
LBKV ₃	0.200	0.540	0.700	0.980	1.240	1.370	1.530	1.76	1.81		1.1253
LBKI ₄	0.190	0.470	0.680	0.822	1.010	1.220	1.45	1.62	1.70		1.018
LF-40	0.220	0.492	0.570	0.730	0.760	0.820	0.83	00.85	0.90		0.685
CH ₁ +LBW	0.230	0.820	1.060	1.190	1.370	1.770	1.87	1.91	1.98		1.3556
Mean P	0.210	0.580	0.752	0.930	1.095	1.295	1.42	1.534	1.597		

* Average titratable acidity values of five replications.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F	Tab.F	Sem.	C.D.
Treatment	3	10.485	3.495	8737.5**	3.24	0.003	0.008
Error (A)	16	0.007	0.0004	-	-	-	-
Period	8	35.223	4.40	15714.28**	2.01	0.0037	0.010
T x P	24	4.219	0.175	625.**	1.6	0.0236	0.0658
Error (B)	128	0.039	0.00028	-	-	-	-
Total	179						

** Rate of acid production of four cultures at different periods is highly significant at 5% level of significance.

APPENDIX - IV

INHIBITION OF ESCHERICHIA COLI DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK (MEAN OF LOG SURVIVORS AT DIFFERENT INCUBATION PERIODS AT 15 C*)

Culture (T)	Incubation Periods (P) at 15 C (Hr)							Mean T
	4	8	12	24	36	48	60	
LBKI ₄	5.65	5.82	6.15	6.164	6.24	6.09	6.01	6.017
LBKV ₃	5.91	6.02	6.28	6.32	6.42	6.37	5.76	6.153
LF-40	5.63	5.73	5.79	5.80	5.82	5.99	6.39	5.878
CH ₁ +LBW	6.24	6.46	6.50	6.61	6.30	5.17	5.11	6.056
Mean P	5.85	6.01	6.18	6.22	6.19	5.91	5.82	

* Average values of five replications.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F	Tab.F	Sem.	C.D.
Treatment	3	150.069	50.023	6252.1**	3.24	0.0015	0.042
Error (A)	16	0.129	0.008	-	-	-	-
Period	6	70.419	11.736	978**	2.19	0.0245	0.07
P x T	18	16.138	0.897	74.79**	1.68	0.0489	0.136
Error (B)	96	1.160	0.012	-	-	-	-
Total	139						

** Differences in log survivors in four cultures at different intervals are highly significant at 5% level of significance.

APPENDIX - XVI

INHIBITION OF ESCHERICHIA COLI DURING PRODUCTION AND STORAGE OF ACIDOPHILUS MILK (MEAN OF TITRATABLE ACIDITY AT DIFFERENT INCUBATION PERIODS AT 15 C)

Cultures (T)	Incubation periods (P) at 15 C (Hr)								Mean T
	4	8	12	24	36	48	60		
LBKV ₃	0.556	0.580	0.590	0.620	0.648	0.650	0.660		0.6149
LBKI ₄	0.544	0.544	0.560	0.564	0.580	0.598	0.610		0.5714
LF-40	0.510	0.530	0.540	0.550	0.550	0.570	0.580		0.5471
CH ₁ +LBW	0.870	0.886	0.898	0.914	0.930	0.940	0.960		0.914
Mean P	0.620	0.6350	0.647	0.662	0.677	0.689	0.702		

* Average values of five replications.

ANOVA Table

Source	D.F.	S.S.	M.S.	Cal.F	Tab.F	Sem.	C.D.
Treatment	3	3.998	1.333	2668**	3.24	0.003	0.01
Error (A)	16	0.008	0.0005	-	-	-	-
Period	6	0.068	0.0113	37.66**	2.19	0.011	0.03
T x P	18	0.016	0.0008	2.666**	1.68	0.0077	0.02
Error (B)	96	0.036	0.0003	-	-	-	-
Total	139						

** Acidity values of four cultures at different periods are significantly different.

APPENDIX - XVII

PERTINENT INFORMATION ON SELECTED LACTOBACILLUS
PREPARATIONS COMMERCIALY AVAILABLE IN
INDIAN MARKET

Product group*	General description	Lactobacillus species and number claimed
Sporolac	180 mg/tablet, method of drying not mentioned.	60 million spores of <u>L. Sporogenes</u> per tablet.
Sporolac	1.8 g/Sachet Method of drying not mentioned.	150 million spores of <u>L. sporogenes</u> per sachet.
Lactobacil	1.0 g/sachet Lyophilized powder.	10 million cells of <u>L. acidophilus</u> per sachet.
Lactisyn	200 mg/ampoule lyophilized powder	<u>L. acidophilus</u> , 490 million cells/ampoule and <u>L. lactis</u> 490 million cells/ampoule.
Vizylac	320 mg/capsule**	<u>L. sporogenes</u> ; 40×10^6 spores/capsule.
Zyspore	250 mg/capsule**	<u>L. sporogenes</u> ; 60×10^6 spores/capsule

* All preparations were for direct consumption as therapeutic agents.

**Method of drying not mentioned