# ANTIMICROBIAL ACTIVITY OF LEUCONOSTOCS

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DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL - 132001 (HARYANA)
YEAR 1991

# ANTIMICROBIAL ACTIVITY OF LEUCONOSTOCS

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FOR THE DEGREE OF

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IN DAIRYING

**DAIRY MICROBIOLOGY**

*By*

**ANJALI**

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(Ext. Examiner)

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WITH DEEPEST REGARDS TO,

MY ELDERS

FOR THEIR NEVER ENDING

LOVE, CARE AND AFFECTION.....

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17th June 1991.

This is to certify that the thesis entitled "ANTIMICROBIAL ACTIVITY OF LEUCONOSTOCS" submitted by MS. ANJALI in Partial fulfilment of the requirement for the award of the MASTER OF SCIENCE in Dairying (Diary Microbiology) of National Diary Research Institute (Deemed university), Karnal (Haryana), India is a bonafide research work carried by her under my supervision and guidence and no part of the thesis has been submitted for any other degree or diploma.

(P.K.AGGARWAL)

# ANTIMICROBIAL ACTIVITY OF LEUCONOSTOCS

By

ANJALI

A thesis submitted to the National Dairy Research Institute ( Deemed University ), Karnal in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN

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Anjali

(ANJALI)

# C O N T E N T S

## 1.0 INTRODUCTION

Lactic acid bacteria are indispensable for the dairy industry because of their role in the manufacture of a wide range of cultured and fermented dairy products. Being highly nutritious, milk products are also liable to be attacked by spoilage and pathogenic organisms. The possibility of pathogens like, *Salmonella*, *S. aureus*, *B. cereus*, *E.coli* etc. in milk and milk products is governed by such factors as their entrance either from cattle or the environment into raw milk, post-pasteurization contamination of milk and also their subsequent growth during different stages of processing.

Apart from carrying out desirable fermentations, lactic acid bacteria play a significant role in the preservation of various fermented foods. It has been very well established that lactic cultures are antagonistic to growth and survival of many micro-organisms, including pathogenic ones. Infact even before their role in milk fermentations was known, they had been used to preserve cultured dairy products and other foods especially beef and pork (Schaack and Marth, 1988). These bacteria create such environment as is detrimental to the survival of food borne spoilage and pathogenic organisms although some of the latter are affected more by this hostile environment than the others.

Leuconostoc spp.have been reported to inhibit the growth of several gram negative and psychrotrophic micro-organisms. The growth of psychrotrophs results in the proteolytic and lipolytic breakdown of numerous milk and meat products at refrigerated storage temperatures, leading to economic losses to the food manufacturer, retailer and consumer.

The inhibitory potential has so far been studied and observed only in the spent medium. The principles responsible for antimicrobial activity of leuconostoncs have not been very well identified. Different workers have attributed this effect to organic acids, hydrogen peroxide and some unidentified biochemical compounds. In Streptococci and Lactobacilli antagonistic activity has been reported to be linked to the plasmids, but no such report is available as far as leuconostocs are concerned.

The use of Leuconostoc cultures to prevent the spoilage of foods has many potentialities. They have been reported to prevent slime formation and proteolytic deterioration due to Pseudomonas spp., prevent defects caused by contaminants in cheese and extend shelf-life of meat products. Use of intact microbial cells, however, poses certain unavoidable problems like textures and odour problems and dextran formation (Teuber and Geis, 1981). Therefore, the separation of the inhibitory action from the cells would be an important step in allowing more purposeful application of these antimicrobials.

To locate the exact harbouring site of the inhibitory principle is all the same more important a pre-requisite to their separation Keeping these factors in view, the present study is a modest maiden attempt to -

i) Screen leuconostocs for their inhibitory effect against different pathogenic and spoilage organisms.

ii) find out the site of production and location of the antimicrobial activity of these lactic cocci, and

iii) look for plasmid linkage, if any, of their antimicrobial activity.

## 2.0 REVIEW OF LITERATURE

The chance isolation of first pure bacterial culture, Streptococcus lactis, from boiled milk by Joseph Lister in 1973 opened the way for isolating and identifying more and more pure cultures. This has finally resulted in the establishment of family Streptococcacea that includes Leuconostoc as one of the genera (Garvie, 1986). Although the most natural habitat of the lactic streptococci is vegetation, many have been isolated from milk and milk products. They, being apathogenic to man and animals, might enter into milk from exterior of the udder and from fodder. However, presently their most important habitat concerns the dairy industry as well as wine industries (Teuber and Geis, 1981).

Leuconostocs play essential role in many fermentations like those of sauerkrauts, cucumbers, whereby L. merenteroides initiates 'lactic fermentations'. wines where L.oenos causes malolactic fermentation; citric acid fermentation in butter, buttermilk, quarg and cheese by L.dextranicum and L.cremoris (i.e. L.citrovorum) to provide delicate flavour of diacetyl fermentation, fermentative removal of sucrose from eggs meant for drying, and also for the production of dextrans from sucrose on an industrial scale etc. (Teuber and Geis, 1981).

In the dairy industry, Leuconostocs are employed in single and mixed cultures for the production of all different kinds of cheeses, fermented milks, cultured butter and casein. These

cultures are desirable for the following changes :

1. Fermentation of lactose to lactate
2. Formation of aroma compounds
3. Ripening of cheeses.

Although, Leuconostocs are mainly known for flavour production, they have been reported to produce substances which have inhibitory effect on other microorganisms. These organisms often predominate in the successive cultures ~~for~~ (or metabiosis) of such fermented products resulting in a decline in many proteolytic, spoilage and pathogenic organisms (Frazier, 1967). These early fermentors are later succeeded by more acid tolerant lactobacilli, hence they play a significant role in the preservation of naturally fermented foods. Their preponderance may atleast partially be due to their production of certain selective antimicrobial substances (Marth and Hussong, 1963; Pinheiro et al; 1968; ~~1990~~).

Among the various antibacterial substances produced by leuconostocs, those of particular interest have hitherto been which inhibit the growth of several psychrotrophic microorganisms and thus prevent economic and quality losses. There are, however, reports on the feasibility of use of Leuconostoc organisms against other spoilage and pathogenic bacteria that may contaminate various foods including milk and fermented milk products. The scanty literature available on this aspect is reviewed under various subheadings :

## 2.1. INHIBITORY EFFECT OF METABOLITES

Not much progress has been made since Mather and Babel (1959) first put to practical use the inhibitory action of L.citrovorum in a creaming mixture against Pseudomonas fraqi, P. fluorescens and coliforms. Marth and Hussong (1963) summarized the then available reports of antibacterial substances, produced by these lactic acid bacteria. The work done in this aspect shows the inhibitory, effect of various leuconstocs or their culture filtrates on many strains of both pathogenic and saprophytic organisms like Enterobacter aeroqenes, E. coli, P.fraqi, P. fluorescens, but not yeasts (Marth and Hussong, 1963), Salmonella gallinarum, S.typhimurium, S.aureus (Sorrells and Speck, 1970). P.putrefaciens at pH below 8.0 (Oliveria, 1969/1970) and Shigella Sonnei, S.flexneri, S. newcastle (Grinewich, 1977)

However, some workers observed a bit conservative behaviour on the part of the leuconostocs towards test microorganisms as they inhibited a lesser number of latter. Commerci. multistrain cultures (lactic acid producing streptococci + L.cremoris) were found to be most effective in restricting psychrotrophic growth (Juffs and Babel, 1975; Go, 1977; Portridge, 1984).

Barnley, Smith et al. (1989) found ~~that~~ L. mesenteroides var. dextranicum (NCIB 8189) to possess greatest inhibitory activity with smallest pH drop among 120 strains of LAB studied. Concentration of inhibitory activity was achieved by freeze-drying of cell-free culture supernatants.

Counts of E.aerogenes and Klebsiella sp. were reduced to less than 10 cfu/ml after 4 days when grown in association with mesophilic lactic cultures (including Leuconostoc species; Reinheimer et al., 1990). However, they found E.coli to be the most resistant coliform bacterium to the inhibitory effect. The counts lower than 10 cfu/ml occurred only after 15 days. But, Salma (1986) found cell-free supernatants of skim milk cultures of L.dextranicum to be antagonistic TOWARDS E.coli. P.fluorescence, P.fragi, P.aeruginosa, P.cepacia, P. mallei and S.aureus. Maximum antimicrobial activity was shown against P.aeruginosa, followed by E.coli.

Some workers, on the other hand, did not find Leuconostoc spp. to have much of the antagonistic effect. Ross (1981) observed that P.aeruginosa was the only spoilage organism to be inhibited to any extent by LAB and inhibition by Leuconostoc spp. was such lower than lactic streptococci. Sixteen strains of Leuconostoc cremoris were studied for their antagonistic properties (Belova et. al, 1982) None of the strains were found to be highly antagonistic. However, thirteen strains exhibited medium antagonistic effect. Ferna' ndez et. al, (1984) on the other hand found that all of the ten strains of Leuconostoc spp. which they studied, either partially or wholly inhibited thirteen strains each of S.aureus, Salmonella (3 sero types) and Shigella (4 spp.)

## 2.2. NATURE OF ANTIMICROBIAL SUBSTANCES PRODUCED BY LEUCONOSTOCS

Attempts made to isolate the antimicrobial substances free of organisms have not been much successful. However, the cell-free spent medium, i.e. culture filtrate normally had potent inhibitory properties (Genske and Branen, 1973).

The inhibitory action of the spent medium is highest at low pH in the range of pH 3.5 to 6.0. The inhibitors are inactive at pH 7.0. Autoclaving at low or high pH does not destroy activity (Genske and Branen, 1973). Reinheimer et al., (1990) reported that factors responsible for antibiosis are, in general, thermostable and active at acid pH values.

### 2.2.1 PROBABLE ANTIMICROBIALS

It is now known that antimicrobial activity results from effects of a combination of biochemical compounds. Organic acids, $H_2O_2$ and other unidentified compounds contribute to inhibitory action.

Marth and Hussong (1962) observed a decrease in intensity of inhibition with an increase in pH. The production of organic acids as inhibitors of other microorganisms by citrate fermenting leuconostocs is supported by many other workers (Pinheiro et al, 1968; Genske and Branen, 1970; Oliveria, 1969; 1970; Sorrells and Speck, 1970; Branen et al. (~~and Genske~~), 1975; Ross, 1981; Barnley - Smith et al., 1989; Reinheimer et al., 1990).

A few workers have demonstrated that leuconostocs produce considerable amounts of $H_2O_2$ which inhibited the spoilage

organisms (Genske and Branen, 1979; Juffs and Babel, 1975; Reinheimer et al; 1990).

Some workers have indicated the presence of other inhibitory factors produced by leuconostocs besides hydrogen peroxide and organic acids (Pinheiro and Parmelee, 1968; Genske and Branen, 1979).

Mather and Babel (1959) reported that total psychrotrophic counts of cottage cheese samples were more effectively reduced by the fermented creaming mix than by the addition of acetic or propionic acids to the same pH level.

## 2.3 HARBOURING SITE OF ANTIMICROBIAL ACTIVITY

The antimicrobial potentiality of the Leuconostoc organisms has so far been explored and observed only in cell-free filtrates of the cultures (Marth and Hussong, 1962; Sorrells and Speck, 1970; Oliveria, 1969/ 1970; Genske and Branen, 1973 Salma, 1986; Reinheimer et al., 1990). Hence, the literature is silent about the exact location of the active principle in or on the cells. It seems that whole washed cells, shells (ghosts) of such cells or their sap (extract) have not been studied to locate the origin of the active ingredients other than organic acids or $H_2O_2$. This aspect surely needs to be investigated.

## 2.4. PLASMID LINKAGE OF ANTIMICROBIAL ACTIVITY

Plasmids have been a turning point in the development of in vitro genetic exchange systems for a number of bacteria. Properties that have been established to be linked to plasmid DNA in different lactic acid bacteria include lactose utilization

bacteriophage resistance, antagonistic properties, restriction/modification systems, resistance to inorganic ions, resistance to nisin and antibiotics, cell aggregation and UV protection. Though, quite a few of these traits have been found to be plasmid encoded in lactic strepotococci, fewer reports are available for lactobacilli but possibly none for leuconostocs. Nisin production in S.lactis was reported to to linked to a 28 M dal plasmid (Le Blanc et.al., 1980). Muriana and Klaen-hammer (1987) first confirmed plasmid control of bacteriocin production and immunity in L.acidophilus 88. No attempts have so far been reported regarding establishing the plasmid linkage of antimicrobial activity of Leuconostoc organisms.

### 2.4.1 CURING OF PHENOTYPES

Some of the important agents used for curing the organisms of plasmids include intercalating dyes, elevated temperature, antibiotics, agitation, U.V. radiation, freezing and thawing and protoplasting (Stanisch, 1984).

#### 2.4.1.1. ELEVATED TEMPERATURE

Use of sub-lethally high temperature incubation of lactic organisms has been reported by many workers. Kozak et al (1974) observed the appearance of Nis strains of S.lactis where $Nis^+$ strains were grown at elevated temperature of 40°C. An increase in $Nis^-$ colonies of S.lactis at 40°C to an extent of 1.4 to 2.0 folds was indivated by Fuchs et al (1975) also. Efstathiou and Mackay (1976) showed that $Lac^+$,

$prt^+$ cells of S.lactis $M_{18}$, $C_{10}$ and $ML_3$ gave rise to $Lac^-$, $prt^-$ cells when grown at 39°C. Similarly, S.cremoris strains were cured of diplococcin production when allowed to grow at 35.5°C for 16 hrs in $M_{17}$ broth (Davey and Pearce, 1980). Higher temperature (48-49°C) was used by Vescovo et al (1982) who found that Lactobacillus reuteri 109 lost all its 6 plasmids coding for antibiotic resistance. In another study, Lactoabcillus strain 100-37 was found to have lost its antagonistic phenotype against Clostridium ramosum (MaCormick and Savage, 1983). Recently, Muriana and Klaenhammer (1987) isolated four lactocin $F^-$ variants following several transfers of L.acidophilus in MRS broth at 30°C.

## 2.5 COMMERCIAL APPLICATIONS OF ANTIMICROBIAL ACTIVITY OF LEUCONOSTOCS

Mather and Babel (1959) developed a special creaming mixture to add and stabilize the diacetyl flavour in cottage cheese. The mixture consisted of three parts of cream and two parts of an acidified cheese, creamed with this mixture and inoculated with P.fragi or P.putrefaciens had less slime formation than control cheese and also lowered coliform counts.

Reddy et al (1970) applied these principles to meat by adding milk and frozen concentrated lactic cultures (S.lactis and L. citrovorum) to ground beef. The addition of 10 percent lactic culture significiantly inhibited the inherent gram negative bacteria in ground beef when held at refrigerated temperatures. Oliveria (1969, 1970) noticed that addition of either of S.diacetylactis or L.citrovorum to fresh salted or unsalted butter made from

cream that had been inoculated with P.putrifaciens prevented proteolytic deterioration even after 90 days at 7°C, whereas, untreated control samples (without cocci) showed proteolytic deterioration after 7-15 days. At 21°C, this fault was found in controls after only 2-4 days but not in treated samples after 15 days.

Belova et al. (1982) suggested that selection of strains antagonistic to E. coli could be employed in the preparation of culture for use in dairy industry, particularly in cheese manufacture to prevent defects caused by this contaminant.

## 2.6 LIMITATIONS

In all the above applications, intact Leuconostoc cells were added to the food produts to inhibit undesirable organisms. The use of these cells, however, limits the application of the antimicrobials to many foods because of the potential production of off-flavours and texture changes resulting from the growth of the cells. Genske and Branen (1973) showed that the addition of one percent of dried cell free medium will prevent the growth of both inherent and inoculated gram negative organisms in ground beef held at refrigerator temperatures. The addition of the medium gave some odour and flavour to the raw ground beef. Leuconostoc species have been isolated from a great variety of spoiled food also. Their tolerance to high sugar concentrations (upto 60% in L.mesenteroides) permits the organism to grow in syrups and ice-cream mixes. Leuconostoc is well known as a spoilage bacterium in sugar refineries. It is well adapted to growth in particular stages of sugar processing,

causing harmful economic effects in the industry. The growth of these microorganisms can result in significant losses of sucrose, corrosion due to acid production, and formation of dextran gums which generate physical troubles in the production process (Tillbury, 1975).

This makes the separation of antimictobials from leuconostocs an important aspect for use in food and does not exclude the direct use of the crude fractions of inhibitors. In the opinion of Genske and Branen (1973), the use of spent whey medium would be inexpensive to produce and would act as both a source of inhibitor and protein as well.

# 3.0 MATERIALS AND METHODS

## 3.1 ORGANISMS USED IN THE STUDY

### 3.1.1 LEUCONOSTOCS

Three cultures of Leuconostoc spp. were procured from National Collection of Dairy Cultures, Dairy Microbiology Division, National Dairy Reserach Institute, Karnal, These were -

Leuconostoc cremoris (NCDC-543)

L. mesenteroides (NCDC - 530)

L. dextranicum (NCDC - 529)

Besides these, six Leuconostoc strains obtained in a previous study (Monika, 1990) were also studied for their antimicrobial activity. These were -

| | Isolate No. | Tentatively identified to be |
|---|---|---|
| 1) | $LD_{10}$ | L. cremoris |
| 2) | $LH_g$ | L. dextranicum |
| 3) | $LM_a$ | L. dextranicum |
| 4) | $LD_3$ | L. lactis |
| 5) | $LH_b$ | L. mesenteroides |
| 6) | $LM_k$ | L. mesenteroides |

3.1.2. Inhibitory effect of the above Leuconostoc organisms was studied on the following 'test' microorganisms obtained from various sources -

| | 'Test' Organism | Sources |
|---|---|---|
| 1) | Bacillus cereus B-48 D. Merrell. | Prof. P.F. Bowntre, USA |
| 2) | E. Coli (745) | NCDC |
| 3) | P. aeruginosa | NCDC |

4) Proteus vulgaris NCDC

5) Salmonella typhi (KIGM) NCDC

6) Shigella dysentriae NCDC

7) Staphylococcus aureus (137) NCDC

8) Streptococcus faecalis (S30) NCDC

Besides, one more cultures Listeria monocytogenes was obtained from Mr.N.K.Goyal, who isolated this strain during his studies on incidence of this organism in milk.

## 3.2 MAINTENANCE OF CULTURES

### 3.2.1. LEUCONOSTOC CULTURES

Leuconostoc cultures were maintained on Reddy's basal agar stabs in screw capped test tubes. These pure organisms were used in the present study.

### 3.2.2 'TEST'CULTURES

Test cultures were maintained on BHI agar slants.

## 3.3. MEDIA, DILUENTS AND REAGENT SOLUTIONS

### 3.3.1 MEDIA

a) Reddy's basal Medium (Reddy et al. 1972)

Reddy's medium with little modification was used for propagation of Leuconstoc cultures. Its agar version was used for the organisms'maintenance. The composition of the medium used was:-

| | |
|---|---|
| Tryptone | - 5.0 g |
| Yeast extract | - 2.5 g |
| Casamino acids (Casein acid-hydrolysate) | - 5.0 g |
| $K_2HPO_4$ | - 1.25 g. |

Calcium citrate - 10.0 g

Carboxy methyl cellulose (CMC) - 15.0 g

Distilled water to make upto - 1000 ml

Agar (whenever gel was required) = 15.0 g

Calcium citrate, 10g and CMC, 15 g were suspended in a glass beaker containing 500 ml of distilled water. The contents were heated while constantly being stirred gently until a homogeneous, white, turbid suspension was formed. This suspension was then transferred to another vessel containing tryptone, 50g; yeast extract, 2.5g; casamino acids, 5.0 g and $K_2HPO_4$, 1.25 g. After adjusting the PH to 6.5 and adding agar, the medium was sterilized by autoclaving at 121°C for 20 minutes (at 1.1 atm. pressure).

b) BHI Broth.

Brain heart infusion (Hi Media) was reconstituted as per the manufacturer's directions as follows:

Brain heart infusion - 37.00 g

Agar (whenever gel was required) - 15.00 g

Distilled water to make upto - 1000 ml

The medium was suspended in screw capped test-tubes (5 ml quantity) and sterilized by autoclaving.

The 'test' microorganisms were passaged three times through BHI broth and maintained on BHI agar slants.

c) Reddy's Milk Medium

Skim milk powder, (spray dried), was reconstituted at 11 percent level and PH adjusted to 6.4. This reconstituted milk was then boiled for 10 minutes, cooled to room temperature, filtered and then sterilized by autoclaving.

This milk was then added at the rate of 10 percent to the flasks containing sterilized Reddy's basal medium.

d) Reddy's whey Medium

Cheese whey powder (spray dried) was reconstituted at 11 percent level and given the same treatment as was given to the reconstituted skim milk above.

The sterilized whey was then added at the rate of 10 percent to the flasks containing sterilized Reddys' basal medium.

e) Litmus Milk

| | |
|---|---|
| Skimmilk powder | -11.0 g/l |
| Litmus solution (sat.aq.) | -15.0 ml/l |

The medium was distributed in 10 ml quantities in test tubes and autoclaved.

f) Yeast Dextrose Milk (Oxoid formulation)

| | |
|---|---|
| Milk | - 100 ml |
| Yeast extract | - 0.5 g |
| Glucose | - 0.5 g |

The ingredients were mixed in 100 ml of milk which was dispensed in 10 ml quantities in test tubes and antoclaved.

g) Yeast Dextrose Broth (Oxoid formulation)

| | |
|---|---|
| Dextrose | - 0.5 g |
| Yeast extract | - 0.5 g |
| Peptone | - 0.5 g |
| Beef extract | - 0.5 g |
| DW | - 1000 ml |
| PH | - 6.8 - 6.9. |

The broth was dispensed in 10 ml quantities in test tubes and autoclaved.

The medium was supplemented with 3.0 or 6.5 percent NaCl wherever required.

h) Arginine Broth

Arginine hydrolysis was tested ~~observed~~ in the broth medium having the following composition:

| | |
|---|---|
| Tryptone | - 0.50 g |
| Dextrose | - 0.50 g |
| Yeast extract | - 0.05 g |
| Sod. acetate | - 2.00 g |
| $K_2HPO_4$ | - 0.10 g |
| Arginine hydrochloride | - 0.50 g |
| Bromocresol purple | - 0.10 ml of 1.5% alc. |
| Distilled water | - 100 ml |
| pH | - 6.2 |

The broth was distributed into ~~the~~ tubes and autoclaved.

i) Carbohydrate Fermentation Medium

The basal medium had the following composition:-

| | |
|---|---|
| Beef extract | - 1.0 g |

| | |
|---|---|
| Peptone | - 10.0 g |
| NaCl | - 5.0 g |
| Phenol red | - 0.01 g |
| Distilled water | - 100 ml |

The medium was distributed in 9 ml quantities in clean test tubes and sterilized by autoclaving. To each of the tubes was added one ml of the Seitz filtered solution of a fermantable carbohydrate. The individual carbohydrates tested were-arabinose fructose, maltose, lactose, sucrose, mannitol, cellobiose, glucose and galactose.

j) Nutrient Agar

| | |
|---|---|
| Tryptone | - 5.0 g |
| Beef extract | - 3.0 g |
| NaCl | - 5.0 g |
| Distilled water | - 1000 ml |
| Agar | - 15.0 g |
| PH | - 7.0 |

The medium was sterilized by autoclaving.

k) Tryptose nutrient agar (TNA): Nutrient agar was eneriched with 1.5% tryptose to support the growth of L.monocytogenes.

3.3.2 DILUENTS

a) Physiological Saline

| | |
|---|---|
| NaCl | - 9.0 g |
| Distilled water | - 1000 ml. |

The saline was sterilized by autoclaving

b) Phosphate Buffered Saline, PBS

| | |
|---|---|
| $KH_2PO_4$ | - 3.4 g |
| NaCl | - 0.9 g |
| Distilled water | - 100 ml. |

## 3.4 TESTING THE PURITY OF LEUCONOSTOC ISOLATES

The purity of the isolates was off and on checked by the criteria suggested by Garvie (1986). The cultures were grown in Reddy's basal medium and used as inoculum for these tests.

### 3.4.1 MICROSCOPIC EXAMINATION

All the Leuconostoc cultures were examined microscopically using gram staining technique for cellular shape, size and arrangement.

### 3.4.2 CATALASE TEST

Two ml of aliquots of young Leuconostoc cultures grown in Reddy's basal medium were transferred to clean test tubes to which 0.5 ml of 3 percent $H_2O_2$ was added and examined for gas production for upto 30 min. lack of effervesence indicated no contamination with catalase positive organisms.

### 3.4.3 GROWTH IN LITMUS MILK

A loopful of Leuconostoc cultures was inoculated into separate litmus milk tubes and incubated at 25-30°C for 24 h. Change in colour, curdling and proteolysis were noted.

Change in colour from blue to pink with or without coagulation indicated growth of the organisms.

### 3.4.4 GROWTH IN YEAST GLUCOSE MILK

Yeast glucose milk was inoculated with one loopful of Leuconostoc cultures and incubated at 25-30°C for 24 h. Acid production evidenced by coagulation and reduction of colour indicated growth of organisms.

### 3.4.5. GROWTH IN 3.0 AND 6.5 PERCENT NaCl

Yeast dextrose broth with 3 percent and 6.5 percent NaCl were separately inoculated with Leuconostoc cultures and incubated at 25-30°C for 24 h. Growth was indicated by appearance of turbidity.

### 3.4.6. GROWTH AT DIFFERENT TEMPERATURES

~~The~~ Leuconostoc cultures were inoculated in yeast dextrose broth and incubated at -

10°C for 48 h,

22°C for 48 h,

37°C for 24 h, and

45°C for 24 h.

Appearance of turbidity showed the occurrence of growth.

### 3.4.7 PRODUCTION OF AMMONIA FROM AGRININE

Arginine broth tubes were inoculated with the flavour organisms. After incubation at 25-30°C for 24 h, one ml of Nessler's reagent was added to one ml of each of the resultant cultures in a clear test tube. Production of ammonia from arginine was indicated by the development of orange colour, absence of which showed that the culture didn't hydrolyse arginine.

### 3.4.8 CARBOHYDRATE FERMENTATION

Individual sugar broth tubes were inoculated with Leuconostoc cultures and incubated at 25-30°C for 24 h. Appearance of turbidity with or with out the development of pink colour indicated a positive reaction.

## 3.5 TESTING THE ANTIBACTERIAL ACTIVITY

### 3.5.1 TREATMENT OF LEUCONOSTOC CELLS

All the cultures to be tested for antibacterial activity were inoculated in three different media -

i) Reddy's basal medium

ii) Reddy's milk medium

iii) Reddy's whey medium

and incubated at 27°C for 36 h. A small portion of each of these resultant cultures(i.e.C)was taken out for testing as such. Rest of the culture was centrifuged at 2500 rpm for 20 min. The centrifugate was Sietz filtered and stored as the cell free culture filtrate (CF). The cell pellet obtained after centrifugation was washed two times with phosphate buffered saline before finally suspending the cells in PBS and labelled as (CS). A portion of this was sonicated at 20,000 cycles per minute. The resulting cell debris (CD) and cell sap/extract (CE) were stored in refrigerator alongwith the products of earlier treatments and all were tested for their anitmicrobial activity as per Fig.1.

### 3.5.2. LOCATING THE SEAT OF ANTIMICROBIAL COMPONENT

The well method of Cruickshank (1970) with some modifications was used to determine antimicrobial activity of Leuconostoc organisms. The 'test' cultures were adjusted to 0.3 O.D. and were then mixed with nutrient agar(TNA for L.monocytogenes) at the rate of 1-2 percent. The inoculated agar was poured into sterile petridishes to a thickness of about 4-6 mm and allowed to set. After incubation at 35°C for 2 h. the plates were transferred into refrigerator for hardening of agar for

15 minutes. Wells of 8 mm diameter were made in the agar. The wells were charged with 0.1 ml of previously prepared Leuconostoc cells and their products from different treatments as in Fig.1. The plates were reincubated at 30°C for 24 h. and observed for the appearance of zone of clearance around the wells. Diameters of the clearance zones, if any, were measured to determine the inhibitory effect.

3.5.3. DETERMINATION OF ROLE OF EXTRACHROMOSOMAL DNA

Curing experiments were performed as per the method of.

Young Leuconostoc cultures in Reddy's basal medium, Reddy's milk medium ~~and Reddy's~~ and Reddy's whey medium (27°C for 18 - 24 h) were reincubated at 40°C for 18 h. after which these were tested for antibacterial activity. The positive samples, if any, were reinoculated into fresh media as usual and tested for their inhibitory effect as described previously.

## TESTING THE ANTIBACTERIAL ACTIVITY

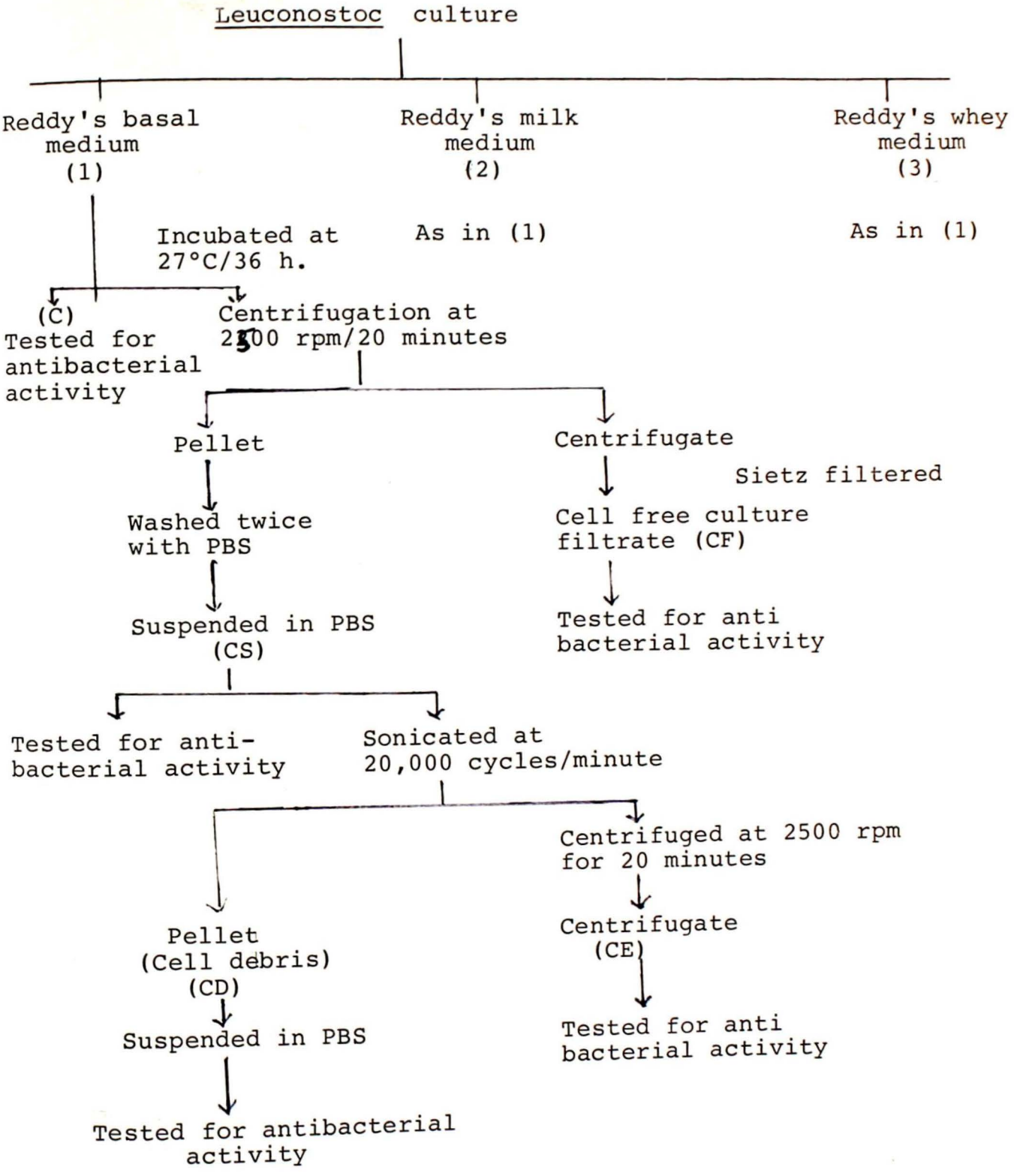


Fig.1 : Schedule for testing the antimicrobial activity of leuconostocs.

CURING EXPERTIMENTS

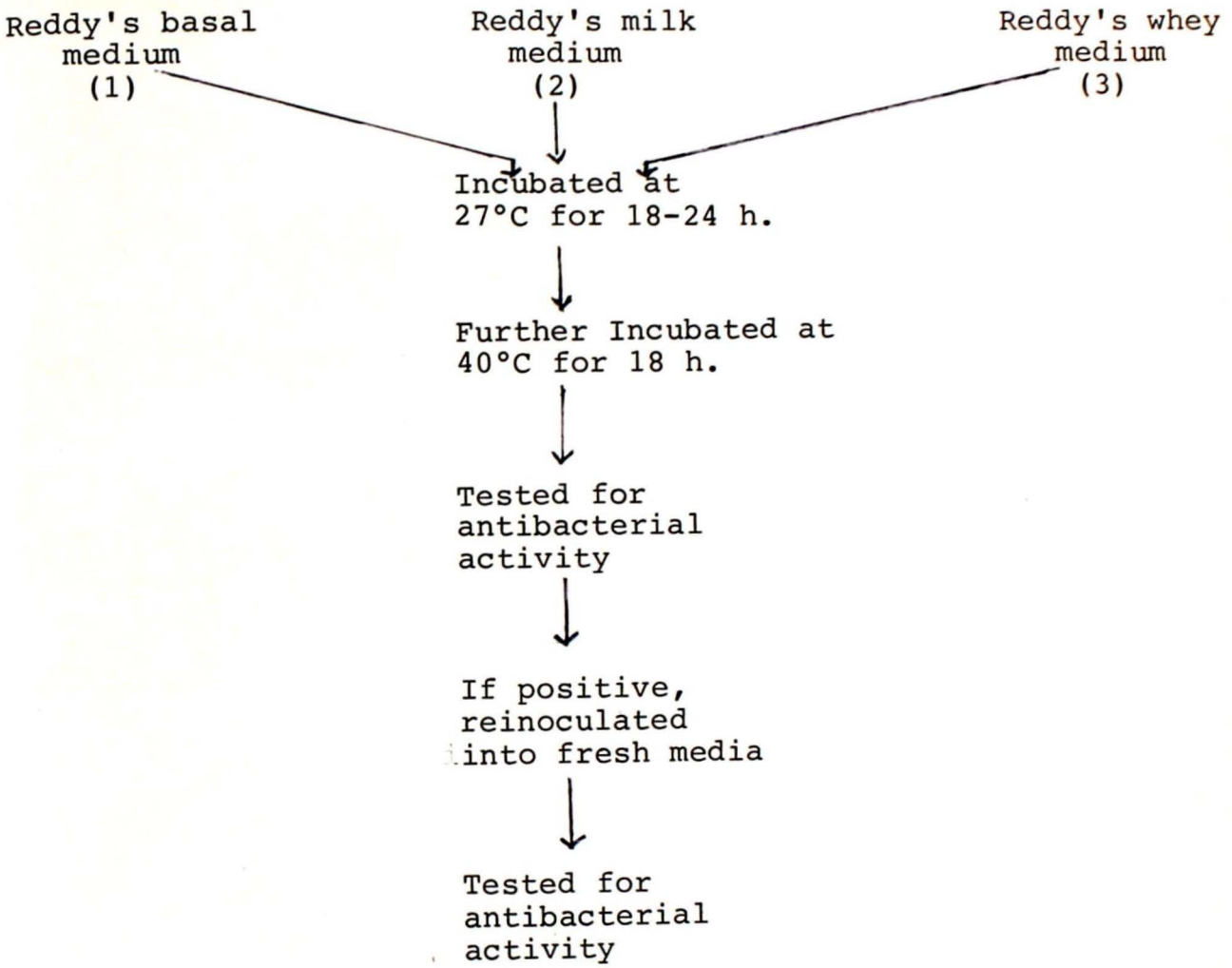


Fig.2: Flow diagram depicting the procedure for curing experiments.

# 4.0 RESULTS AND DISCUSSION

## 4.1 MAINTENANCE OF LEUCONOSTOC CULTURES

Different workers have used various media for isolation and maintenance of leuconostocs. These include MRS fermentation medium (deMan et al, 1960), glucose yeast extract agar (Whittenbury, 1965), acidic tomato broth (Garvie, 1967), differential agar for aroma producing cocci (Reddy et al, 1972), citrate differential agar (Kempler and McKay, 1980) and modified MRS agar (Salma, 1986). Monika (1990) found that isolation and maintenance of these organisms was best accompalished on the differential medium for aroma producing cocci (herein after called Reddy's basal medium) with certain modifications and the medium was made specific by the addition of doxycyline at a final concentration of 0.13 ug/ml of the medium to inhibit the growth of streptococci and lactobacilli. Since the present study was on pure Leuconostoc cultures, Reddy's basal medium was used without doxycycline for the growth and maintenance of these cultures. For the same reason arginine hydrochloride was also deleted from the medium proposed by Monika (1990). Substantial growth was obtained in this modified Reddy's basal medium after incubation at 30°C for 36 h. and it was comparable to that obtained in the original supplemented medium, (Table 1). Leuconostocs are known to grow optimally at 22-30°C in 36 h. (Reddy et al 1972)

## 4.2 CHECKING THE PURITY OF LEUCONOSTOC CULTURES

The purity of Leuconostoc strains used in the present study was initially and occasionally ascertained by some selected tests as proposed by Garvie (1986). These tests and their results have been illustrated in Table 2. The isolates

*Table - 1* Growth of Leuconostocs at different temperatures in Reddy's basal medium with and without arginine hydrochloride and doxycycline

| Incubation Temperature (°C) | Medium | Strains | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 529 | 530 | 543 | $LD_{10}$ | LMg | Lma | $LD_3$ | LHb | LMk |
| 22 | Basal | + | + | + | + | + | ++ | + | + | ++ |
| | Supplemented | + | + | + | + | + | ++ | + | + | ++ |
| 30 | Basal | ++ | ++ | + | + | ++ | ++ | + | + | + |
| | supplemented | + | + | + | + | + | + | + | + | + |
| 37 | Basal | + | + | + | + | + | + | - | + | + |
| | Supplemented | + | + | + | + | + | + | - | + | + |

NOTE : The positive signs denote only the comparative growth in the two types of media; these don't compare growth at different temperatures.

were found to be pure and retained their activity in terms of results obtained by Monika(1990).

## 4.3 PROPAGATION AND HARVESTING OF LEUCONOSTOCS FOR STUDYING THEIR ANTIMICROBIAL ACTIVITY.

Skim milk or whey have been reported to be good media for production of inhibitory activity by Leuconostoc spp. Branen et al.,(1975) reported 5 percent spray dried whey supplemented with 0.5 percent yeast extract as an excellant medium for the production of antimicrobial activity. Salma (1986) used plain skim milk and observed good amount of antimicrobial activity of some of Leuconostoc strains; however, she cultivated these organisms with lactic strepto - cocci. However, Jenske and Branen (1973) recommended either skim milk or whey supplementation. Therefore, supplementation with whey or skim milk was studied in the present investigation the results of which have been reported in appropriate sections.

## 4.4 TESTING OF ANTIMICROBIAL ACTIVITY OF THE LEUCONOSTOCS

Varies spoilage and pathogenic organisms were grown on nutrient agar (Salma, 1986). Specific media for such test organisms were not used because of the apprehension that these might not support or rather inhibit in general the growth and activity of Leuconostoc cultures as happens with the media made selective for leuconostocs. (Teuber and Geis 1981; Monika, 1990). For the growth of L.monocytogenes nutrient agar was supplemented with tryptose which is an essential nutrient for its growth (Seeliger and Jones, 1986).

*Table 2* : *Checking the purity of* Leuconostoc isolates by morphological and biochemical characteristi

| Character | Strains | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 629* | 530* | 543* | LD10 | LMg | LMa | LD3 | LMb | LMk |
| I. Morphological | | | | | | | | | |
| Shape | S | S | S | L,S | S | L | L | L | S |
| Arrangement | P | P | LC | LC | SC | P | SC | P | SC |
| Gram reaction | + | + | + | + | + | + | + | + | + |
| II. Cultural | | | | | | | | | |
| i) Growth in 3.0% NaCl | d | + | + | + | d | d | + | + | + |
| 6.5% NaCl | - | d | - | - | - | - | d | d | d |
| ii) Growth at | | | | | | | | | |
| 45°C | - | - | - | - | - | - | - | - | - |
| 22°C | + | + | + | + | + | + | + | + | + |
| 10°C | + | - | + | + | + | + | + | - | - |
| III. Biochemical | | | | | | | | | |
| i) Catalase | - | - | - | - | - | - | - | - | - |
| ii) Litmus milk reduction | + | fr | fr | + | fr | + | fr | fr | fr |
| acid clot | - | - | - | - | - | - | - | - | - |

*Contd.., Table 2*

| Character | Strains | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 520* | 530* | 543* | LD10 | LHg | LMa | LD3 | LHb | LMk |
| iii) Yeast dextrose milk | | | | | | | | | |
| Reduction | d | d | d | d | - | d | - | - | - |
| Acid clot | d(w) | d(w) | d(w) | d(w) | d(w) | d(w) | d(w) | d(w) | d(w) |
| Gas | - | - | - | - | - | - | - | - | - |
| iv) $NH_3$ from Original | - | - | - | - | - | - | - | - | - |
| IV. Acid production from carbohydrates (at 25°C/24h) | | | | | | | | | |
| Arabinose | + | + | - | - | - | - | + | + | + |
| Fructose | + | + | - | - | + | + | + | + | + |
| Galactose | +,- | + | + | + | + | +,- | + | + | + |
| Glucose | + | + | + | + | + | + | + | + | + |
| Cellobiose | + | +,- | - | - | +,- | +,- | - | + | + |
| Lactose | + | d | + | + | + | + | - | d | - |
| Maltose | + | + | +,- | +,- | + | + | + | + | + |
| Mannitol | + | + | - | - | + | + | + | + | + |
| Sucrose | + | + | - | - | + | + | + | + | + |

Legend : * - Reference cultures obtained from NCDC, Karnal; S-Spherical; L-Lenticular; P-Pairs; L-Long Chains; S-Short Chains; d-Delayed reaction accuring in 40-48h; (w) - Weak acid clot; fr - Faint reaction

\+ - positive reaction

\- - negative reaction

## 4.5 INHIBITORY EFFECT OF LEUCONOSTOCS

### 4.5.1 SPECTRUM OF ANTIMICROBIAL ACTIVITY

Seven out of nine Leuconostoc cultures showed antibacterial effect on various spoilage and pathogenic organisms. As presented in tables 3,4 & 5 maximum number of strains i.e. 543, LMa, LMk, LHg, LD3 were antagonistic to S.faecalis with strain LMk elaborating maximum activity against this 'test' organisms. Our results are at variance with the findings of Genske and Branen (1973) who observed no inhibition of S.faecalis by L.citrovorum. These workers also reported inhibition of P.aeruginosa by L.citrovorum (L.cremoris). However, Leuconostoc strain 543 used in the present study did not affect P.aeruginosa. On the other hand L.dextranicum strain 529 adversely affected the growth of this test organism. These results are in agreement with those of Ross (1981) and Salma (1986) who found that L.dextranicum strain 529 inhibited P.aeruginosa. The results presented in tables 9-11 are in agreement with those of Ferna'ndez et al (1984) and Salma (1986) who noted inhibition of S.aureus by L.citrovorum. S. aureus in the present investigation was found to be inhibited by Leuconostoc strain LD10 (table 9-11) which had tentatively been found to be L.cremoris. Salma (1986) reported inhibition of S.aureus by L.dextranicum also, which on the contrary was not observed in the present study.

Different strains of Leuconostoc spp. failed to produce any demonstrable inhibition of other 'test' organisms including E.coli, B.cereus, L.monocytogenes, P.vulgaris,

Table 3 : Effect of Leuconostoc strains grown in Reddy's basal medium on the growth of S. faecalis S30

| Leuconostoc culture | Leuconostoc culture products (Diameter (cm) of inhibition zone) | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| 529 | NE | NE | | | |
| 530 | NE | NE | | | |
| 543 | 1.0-1.3 (1.1) | 1.1-1.4 (1.2) | | | |
| $LD_{10}$ | NE | NE | | | |
| L Hg | 1.1-1.2 (1.1) | 1.3-1.6 (1.5) | | | |
| L Ma | 1.2-1.5 (1.4) | 1.2-1.6 (1.5) | | NE | |
| $LD_3$ | 1.3-1.5 (1.4) | 1.4-1.5 (1.55) | | | |
| LHb | NE | NE | | | |
| LMk | 1.5-1.7 (1.6) | 1.6-1.8 (1.7) | | | |

NE - No effect observed

Figures in parentheses denote averages of three trials

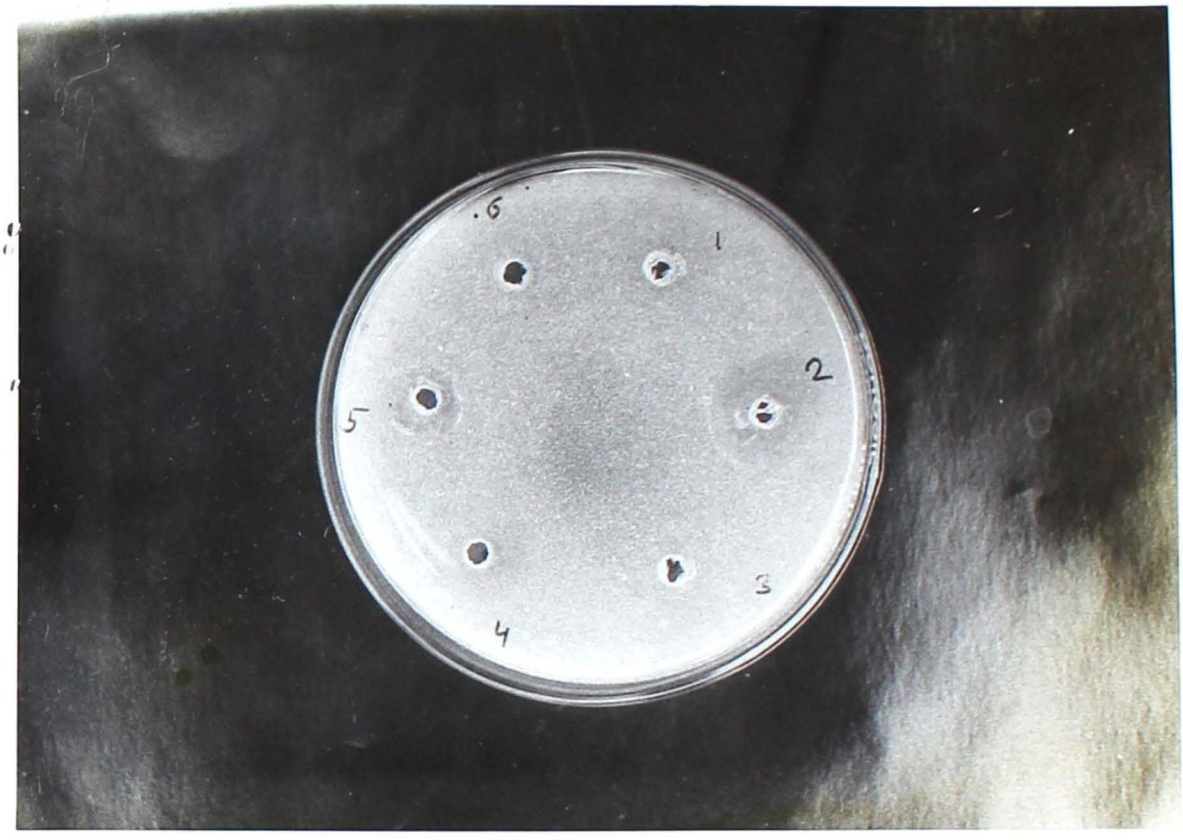


Plate 1 : Showing the effect of Leuconostoc cultures grown in Reddy's milk medium on the growth of S. faecalis S 30,1-530, 2-LMR, 3 - 529, 4 - LMa, 5 - $LD_3$, 6 - medium.

S.typhi and S.dysentriae as was evident from the abscence of inhibitory zones.

### 4.5.2 EFFECT OF MEDIUM ON ANTIMICROBIAL ACTIVITY

Tables 3,4 and 5 present the antibacterial effect of leuconostocs on S.faecalis when grown in RBM, RMM and RWM respectively. As is evident from the diameters of inhibition zones, supplementation with skim milk or whey didn't make any appreciable difference in the inhibitory effect of leconostocs whose growth in RBM showed marginally larger zones. However supplimentation of RBM with whey slightly increased the antibacterial effect of Leuconostoc strains 529 and LD10 against P.aeruginosa and S.aureus, respectively (tables 6-11).

This indicates that not only the production of antimicrobials is an inherent property of these lactic cocci, but also it is not much affected by the presence of nutrients in the medium. However, earlier workers found that supplementation of basal medium with skim milk or whey enhanced the potentiality of leuconostocs (Branen et al, 1975; Genske and Branen, 1973), the present study did not corroborate the same.

### 4.5.3 HARBOURING SITE OF ANTIMICROBIAL FACTOR(S)

Results depicted in tables 3-11 have indicated that antimicrobial factors were elaborated extracellularly and hence detectable in the growth menstruum. The whole culture (i.e.C) of the positive leuconostocs produced inhibition zones of diameter 1.0 to 1.6 cm on the susceptible three test organisms. Besides only cell free Seitz filterates (CF; zone dia 1.1-1.7cm),

Table 4 : Effect of Leuconostoc strains grown in Reddy's milk medium on the growth of S. faecalis S30

| Leuconostoc culture | Leuconostoc culture products | | | |
|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | |
| 529 | NE | NE | | |
| 530 | NE | NE | | |
| 543 | 1.0-1.3 (1.2) | 1.1-1.3 (1.2) | | |
| $LD_{10}$ | NE | NE | | |
| LHg | 1.0-1.1 (1.0) | 1.2-1.5 (1.4) | | |
| LMa | 1.3-1.4 (1.4) | 1.1-1.5 (1.4) | | NE |
| LD3 | 1.1-1.4 (1.3) | 1.2-1.4 (1.4) | | |
| LHb | NE | NE | | |
| LMk | 1.3-1.6 (1.5) | 1.4-1.7 (1.6) | | |

NE - No effect observed

Figures in parentheses denote averages of three trials

Table 5 : Effect of Leuconostoc strains grown in Reddy's whey medium on the growth of S. faecalis S30

| Leuconostoc culture | Leuconostoc culture products | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | | |
| 529 | NE | NE | | | |
| 530 | NE | NE | | | |
| 543 | 1.1-1.3 (1.2) | 1.1-1.3 (1.2) | | | |
| $LD_{10}$ | NE | NE | | | |
| LHg | 1.2-1.6 (1.5) | 1.3-1.5 (1.45) | | NE | |
| LMa | 1.2-1.4 (1.35) | 1.3-1.6 (1.4) | | | |
| $LD_3$ | 1.3-1.5 (1.4) | 1.4-1.6 (1.55) | | | |
| LHb | NE | NE | | | |
| LMk | 1.4-1.5 (1.45) | 1.5-1.55 (1.5) | | | |

NE - No effect observed

Figures in parentheses denote averages of three trails

ghosts or debris of broken cells (CD; zone dia 1.2-1.8 cm) and washed cells suspended in PBS (CS;zone dia 1.2 - 1.6 cm) showed such effect for different test organisms. In no case could the activity be detected in the cell sap or extract (CE) of any of the leuconostocs.

4.5.3.1 Factors against S.faecalis

Five Leconostoc cultures (tables 3-5) exclusively inhibited S.faecalis (Zone dia 1.0 - 1.6 cm) and the culture filterates of the same organisms had similar activity, which was occasionally marginally higher (zone dia. 1.2 - 1.7 cm). The rest three treatments of leuconostocs did not produce any demonstrable inhibition of S.faecalis. No preparation of other Leconostoc strains i.e. 529, 530, LHb and LD10 showed any effect on the growth of S.faecalis.

4.5.3.2 Factor against P.aeruginosa

Only one Leconostoc strain 529 was found to be inhibitory to P.aeruginosa (tables 6-8). However, different preparations of strain 529 differed in their activity with changed media. In RBM the CF showed the maximum activity (Zone dia 1.35 cm) and the cell debris followed it very closely (zone dia 1.33cm) This indicated that the active principle was not fully liberated into the medium but almost a similar quantity was also held bound to the cell wall. Furthermore, washed cells also possessed same activity as that of whole culture (Zone dia 1.3 cm) indicating that the active principle might be embedded deep in the outer surface layers of the wall. Almost similar

Table 6 : Effect of Leuconostoc strains grown in Reddy's basal medium on the growth of P. aeruginosa

| Leuconostoc culture | Leuconostoc culture products | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | | |
| 529 | 1.2-1.4 (1.3) | 1.3-1.5 (1.35) | 1.2-1.5 (1.3) | 1.2-1.35 (1.33) | NE |
| 530 | | | | | |
| 543 | | | | | |
| $LD_{10}$ | | | | | |
| Lhg | | NE | | | |
| LMa | | | | | |
| $LD_3$ | | | | | |
| LHb | | | | | |
| LMk | | | | | |

NE - No effect observed

Figures in parentheses denote averages of three trials

Table 7 : Effect of Leuconostoc strains grown in Reddy's milk medium on the growth of P. aeruginosa

| Leuconostoc culture | Leuconostoc culture products | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | | |
| 529 | 1.1-1.4 (1.3) | 1.2-1.55 (1.4) | 1.0-1.3 (1.2) | 1.0-1.4 (1.3) | NE |
| 530 | | | | | |
| 543 | | | | | |
| $LD_{10}$ | | | | | |
| LMg | | NE | | | |
| LMa | | | | | |
| $LD_3$ | | | | | |
| LHb | | | | | |
| LMk | | | | | |

NE : No effect observed

Figures in parentheses denote averages of three trials

Table 8 : Effect of Leuconostoc strains grown in Reddy's whey medium on the growth of P. aeruginosa

| Leuconostoc culture | Leuconostoc culture products | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | | |
| 529 | 1.3-1.6 (1.4) | 1.3-1.4 (1.35) | 1.1-1.3 (1.25) | 1.1-1.3 (1.2) | NE |
| 530 | | | | | |
| 543 | | | | | |
| $LD_{10}$ | | | | | |
| LMg | | | | | |
| LMa | | NE | | | |
| $LD_3$ | | | | | |
| LHb | | | | | |
| LMk | | | | | |

NE - No effect observed

Figures in parentheses denote averages of three trials

hypothesis could be explained for RMM and RWM, the only difference being that these included an increased liberation of the inhibitory principle from cell wall into the spent medium as was seen from the inhibition zone diameters.

4.5.3.3. Factor against S.aureus

A major departure from the above findings was noted with S.aureus as 'test' organism. Leuconostoc strain LD10 whole culture, washed cells and broken cells exhibited constantly increasing inhibitory effect from 1.4cm to 1.75cm zone dia. in the three media (tables 9-11). The particular active principle seemed to be linked, firmly bound and embedded in the cell wall because of its conspicuous abscence from the cell free spent medium and cell extract.

Although antagonistic effect of culture filterates of leuconostocs is in close proximity with the reports of other workers (Marth and Hussong, 1962; Sorrells and Speck, 1970; Oliveria, 1969/1970; Genske and Branen, 1973; Salma,1986, Reinheimer, 1990), the present work supplements the field with a new feature indicating that the antimicrobial principle may not only be some metabolic product of the organism, but also some complex compound attached to and be a part of the outside of S.aureus cell wall. Furthermore the metabolite may be partly excreted and partly get bound to the outer surface layers of the cell wall.



Another important observation of this study was that one Leuconostoc strain was found to be inhibitory towards only one 'test' organism. This indicates that inhibition may be


Plate 2 : Showing the effect of Leuconostoc cell products of Strain $LD_{10}$ in Reddy's whey medium on the growth of S. aureus 137.

A, B - Washed cell suspension

C, D - Sonicated cell debris

E, F - Cell sap

Table 9 : Effect of Leuconostoc strains grown in Reddy's basal medium on the growth of S. aureus 137.

| Leuconostoc culture | Leuconostoc culture products | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | | |
| 529 | | | | | |
| 530 | | NE | | | |
| 543 | | | | | |
| $LD_{10}$ | 1.3-1.6 (1.4) | NE | 1.4-1.6 (1.5) | 1.6-1.8 (1.75) | NE |
| LHg | | | | | |
| LMa | | | | | |
| $LD_3$ | | NE | | | |
| LHb | | | | | |
| LMk | | | | | |

NE - No effect observed

Figures in parenthesis denote averages of three trials

Table 10 : Effect of Leuconostoc strains grown in Reddy's milk medium on the growth of S. aureus 137.

| Leuconostoc culture | Leuconostoc culture products | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | | |
| 529 | | | | | |
| 530 | NE | | | | |
| 543 | | | | | |
| $LD_{10}$ | 1.3-1.5 (1.4) | NE | 1.5-1.6 (1.45) | 1.6-1.8 (1.7) | NE |
| LHg | | | | | |
| LMa | | | | | |
| $LD_3$ | | NE | | | |
| LHb | | | | | |
| LMk | | | | | |

NE - No effect observed

Figures in parentheses denote averages of three trials

Table 11 : Effect of Leuconostoc strains grown in Reddy's whey medium on the growth of S. Aureus 137.

| Leuconostoc culture | Leuconostoc culture products | | | | |
|---|---|---|---|---|---|
| | C | CF | CS | CD | CE |
| | (Diameter (cm) of inhibition zone) | | | | |
| 529 | | | | | |
| 530 | | NE | | | |
| 543 | | | | | |
| $LD_{10}$ | 1.4-1.5 (2.45) | NE | 1.4-1.7 (1.6) | 1.6-1.8 (1.75) | NE |
| LHg | | | | | |
| LMa | | | | | |
| $LD_3$ | NE | | | | |
| LHb | | | | | |
| LMk | | | | | |

NE - No effect observed

Figures in parentheses denote averages of three trials

affected by various factors including the chemical nature of the 'test' organism as well as that of Leuconostoc strains and other physicochemical forces taking part in the phenomenon.

## 4.6. STIMULATORY EFFECT ON THE GROWTH OF S.DYSENTRIAE

Besides the antibacterial effect of leuconostocs on the aforementioned organisms a peculiar phenomenon also was observed. S.dysentriae showed extensive growth around the wells which were charged with culture and culture filterates of strains 529, LMk and LHg. This may be due to some metabolic product of these Leuconostoc strains which instead of being inhibitory rather turned out to be growth promoting for S.dysentriae. It infers that an utmost care has to be taken when fermentation is carried out with the help of Leuconostoc sp. There are chances of extensive growth of Shigella in case such contamination, which may result in higher incidences of food poisoning This aspect, however, needs to be investigated further to identify the factor(s) which supported the growth of S.dysentriae

## 4.7 PLASMID LINKAGE OF ANTIMICROBIAL ACTIVITY.

For curing the lactic acid bacteria of their plasmids, different workers used a wide range of sublethally higher incubation temperatures. Generally, a temperature of around 40°C has been used for S.lactis and other lactic cocci (Kozak et al, 1974; Fuchs et al, 1975) for curing them of their nisin/diplococcin production and lactose utilization coding plasmids and a temperature range of 47-49°C for lactobacilli (Vescovo et al, 1982; MaCormick and Savage, 1983)

Table 12 : Summary of the inhibitory effect of *Leuconostoc* spp. on spoilage and pathogenic batcteria in Reddy's basal and supplemented media.

| Medium | *Leuconostoc* strain | 'Test' organism | Leuconostoc cutlure products | | | | |
|---|---|---|---|---|---|---|---|
| | | | C | CF | CS | CD | CE |
| | | | (Diameter (cm) of inhibition zone) | | | | |
| RM | 529 | *P.aeruginosa* | 1.3 | 1.35 | 1.3 | 1.33 | NE |
| RMM | | | 1.3 | 1.4 | 1.2 | 1.3 | |
| RWM | | | 1.4 | 1.35 | 1.25 | 1.2 | |
| RM | $LD_{10}$ | *S.aureus* | 1.4 | NE | 1.5 | 1.75 | NE |
| RMM | | | 1.4 | NE | 1.45 | 1.7 | |
| RWM | | | 1.45 | NE | 1.6 | 1.&5 | |
| RM | 543 | *S.faecalis* | 1.1 | 1.2 | | | |
| RMM | | | 1.1 | 1.2 | | | |
| RWM | | | 1.1 | 1.2 | | | |
| RM | LHg | *S.faecalis* | 1.1 | 1.5 | | | |
| RMM | | | 1.0 | 1.4 | | | |
| RWM | | | 1.5 | 1.45 | | | |
| RM | LMa | *S.faecalis* | 1.4 | 1.45 | | | |
| RMM | | | 1.4 | 1.4 | | | |
| RWM | | | 1.35 | 1.4 | | NE | |
| RM | $LD_3$ | *S.faecalis* | 1.4 | 1.55 | | | |
| RMM | | | 1.3 | 1.4 | | | |
| RWM | | | 1.4 | 1.55 | | | |
| RM | LMk | *S.faecalis* | 1.6 | 1.7 | | | |
| RMM | | | 1.5 | 1.6 | | | |
| RWM | | | 1.45 | 1.5 | | | |

NE - No effect observed

for knocking out their plasmids coding for their antibiotic resistance and antagonistic characterstics. Considering these facts, the temperature of 40°C was selected for curing leuconostocs of any possible plasmids linked with their antimicrobial activity.

Incubation of young Leuconostoc cultures at 40°C for 18 h. did not cause any loss of their inhibitory effect towards the three suceptible 'test' organisms as was evident from their usual zones of inhibition. This indicated that antimicrobial activity of the Leuconostoc strains used was not linked to any extrachromosomal DNA. Orberg et al. (1984) demonstrated that Leuconstoc strain PO184, was resistant to vancomycin, sulfa-thiazole and trimethoprim and showing antibacterial activity against S.cremoris strain U134. Although they reported the occurence of four different plasmids in this strain, they didn't mention if these traits were plasmid linked. The present study also could not establish any link between antimicrobial activity of the Leuconostoc strains used and some prospective plasmids, if any, carried by these organisms under the conditions of investigation using elevated growth temperature. Davey and Pearce (1980) incubated S.cremoris at 35.5°C and could cure the organisms of their plasmid that coded for the production of diplococcin. Therefore, a growth temperature of 40°C used in the present study was selected so that the antibacterial Leuconostoc organisms were not killed and at the same time should get cured of plasmids, if any, linked with their antibacterial activity.

## 5.0 SUMMARY AND CONCLUSION

1. The present study was undertaken with a view to screen some Leuconostoc isolates for their antimicrobial potential and determining the site of production and location of such active principle(s) against certain spoilage and pathogenic bacteria.

2. The nine isolates included L.dextranicum (strains 529, LMk and LHg), L.mesenteroides (strains 530, LMk and LHb), L.lactis strain LD3 and L.cremoris (L.citrovorum) strains 543 and LD10 obtained from National Collection of Dairy Cultures, NDRI,Karnal and from a previous study.

3. Nine test organisms used in the study procured from different sources were - B.cereus B-48 D.Merrell, E.coli 745, L.monocytogenes, P.aeruginosa, P.vulgaris, S.faecalis S30, S.typhi KIGM, S.dysentiriae and S.aureus 137.

4. The antibacterial activity of Leuconostoc cultures,after growing them in Reddy's basal broth medium and that supplemented with skim milk or whey, their culture filterates, washed cells, sonicated cell debris and cell sap was tested by the agar well technique on different test organisms using nutrient agar (or NA) supplemented with tryptose (for L.monocytogenes).

5. In all seven Leuconostoc strains exihibited antibacterial activity. Five of them (strains 543, LD3, LHg, LMa and LMk) were inhibitory to S.faecalis strain 529 to P.aeruginosa and strain LD10 to S.aureus. Evidently Leuconostoc strains 530 and LHb did not affect the growth of any of the test organisms adversely. Strain LM

produced the widest zone among the positive leuconostocs.

6. Supplementation of Reddy's basal medium with skim milk or whey did not make much difference in the inhibitory potentials of the leuconostos.

7. Besides whole cultures of all the potential strains, culture filterates of six strains (i.e. except LD10), washed whole cells and brocken cells of two strains (529 and LD10) also were more or less inhibitory to various test organisms.

8. The target and the inhibitor organisms seemed to be specific as only one Leuconostoc strain was active against a given test bacterium.

9. The specific inhibitory principle for each organism appeared to be localized specifically on or outside the leuconostoc cells. The factor against S.faecalis was secreted out of the inhibitory strains. It was exreted as well as found adhering to cell wall of strain 529 active against P.aeruginosa while it was supposedly embedded in and on the cell wall of Leuconostoc strain LD10 active against S.aureus ,

10. Growing the positive Leuconostoc strains at elevated temperatures of 40°C for 18 h did not decrease their antibacterial activity thereby indicating the absence of any plasmid linkage.

11. The present study was a modest and maiden attempt to unearth the hitherto unknown facts about the positioning of the antimicrobial activity of Leconostoc species in these cultures. The previous studies have been made only on qualitative screening of these cultures for their overall

antibacterial activity. The present investigation has been able to throw useful light on the probable site of production as well as concentration of the inhibitory factor(s). Thus, the study has opened new vistas concerning the antimicrobial properties of this group of lactic cocci which has since been known only for their flavour production ability. The probability that the antimicrobial {factor(s)} is localised in the cell wall of some of the Leuconostocs species makes its isolation and purification easier and can be commercially exploited for preservation of a variety of food stuffs. However, stimulation of some pathogens by the Leuconostoc metabolites also demands attention.

There is a great scope of further investigation, as to the effect of mutations on the production and study of the physicochemical aspects of purified factor(s) responsible for inhibition.

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