STUDIES ON LACTIC ACID BACTERIA FROM TROPICAL FISH AND SHELLFISH

Thesis submitted to

The Cochin University of Science and Technology

Cochin – 682 022

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Microbiology

(Under the Faculty of Marine Sciences)

SEEMA NAIR P.

Microbiology, Fermentation and Biotechnology Division
Central Institute of Fisheries Technology, Matsyapuri P.O.
Kochi – 682 029

November 2000

CERTIFICATE

CENTRAL INSTITUTE OF FISHERIES TECHNOLOGY

(Indian Council of Agricultural research)

Willingdon Island, Matsyapuri. P.O., Cochin- 682 029.

Dr. P.K.Surendran, M.Sc., Ph.D., F.S.F.T., ARS,
Principal Scientist & Head of Division,
Microbiology, Fermentation & Biotechnology Division.

This is to certify that this thesis entitled "**Studies on Lactic Acid Bacteria from Tropical Fish and Shellfish** " embodied the results of the original research work done by **Smt. Seema Nair, P** under my supervision and guidance from 11- 10- 1995 to 02-10-1999. I further certify that no part of this thesis has previously formed the basis of the award of any Degree, Diploma, Associate-ship, and Fellowship or other similar titles of this or any other University or Societies. She has passed the Ph.D. Qualifying Examinations of the Cochin University of Science and Technology in August, 1999.

Cochin-682 029.
20th November, 2000.

(Dr. P.K.Surendran)

DR. P.K. SURENDRAN
PRINCIPAL SCIENTIST & HEAD OF DIVISION
Microbiology, Fermentation & Biotechnology

Contents

	Page no
CHAPTER 1	
INTRODUCTION	1
CHAPTER 2	
REVIEW OF LITERATURE	3
2.1 Fish Bacteriology	**3**
2.1.1 Bacterial flora of fresh fish and shellfish in temperate region	4
2.1.1.1 Quantitative aspects	4
2.1.1.2 Qualitative aspects	5
2.1.2 Bacterial flora of fresh fishes and shellfish in tropical region	6
2.1.2.1 Quantitative aspects	6
2.1.2.2 Qualitative aspects	8
2.1.3 Bacteriology of frozen fish and shellfish	9
2.1.3.1 Quantitative aspects	9
2.1.3.2 Qualitative aspects	10
2.2 Bacterial spoilage of Fish	**11**
2.3 Fish preservation	**14**
2.4 Pathogenic bacteria associated with fish	**21**
2.5 Listeria in seafoods	**25**
2.6 Lactic Acid Bacteria	**27**
2.6.1.1 *Streptococcus*	29
2.6.1.2 *Lactococcus*	30
2.6.1.3 *Enterococcus*	31
2.6.1.4 *Carnobacterium*	32
2.6.1.5 *Leuconostoc*	33
2.6.1.6 *Pediococcus*	34
2.6.1.7 *Lactobacillus*	34
2.6.2 *Lactobacillus* from fish and fishery products	36

2.6.3 *Lactobacillus* as probiotics 38
2.6.4 Pathogenic Lactic acid bacteria 38
2.6.5 Lactic acid bacteria in Biopreservation 39
2.7 Bacteriocins **41**
2.7.1 Bacteriocins of Lactic acid bacteria 41
2.7.1.1 Bacteriocins of *Lactococcus* 41
2.7.1.2 Bacteriocins of *Pediococcus* 42
2.7.1.3 Bacteriocins of *Leuconostoc* 43
2.7.1.4 Bacteriocins of *Carnobacteria* 43
2.7.1.5 Bacteriocins of *Lactobacillus* 44
2.7.2 Non bacteriocin-inhibiting compounds 46
2.7.2.1 Diacetyl 46
2.7.2.2 Acetaldehyde 46
2.7.2.3 Hydrogen peroxide 46

CHAPTER 3
MATERIALS AND METHODS 47

3.1 Materials **47**
3.1.1 Bacterial strains 47
3.1.2 Fish 47
3.1.3 Media 54
3.1.4 Dehydrated media 54
3.1.5 Compounded media 54
3.1.4 Indicator solutions 60
3.1.5 Test reagents 60
3.2 Methods **62**
3.2.1 Sampling of fish/prawn for Total Plate Count 62
3.2.1.1 Pour plate method 62
3.2.1.2 Spread plate method 62
3.2.2 Enumeration of Lactic acid bacteria 63
3.2.3 Isolation of Lactic acid bacteria 63

3.2.4 Maintenance of Lactic acid bacteria 63
3.2.5 Identification of Lactic acid bacteria 64
3.2.6 Factors influencing the growth of Lactic acid bacteria 67
3.2.6.1 Effect of temperature on growth 67
3.2.6.2 Effect of pH on growth 67
3.2.6.3 Effect of NaCl on growth 67
3.2.6.4 Effect of glucose concentration on the growth and acidity produced in the medium 68
3.2.7 Titrable acidity 68
3.2.8 Water activity 68
3.2.9 Moisture content 68
3.2.10 Salt content 69
3.2.11 Detection of antibacterial activity 69
3.2.12 Characterization of antibacterial components produced by Lactic acid bacteria 70
3.2.12.1 Organic acids 70
3.2.12.2 Hydrogen peroxide producxtion 71
3.2.13 Purification of bacteriocins 71
3.2.14 Characterization of bacteriocins 72
3.2.14.1 Heat sensitivity 72
3.2.14.2 Protease sensitivity 72

CHAPTER 4
RESULTS AND DISCUSSION **73**

4.1 Total bacterial count vis-a-vis Lactic acid bacterial count in fresh fish **73**
4.1.1 Total bacterial count in fresh fish 73
4.1.2 Lactic acid bacterial count in fresh fish 74
4.2 Total bacterial count vis-a-vis Lactic acid bacterial count in fresh prawn **76**
4.2.1 Total bacterial count in fresh prawn 76
4.2.2 Lactic acid bacterial count in fresh prawn 77
4.3 Total bacterial count vis-a-vis Lactic acid bacterial count in frozen fish **78**

4.3.1 Total bacterial count in frozen fish 78
4.3.2 Lactic acid bacterial count in frozen fish 79
4.4 Total bacterial count vis-a-vis Lactic acid bacterial count in frozen prawn 79
4.4.1 Total bacterial count in frozen prawn 79
4.4.2 Lactic acid bacterial count in frozen prawn 80
4.5 Total bacterial count vis-a-vis moisture content, water activity and salt content in cured fishes of commerce 80
4.6 Total bacterial count in Ocean fresh fish 81
4.7 Total bacterial count vis-a-vis Lactic acid bacterial count in farmed fish/prawn 82
4.8 Total bacterial count in pickled fish/prawn 83
4.9 Studies on Lactic Acid Bacteria 84
4.9.1.1 Isolation of LAB from fresh fish 84
4.9.1.2 Isolation of LAB from fresh prawn 84
4.9.1.3 Isolation of LAB from frozen fish 84
4.9.1.4 Isolation of LAB from frozen prawn 85
4.9.2 Morphological characteristics of LAB cultures 85
4.9.3 Homofermentative and heterofermentative nature of LAB 86
4.10 Studies on *Lactobacillus* isolated from fish and shellfish 88
4.11 Characterization of *Lactobacillus* species 89
4.11.1 *L.plantarum* 89
4.11.2 *L.brevis* 90
4.11.3 *L.divergens* 90
4.11.4 *L.gasseri* 90
4.11.5 *L.casei ssp rhamnosus* 91
4.11.6 *L.fermentum* 91
4.11.7 *L.viridescens* 91
4.11.8 *L.farciminis* 92
4.11.9 *L.buchneri* 92
4.11.10 *L.acidophilus* 92
4.11.11 *L.alimentarius* 92
4.11.12 *L.animalis* 93

4.11.13 *L.reuteri* 93

4.12 Distribution of *Lactobacillus* in fresh and frozen fish and prawn **93**

4.12.1 *Lactobacillus* in fresh fish 93

4.12.2 *Lactobacillus* in fresh prawn 93

4.12.3 *Lactobacillus* in frozen fish 94

4.12.4 *Lactobacillus* in frozen prawn 94

4.13 The Biochemical and antibacterial investigations of *Lactobacillus* cultures **96**

4.13.1 Fermentation of sugars 96

4.13.1.1 Sugar fermentation by *L.plantarum* 97

4.13.1.2 Sugar fermentation by *L.brevis* 98

4.13.1.3 Sugar fermentation by *L.divergens* 98

4.13.1.4 Sugar fermentation by *L.fermentum* 98

4.13.2 Studies on effect of glucose, NaCl and incubation temperature on growth and bacteriocin production by *Lactobacillus* cultures 100

4.13.2.1 Effect of glucose concentration on lowering of pH of the culture media by *Lactobacillus* cultures 100

4.13.2.1.1 Titrable acidity by *Lactobacillus* species 101

4.13.2.2 Effect of NaCl in culture media on the growth of *Lactobacillus* cultures 102

4.13.2.3 Effect of incubation temperature on the growth of *Lactobacillus* cultures 103

4.13.2.4 Effect of pH of the media on the growth of *Lactobacillus* cultures 104

4.13.3 Studies on antibacterial activity of *Lactobacillus* cultures 104

4.13.3.1 Screening of *Lactobacillus* cultures for antibacterial activity against indicator/pathogenic bacteria 104

4.13.3.1.1 Inhibition by *L. plantarum* 105

4.13.3.1.2 Inhibition by *L.brevis* 106

4.13.3.1.3 Inhibition by *L. divergens* 106

4.13.3.1.4 Inhibition by *L. casei ssp rhamnosu* 106

4.13.3.1.5 Other *Lactobacillus* groups 106
4.13.3.2 Nature of antibacterial activity by *Lactobacillus* 107
4.13.3.3 Characterization of antibacterial activity by *Lactobacillus* cultures 107
4.13.3.4 Antibacterial activity of bacteriocins against other *Lactobacillus* cultures 109
4.13.3.5 Antibacterial activity of lactic acid and acetic acid 110
4.13.3.6 Antibacterial activity due to Hydrogen peroxide 111
4.13.3.7 Antibacterial activity due to bacteriocins 111
4.13.4 Antibacterial activity of bacteriocin concentrates from *Lactobacillus* cultures 112
4.13.4.1 Antibacterial activity of bacteriocin concentrates against pathogenic/indicator bacteria 112
4.13.4.1.1 Action of bacteriocins against *Listeria monocytogenes* 112
4.13.4.1.2 Action of bacteriocins against *Bacillus cereus* 112
4.13.4.1.3 Action of bacteriocins against *Staphylococus aureus* 113
4.13.4.1.4 Action of bacteriocins against *Salmonella typhimurium* 113
4.13.4.1.5 Action of bacteriocins against *Escherichia coli* 113
4.13.4.1.6 Action of bacteriocins against *Vibrio cholerae* and *Vibrio vulnificus* 114
4.13.5 Effect of salt, pH, incubation temperature and glucose concentration on the antibacterial activity of *Lactobacillus* cultures 114
4.13.5.1. Effect of salt 114
4.13.5.2. Effect of pH 115
4.13.5.3.Effect of temperature of incubation 115
4.13.5.4.Effect of glucose concentration in the culture media 116
CHAPTER 5
SUMMARY AND CONCLUSION 119
CHAPTER 6
REFERENCES 125

Chapter .1

INTRODUCTION

1. Introduction

Lactic acid Bacteria (LAB) are characterized as Gram-positive, usually non-motile, non-sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism. Members of this group contain both rods (*Lactobacillus* and *Carnobacterium*) and cocci (*Streptococcus, Leuconostoc, Pediococcus, Lactococcus, Weissella, Oenococcus, Vagococcus* and *Tetragenococcus*); they are generally catalase-negative and lack cytochromes. LAB are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins. The LAB of importance in foods mainly belong to the genera: *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus* and *Streptococcus.*

Lactic Acid Bacteria (LAB) have for centuries been responsible for the fermentative preservation of many foods. They are used to retard spoilage and preserve foods through natural fermentations. They have found commercial applications as starter cultures in the dairy, baking, meat, fish, and vegetable and alcoholic beverage industries. They are industrially important organisms recognized for their fermentative ability as well as their nutritional benefits. These organisms produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocins or bactericidal proteins during lactic fermentations. Not only are these components desirable for their effects on food flavour and texture, but they also inhibit undesirable microflora. LAB and their products give fermented foods distinctive flavours, textures and aromas while preventing spoilage, extending shelf-life and inhibiting pathogenic organisms. Currently, there is some interest in the possible use of LAB and their metabolic products as biocontrol agents, biopreservatives for non-fermented foods and probiotics.

Biopreservation of foods using bacteriocin producing LAB cultures is becoming widely used. The antimicrobial effect of bacteriocins and other compounds produced during fermentation of carbohydrates are well known to inhibit the growth of certain food spoiling bacteria as well as a limited group of food poisoning and pathogenic bacteria. Eventhough lactic acid bacteria are commonly used in South East Asia for the production of fermented fish products, such preservation methods are not very popular in India.

LAB like *Lactobacillus plantarum* are widely used as starter cultures for the production of fish ensilage, in which, low value fish and waste are mixed with carbohydrates like cereals and molasses. Fish ensilage is a good source of protein as cattle and poultry feed.

Reports on the occurrence, distribution and biochemical studies on LAB from tropical fish and shellfish are very scanty. In this study, an attempt has been made to gather enough information regarding lactic acid bacteria from fish and shellfish of tropical regions. The occurrence and distribution of lactic acid bacteria in fresh and frozen marine fish and shellfish, farmed fish and shellfish, cured and pickled fish and shellfish have been investigated in detail. The *Lactobacillus* cultures from these fish and shellfish samples were isolated and their biochemical characteristics were studied. The effects of different physical and chemical parameters like pH, temperature,, NaCl concentration and glucose concentration on the growth of *Lactobacillus* cultures have been studied in detail. Investigations have also been made on the antibacterial effect of bacteriocins from LAB against pathogenic and food poisoning bacteria.

Chapter.2

REVIEW OF LITERATURE

2. Review of Literature

2.1 Fish Bacteriology

Bacteriologists focused on human and animal disease around the turn of the century. They recognized the involvement of shellfish as carriers of human enteric disease. This led to an extensive investigation of the enteropathogenic bacteria in fish and other aquatic animals. Anderson (1907) clearly showed that spoilage was dominantly caused by bacteria. Investigators in different countries established that the bacteria most apparently involved in spoilage were those found normally on fresh fish. This sparked a whole series of studies on the bacteriology of fresh and spoiling fish with attention being concentrated on those species of fish or shellfish of commercial significance.

For the period from the 1920's to 1945, the results of studies on fish bacteria and their involvement in spoilage was rather completely covered in the review by Reay and Shewan (1949). The early researchers had shown that fish muscle tissues are sterile in healthy animals, while more or less large populations of bacteria are associated with the external surfaces, gills and intestine of fish. Estimates were quoted of 10^2-10^6 bacteria/cm^2 skin surface, similar or slightly higher gill counts and intestinal counts of very few to in excess of 10^8/g depending on whether or not the fish were feeding. During spoilage, skin counts increased to 10^7organisms/cm^2 or more and gill counts increased comparably. Liston (1980) has completely reviewed the bacteriology of fish and shellfish.

2.1.1. Bacterial flora of fresh fish and shellfish in temperate region

Several scientists have studied the bacterial flora of cold water fish species (Shewan, 1962; Shewan and Hobbs, 1967; Shewan, 1977; Horseley, 1977; Liston, 1980). The composition of the microfloras of the different species of fish tested was mostly reported to be dominated by gram-negative bacteria usually identified as *Achromobacter, Flavobacterium, Pseudomonas* or less frequently *Vibrio* or Enterobacteriaceae (Liston, 1980). It was noted that fish bacteria are mostly psychrotrophs growing between 0°C and about 30°C with some strains growing as low as -7.5°C.

2.1.1.1. Quantitative aspects

The number of viable, aerobic, heterotrophic bacteria on the skin surface (per cm^2) of the fish from temperate zone at 20°C consisted of 10^2-10^5 bacteria, while gills and gut contents carried 10^3-10^7 and 10^3-10^8 bacteria per gram of tissue respectively (Shewan, 1962). The flesh and body fluids of newly caught healthy fish are generally considered to be free from any bacteria (Shewan, 1961). But the skin and adhering slime, gills and in the case of recently feeding fish, intestines carry heavy loads of bacteria. Organisms in the order of 10^2-10^7/ cm^2 on the skin have been recorded. The gills and intestines usually carry bacterial loads of the order of 10^3-10^8 and 10^3-10^9/g respectively. Somewhat higher loads on marine fish from tropical and subtropical areas, compared with the colder areas have been recorded. Accordingly the North sea fish has a bacterial load of 10^2-10^5/cm^2 of skin, 10^3-10^7/g of gills and 10^3-10^8/g of intestinal contents (Shewan, 1962; Georgala, 1958; Liston, 1956) while for tropical fish, a bacterial load of 10^3-10^7/cm^2 of skin, 10^5-10^8/g of gills and 10^5-10^9/g of intestines have been reported (Karthiayani and Iyer, 1967, 1971). Dyer (1947) recorded a bacterial load

of 10^4-10^5/g of slime of Atlantic cod and for Japanese flat fish, a count of $10^4/cm^2$ of skin, 10^3-10^5/g of gills and 10^3-10^7/g of intestines have been reported (Simidu et al. 1969).

Liston (1956, 1957) observed two peaks of bacterial loads at 0°C and 20°C in the flora of slime and gills of Sole and Skate in the late spring and autumn. Georgala (1958) had reported similar seasonal changes in the bacterial loads of cod.

The total bacterial count reported for shellfish varied from place to place (10^3-10^7/g muscle). Thus, Cobb and Vanderzant (1971) reported counts at 20°C in the range of 10^4-10^7/g for shrimp from Gulf of Mexico, 10^3-10^7/g for Louisiana crab, 10^3-10^6/g for Scampi, (*Nephrops norvegicus)* (Walker et al.1970., Cann et al.,1971), 10^3-10^6/g for some edible varieties of crab (Early,1967), 10^4-10^7 for deep water shrimp, (*Pandalus borealis)* and 10^4-10^5/g for inshore shrimp (*Pandalus montagui* and *Crangon crangon)* (Cann,1977).

2.1.1.2. Qualitative aspects

Evidences showed that the flora of fish were directly related to its aquatic environment (Wood, 1953). In the warmer waters off the east coast of South Africa, Australia and the Adriatic, a greater percentage of mesophiles (*Bacillus spp,* Coryneforms and *Micrococcus*) are found. Other factors like type of media used (Liston, 1955), temperature of incubation (Georgala 1957, 1958), seasonal variation (Liston, 1955; Shewan 1949) affected the bacterial flora found in the fish. The bacteria found most frequently by Torry workers on North sea fish were *Pseudomonas spp, Achromobacter, Vibrio, Flavobacterium* and *Corynebacterium.* Strict anaerobes appear

to be absent from the slime of newly caught fish, although they are usually encountered in the intestines. *Clostridium spp* were also reported (Shewan 1938, 1949).

The main groups of bacteria comprising the flora of crustacean shellfish were *Micrococcus*, Coryneforms, *Achromobacter* and *Pseudomonas* together with smaller numbers of *Flavobacterium, Cytophaga* and *Bacillus* (Cann, 1977). The proportional composition varies with the temperature of the water in which the animals lived, cold-water species having largely *Pseudomonas* and *Achromobacter* (Liston, 1980), while *Micrococcus* and Coryneforms dominated in crustaceans from warmer waters. Gulf shrimp contained largely *Pseudomonas, Achromobacter, Micrococcus* and *Bacillus* (Williams et al.1952). *Micrococcus* predominated in the flora of Loiusiana crab (Alford et al.1942), while *Achromobacter* was dominant in both flesh and gut of North sea (Early, 1967) and Pacific crab (Lee and Pfeifer, 1975).

2.1.2. Bacterial flora of fresh fish and shellfish in tropical region

2.1.2.1. Quantitative aspects

Bacteriology of fish and shellfish from tropical marine regions have been studied more or less extensively (Venkataraman and Sreenivasan, 1953, Shaikmahmud and Magar, 1956; Pawar and Magar, 1966; Jadhav and Magar, 1970; Karthiayani and Iyer, 1967; Anand and Setty, 1977; Surendran, 1980; Surendran and Gopakumar, 1981; Surendran et al. 1989; Lima dos Santos, 1981).

Karthiayani and Iyer (1975) have studied the qualitative and quantitative aspects of the bacterial flora of certain marine fishes and prawns in Cochin waters in relation to their environments. Quantitatively the bacterial density of the seawater increased with depth, the highest aerobic count being shown by bottom mud ($2.7x10^4$

organisms/g) and the least by surface water upto 14m depth (0-90 organisms/g). But compared to bottom mud, a 10 to 100 fold increase in the bacterial count was observed on the body of the fishes in general and a higher increase (10^3-10^4 fold) at the gills and the highest (10^4-10^5 fold) in the guts of sardines have been recorded.

Surendran and Iyer (1980) have studied the qualitative and quantitative aspects of the bacterial flora of freshly caught Pearlspot (*Etroplus suratensis*). The aerobic bacterial count on the surface of the fish varied between 10^3-10^4/cm^2 of gills, between 10^4-10^6/g of intestines with contends, between 10^5-10^8/g. In the case of tropical fishes, high peaks in the bacterial counts on skin of oil sardine have been noticed during the months of June and September. Intestinal flora of oil sardine showed peak counts in June and October (Karthiayani and Iyer, 1967). Nambiar and Iyer (1990) have reported that 69.9% of fresh fish samples obtained from retail trade in Kochi had TPC more than $1x10^5$/g and 49.4% had counts more than $5x10^5$/g. Lakshmanan et al.(1984) have reported that 66.7% of the fish collected from landing centres in Kochi had TPC more than $1x10^5$/g and only 8.5% had counts more than $5x10^5$/g.Thampuran (1987) had reported TPC in the range of $8.9x10^3$-$4.1x10^5$/g in skin with muscle of fresh Indian Mackerel. The bacterial count reported for Oil sardine at room temperature (RT) on Sea water agar was in the range of 10^3-10^7/g according to Karthiayani and Iyer (1967) and Surendran and Gopakumar (1982). For Mackerel, the latter reported counts in the range of 10^4-10^6/g. The data compiled by Lima dos Santos (1981) for tropical fish showed that TPC was in the range of 10^3-10^7 organisms /cm^2 of skin.

Venkataraman and Sreenivasan (1953) observed a TPC of $1.82x10^6$/g muscle for fresh prawn. The average bacterial counts reported for prawns from tropical sea ranged from 10^3 to 10^7/g of muscle (Liston,1980). According to the studies

conducted by Surendran (1980), the TPC of prawn *Penaeus indicus* at RT ranged from 10^2-10^5/g, that of *Metapenaeus dobsoni* from 10^3-10^6/g and *M.affinis* from 10^2-10^6/g. Thampuran (1987) reported a TPC of 5.2x10^3-9.4x10^6 /g of muscle in fresh prawn (*M.dobsoni*) at 37°C. Most of the data available on tropical prawn from India pertains to the freshly landed material (Pillai et al. 1961; Jacob et al.1962 and Lakshmanan et al.1984).The TPC of the shrimp from Thailand was found to be in the range of 10^3-10^6/g (Cann et al.1971). Most of the samples of shrimp studied in Northern Australia were found to harbour a bacterial load in the range of 10^3-10^5/g (Ruello,1974).

2.1.2.2. Qualitative aspects

Karthiayani and Iyer (1967) have studied the qualitative aspects of the bacterial flora of fresh sardines (*Sardinella longiceps*) caught off Cochin. There was a majority of gram-negative rods mainly of *Achromobacter, Vibrio* and *Pseudomonas* groups. The percentage of gram-positive organisms was very low or nil at times in the ocean fresh sardines. Surendran and Iyer (1976) have reported the presence of *Vibrio, Pseudomonas, Achromobacter, Flavobacterium, Corynebacterium, Micrococcus, Bacillus* and yeast in the natural flora of the Indian Mackerel. The flora on the skin and of gills and intestines consisted mainly of asporogenous rods. The predominating flora on the skin surface consisted of *Pseudomonas* species (25%), *Alcaligenes* spp. (10%), *Micrococcus* spp. (20%), *Flavobacterium* spp. (5%), *Bacillus* spp. (5%) and Coliforms (5%). The flora of the gill tissue comprised of mainly *Pseudomonas* spp. (35%), *Alcaligenes* (25%) and *Micrococcus* spp. (15%). The intestinal flora consisted of *Pseudomonas* spp. (50%), Coliforms (20%), *Flavobacterium* spp. (8%) and *Alcaligenes* (6%). *Micrococcus* and coryneforms were found to dominate in Indian prawns from

warmer waters. Gulf shrimp contained largely *Achromobacter, Micrococcus, Pseudomonas* and *Bacillus* (Williams et al. 1952).

2.1.3. Bacteriology of frozen fish and Shellfish

2.1.3.1 Quantitative aspects

The salient features of the microbiology of freezing of fish was discussed by Tressler and Evers (1957). Freezing does not sterilize the fish, nevertheless there is considerable reduction in bacterial population due to freezing. Pivnick (1949) noted a reduction of 40-60% in bacterial population in Mackerel. Similar values were reported for tropical fishes also (Bose, 1969; Jadhav and Magar, 1970; Cann, 1974. and Thampuran,1987). While rate of freezing had no effect on bacterial death, repeated freezing and thawing was found to be more lethal to bacteria. Nambiar and Iyer (1990) noted a total bacterial count ranging from $1.45x10^2$ to $4.0x10^6$/g and the highest count was noted in Pearlspot. It was noted that 4.7% of the frozen fish samples analyzed showed counts more than $5.0x10^5$/g. Thampuran and Iyer (1985) have analyzed different samples of frozen fish, prawns, fish fillets and minced fish for TPC. They obtained a TPC of $5.2x10^4$ - $4.5x10^5$/g at RT ($30\pm$ 1°C).

The method of packing, freezing and storage of headless shrimp was investigated by Fieger et al.(1956). The study indicated that freezing caused a greater reduction in bacteria in peeled shrimp than in unpeeled. Pillai et al (1965) surveyed the microbiological quality of commercial frozen prawn products. The standard plate count varied between $1x10^4$ and $1x10^6$/g for headless prawn and between $1x10^4$ and $1x10^7$ for peeled and deviened and cooked frozen samples. Majority of the samples had bacterial loads well within the limits prescribed for such products.

Effect of immediate icing on bacterial numbers after freezing was studied for tropical prawns (Lekshmy et al.1962; Bose, 1969). The difference between laboratory scale and commercial scale samples of frozen prawn with regard to the number of bacteria was studied by Novak (1973). The percentage reduction of bacteria in laboratory frozen samples were fairly constant (89-99%), whereas in commercial samples, large variations were noted. Thampuran (1987) has studied the quantitative and qualitative aspects of frozen fish and prawns. The total bacterial count of skin and muscle of mackerel (*R.. kanagurta*) just before freezing was in the range of 9.8×10^3/g - 6.4×10^5/g which decreased to 9.8×10^3- 1.1×10^5/g after freezing at -40°C for 6 hrs. Jadhav and Magar (1970) noted 60% reduction in the initial bacterial population for mackerel after freezing at -40°C for two and a half months. Changes in the total bacterial count of the muscle of prawn (*Metapenaeus dobsoni*) was studied by Thampuran (1987). The total bacterial count of the muscle of the prawn ranged from 5.33×10^5- 1.82×10^6/g just before freezing. However, freezing at -40°C for six hrs decreased the TPC of muscle of prawn to 3.1×10^4- 5.27×10^5/g.

2.1.3.2 Qualitative aspects

Earlier studies indicated that freezing imparted a selective action on the microbial flora of fish and various species were affected at different levels. The Gram-positive bacteria were found to be more resistant to freezing and frozen storage. Kiser and Beckwith (1942) observed that *Micrococcus* and *Achromobacter* were frequently encountered in frozen mackerel. Among Gram-positives, *Bacillus, Lactobacillus* and *Micrococcus* species were most susceptible and Coryneforms least affected.

Jadhav and Magar (1970) studied the bacterial flora of tropical fishes from Bombay coast and found that spore formers like *Bacillus mesentericus* group were

resistant to freezing. Surendran et al. (1989) have reported that the pattern of change in the flora of the tropical fishes during iced storage is similar to those of the fish of temperate waters. In the initial flora *Moraxella, Acinetobacter* and *Vibrio* groups accounted for 58-76% in the marine fishes and 25-45% in the brackish and freshwater fishes. *Pseudomonas* formed 10% in the flora of Indian Mackerel and 20% of oil sardine, but in Tilapia, Pearlspot and milkfish, the *Pseudomonas* accounted for 20-25% of their flora. As the days of storage progressed, *Pseudomonas* spp. emerged as the dominant group. Kawabata et al. (1975) investigated microbiological characteristics of the frozen prawn imported from tropical countries. The study revealed that for shrimp also 70% of the flora constituted of Gram-positives belonging to genera *Micrococcus, Streptococcus, Staphylococcus, Microbacterium* and *Corynebacterium.* The Gram-negatives were few in number and belonged to the genera *Flavobacterium/Cytophaga, Pseudomonas, Moraxella* and *Acinetobacter*.

2.2. Bacterial spoilage of Fish

Several reports are available on the bacterial flora and spoilage characteristics of fishes from cold and temperate waters (Shewan, 1961, 1977; Lima dos Santos, 1981). However information regarding the spoilage characteristics of tropical fishes is rather limited (Surendran and Iyer, 1973, 1976; Surendran, 1980; Curran and Disney, 1979). Surendran et al. (1989) have studied the spoilage of commercially important Tropical fishes under iced storage. In all these fishes (Oil sardine, Indian Mackerel, Pearl spot, Milkfish and Tilapia), the spoilage flora were composed mainly of a single genus of bacteria, namely *Pseudomonas*. Studies on identification of bacteria involved in fish spoilage, type of end products responsible for putrid and offensive odors, substrates utilized by these bacteria and rate of degradation among different

spoilage groups, are available, (Herbert et al.1971; Miller et al.1973; Shewan,1977; Chung,1968).

Fish spoilage bacteria bring about the reduction of TMAO to trimethyl amine by a coupled reaction involving oxidation probably of lactate to acetic acid, CO_2 and H_2O through an inactivation step involving a triamine-oxidase.

The work at Torry had repeatedly shown that excised fish muscle tissue held under sterile conditions for several weeks at refrigerated temperatures did not develop objectionable odors or appearance. Endogenous biochemical changes which occurred in fish *post mortem* were important in conditioning the substrate for bacterial action and autolysis as such was insignificant as a spoilage mechanism (Shewan, 1971). In Pacific cod and Yellow tail, the TVB-N (Total volatile base nitrogen) in aseptic muscles of both species increased to about 4mg/100g during a storage period of 12-14days; which was attributed to deamination of adenine nucleotides. TMAN (Trimethyl amine nitrogen) in both species changed little during the storage; whereas ATP (Adenosine triphosphate) and its related compounds in both species changed remarkably during storage and values were nearly identical with those of non-aseptic muscles of the respective species.

Liston (1980) summarizing the above work said that *Pseudomonas* types having the shortest generation time at temperatures in the 0-5°C range and with an enhanced capability to utilize NPN components of the muscle fluids as growth substrates, rapidly outgrew the other bacteria present and become the dominant organisms in a population which increases to 10^8 bacteria /cm^2. This population oxidized amino acids and lactic acids, reduced trimethyl amine oxide and in later stages produced proteinases and variety of S-containing compounds. Dominant in the

production of spoilage odors was low GC *Pseudomonas*, probably *Alteromonas*, though other *Pseudomonas* also might be involved.

Surendran et al. (1989) have studied the spoilage of commercially important tropical fishes under iced storage. The pattern of change in the flora of the tropical fishes during iced storage was similar to that of the fish of temperate waters. In the initial flora, *Moraxella, Acinetobacter* and *Vibrio* groups accounted for 58-76% in the marine fishes and 24-45% in the brackish and freshwater fishes. *Pseudomonas* formed 10% in the flora of Indian mackerel and 20% of oil sardine, but in Pearlspot, Tilapia and Milkfish, the *Pseudomonas* accounted for 20-25% of their flora. As the days of storage progressed, *Pseudomonas* spp. emerged as the dominant group. As observed by Shewan (1977), "irrespective of the initial flora of fish, *Pseudomonas* and *Alteromonas* groups emerged as the predominant genera during spoilage of fish in ice". The spoilage of fish when stored in ice is mainly caused by psychrotrophic bacteria (Shewan, 1977; Lima dos Santos, 1981; Surendran and Gopakumar, 1981, 1982.) which are capable of growth and multiplication at low temperatures.

Instances where *Achromobacter (Moraxella)* and *Corynebacterium* dominating the spoilage of shrimps (Walker et al.1970), Gram-positive bacteria involving in the spoilage of crab meat, and *Moraxella* and yeast dominating in irradiated fish (Liston, 1980) had also been reported. Control of spoilage organism in fish is mainly a deal with psychrophiles/psychrotrophs/facultative psychrophiles. Some of the methods of fish preservation are elaborated below.

2.3. Fish preservation

The basic principle of preservation is to affect the homeostasis of a microorganism, such that its ability to survive and grow in a food is compromised. Acidification to pH 2.0, for example, is used to make a food safe by inhibiting the growth of microorganisms. Acidification exposes the cell to a low pH environment, reduces the internal pH, and causes an excess of H+ ions to accumulate, which have to be removed in order for the internal pH to be raised. Similarly, when low water activity (a_w) is used for preservation, it affects the ability of the cell to maintain its turgor pressure. The only way an organism can cope with this stress is by increasing its osmotic potential. The use of a preservative system with adjunct such as vacuum packing puts additional stresses on the microorganism and can lead to effective inhibition of growth.

Target organisms in a food may have varying rates of growth under various conditions. It is well recognized that microorganisms can survive over a wide range of pH conditions, temperature, and water activity (a_w). Proteolytic *Clostridium botulinum* for example, can grow at temperatures as low as 3°C. *Salmonella* will not grow much below pH 3.8, but other organisms like yeasts and molds will grow at very low pH values. The preservative systems used must be tailored to suit the type of food, the target organisms of concern, and the conditions of preparation of that food. At the same time, the preservative system must not affect the taste and organoleptic qualities of the food. It is very important to specify the target microorganisms while choosing the preservative system (Brown and McClure, 1998).

Fair flow reports (1994) discuses the use of Lactic acid bacteria (LAB) in the preservation of fish and fish products. Isolates of *Carnobacterium* and *Lactococcus*

that produced acid at low temperature were selected for study. The *Lactococcus* strains produced compounds, which would be undesirable in fish products (e.g. histamine from histidine). One strain of *Carnobacterium* produced a bacteriocin that was active against *Listeria* spp. After growth in sterile fish broth, *C. piscicola* strains produced acetic and pyruvic acids, and slightly lowered the pH. When glucose was added, final pH was lower and lactic acid was produced. The LAB adapted well to growth on fish mince. They found that there was little or no effect of adding high concentration of LAB on chemical or sensory properties of refrigerated minced whiting, and the effect on the spoilage flora was generally weak. When glucose was added to the minced whiting, acidification occurred and there was a reduction in the spoilage flora. Low pH adversely affected both the water holding capacity and water uptake and the mince was also bleached. It was concluded in the report that lactic acid bacteria technology could be applied to fish mince intended for further processing or to cold-smoked fish fillets.

The present methods of fish preservation include Freezing, Canning, Salting, Drying, Smoking, Irradiation, Chemical preservation and Fermentation.

Freezing is an effective method of halting bacterial action. Freezing fish and storing them at -20°C or higher has more or less solved the problems of microbial spoilage. When frozen seafoods are defrosted and stored at refrigerator temperature they seem to undergo bacterial spoilage similar to that of unfrozen products. *Salmonella* and the other members of the Enterobacteriaceae were among the sensitive bacteria (Raj and Liston, 1961). In general, gram-negative bacteria were more sensitive to freezing than gram-positive bacteria and bacterial spores were highly resistant.

Canned seafoods fall into two categories from a bacteriological point of view, these are fully processed commercially sterile products and semi-preserved

products. The fully processed products would include canned tuna, salmon, shrimp, crab, sardines and other fish, fish balls, etc. The heating process applied to these products was designed to destroy all pathogenic bacteria and normal numbers of other organisms. *Bacillus stearothermophilus* and *Clostridium* spp. were mainly responsible for spoilage of canned seafoods (Shewan and Liston, 1955). Improper seaming and contaminated cooling water were the primary source of spoilage of canned foods. It has been reported that some canned fish products are not always sterile (Neufeld, 1971). This may be due to the oil pack used since oil will protect bacterial spores against heat to some extent.

Salting and drying have been two age-old methods of preserving surplus fish practised by the fishermen. It is a method of preservation based on the penetration of salt into the tissues and governed by the various physical and chemical factors such as diffusion, osmosis and a series of chemical and biochemical processes associated with changes in various constituents of fish. There are three basic methods used in salting of fish. They are 1) dry salting, 2) wet salting and 3) mixed salting. Preservation of fish and shrimp by drying or by salting or by a combination of these processes is still widely practiced throughout the world, though this method is less important in industrialized countries. It is estimated (CIEO,1991) that around 14% of the catch is preserved by curing techniques on a global basis (Gopakumar,1997).

The principle effect on microorganisms is due to the lowering of aw though NaCl itself in higher concentrations may be lethal for some bacteria and yeasts due to osmotic effects. The final population on salted and dried fish is generally dominantly composed of micrococci and Gram-positive rods (Liston and Shewan, 1958; Rudra Setty, 1985).

Spoilage of salted fish may occur due to growth of halophilic bacteria or halotolerant moulds. The halophilic bacteria are frequently derived from contaminated solar salts (Bain et al.1958). They include rod shaped *Halobacterium* and coccal, *Halococcus* forms (Gibbons, 1974).

Smoking is a traditional food processing method used for preservation of fish. Thermal cracking of wood under reduced oxygen supply result in smoke containing several compounds (Gopakumar, 1997). Smoked fish products are not popular in India, but they are, in many parts of the world. In the industrialized countries, this process is primarily designed to produce a product of desirable appearance, odour and flavour, but in much of the world smoking is used as a preservative process. Brining fish before smoking is a common practice and this alters the microflora on fish. Secondly the survivors on fish, depend on whether the fish is hot smoked (above 60°C) or cold smoked (below 35°C), preservation is achieved essentially by drying.

Generally, smoking extends the shift from a Gram-negative to a Gram-positive microflora. Coryneform bacteria and micrococci and *Bacillus* are frequently the dominant forms present (Lee and Pfeifer, 1975). However, in cold-smoked fish a typical pseudomonad spoilage flora develops during subsequent storage. These regulations require that the fish be brought to an internal temperature of 82°C for 30min if brined to contain 3.5% water phase salt or not less than 65°C for 30min, if salt content is 5.0% in the water phase.

In a study of smoked and dried fish from tropical regions, Phillips and Wallbridge (1977) reported isolating *Aspergillus* (six spp.), *Wallemia (*Sporendonema*)*, *Penicillium (*five spp.*)*, *Acremonium* and *Rhizopus*. Most of these fungi are capable of growth, if the relative humidity rises above 70%.

Ionizing radiations such as gamma rays (wavelengths <2A°) emitted from the excited nucleus of Cobalt-60 or electrons emitted from a hot cathode and accelerated to a very high velocity have been studied extensively with the objective of producing foods that are free of spoilage microorganisms (Radappertization) or pathogens (Radicidation) or contain a greatly diminished content of spoilage organisms (Radurization). Studies on the use of **high-energy radiation** (Gamma-radiation and high-energy electron beams) to destroy bacteria and thereby extend the shelf- life of fish and other seafoods began in the 1940's and have continued intermittently ever since (Hayner and Proctor, 1953; Hannan, 1956). One of the benefits of irradiation is that spores can be sensitized to heat treatment. Dose levels of 100 Krad or greater are highly effective in destroying common spoilage bacteria on fish (Liston and Matches, 1968).

Yersinia, Pseudomonas, Campylobacter, Aeromonas spp. and the vegetative cells of *B.cereus* are the most radiation sensitive vegetative bacteria with D10- value of between 0.04 and 0.20 KGy in non-frozen foods. *E.coli* and *Arcobacter butzleri* are also quite radiation sensitive, with D10- values in the range 0.24 - 0.40 KGy in non-frozen products. *Staphylococcus aureus, Salmonella* spp. and *Listeria monocytogenes* are relatively more radiation resistant when compared to other non-sporeforming pathogenic bacteria, with most reported D10-values being in the range of 0.4- 0.8 KGy in non-frozen food, somewhat similar to the vegetative cells of *Clostridium perfringens*. One major problem of radiation pasteurized fish is *C.botulinum* growth. This is well reviewed by Hobbs (1977).

Fermentation has been a popular technology for the preservation of fish in Southeast Asian countries from time immemorial. Fermentation is practiced as a means of preserving or altering the flavor of fish products to greater degree in the orient than in

any other country. Fermented fishery products are generally classified into high-salted fermented, low-salted fermented and unsalted fermented. Fermentation of fish takes place as a result of the action of exogenic and endogenic enzymes.

Of the major products, fish sauces, which are produced by allowing fish, small crustaceans or squid to digest in brine formed by adding 20-25% salt to the raw fish, seem to be mainly the product of fish enzyme action. Bacteria rapidly diminish in number during the fermentation period. Some studies suggest that bacteria are involved in flavor development (Saisithi et al.1966). Specifically, *Micrococcus,* Clostridia and *Pediococcus* have been separately indicated as flavor producers.

Biopreservation systems using bacteriocinogenic LAB cultures and/or their bacteriocins are becoming widely popular these days. The antimicrobial compounds, low water activity and pH produced by LAB prevent the growth of other pathogenic bacteria. Lactic fermented fish products are common in South East Asia. The composition and the quality of the products vary considerably since they are produced on a small scale and the fermentation of the fish-salt-carbohydrate mixtures depends on the natural microflora (Adams et al. 1987). LAB and its bacteriocins have been used in extending the shelf life of Shrimp (Moon et al. 1982) and brined shrimp (*Pandalus borealis*) (Einarsson and Lauzon,1995).

There are several reports on fermented products (Hilmasdottir and Karmas.1984).Indonesian fermented fish sauce "bakasang" (Ijong and OhtaYoshiyuki,1995) "Shiokara" (Morishita et al.1994), "Ika-shiokara" (Morishita et al.1995). "Burong bangus" from Philippines (Olympia et al,1992). Mold fermented seafood products are popular in Japan. The category known as Koji-zuki (Tanikawa,

1965) is prepared by adding a Koji to salted fish or roe. Another favorite Japanese product, which involves molds, is Katsuobushi.

Fish ensilage involves a lactic fermentation. Lactic starter cultures including *L.plantarum, Pediococcus* and others are added to the mixture of fish and carbohydrate source such as cereal meals, cassavaa, molasses etc and a controlled digestion proceeds (Disney et al.1977). This microbial process shows great promise as a means of utilizing fish, which might otherwise be wasted to produce a high quality animal food.

The desire for high quality products has led to the emergence of several new preservation technologies (Brown and McClure,1998). **Natural antimicrobials** like Lysozyme, nisin and other bacteriocins of LAB are being used in foods. The bacteriocins and their method of preservation will be elaborated later on in the review. **Hydrostatic pressure** in the range of 400- 600 MP (megapascals) is used to inactivate vegetative organisms. Hydrostatic pressure can be effectively used in combination with other preservatives. For example, the use of nisin or lysozyme in combination with high-pressure treatment can lead to the inactivation of spores at much lower pressures. Other technologies are **Mano-Thermal Sonication (MTS)**, which is a combination of sonication, temperature and pressure. **Electric pulse** uses high pulses of energy for inactivation of microbes. **Light pulse technology** uses high intensity light pulse to inactivate cells. **High magnetic Pulse** technology is another new technology showing potential for inactivating microorganisms.

2.4. Pathogenic bacteria associated with fish

Generally marine fish are free from pathogens except certain halophilic *Vibrio* spp. like *V. vulnificus*, *V. parahaemolyticus* and *Clostridium botulinum* type E.

Clostridium botulinum type E has been called the fish botulism organism and has been shown to occur in marine and lake sediments and fish intestine. It is unusual in being able to grow and produce toxin at low temperatures (Hobbs, 1976). However, it does not seem to produce toxin in living fish, but is carried passively. *Clostridium botulinum* is the causative agent of a fatal form of food poisoning, namely botulism, in Canada, USA, USSR and Japan (Sakaguchi, 1979). *C.botulinum* has been isolated from fish from UK, Denmark, USA, Indonesia and Thailand (Craig et al.1968; Mortojudo et al.1973; Huss et al.1974; Cann et al.1975; Suhadi et al. 1981).

There are very scanty reports of *C.botulinum* from Indian fish. Lalitha and Iyer (1990) have conducted a survey on the occurrence of *C.botulinum* in fish and shellfish landed at Kochi, India. They could isolate *C.botulinum* type B from Trichurus spp. Nambiar and Iyer (1973) have reported the incidence of 6.9% *Clostridium perfringens* in fish processing areas and processed products. Eighty percent of the bacteria was isolated from prawn guts, followed by 50% from soil, 38% prawn, 33.3% ice, 11% frozen prawn, 5% swab and 1.1% water.

Vibrios are widely distributed in aquatic environments, especially in the coastal seawater of tropics (Oliver et al.1983; Sakazaki and Shimada, 1986). *Vibrio parahaemolyticus* is perhaps more truly an indigenous organism, because it is halophilic and is known to grow in marine environment and on fish and shellfish when temperatures are high enough. The organism is distributed widely in inshore marine areas and can be readily isolated all year round from marine animals in tropical areas

(Liston, 1976), but is only found on animals during summer months in temperate zones. When *V. parahaemolyticus* is ingested at a level of 10^6 cells or more, it frequently causes a characteristic food poisoning syndrome. However, not all *V.parahaemolyticus* are pathogenic and since the level of natural occurrence only rarely approaches infective numbers, food poisoning from this organism again normally involves mishandling of seafood products. It is very sensitive to heat above 48°C and to cold particularly 0-5°C and is killed by exposure to freshwater. Most outbreaks in Japan derive from consumption of raw fish (Okabe, 1974) and elsewhere from eating shrimp and crab recontaminted after cooking and held at temperature permitting rapid growth (Barker et al.1974).

Vibrios are primarily aquatic, associated with fish and other poikilothermic animals, where they are present as the normal bacterial flora as well as pathogens (Cahill, 1990; Austin and Austin, 1993). The vibrios isolated from fish and fishery products are *V. parahaemolyticus, V.fluvialis, non-O1 V.cholerae, V.fischeri, V.harveyi, V.alginolyticus and V.vulnificus* (Lee et al. 1981; West et al. 1980; Thampuran and Surendran, 1998.).

The vibrios found in shellfishes belong to the species *V. parahaemolyticus, V.cholerae, V.vulnificus, V,mytili, V.tubiashii, V.ichthyoenterii, V.penaeicida, V.esturianus* (Thampuran and Surendran,1998; Wong et al. 1995, Singh et al.1996). Thampuran and Surendran (1998) studied the incidence and distribution of *Vibrio vulnificus* in marine and brackish water fish and shellfish from coastal areas of Cochin on the West Coast of India.

Wong et al. (1995) have studied the occurrence of vibrios in frozen seafoods and have isolated several species of *Vibrio* from peeled shrimps, fish and shrimp dumplings. Shih et al. (1996) have studied the occurrence of *V.parahaemolyticus* in

imported aquatic foods from Manila and China. The overall incidence of *V.parahaemolyticus* was found to be 16.9%,whereas 50% in clam, 25% in shrimp and 14.9% in fishes. Singh et al.(1996) have studied the prevalence of *V.parahaemolyticus* and *V.cholerae* in fishes, crustaceans, molluscs from various Indian markets.

The importance of ***Salmonella*** as a cause of foodborne illness is well recognized. The occurrence of the organism in fish and shellfish, both freshwater and marine is normally associated with the fecal contamination of the environment from which they are harvested. Heinitz et al. (2000) tested 11,312 imported and 768 domestic seafood samples over a 9-year period (1990 to 1998) for the presence of *Salmonella*. The overall incidence of *Salmonella* was 7.2% for imported and 1.3% for domestic seafood. Mohamed Hatha and Lakshmanaperumalsamy (1977) have studied the prevalence of *Salmonella* spp. in fish and crustaceans (prawns and crabs) sold in fish markets in Coimbatore. *Salmonella* spp. were detected in 17.39 and 14.25% of crustaceans and fish samples, respectively. The highest incidence of *Salmonella* in crustacean species tested was in *Portunus pelagicus* (33.33%) . In fishes, *Salmonella* were more prevalent in the Trachnidae (26%) and Scopelidae (28%) families. Incidence of *Salmonella* was higher during monsoon season. *Salmonella typhi* was isolated from both groups.

Nambiar and Iyer (1991) have studied the distribution of *Salmonella* serotypes in fresh and frozen fish in retail trade in Kochi. The fresh fish analyzed contained 5.76% of *Salmonella* and frozen fish samples were contaminated with 8.66% *Salmonella*. The *Salmonella* serotypes isolated were *S.adelaid, S.barendrup, S.chingola, S.cerro, S.nchanga, S.oslo, S.emek* and *S.mbandaka.* Nambiar and Surendran (1998) have studied the factors affecting the growth and survival of *Salmonella* serotypes in fish. *Salmonella* was found to grow at refrigerated temperatures, at a pH range of 3.5-

10.6 and in 8% salt concentration of the media. Iyer and Srivastava (1989) have reported the occurrence of *Salmonella* in fishery products.

The main reservoir of ***Staphylococcus aureus*** is man: hands, face, sweat, boils, ulcers, nasal cavities, throat, eargum and post nasal drips of man contain this organism in considerable numbers. About 30% of human population is known to be nasal carriers of this organism. Staphylococcal food poisoning is one of the most common types of foodborne illness and results from the ingestion of enterotoxins produced during growth of enterotoxigenic strains of *S.aureus* in food (Anon,1986). Published reports on Staphylococcal enterotoxins from fish and fishery products are rare. Sanjeev and Surendran (1992) have studied the incidence of Staphylococcal enterotoxins A,B,C and D and enterotoxigenic strains of Staphylococci in frozen fish and fish products. All the frozen fish products they studied were free from enterotoxins. *S.aureus* was isolated from 79.63% of the samples (cooked, picked crab meat, prawns, fish cutlet and frozen fish products) and its load varied from 55 to $1.1x\ 10^6$ cfu/g. Ninety eight *S.aureus* strains out of one hundred and forty strains tested were found enterotoxigenic and produced enterotoxins A,B,C and D either singly or in combinations.

Aerobic endospore forming bacteria have been implicated as an agent of food poisoning since 1906, but it is only in recent times that ***Bacillus*** species have been given increasing attention as a food -borne enteropathogen. The spores of *B.cereus* are relatively heat resistant and food poisoning may occur when foods prepared and held without adequate refrigeration for several hours is consumed. Under favourable conditions, the surviving heat resistant spores can germinate enabling the organisms to multiply and produce toxin. Consumption of foods that contain more than 10^6 cells of *B.cereus* per gram may result in food poisoning. *Bacillus* spp. produces a diarrhoeal

enterotoxin. Thampuran and Surendran (1996) have reported the isolation of several species of *Bacillus (B.coagulans, B.circulans, B.alvei* and *B.subtilis*) from pickled or cured fish samples. Nambiar and Surendran (1999) have reported the incidence of enterotoxigenic *B.cereus* in fresh and frozen fish. 13% of the 200 fresh fish and 20% of the 200 frozen fish samples analyzed were found to harbour *B.cereus* and 84% of the strains were found to produce diarrhoeal enterotoxins.

Velazquez et al.(1996) havestudied the prevalence of ***Yersinia enterocolitica*** in refrigerated hake (Merluccius hubbis) fillets from retail markets to determine the degree of pathogenicity. *Yersinia enterocolitica* is a human pathogenic bacterium transmitted by water and contaminated food. They reported the isolation of potentially pathogenic strains of *Yersinia* spp. which represent a risk to human health.

2.5 *Listeria* in Seafoods

Seafoods are involved in listeriosis (human foodborne illness). Outbreak of listeriosis has been linked to the consumption of shellfish and raw fish (Lennon et al. 1984). Buchanan et al. (1989) isolated the pathogen from uncooked seafood samples. Dillon and Patel (1992) have reviewed the incidence of *L.monocytogenes* in seafoods. They have isolated *L.monocytogenes* strains from 10% of fresh trout samples including serovar 4b which is involved in human infections. Also analysis of cooked shrimps, crab and smoked salmon showed that *L.monocytogenes* contaminated half of samples. Although *L.monocytogenes* was present at low levels, studies show that organisms are able to grow during storage at 4°C. *L.monocytogenes* have been isolated from ten different fish products by Boerlin et al.(1997). The presence of *Listeria monocytogenes* in frozen seafoods imported to the U.S has been recorded by Weagent et al. (1988), to the extent of 28% of the samples examined by them. Fuchs and Surendran (1989); Kamat and Nair (1994) could not detect any *L.monocytogenes* from Indian seafoods.

However, Jeyasekaran et al. (1996) have reported the incidence of *L.monocytogenes* in Indian seafoods.

The characteristics of *L.monocytogenes* are different from those of other pathogens of fish. It can grow at refrigerated temperatures and is known to survive in frozen products for a considerable length of time. Glass and Doyle (1989) reported that *L.monocytogenes* is able to grow on various meat products at refrigeration temperatures.

Listeria spp. are considered to be very resistant to high salt concentrations and can easily survive in a marinade with 6% NaCl (Guyer and Jemmi, 1991). Seeliger (1961) stated that *Listeria* could multiply in a pH interval from 5.6-9.6, while Conner et al.(1986) observed a multiplication even at a pH of 5.0.

Smoking had an antibacterial effect on sardine fillets. Studies by Messina et al.(1988) showed that liquid smoke preparations used in manufacture of frankfurters had antimicrobial activity against *L.monocytogenes*. Cold smoked fishes are more frequently contaminated with *L.monocytogenes* than hot smoked fishes. Smoking temperature below 30°C cannot eliminate *L.monocytogenes*, but conditions during smoking prevented *L.monocytogenes* from growing (Guyer and Jemmi, 1991). Studies conducted by Guyer and Jemmi (1991) show that refrigeration at 4°C to 10°C will not prevent the growth of *L.monocytogenes* in smoked salmon.

Control of *L. monocytogenes* in foods is difficult due to its ability to grow at refrigeration temperature and its tolerance to low pH. *L. monocytogenes* also tolerates certain levels of NaCl and $NaNO_2$, which are inhibitory to other food pathogens (Buchanan et al. 1989; Farber and Peterkin, 1991).

2.6 LACTIC ACID BACTERIA

The concept of Lactic acid bacteria (LAB) as a group of organisms wasdeveloped in the beginning of the 1900s. A significant contribution in this field was by Pasteur on lactic acid fermentation in 1857, followed by the first isolation of a pure bacterial culture, *Bacterium lactis*, by Lister in 1873. *Lactobacillus* organisms were described as Gram-positive bacteria by Beijerinck in 1901. The influence of selected lactobacilli in various food fermentations has been well established since then. According to Orla-Jensen (1919) the 'true lactic acid bacteria' form a natural group of gram-positive, nonmotile, non-sporeforming, rod-and coccus shaped organisms that ferment carbohydrates and higher alcohols to form chiefly lactic acid.

The classical approach to bacterial taxonomy was based on morphological and physiological features. This was expanded to include the cell wall composition, cellular fatty acids, isoprenoid quinones and other characteristics of the cells. Molecular characteristics have become important taxonomic tools, such as the mol% G+C content of the DNA, electrophoretic properties of the gene products, DNA: DNA hybridisation studies and structures and sequence of ribosomal RNA (rRNA). This has resulted in dramatic changes in taxonomy of the LAB (Schleifer, 1987). The classification of LAB remains volatile and it forms the focus of intense taxonomic study with an increasing urgency for a polyphasic approach involving both phenotypic and phylogenetic characterization of bacteria (Vandamme et al. 1996).

Lactic acid bacteria (LAB) are characterized as gram-positive, usually non-motile, non-sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism (vide infra). Members of this group contain rods, cocci and coccobacilli and have less than 55 mol% G+C content in their DNA. They are generally

catalase negative and lack cytochromes. LAB are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins. The LAB of importance in foods belong to the genera: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Vandamme et al.1996).

The classical division of the lactobacilli into (1) obligately homofermentative: (2) facultatively heterofermentative: and (3) obligately heterofermentative was based on their fermentative characteristics. Homofermenters are LAB that produce only acid and no gas after sugar fermentation, while heterofermenters produce gas from sugars. Several lactobacilli of groups 1 and 2 and some of the hetero fermentative group 3 lactobacilli are either used in fermented foods, but group 3 are also commonly associated with food spoilage.

Ringoe and Gatesoupe, (1998) have presented a review of the LAB present in fish. They have reported that *Streptococcus, Leuconostoc, Lactobacillus and Carnobacterium* belong to the normal microbiota of the gastrointestinal tract in healthy fish. The population level of LAB associated with the digestive tract is affected by nutritional and environmental factors like dietary polyunsaturated fatty acids, chromic oxide, stress and salinity. Pathogenic LAB such as *Streptococcus, Enterococcus, Lactobacillus, Lactococcus* and *Carnobacterium* have been detected from ascites, kidney, liver, heart and spleen.

Although fish has a very low carbohydrate content, LAB have been isolated from fresh and sea water fresh fish (Ross and Toth, 1974; Schroder et al.1979; Cone, 1982; Okafor and Nzeako, 1985). It has often been suggested that lactic acid bacteria found on fish might be regarded as land-borne contaminants (Kandler and Weiss, 1986).

Their growth on fish can be stimulated by the drying processes (Valdimarsson and Gudbjornsdottir, 1984). They constitute the major part of the end microflora of the marinated fish (Erichson, 1973; Blood, 1975). Studies leading to extend shelf-life of fresh fish under modified atmosphere revealed the importance of lactic acid bacteria (Lannelongue et al.1982; Oberlander et al. 1983; Molin et al.1983; Wang and Ogrydziak, 1986).

Lactic acid bacteria isolated from seafood are mostly unidentified: most isolates have been described as atypical *Lactobacillus.* Mauguin and Novel (1994) isolated Lactic acid bacteria from various samples of seafood: fresh pollock, brine shrimp, gravad fish, vacuum packed seafood (surimi, smoked tuna, salted cod) and fish stored under 100% CO_2 at 5°C (smoked tuna, fresh and salted cod, salmon). They isolated 54 strains of *L.lactic subsp.lactis*, four strains of *L.plantarum*, eight strains of *Leuconostoc* and sixteen strains of *Carnobacterium* from various samples of seafood. The 32 strains of *Lactococcus lactis subsp lactis* were found in all samples except surimi. *Leuconostoc mesenteroides* strains were found only on non-salted samples and on surimi. *Carnobacterium* strains were isolated from salmon, cod and pollock. *L.plantarum* was the only *Lactobacillus* strain identified from gravad fish and brined shrimp. Sixty percent of LAB isolated from fish were nonacidic. Most strains isolated from seafood were atypical.

Stiles and Holzapfel (1997) have conducted a review on the Lactic acid bacteria from foods and their current taxonomy, which is as given below.

2.6.1.1 *Streptococcus*

The generic name *Streptococcus* was first used by Rosenbach (1884) to describe the chain-forming, coccus-shaped homofermentative bacteria associated with

wound infections. Lancefield (1933) proposed the serological differentiation of the streptococci into groups A - E and N based on their 'C substances'(capsular substance). Sherman (1937) proposed the first systematic classification of the *Streptococcus*. He classified them into four groups, Pyogenic, Viridans, Lactic and Enterococcus. The genus *Streptococcus* comprises a wide range of organisms *S.pneumoniae, S.pyogenes, S.agalactiae, S.faecalis, S.faecium*. The economically important group N starter bacteria are *S.cremoris , S.lactis and S.thermophilus.*

The *Streptococcus* have complex nutritional requirements and thrive in environments with a good supply of carbohydrate and protein including tissues and intestinal tracts of animals, milk, dairy products, vegetable material and other foods (Jones, 1978).

2.6.1.2 *Lactococcus*

Lancefield's group N lactic streptococci have been transferred to the new genus *Lactococcus* (Schleifer et al. 1985). The cells of *Lactococcus* are sometimes elongated in the plane of chain formation appearing as coccobacilli. The genus *Lactococcus* includes several uncommon species: *L.garvieae* associated with mastitis in cows, *L.piscium* from salmonid fish, *L.plantarum* from frozen peas and *L.raffinolactis* from raw milk (Schleifer et al.1985; Williams et al.1990). Based on the studies of Schleifer et al. (1985), there was the revolutionary reclassification of *L. xylosus* as *L.lactis subsp.lactis* and *L.hordinae* as *L.lactis subsp hordinae*.

The subspecies of *L.lactis* are of great economic importance and have been extensively studied for their biochemical and physiological characteristics and their effect on foods (Teuber et al.1991). The subspecies of *L. lactis* are the most important of the commercially used LAB. *L.lactis* is commonly isolated from plant material

(Sandine et al.1972), but the most recognized habitat for the *Lactococcus* is dairy products. They are non-motile, coccus-shaped homofermentative bacteria that grow at 10°C but not at 45°C and produce L(+)-lactic acid from glucose. The use of *Lactococcus* is widespread and has the longest tradition in industrial starter culture technology. Genetic studies on *Lactococcus* have focused on the lactic fermentation, caesin breakdown, and diacetyl production from citrate and resistance to phage attack. Production of inhibitory substances (bacteriocins) by LAB is an area of increasing interest. Strains of *L.lactis* produce a range of bacteriocins the most important of which is the lantibiotic, nisin, which is a relatively broad spectrum bacteriocin that is active against gram-positive bacteria, including *Clostridium botulinum* and its spores. Pilet et al. (1995) have isolated *Lactococcus* from marinated fish. *Lactococcus* strains were the major flora isolated from fish by Maugin and Novel (1994). That is in contradiction with published reports (Erichsen, 1973; Lannelongue et al.1982; Oberlander et al. 1983; Molin et al.1983; Wang and Ogrydziak, 1986; Valdimarsson and Gudbjornsdottir, 1984).

2.6.1.3. *Enterococcus*

Sherman (1937) separated the Lancefield group D streptococci into a new genus *Enterococcus*. The *Enterococcus* were described as those organisms that grow both at 10°C and 45°C, in 6.5% NaCl and at pH 9.6, survive heating at 60°C for 30min, and react with Lancefield group D antisera. *Enterococcus* produce L(+)-lactic acid homofermentatively from glucose and derive energy from degradation of amino acids. They have a PEP-PTS system for uptake of lactose and other carbohydrates, including gluconate (Bernsmann et al., 1982). The species included in the genus *Enterococcus* include, *E.faecalis*, *E.faecium*, *E.avium*, *E.columbae*, *E.dispar*, *E.durans*, *E.fallox*,

E.flavescens, E.gallinarum, E.hirae, E.mundtu, E.malodoratus, E.pseudoavium, E.raffinsus, E. saccharolyticus, E.seriolicida and *E.solitarus.*

The importance of the *Enterococcus* for food and public health microbiologists is related to their external habitat, their use as indicators for food safety and their possible involvement in foodborne illness (Stiles, 1989). The value of *Enterococcus* as indicators of faecal contamination of foods is favoured by their ability to survive in the extra enteral environment, their relatively high heat resistance and the fact that they can dominate the microbial population of heat treated foods.

2.6.1.4 *Carnobacterium*

Lactobacilli that did not grow in acetate medium were grouped as a new genus *Carnobacterium.* Collins et al. (1987) proposed this genus and therefore it did not appear in 'Bergey's Manual of Systematic Bacteriology' published in 1986. Thornley (1957) reported the isolation of gram-positive, catalase-negative, non-sporeforming rods from poultry meat stored at low temperature. A similar group of bacteria isolated by Shaw and Harding (1984) from vacuum-packaged chill stored meats was referred to as the 'non-aciduric lactobacilli'. Two groups of non-aciduric lactobacilli had previously been described and proposed as the new species *L.divergens* and *L.carnis*. Originally thought to be heterofermentative, these organisms were shown to follow the glycolytic pathway of glucose fermentation with small amounts of CO_2 being produced by some form of endogenous metabolism (De Bruyn et al.1988). Because these two species were not included in earlier comparative studies, Collins et al. (1987) studied them in association with *L.piscicola*, the pathogen of salmonid fish (Hiu et al.1984), which shares many properties with the non-aciduric (atypical) lactobacilli from meat.

The genus *Carnobacterium* was proposed by Collins et al. (1987). *Carnobacteria* are present in meat, poultry, surface ripened mould cheeses, salmonid fish and Antarctic lake (Collins et al.1987). *Carnobacterium* have been isolated from kidney tissues or visceral organs of Salmonids (Cone, 1982; Hiu et al. 1984). Pilet et al. (1995) has isolated *Carnobacterium piscicola* and *C.divergens* from fish intestine. The species present in the genus *Carnobacterium* are *C.divergens, C.galinarum, C.mobile, C.piscicola, C.funditum* and *C.alterfunditum.*

2.6.1.5. *Leuconostoc*

Heterofermentative cocci formerly called the betacocci by Orla Jensen was classified as *Leuconostoc*. They produce D(-) lactate from glucose as opposed to L(+) lactate that is produced by the *Lactococcus* and DL-lactate by the heterofermentative *Lactobacillus* with whom they share many characteristics. *Leuconostoc* is the predominant genus among the LAB on plants, with *Leuconostoc mesenteroides subsp mesenteroides* as the principal isolate. In fermented foods of plant origin, *Leuc.mesenteroides* is generally the first organism to grow and it is succeeded by the more acid tolerant lactobacilli. The *Leuconostoc* group has considerable species-specific commercial importance, including spoilage in sugar processing by production of dextrans, the malolactic fermentation in wine making, production of flavor components from citrate in dairy fermentations and production of dextrans that have wide applications in research, industry and medicine. The species belonging to the genus *Leuconostoc* are *Leuc.mesenteroides, Leuc.mesenteroides* ssp *cremoris, Leuc.mesenteroides* ssp *dextranicum, Leuc.lactis, Leuc.carnosum, Leuc.gelidium, Leuc.amelibiosum* and *Leuc.fallax.*

2.6.1.6 *Pediococcus*

They are homofermentative cocci. Tetrad formation and spherical shape served as key characteristics for their early recognition. They were the only LAB that divided in two planes to produce tetrads or pairs. The *Pediococcus* are salt tolerant, homofermentative and produce 'pseudocatalase'. All species produce DL-lactate from glucose. In Bergey's Manual of Systematic Bacteriology, eight species are recognized. Collin s et al (1990) clearly demonstrated a relationship between the *pediococci* and *lactobacilli* of the *L.casei* group. *P.acidilactici, P.damnosus, P.parvulus and P.pentosaceous* form an evolutionary grouping with *L.pentosus, L.brevis and L.buchneri*. The species grouped under the genus *Pediococcus* are *P.damnosus, P.dextrinicus, P.parvulus, P.inopintus, P.pentosaceous* and *P.acidilactici. Pediococcus* were isolated from breweries, wine, cider beer, silage, sauerkraut, vegetable material, fermented sausages, milk and dairy products.

2.6.1.7 *Lactobacillus*

In Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986) the genus *Lactobacillus* was described with a heterogeneous group of 'regular non-sporing gram-positive rods'. Lactobacilli are rod shaped homofermentative and heterofermentative LAB. The genus *Lactobacillus* is heterogeneous with 33-55 mol% G+C content in the DNA (Collins et al. 1991; Hammes and Vogel. 1995). *Lactobacillus* grow under anaerobic conditions or atleast under reduced oxygen tension in all habitats providing ample carbohydrates breakdown products of protein and nucleic acids, and vitamins. A mesophilic to slightly thermophilic temperature range is favorable. However, strains of some species *(L.viridescens, L.sake, L.curvatus, and L.plantarum*) grow even at low temperatures close to freezing point. Lactobacilli are generally

aciduric or acidophilic. They decrease the pH of their substrate to below 4.0 by lactic acid formation, thus preventing or severely delaying, growth of virtually all other competitors except other lactic acid bacteria and yeasts. These properties make lactobacilli valuable inhabitants of the intestinal tract of man and animals and important contributors to food technology.

Different species of *Lactobacillus* have adapted to grow under widely different environmental conditions, and they are widespread in nature. *Lactobacillus* are commonly found in the gastrointestinal tract of various endothermic animals (mice, rats, pigs, fowl and humans) (Tannock et al.1982; Finegold et al.1983; Tannock, 1988); in milk and dairy products (Sharpe, 1981); seafood products (Maugin and Novel, 1994; Gancel et al.1997) and on some plant surface (Keddie, 1959). They are generally used in the production and preservation of food products like cheese, sauerkraut, meat, yogurt and silage (Gibbs, 1987; Mckay and Baldwin, 1990). The role of *Lactobacillus* within the digestive tract of endothermic animals has been extensively investigated and reviewed (Conway, 1980; Sissons, 1989; Gorbach, 1990; Goldin and Gorbach, 1992; Jonson and Conway, 1992).

Lactobacillus occur in nature in low numbers at all plant surfaces and together with other lactic acid bacteria grow luxuriantly in all decaying plant material, especially decaying fruits. Species chiefly isolated from plants have been *L.plantarum, L.brevis, L.coryniformis, L.casei, L.curvatus, L.fermentum* and *L.sake.*

Milk contains no lactobacilli when it leaves the udder, but becomes very easily contaminated with lactobacilli by dust, dairy utensils, etc. The common *Lactobacillus* species isolated from milk and dairy products are *L.helveticus, L.delbrueckii subsp.bulgaricus*, . *L.delbrueckii subsp.lactis, L.plantarum, L.brevis,*

L.casei and *L.kefir*. Although several species of lactobacilli may contribute to spoilage of dairy products by slime or gas production, only two species cause specific spoilage, *L.maltaromicus* and *L.bifermentans*.

Lactobacillus plays an important role in the curing process of fermented sausages containing added sucrose. The most common naturally occurring species that is found in ripening raw sausages are *L.plantarum, L.brevis, L.farciminis, L.alimentarius, L.sake* and *L.curvatus*. Various species of *Lactobacillus* multiply during cold storage of meat products. This delays spoilage by proteolytic bacteria, but may also lead to spoilage by producing off flavor, acid taste, gas, slime or greening.

The intestinal tract of man and animals harbors many species of *Lactobacillus* living as commensals intimately associated with the mucous surface epithelium. *L.salivarius* may be the most typical species of the mouth flora, although it is also found in the intestinal tract. The most prominent species, probably indigenous to the intestine, is *L.acidophilus*, which is believed to exert a beneficial effect on human and animal health. It is used on an industrial scale in preparing acidophilus sour milk and producing pharmaceutical preparations for restoring the normal intestinal flora after disturbance caused by disease or treatment with antibiotic. The most common species isolated are *L.gasseri, L.fermentum, L.animalis, L.reuteri, L.murinus, L.ruminis* and *L.vitulinus*.

2.6.2 Lactobacillus from fish and fishery products

Lactobacillus are not considered to be indigenous in the marine environments (Kandler and Weiss, 1986), but occur as spoilage organisms in fish and fish products (Shewan, 1977; Magnusson and Traudottir, 1982; Hobbs, 1983). However

recent studies show that *Lactobacillus* are present in fish and fish products. Although fish has a very low carbohydrate content, *Lactobacillus* has been isolated from fresh and seawater fresh fish (Ross and Toth, 1974; Schroder et al.1979; Cone, 1982; Okafor and Nzeako, 1985). *Lactobacillus* has been isolated from intestines of Herring (Kraus, 1961); *L. plantarum* has been identified from gut of Saithe (Schroder et al 1979).

L.plantarum predominated the bacterial flora of cod (*Gadus morhua*) and saithe (*Pollachius virens*) during the drying process (Vladimarsson and Gudbjornsdottir, 1984). Gancel et al.(1997) have isolated 78 strains belonging to the genus *Lactobacillus* from fillets of vacuum packed smoked and salted herring (*Clupea harengus*). Growth of LAB was stimulated in marinated fish (Erichsen, 1973; Blood, 1975). Studies leading to extend shelf- life of fresh fish under modified atmospheres revealed the importance of LAB (Lannelongue et al.1982; Oberlander et al.1983; Molin et al.1983; Wang and Ogrydziak, 1986).

LAB has been found to occur in marinated herring (Blood, 1975), herring fillets (Molin et al. 1983), cured stockfish (Vladimarsson and Gudbjornsdottir ,1984). In marinated or dried fish, the lactic acid flora maybe quite diverse since the presence of *Lactobacilli* and *Pediococci* has been reported (Blood, 1975; Erichsen 1973, Vladimarsson and Gudbjornsdottir, 1984).

Thai fermented fishery products were screened for the presence of LAB by Ostergaard et al. (1998). LAB was found to occur in the low salted fermented products in the range of 10^7-10^9 cfu/g. The high salt product hoi dorng had a lower LAB count of 10^3-10^5 cfu/g. Olympia et al. (1992) have isolated 10^8 LAB/g from a Philippine low salt rice-fish product burong bangus. The bacterial flora of stored smoked fish consists mainly of *Lactobacillus* spp. (Guyer and Jemmi,1991).

2.6.3 *Lactobacillus* as Probiotics

Interest in the lactobacilli in the human diet was great at the turn of the 20th century when Elie Metchnikoff at the Pasteur Institute in Paris promoted their use in the diet for bacterioprophylaxis and bacteriotherapy. Fuller (1989) has defined probiotics as " a live microbial feed supplement which beneficially affects the host animals by improving its intestinal microbial balance". The beneficial effects of probiotics are well established for terrestrial animals, whose flora is composed of about 90% of facultative lactic acid producing bacteria (Fuller, 1989; Sissons, 1989; Tannock, 1988; Aguirre and Collins, 1993; Montes and Pugh, 1993).

2.6.4 Pathogenic Lactic Acid Bacteria

Apart from dental caries, Lactobacilli are generally considered to be non-pathogenic. However, streptococcosis outbreaks have occurred for more than three decades in Japanese farms culturing rainbow trout (Hoshina,1956).

The pathogen initially identified as *Streptococcus* species has been reclassified as *Enterococcus seriolicida* (Kusuda, 1992), then as *Lactococcus gravieae* (Domenech et al.1993; Eldar et al.1996). The genus *Lactobacillus* has been reported to cause kidney disease in Pacific salmon (Earp et al.1953; Rucker et al.1953). Nowadays, a growing number of diseases that appeared with the world- wide development of aquaculture may be assigned to distinct bacteria belonging to the genera *Streptococcus, Lactococcus, Vagococcus* and *Carnobacterium. L.casei subsp.rhamnosus, L.acidophilus, L.plantarum* and occasionally *L.salivarius* have been found to be associated with subacute bacterial endocarditis, systemic septicemia and abscesses. A homofermentative lactobacilli was the only organism isolated in pure culture from a case of chorioamnionotis and *L.gasseri* was found in a case of urosepsis. The many

cases in which lactobacilli have been isolated from diseased tissue indicate their potential pathogenicity. However, the biochemical basis of such pathogenicity is unknown (Kandler and Weiss, 1986). Aguirre and Collins (1993) have reported the involvement of LAB in human clinical infection. They also focussed attention on the importance of Enterococci in clinical infection.

2.6.5 LAB in Biopreservation

LAB has for centuries been responsible for the fermentative preservation of many foods. Currently there is interest in possible use of LAB as biocontrol agents. The earliest reference to competitive inhibition of pathogenic bacteria by competing microbial cultures relate to clinical applications that date back to the end of the 19th century and the beginning of the science of microbiology (Florey, 1946). One of the first reports of the use of LAB as biocontrol agents in foods was the work of Saleh and Ordel (1955), who demonstrated the antagonistic effects of a *Lactococcus lactis* (formerly *Streptococcus lactis*) inoculum on the growth, and formation of toxin by *Cl.botulinum* in a frozen chicken '*a la king*' product. A more recent example is the Wisconsin process for ensuring the safety of bacon (Tanaka et al.1985). Protective LAB cultures in bacon was found to be more effective than 120ppm of sodium nitrite, the maximum amount of nitrite allowed on cured meats. The use of biocontrol LAB has been investigated for a variety of applications with refrigerated meat products (Holzapfel et al.1995).

In addition to being used as a competitive biocontrol cultures, LAB has also been added to non-fermented foods to extend shelf- life. Gilliland and Speck (1975) and Raccah et al. (1979), both used large numbers of LAB (10^8 or more) inoculated on meats to extend shelf life and prevent the growth of pathogens.

LAB species can produce a variety of metabolites (Table2.1), including lactic and acetic acids which lower pH, that are inhibitory to competing bacteria, including psychrotrophic pathogens (de Vyust and Vandamme, 1992; Vandenberg,1993). The inhibition by organic acids has been attributed to the protonated form of these acids, which are uncharged and may therefore cross biological

membranes. The resulting inhibition of growth maybe due to acidification of the cytoplasm and/or accumulation of anions inside the cell (Russel,1992). LAB can inhibit growth of other bacteria by lowering the pH (Tramer,1966), by producing H_2O_2 (Dahiya and Speck,1968; Gilliland and Speck,1961), and by antibiotics (de Klerk and Coetzee,1961; Reeves,1972; Upreti and Hinsdill,1973; Lindgren and Clenstrom,1978). Stimulation of the growth of LAB is therefore a method of preserving food or feed.

Table 2.1 : Inhibitory metabolites of LAB (adapted from Holzapfel et al.1995)

Product	Main target organisms
Organic acids	
Lactic acid	Putrefactive and G-negative bacteria, some fungi.
Acetic acid	Putrefactive bacteria, Clostridia, some fungi and yeast.
Hydrogen peroxide	Pathogens and spoilage organisms especially in protein-rich foods.
Enzymes	
Lactoperoxidase system with hydrogen peroxide	Pathogens and spoilage bacteria (milk and dairy products).
Lysozyme (by Recombinant DNA)	Undesired G-positive bacteria
Low-molecular-weight metabolites	
Reuterin	Wide spectrum of bacteria,yeasts and molds.
Diacetyl	G-negative bacteria
Fatty acids	Different bacteria
Bacteriocins	
Nisin	Some LAB and G-positive bacteria, notably endospore-formers
Other	G-positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type.

2.7 Bacteriocins

Bacteriocins are proteinaceous compounds produced by bacteria that exhibit a bactericidal or bacteriostatic mode of action against sensitive bacterial species (Klaenhammer, 1988; Tagg et al. 1976). Lactic acid bacteria have been studied extensively for bacteriocinogenicity. Considerable emphasis has been placed on the identification of bacteriocins from lactic acid bacteria associated with dairy products. Researchers presently are isolating strains of lactic acid bacteria associated with meat and vegetable fermentations and characterizing their bacteriocins. Methods for detection, characterization, purification and identification of genetic determinants of bacteriocins from Gram-positive (Tagg et al. 1976) and Gram-negative organisms are addressed in several reviews (Reeves, 1965; Konisky, 1978; Hardy, 1982).

2.7.1 Bacteriocins of Lactic Acid Bacteria

2.7.1.1 Bacteriocins of *Lactococcus*

Lactococcus are widely used as starter cultures in dairy industry. Several strains of dairy species produce bacteriocins. *Lactococcus lactis* and subspecies produce diplococcin, lactococcin, lactostrepcins or nisin. Diplococcin is one of the earliest bacteriocins identified in LAB. It inhibits DNA and RNA synthesis in sensitive cells of selected strains of *L.lactis* resulting in death without lysis. Lactostrepcins are group of acid tolerant bacteriocins produced by non-nisin producing strains of *L.lactis biovar diacetylactis*, and some strains of *L.lactis subsp cremoris*. Antagonistic activity has been detected against other lactococci group A,C and G streptococci, *Bacillus cereus*, *L.helveticus*, *Leuconostoc mesenteroides subsp.cremoris* and *Leuc.paraacitrovorum*. Lactostrepcin 5 disrupts the integrity of the cell membrane.

Nisin is the most extensively characterized bacteriocin of the antimicrobial proteins produced by LAB. Nisin belongs to the 'lantibiotics'. It is active against most Gram-positive bacteria including lactococci, bacilli, micrococci, *Staph.aureus*, *L.monocytogenes* and *Cl.botulinum*. At 10°C, the shelf life of cod, hot smoked mackerel and herring was extended through the use of nisin and packaging in a CO_2 atmosphere (Taylor et al.1990). However, toxin production by *Cl.botulinum* was observed in fish that were subjected to temperature abuse above 26°C. Therefore, the CO_2 could be creating an environment favourable for the outgrowth of *Cl.botulinum or Cl.botulinum* spores might be resistant to nisin at the concentration used.

2.7.1.2 Bacteriocins of *Pediococcus*

Pediococci are involved with the fermentation of many vegetables, cheeses, meats and sausage products. Pediocins are bacteriocins produced by three species within the genus *Pediococcus, P.acidilactici, P.cerevisiae and P.pentosaceus.* Pediocins exhibit a wide spectrum of activity against Gram-positive organisms including *LAB, L.monocytogenes, Staph.aureus and Clostridium.*

Pediocin AcH produced by *Pediococcus acidilactici* strain H, was isolated from fermented sausage. It is active against many lactobacilli, *Leuconostoc* , *L.monocytogenes, S .aureus, Cl.perfringens,* and *Pseudomonas putida*. The mode of action of this bacteriocin is thought to be related to inhibition of ATP synthesis, impairment of transport systems, or damage to permeability barriers of the cytoplasmic membrane.

Pediocin PA-1 is produced by *P.acidilactici* strain PAC-10. It inhibits other pediococci, lactobacilli, leuconostoc, *L.monocytogenes.*

2.7.1.3 Bacteriocins of *Leuconostoc*

Leuconostoc spp. are lactic acid bacteria found in raw foodstuffs, dairy products and wine fermentations. Most often the antagonistic compounds are not bacteriocins, but acetate or diacetyl. Studies show that, wine and dairy isolates of *Leuconostoc* spp. produced bacteriocin-like compounds active against selected strains of *L.lactis subsp.lactis* (Orberg and Sandine,1984).

Mesenterocin 5 is produced by *Leuconostoc mesenteroides* UL5, isolated from Swiss-type cheese. It inhibits gram-positive bacteria such as *L.monocytogenes, Streptococcus faecalis, Brevibacterium linens* and *Pediococcus.pentosaceus*. Leucocin A is produced by *Leuconstoc gelidium* isolated from meat that was packaged under 30% CO_2. It produces antimicrobial activity that was unrelated to acid or hydrogen peroxide. It inhibits *Leuconostoc*, lactobacilli, pediococci, and *L.monocytogenes*. Leuconocin S is produced by *L.paramesenteroides* OX isolated from retail lamb. It was found to be active against *L.monocytogenes, S.aureus, Lactobacillus sake, Aeromonas hydrophila, Yersinia enterocolitica* and some strains of *Cl.botulinum*. *L.carnosum* LA44A isolated from vacuum packaged vienna-type sausage was found to produce the bacteriocin designated carnocin (Van Laack et al.1992). It was active against lactobacilli, carnobacteria, enterococci, pediococci, leuconostoc and *Listeria* spp.

2.7.1.4 Bacteriocins of *Carnobacteria*

The newly named genus *Carnobacterium* spp. includes nonaciduric, heterofermentative lactobacilli isolated from poultry, fish and vacuum-packaged meat. While identification and classification of Carnobacteria is relatively recent, bacteriocins associated with this genus have been reported.

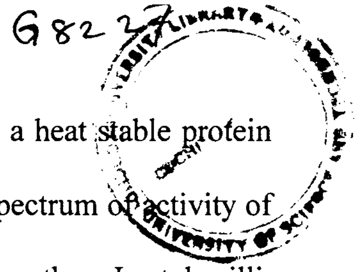
Carnobacteriocins A1, A2, A3 produced by *Carnobacterium piscicola* LV17 isolated from meat active against other LAB. Carnobacteriocins B1 and B2 produced by *C.piscicola* LV17B. Carnocin V149 is produced by *C.piscicola* isolated from fish, with activity against Carnobacteria, lactobacilli, pediococci and lactococci.

2.7.1.5 Bacteriocins of *Lactobacillus*

Antagonism by Lactobacilli has been attributed to metabolic end products such as acid, lactoperoxidase, diacetyl and H_2O_2. . Elimination of these inhibitory compounds from sample preparations has facilitated the detection of bactericidal proteins. Lactobacilli that produce bacteriocins have been cultured from naturally fermented dairy products, nondairy fermentation (plant and meat) starter cultures and plant, animal or human isolates.

In 1961, Fermenticin was isolated from *Lactobacillus fermentii* cultures. Strains of *L.plantarum*, a culture associated with silage and/or vegetable fermentations, produce the bacteriocins plantaricinA and plantacin B. Plantacin B, a bacteriocin-like inhibitor produced by *L.plantarum* NCDO 1193 is a protein with a narrow spectrum of activity against other strains of *L.plantarum*, *Leuconostoc mesenteroides* and *Pediococcus damnosus*.

L.sake present in fermented sausage produces sakacin A, sakacin M and lactocin S. They inhibit other lactobacilli as well as *L.monocytogenes*. Sakacin P produced by *L.sake* LTH 673, a meat isolate inhibits lactobacilli including meat starter cultures and spoilage organisms, *Leuconostocs*, carnobacteria, enterococci, *Brochothrix thermosphacta* and *Listeria* spp.


G 82

Lactocin S, the bacteriocin produced by *L.sake* L45 is a heat stable protein active against *Pediococcus, Leuconostoc and Lactobacillus*. The spectrum of activity of curvacin A from *L.curvatus* LTH 1174 is directed towards other Lactobacilli, *Leuconostoc*, Carnobacteria, *L.monocytogenes*, *L.ivano*vii as well as weakly inhibiting micrococci and staphylococci. Brevicin 37 produced by *L.brevis* B37 is active against other LAB. Caesicin 80 produced by *L.casei* B 80 exerts a weak bactericidal effect on sensitive cells. Plantaricin BN and bavaricin MN isolated from *L.plantarum* BN and *L.bavaricus* MN from retail beef.

Table 2.2 : Bacteriocins of Lactic Acid Bacteria

Lactic Acid Bacteria	**Bacteriocins**
Lactococci	Diplococcin, Lactostrepcins, Lactostrepcin 5, Lactococcin I, Lactococcin A, Lactococcin M and N Lactococcin B, nisin, Lacticin 481.
Pediococci	Pediocin AcH, Pediocin PA-1, Pediocin A.
Leuconostoc	Mesenterocin 5, Leucocin A, Leuconocin S Carnocin.
Carnobacteria	Carnobacteriocin A1, A2, A3 Carnobacteriocin B1, B2 Carnocin U149, Piscicocin V1, Divercin V41.
Lactobacilli	Fermenticin, Plantaricin A, Plantaricin B, Plantaricin BN, Sakacin A, Sakacin M, Sakacin P, Lactocin S, Curvacin A, Brevicin, Caseicin 80, Bavaricin MN, Lactocin 27, Helveticin J, Helveticin V-1829, Lactacin F, Lactacin B.

2.7.2 Nonbacteriocin - inhibiting compounds

2.7.2.1 Diacetyl

Diacetyl is best known as the compound responsible for the characteristic aroma and flavor of butter. The compound is produced by a variety of genera including *Lactococcus, Leuconostoc, Lactobacillus* and *Pediococcus. Lactococcus lactis subsp. Lactis biovar diacetylactis* and *Leuconostoc sp.* can use citrate present in milk to produce diacetyl. This dicarbonyl was first identified as a flavor compound in 1929 and its antibacterial properties were observed in 1936 (Hano, 1936). The compound was more active against Gram-negative than Gram-positive bacteria.

2.7.2.2 Acetaldehyde

Acetaldehyde is produced in certain dairy fermentations, notably yoghurt, where it arises from the activity of the enzyme threonine aldolase in *L.bulgaricus*. It has been reported by Egyud that 44ppm of acetaldehyde would inhibit cell division in *E.coli* (Egyud, 1967). Therefore, this compound might play a small role in controlling the growth of contaminants in this food system.

2.7.2.3 Hydrogen peroxide

Gunsalus and Umbreith first observed the production of bacterial hydrogen peroxide in 1945. They observed that *Lactobacillus delbrueckii subsp. bulgaricus* and *L.helveticus* produced hydrogen peroxide. Later, hydrogen peroxide production was observed among several genera of Lactics including lactobacilli, lactococci and pediococci. The lactobacilli have been identified as producing more hydrogen peroxide than other starter culture bacteria (Gilliland and Speck, 1975). Studies demonstrated that *L.delbrueckii subsp. bulgaricus* could inhibit *Pseudomonas fluorescens*, at refrigeration temperatures through the production of hydrogen peroxide (Ross, 1981). The production of hydrogen peroxide and its application to the inhibition of a variety of spoilage agents has been the subject of various patent applications (Matrozza et al. 1988).

Chapter.3

MATERIALS AND METHODS

3. Materials and Methods

3.1. Materials

3.1.1. Bacterial Strains

Listeria monocytogenes (ATCC 19111), *Salmonella typhimurium* (ATCC 14028), *Vibrio vulnificus* (ATCC 2406) were obtained from the American Type Culture Collection (Maryland, USA). *Vibrio cholerae* (Vc-7), *Bacillus cereus* (B3/3), *Staphylococcus aureus* (SA3B), *Escherichia coli* (Ec101) were from CIFT type culture collection National Collection of Aquatic and Fish Bacteria (NCAFB). *Lactobacillus casei* (NCIM 2165) obtained from National Collection of Industrial Microorganisms (NCIM, Pune). All other cultures of LAB used for analysis were isolated from various sources as a part of the study and identified by standard methods. (USFDA, 1995 and Bergey's Manual of Systematic Bacteriology, Kandler and Weiss, 1986).

3.1.2. Fish

Fresh Fish were procured from retail markets of Polakandam (Kochi), Thevara, Fortkochi and Kaloor. Ocean fresh fish were procured from fish landing centers in Fortkochi. Farm fresh fish/prawn were obtained from aquaculture farms at Chellanam and Thrissur. The freshly caught fish were packed in iceboxes and transferred to the laboratory within 2h. The following fish species were sampled.

Table 3.1.A List of fresh fishes from local markets used in the study

Scientific name	Common name
1. *Acanthopagrus berda*	Picnic silver-bream
2. *Caranx malabaricus*	Malabar trevally
3. *Chanos chanos*	Milk fish
4. *Euthynnus affinis*	Little tuna
5. *Lactarius lactarius*	White fish/Big-jawed jumper
6. *Lepturacanthus savala*	Silver ribbon-fish
7. *Lutjanus malabaricus*	Malabar red-snapper
8. *Lobotes surinamiensis*	Brown tripletail
9. *Nemipterus japonicus*	Japanese thread-fin-bream
10. *Oreochromis mossambicus*	Tilapia
11. *Otolithus cuvieri*	Lesser tiger-toothed croaker

Table 3.1.B. List of fresh fishes from local markets used in the study

Scientific name	Common name
12. *Pampus argenteus*	Silver pomfret
13. *Parastromateus niger*	Black pomfret
14. *Protonibea diacanthus*	Jewfish
15. *Rastralliger kanagurta*	Indian mackerel
16. *Sardinella longiceps*	Indian oil sardine
17. *Scatophagus argus*	Spotted butter-fish
18. *Scomberomorus commersoni*	Narrow-barred seer-fish
19. *Arius dussumieri*	Dussumier's cat-fish
20. *Mugil cephalus*	Flat head grey mullet
21. *Sphyraeno jello*	Banded barracuda
22. *Etroplus suratensis*	Pearlspot

Table 3.2. List of ocean fresh fishes used for the study

Scientific name	Common name
1.*Rastralliger kanagurta*	Indian Mackerel
2.*Sardinella longiceps*	Oil sardine

Table 3.3. List of farmed fishes/prawn used for the study

Scientific name	Common name
1. *Labeo rohita*	Rohu
2. *Catla catla*	Catla
3. *Etroplus suratensis*	Pearl spot
4. *Penaeus monodon*	Giant tiger Prawn

Frozen fish were obtained from local retail cold storages in and around Kochi. They were transported to the laboratory in ice- boxes within 2h. The following frozen fishes were analysed.

Table 3.4. List of frozen fish used for the study

Scientific name	Common name
1. *Acanthopagrus berda*	Picnic silver-bream
2. *Alepes djeddaba*	Djeddaba trevally
3. *Etroplus suratensis*	Pearl spot
4. *Epinephleus malabaricus*	Malabar reef-cod
5. *Euthynnus affinis*	Little tuna
6. *Lepturacanthus savala*	Silver ribbon-fish
7. *Lobotes surinamiensis*	Brown tripletail
8. *Oreochromis mossambicus*	Tilapia
9. *Otolithus cuvieri*	Lesser tiger-toothed croaker
10. *Pampus argenteus*	Silver pomfret
11. *Parastromateus niger*	Black pomfret
12. *Sardinella longiceps*	Indian oil sardine
13. *Scatophagus argus*	Spotted butter-fish
14. *Scomberomorus commersoni*	Narrow-barred seer-fish
15. *Sphyraeno jello*	Banded barracuda

Salted and dried fishes/prawns were obtained from local markets in Polakandam, Vyttila, Pachalam, Palluruthy and Kaloor. The samples were packed in sterile plastic bags and brought to the laboratory. The following fish species were analysed.

Table 3.5.List of cured fish/prawn used for the study

Scientific name	Common name
1. *Cynoglossus macrostomus*	Malabar tongue-sole
2. *Escualosa thoracata*	White sardine
3. *Johnieops aneus*	Grey –fin croaker
4. *Lactarius lactarius*	White fish/Big-jawed jumper
5. *Leiognathus splendens*	Pony fish
6. *Lepturacanthus savala*	Silver ribbon-fish
7. *Otolithus cuvieri*	Lesser tiger-toothed croaker
8. *Penaeus indicus*	Indian white-prawn
9. *Rachycentron canadus*	Blank King-fish/cobia
10. *Rastralliger kanagurta*	Indian mackerel
11. *Sardinella longiceps*	Indian oil sardine
12. *Scoliodon spp*	Yellow dog-shark
13. *Thryssa malabarica*	Malabar anchovy

Pickled fish/prawn in glass jars were obtained from the local retail shops in Kochi. The following fish spp were analysed.

Table 3.6. List of pickled fish/prawn used for the study

Scientific name	Common name
1. *Thunnus albacares*	Little tuna
2. *Scomberomorus commerson*	Narrow-barred seer fish
3. *Penaeus indicus*	Indian white prawn

Fresh/frozen shellfish were obtained from retail markets in Kochi packed in iceboxes and transferred to the laboratory within 2h. The following shellfishes were analysed.

Table 3.7. List of fresh/frozen shellfish used for the study

Scientific name	Common name
1. *Metapenaeus affinis*	Brown shrimp (Kazhanthan prawn)
2. *Metapenaeus dobsoni*	Flower tail prawn (Poovalan)
3. *Macrobrachium rosenbergii*	Giant freshwater scampi
4. *Parapenaeopsis stylifera*	Kiddi prawn
5. *Penaeus indicus*	Indian white prawn (Naran)

3.1.3. Media

The bacteriological media used in the study were of two categories.

3.1.3.1. Dehydrated Media

The following dehydrated media from Oxoid, England and Difco laboratories, USA were used.

1. Brain Heart Infusion **(BHI)**
2. Brain Heart Infusion Agar **(BHIA)**
3. Tryptone Soya Agar **(TSA)**
4. Trypticase Soy Broth **(TSB)**

3.1.3.2. Compounded Media

1.Gibsons Liquid Tomato Juice Media

Peptone	10g
Yeast extract	2.5g
Glucose	5g
Manganese sulphate	0.4%
Reconstituted skim milk	10%
Tomato juice	100ml

pH – 6.5 ; Sterilized at 0.7Kg/cm^2 for 20min.

2. All Purpose Tween-80 medium (APT)

Tryptone	12.5g
Yeast extract	7.5g
Dextrose	10g
Tween-80	2ml
Dipotassium hydrogen phosphate	5g
NaCl	5g
Tri sodium citrate	5g
Magnesium sulphate	0.8g
Thiamine dichloride	0.001g
Ferrous sulphate	0.8g
Manganese sulphate	0.02g
Agar-agar	15g

pH- 6.2 $\pm$ 0.2 ; Sterilized at 0.7Kg/cm^2 for 20min

3. Hugh & Leifson's O/F Medium (H&L)

Peptone	10g
Sodium chloride	5g
Glucose	10g
Dipotassium hydrogen phosphate	3g
Agar-agar	3g
Phenol red	0.1%
DW	1L

pH – 7.2+/- 0.2 ; Sterilized at 0.7Kg/cm^2 for 20 min.

4. LAB Maintenance Media

Glucose	0.5%
Lactose	0.5%
Beef extract	0.6%
Sodium acetate	0.6%
Yeast extract	0.5%
Salt solution A	0.5ml
Salt solution B	0.5ml

pH – 7.6 ; Sterilize at 1.05Kg/cm^2 for 15min.

Solution A (Phosphate buffer)

K_2HPO_4	25g
KH_2PO_4	25g
DW	250ml

Solution B

$MgSO_4.7H_2O$	10g
NaCl	500mg
$MnSO_4.4H_2O$	500mg
$FeSO_4.7H_2O$	500mg
DW	250ml

A few drops of 2N Dil.HCl was added to dissolve the solution and kept in water-bath. Filtered and stored in reagent bottle.

5. de Man, Rogosa and Sharpe Agar (MRS)

Bacteriological peptone	10g
Beef extract	8g
Yeast extract	4g
Dextrose	20g
Tween-80	1ml
Dipotassium hydrogen phosphate	2g
Sodium acetate	5g
Tri ammonium citrate	2g
Magnesium sulphate	0.2g
Manganese sulphate	0.05g
Agar-agar	15g
Distilled water (DW)	1L

pH - 6.2±0.2 ; Sterilized at 0.7 Kg/cm^2 for 20 min.

6. Methyl Red Voges Proskaeur Medium (MRVP)

Peptone	5g
Glucose	5g
Dipotassium hydrogen phosphate	5g
DW	1L

pH - 7.2+/- 0.2 ; Sterilized at 0.7Kg/cm^2 for 20 min.

7. Nitrate broth

Peptone	10 g
NaCl	5 g
Potassium nitrate	1 g
DW	IL

pH – 7.1±0.1; Sterilized at 1.05Kg/cm^{2}for 15 min.

8. Normal Saline (NS) (Physiological saline)

NaCl – 8.5g

DW – 1L

Sterilize at 15lbs for 15 min.

9. Nutrient Agar (NA)

Peptone	10g
Sodium chloride	5g
Beef extract	3g
Agar-agar	15g
DW	1L

pH – 7.1+/- 0.2 ; Sterilized at 1.05Kg/cm^2 for 15min

10. Selective SL Medium (Acetate Agar)

Caesin Peptone	10 g
Yeast extract	5g
KH_2PO_4	6.0 g

Diammonium citrate	2.0g
$MgSO_4 . 7H_2O$	0.5g
$MnSO_4 . 4H_2O$	0.5g
$FeSO_4 . 7H_2O$	0.04g
Tween 80	1.0g
Glucose	20 g
Sodium acetate	25g
DW	1L
Agar	15.0g

pH-5.4 ; Sterilization at 0.7Kg/$cm^2$for 20 min.

11. Tryptone Broth

Tryptone	10g
Sodium chloride	5g
DW	1L

pH – 7.1+/- 0.2 ; Sterilized at 1.05Kg/cm^2 for 15min

12. Tryptone Glucose Agar (TGA)

Tryptone	5g
Glucose	1g
Beef extract	3g
Sodium chloride	5g
Agar-agar	15g
DW	1L

pH – 7.2 $\pm$ 0.2 ; Sterilized at 1.05Kg/cm^2 for 15 min.

3.1.4. Indicator solutions

1. Chlorophenol red indicator (0.2%)

Chloro phenol red	200mg
Alcohol	10ml
DW	90ml

2. Methyl Red indicator

Methyl red	50mg
Alcohol	150ml
DW	100ml

Methyl red was dissolved in alcohol and diluted with DW .The solution was filtered.

3.1.5. Test Reagents

1. Greiss Reagent (Nitrate test)

Solution A

Sulphanilic acid	8g
Conc.H_2SO_4	48ml
DW	952ml

Solution B

a-naphthylamine	5g
Conc.H_2SO_4	8ml
DW	992ml

Dissolved and filtered. Kept in coloured bottles.

2. Kovac's Indole reagent

p-dimethyl amino benzaldehyde	5g
n-butyl alcohol	75ml

p-dimethyl amino benzaldehyde was dissolved in n-butyl alcohol. To the dissolved solution, 25 ml Conc.HCl was added and filtered.

3. Kovac's cytochrome oxidase reagent

N:N:N:N.Tetramethyl-p-phenylene diamine-hydrochloride	100mg
DW	10ml

4. VP reagents

Solution A

a-naphthol	0.25g
Alcohol	5ml

Solution B

KOH	2g
DW	5ml

3.2. Methods

3.2.1 Sampling of fish /prawn for Total Plate Count

3.2.1.1 Total Plate Count

Total plate count was determined using Tryptone Glucose Agar (TGA). About 10g of skin with muscle was cut aseptically from a given fish sample into a sterile sample dish. The sample was homogenized with 90ml diluent (Normal Saline/NS) in a homogeniser and serially and decimally diluted to 10^2, 10^3, 10^4 etc, as required in NS. The sample dilutions plated by pour plate and spread plate method as given below in duplicates.

The plates were incubated at room temperature (RT, 28+/-2^{0}C) for 48hrs and counted using a Quebec colony counter. TPC/g sample is calculated using the relation

TPC/g sample = Average count X Dilution factor

In the case of colony counts from spread plates, the average count has to be doubled before calculation of TPC.

3.2.1.1.1 Pour plate Method

About 1ml of the appropriate dilution of the sample was pipetted into appropriately labeled sterile petridishes. The molten media was cooled to 45°C and about 15-18ml poured to each plate and mixed well with the inoculum by rotating in clockwise and anticlockwise directions. Allowed to set and incubated.

3.2.1.1.2 Spread plate Method

For spread plate method (surface plate method), agar plates have to be poured and dried in advance. The agar media is melted in water bath and cooled to

45°C. The melted media poured into sterile petridishes and allowed to set. The surface of the media dried in a 56°C incubator or in a laminar flow chamber for 45 min. The plates are cooled to room temperature. About 0.5 ml of the sample of appropriate dilution was pipetted on to the surface of the plate. Using a sterile bent glass rod, the inoculum was spread uniformly on the surface of each plate. Plates were incubated as described earlier.

3.2.2 Enumeration of Lactic Acid Bacteria

de Man, Rogosa and Sharpe agar (MRS) was used for enumeration of Lactic Acid Bacteria (LAB) (de Man et al. 1960). Fish/prawn sample was aseptically cut and blended in NS as described in 3.2.1.1. From the appropriate dilutions, 1ml was taken and pour plated with MRS agar. After the plates had set, an overlay of MRS agar, molten and cooled to 45°C was poured. The set plates were incubated at 37° C with 5% CO_2 (NAPCO automatic CO_2 incubator, precision scientific, USA.) for 48 to 72 hrs. Pure white colonies (2-3mm dia) were counted as LAB.

3.2.3 Isolation of Lactic Acid Bacteria

About 15-20 colonies, pure white and 2-3mm diameter were picked from MRS plates. These colonies were inoculated to MRS broth and purified by streaking on MRS agar plates. Individual colonies were maintained in MRS broth for further studies.

3.2.4 Maintenance of LAB

The cultures were maintained in MRS broth upto 2 weeks at 10°C for regular studies. The stock cultures were maintained as agar stabs in MRS agar with an

overlay of liquid paraffin. The stock cultures were maintained below 10°C in the refrigerator.

3.2.5 Identification of LAB

Gram Reaction : Young cultures (18-24hrs) grown in MRS agar slants were stained by Grams method (Hucker Modification). (Salle,1954).

Test for Catalase : A speck of young culture was placed on a clean glass slide and flooded with 2 drops of 30% H_2O_2. Evolution of gas from the culture indicated positive test for catalase.

Test for Cytochrome oxidase : A little of the young culture was smeared on a filter paper impregnated with Kovac's cytochrome oxidase reagent. Development of a blue colour in a few seconds indicated the presence of cytochrome oxidase.

Test for Motility : Motility was tested by the hanging drop method. A small drop of distilled water (DW) was placed on the middle of a cover slip and mixed with the young culture. The margin of a cover slip was smeared with a little paraffin jelly. The cavity slide was inverted on the cover slip in such a way that the cover slip got attached to the slide and on turning upside down, the culture drop hangs into the cavity. Motility was observed under the microscope (10X eyepiece and 45X ocular).

Hydrogen peroxide Production : On a clean glass slide, a speck of the culture was placed and catalase reagent (5mg/ml DW) was added The slide was observed under low power microscope for effervescence. Evolution of gas indicated the production of H_2O_2.

Hugh & Leifson's Oxidation/Fermentation reaction : A little of the culture is stab inoculated into the H & L glucose O/F medium, leaving a 2cm long column at the bottom of the tube. The culture is incubated at 37°C for 18-24h. A yellow colour through out the medium indicated fermentative reaction. If gas bubbles are also trapped in medium, reaction is fermentative with gas production. A yellow colour only at the top part of the medium indicated an oxidation reaction.

Fermentation of glucose (Durham's tube method): MRS broth without beef extract is used for the reaction. Chlorophenol red indicator in the medium indicated the acid produced in the medium. The young culture is inoculated to the medium and incubated at 37°C in 5% CO_2 atmosphere in a CO_2 incubator for 48h. A yellow colour in the medium indicated acid production and gas bubbles in the durham's tube showed fermentation of glucose with gas production.

Growth at 15°C and 45°C : The young cultures were inoculated to MRS broth and incubated at 15°C and 45° C for 1 week. Turbidity indicated bacterial growth.

Sugar fermentation: MRS broth without beef extract and glucose, with chlorophenol red is the basal medium. To the basal medium 0.5% - 1.0% of the sugars were added and autoclaved, except for xylose, mannose and maltose, which were filter-sterilized and added into the basal medium after sterilisation.. The inoculum was prepared by separating cells from a 24h old culture and diluted in MRS broth. The test cultures were incubated at 37°C in 5% CO_2 atmosphere for a week. A yellow colour and turbidity indicated fermentation of the sugars by bacteria. The following sugars were studied.

Table 3.2.1 List of sugars used for fermentation studies

1. Arabinose	6. Esculin	11. Mannitol	16. Salicin
2. Cellobiose	7. Lactose	12. Mannose	17. Sorbitol
3. Fructose	8. Maltose	13. Raffinose	18. Sucrose
4. Glucose	9. Melebiose	14. Rhamnose	19. Trehalose
5. Galactose	10. Melezitose	15. Ribose	20. Xylose

Production of Ammonia from Arginine: Arginine degradation was tested with Niven's medium modified by Hitchener et al. (1982) and two concentrations of glucose; 0.5% and 2% (w/v).

Growth on Acetate Agar: Young cultures were inoculated to Acetate agar (Rogosa) and incubated at 37°C in 5% CO_2 for 2-3 days. *Carnobacterium* spp. do not grow in Acetate agar while *Lactobacillus* grows on Acetate agar.

Nitrate Reduction: The 24h old test culture was inoculated to Nitrate broth and incubated at 37° C for 3-4 days. Reduction of nitrate was tested by the sulphanilic acid-a-naphthylamine reagent.

Indole Production: Tryptone broth was inoculated with the test culture and incubated at 37°C for 48-72hrs. 0.5ml of Kovac's indole reagent was added to each tube and shaken. A red/pink layer at the top indicated indole production. (Cowan and Steel,1965)

Methyl Red test: To a tube of 48h old bacterial culture in MR-VP medium, 5 drops of Methyl red indicator was added and shaken. A red colour is positive test, while yellow colour gives a negative result.

Voges-Proskauer test: In a small test tube, to 1ml of 48h old bacterial culture grown in MRVP medium,0.6ml solution A (5% a-naphthol) & 0.2ml Solution B (40% KOH) were added. Shaken well; a small crystal of creatine was added and allowed to stand for upto 4h. Development of an eosine pink colour indicated a positive VP test.

3.2.6 Factors influencing the growth of LAB

3.2.6.1 Effect of Temperature on growth

The effect of various incubation temperatures on the growth of LAB cultures was studied. The test organisms grown in MRS broth was inoculated to fresh MRS broth tubes and incubated at 10°C, 15° C, 28° C, 37° C and 45° C for 3-5days. After 7days, the test cultures were observed for growth, indicated by turbidity in the medium.

3.2.6.2 Effect of pH on growth

Growth of the test organism at different pH of media was studied. MRS broth at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 were used for the study. The test organisms were inoculated from a 24h old culture. The test cultures were incubated at 37°C in 5% CO_2 atmosphere for 3-5days. Growth was indicated by turbidity in the medium.

3.2.6.3 Effect of NaCl on growth

Growth at different concentrations of NaCl was tested by observing growth in MRS broth at pH 6.5 $\pm$ 0.2 containing various amounts of analytical grade NaCl. The MRS broth containing 0%, 5%, 6%, 7%, 8%, 9% and10% NaCl were inoculated with 24h old test organism and incubated at 37°C in a CO_2 incubator (5% CO_2) for 3-5 days. Growth was detected visually by observing turbidity.

3.2.6.4 Effect of Glucose concentrations on the growth and acidity produced in the medium

The ability of the test organism to grow at different glucose concentrations and produce acid was studied. MRS broth with different glucose concentrations of 0%, 0.2%, 0.4%, 0.6%, 0.8%, 1%, 1.2%, 1.4%, 1.6%, 1.8% and 2% were used for the study. The test organisms were inoculated from a 24h old culture and incubated at 37°C in a CO_2 incubator. Growth was indicated by turbidity in the medium. The acidity of the culture broth with different glucose concentrations were studied by measuring the titrable acidity.

3.2.7 Titrable acidity (AOAC,1975)

One millilitre of the sample LAB culture was taken in a conical flask and diluted with distilled water twice its volume. One to two drops of phenophthalene was added and titrated against 0.1N NaOH to first persistant pink. Acidity was reported as % lactic acid by wt (1ml 0.1N NaOH = 0.0090g lactic acid).

3.2.8 Water activity

Water activity of the cured/dried fish was measured in a water activity meter (Wert-Messer, Germany).

3.2.9 Moisture content

Moisture content of cured/dry fish was determined as per method of Gould (1989). Ten grams of the sample was cut into small pieces and spread evenly on the pre weighed petridish. The petridish with sample was weighed accurately. Duplicates were done. The petridish was kept at 100°C in hot air oven for 18-24h. The petridish was then

slowly cooled in the dessicator and weighed. The moisture content of the sample was expressed as the percent of the fresh weight.

$$\% \text{ Moisture} = \frac{\text{Wt of sample before drying} - \text{wt of sample after drying}}{\text{Wt of sample before drying}} \times 100$$

3.2.10 Salt content

Salt content of cured/dry fish was determined as per method of Gould (1989). Twenty five gram of the sample was cut into small pieces and ground to a paste using pestle and mortar. The ground sample was triturated in 50ml of dist. water and filtered through a Whatman No.1 filter paper. The filtrate was made upto 100ml in a standard flask. One millilitre of the clear filtrate was taken in a conical flask and diluted with water. Two-three drops of potassium chromate solution was added as indicator. Then titrated against 0.1N silver nitrate until the solution just changes to a brick red color. Salt content was calculated .

$$\text{Percentage of salt} = \frac{\text{Volume of } AgNO_3 \times \text{Normality of } AgNO_3}{\text{Weight of sample}} \times 100$$

3.2.11 Detection of antibacterial activity

Agar well diffusion method

Antibacterial activity was determined by agar well diffusion method as per Toba et al.(1991). BHI, TSA or MRS agar plates were overlaid with 10ml BHI,TSA or MRS soft agar (0.75% agar) lawn containing an indicator bacterial strain. The indicator lawns were prepared by adding 0.25ml of a 10^{-1} dilution from overnight cultures of bacteria. Wells, 8mm in diameter were cut into the agar using a sterile cork borer, then

100-200ul of culture supernatant fluids of test lactobacilli strains were placed into each well. The plates were incubated at 37°C for 24h and examined for zones of inhibition.

Agar spot method

Agar spot method was done as per Fricourt et al.(1994) to screen the LAB cultures isolated from fresh and frozen fish & shellfish for antibacterial activity. TGA/BHIA/TSA agar plates depending on the indicator bacteria used were overlaid with 6ml of soft agar medium (0.75% agar) seeded with 20ul of a log phase culture (O.D=0.2) of each indicator bacteria. Crude cell free extract (20ul) of the LAB culture was then spotted on the surface of the overlaid plate and incubated overnight at the optimum growth temperature for each indicator bacterium. Relative inhibition was determined by the size of the zone clearance in the indicator lawn where the crude cell free extract was spotted. Control plates were also spotted with sterile lactic acid (pH-3.5) and Acetic acid (pH-3.5), in order to ascertain the contribution of these acids in the antibacterial activity.

Measurement of Optical density

The test cultures were grown in MRS broths and incubated at 37°C in CO_2 Incubator (5%CO_2) for 24h. The optical density was measured at 650nm in a spectrophotometer (Spectronic 20, Milton Roy) for assessing the growth.

3.2.12 Characterization of antibacterial components produced by LAB

3.2.12.1 Organic acids

The LAB cultures were grown in 200ml of MRS broth in a conical flask. The cultures were centrifuged at 10,000 rpm in a high speed centrifuge (REMI). The

culture supernatants were neutralized to pH-6.5 with the addition of sterile 1N NaOH so as to neutralize the effect of organic acids produced during growth of LAB in MRS. The neutralized culture supernatant was tested for antibacterial activity by the agar well diffusion method. Controls were run with the supernatant, which was not neutralized with NaOH. The inhibition zones were measured. Difference in the zone diameter was a measure of the effect due to organic acid produced.

3.2.12.2 H_2O_2 production

Culture supernatants after centrifugation was used for this experiment also. The culture supernatants were treated with catalase (5mg/ml culture) and incubated at 37°C for 3-4 h in order to decompose any hydrogen peroxide present. The antibacterial activity of the control and treated supernatant were determined by agar well diffusion method. Difference in zone size gave the effect due to hydrogen peroxide.

3.2.13 Purification of Bacteriocins

Purification of Bacteriocin was done as follows (Scopes, 1982). The test culture (1%) was inoculated to 200ml of MRS broth and incubated at 37°C in a CO_2 incubator (5% CO_2) for 24-48h. The culture was centrifuged at 10,000 rpm for 10min at 4°C. The cell free supernatant was separated for ammonium sulfate precipitation. Bacteriocins were precipitated from the supernatant by gradually dissolving ammonium sulfate (to obtain 40-60% wt/v saturation) and stirring continuously and keeping the beaker containing supernatant in ice bath, for 1h. The supernatant was centrifuged at 10,000 rpm for 30min at 4°C. The pellet and the precipitate on the surface were collected and washed two to three times in sterile distilled water. The washed pellet was dissolved in 1ml of glycine-NaOH buffer and kept at 4°C. The bacteriocin dissolved in

glycine-NaOH buffer (pH - 7.5) was tested for antibacterial activity against indicator /pathogenic bacteria by agar well diffusion method.

3.2.14 Characterization of Bacteriocin

3.2.14.1 Heat sensitivity

The LAB culture supernatant was divided into four sets. One set was kept as control, the other three were respectively maintained at 80°C for 30min, 100°C for 10min and 121°C for 10min. Heated samples were cooled to ambient temperature. Antibacterial activity was determined by agar well diffusion method, using a test bacterium. The inhibition zones were measured. Decrease in antibacterial activity from the control was due to the effect of temperature.

3.2.14.2 Protease sensitivity

The LAB culture supernatant was divided into five sets. One control, the others were treated with a-chymotrypsin (Sigma) (1mg/ml), trypsin (SD fine chem) (1mg/ml), Pepsin (Sigma) (1mg/ml) and Protease (Sigma) (1mg/ml) and incubated at 37°C for 24h. The antibacterial activity was assayed by agar well diffusion method. Some of the LAB culture supernatant treated with proteases showed no antibacterial activity as the proteinaceous bacteriocins were destroyed.

Chapter.4

RESULTS AND DISCUSSION

4. Results & Discussion

4.1 Total bacterial count vis-a-vis Lactic acid bacterial count in fresh fish

4.1.1 Total bacterial count in fresh fish

The total bacterial counts (TPC) and the Lactic acid bacterial (LAB) counts of 22 different species of fresh fishes from the local markets in Cochin are presented in Table 4.1.A and 4.1.B. A total of 211 fresh fish samples were analyzed. The values shown are averages of the corresponding bacterial counts for each species analyzed.

As a whole, the TPCs of the fishes examined were in the range of 1.06×10^5-9.07×10^6/g of skin with muscle. The lowest average TPC of 1.06×10^5 were recorded for Japanese thread-fin-bream (*Nemipterus japonicus*). A similarly lower average TPC of 1.45×10^5/g was obtained for tilapia (*Oreochromis mossambicus*). The banded barracuda (*Sphyraeno jello*) showed the highest average TPC of 9.07×10^6/g, while picnic silver bream (*Acanthopagrus berda*) harboured a total bacterial population of 7.08×10^6/g.

The total bacterial counts of the fresh fish samples is usually considered as one of the indices for quality and generally a TPC of more than million per gram is an indication that the fish could be in the state of incipient spoilage. In the case of the present study, 211 fresh fish samples belonging to 22 different species had a maximum TPC of a million per gram. Nambiar and Iyer (1990) has made a detailed study of the microbial quality of 156 samples comprising about 12 different species of fish in retail trade in Cochin. They have reported the lowest TPC of 10^3/g and the highest 10^7/g. In their studies, less than 40% of the samples on an average had a TPC of 5×10^5 and the

Table 4.1.A Total bacterial count (TPC) vis-a-vis Lactic acid bacterial count in fresh fishes from local markets in Kochi **

Sl.No.	Fish spp.	Average TPC/g	Average LAB count/g
	Japanese thread -fin –bream (8)* *Nemipterus japonicus*	**1.06×10^5**	**6.43×10^6**
	Little tuna (19) *Euthynnus affinis*	1.12×10^6	1.87×10^3
	Jewfish (11) *Protonibea diacanthus*	5.67×10^6	9.34×10^4
	Indian mackerel (15) *Rastralliger kanagurta*	8.76×10^5	1.12×10^4
	Banded barracuda (8) *Sphyraeno jello*	**9.07×10^6**	8.67×10^4
	Silver pomfret (6) *Pampus argenteus*	9.02×10^5	8.99×10^4
	Tilapia (5) *Oreochromis mossambicus*	1.45×10^5	4.55×10^3
	Pearl spot (10) *Etroplus suratensis*	3.31×10^5	2.64×10^3
	Picnic silver bream(6) *Acanthopagrus berda*	7.08×10^6	4.02×10^4
	Black pomfret (5) *Parastromateus niger*	2.28×10^6	1.62×10^5

*- Values in parenthesis is the number of samples analyzed.

**- Values given are averages of the values from the samples analyzed.

Table 4.1.B Total bacterial count (TPC) vis-a-vis Lactic acid bacterial count in fresh fishes from local markets in Kochi**

Sl.No.	Fish spp*	Average TPC/g	Average LAB count/g
11.	White fish (9) *Lactarius lactarius*	4.07×10^5	2.28×10^4
12.	Milk fish (7) *Chanos chanos*	1.72×10^6	6.40×10^3
13.	Indian oil sardine (26) *Sardinella longiceps*	4.11×10^5	7.02×10^3
14.	Spotted butter fish (8) *Scatophagus argus*	5.57×10^6	8.23×10^3
15.	Lesser tiger toothed croaker (24) *Otolithus cuvieri*	3.34×10^5	**4.71×10^2**
16.	Silver ribbon fish (10) *Lepturacanthus savala*	2.09×10^6	1.67×10^3
17.	Malabar trevally(5) *Caranx malabaricus*	1.38×10^6	2.50×10^4
18.	Narrow barred seer fish (8) *Scomberomorus commersoni*	1.90×10^5	1.00×10^3
19.	Malabar red snapper(7) *Lutjanus malabaricus*	2.40×10^5	2.10×10^3
20.	Brown triple tail (6) *Lobotes surinamiensis*	2.60×10^6	1.90×10^4
21.	Flat head grey mullet(4) *Mugil cephalus*	6.20×10^6	1.10×10^3
22.	Dussumier's cat –fish (4) *Arius dussumieri*	3.10×10^6	2.00×10^3

*- Values in parenthesis is the number of samples analyzed.

**- Values given are averages of the values from the samples analyzed.

rest of the samples had above this TPC level. Of the samples showing TPC above $5x10^5/g$, majority had TPCs above 1 million/g mark. However,only about 10% of the samples examined by them indicated a TPC above 10 million/g.

Iyer et al. (1986) had made a bacteriological examination of the fishes from the retail markets in Bombay. They found that nearly 75% of the samples had a TPC less than 1 million per gram. A study by Lakshmanan et al. (1984) on the bacterial counts of fresh fish collected from the fish landing centers in and around Cochin revealed that 90% of the samples had a total bacterial count of above $10^5/g$. However, Thampuran (1987) reported that in the case of fresh Indian Mackerel (*Rastralliger kanagurta*) from the retail markets in Cochin, TPCs were in the range of $8.9x10^3$-$4.1x10^5/g$ of skin with muscle. A compilation of the microbiological data for tropical fishes by Lima dos Santos (1981) gave that for fresh fish TPCs were in the range of 10^3-10^7/sq.cm of the skin. In the case of cold water fishes, the TPCs on the skin surface were in the lower range of 10^2-10^5 per sq.cm (Shewan and Hobbs,1967 and Shewan,1977). The total bacterial counts for fresh fish from local markets in this study is more or less in agreement with the TPCs reported for tropical fresh fishes.

The TPC has an important role in any microbiological standards for food materials. In the case of fish, the value of TPC reflected on the hygienic conditions under which the fish had been handled after catch (Abrahamson,1960, Dack al,1960, Thatcher,1960). Elliot and Michener (1961) have emphasized the significance of TPC in quality control programs of fish.

4.1.2 Lactic acid bacterial count in fresh fish

The total LAB counts of fresh fishes from retail markets in Cochin are also presented in Table.4.1.A and Table.4.1.B. Data for a total of 211 fresh fishes belonging

to 22 different species are presented. The highest average total LAB count of 4.71×10^2/g is for lesser tiger toothed croaker (*Otolithus cuiveri*) and the highest average LAB count of 6.43×10^6/g was obtained for Japanese thread-fin-bream (*Nemipterus japonicus*). Most of the other fishes harboured LAB counts in the range of 10^3-10^4/g of skin with muscle. It is interesting to note that LAB was invariably found in the total bacterial population of the fresh fishes available in our local markets. Further, it would appear ambiguous that in the case of Japanese thread-fin-bream (*Nemipterus japonicus*) the average TPC/g (1.06×10^5) was less than the total average LAB count of 4.43×10^6/g. In all other fishes, the LAB counts are well below the total bacterial count. The TPC per gram shown in the table is the total aerobic count recovered on Tryptone Glucose Agar (TGA). The LAB are not reflected in the total aerobic count on TGA, because recovery of LAB required incubation of the culture plates in a controlled atmosphere, and also that the recovery media needed to be sugar rich. The LAB counts reported in Table.4.1.A and 4.1.B are recovered on MRS agar with the culture plates incubated at 5% Carbon dioxide atmosphere (in a CO_2 incubator).

The recovery of LAB from fresh fishes from tropical regions are very rare. Probably this report is a pioneering one. However, there are a few reports on isolation of LAB from fresh fish. Knochel (1981) sampled various species of iced fish from North Sea. He obtained a LAB count of 40-2700/g from the skin on fillets and 30-2000/g from the gut. Similarly, Kraus (1961) has reported the isolation of LAB from the intestine of herring and Schroder et al. (1979) from the gut of freshly caught Saithe (*Gadus virens*). Baya et al. (1991) and Pilet et al. (1995) have also reported the isolation of LAB from fresh fishes in temperate regions. There are also a few reports of isolation of LAB from diseased rainbow trout and from salmonid fishes (Hiu et al. 1984). But, in none of these reports there is any mention about the quantitative recovery of LAB from

fish. Hence, the present report appears to be the first quantitative study on the incidence of LAB in fresh fish.

4.2 Total bacterial count vis-a-vis Lactic acid bacterial count in fresh prawns

4.2.1 Total bacterial count in fresh prawn

Total plate count of 40 fresh prawn samples comprising of 5 species obtained from the local retail markets in Cochin is presented in Table.4.2. Of the 5 species studied, 4 species were marine and the fifth one *Macrobrachium rosenbergii* was of fresh water origin. It is interesting to note that the average TPC/g muscle for the prawns were in the range of 10^6-10^7, indicating that the prawn muscle harboured a higher bacterial population. The lowest average TPC of $5x10^6$/g was for *Metapenaeus affinis*, while the highest average TPC of $1.76x10^7$/g was recorded for *Penaeus indicus* among the marine prawns. But the freshwater prawn (Scampi), *Macrobrachium rosenbergii* recorded the highest average TPC of 1.88x 10^7/g.

Surendran (1980) recorded a TPC of 10^2-10^5/g for *P.indicus*, 10^3-10^6/g for *Metapenaeus dobsoni* and 10^2-10^6/g for *M.affinis*. Thampuran (1987) reported TPC in the range of $5.2x10^3$-$9.4x10^6$/g muscle of fresh prawn (*M.dobsoni*). A series of other reports (Pillai et al.1961; Jacob et al. 1962 and Lakshmanan et al.1984) also indicated similar bacterial counts on freshly landed tropical prawns. Cann et al. (1971) found a TPC of Thailand shrimp to be in the range of 10^3-10^6/g while the TPC of the Malaysian prawns were in the range of 10^6-10^7/g, even when they were collected directly from the nets (Cann,1977). In the case of tropical prawns from Northern Australia, the bacterial counts were in the range of 10^3-10^5/g (Ruello, 1974). Vanderzant et al. (1974) found that the aerobic plate count of fresh gulf shrimp varied between $8.7x10^2$ to $1.3x10^6$/g,

Table 4.2 Total bacterial count (TPC) vis-a-vis Lactic acid bacterial count in fresh prawn from local markets in Kochi **

Sl.No.	**Prawn spp/#**	**Average TPC/g muscle**	**Average LAB count/g muscle**
1.	*Parapenaeopsis stylifera* (7)*	6.00×10^{6}	3.32×10^{2}
2.	*Metapenaeus affinis*(5)	5.00×10^{6}	$\mathbf{2.80 \times 10^{2}}$
3.	*Metapenaeus dobsoni*(10)	$\mathbf{2.90 \times 10^{6}}$	5.18×10^{2}
4.	*Penaeus indicus*(8)	1.76×10^{7}	$\mathbf{2.60 \times 10^{3}}$
5.	*Macrobrachium rosenbergii* (10)	$\mathbf{1.88 \times 10^{7}}$	5.10×10^{2}

- The common names of prawns are given in Table 3.7 in Materials and Methods

* - Values in parenthesis is the number of samples analyzed

** - Values given are averages of the values from the samples analyzed

while in the case of pond reared shrimp of Texas, Christopher et al. (1978) have reported a TPC of $1.5x10^3$ to $1.3x10^4$/g.

The data on the TPCs of prawns from the temperate regions indicated that the TPCs were in the range between 10^3-10^6/g. Walker et al. (1970) and Cann et al. (1971) found the bacterial count of North sea scampi (*Nephrops norvegicus*) to be 10^3-10^6/g. Lee and Pfiefer (1975) have recorded a TPC of $1.6x10^5$/g to $3.2x10^6$/g for Pacific shrimp (*Pandalus jordani*) as the prawns were landed at the dock site.

Comparing the bacterial counts of the 40 samples of fresh prawns in the present study to those discussed earlier in the case of tropical prawns indicated that the TPCs obtained in this study were more or less on the higher side. But, eventhough the prawn samples we chose from the market were very fresh, they had naturally undergone some stages of handling and consequent increase in the TPCs.

4.2.2 Lactic acid bacterial count in fresh prawn

Table 4.2 also gives the average total LAB counts in 40 samples of fresh prawn comprising of 4 marine species and 1 freshwater species. All the samples harboured LAB in the range of 10^2-10^3/g muscle, maximum LAB count being in *P.indicus*. Compared with the total aerobic bacterial count, the LAB counts are insignificant. In the literature we have surveyed, there were no reports of any previous study on the distribution of LAB in prawns or any other shellfishes. As such,this is the first report on occurrence of LAB in prawns both marine species and freshwater species.

4.3 Total bacterial count vis-à-vis Lactic acid bacterial count in frozen fish

4.3.1 Total bacterial count in frozen fish

The average total bacterial counts of 128 frozen fish samples belonging to 16 different species are presented in Table 4.3. The highest average TPC per gram of skin with muscle was for pearlspot (*Etroplus suratensis*) samples, while the lowest average TPC was recorded for little tuna (*Euthynnus affinis*). Generally the average TPC of frozen fishes varied between 10^4/g to 10^6/g, most of the samples harbouring a total bacterial population in the range of 10^5/g.

Based on the TPC of the frozen fish samples examined, samples were in microbiologically acceptable limits. Nambiar and Iyer (1990) have examined 126 frozen fish samples belonging to 9 different species from cold storage in Cochin. They have reported the average plate counts ranging from $1.45x10^2$ to $4x10^6$/g. In their study, more than 80% of the total samples showed a TPC less than 10^5/g and only 2% of the samples had TPCs above 1 million per gram. Thampuran and Iyer (1985) have analyzed different frozen fish and fish fillets for TPC and have reported a total bacterial count of $5.2x10^4$ to $4.5x10^5$/g. Thampuran (1987) has found that the bacterial counts of frozen Mackerel (*R.kanagurta*) were in the range of $9.8x10^3$-$1.1x10^5$/g. Girija (1993) has made a detailed study on freezing and frozen storage of thread fin-bream. She has reported the bacterial load of frozen fish to be in the range of $2.46x10^3$ to $9.2x10^4$/g.

In this study, 10% of the frozen fish samples showed an average TPC of 10^4/g, 65% of the samples had an average TPC of 10^5/g and 24% had 10^6/g. According to the European Union standards for frozen fish, maximum allowable TPC is $5x10^5$/g.

Table 4.3. **Total bacterial count (TPC) vis-a-vis Lactic acid bacterial count in frozen fishes from local cold storages****

Sl. No	**Fish spp**	**Average TPC/g**	**Average LAB count/g**
1.	Malabar reef cod (4)* *Epinephleus malabaricus(4)*	4.12×10^5	2.23×10^3
2.	Little tuna (10) *Euthynnus affinis*	$\mathbf{2.10 \times 10^4}$	5.13×10^3
3.	Black pomfret (10) *Parastromateus niger*	1.24×10^5	5.00×10^3
4.	Djeddaba trevally (4) *Alepes djeddaba*	5.00×10^5	1.30×10^2
5.	Banded barracuda (6) *Sphyraeno jello*	4.70×10^5	2.10×10^4
6.	Silver pomfret (7) *Pampus argenteus*	1.24×10^5	5.13×10^3
7.	Tilapia (5) *Oreochromis mossambicus*	6.40×10^5	$\mathbf{2.50 \times 10^5}$
8.	Pearl spot (18) *Etroplus suratensis*	$\mathbf{2.67 \times 10^6}$	7.07×10^4
9.	Indian oil sardine(15) *Sardinella longiceps*	2.70×10^5	1.30×10^4
10.	Spotted butter fish(12) *Scatophagus argus*	1.59×10^5	1.39×10^4
11.	Lesser tiger toothed croaker(10) *Otolithus cuvieri*	1.75×10^6	1.60×10^5
12.	Silver ribbon fish (3) *Lepturacanthus savala*	2.02×10^6	3.20×10^3
13.	Malabar trevally(4) *Caranx malabaricus*	4.50×10^4	$\mathbf{1.30 \times 10^2}$
14.	Narrow-barred seer fish (11) *Scomberomorus commersoni*	9.10×10^5	2.50×10^3
15.	Picnic silver-bream (5) *Acanthopagrus berda*	1.76×10^5	2.00×10^3
16.	Brown triple tail (4) *Lobotes surinamiensis*	2.30×10^5	2.28×10^3

*- Values in parenthesis is the number of samples analyzed.
** - Values given are averages of the values from the samples analyzed.

As such 24% of frozen fish samples from local cold storages of Cochin do not meet the bacterial quality standards of the EU.

4.3.2 Lactic acid bacterial count in frozen fish

The Table 4.3 also presents the average total LAB counts in 128 samples of frozen fish belonging to16 different species. The average total LAB counts ranged between 1.30x 10^2/g for Malabar trevally to 2.50x 10^5/g for Tilapia. LAB were present in all samples of frozen fish analyzed. A similar observation has been obtained in the case of fresh fish samples from local markets. However, compared with the average total LAB counts of fresh fish, the average total LAB count of frozen fish were substantially less in number. This would indicate that freezing has caused considerable destruction of the LAB in fish. It can be seen from the Table 4.3 that nearly 40% of the frozen fishes examined had total LAB count of 10^4 /g and 10^5/g, 42% of the samples had a LAB count between 10^3 /g and 10^4/g nearly 12% had average LAB count in the range of 10^5/g and only 6% had average total LAB count between 10^2 /g and 10^3/g. There is no record on the LAB population in frozen fish samples of commerce. So it was not possible to discuss our observation in relation to published reports.

4.4 Total bacterial count vis-a-vis Lactic acid bacterial count in frozen prawn

4.4.1 Total bacterial count in frozen prawn

In Table 4.4, the average total bacterial count of 26 frozen prawn samples belonging to five different species of prawns are presented. It is to be noted that the frozen prawn samples from local cold storages had a very high bacterial population in the range of 10^6-10^8/g muscle. This is atleast two log cycles higher than the

Table 4.4 Total bacterial count (TPC) vis-a-vis Lactic acid bacterial count in frozen prawn from local cold storages **

Sl.No.	**Prawn spp.**	**TPC/g**	**LAB/g**
1.	*Parapenaeopsis stylifera # (5)**	3.70 $\times 10^{7}$	**2.30 $\times 10^{3}$**
2.	*Metapenaeus affinis(3)*	3.80 $\times 10^{7}$	3.14 $\times 10^{4}$
3.	*Metapenaeus dobsoni(8)*	**3.40 $\times 10^{6}$**	4.10 $\times 10^{3}$
4.	*Penaeus indicus (5)*	2.10 $\times 10^{7}$	2.80 $\times 10^{3}$
5.	*Macrobrachium rosenbergii(5)*	**2.50 $\times 10^{8}$**	**3.20 $\times 10^{4}$**

- The common names of prawns are given in Table 3.7 in Materials and Methods

* - Values in parenthesis is the number of samples analyzed

** - Values given are averages of the values from the samples analyzed

corresponding values for frozen fishes of local trade. It is significant that the prawns harbour a higher bacterial population compared to fish as is revealed in Table 4.4. But the expected decline in total count was apparent in frozen prawn samples analyzed in this study. Thampuran (1987) has reported the average total plate count of frozen prawns in the range of 3.1 x 10^4 to 5.27 x 10^5/g. Pillai et al. (1965) has reported the total bacterial population of commercially frozen prawn products. They found that the TPC varied between $1x10^4$ and $1x10^7$ for peeled deviened and cooked frozen prawns. In comparison, the average TPC obtained in the case of samples analyzed in this study are very much on the higher side.

4.4.2 Lactic acid bacterial count in frozen prawn

Table 4.4 also gives the average total LAB counts of the 26 frozen prawn samples belonging to 5 different species. The average total LAB counts were in the range of 10^3 to 10^4/g. Compared with the population of LAB in fresh prawn samples from local markets, the average total LAB count of frozen samples are 1-2 log cycles higher. This could be only a reflection of the fact that the total LAB/g of frozen prawn were high. Just like in the case of frozen fish, there is no reported work on the LAB count in frozen prawns and hence it was not possible to discuss our results with those in literature.

4.5 Total bacterial count vis-a-vis moisture content, water activity and salt content in cured fishes of commerce

In Table 4.5 the average total bacterial count of dry/salted fishes (12 species) of local markets along with the moisture content, salt content and water activity (a_w) is presented. The average TPC were in the range of $1.2x10^2$ to $6x10^4$/g. About 62% of the samples had an average TPC between 10^2/g and 10^3/g, 15% 10^3/g and 10^4/g and 24% had 10^4/g and above. The moisture content of cured fishes was between 36.19% to

Table 4.5 Total bacterial count in cured fishes with relation to water activity, moisture content and salt content **

Sl.No	Fish spp#	Average TPC/g	Water activity	Moisture content in %	Salt content in %
1.	Lesser tiger toothed croaker(5) *	**$1.2x10^2$**	**0.776**	42.06	**16.85**
2.	Pony fish (2)	$3.1x10^2$	0.774	40.82	16.08
3.	Dog shark (5)	$1.3x10^2$	0.770	50.5	11.05
4.	Malabar tongue-sole(8)	$3.4x10^2$	0.764	45.18	13.3
5.	Silver ribbon fish (3)	$2.5x10^3$	0.762	52.4	12.65
6.	Grey-fin croaker (3)	$6.7x10^3$	0.762	46.4	11.9
7.	Malabar anchovy (4)	$2.0x10^2$	0.762	36.19	9.79
8.	Indian oil sardine (8)	**$6.0x10^4$**	0.757	**58.88**	12.3
9.	White fish (2)	$4.0x10^3$	0.757	50.04	14.2
10.	Blank king-fish (3)	$3.0x10^2$	0.752	50.59	13.3
11.	Indian mackerel (5)	$3.5x10^4$	0.752	41.57	14.24
12.	White sardine (7)	$5.2x10^2$	**0.64**	**31.47**	**8.05**

#- The scientific names of fishes are given in Table 3.5 in Materials and Methods
*- Values in parenthesis is the number of samples analyzed
**- Values given are averages of the values from the samples analyzed

58.88% and a_w is more or less above 0.6 and below 0.8. The salt content varied between 8% and 16%. LAB was not detected in any of these samples. This may be due to the fact that the high salt content and lower a_w were not favorable for the survival of LAB. The TPC also are very low in the cured fishes and this is to be expected because of the high salt content and lower a_w. Bacteria, in general, would prefer a_w above 0.9 and a salt content about 1%.

In the case of cured samples, the lowest salt content was 8% which would affect the survival of most of the known halo-tolerant bacteria. Further, a_w in the range of 0.7 is also not conducive for survival of most of the bacteria. The TPC that had been recorded therefore might be contributed mainly by halotolerant/halophilic bacteria as well as some Gram positive bacteria, which may be just haloduric.

Most of the work reported on the bacterial flora of salted fish indicated that only halophilic bacteria belonging to *Halobacterium* and *Halococcus* survived in salted cured fishes (Bains *et al.* 1958 and Gibbons 1974). Rudrasetty (1985) has reported that the bacterial survival in salt cured fish is limited by a_w of 0.75.According to ICMSF (International commission on microbiological specifications for foods) (1980), LAB were absent in cured fish due to their very low a_w . It has been reported that a_w of about 0.94 limits the growth of *L.plantarum* and *Pediococcus pentosaceus*. Mugula and Lyimo (1992) had tested the microbiological quality of traditionally cured fish in retail markets. The investigations were conducted over a 5 month period on six species of cured fish. They obtained an average TPC of 1.7 x 10^6 cfu/g of fish.

4.6 Total bacterial count in Ocean fresh fish

Average total bacterial counts in 14 samples of Ocean fresh fish belonging to two species are presented in Table 4.6. In the case of ocean fresh Indian mackerel, the

Table 4.6 **Total bacterial count vis-a-vis Lactic acid bacterial count in Ocean fresh, farmed and pickled fishes/prawns** **

Sl.No.	Source #	Average TPC/g	Average LAB count/g
	Ocean fresh fish		
1.	Indian mackerel (8)	2.50 x10^3	ND
2.	Indian oil sardine (6)	4.63 x10^4	ND
	Farmed fishes		
1.	Rohu(5)	2.08 x10^4	1.32 x10^2
2.	Catla (4)	7.24 x10^3	2.71 x10^2
3.	Pearl spot (7)	4.30 x10^4	6.63 x10^3
4.	Giant tiger prawn (4)	1.37 x10^5	4.14 x10^3
	Pickled fishes/prawn		
1.	Little tuna (5)*	2.00 x10^3	ND
2.	Narrow barred seer fish (5)	8.00 x10^2	ND
3.	White prawn (5)	1.10 x10^2	ND

#- The scientific names of fishes are given in Tables 3.2, 3.3 and 3.6 in Materials and Methods

*- Values in parenthesis is the number of samples analyzed

**- Values given are averages of the values from the samples analyzed

average TPC was in the range of 2.4×10^3/g while for 6 samples of Indian oil sardine, the average TPC was 4.63×10^4/g. In both cases, LAB were not detected.

Surendran (1980) has reported a TPC of skin of oil sardine between 2.15×10^3 and 2.5×10^7/g and that of Indian Mackerel between 4.63×10^4 and 8.35×10^6. In comparison to the above findings, data in the present study show that the TPC counts in the ocean fresh fish samples analyzed were in the lower range. Also our data is in agreement with the results of Liston (1956), who had recorded a total bacterial count of 10^3 to 10^5/sq. cm. of skin. Similar results were also obtained by Shewan (1962) and Georgala (1958) in the case of North Sea cod.

4.7 Total bacterial count vis-a-vis Lactic acid bacterial count in farmed fish/prawn

Table 4.6 also gives the average TPC and average LAB count in 16 samples of farmed fishes belonging to 3 species and 4 samples of Tiger prawn. The farmed fishes analyzed were Rohu, Catla and Pearlspot. The average TPC was in the range of 7.24×10^3 – 4.3×10^4/g. LAB were recovered from all the samples of farmed fishes and were in the range of 10^2-10^3/g.

Surendran and Gopakumar (1991) have reported a TPC of 2.08×10^4/g on skin surface of farmed Rohu and 7.24×10^3/g for farmed Catla. They have noted that the total bacterial populations of Catla and Rohu were more or less similar and the TPC at 37°C were about 20 to 50% of those at RT (29$\pm$2°C). This indicated that the sizeable portion of the bacterial flora of these fishes are mesophilic in character. A similar study on the TPC of farmed fishes (*Chanos chanos*, *Mugil cephalus* and *Oreochromis mossambicus*) by Lalitha and Gopakumar (1996) recorded counts between 10^4 and 10^6/g in skin with

muscle. In their studies, they have also reported a TPC count of 8.3×10^4 to 3.2×10^5/g for whole shrimp (*Penaeus indicus* and *P. monodon*).

It is interesting to note that LAB are present in farmed fishes, invariably indicating that they are integral part of the microflora of inland fishes as well as inland water bodies. The data in the case of farmed tiger prawn also support this observation.

4.8 Total bacterial count in pickled fish/prawn

Fifteen samples of commercial pickled fish/prawn were tested for total bacterial count and total LAB count (Table 4.6). The average TPC of commercial pickled fish/prawn were in the range of 10^2-10^3/g and there was no LAB in any of the samples. Since pickles are heat -processed products with added salt and spices, one shall not expect a higher bacterial population in them. LAB will also die in the heat processing temperature. The bacterial counts of few hundreds to thousand obtained will be due to the spores, which survived the heat process.

There are no reports on the total bacterial counts and Lactic acid bacterial counts in the commercially pickled prawns/fish. However, few reports are available on the total bacterial counts in the indigenously prepared pickled fish. The changes in the chemical and bacteriological parameters of the pickled fish product were studied by Chattopadhyay *et al.* (1986). They have reported an initial TPC of 1.85×10^3/g and a TPC of 4.5×10^3/g pickle after six months of storage at room temperature. Another study conducted by Behanan *et al.* (1992) on pickled fish was based on the effect of preservatives on the chemical and bacteriological parameters. The pickled fish (*Epinephleus spp.*) recorded a TPC of 1.85×10^2/g after storage at room temperature. The observations on the total bacterial counts in our study on commercially pickled fish were more or less in the same range as those in indigenously pickled fish.

4.9 STUDIES ON LACTIC ACID BACTERIA (LAB)

4.9.1 Isolation of LAB from fish and prawn

4.9.1.1 Isolation of LAB from fresh fish

LAB cultures were isolated from the MRS plates in which sampling of fish for Total LAB count was done. Thirty to sixty well isolated colonies with typical characteristics namely pure white, small (2-3 mm diameter), with entire margins were picked and transferred to MRS broth. Around 1500 LAB cultures were studied from fresh fish. The cultures giving positive growth in MRS broth were purified by streaking on preset MRS agar. The purified cultures were maintained by stab culture method in MRS soft agar containing 0.2% Agar for biochemical characterization.

4.9.1.2 Isolation of LAB from fresh prawn

Forty samples of fresh prawn were analyzed for LAB in MRS plates. Twenty to thirty LAB colonies with typical characteristics were isolated from each sample into MRS broth. The LAB cultures were purified on MRS agar plates. The pure colonies were maintained as stab culture in MRS soft agar (0.2% agar). The pure cultures were studied in detail for their biochemical characters. Around 800 LAB cultures were isolated from fresh prawn.

4.9.1.3 Isolation of LAB from frozen fish

The frozen fish samples were analyzed for the enumeration of LAB in MRS agar and from these plates isolated LAB colonies were picked and inoculated to MRS broth. The LAB cultures purified in MRS agar by streaking were maintained in MRS soft agar by stab culture method. An approximate number of 1000 LAB cultures were isolated from frozen fish and studied for their biochemical characters.

4.9.1.4 Isolation of LAB from frozen prawn

Frozen prawns belonging to five different species were used for the enumeration of LAB on MRS agar. Around 500 LAB cultures were isolated, purified, maintained and studied for their biochemical characters.

4.9.2 Morphological characteristics of LAB cultures

The purified LAB cultures from fresh and frozen fish/prawn were stained by Grams method and examined for their morphological characteristics under the microscope. Morphologically the cultures were found to fall into the following groups.

Group I - Gram positive cocci in pairs or chains

Group II - Gram positive cocci in tetrads or clusters

Group III - Gram positive rods singly or in chains

Some cultures were having shapes in between cocci and rods (Coccobacilli), which are arranged either in chains or clusters and grouped under Group III.

Figure.4.1 shows the distribution of different morphological groups of LAB in fresh and frozen fish /prawn samples. Among the 1500 LAB cultures isolated from fresh fish, 30% belonged to the Group I (Gram positive cocci in pairs or chains), 10% belonged to Group II (Gram positive cocci in tetrads or clusters) and 60% of the LAB cultures belonged to Group III (Gram positive rods singly or in chains).

From the 800 LAB cultures isolated from fresh prawn, 65% belonged to Group III, 5% belonged to Group II and 30% belonged to Group I. Among the 1000 LAB cultures isolated from frozen fish, 10% belonged to Group I and Group II while rest of the 80% of LAB cultures belonged to Group III (Gram positive rods singly or in

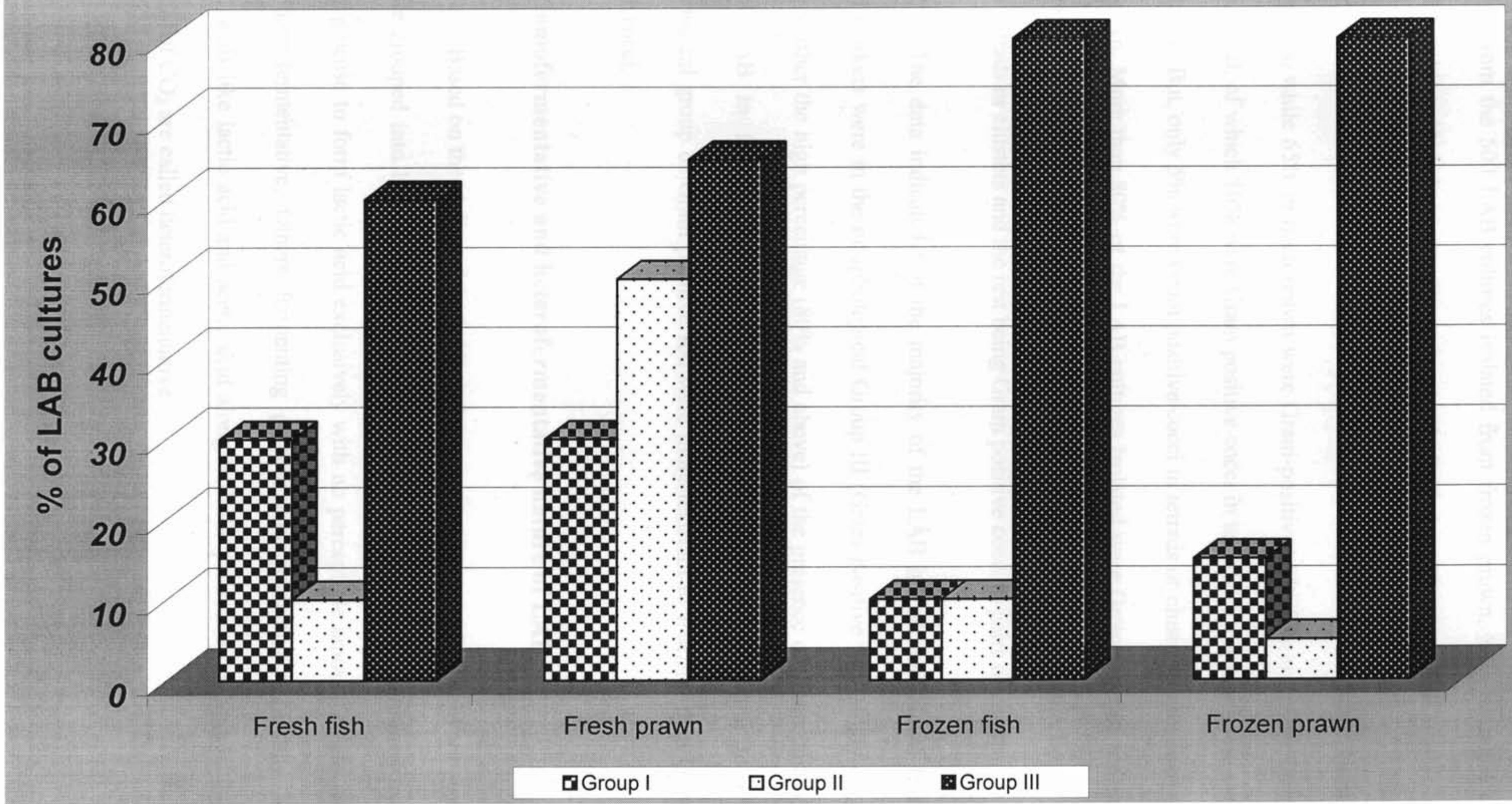


Figure.4.1. The distribution of different morphological groups of LAB in fresh and and frozen fish/prawn
% of LAB cultures
0
10
20
30
40
50
60
70
80
Fresh fish
Fresh prawn
Frozen fish
Frozen prawn
Group I
Group II
Group III

chains). From the 500 LAB cultures isolated from frozen prawn, 80% belonged to Group III, while 15% belonged to Group I and only 5% belonged to Group II.

In the case of fresh fish, 60% of the LAB cultures isolated were Gram-positive rods, while 65% in fresh prawn were Gram-positive rods. The rest were Gram positive cocci, of which 10% were Gram positive cocci in tetrads or clusters in the case of fresh fish. But, only 5% were Gram positive cocci in tetrads or clusters in the case of fresh prawn. More than 80% of the LAB cultures isolated were Gram positive rod sin the case of frozen fish as well as frozen prawns while nearly10% were Gram positive cocci in tetrads or clusters and the rest being Gram positive cocci in pairs or chains.

The data indicated that the majority of the LAB in fish and prawn from internal markets were in the morphological Group III (Gram positive rods singly or in chains), further the high percentage (80% and above) of the presence of Gram positive rods of LAB in frozen fish and prawn indicated that LAB belonging to the morphological group of Gram positive rod were more resistant to freezing than Gram positive cocci.

4.9.3 Homofermentative and heterofermentative nature of LAB

Based on the ability of the LAB to ferment glucose to produce acid and gas, LAB are grouped into homofermentative and heterofermentative. Those cultures that ferment glucose to form lactic acid exclusively with no perceptible amount of gas are called homofermentative. Others fermenting glucose and producing a mixture of organic acids like lactic acid and acetic acid along with the production of appreciable quantities of CO_2 are called heterofermentative.

The LAB cultures isolated from fresh and frozen fish and fishery products were studied for fermentation in MRS broth containing 2% glucose by incubating in a CO_2 incubator in which 5% CO_2 atmosphere was maintained. Figure 4.2 shows the distribution of homofermentative and heterofermentative LAB in different samples analyzed.

Out of the 3800 LAB isolates, 60% were homofermentative and the rest 40% heterofermentative. Among the 1500 LAB isolates from fresh fish alone, 70% were homofermentative and 30% heterofermentative. The corresponding values for frozen fish were 60% and 40%. In the case of fresh prawns, 55% of the 800 LAB isolates studied were homofermentative and 45% were heterofermentative, while for frozen prawns, 50% of the 500 LAB cultures were equally homofermentative and heterofermentative.

An analysis of the relative distribution of the morphological groups of LAB in the homofermentative and heterofermentative groups are presented in Table 4.7. As already stated, homofermentative groups prepondered in the LAB cultures isolated from fish and prawn both fresh and frozen. But, it is to be noted that among the Gram positive rods isolated from fresh and frozen fish, a significant majority is homofermenters. Probably the presence of a very small quantity of carbohydrate in fish (1%) tissue might have selectively favoured the occurrence of homofermentative LAB in fish. Since the shrimp tissue has a higher percentage of carbohydrates, heterofermentative groups of LAB are also equally favoured along with homofermenters.

The morphological groups of LAB fall under four genera based on the scheme of Sharpe *et al.* (1979). Morphological distribution of LAB according to Sharpe

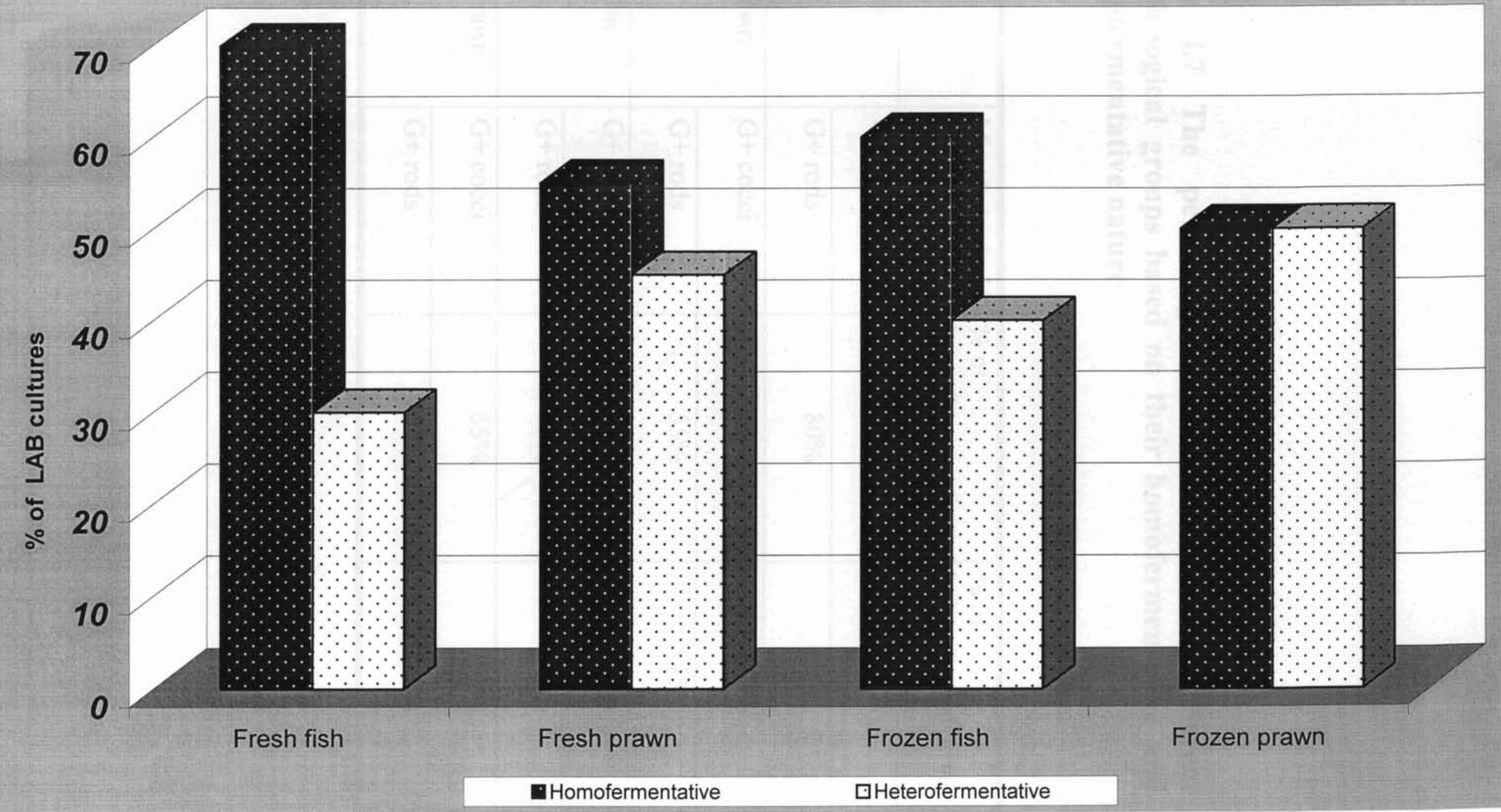


Fig.4.2. The percentage of homofermentative and heterofermentative LAB cultures in different fresh and frozen fish/prawn samples

Table 4.7 The percentage occurrence of the different morphological groups based on their homofermentative and heterofermentative nature

Sample	Morphological group	% of homofermentative	% of heterofermentative
Fresh fish	G+ cocci	75%	25%
	G+ rods	80%	20%
Fresh prawn	G+ cocci	50%	50%
	G+ rods	55%	45%
Frozen fish	G+ cocci	65%	35%
	G+ rods	60%	40%
Frozen prawn	G+ cocci	65%	35%
	G+ rods	50%	50%

Table 4.8 Morphological distribution of LAB according to Sharpe et al (1979)

	Genus of Lactic acid bacteria	Morphological character
I	*Streptococcus*	Homofermentative cocci in pairs or chains.
II	*Leuconostoc*	Heterofermentative cocci in pairs or chains.
III	*Pediococcus*	Homofermentative cocci dividing in two planes to give tetrads which may appear as clusters.
IV	*Lactobacillus*	Homofermentative or heterofermentative rods.

et al. (1979) is presented in Table 4.8. Accordingly,homofermentative Gram positive cocci fall into two genera namely *Streptococcus* and *Pediococcus*. All those Gram positive homofermentative cocci which are in pairs or chains are *Streptococcus* and others *Pediococcus*. Heterofermentative Gram positive cocci are *Leuconostoc*. Both homofermentative and heterofermentative Gram positive rods are grouped into the genera *Lactobacillus*. The 3800 LAB cultures isolated in this study were grouped into these four groups as presented in Table 4.8.

The share of *Lactobacillus* was 60% in fresh fish, 65% in fresh prawns and 80% each in frozen fish and prawns. This was followed by *Streptococcus*, whose presence was 20% each in fresh fish and fresh prawns, 5% in frozen fish and 10% in frozen prawns. *Leuconostoc* and *Pediococcus* were between 5-10% in these fishes.

Data presented in Figure 4.3 indicated that *Lactobacillus* was the most predominant group of LAB present in fish and shellfish. Accordingly,*Lactobacillus* was chosen for further detailed studies.

4.10 Studies on *Lactobacillus* isolated from fish and shellfish

The list of biochemical reactions used for characterization of *Lactobacillus* isolated from fish and shellfish is presented in Table 4.10. The genus *Lactobacillus* were classified into species according to the latest edition of Bergeys manual Vol-2 (Kandler and Weiss,1986).

The differentiating characteristics of the species of the genus *Lactobacillus* is given in Table.4.11.Of the nearly 1000 *Lactobacillus* cultures isolated during the entire course of the study, many cultures lost their viability during different stages of biochemical characterization and maintenance in the laboratory. Nearly 600 cultures

Figure.4.3 The percentage distribution of different genus of LAB in fresh and frozen fish/prawn samples

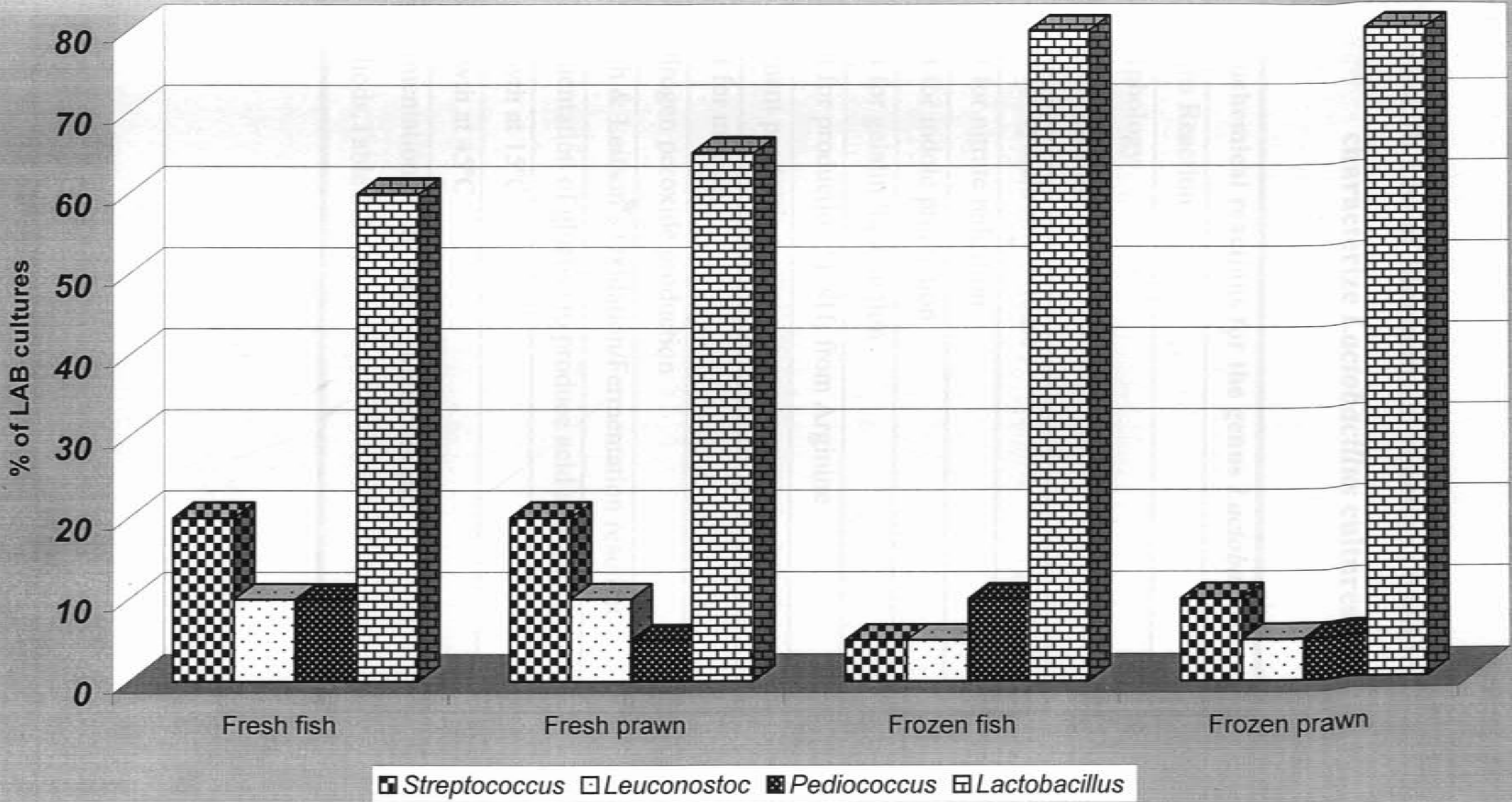


Table 4.10 List of Biochemical reactions used to characterize *Lactobacillus* cultures

Biochemical reactions for the genus *Lactobacillus*
1. Grams Reaction
2. Morphology
3. Test for catalase
4. Test for cytochrome oxidase
5. Test for nitrate reduction
6. Test for indole production
7. Test for gelatin liquefaction
8. Test for production of NH_3 from Arginine
9. Pigment production
10.Test for motility
11.Hydrogen peroxide production
12.Hugh & Leifson's Oxidation/Fermentation reaction
13.Fermentation of glucose to produce acid and gas
14.Growth at 15°C
15.Growth at 45°C
16. Fermentation of 20 sugars (Materials and methods,Table 3.2.1)

Table 4.11 Differentiating characteristics of the species of the genus *Lactobacillus*

No	*Lactobacillus* spp.	Morphology	Growth at			Acid & gas from glucose	Ammonia from	Sugar fermentation													
			15ºC only	45ºC only	15 & 45ºC			Arabinose	Cellobiose	Mannitol	Mannose	Melebiose	Raffinose	Ribose	Salicin	Lactose	Melezitose	Rhamnose	Sorbitol	Xylose	Trehalose
1.	*L.plantarum*	SR	+	-	+	-	-		+	+	+	+	+	+	+						
2.	*L.brevis*	SR	+	-	+	+	+	+						+		+	-	-	-	+	
3.	*L.divergens*	SR	+	-		+	+		+		+	-	-		+	-				+	+
4.	*L.gasseri*	SR	-	+		-	-	-	+		+			-	+			-	+		+
5.	*L.casei ssp rhamnosus*	SR	-	-	+	-	-		+	+	+	-			+	+	+		+	+	+
6.	*L.fermentum*	SR			+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
7.	*L.viridescens*	SR			+			-	+	+	+	-	-	-	-	+	+	-	-	-	+
8.	*L.farciminis*	SR			+		+	-	+	+	+	+	-	+	+	-	+	-	+	+	+
9.	*L.buchneri*	SR	+			+	+	-	+	-	-	+	-	+	-	+	-	-	-	+	-
10.	*L.acidophilus*	SR	-	-				+	+	+	+	-	-	-	+	+	+	-	-	+	+
11.	*L.alimentarius*	C	+					+	+	+	+	+	-	+	+	-	+	-	+	+	+
12.	*L.animalis*	C	-	-				+	+	+	+	+	+	-	+	+	-	+	+	-	+
13.	*L.reuteri*	R			+	+	+	+	-	-	-	+	-	+	-	+	-	-	-	+	-

<u>Key</u>

'+' – 80% or more of strains are positive, '-' means 80% or more of strains are negative

could be studied completely and characterized to the genus level. These cultures were grouped into 13 different species as presented in Table 4.12.

L.plantarum formed 23% of the 554 *Lactobacillus* cultures isolated and identified, followed by *L.brevis 11%, L.divergens 4.6%, L.gasseri 4%, L.casei ssp rhamnosus 3.5%, L.fermentum 3.3%, L.viridescens 1.7%, L.farciminis 1.2%, L.buchneri 1.2% and L.acidophilus, L.animalis, L.alimentarius* and *L.reuteri* all less than 1%. A significant fact is that 217 cultures (36.2%) were found to be atypical *Lactobacillus*. They are grouped as atypical because they exhibited characteristics common to many of the above 13 *Lactobacillus spp.*

Our observation that amongst the LAB, the *Lactobacillus* formed the major flora in fish has been substantiated by the observations of Erichsen (1973); Schroder et al,(1979); Lannelogue et al.(1982); Oberlender et al(1983); Valdimarsson and Gudbjornsdottir (1984) and Wang and Ogrydziak (1986). However, Maugin and Novel (1994) found that *Lactococcus* was the major flora isolated from fish. Magnusson and Trautadottir (1982) and Molin et al.(1984) had isolated *Lactobacillus* spp. from herring as the major group. Sarkar and Banerjee (1996) found that out of the 12 LAB cultures isolated from fish, 10 belonged to *Lactobacillus* and two to *Lactococcus*. Gancel et al (1997) have reported that all their isolates from fillets of vacuum packed smoked and salted herring belonged to a heterofermentative group of *Lactobacillus*.

4.11 Characterization of *Lactobacillus* species

4.11.1 Lactobacillus plantarum

Nearly 138 *L.plantarum* cultures were isolated from fish and shellfish. The cells showed varied shapes from coccoid, short rods to long slender rods. However,

Table 4.12 *Lactobacillus* species isolated from fish and shellfish

No.	***Lactobacillus* spp.**	**Number of isolates**	**% of isolates**
1.	*L.plantarum*	138	23%
2.	*L.brevis*	66	11%
3.	*L.divergens*	28	4.6%
4.	*L.gasseri*	24	4.0%
5.	*L.casei ssp rhamnosus*	21	3.5%
6.	*L.fermentum*	20	3.3%
7.	*L.viridescens*	10	1.7%
8.	*L.farciminis*	7	1.2%
9.	*L.buchneri*	7	1.2%
10.	*L.acidophilus*	5	>1%
11.	*L.alimentarius*	4.	>1%
12.	*L.animalis*	4	>1%
13.	*L.reuteri*	3	>1%
14.	Atypical *Lactobacillus* cultures	217	36.2%
	Total cultures	554	

majority of the strains exhibited short rod shape. None of the strains was able to hydrolyze arginine. All the cultures were homofermentative of glucose and nearly half of the strains showed growth at 15°C and 45°C. All the strains fermented esculin, fructose, galactose, glucose, maltose, mannose and sucrose.

4.11.2 L.brevis

Sixty-six isolates from fresh and frozen fish were identified as *L.brevis*. Majority of the heterofermentative species isolated from fresh and frozen fish belonged to this group. The morphology of the cells varied from coccoid to short rods to long slender rods. All the strains were heterofermentative, hydrolyzed arginine and grew at 15°C. Only a small percentage could grow at 45°C. All the strains fermented esculin, fructose, galactose, glucose, maltose, ribose and sucrose.

4.11.3 L.divergens

Twenty-eight isolates from fresh fish and shellfish were identified as *L.divergens*. In all the strains of *L.divergens* studied, the cells were short rods and heterofermentative. Except for a few strains, all the other strains hydrolyzed arginine. All the strains showed growth at 15°C, while only a few strains showed growth at 45°C. All the strains fermented cellobiose, fructose, galactose, glucose, mannose, salicin, sucrose and trehalose. Lactose, melebiose, raffinose and rhamnose were fermented by only a few strains. None of the strains could ferment sorbitol.

4.11.4 L.gasseri

Twenty four isolates were *L.gasseri*. The cells were short rods and one strain showed rods in chains. A few of the strains did not grow at 15°C and 45°C. While a few other strains showed growth only at 45°C. None of the cultures hydrolyzed

arginine. All the strains fermented cellobiose, fructose, galactose, glucose, maltose, mannose, salicin, sorbitol, sucrose and trehalose. None of the strains could ferment arabinose, rhamnose and ribose.

4.11.5 L.casei subsp.rhamnosus

The 21 strains that were identified as *L.casei subsp.rhamnosus* showed varied cell morphology. Some of the strains were coccoid shaped, short rods singly and in chains and typical rods. All the strains were homofermentative and grew at both 15°C and 45°C. These organisms are the only homofermentative lactobacilli which grew well at both 15°C and 45°C. None of the cultures hydrolyzed arginine. They could ferment cellobiose, fructose, galactose, glucose, lactose, maltose, mannitol, salicin, sorbitol, sucrose and trehalose. None of the strains could ferment melebiose. *L.casei subsp.rhamnosus* is now called *L.rhamnosus* (Stiles and Holzapfel, 1997).

4.11.6 L.fermentum

Twenty strains of *L.fermentum* were identified. The cells were coccoid to short rods. All the strains were heterofermentative, hydrolyzed arginine and grew at both 15°C and 45°C. All the strains isolated could ferment cellobiose, esculin, fructose, glucose, galactose, lactose, maltose, mannitol, ribose, salicin, sorbitol, sucrose and xylose. Except for a few strains, none of the strains could ferment melezitose.

4.11.7 L.viridescens

Ten isolates of *L.viridescens* were identified from fresh fish. It had short rod shaped cells. It was homofermentative, did not hydrolyze arginine and grew at both 15°C and 45°C. *L.viridescens* strains studied could not ferment arabinose, esculin, melebiose, raffinose, rhamnose, ribose salicin, sorbitol and xylose.

4.11.8 L.farciminis

Seven strains of *L.farciminis* were isolated from fresh fish. The cells were short rods. It was homofermentative, hydrolysed arginine and grew at 15°C and 45°C. None of the strains could ferment arabinose, lactose, raffinose and rhamnose.

4.11.9 L.buchneri

Seven strains of *L.buchneri* were isolated. All the cells were short rods. The strains isolated from fresh fish were heterofermentative, hydrolyzed arginine and grew at only 15°C. The strains could not ferment arabinose, mannose, mannitol, melezitose, raffinose. rhamnose, salicin, sorbitol and trehalose.

4.11.10 L.acidophilus

Five rod shaped *L.acidophilus* strains were isolated. These strains did not hydrolyze arginine and grew only at 37°C and ambient temperature (28±2°C). It varied from the typical strains in not being able to grow at 45°C. None of the strains could ferment arabinose, galactose, melebiose, raffinose, ribose, rhamnose and sorbitol.

4.11.11 L.alimentarius

Four strains of *L.alimentarius* were isolated from fresh and frozen fish. None of the strains hydrolyzed arginine. All the strains grew at 15°C, fermented arabinose, fructose, cellobiose, galactose, glucose, maltose, mannitol, mannose, melebiose, salicin, sucrose and trehalose. None of the strains fermented raffinose and rhamnose.

4.11.12 L.animalis

Coccoid shaped four strains of *L.animalis* cultures were isolated. None of the strains hydrolyzed arginine. *L.animalis* strains did not produce gas from glucose and neither did it grow at 15°C nor at 45°C.However they could ferment all the sugars except melezitose, ribose and xylose.

4.11.13 L.reuteri

The cells of *L.reuteri* (3 strains) isolated were short rods occurring singly and in chains. These strains could not hydrolyze arginine, neither produce gas from glucose. None of the strains was able to grow both at 15°C and 45°C. They fermented arabinose, fructose, galactose, glucose, lactose, maltose, melebiose, ribose, sucrose and xylose.

4.12 Distribution of *Lactobacillus* in fresh and frozen fish and prawn

4.12.1 *Lactobacillus* spp. in fresh fish

Tables 4.13.1 and 4.13.2 gives the relative distribution of the *Lactobacillus* spp. in the 22 different species of fresh fish studied. The most frequently isolated *Lactobacillus* spp. from fresh fish are *L.plantarum* and *L.brevis*. *L.gasseri* and *L.casei ssp rhamnosus* are also found in many of the fresh fish spp. while other *Lactobacillus* spp. isolated only from a few species of fresh fish.

4.12.2 *Lactobacillus* spp. in fresh prawn

Table 4.14 shows the distribution of different *Lactobacillus* spp. in 5 species of prawns both marine and freshwater. *L.plantarum*, *L.brevis* and *L.divergens* are the

Table 4.13.1 Distribution of different *Lactobacillus* species in fresh fish

No.	Fish	*L.plantarum*	*L.brevis*	*L.divergens*	*L.gasseri*	*L.casei ssp rhamnosus*	*L.fermentum*	*L.viridescens*	*L.farciminis*	*L.buchneri*	*L.acidophilus*	*L.alimentarius*	*L.animalis*	*L.reuteri*
1.	Picnic silver-bream	2	1	0	0	0	0	0	0	0	0	0	0	0
2.	Malabar trevally	3	1	0	2	1	0	0	0	0	0	0	0	0
3.	Milkfish	2	4	0	0	2	0	0	0	0	0	0	0	0
4.	Little tuna	6	0	2	0	0	0	0	0	0	0	1	0	0
5.	White fish	5	0	0	3	0	3	0	3	0	0	1	0	0
6.	Silver ribbon fish	3	3	0	1	0	0	2	0	0	0	0	0	0
7.	Malabar red snapper	3	0	0	1	0	1	0	0	0	0	0	1	0
8.	Brown triple tail	2	0	0	0	2	0	0	1	0	0	0	0	0
9.	Japanese thread fin bream	2	1	0	0	0	1	0	1	0	1	0	0	0
10.	Tilapia	4	0	0	0	1	0	0	0	0	0	0	1	0
11.	Tiger toothed croaker	1	2	0	0	1	0	0	0	0	0	0	0	0
12.	Silver pomfret	1	1	0	0	0	1	0	0	0	0	0	1	0

Table 4.13.2 Distribution of different *Lactobacillus* species in fresh fish

No.	Fish	*L.plantarum*	*L.brevis*	*L.divergens*	*L.gasseri*	*L.casei ssp rhamnosus*	*L.fermentum*	*L.viridescens*	*L.farciminis*	*L.buchneri*	*L.acidophilus*	*L.alimentarius*	*L.animalis*	*L.reuteri*
13.	Black pomfret	0	0	0	1	0	0	0	0	0	2	0	0	1
14.	Jewfish	0	2	0	0	1	0	0	0	0	0	0	0	0
15.	Indian mackerel	4	1	0	0	0	0	0	0	2	0	0	0	0
16.	Indian oil sardine	5	1	2	1	0	0	3	0	2	1	0	0	1
17.	Spotted butter fish	2	0	0	1	0	0	0	0	0	0	0	0	0
18.	Seer fish	1	1	0	0	0	0	0	1	0	0	0	0	0
19.	Dussumiers catfish	0	0	0	0	1	1	0	0	0	0	0	0	0
20.	Grey mullet	0	0	0	0	1	1	0	0	0	0	0	0	0
21.	Banded barracuda	1	1	0	0	0	0	0	0	0	0	0	0	0
22.	Pearlspot	2	1	0	0	0	0	0	0	0	1	0	0	0

Table 4.14 Distribution of different *Lactobacillus* species in fresh prawn

No.	Prawn	*L.plantarum*	*L.brevis*	*L.divergens*	*L.gasseri*	*L.casei ssp rhamnosus*	*L.fermentum*	*L.viridescens*	*L.farciminis*	*L.buchneri*	*L.acidophilus*	*L.alimentarius*	*L.animalis*	*L.reuteri*
1.	Brown shrimp	6	4	1	0	0	0	0	0	1	0	0	0	0
2.	Flower tail prawn	9	2	2	0	0	0	0	0	0	0	1	0	0
3.	Giant freshwater scampi	5	5	4	2	0	1	1	0	0	0	0	0	0
4.	Kiddi prawn	5	1	1	0	0	0	0	0	0	0	0	1	1
5.	Indian white prawn	3	1	2	0	1	1	0	1	0	0	0	0	0

major species universally present in these species of fresh prawn. Of these three species *L.plantarum* are isolated in more numbers followed by *L.brevis* and *L.divergens*. Other species are detected only very occasionally. Further, it is interesting to note that the Giant freshwater scampi (*Macrobrachium rosenbergii*) and Indian white prawn (*Penaeus indicus*) harboured 6 species each of *Lactobacillus*, other species had mostly 4-5 species of *Lactobacillus*. Among the rarer species of *Lactobacillus*, *L.gasseri* was isolated only from the Giant freshwater scampi, while *L.casei ssp rhamnosus* and *L.farciminis* were detected only from marine shrimp.

4.12.3 *Lactobacillus* spp. in frozen fish

The distribution of six species of commercially frozen fishes are presented in Table 4.15. *L.plantarum* and *L.brevis* were found in most of the species while other species are detected in less number of samples. Most of the frozen fish samples had only two or three *Lactobacillus* spp. including *L.plantarum* and *L.brevis*. Frozen seer fish was an exception from which six *Lactobacillus* spp. were detected. Certain species like *L.acidophilus*, *L.farciminis*, *L.animalis* and *L.reuteri were* not at all detected in any of the frozen fish samples.

4.12.4 *Lactobacillus* spp. in frozen prawn

Table 4.16 presents the distribution of *Lactobacillus* spp. were detected species in frozen prawns. *L.plantarum* and *L.divergens* were isolated from all the 5 species of prawns. While *L.brevis*, *L.gasseri* and *L.casei ssp rhamnosus* were detected in 3 prawn species and *L.fermentum* in 2 prawn species. Other *Lactobacillus* spp. were not detected in any of the frozen prawns. An interesting observation in the case of frozen prawn is that *L.divergens* were universally present in the frozen prawn species while *L.brevis* was found only in three species of frozen prawn. This is at variation from

Table 4.15 Distribution of different *Lactobacillus* species in frozen fish

No.	Fish	*L.plantarum*	*L.brevis*	*L.divergens*	*L.gasseri*	*L.casei ssp rhamnosus*	*L.fermentum*	*L.viridescens*	*L.farciminis*	*L.buchneri*	*L.acidophilus*	*L.alimentarius*	*L.animalis*	*L.reuteri*
1.	Picnic silver bream	4	3	0	0	0	1	0	0	0	0	0	0	0
2.	Djeddaba trevally	2	2	0	0	2	1	0	0	0	0	0	0	0
3.	Pearlspot	1	1	0	0	1	0	0	0	0	0	0	0	0
4.	Malabar reef cod	0	1	0	1	0	0	1	0	0	0	0	0	0
5.	Little tuna	2	1	0	2	0	0	0	0	0	0	0	0	0
6.	Silver ribbon fish	6	2	0	0	0	2	0	0	0	0	0	0	0
7.	Brown triple tail	1	3	0	0	0	0	1	0	0	0	0	0	0
8.	Tilapia	1	1	0	0	2	0	0	0	0	0	0	0	0
9.	Tiger toothed croaker	5	1	0	3	2	1	0	0	0	0	0	0	0
10.	Silver pomfret	0	4	2	0	0	1	0	0	0	0	0	0	0
11.	Black pomfret	6	0	0	0	0	0	0	0	0	0	0	0	0
12.	Indian oil sardine	8	5	0	0	0	0	0	0	0	0	0	0	0
13.	Spotted butter fish	5	3	0	0	0	0	0	0	0	0	0	0	0
14.	Seer fish	3	2	0	2	0	0	1	0	2	0	1	0	0
15.	Banded barracuda	5	1	0	1	0	0	0	0	0	0	0	0	0
16.	Malabar trevally	2	0	0	0	0	0	1	0	0	0	0	0	0

Table 4.16 Distribution of different *Lactobacillus* species in frozen prawn

No.	Prawn	*L.plantarum*	*L.brevis*	*L.divergens*	*L.gasseri*	*L.casei ssp rhamnosus*	*L.fermentum*	*L.viridescens*	*L.farciminis*	*L.buchneri*	*L.acidophilus*	*L.alimentarius*	*L.animalis*	*L.reuteri*
1.	Brown shrimp	3	1	3	0	0	0	0	0	0	0	0	0	0
2.	Flower tail prawn	2	0	2	1	0	2	0	0	0	0	0	0	0
3.	Giant freshwater scampi	2	1	2	1	1	2	0	0	0	0	0	0	0
4.	Kiddi prawn	1	0	3	1	1	0	0	0	0	0	0	0	0
5.	Indian white prawn	2	1	2	0	1	0	0	0	0	0	0	0	0

the observations made in fresh fish and frozen fish species in which *L.brevis* were more prevalent, while *L.divergens* were detected in a few species only. However the distribution of *Lactobacillus* spp. in frozen prawn appears to be very similar to the distribution in fresh prawns.

It is interesting to note that majority of the *Lactobacillus* spp that had been isolated from fresh and frozen fish/prawns were those species which are commonly found on meat, animals and human. There are a few reports of isolation of LAB from fresh and seawater fish (Ross and Toth,1974; Schroder et al,1979; Cone,1982 and Okafor and Nzeako,1985). Maugin and Novel (1994) examined various samples of seafood, fresh Pollock, brined shrimp, gravid fish and vacuum packed seafood. Of the LAB cultures they isolated, 54 belonged to *Lactobacillus lactis ssp lactis*, four cultures belonged to *Leuconostoc* spp. and 16 cultures belonged to *Carnobacterium*. Evelyn and McDermot (1961) have reported the isolation of *Lactobacillus* from freshwater, salmonid fishes. Kraus (1961) and Schroder et al (1979) have isolated *L.plantarum* from herring and Artic krill. Fricourt et al (1994) have reported the isolation of *L.plantarum* from chilled channel catfish fillets. Ostergaard et al (1998) have reported that *L.plantarum* predominated among the *Lactobacillus* spp. isolated from low salt fermented fish products.

Kandler and Weiss (1986) have reported the isolation of *L.brevis* from different sources including environmental samples. However, there is no published report on the isolation of *L.brevis* from fish and fishery products, as such this report on the detection and isolation of *L.brevis* from fresh and frozen fish/prawn appears to be a pioneering one.

The *L.divergens* is now classified as *Carnobacterium divergens* (Collins et al. 1987). According to them, the *Carnobacterium* cultures will not grow on acetate agar. However, the *L.divergens* cultures we have isolated grew well on acetate agar and so do not conform to the classification adopted by Collins et al (1987) for *C.divergens*. Hence, we retained the classification of our *Lactobacillus* cultures as *L.divergens* based on the classification scheme adopted by the Bergeys Manual (Kandler and Weiss,1986). There are recent reports (Pilet et al,1995) of isolation of *C.divergens* and *C.piscicola* from fresh fish products. From the tropical fish and prawn, isolation of *L.divergens* in this study is also first its kind. Similarly, the isolation of *L.gasseri, L.casei ssp rhamnosus, L.fermentum, L.viridescens, L.farciminis, L.acidophilus, L.alimentarius, L.animalis* and *L.reuteri* from fresh and frozen fish and shellfish is also the first of its kind.

4.13 The Biochemical and antibacterial investigations of *Lactobacillus* cultures

Amongst the nearly 600 *Lactobacillus* cultures, 47 well-characterized cultures were chosen for further detailed biochemical and antibacterial studies. The chosen cultures were typical of the species to which they belonged.

Fermentation of sugars, detailed studies on the fermentation of glucose and antibacterial activity against fish pathogenic/indicator bacteria and the nature of bacteriocin produced were investigated.

4.13.1 Fermentation of sugars

A set of 20 sugars consisting of 8 monosaccharides,6 disaccharides, 2 trisaccharides, 2 sugar alcohols and 2 glucosides as shown in materials and methods

Table 4.17.Fermentation of sugars, alcohols and glucosides by *Lactobacillus* cultures (Total cultures tested = 47)

Sl.No.	Sugars	% of cultures capable of fermentation
1.	Fructose (M)	100
2.	Glucose (M)	100
3.	Galactose (M)	100
4.	Mannose (M)	78.7
5.	Xylose (M)	74.5
6.	Ribose (M)	70.2
7.	Arabinose (M)	57.4
8.	Rhamnose (M)	25.5
9.	Sucrose (D)	100
10.	Maltose (D)	95.7
11.	Cellobiose (D)	83
12.	Trehalose (D)	72.3
13.	Lactose (D)	68.1
14.	Melebiose (D)	63.8
15.	Raffinose (T)	51.1
16.	Melezitose (T)	44.7
17.	Mannitol (SA)	83
18.	Sorbitol (SA)	40.4
19.	Esculin (G)	83
20.	Salicin (G)	76.6

(M) – Monosaccharide (D) – Disaccharide (T) – Trisaccharide

(SA) - Sugar alcohol (G) - Glucoside

(Table 3.2.1) were chosen for the fermentation study. Table 4.17 gives the summary of the fermentation characteristics of the 47 *Lactobacillus* cultures. All the cultures fermented monosaccharides, fructose, glucose, galactose, while the monosaccharide mannose, xylose and ribose were fermented by more than 70% of the cultures. Fifty-seven percent of the cultures fermented arabinose, while only 25% of the cultures fermented rhamnose.

Among the disaccharides studied, all the 47 cultures fermented sucrose, while 96% of the cultures fermented maltose. Cellobiose was fermented by 83% of the cultures, while trehalose and lactose were fermented by nearly 70% of cultures. The disaccharide, melebiose was fermented by 64% of the cultures. Of the 2 trisaccharides tested, namely raffinose and melezitose, nearly half of the *Lactobacillus* strains fermented them. Among the 2 sugar alcohols, mannitol was fermented by 83% of the cultures, but only 40% of the cultures fermented sorbitol. Both the glucosides namely esculin and salicin were fermented by majority of the *Lactobacillus* tested. The detailed breakup of the fermentation characters of major *Lactobacillus* spp. isolated from fish and prawn are represented in Tables 4.18 to 4.21.

4.13.1.1 Sugar fermentation by *Lactobacillus plantarum*

Table.4.18 shows the fermentation profile of 12 *L.plantarum* strains. Esculin, fructose, galactose, mannose and sucrose were fermented by all the 12 *L.plantarum* strains, while more than 90% of the cultures fermented cellobiose, mannitol, raffinose and salicin. Eighty-three percent cultures fermented melebiose and ribose, nearly 70% of the *L.plantarum* strains fermented melezitose, trehalose and xylose, and 58% cultures fermented arabinose and lactose. But, rhamnose, eventhough a monosaccharide, was fermented only by 33% of the *L.plantarum* cultures.

Table 4.18 Fermentation of sugars by *L.plantarum* cultures

No	LAB Strain	Arabinose	Cellobiose	Esculin	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Mannose	Melezitose	Melebiose	Raffinose	Rhamnose	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
1.	Esu-120	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+
2.	Lla-510	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
3.	Mce-43	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-
4.	Fpar-121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.	AV-2141	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+
6.	FE-136	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.	FS-521	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	-	-
8.	Lsa-71	-	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	-	+	-	+
9.	Lsa-108	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	-	+
10.	Lsa-127	-	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+
11.	Slo-621	-	+	+	+	+	+	-	+	+	+	-	-	+	-	+	+	-	+	+	+
12.	Slo-846	-	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-	+	-	+
Percentage of Cultures fermenting		58	92	100	100	100	100	58	100	92	100	67	83	92	33	83	92	25	100	67	75

4.13.1.2 Sugar fermentation by *Lactobacillus brevis*

The sugar fermentation pattern of 10 strains of *L.brevis* is given in Table 4.19. All the strains fermented esculin, fructose, galactose, glucose, maltose, ribose and sucrose. Nearly 80% of the cultures fermented arabinose, lactose, melebiose and xylose. Mannitol was fermented by 70% of the cultures followed by raffinose, which was fermented by 60% of the cultures. Only 10% of the *L.brevis* cultures could ferment the trisaccharide, melezitose. The rest of the sugars were fermented by less than 50% of the *L. brevis* cultures.

4.13.1.3 Sugar fermentation by *Lactobacillus divergens*

Table.4.20 shows the sugar fermentation profile of six *L.divergens* strains. All the cultures fermented cellobiose, fructose, galactose, glucose, mannose, salicin, sucrose and trehalose. None of the cultures fermented sorbitol. Sixty-seven percent of the cultures fermented esculin, maltose, mannitol, followed by 60% of the cultures fermenting xylose. Arabinose was fermented by 50% of the cultures, while less than 50% of the cultures fermented lactose, melezitose, melebiose, raffinose, rhamnose and ribose.

4.13.1.4 Sugar fermentation by *Lactobacillus fermentum*

Table.4.21 shows the sugar fermentation profile of 5 strains of *L.fermentum*. All the strains of *L.fermentum* tested were able to ferment cellobiose, fructose, glucose, galactose, lactose, maltose, mannitol, salicin and sucrose. Eighty percent of the cultures fermented arabinose, esculin, mannose, melebiose, ribose, sorbitol, trehalose and xylose. Melezitose and rhamnose were fermented by 20% of the cultures.

Table 4.19.Fermentation of sugars by *L.brevis* cultures

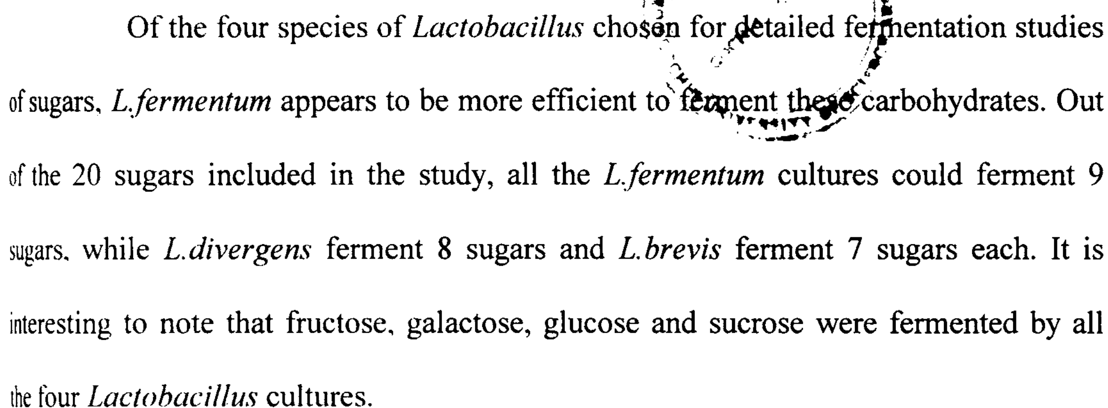
No	Culture No.	Arabinose	Cellobiose	Esculin	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Mannose	Melezitose	Melebiose	Raffinose	Rhamnose	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
1.	Par-356	-	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-	-	+	-	+
2.	Ocu-177	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	+	-	+
3.	Ocu-46	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	+	-	+
4.	Ocu-611	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
5.	Ocu-933	+	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+
6.	Eaf-970	+	-	+	+	+	+	+	+	+	+	-	+	-	-	+	+	-	+	+	+
7.	Adu-55	+	-	+	+	+	+	+	+	+	-	-	+	+	-	+	-	-	+	+	-
8.	FK-2	+	-	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
9.	Ftu-6	+	-	+	+	+	+	-	+	+	+	-	+	+	-	+	-	-	+	-	+
10.	Ftu-9	+	-	+	+	+	+	-	+	+	+	-	+	-	-	+	-	-	+	-	+
Percentage of cultures fermenting		80	40	100	100	100	100	80	100	70	50	10	80	60	20	100	30	20	100	40	80

Table 4.20. Fermentation of sugars by *L.divergens* cultures

No	Culture No.	Arabinose	Cellobiose	Esculin	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Mannose	Melezitose	Melebiose	Raffinose	Rhamnose	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
1.	Par-145	-	+	-	+	+	+	-	+	-	+	-	-	-	+	+	+	-	+	+	-
2.	Ocu-514	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+
3.	Lla-160	-	+	-	+	+	+	-	+	+	+	-	-	-	-	-	+	-	+	+	+
4.	Pm-112	+	+	+	+	+	+	-	+	-	+	-	+	-	-	-	+	-	+	+	-
5.	Pm-234	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	-	+	+	+
6.	Pm-300	+	+	+	+	+	+	-	-	+	+	-	-	+	-	-	+	-	+	+	+
Percentage of cultures fermenting		50	100	67	100	100	100	17	67	67	100	33	17	17	17	33	100	0	100	100	67

Table 4.21. Fermentation of sugars by *Lactobacillus fermentum* cultures

No	Culture No.	Arabinose	Cellobiose	Esculin	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Mannose	Melezitose	Melebiose	Raffinose	Rhamnose	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
1.	Ocu-75	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+
2.	Ocu-89	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+
3.	Ocu-10	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+
4.	Ocu-11	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+
5.	FS-11	+	+	-	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	-
Percentage of cultures fermenting		80	100	80	100	100	100	100	100	100	80	20	80	60	20	80	100	80	100	80	80



G18227

Of the four species of *Lactobacillus* chosen for detailed fermentation studies of sugars, *L.fermentum* appears to be more efficient to ferment these carbohydrates. Out of the 20 sugars included in the study, all the *L.fermentum* cultures could ferment 9 sugars, while *L.divergens* ferment 8 sugars and *L.brevis* ferment 7 sugars each. It is interesting to note that fructose, galactose, glucose and sucrose were fermented by all the four *Lactobacillus* cultures.

Maltose was fermented by 3 species namely *L.plantarum*, *L.brevis* and *L.fermentum*. mannose by all the strains of *L.plantarum* and *L.divergens*. Esculin was fermented by all the strains of *L.plantarum* and *L.brevis*, cellobiose by all the strains of *L.divergens* and *L.fermentum*.

Usually carbohydrates are included in the culture media to serve as an easy source of energy and to help in the identification and classification of bacteria. Carbohydrates so added are aldoses (eg. glucose, mannose), ketoses (eg. fructose), sugar alcohols (eg. mannitol) and glucosides (eg. esculin). Among the aldoses and ketoses, there are monosaccharides, disaccharides and trisaccharides. Microorganisms in general preferentially ferment simple sugars like monosaccharides. In the case of 4 species of *Lactobacillus* studied, it can be seen that almost all the cultures fermented the monosaccharides completely and certain disaccharides like sucrose was also completely fermented. Interestingly the glucosides, salicin and esculin have also been fermented either by all cultures or a substantial percentage of cultures of the *Lactobacillus*. Similar observation is also seen in the case of some rare disaccharides like cellobiose and trehalose. So, these observations suggest that the *Lactobacillus* spp. also possess the necessary metabolic pathways for fermenting certain carbohydrates with complex molecular structures apart from fermenting the common monosaccharides and disaccharides.

4.13.2 Studies on the effect of glucose, sodium chloride and incubation temperature on growth and bacteriocin production by *Lactobacillus* cultures

4.13.2.1 Effect of glucose concentration on the lowering of pH of the culture media by *Lactobacillus* culture

For growth studies, MRS broth was chosen and the basal MRS broth without sugar was enriched with different concentration of glucose namely 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.5%, 2.0%, 3.0% and 4.0%. Six typical *Lactobacillus* cultures belonging to six different **species** isolated from fish and shrimps were used for the study. The lowering of pH of the culture broth upto 10 days of incubation was determined. Table 4.22 A and 4.22 B show the effect of glucose concentration in MRS broth on the pH of the culture media. The initial pH of the MRS broth before inoculation was 6.5. It can be seen from the Tables that within 24h of growth, the pH of the culture media was lowered to about 5 to 5.4 in the case of 0.2% glucose in the medium. As the concentration of glucose increases to 2% ,there was a steady decrease of the pH of the medium to a level below pH 4.0 or near within 24h of incubation. Further increase in the concentration of glucose did not bring down the pH appreciably. Similarly, continued incubation upto 10 days did not materially decrease the pH of the culture medium. Hence it could be assumed that the *Lactobacillus* utilized the carbohydrate in the initial days of incubation and reached the plateau of their logarithmic growth.

Among the different *Lactoabcillus* spp, *L.plantarum, L.casei ssp rhamnosus* and *L.viridescens* appeared to be more efficient in fermenting glucose and bringing down the pH to very low level ie., below pH 4.0 with 2% glucose in growth medium. *L.plantarum* and *L.viridescens* attained the lowest pH of 3.6 in 48 h time with 2% glucose in the growth medium. On further incubation, no appreciable lowering of

Table 4.22. A. Effect of glucose concentration in MRS broth on pH of culture media

No	*Lactobacillus* cultures	Glucose conc. in %	pH				
			I day	II day	III day	IV day	X day
	L.casei (NCIM 2165)	0.2	5.20	5.00	5.30	5.25	5.20
		0.4	4.40	4.40	4.35	4.30	4.30
		0.6	4.50	4.45	4.45	4.30	4.30
		0.8	4.45	4.40	4.36	4.35	4.30
		1.0	4.00	4.00	4.00	3.95	3.90
		1.5	3.90	3.85	3.85	3.80	3.75
		2.0	3.75	3.60	3.60	3.58	3.50
		3.0	3.73	3.54	3.53	3.51	3.45
		4.0	3.72	3.45	3.45	3.43	3.42
	L.plantarum (Esu-120)	0.2	5.00	5.20	5.30	5.30	5.25
		0.4	4.75	4.75	4.60	4.50	4.48
		0.6	4.30	4.25	4.20	4.00	4.00
		0.8	4.30	4.20	4.00	3.95	3.90
		1.0	4.00	4.00	4.00	3.90	3.90
		1.5	4.00	3.98	3.96	3.70	3.68
		2.0	3.98	3.85	3.81	3.45	3.44
		3.0	3.96	3.94	3.80	3.42	3.41
		4.0	3.94	3.80	3.75	3.40	3.40
	L.divergens (Lla-160)	0.2	5.35	5.35	5.40	5.60	5.60
		0.4	5.30	5.25	5.10	4.90	4.87
		0.6	4.70	4.75	4.75	4.60	4.59
		0.8	4.58	4.58	4.20	4.20	4.18
		1.0	4.22	4.22	4.24	4.10	4.10
		1.5	4.25	4.25	3.97	3.80	3.78
		2.0	3.83	3.81	3.75	3.72	3.60
		3.0	3.81	3.78	3.74	3.71	3.58
		4.0	3.78	3.76	3.74	3.70	3.55

Table 4.22.B. **Effect of glucose concentration in MRS broth on pH of culture media**

No.	***Lactobacillus* cultures**	**Glucose conc. in %**	pH				
			I day	II day	III day	IV day	X day
.	*L.gasseri* (Par-276)	0.2	5.25	5.25	5.25	5.25	5.30
		0.4	4.89	4.78	4.76	4.75	4.70
		0.6	4.86	4.75	4.71	4.70	4.68
		0.8	4.77	4.74	4.70	4.67	4.66
		1.0	4.73	4.72	4.69	4.65	4.64
		1.5	4.58	4.50	4.48	4.47	4.45
		2.0	4.39	4.35	4.33	4.20	4.10
		3.0	4.37	4.34	4.32	4.00	3.95
		4.0	4.35	4.33	4.32	3.95	3.80
:	*L.alimentarius* (Ocu-12)	0.2	5.40	5.30	5.50	5.50	5.60
		0.4	4.50	4.50	4.40	4.40	4.38
		0.6	4.50	4.48	4.48	4.20	4.18
		0.8	4.35	4.20	4.20	4.10	4.08
		1.0	4.30	4.30	4.31	4.00	4.00
		1.5	4.30	4.30	4.26	3.90	3.89
		2.0	3.90	3.90	3.89	3.85	3.84
		3.0	3.87	3.85	3.84	3.83	3.82
		4.0	3.85	3.83	3.80	3.78	3.76
	L.viridescens (Par-620)	0.2	5.05	5.05	5.05	5.05	5.10
		0.4	4.75	4.75	4.10	4.00	3.99
		0.6	4.75	4.65	4.20	3.90	3.87
		0.8	4.65	4.65	4.00	3.88	3.87
		1.0	4.00	4.00	4.00	3.87	3.86
		1.5	3.86	3.70	3.70	3.68	3.68
		2.0	3.80	3.65	3.56	3.54	3.54
		3.0	3.78	3.60	3.60	3.53	3.50
		4.0	3.75	3.58	3.54	3.53	3.48

pH was noticed. Also, an increase in the concentration of glucose above 2% did not substantially lower the pH produced in the culture medium.

4.13.2.1.1 Titrable acidity of *Lactoabcillus* spp.

The lowering of pH by *Lactobacillus* during fermentation of glucose or any carbohydrate is due to the acids produced by the fermentation process. Usually lactic acid and acetic acid are produced in the growth medium by *Lactobacillus*. The amount of acidity produced in the growth medium in relation to the growth of *Lactoabcillus* strains in MRS broth containing 2% glucose is presented in Table.4.23. A total of 23 *Lactobacillus* cultures (10 species) were included in the study.

Growth was measured as absorbance at 660nm in a spectrophotometer and the acid produced was determined as titrable acidity and calculated as percentage of lactic acid. In 24h growth attained by *Lactobacillus* cultures were more or less 1.5 optical density (O.D) units, but the titrable acidity produced varied between 0.45 to 0.9 g of lactic acid/100ml of the culture indicating that the titrable acidity produced has no direct correspondence to the growth. When *L.plantarum* culture (Lla-510) with an O.D of 0.95 could produce a titrable acidity of 0.45%, *L.fermentum* with a growth of 1.5 O.D produced the same quantity of acidity. Among the same *Lactoabcillus* spp. different strains produced different quantity of titrable acidity, even when they had the same growth measured as O.D. For eg. While *L.fermentum* (Ocu-11) produced 0.45% titrable acidity, *L.fermentum* (Ocu-75) produced 0.72% titrable acidity and *L.fermentum* (Ocu-89) produced 0.9% titrable acidity and *L.fermentum* (Ocu-89) produced 0.9% titrable acidity. Similar instances were also available in the case of *L.divergens* and *L.brevis*, but in the case of *L.plantarum*, except one strain others produced higher quantity of titrable acidity.

Table 4.23. **Titrable acidity produced by *Lactobacillus* strains in MRS** broth **with 2% glucose**

No.	***Lactobacillus* strain**	**Growth as absorbance at 660nm (Optical density) in 24 hrs**	**Titrable acidity As % of lactic acid**
1.	*L.plantarum* (Lla-510)	0.95	0.45
2.	*L.plantarum* (AV-2141)	1.3	0.77
3.	*L.plantarum* (Esu-120)	1.5	0.81
4.	*L.fermentum* (Ocu-10)	1.45	0.45
5.	*L.fermentum* (Ocu-11)	1.5	0.45
6.	*L.fermentum* (Ocu-75)	1.5	0.72
7.	*L.fermentum* (Ocu-89)	1.5	0.9
8.	*L.divergens* (Pm-112)	1.5	0.5
9.	*L.divergens* (Pm-234)	1.5	0.5
10.	*L.divergens* (Ocu-514)	1.5	0.59
11.	*L.divergens* (Pm-300)	1.5	0.59
12.	*L.divergens* (Par-145)	1.45	0.72
13.	*L.brevis* (Ocu-933)	1.5	0.59
14.	*L.brevis* (Ocu-611)	1.5	0.68
15.	*L.brevis* (Ocu-177)	1.5	0.72
16.	*L.brevis* (Ocu-46)	1.5	0.77
17.	*L.brevis* (Par-356)	1.5	0.81
18.	*L.farciminis* (Ocu-28)	1.5	0.59
19.	*L.animalis* (Lla-476)	1.2	0.63
20.	*L.buchneri* (Ocu-31)	1.5	0.68
21.	*L.viridescens* (Par-620)	1.5	0.72
22.	*L.casei subsp rhamnosus* (Cma-611)	1.5	0.77
23.	*L.gasseri* (Par-276)	1.5	0.77

Organic acid, particularly acetic and lactic acid produced by *Lactobacillus* in culture media by the fermentation of carbohydrate has antimicrobial properties due to the undissociated acid molecules as well as to the lowered pH (Ray and Sandine, 1992). *Lactobacillus* cultures which lower the pH to 4.5 and below and produce titrable acidity above 1% as lactic acid are used to produce fermented fish products. Jamias-Apilado and Mabesa (1992) used starter cultures of *L.plantarum* and *Pediococcus cereviseae* for production of fermented rice fish products. A titrable acidity of 1.03 to 1.42% was achieved in a week time. Abraham and Setty (1994) studied the acid producing potential of *L.plantarum* cultures during fish fermentation with added glucose and sucrose at 4% level. They obtained a titrable acidity of 0.88% lactic acid with 4% glucose in the medium. However, the *L.plantarum* culture (Esu-120) used in this study could produce 0.81% lactic acid in MRS broth with 2% glucose.

4.13.2.2 Effect of sodium chloride in culture media on the growth of *Lactobacillus* culture

The salt tolerance of LAB cultures is very important when they are chosen for biopreservation of fish products. In the production of fermented fish products, NaCl at 4-8% is used to check the growth of native spoiling microorganism and also to prevent the growth of *Clostridium botulinum* type E. Naturally, the *Lactobacillus* cultures should be high salt tolerating as well as capable of growing in the presence of high salt content. Table.4.24 A and 4.24 B show the potential of 30 *Lactobacillus* cultures belonging to 13 species to grow in presence of high salt concentration in growth media. NaCl was incorporated from 4 to 10% concentration level in MRS broth. The growth was measured in terms of O.D at 660nm. All the 30 cultures showed excellent growth upto 5% NaCl in the medium and moderate growth at 6% and 7% NaCl concentration. However, only 8 cultures showed growth at 8% salt and none grew

Table 4.24.A Effect of NaCl in growth medium on *Lactobacillus* cultures

Lactobacillus cultures	NaCl concentration						
	4%	5%	6%	7%	8%	9%	10%
L.gasseri (Lla-22)	++	++	+	+	-	-	-
L.gasseri (Rka-2)	++	++	+	+	-	-	-
L.gasseri (Par-276)	++	++	+	+	-	-	-
L.brevis (Par-356)	++	++	+	+	-	-	-
L.brevis (Ocu-177)	++	++	+	+	-	-	-
L.brevis (Ocu-46)	++	++	+	+	-	-	-
L.brevis (Ocu-611)	++	++	+	+	-	-	-
L.brevis (Ocu-933)	++	++	+	+	-	-	-
L.brevis (Eaf-970)	++	++	+	+	-	-	-
L.viridescens (Par-620)	++	++	+	+	-	-	-
L.casei (Cma-611)	++	++	+	+	-	-	-
L.casei (Par-78)	++	++	+	+	-	-	-
L.farciminis (Ocu-28)	++	++	+	+	-	-	-
L.buchneri (Ocu-31)	++	++	+	+	-	-	-
L.acidophilus (Rka-312)	++	++	+	+	+	-	-

-indicates optical density at 24h = 0 (zero)

· -indicates optical density between 0-1.5 at 24h.

-'-indicates optical density above 1.5 at 24h.

Table 4.24.B. **Effect of NaCl in growth medium on *Lactobacillus* cultures**

No.	*Lactobacillus* cultures	NaCl concentration						
		4%	5%	6%	7%	8%	9%	10%
6.	*L.fermentum* (Ocu-75)	++	++	+	+	+	-	-
7.	*L.fermentum* (Ocu-89)	++	++	+	+	+	-	-
8.	*L.fermentum* (Ocu-10)	++	++	+	+	+	-	-
9.	*L.fermentum* (Ocu-11)	++	++	+	+	-	-	-
0.	*L.alimentariu* (Ocu-12)	++	++	+	+	-	-	-
1.	*L.animalis* (Lla-476)	++	++	+	+	-	-	-
2.	*L.plantarum* (Esu-120)	++	++	+	+	+	-	-
3.	*L.plantarum* (Lla-510)	++	++	+	+	+	-	-
4.	*L.reuteri* (Eaf-611)	++	++	+	+	-	-	-
5.	*L.divergens* (Lla-160)	++	++	+	+	-	-	-
6.	*L.divergens* (Ocu-514)	++	++	+	+	-	-	-
7.	*L.divergens* (Par-145)	++	++	+	+	-	-	-
8.	*L.divergens* (Pm-112)	++	++	+	+	+	-	-
9.	*L.divergens* (Pm-234)	++	++	+	+	+	-	-
0.	*L.divergens* (Pm-300)	++	++	+	+	-	-	-

- indicates optical density at 24h = 0 (zero)

· indicates optical density between 0-1.5 at 24h.

·· indicates optical density above 1.5 at 24h.

beyond this level of salt in the growth medium. Among the 8 cultures showing growth at 8% salt, 3 belong to *L.fermentum*, 2 *L.plantarum, 2 L.divergens* and 1 *L.acidophilus*. The study indicated that only a small percentage of *Lactobacillus* cultures have the potential to grow in salt levels above 7% and none of the 30 cultures could tolerate salt levels beyond 8%.

Adams et al (1987) studied the effect of salt on the growth of *L.plantarum* cultures. They found that increase in the salt concentration above 6% affected the growth and fermentation rate of *L.plantarum*. Maugin and Novel (1994) while characterizing the LAB isolated from seafood, found that 69 out of 72 LAB cultures grew in presence of 8% salt and 6 of them could tolerate 10% salt. However, none of the cultures in the present study could tolerate 10% salt in growth media. Only 8 out of 30 of the cultures could grow in presence of 8% NaCl. Study indicated that the *Lactobacillus* cultures are capable of luxuriant growth only upto 5% salt in the growth media.

4.13.2.3 Effect of incubation temperature on the growth of *Lactobacillus* culture

Lactobacillus cultures that can grow and survive at very low temperatures are likely candidates for fish preservation. Tables 4.25 A and 4.25 B show the effect of incubation temperature on the growth of selected *Lactobacillus* cultures. About thirty *Lactobacillus* cultures (13 species) were studied. The optimum temperature for growth of all the cultures were between 28°C to 37°C. Except for 3 cultures of *L.gasseri,* 1 of *L.acidophilus* and 1 of *L.animalis*, all the others could grow at very low temperatures between 10-15°C. However, only 16 out of the 30 *Lactobacillus* cultures studied could survive at 45°C. It was observed that 3 cultures (*L.acidophilus, L.gasseri (Lla-22)* and *L.animalis*) could not grow at temperatures less than or greater than 28°C and 37°C,

Table 4.25.A. Effect of Temperature on the growth of *Lactobacillus* cultures

No.	***Lactobacillus* cultures**	**Incubation Temperature**				
		10°C	15°C	28°C	37°C	45°C
	L.gasseri (Lla-22)	-	-	++	++	-
	L.gasseri (Rka-2)	-	-	++	++	+
	L.gasseri (Par-276)	+	+	++	++	+
	L.brevis (Par-356)	+	+	++	++	+
	L.brevis (Ocu-177)	+	+	++	++	-
	L.brevis (Ocu-46)	+	+	++	++	-
	L.brevis (Ocu-611)	+	+	++	++	-
	L.brevis (Ocu-933)	+	+	++	++	+
	L.brevis (Eaf-970)	+	+	++	++	+
	L.viridescens (Par-620)	+	+	++	++	+
	L.casei (Cma-611)	+	+	++	++	+
	L.casei (Par-78)	+	+	++	++	+
	L.farciminis (Ocu-28)	+	+	++	++	+
	L.buchneri (Ocu-31)	+	+	++	++	-
	L.acidophilus (Rka-312)	-	-	++	++	-

- indicates optical density at 24 h = 0 (zero)
+ indicates optical density between 0-1.5 at 24 h.
++ indicates optical density above 1.5 at 24 h.

Table 4.25.B. Effect of Temperature on the growth of *Lactobacillus* cultures

No.	*Lactobacillus* cultures	Incubation Temperature				
		10°C	15°C	28°C	37°C	45°C
	L.fermentum (Ocu-75)	+	+	++	++	+
	L.fermentum (Ocu-89)	+	+	++	++	+
	L.fermentum (Ocu-10)	+	+	++	++	+
	L.fermentum (Ocu-11)	+	+	++	++	+
	L.alimentarius (Ocu-12)	+	+	++	++	-
	L.animalis (Lla-476)	-	-	++	++	-
	L.plantarum (Esu-120)	+	+	++	++	-
	L.plantarum (Lla-510)	+	+	++	++	+
	L.reuteri (Eaf-611)	+	+	++	++	+
	L.divergens (Lla-160)	+	+	++	++	-
	L.divergens (Ocu-514)	+	+	++	++	-
	L.divergens (Par-145)	+	+	++	++	+
	L.divergens (Pm-112)	+	+	++	++	-
	L.divergens (Pm-234)	+	+	++	++	-
	L.divergens (Pm-300)	+	+	++	++	-

- indicates optical density at 24h = 0 (zero)
· indicates optical density between 0-1.5 at 24h.
·· indicates optical density above 1.5 at 24h.

respectively. There is no available data on studies related to the effect of temperature on the growth of LAB. Our study shows that *Lactobacillus* cultures can be used to preserve chilled foods as they grow well below 15°C but upto 10°C.

4.13.2.4 Effect of pH of the media on the growth of *Lactobacillus* culture

Table 4.26A and 4.26B show the effect of initial pH of MRS media on the growth of *Lactobacillus* cultures. The growth was measured spectrophotometrically at 660nm after 24h. None of the 30 strains studied recorded any growth at pH 4.0 and 4.5 of the media. Almost all the *Lactobacillus* cultures showed reasonably good growth at pH 5.0, 5.5 and 7.0. Exceptions were few strains of *L.plantarum* (Esu-120,Lla-510) , *L.fermentum* (Ocu-10,Ocu-7,Ocu-8), *L.gasseri* (Lla-22) and *L.brevis* (Eaf-9), which did not grow at pH 5.0. The optimum growth of the *Lactobacillus* strains was between pH 6 and 6.5 pH of MRS media. All the cultures studied grew at pH between 5.5-7.0. According to Kandler and Weiss (1986), lactobacilli grow best in slightly acidic media with an initial pH of 6.4 to 4.5. In our studies, the optimum pH range was found to be between 6.0 and 6.5.

4.13.3 Studies on antibacterial activity of *Lactobacillus*

4.13.3.1 Screening of *Lactobacillus* cultures for antibacterial activity against indicator/pathogenic bacteria

All the *Lactobacillus* cultures isolated from fresh and frozen fish/prawn were screened for their antibacterial activity by the agar spot test against

1. Faecal indicator organism – *E.coli*
2. Pathogenic organism – *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*.

Table 4.26.A. Effect of pH of culture media on the growth of *Lactobacillus* cultures

No.	*Lactobacillus* cultures	pH of media						
		4.0	4.5	5.0	5.5	6.0	6.5	7.0
	L.gasseri (Lla-22)	-	-	-	+	++	++	+
	L.gasseri (Rka-2)	-	-	+	+	++	++	+
	L.gasseri (Par-276)	-	-	+	+	++	++	+
	L.brevis (Par-356)	-	-	+	+	++	++	+
	L.brevis (Ocu-177)	-	-	+	+	++	++	+
	L.brevis (Ocu-46)	-	-	+	+	++	++	+
	L.brevis (Ocu-611)	-	-	+	+	++	++	+
	L.brevis (Ocu-933)	-	-	+	+	++	++	+
	L.brevis (Eaf-970)	-	-	-	+	++	++	+
	L.viridescens (Par-620)	-	-	+	+	++	++	+
	L.casei (Cma-611)	-	-	+	+	++	++	+
	L.casei (Par-78)	-	-	+	+	++	++	+
	L.farciminis (Ocu-28)	-	-	+	+	++	++	+
	L.buchneri (Ocu-31)	-	-	+	+	++	++	+
	L.acidophilus (Rka-312)	-	-	+	+	++	++	+

- indicates optical density at 24h = 0 (zero)
- indicates optical density between 0-1.5 at 24h.
- indicates optical density above 1.5 at 24h.

Table 4.26.B. Effect of pH of culture media on the growth of *Lactobacillus* cultures

No.	*Lactobacillus* cultures	pH of media						
		4.0	4.5	5.0	5.5	6.0	6.5	7.0
6.	*L.fermentum* (Ocu-75)	-	-	-	+	++	++	+
7.	*L.fermentum* (Ocu-89)	-	-	-	+	++	++	+
8.	*L.fermentum* (Ocu-10)	-	-	-	+	++	++	+
9.	*L.fermentum* (Ocu-11)	-	-	+	+	++	++	+
[illegible]	*L.alimentariu* (Ocu-12)	-	-	+	+	++	++	+
[illegible]	*L.animalis* (Lla-476)	-	-	+	+	++	++	+
[illegible]	*L.plantarum* (Esu-120)	-	-	-	+	++	++	+
[illegible]	*L.plantarum* (Lla-510)	-	-	-	+	++	++	+
[illegible]	*L.reuteri* (Eaf-611)	-	-	+	+	++	++	+
[illegible]	*L.divergens* (Lla-160)	-	-	+	+	++	++	+
[illegible]	*L.divergens* (Ocu-514)	-	-	+	+	++	++	+
[illegible]	*L.divergens* (Par-145)	-	-	+	+	++	++	+
[illegible]	*L.divergens* (Pm-112)	-	-	+	+	++	++	+
[illegible]	*L.divergens* (Pm-234)	-	-	+	+	++	++	+
[illegible]	*L.divergens* (Pm-300)	-	-	+	+	++	++	+

- indicates optical density at 24h = 0 (zero)
· indicates optical density between 0-1.5 at 24h.
·· - indicates optical density above 1.5 at 24h.

Typical results are presented in Tables 4.27A and 4.27B. The *Lactobacillus* strains belonging to *L.plantarum, L.brevis, L.divergens, L.fermentum, L.gasseri, L.acidophilus, L.animalis, L.alimentarius, L.casei ssp rhamnosus, L.buchneri, L.farciminis, L.reuteri* and *L.viridescens* were included in the preliminary screening process. *Listeria monocytogenes* was ATCC culture (ATCC 19111) while *Bacillus cereus* (B3/3), *Staphylococcus aureus* (SA3B) and *E.coli* (Ec101) were well characterized cultures from the National Collection of Aquatic and Fish Bacteria (NCAFB) at CIFT, Cochin. Almost all the *Lactobacillus* cultures inhibited *L.monocytogenes*, eventhough with varying degrees as measured by the diameter of zone of inhibition. Similarly, *B.cereus* was also inhibited by most of the *Lactobacillus* cultures. In the case of *S.aureus*, the level of inhibition was less and only about 50% of *Lactobacillus* cultures inhibited the growth of *S.aureus*.

In the case of *E.coli*, the inhibition by *Lactobacillus* was limited and only less than 50% of the *Lactobacillus* could inhibit the growth of *E.coli*.

4.13.3.1.1 Inhibition by *Lactobacillus plantarum*

Among the *L.plantarum*, most of the cultures inhibited the growth of *L.monocytogenes* and *B.cereus* very effectively. The zone of inhibition were always very large indicating the susceptibility of both *L.monocytogenes* and *B.cereus* to *L.plantarum*. But, the inhibitory effect of *L.plantarum* was not very appreciable in the case of *S.aureus*, eventhough most of the *L.plantarum* cultures inhibited the *S.aureus* with a comparatively smaller zone size. In the case of *E.coli*, though *L.plantarum* exhibited antibacterial activity, the inhibition was not universal.

Table 4.27.A. Screening of *Lactobacillus* cultures for antibacterial activity

Lactobacillus strains	Inhibition measured as diameter of zone of inhibition (mm)			
	L.monocytogenes	*B.cereus*	*S.aureus*	*E.coli*
L.plantarum (Esu-120)	++	++	+	+
L.plantarum (Lla-510)	++	++	+	+
L.plantarum (Mce-43)	++	++	+	+
L.plantarum(Fpar-121)	++	++	-	-
L.plantarum (AV-2141)	++	++	+	-
L.plantarum (FE-136)	++	++	+	-
L.plantarum (FS-521)	++	++	+	-
L.plantarum (Lsa-71)	++	++	+	+
L.plantarum (Lsa-108)	++	++	+	-
L.plantarum (Lsa-127)	++	++	+	+
L.plantarum (Slo-621)	++	++	+	+
L.plantarum (Slo-846)	++	++	+	+
L.brevis (Par-356)	+	+	-	-
L.brevis (Ocu-177)	++	++	+	+
L.brevis (Ocu-46)	++	++	+	+
L.brevis (Ocu-611)	++	++	+	+
L.brevis (Ocu-933)	++	++	+	+
L.brevis (Eaf-970)	++	++	+	+
L.brevis (Adu-55)	++	++	+	+
L.brevis (FK-2)	+	+	-	-
L.brevis (Ftu-6)	+	+	-	-
L.brevis (Ftu-9)	+	+	-	-
L.divergens (Par-145)	++	++	-	-
L.divergens (Ocu-514)	++	++	-	-
L.divergens (Lla-160)	+	+	-	-

-' - indicates inhibition zone diameter above 15mm

' - indicates inhibition zone diameter between 10-15mm

' - indicates no inhibition zone

Table 4.27.B Screening of *Lactobacillus* cultures for antibacterial activity

No	*Lactobacillus* strains	Inhibition measured as diameter of zone of inhibition (mm)			
		L.monocytogenes	*B.cereus*	*S.aureus*	*E.coli*
1.	*L.divergens* (Pm-112)	++	++	-	-
2.	*L.divergens* (Pm-234)	++	++	-	-
3.	*L.divergens* (Pm-300)	++	++	+	+
4.	*L.gasseri* (Par-276)	++	++	+	+
5.	*L.gasseri* (Rka-2)	++	++	+	+
6.	*L.gasseri* (Lla-22)	++	++	+	+
7.	*L.casei subspp.rhamnosus* (Par-78)	+	+	+	+
8.	*L.casei subspp.rhamnosus* (Cma-611)	+	+	+	+
9.	*L.casei subspp.rhamnosus* (Fpar-9)	+	+	-	-
10.	*L.casei subspp.rhamnosus* (Frcu-6)	++	++	-	-
11.	*L.viridescens* (Par-620)	+	+	-	-
12.	*L.farciminis* (Ocu-28)	+	+	-	-
13.	*L.buchneri* (Ocu-31)	+	+	-	-
14.	*L.acidophilus*(Rka-312)	++	++	+	+
15.	*L.alimentarius* (Ocu-12)	+	++	-	-
16.	*L.alimentarius* (FS-11)	+	+	-	-
17.	*L.reuteri* (Eaf-611)	++	++	-	-
18.	*L.fermentum* (Ocu-75)	+	+	-	-
20.	*L.fermentum* (Ocu-89)	+	+	-	-
21.	*L.fermentum* (Ocu-10)	+	+	-	-
22.	*L.fermentum* (Ocu-11)	++	++	+	+
23.	*L.animalis* (Lla-476)	++	++	+	+
24.	*L.casei* (NCIM 2165)	+	++	++	-

'++' - indicates inhibition zone diameter above 15mm

'+' - indicates inhibition zone diameter between 10-15mm

'-' - indicates no inhibition zone

4.13.3.1.2 Inhibition by *Lactobacillus brevis*

L.brevis cultures exhibited very good antibacterial activity against both *L.monocytogenes* and *B.cereus*, eventhough the extend of antibacterial effect was not as appreciable as in the case of *L.plantarum* against *S.aureus* and *E.coli*, the antibacterial activity was not very appreciable and in many cases *L.brevis* did not show any inhibitory effect at all.

4.13.3.1.3 Inhibition by *Lactobacillus divergens*

Six strains of *L.divergens* were studied for their antibacterial activity. Except for one strain (Lla-160), all the others showed inhibition zones above 15mm diameter against *L.monocytogenes* and *B.cereus*. *L.divergens* cultures did not exhibit antibacterial activity against *E.coli* and *S.aureus* except for one strain, which showed weak inhibition. Compared to *L.plantarum* cultures, *L.divergens* showed a narrow range of inhibition.

4.13.3.1.4 Inhibition by *Lactobacillus casei ssp rhamnosus*

Among the four strains of *L.casei ssp rhamnosus* screened for antibacterial activity, all the four inhibited *L.monocytogenes* and *B.cereus* with an inhibition zone diameter less than 15mm. However, only two strains could inhibit *S.aureus* and *E.coli*. *L.casei ssp rhamnosus* (Freu-6) showed very good antibacterial activity against *L.monocytogenes* and *B.cereus* compared to other strains of *L.casei ssp rhamnosus*.

4.13.3.1.5 Other Lactobacillus groups

Among the other *Lactobacillus* cultures tested, *L.gasseri* cultures exhibited excellent antibacterial activity against *L.monocytogenes* and *B.cereus* and limited

activity against *S.aureus* and *E.coli*. *L.viridescens*, *L.farciminis*, *L.buchneri*, *L.fermentum* and *L.alimentarius* showed antibacterial activity against *L.monocytogenes* and *B.cereus* but, the extend of inhibitory property was not very appreciable.These *Lactobacillus* spp. were more or less inactive against both *S.aureus* and *E.coli*.

4.13.3.2 Nature of antibacterial activity by *Lactobacillus*.

The antibacterial activity by *Lactobacillus* cultures could be mainly due to the

1. Organic acids produced
2. Hydrogen peroxide production
3. Bacteriocin production

Usually lactic acid and acetic acid are the two major organic acids produced by *Lactobacillus* by fermentation of glucose in culture media.

4.13.3.3 Characterization of antibacterial activity by *Lactobacillus* cultures

Table.4.28 presents the data from 10 selected *Lactobacillus* cultures regarding characterization of antibacterial activity against *L.monocytogenes*. During the screening of *Lactobacillus* cultures for antibacterial activity against chosen pathogenic/indicator bacteria, *L.monocytogenes* was found to be the most susceptible strain against *Lactobacillus* cultures. Hence, *L.monocytogenes* is used as the candidate strain for characterisation of antibacterial activity.

In the culture filtrate of *Lactobacillus* strains, the zone of inhibition of the culture filtrate as such , after neutralization with alkali, after treatement with four protein hydrolyzing enzymes namely protease, trypsin, chymotrypsin and pepsin are presented in Table 4.28.

Table 4.28 Characterization of antibacterial activity of *Lactobacillus* cultures against *Listeria monocytogenes*

No	*Lactobacillus* cultures	BC	BC + neutralized	BC + neutralized +catalase	Heat treatment			Proteinase treatment			
					80°C 30 min	100°C 10 min	121°C 10 min	Protease	Trypsin	Chymo-trypsin	Pepsin
1.	*L.divergens* (Par-145)	20mm	18mm	18mm	18mm	15mm	NZD	NZD	NZD	14mm	14mm
2.	*L.divergens* (Pm-234)	20mm	18mm	18mm	20mm	20mm	NZD	NZD	NZD	NZD	NZD
3.	*L.gasseri* (Par-276)	24mm	18mm	18mm	18mm	18mm	16mm	NZD	NZD	NZD	NZD
4.	*L.gasseri* (Lla-22)	20mm	16mm	16mm	18mm	16mm	NZD	NZD	NZD	NZD	NZD
5.	*L.brevis* (Ocu-933)	26mm	22mm	22mm	22mm	22mm	NZD	NZD	NZD	NZD	NZD
6.	*L.fermentum* (Ocu-11)	24mm	20mm	20mm	18mm	18mm	NZD	NZD	12mm	12mm	NZD
7.	*L.animalis* (Lla-476)	20mm	18mm	18mm	18mm	18mm	NZD	NZD	NZD	NZD	NZD
8.	*L.plantarum* (Lla-510)	22mm	20mm	20mm	20mm	14mm	NZD	NZD	NZD	NZD	10mm
9.	*L.plantarum* (Esu-120)	24mm	22mm	22mm	22mm	15mm	NZD	NZD	NZD	NZD	10mm
10.	*L.reuteri* (Eaf-611)	24mm	22mm	22mm	22mm	22mm	NZD	NZD	NZD	NZD	NZD

BC – Bacteriocin Concentrate   NZD – No Zone Detected

The neutralization of culture filtrate with alkali neutralizes the organic acid and thereby neutralizes the antibacterial activity due to organic acids. Catalase treatment deactivates H_2O_2, if at all formed in the culture media.

Heat treatment in 80°C, 100°C and 121°C has been done with a specific purpose. Heating at 80°C for 30 min denatures proteins other than bacteriocins. Bacteriocins withstand to a considerable degree at 100°C for 10 min and are generally denatured at 121°C in 10 min. Treatment with four different proteolytic enzymes has been done to evidently prove the protein nature of the bacteriocin. A typical photograph is presented in Figure 4.4.

The antibacterial activity of all 10 cultures were partially due to the organic acids produced by the *Lactobacillus*. So, the resultant antibacterial activity is due to either bacteriocin, H_2O_2 if produced or due to both. The antibacterial activity after treatment with excess catalase, is entirely due to bacteriocin alone. The data presented in the Table 4.28 definitely indicated that there was no H_2O_2 produced by the *Lactobacillus* cultures in the MRS broth and the remaining antibacterial activity was entirely due to bacteriocin. Heat treatment at 80°C for 30min has not affected the antibacterial activity indicating that the bacteriocins withstood heat treatment at 80°C for 30 min. However, heating at 100°C has diminished antibacterial activity implying that there was partial denaturation of the bacteriocin at 100°C. Treatment at 121°C for 10 min deactivated all the bacteriocins, except the one produced by *L.gasseri* (Par-276).

The protein hydrolyzing enzymes generally deactivated bacteriocins produced by all the 10 cultures, while treatment with trypsin destroyed the bacteriocins from all the nine cultures except the one produced by *L.fermentum* (Ocu-11). Similarly, treatment with chymotrypsin could destroy the bacteriocins from 8 cultures. The bacteriocins produced by *L.divergens* (Par-145) and *L.fermentum* (Ocu-11) survived the

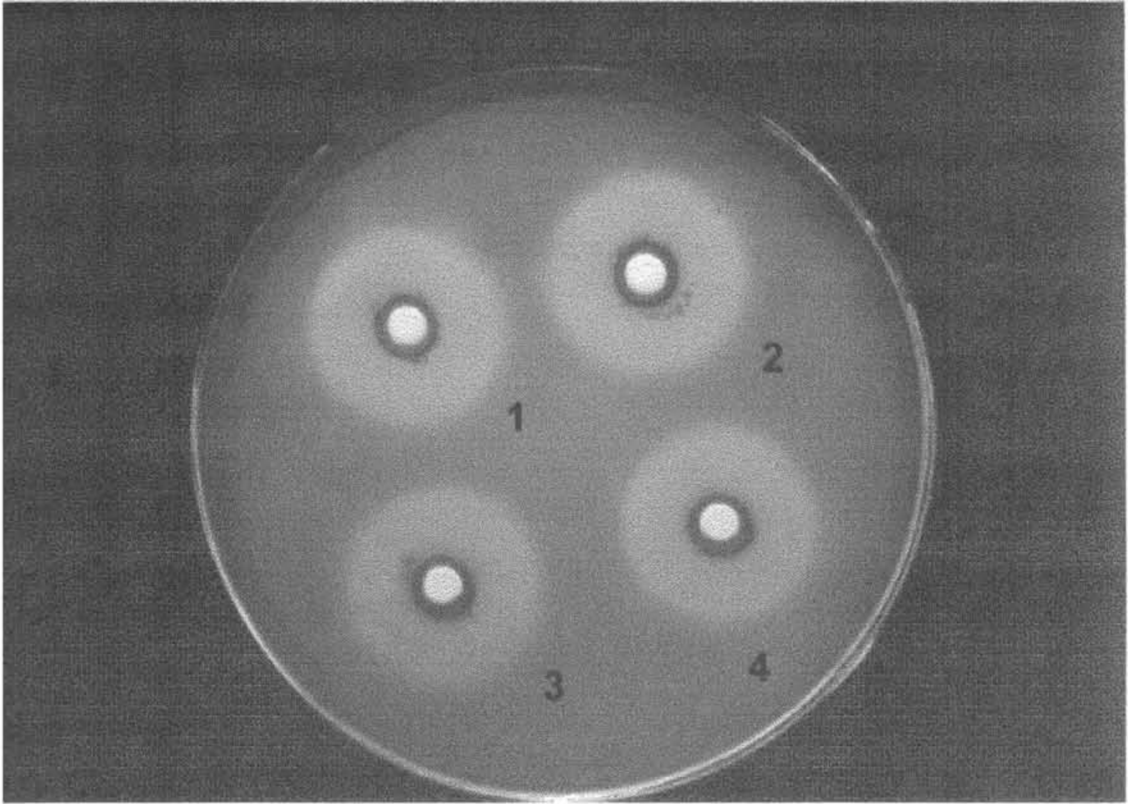


Fig.4.4 Inhibition of *Listeria monocytogenes* (ATCC 19111) by agar well diffusion assay. Wells contained *Lactobacillus divergens* (Par-145) culture bacteriocin concentrate (well 1), neutralized (well 2), treated with catalase (well 3) and heated for 30 min at 80 ^{0}C (well 4).

proteinase activity of chymotrypsin. Pepsin also had no action on the bacteriocins produced by *L.divergens* (Par-145) and *L.plantarum* (Lla-510 and Esu-120). The fact that all the bacteriocins were deactivated by the general proteolytic enzyme, protease proved that the bacteriocins were proteins. The trypsin, chymotrypsin and pepsin are not general proteolytic enzymes, but acts only if there are certain specific groups in the protein substrate. Hence, the resistance of some of the bacteriocins against deactivation by these proteolytic enzymes only indicated that some bacteriocin did not have the enzyme specific groups. Hence, the antibacterial activity of *Lactobacillus* cultures has been proved to be due to

1. Organic acids produced
2. Bacteriocins produced

4.13.3.4 Antibacterial activity of bacteriocins against other *Lactobacillus* cultures

The bacteriocins produced by LAB will be effective against other *Lactobacillus* cultures, which are closely related to them. Further, this is a confirmation of the bacteriocins produced by LAB. The antibacterial activities of the 10 chosen *Lactobacillus* cultures are presented in Table 4.29. It can be seen from the Table that bacteriocins from most of the cultures were inhibitory to the other six closely related *Lactobacillus* with a few exceptions. Bacteriocins from two *L.plantarum* cultures and one *L.animalis* culture showed antagonistic activity against all the test *Lactobacillus* cultures. These observations confirmed the nature of bacteriocins produced by the 10 *Lactobacillus* cultures included in the study.

Halami et al (1999) had reported the isolation of LAB from fish intestine, which were identified and characterized with reference to the production of bacteriocins. One isolate of *L.casei ssp casei* (C-40) was found to be a potent bacteriocin producer active against other LAB cultures.

Table 4.29 Antibacterial activity of bacteriocin concentrate against other *Lactobacillus* cultures

No.	*Lactobacillus* strains	*L.plantarum* (AV 2141)	*L.brevis* (Ocu 611)	*L.fermentum* (Ocu-75)	*L.lactis* (Cma-7)	*L.casei* (NCIM 2165)	*L.acidophilus* (Rka-312)
1.	*L.divergens* (Par-145)	NZD	NZD	10mm	10mm	10mm	10mm
2.	*L.divergens* (Pm-234)	NZD	NZD	12mm	10mm	12mm	NZD
3.	*L.gasseri* (Par-276)	16mm	NZD	NZD	NZD	NZD	NZD
4.	*L.gasseri* (Lla-22)	NZD	NZD	12mm	12mm	10mm	10mm
5.	*L.plantarum* (Esu-120)	14mm	16mm	10mm	10mm	10mm	10mm
6.	*L.plantarum* (Lla-510)	12mm	12mm	10mm	10mm	10mm	12mm
7.	*L.reuteri* (Eaf-611)	12mm	14mm	10mm	NZD	NZD	NZD
8.	*L.animalis* (Lla-476)	12mm	12mm	10mm	10mm	10mm	10mm
9.	*L.brevis* (Ocu-933)	12mm	NZD	NZD	NZD	10mm	10mm
10.	*L.fermentum* (Ocu-11)	12mm	NZD	NZD	NZD	10mm	10mm

NZD – No Zone Detected

Toba *et al.* (1991) isolated *L. gasseri* from infant faeces, which inhibited several other *Lactobacillus* cultures by agar well diffusion method. The bacteriocins from *L. gasseri* inhibited *L. acidophilus, L. casei* and *L. brevis* cultures. *L. gasseri* cultures did not inhibit any of the test *L. plantarum* cultures. These results agree with our findings, that the *L. gasseri* cultures were inhibitory to *L. acidophilus* and *L. casei.* However,the *L. gasseri* isolated in this study also inhibited *L. plantarum* cultures.

Plantaricin F from *L.plantarum* inhibited all genera of LAB tested, *L.acidophilus, L.bulgaricus, L.casei, L.fermentum, L.helveticus, L.plantarum, Pediococcus acidilactici, P.pentosaceous, Streptococcus faecalis, S.pyogenes, Lactococcus lactis* and *Leuconostoc dextranicum*. Certain strains of *L.lactis* and *P.pentosaceous* were not inhibited (Fricourt et al. 1994).

4.13.3.5 Antibacterial activity of lactic acid and acetic acid

In order to ascertain the inhibitory effect of lactic acid and acetic acid on pathogenic/indicator bacteria, antibacterial activity of these acids at 0.75% and 1.5% were determined by agar diffusion method. Results are presented in Table 4.30. At 0.75% and 1.5% levels, both lactic and acetic acid adjusted to a pH of 3.5 were effective against *B.cereus* , *L.monocytogenes, S.aureus, Vibrio vulnificus, V.cholerae* and *E.coli.* The antibacterial activity was more apparent against Gram positive bacteria like *B.cereus* and *L.monocytogenes*, as was observed in the case of antibacterial activity by *Lactobacillus* cultures.

In the case of gram negative group, namely *Salmonella typhimurium, Vibrio vulnificus, V.cholerae* and *E.coli*, both lactic acid and acetic acid exhibited their antibacterial activity with varying degrees. While *S.typhimurium* and *V.vulnificus* were significantly inhibited, *V.cholera* and *E.coli* were inhibited to a comparatively smaller level by lactic acid. Acetic acid showed appreciable inhibition on *V.cholerae*, but *E.coli* was not significantly affected.

Table 4.30. Antibacterial activity of Lactic acid (pH 3.5) and Acetic acid (pH 3.5) measured as zone of inhibition in mm

No.	Pathogenic/ Indicator bacteria	Lactic acid		Acetic acid	
		0.75%	1.5%	0.75%	1.5%
1.	*Bacillus cereus* (B3/3)	28	28	28	28
2.	*Listeria monocytogenes* (ATCC 19111)	22	24	26	28
3.	*Staphylococcus aureus* (SA3B)	14	16	16	16
4.	*Salmonella typhimurium* (ATCC 14028)	20	22	24	26
5.	*Vibrio vulnificus* (ATCC 2046)	16	20	24	24
6.	*Vibrio cholerae* (Vc-7)	12	18	20	22
7.	*Escherichia coli* (Ec101)	12	16	14	16

S.aureus was an exception in the case of the Gram positive group. Both acetic and lactic acid showed only marginal antibacterial activity against *S.aureus*.

Both lactic and acetic acids are produced by LAB from carbohydrates and also there was a corresponding decrease in pH of media (Schillinger and Lucke, 1989). As mentioned earlier, the decrease in pH was one of the factors that accounted for antibacterial activity of LAB. The antibacterial activity of the *Lactobacillus* cultures against pathogenic/indicator bacteria could in part be due to the organic acids produced by them (Tables.4.27A and 4.27B). Hechard et al. (1990) found that *Lactobacillus* cultures isolated from goat milk inhibited the growth of *L.monocytogenes* and *S.aureus* through the production of organic acid. Similar observations on antibacterial activity of *Lactobacillus* cultures had been made by Jin et al. (1996), Ostergaard et al. (1998) and Conner et al. (1990).

4.13.3.6 Antibacterial activity due to Hydrogen peroxide

None of the 30 *Lactobacillus* cultures included in the study on antibacterial activity produced H_2O_2 when tested with catalase. Hence, the antibacterial activity due to the production of H_2O_2 had no relevance in this study.

4.13.3.7 Antibacterial activity due to bacteriocins

Twenty *Lactobacillus* cultures listed in Table 4.31 were used for studies on antibacterial activity and production of bacteriocins.

The *Lactobacillus* cultures were grown in 200ml each MRS broth for 3 days and centrifuged at 10,000 rpm for 10 min at 4°C to get cell free supernatant containing the bacteriocins. The supernatants were neutralized with alkali and treated with catalase in order to eliminate any antibacterial activity due to organic acids and H_2O_2. Hence,

Table 4.31. List of LAB cultures from fish/prawn selected for studies on antibacterial activity

No.	*Lactobacillus* cultures	Strain
1.	*L.divergens*	Par-145
2.	*L.divergens*	Lla-160
3.	*L.divergens*	Pm-112
4.	*L.divergens*	Pm-234
5.	*L.divergens*	Pm-300
6.	*L.gasseri*	Par-276
7.	*L.gasseri*	Lla-22
8.	*L.brevis*	Par-356
9.	*L.brevis*	Eaf-970
10.	*L.brevis*	Ocu-177
11.	*L.brevis*	Ocu-46
12.	*L.brevis*	Ocu-933
13.	*L.viridescens*	Par-620
14.	*L.casei.ssp.rhamnosus*	Par-78
15.	*L.plantarum*	Esu-120
16.	*L.plantarum*	Lla-510
17.	*L.alimentarius*	Mce-43
18.	*L.animalis*	Lla-476
19.	*L.farciminis*	Ocu-28
20.	*L.fermentum*	Ocu-11

any antibacterial activity exhibited by the supernatant could be only due to the bacteriocins produced by *Lactobacillus*. The bacteriocins in the supernatant were precipitated by ammonium sulphate and redissolved in small quantities of glycine-NaOH buffer (pH 7.5) as described in 3.2.16 (Materials and methods). Only these purified bacteriocins were used for studies on antibacterial activity against selected pathogenic/indicator bacteria.

4.13.4 Antibacterial activity of bacteriocin concentrates from *Lactobacillus* cultures

4.15.4.1 Antibacterial activity of bacteriocin concentrates against pathogenic / indicator bacteria

The antibacterial activity of bacteriocin concentrates from the selected *Lactobacillus* spp. against pathogenic/indicator bacteria is presented in Figures 4.5 and 4.6.

4.13.4.1.1 Action of bacteriocins against *Listeria monocytogenes*

L.monocytogenes was highly inhibited by the bacteriocins from 18 *Lactobacillus* cultures out of the 20 tested. Bacteriocins from *L.plantarum, L.divergens, L.gasseri, L.brevis, L.casei ssp rhamnosus, L.fermentum* and *L.reuteri* exhibited antibacterial activity against *L.monocytogenes*. Only bacteriocins from *L.viridescens* and *L.farciminis* could not inhibit *L.monocytogenes*.

4.13.4.1.2 Action of bacteriocins against *Bacillus cereus*

B.cereus was inhibited by 18 out of 20 bacteriocins. Bacteriocins from *L.viridescens* and *L.farciminis* could not exhibit antibacterial activity against *B.cereus*. *B.cereus* behaved very similar to *L.monocytogenes* with respect to the antibacterial activity of the bacteriocins from the above 20 *Lactobacillus* cultures.

Fig.4.5. Antibacterial activity of bacteriocin concentrate against pathogenic/indicator bacteria

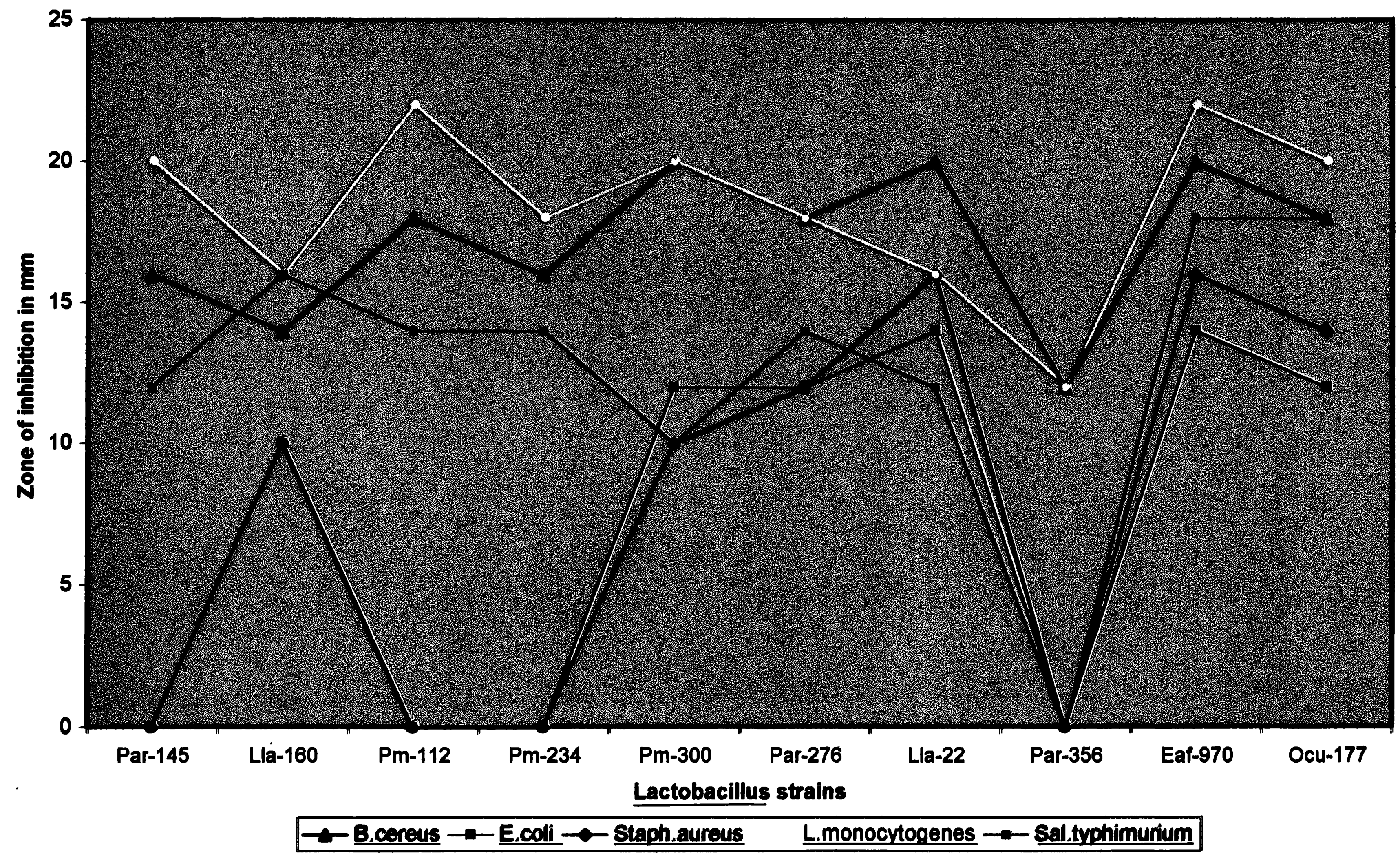
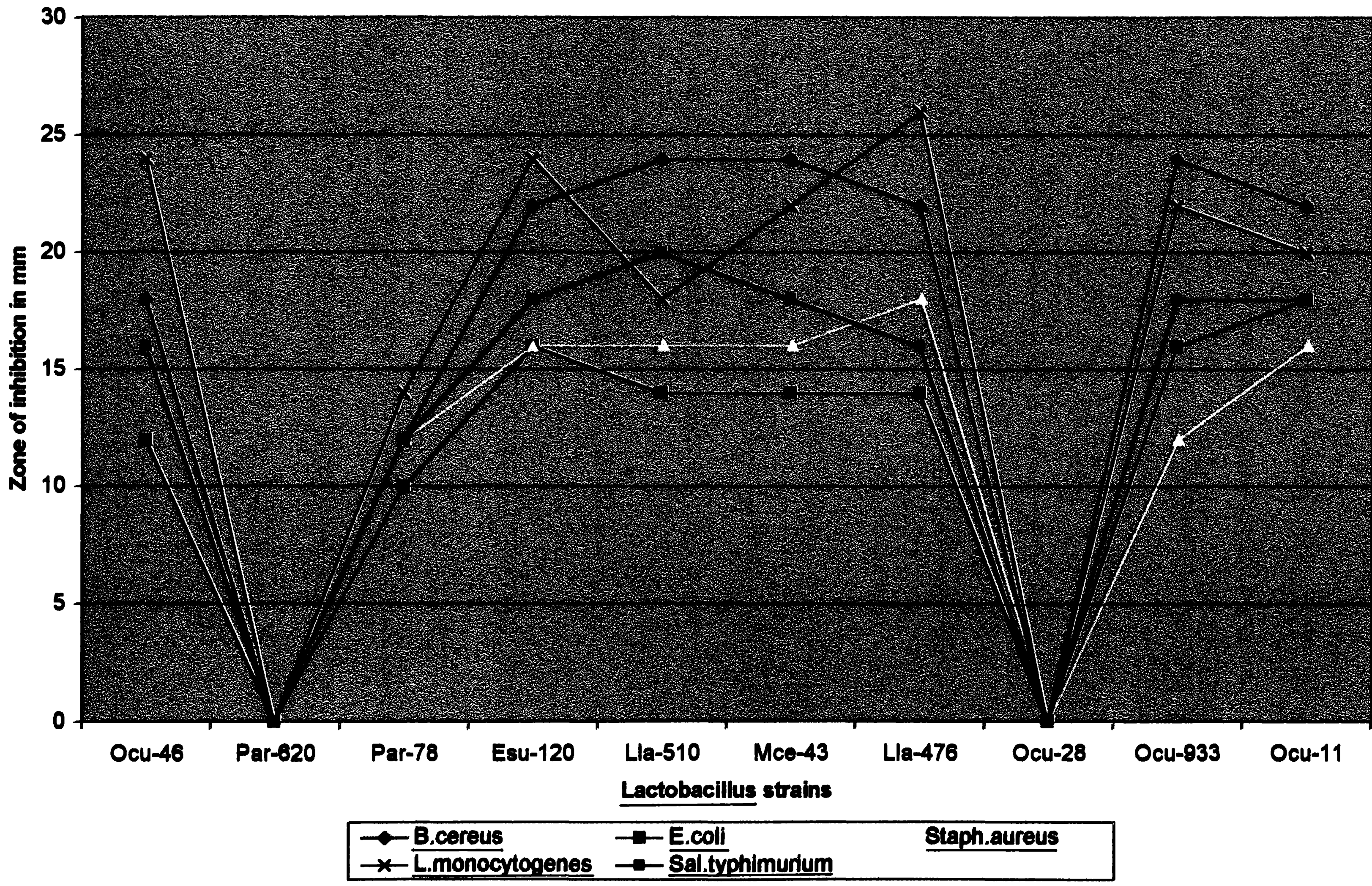


Fig.4.6. Antibacterial activity of bacteriocin concentrate against pathogenic/indicator bacteria



4.13.4.1.3 Action of bacteriocins against *Staphylococcus aureus*

Thirteen out of 20 bacteriocins from *Lactobacillus* tested exhibited antibacterial activity against *S.aureus*. Bacteriocins from 4 out of 5 *L.brevis*, 1 *L.viridescens* and 1 *L.farciminis* did not show any antibacterial activity against *S.aureus*, while the bacteriocins from *L.plantarum*, 4 out of 5 *L.brevis, L.gasseri, L.casei ssp rhamnosus, L.animalis* and *L.fermentum* showed very good antibacterial activity against *S.aureus*.

4.13.4.1.4 Action of bacteriocins against *Salmonella typhimurium*

Out of the bacteriocins from 20 *Lactobacillus* cultures, 17 showed antibacterial activity against *S.typhimurium*. Bacteriocins from *L.plantarum*, *L.brevis, L.gasseri, L.casei ssp rhamnosus, L.animalis* and *L.fermentum* were inhibitory to *S.typhimurium*, while 3 out of 5 *L.divergens*, *L.viridescens* and *L.farciminis* could not exhibit antibacterial activity against this strain.

4.13.4.1.5 Action of bacteriocin against *Escherichia coli*

The behavior of *E.coli* to the action of bacteriocins from *Lactobacillus* was more or less similar to that of *S.typhimurium*. To some extent, *E.coli* were insensitive to the antibacterial activity of more number of bacteriocins than *S.typhimurium*. Out of the 20 bacteriocin concentrates tested, *E.coli* was inhibited only by 14 bacteriocins. These inhibitory bacteriocins were from *L.plantarum*, *L.gasseri,*3 out of 4 *L.brevis, L.casei ssp rhamnosus, L.animalis,L.fermentum* and 2 out of 5 *L.divergens*.

4.13.4.1.6 Action of bacteriocin against *Vibrio cholerae* and *V.vulnificus*

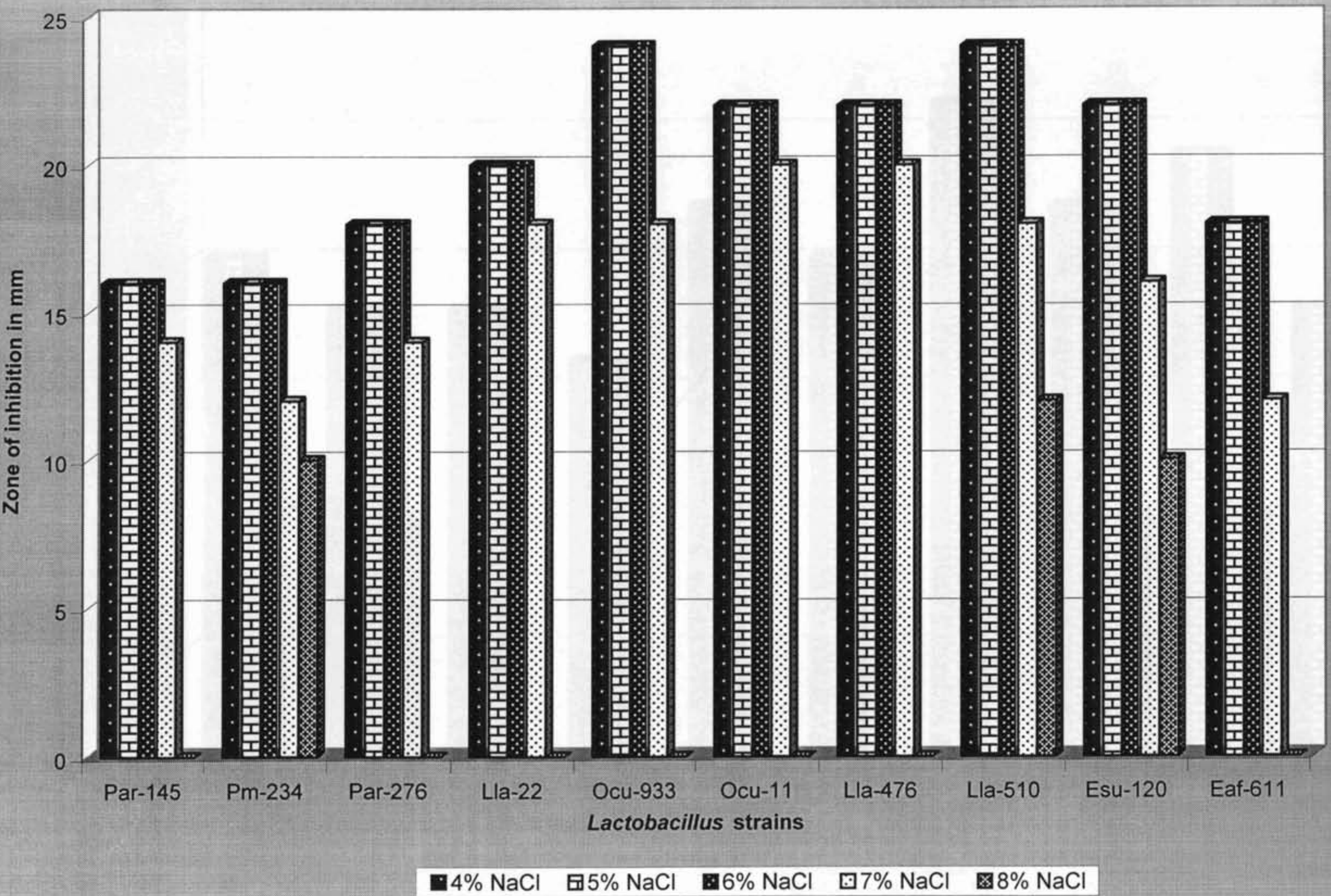
None of the bacteriocins produced by the 20 *Lactobacillus* cultures showed any antibacterial activity against *V.vulnificus* and *V.cholerae*. However, it was observed that the organic acids produced by the *Lactobacillus* cultures inhibited the vibrios effectively.

4.13.5 Effect of salt, pH, incubation temperature and glucose concentration on the antibacterial activity of *Lactobacillus* cultures.

4.13.5.1 Effect of salt

Effect of salt on the antibacterial activity of 10 *Lactobacillus* strains against *B.cereus* and *L.monocytogenes* are presented in Figures 4.7 and 4.8. Sodium chloride at 4%, 5%, 6%, 7% and 8% were added in the MRS broth used for growing the *Lactobacillus* cultures. From the cell free supernatant, bacteriocins were precipitated by ammonium sulphate and redissolved in buffer as described in Materials and methods (3.2.16). Antibacterial activity against *B.cereus* and *L.monocytogenes* were determined by agar well diffusion method and the zone of inhibition obtained at each salt level. A comparison of histograms show that the NaCl at 4%, 5% and 6% did not materially affect the antibacterial activity of the *Lactobacillus* cultures against *B.cereus* and *L.monocytogenes*, but NaCl at 7% resulted in decrease in the antibacterial activity. At 8% NaCl in the medium, the bacteriocin production was limited to only 3 cultures, *L.divergens* (Pm-234) and two strains of *L.plantarum* (Lla-510 and Esu-120). Other cultures did not exhibit any antibacterial activity at all at 8% NaCl in the medium. It has to be noted that at salt concentrations above 6% ,only limited growth was noticed for most of the *Lactobacillus* strains. At 8% salt in the media, only the three *Lactobacillus* cultures that showed growth at 8% salt produced bacteriocins. Hence, it is concluded

Fig. 4.7. Effect of salt on antibacterial activity of *Lactobacillus* strains against *B.cereus*



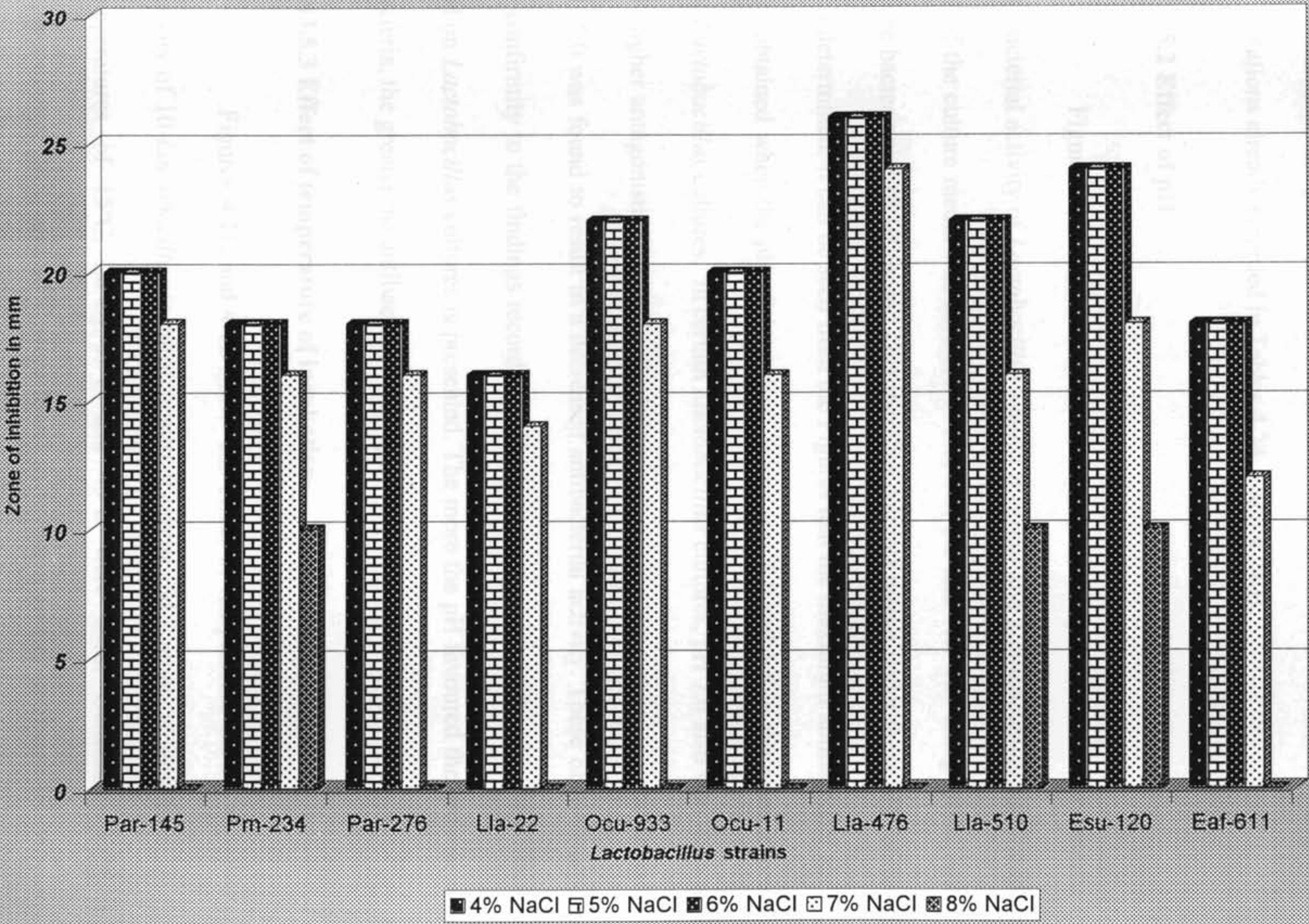


Fig.4.8. Effect of salt on antibacterial activity of LAB cultures against *L.monocytogenes*

that the antibacterial activity, namely bacteriocin production is directly related to the growth of *Lactobacillus* cultures in the medium. Salt concentrations above 6% were detrimental to the growth of *Lactobacillus*. This observation is in conformity to the observations already recorded in Tables 4.24.A and 4.24.B.

4.13.5.2 Effect of pH

Figures 4.9 and 4.10 show the effect of the pH of the culture media on antibacterial activity of *Lactobacillus* against *B.cereus* and *L.monocytogenes*. The initial pH of the culture media was adjusted to 5.0, 6.0, 6.5 and 7.0. The antagonistic activity of the bacteriocins of 10 chosen *Lactobacillus* cultures grown under these pH conditions was determined. It can be seen from the Figures that the maximum antibacterial activity was obtained when the pH of the growth media was 6.0 and 6.5 in the case of most of the *Lactobacillus* cultures. In certain *Lactobacillus* cultures, pH 7.0 also was favourable for higher antagonistic activity by the bacteriocin against the test organisms. Generally, pH 5.0 was found to result in a decreased antibacterial activity. These observations are in confirmity to the findings recorded in Tables 4.26.A and 4.26.B, where the effect of pH on *Lactobacillus* cultures is presented. The more the pH favoured the growth of the bacteria, the greater the antibacterial activity.

4.13.5.3 Effect of temperature of incubation

Figures 4.11. and 4.12 show the effect of temperature on the antibacterial activity of 10 *Lactobacillus* cultures against *B.cereus* and *L.monocytogenes*. Incubation temperatures of 15°C, 28°C, 37°C and 45°C were used. Generally, incubation temperature of 28°C and 37°C were found to favour a greater antibacterial activity. In the case of *Lactobacillus* culture, *L.gasseri* (Par-276), antibacterial activity was exhibited at all the four incubation temperatures. However, incubation at 15°C produced

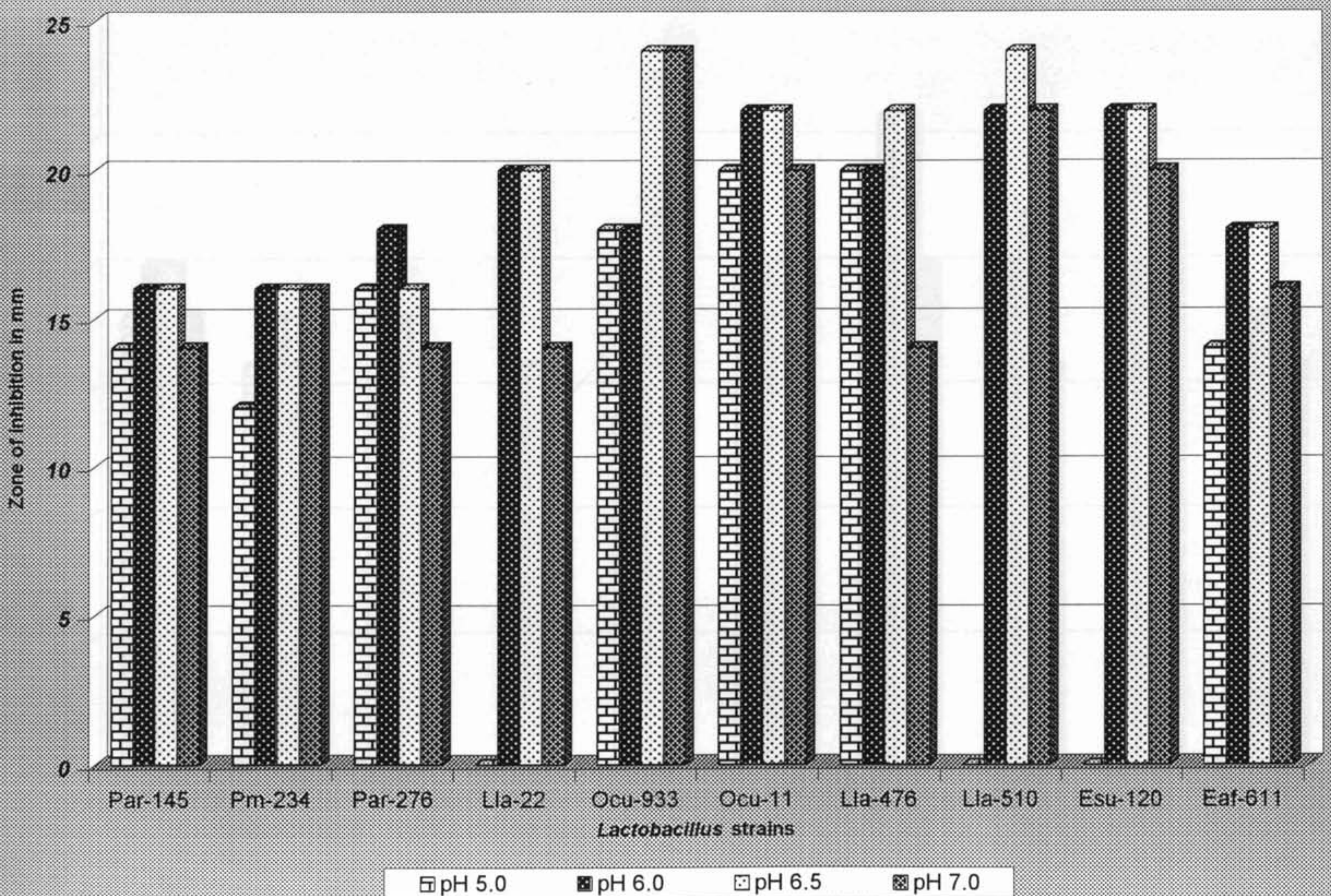
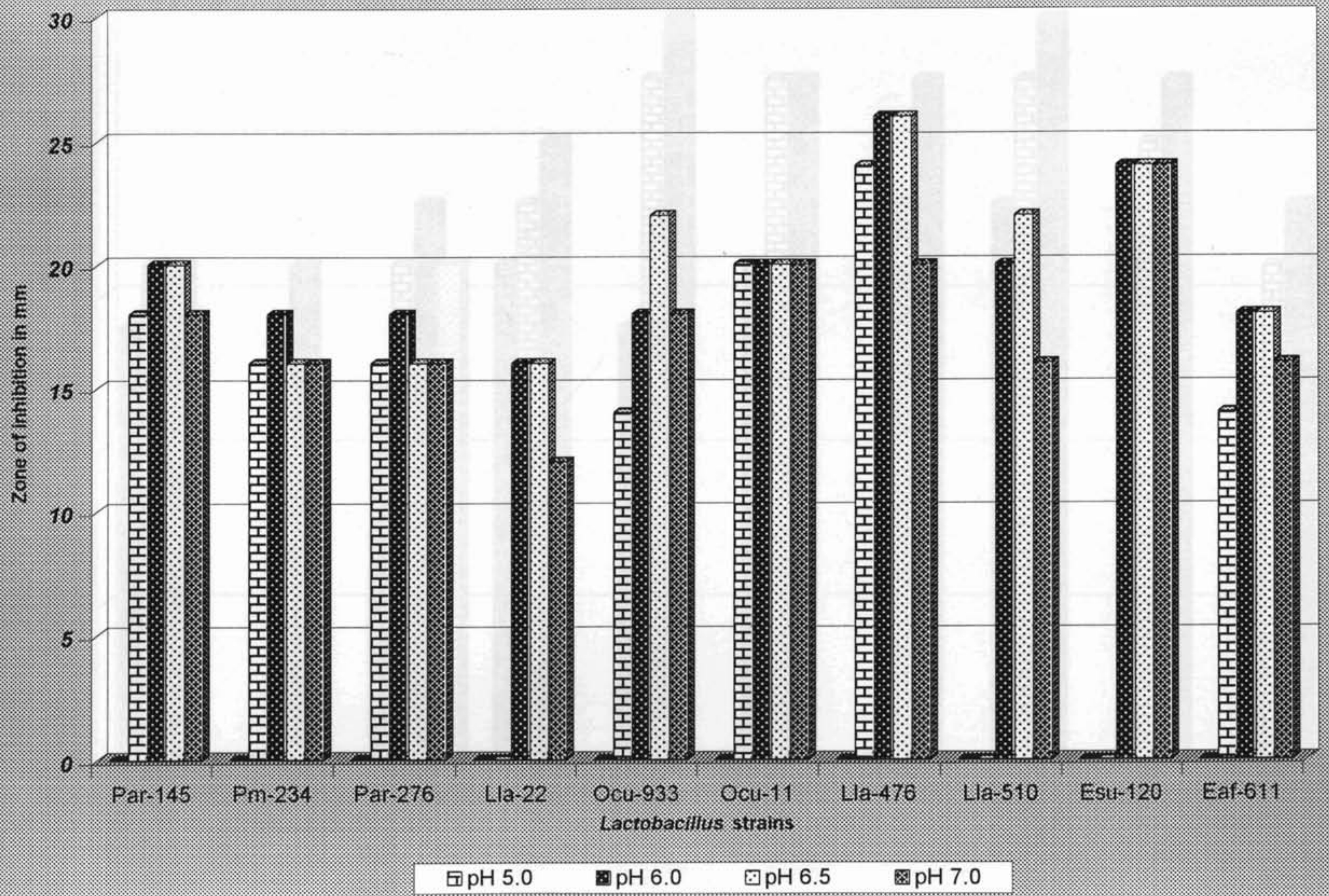


Fig.4.9. Effect of pH of media on antibacterial activity of *Lactobacillus* cultures against *B.cereus*


L.monocytogenes
30
25
20
15
10
5
0
Zone of inhibition in mm
Par-145
Pm-234
Par-276
Lla-22
Ocu-933
Ocu-11
Lla-476
Lla-510
Esu-120
Eaf-611
Lactobacillus strains
pH 5.0
pH 6.0
pH 6.5
pH 7.0

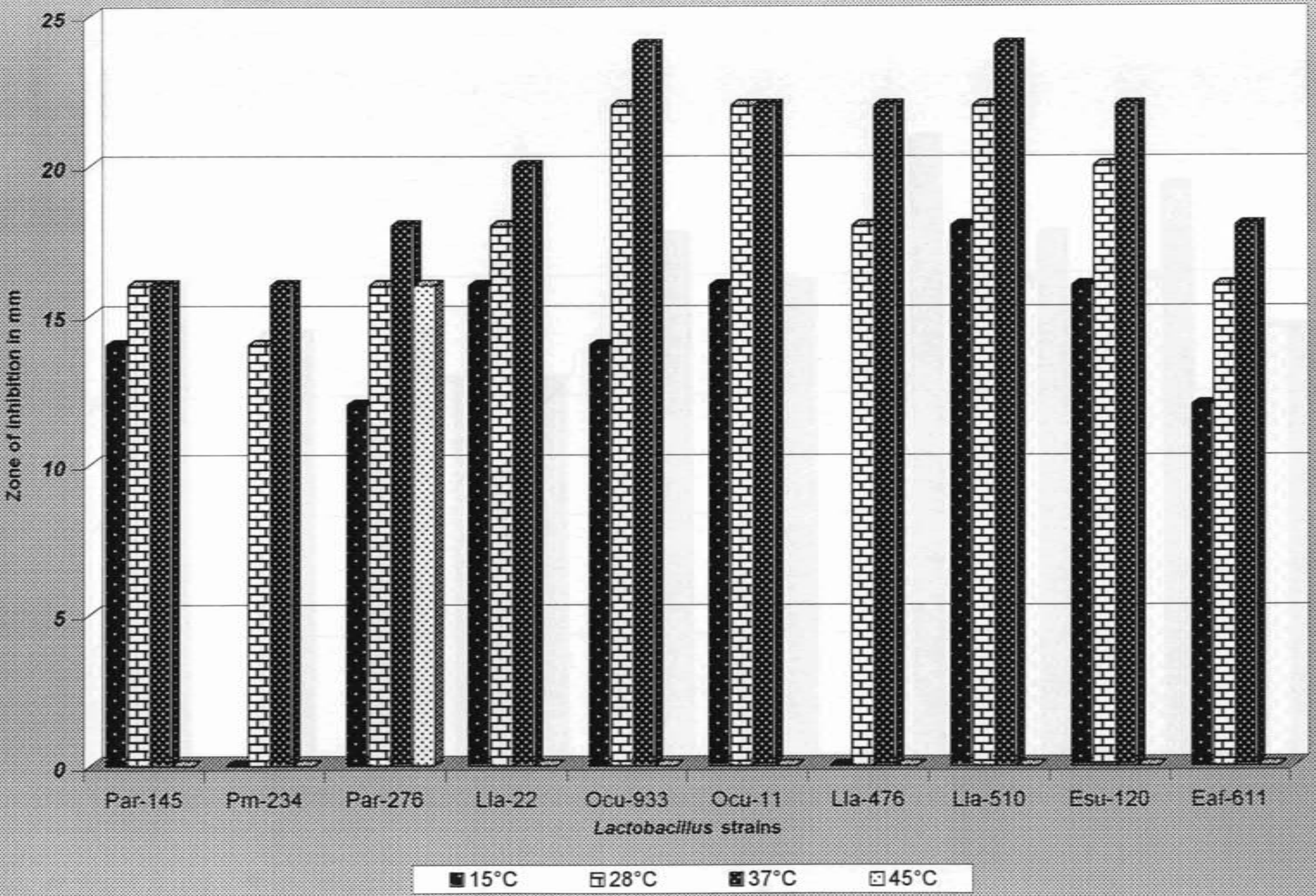


Fig.4.11. Effect of Temperature on the antibacterial activity of *Lactobacillus* cultures against *B.cereus*
25
20
15
10
5
0
Zone of inhibition in mm
Par-145
Pm-234
Par-276
Lla-22
Ocu-933
Ocu-11
Lla-476
Lla-510
Esu-120
Eaf-611
Lactobacillus strains
15°C
28°C
37°C
45°C

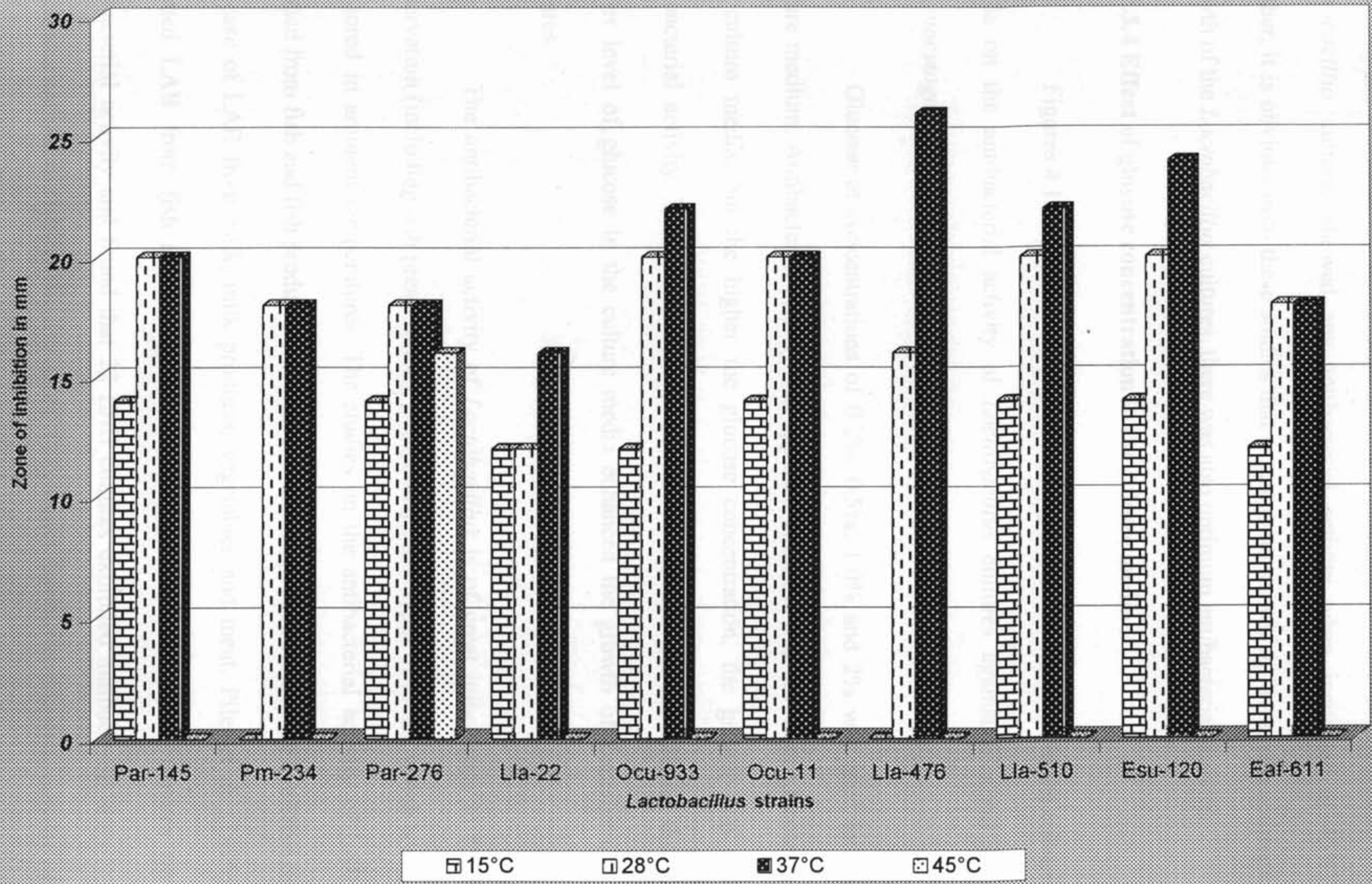


Fig.4.12. Effect of temperature on antibacterial activity of Lactobacillus cultures against L.monocytogenes
Zone of inhibition in mm
30
25
20
15
10
5
0
Par-145
Pm-234
Par-276
Lla-22
Ocu-933
Ocu-11
Lla-476
Lla-510
Esu-120
Eaf-611
Lactobacillus strains
15°C
28°C
37°C
45°C

a decreased antagonistic activity. Except for *L.gasseri* (Par-276), none of the *Lactobacillus* cultures showed any antibacterial activity when incubated at 45°C. Further, it is obvious from these studies that at temperatures which favoured maximum growth of the *Lactobacillus* cultures, there was also optimum antibacterial activity.

4.13.5.4 Effect of glucose concentration in the culture media

Figures 4.13 and 4.14 show the effect of glucose concentration in the culture media on the antibacterial activity of *Lactobacillus* cultures against *B.cereus* and *L.monocytogenes*.

Glucose at concentrations of 0.2%, 0.5%, 1.0% and 2% was used in the culture medium. Antibacterial activity was apparent at all the four levels of glucose in the culture media, but the higher the glucose concentration, the greater was the antibacterial activity. This observation is also in confirmity with the finding that a higher level of glucose in the culture media enhanced the growth of *Lactobacillus* cultures.

The antibacterial activity of *Lactobacillus* is of great importance in food preservation (including fish preservation), particularly those types of foods, which are to be stored in ambient temperatures. The studies on the antibacterial activity of LAB isolated from fish and fish products are limited, eventhough such studies are available in the case of LAB from milk, milk products, vegetables and meat. Pilet et al. (1995) isolated LAB from fish and fish products. They screened 338 LAB isolates for antibacterial activity and found that 22 LAB cultures exhibited antibacterial activity, which was not due to H_2O_2 or organic acids. They found that the antibacterial activity of the cell free supernatant of these cultures were lost after protease treatment and

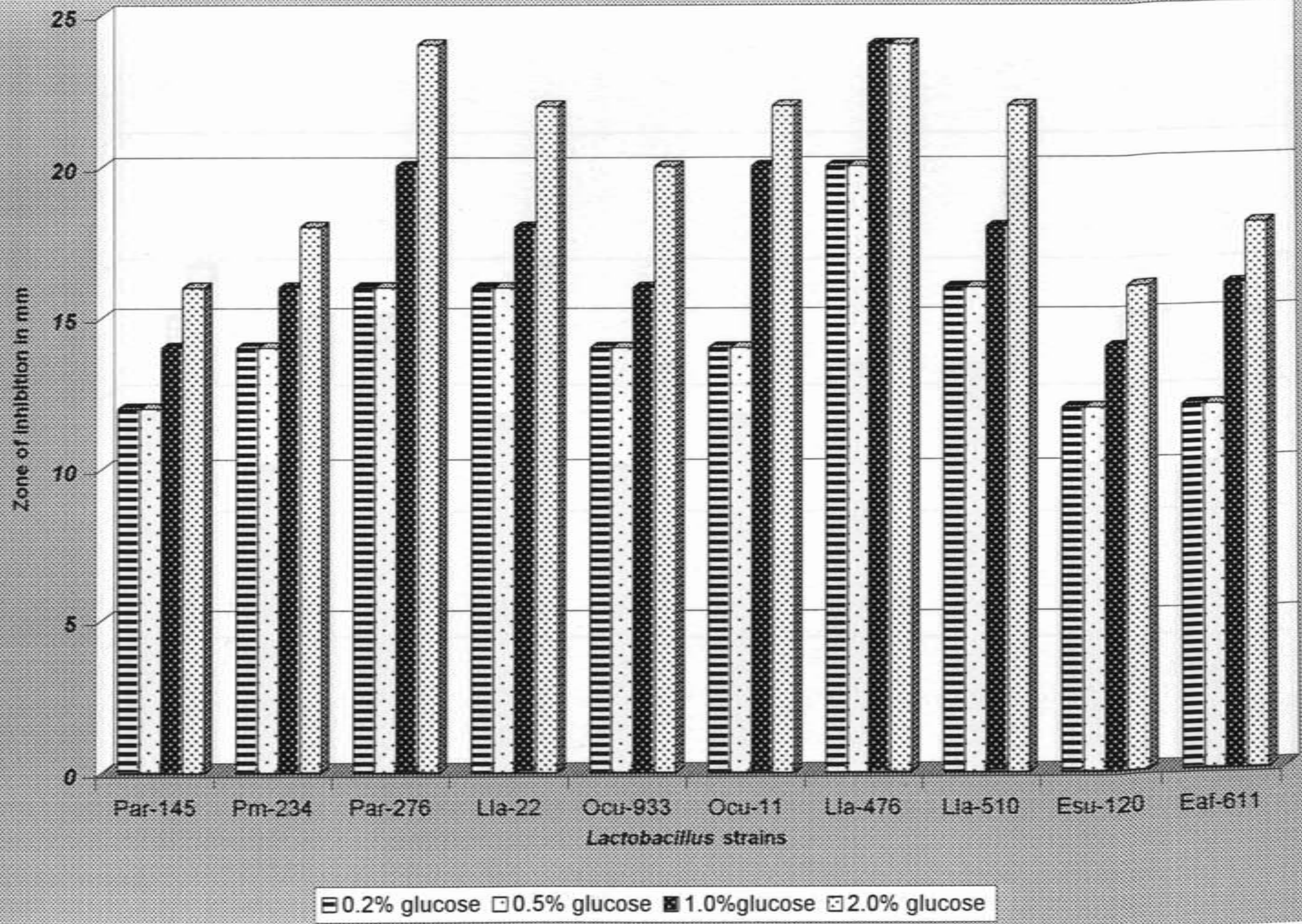


Fig.4.13. Effect of glucose concentration on antibacterial activity of *Lactobacillus* cultures against *B.cereus*

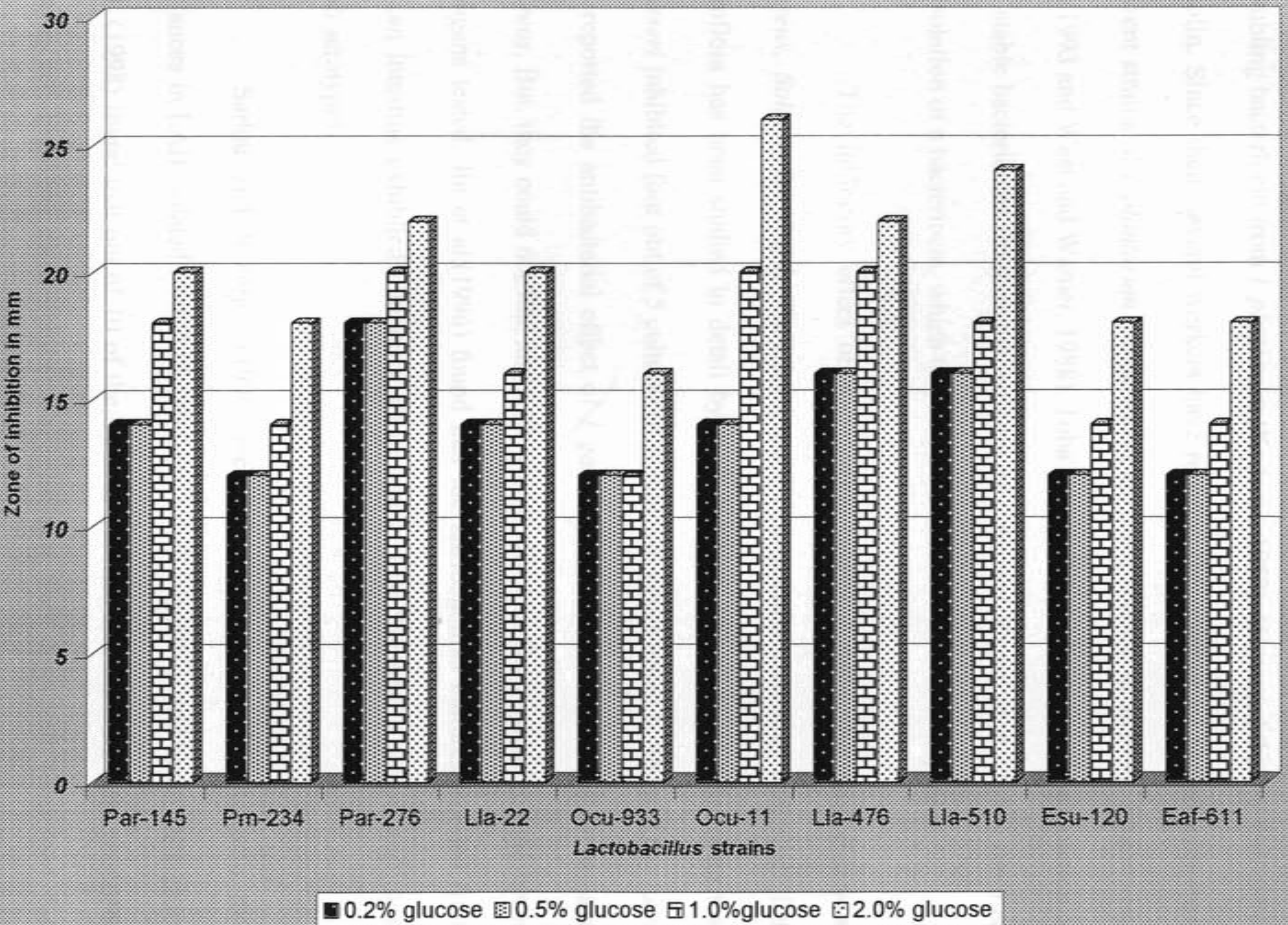


Fig.4.14. Effect of glucose concentration on the antibacterial activity of *Lactobacillus* cultures against *L.monocytogenes*

consequently were due to the bacteriocins produced and those belonged to the genera *Carnobacterium (L.divergens)*, *Enterococcus* and *Lactococcus*.

There were similar reports of isolation of antibacterial substances resembling bacteriocin from *L.plantarum* (Kodama, 1952). He named this bacteriocin as Lactolin. Since then, several workers have reported the isolation of bacteriocins from different strains of *L.plantarum* (Anderson et al. 1988; Daescel et al.1990; Jimnez-diaz et al.1993 and West and Warner, 1988). Toba et al. (1991) have reported the isolation of heat stable bacteriocins from several cultures of *L.gasseri*. Kawai et al. (1994) reported the isolation of a bacteriocin, which they named acidocin A from *L.acidophilus*.

The inhibitory effect of bacteriocins against *L.monocytogenes, B.cereus, S.aureus, Salmonella, E.coli, Pseudomonas, Vibrio parahaemolyticus* and other fish microflora has been studied in detail by many workers. Itoh et al. (1995) found that *L.gasseri* inhibited four out of 5 cultures of *L.monocytogenes* tested by them. They have also reported the antibacterial effect of *L.gasseri* and *L.reuteri against B.cereus* and *S.aureus*. But, they could not find any antibacterial effect on Gram-negative food-borne pathogens tested. Jin et al.(1996) found that the *Lactobacillus* cultures isolated from chicken intestine exhibited inhibitory effect against five *Salmonella* strains and three *E.coli* serotypes.

Sarkar and Banerjee (1996) recorded the presence of bacteriocin like substances in LAB isolated from milk products, rotten vegetables and meat. Ostergaard et al. (1998) found that out of 10 of the 44 LAB cultures examined by them produced bacteriocins. Eight of these cultures were isolated from high salt fermented fish products. From these reports, it is evident that many of the LAB cultures produced bacteriocins capable of inhibiting pathogenic bacteria. In the investigation reported in

this thesis, it is found that majority of the *Lactobacillus* cultures isolated from fish and shellfish were inhibitory to both Gram positive and Gram negative pathogenic/indicator bacteria. Among them, *L.monocytogenes* and *B.cereus* were predominantly susceptible to the inhibitory effect of *Lactobacillus*. It is found that the inhibitory effect is contributed partly by organic acid formed by fermentation of sugars and partly by the bacteriocins produced by them. In the case of most of the *Lactobacillus* cultures, when the contribution of organic acid in the antibacterial activities is excluded by neutralization, the inhibitory effect due to bacteriocin was found to be universal in all the species of *Lactobacillus* tested. Further studies on the effect of different parameters on the antibacterial activity of bacteriocins produced by *Lactobacillus* have conclusively proved that the amount of bacteriocin produced as measured in terms of antibacterial activity was directly related to the growth of the *Lactobacillus* in the culture media. Parameters like salt content, pH of the media, incubation temperature and the concentration of glucose in the growth medium influenced the bacteriocin production and conditions or levels favourable for maximum growth of *Lactobacillus* also favoured the production of maximum antimicrobial activity.

Chapter.5

SUMMARY AND CONCLUSION

5. Summary and Conclusion

The occurrence and distribution of lactic acid bacteria in fresh and frozen marine fish and shellfish, farmed fish and shellfish, cured and pickled fish and shellfish *have been investigated in detail. The Lactobacillus cultures from these fish and shellfish* samples were isolated and biochemical characteristics investigated. The antibacterial properties of *Lactobacillus* cultures on pathogenic and indicator bacteria have also been studied in detail.

- The total plate count (TPC) vis-a-vis LAB counts was determined in fresh and frozen fish and shellfish, ocean fresh fish, pickled and cured fish and shellfish. The TPC ranged between 10^4-10^7/g in different samples of fresh and frozen fish and shellfish, while the LAB count ranged between 10^2-10^5/g. In farmed fish and shellfish, the TPC ranged between 10^3-10^5/g and LAB count varied from 10^2 to 10^3/g.

- LAB was absent in ocean fresh fish, cured fish and shellfish and pickled fish and shellfish. LAB is not present in the marine ecosystem, also they cannot survive high salt content and low water activity in the environment. LAB is a part of the terrestrial environment and enters fresh and frozen fish during handling.

- The LAB isolated from these different samples were characterized by biochemical and cultural properties. The *Lactobacillus* cultures were identified to the species level mainly based on sugar fermentation capacity. The following sugars were studied, arabinose, cellobiose, glucose, galactose, fructose, maltose, mannitol,

mannose, melebiose, melezitose, esculin, lactose, ribose, salicin, sorbitol, sucrose, trehalose, raffinose, rhamnose and xylose.

The *Lactobacillus* species identified in fresh fish and shellfish are *Lactobacillus gasseri, L.plantarum, L.divergens, L.brevis, L.viridescens, L.casei subsp rhamnosus, L.farciminis, L.buchneri, L.alimentarius, L.fermentum, L.acidophilus, L.animalis and L.reuteri.* The *Lactobacillus* species identified in frozen fish and shellfish are *L.plantarum, L.brevis, L.casei subsp rhamnosus and L.alimentarius. L.plantarum* was the predominant species in fresh and frozen fish and shellfish.

Based on the ability to ferment glucose and produce gas, LAB are divided into homofermentative and heterofermentative. In the different fish and shellfish species analyzed, homofermentative LAB predominated while the heterofermenters formed only a minority. The ability of LAB to ferment different sugars, alcohols and glucosides were studied in detail. It was found that fructose, glucose and galactose was fermented by all the cultures studied. However, the more complex sugars like raffinose, melezitose were fermented by only 50% of the cultures. Mannitol was fermented by 83% of the cultures, sorbitol by 40%, Esculin by 83%and salicin by 77% of the cultures. Irrespective of the complexity of the sugars, the ability of lactobacilli to ferment them varied from strain to strain.

The capacity of selected *Lactobacillus* cultures to produce lactic acid from glucose after 24 h was studied. The cultures were grown to an optical density of 1.5 and the amount of lactic acid produced in the culture broth was measured as grams of lactic acid in 100ml of the culture. The cultures produced titrable acidity in the range of

0.45-0.9g lactic acid/100ml culture. It was observed that the amount of acid produced was different in strains of the same species.

The effect of different parameters like pH, temperature, salt concentration and glucose concentration on LAB growth was studied using selected cultures. The optimum pH of the culture media for growth of Lactobacillus cultures was between 6.0-6.5. It was observed that *Lactobacillus* cultures could tolerate upto 7% salt in the culture media. Some strains of *L.plantarum* (Esu-120), *L.acidophilus* (Rka-312) and *L.fermentum* (Ocu-75) could grow even at a salt concentration of 8% in the culture media. The optimum temperature for growth of lactobacilli was found to be between 28- 37°C. The *Lactobacillus* cultures studied could utilize 0.2-2.0% glucose in the culture media rapidly in 24-48 h and decrease the pH to 3.8-3.4. *L.plantarum* culture (Esu-120) was most efficient in utilizing glucose and decreasing the pH to 3.44 in 4d.

Lactobacillus cultures were screened for their antibacterial activity using agar spot method. The inhibition was measured as diameter of zone of inhibition in mm. The pathogenic bacteria used as test organisms for the study were *Listeria monocytogenes (ATCC 19111), Salmonella typhimurium (ATCC 14028), Bacillus cereus (B3/3), Escherichia coli (Ec 101), Staphylococcus aureus (SA3B), Vibrio cholerae (Vc-7) and V.vulnificus (ATCC 2046).* The *Lactobacillus* cultures showed highest antibacterial activity against *L.monocytogenes* and *B.cereus*.

Selected *Lactobacillus* cultures, which showed consistently high antibacterial activity, were grown in large scale. The cell free supernatant was neutralized to a pH of 6.5 (using 1N NaOH), treated with catalase (5mg/ml) and the bacteriocins were

precipitated by ammonium sulphate. The bacteriocin was redissolved in glycine-NaOH buffer and the antibacterial activity was again assayed. *L.plantarum* (Lla-510) showed maximum antagonistic activity against *B.cereus* and *S.typhimurium*. *L.animalis* (Lla-476) showed maximum antibacterial activity against *L.monocytogenes* and *S.aureus*. *L.fermentum* (Ocu-11) showed maximum activity against *E.coli*. None of the bacteriocins from LAB were active against *V.cholerae* and *V.vulnificus*, however lactic acid was inhibitory to both pathogens. The bacteriocins from LAB cultures were active against other LAB cultures.

The proteinaceous nature of the bacteriocins produced by LAB cultures were analyzed by heat inactivation studies and protease treatment. The bacteriocins were exposed to a temperature of 80°C for 30min, 100°C for 10min and 121°C for 10min. The bacteriocins were inactivated above 100°C. Different proteinases tested were trypsin (1mg/ml), chymotrypsin (1mg/ml), pepsin (1mg/ml) and protease (1mg/ml). Except for bacteriocins from *L.divergens* (Par-145), *L.fermentum* (Ocu-11) and *L.plantarum* (Esu-120, Lla-510), all the other bacteriocins studied were inactivated by the protease. The characterization of the antibacterial factor confirms the presence of bacteriocins in LAB cultures from fresh and frozen fish and shellfish.

The effects of different parameters like pH of growth media, glucose concentration, salt concentration and temperature of incubation on antibacterial activity was studied. It was observed that the optimum conditions for growth was also the optimum conditions for antibacterial activity. Also, the antibacterial activity was partially due to organic acids produced and bacteriocin production.

The present study is the first quantitative and qualitative study on the occurrence and distribution of lactic acid bacteria in fresh and frozen fish and prawn. It is concluded that *Lactobacillus plantarum* was the predominant *Lactobacillus* species in fresh and frozen fish and shellfish. The ability of selected *Lactobacillus* cultures to grow at low temperatures, high salt content, produce bacteriocins, rapidly ferment sugars and decrease the pH make them potential candidates for biopreservation of fish and shellfish. Many *Lactobacillus* species present in fish and prawn also produce bacteriocins active against pathogenic and indicator bacteria present in fish and shellfish.

Research papers published by the Author

1. Seema Nair. P., P.K. Surendran and K. Gopakumar. (1997). "Occurrence and Distribution of Lactic acid bacteria in Fish and fishery environments". **Fishery Technology**. 34, 34-39.

Abstracts presented in scientific symposia and seminars

2. Leema Jose. P., P.Seema Nair and M.R.Raghunath.(1998). "Changes in the Tissue Proteinase activity upon curingin Brine and salt". Paper presented at the 26th APFIC session of the FAO Rome, held at Beijing, China, 24-26 September 1998.

3. Seema Nair.P. and P.K. Surendran. (1999). "Lactic acid bacteria from Frozen fish and their antibacterial activity against *Listeria monocytogenes* and *Salmonella typhimurium*". Paper presented at the National Symposium on Sustainable Development of Fisheries Toward 2020 AD. 21-23April 1999 held at Cochin.

Chapter.6

REFERENCES

6. REFERENCES

Abraham,T.J. and T.M.R.Setty.(1994). Effect of sodium benzoate on the fermentative activity of *L.plantarum* in fermented prawn pickle.*Fish.Technol.*31,48-51.

Abrahamson, A.E.* (1960). *J. Milk. Fd. Technol.* 23, 72.Quoted from Thampuran,N. (1987). Ph.D. Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology, Cochin-16.

Adams,M.R., R.D., Cooke. and D.R. Twiddy. (1987). Fermentation parameters involved in the production of lactic acid preserved fish-glucose substrates. *Int.J.Food.Sci.Technol.*22,105-114.

Aguirre, M. and M.D, Collins. (1993). Lactic acid bacteria and human clinical infection. *J. Appl. Bacteriol.* 75, 95-107.

Alford, J.A., L.Tobin. and C.S.McCleskey. *(1942). Bacterial spoilage of iced fresh crab meat. Food Research, 7, 353.

Anand.C.P. and T.M.R.Setty.* (1977).*Fish Technol.*3.:26. Quoted from Surendran, P.K. (1980). Chemical preservatives in relation to control of microbial changes in fishery products. Ph.D. thesis, University of Kerala, Trivandrum. India.

Anderson,A.G.(1907). On the decomposition of fish. *Ann.Rept.Fish.Bd.(scot).Part III. Scient. Invest.*, 26,13-39.

* Not referred in original

8. Anderson,R., M.A, Daeschel. and H.M, Hassan. (1988). Antibacterial activity of Plantaricin SIK-83, a bacteriocin produced by *L.plantarum.BioChimie*,70, 381-390.

9. Anon (1986). Food borne disease surveillance in England and Wales, 1984. *Br. Med. J.* 293, 1424-7.

10. AOAC (1975). Official Methods of Analysis (Horwitz,W.,Ed.) 12th edn.Association of Official Analytical Chemists, Washington, 1094.

11. Austin,B. and D.A.Austin.(1993). Disease in farmed and wild fish, In bacterial fish pathogens. (L.M.Laird,ed.) 2nd edition, Chichester,Ellis Norwood. 346.

12. Bains,N., W,Hodgkiss. and J.M,Shewan.(1958). The bacteriology of salt used in fish curing.In:The Microbiology of fish and meat curing brines.Ed.B.P. Eddy,HMSO,London.1-11.

13. Barker,W.H., R.E,Weaver., G.K,Morris. and W.T,Martin.(1974). Epidemiology of *Vibrio parahaemolyticus* infections in humans. In:Microbiology Ed, D.Schlessinger.USA.American Society for Microbiology, Washington.D.C. 257-262.

14. Baya,A.M., A.E.Toranzo., B.Lupiani., T.Li., B.S.Roberson. and F.M.Hetrick. (1991). Biochemical and serological characterisation of *Carnobacterium* spp. isolated from farmed and natural population of striped bass and catfish. *Appl.Environ.Microbiol.*57, 3114-3120.

15. Behanan,L., S.Mathew., D.Sudharma, M.K.Mukundan. and V.Malika. (1992). Effect of fruit juices with acetic acid on the quality and storage stability of pickled fish. *Fish Technol.*29,40-44.

16. Bernsmann, P., C.A.Alpert., P.Muss., J.Deutscher. and W.Hengstenberg. (1982) The bacterial PEP-dependant phosphotransferase system mechanism of gluconate phosphorylaation in *Streptococcus faecalis*. *FEMS Lett.*138,101-103.

17. Blood, R.M.(1975). Lactic Acid Bacteria in marinated herring. pp.195-208. In J.G.Carr, C.V.Cutting and G.C.Whiting (eds) Lactic Acid Bacteria in Beverages and Food. Academic Press. London.

18. Boerlin, P., F.Boerlin-Petzold., E.Bennerman., J.Bille., T.Jemmi. (1997). Typing *Listeria monocytogenes* isolates from fish products and human Listeriosis cases. *Appl. Environ. Microbiol.* 63, 1338-1343.

19. Bose.A.N. *(1969). In freezing and Irradiation of fish Rudolf Kreuzer (ed.) p.179.Fishing News (Books), Limited London. Quoted from Surendran, P.K. (1980). Chemical preservatives in relation to control of microbial changes in fishery products. Ph.D. thesis, University of Kerala, Trivandrum. India.

20. Brown, M.H. and P.J.McClure. (1998). Emerging preservation technologies. The microbiological safety of processed foods. Edited by Crowther J.S. and B. Marthi, Oxford & IBH Publishing Co., Pvt. Ltd., New Delhi (Publ.) pp.68-74.

21. Buchanan,R.L., H.G.Stahl., M.M.Bencivengo. and Fernado Del Corral.(1989). Comparison of lithium chloride-phenyl ethanol moxalactam and modified Vogel Johnson agars for detection of *Listeria* spp in retail-level meats,poultry and seafood. *Appl.Environ.Micobiol.*55,599-603.

22. Cahill,M.M. (1990). Bacterial flora of fishes: a review .*Microbiol.Rev.*19,21-41.

23. Cann, D.C. (1977). Proceedings of Conference on *Handling, processing and marketing tropical fish* (Eds. Penetope Suteliffe and J.Disney), Tropical Products Institute, London, pp. 377-94.

24. Cann, D.C., G.Hobbs, B.B.Wilson and R.W.Horsley. *(1971). The bacteriology of "scampi" (*Nephrops norvegicus*) II. Detailed investigation of the bacterial flora of freshly caught samples. *J. Food. Technol.* 6, 153-161.

25. Cann,D.C., Taylor,L.Y. and Hobbs,G.(1975). The incidence of *C.botulinum* in farmed trout raised in Great Britain.*J.Appl.Bacteriol.*39, 331-336.

26. Cann.D.C. (1974).Bacteriological aspects of tropical shrimp. In Fishery products Rudolf Kreuzer (ed.) Fishing News (Books)Ltd.West Byfleet, Surrey, England,338-344.

27. Chattopadhyay,A.K., J.K.Bandhopadhyay. and S.K.Battacharya.(1986). Lowcost fresh water fish pickle using cheap citrus fruit *(C.reticulata). Fish.Technol.*23 ,171-173.

28. Christopher.F.M., C.Vanderzant., J.D.Parker. and F.S.Conte. (1978). Microbial flora of pond reared shrimp *Penaeus stylirostris, Penaeus vannamei* and *Penaues setiferus.J.Food.Prot.*41,20-23.

29. Chung,J.* (1968).Ph.D.Thesis, University of Washington,Seattle,W.A. Quoted from Shewan.J.M. (1961)-Fish as Food.Vol.I.

30. CIEO, (1991). World Fisheries Atlas. Halifax, Nova Scotia, Canada.

31. Cobb.B.F. and C.Vanderzant.* (1971).*J.Milk.Food.Technol.*34,77. Quoted from Surendran, P.K. (1980). Chemical preservatives in relation to control of microbial changes in fishery products. Ph.D. thesis, University of Kerala, Trivandrum. India.

32. Collins, M.D., J.A.E.Farrow., B.A.Phillips., S.Ferusu. and D.Jones. (1987). Classification of *Lactobacillus divergens, Lactobacillus piscicola*, and some catalase-negative, asporogenous, rod-shaped bacteria from Poultry in a new genus, *Carnobacterium. Int. Journ. Syst. Bacteriol.* 37, 310 - 316.

33. Collins, M.D., U.Rodrigues., C.Ash., M.Auguirre., J.A.E.Farrow., A.Martinez-Murcia., B.A.Phillips., A.M.Williams. and S.Wallbanks. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16s rRNA. *FEMS.Microbiol.Lett.*77, 5-12.

34. Collins,M.D., A.M.Williams. and S.Wallbanks.(1990). The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis description of *Tetragenococcus* gen.nov.*FEMS Microbial.Lett.*70,255-262.

35. Cone, D.K.(1982). A *Lactobacillus* spp. from diseased female rainbow trout, *Salmo gairdneri* Richardson in Newfoundland Canada. *J. Fish. Diseases* .5, 479-485.

36. Conner, D.E., R.E.Brackeff. and L.R.Beuchat. (1986). Effect of temperature, sodium chloride, and pH on growth of *L. monocytogenes* in cabbage juice. *Appl. Env. Microbiol.* 52, 59-63.

37. Conner.D.E.,V.N.Scott. and D.T.Bernard.(1990). Growth, inhibition and survival of *L.monocytogenes* as affected by acidic conditions.*J.Food.Prot.*53,652-655.

38. Conway, P. (1980). *Lactobacilli* fact and fiction. In: Grubb, R., Midtvedt, T.M., Norin, E., (Eds.). The Regulatory and Protective role of the Normal Microflora. Stockton Press, New York. pp. 263-281.

39. Cowan,S.T. and K.J.Steel (1965). Manual for the Identification of medical bacteria,Cambridge University Press. 253.

40. Craig, J.M., S.Hayes. and K.S.Pilcher. (1968). Incidence of *C. botulinum* type E in salmon and other marine fish in the Pacific North west. *Appl. Microbiol.* 16, 553-557.

41. Curran, C.A. and J.G.Disney. (1979). The iced storage life of tropical fish. Paper presented at the Indo-Pacific Fisheries Commission Working Party on Fish Technology, Jakarta, Indonesia, 19.

42. Dack, G.M., E.Wheaton., M.N.Mickelson. and M.N.Shulder. (1960) Quick Frozen Foods, 22, 44. Quoted from Thampuran,N. (1987). Ph.D. Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology, Cochin-16.

43. Daeschel, M.A., M.C.McKenny. and L.C.McDonald. (1990) Bacterial activity of *Lactobacillus plantarum* C-11. *Food Microbiology*,7, 91-98.

44. Dahiya, R.S. and M.L.Speck. (1968). Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *J. Dairy Science*, 51, 1568-1972.

45. De Bruyn,I., W.H.Holzapfel., L.Visser., and A.I.Louw. (1988). Glucose metabolism by *Lactobacillus divergens.J.Gen.Microbiol.* 134, 2103-2109.

46. de Man., J.C.Rogosa. and M.E.Sharpe. (1960). A medium for the cultivation of lactobacilli.*J.Appl.Bacteriol.*23, 130-135.

47. De Vuyst,L. and E.J.Vandamme. (1992). Influence of the carbon source on nisin production in *Lactococcus lactis ssp. lactis* batch fermentations. *Journal of General Microbiology*. 138: 571-578.

48. DeKlerk, H.C.C. and J.N.Coetzee. (1961). Antibiotics among lactobacilli. *Nature*, 192, 340 pp.

49. Dillon, R.M. and T.R.Patel .(1992). *Listeria* in seafoods: a review. *J. Food. Prot.* 55, 1009-1015.

50. Disney.J.G., I.N.Tatterson. and J.Olley. (1977). Recent developments in fish silage. In *Handling, Processing and Marketing tropical fish.* (Eds. Penetope Suteliffe and J.Disney), Tropical products Institute,London-231-240.

51. Domenech, A., J.Prieta., J.F.Fernandez-Garayzabal., M.D.Collins., D.Jones. and L.Dominguez. (1993). Phenotypic and phylogenetic evidence for a close relationship between *Lactococcccus gravieae* and *Enterococcus seriolicida. Microbiologia* 9, 63-68.

52. Dyer, F.E.* (1947). Microorganisms from Atlantic Cod. *J. Fish .Res. Bd.* Canada 7, 128-136.

53. Early, J.C. *(1967). MSc. Thesis, University of Nottingham. Quoted from Shewan.J.M. (1961)-Fish as Food.Vol.I.

54. Earp, B.J., C.H.Ellis. and E.J.Ordal. (1953). Kidney disease in young salmon. State Washington Department of Fisheries, Special Report No.1.

55. Egyad, L.G. (1967). Studies on cell division and the effect of aldehydes, ketones and ketoaldehydes on the proliferation of *E. coli. Curr. Med. Biol.* 1, 14-20.

56. Einarsson,H. and H.L.Lauzon. (1995). Biopreservation of brined shrimp (*Pandalus borealis*) by bacteriocins from lactic acid bacteria. *Appl.Environ.Microbiol.* 61,669-676.

57. Eldar, A.C., L.Ghittino., C.Asanta., L.Bozzetta., E.Goria., M.Prearo. and M.Bercovier. (1996). *Enterococcus seriolicida* is a junior synonym of *Lactococcus*

gravieae, a causative agent of septicemia and meningo encaphalitis in fish. *Curr. Microbiol.* 32, 85-88.

58. Elliot, P. R. and D.Michener.* (1961). *J. Appl. Microbiol.* 9, 452. Quoted from Thampuran,N. (1987). Ph.D. Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology, Cochin-16.
59. Erichsen, I. (1973). The microflora of semi-preserved fish. *Process Biochemistry* 8, 21-28.
60. Evelyn,T.P.T. and L.A.McDermott. (1961). Bacteriological studies of freshwater fish.*Can.J.Microbiol.*7,375-382.
61. Fair-Flow-Reports. (1994). Lactic acid bacteria in fish preservation. F-FE 143/94, 1p.
62. Farber,J.M. and P.I.Peterkin.(1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev.*55,476-511.
63. Fieger, E.A., M.E.Bailey. and A.F. Novak. *(1956). Chemical ices for shrimp preservation. *Food Technol.* 10, 578-583.
64. Finegold, S.M., V.L.Sutter. and G.E.Mathisen. (1983). Normal indigenous intestinal flora. In. Hentgens, D.J. (Ed.) Human intestinal microflora in health and disease. Academic Press, New York, pp. 3-31.
65. Florey, H.W. (1946). The use of microorganisms for therapeutic purposes. *Yale. J. Biol. Med.* 19, 101-1117.
66. Fricourt, B.V., S.F.Barefoot., R.F.Jestin. and S.S.Hayasaka. (1994). Detection and Activity of Plantaricin. F. an antibacterial substance from *Lactobacillus plantarum* BF 001 isolated from Processed Channel Catfish. *J. Food. Prot.* 57, 698 - 702.

67. Fuchs, R.S. and P.K.Surendran. (1989). Incidence of *Listeria* in tropical fish and fishery products. *Lett. Appl. Microbiol.*. 9, 49-51.

68. Fuller, R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol. Sppl.* 76,

69. Gancel, F., F.Dzierszinski. and R. Tailliez. (1997). Identification and characterization of *Lactobacillus* species isolated from fillets of vaccum - packed smoked and salted herring (*Clupea harengus*). *J. Appl. Microbiol.* 82,722-728.

70. Georgala, D.* (1957). Changes in the skin flora of cod after washing and icing. *J. Appl. Bact.* 20, 23-29.

71. Georgala, D.L.* (1958). The bacterial flora of the skin of North Sea Cod. *J. Gen. Microbiol.* 18, 84-91.

72. Gibbons,N.E.(1974).Halobacteriaceae In: Bergeys Manual of Determinative Bacteriology, Eds.R.E. Buchanan and N.E.Gibbons.Williams and Wilkins Co, Baltimore,USA-269-273.

73. Gibbs, D.A. (1987). Novel uses for lactic acid fermentation in food preservation. *J. Appl. Bacteriol. Symp. Supp.*)515-525.

74. Gilliland, S.E. and M.L.Speck. (1975). Inhibition of psychrotrophic bacteria by lactobacilli and pediococci in nonfermented refrigerated foods. *J. Food. Sci.* 40, 903-905.

75. Gilliland, S.E. and M.L.Speck. (1961). Antagonistic action of *Lactobacillus acidophilus* toward intestinal and food borne pathogens in associative cultures. *J. Food .Prot.* 40, 820-823.

76. Girija,S. (1993). Studies on Minced fish Technology. Ph.D.Thesis. Cochin University of Science and Technology ,Cochin.

77. Glass, K.A. and M.P.Doyle. (1989). Fate of *L. monocytogenes* in processed meat products during refrigerated storage. *Appl. Environ. Microbiol.* 55, 1565-1569.

78. Goldin, B.R. and S.L.Gorbach. (1992). Probiotics for humans. In: Fuller, R. (Ed.) Probiotics. The Scientific Basis. Chapman & Hall, London, U.K., pp.355-376.

79. Gopakumar.K (1997). Tropical Fishery products. Oxford & IBH Publishing Co.Pvt.Ltd,66 Janpath, New Delhi 110 001. 190.

80. Gorbach, S.L. (1990). Lactic acid bacteria and human health. *Ann. Med.* 22, 7-41.

81. Gould,G.W.(1989). Mechanism of action of food preservation. Elseiver Appl.Science, London.

82. Guyer,S. and T.Jemmi.(1991). Behaviour of *Listeria Monocytogenes* during fabrication and storage of experimentally contaminated smoked salmon *Appl.Environ.Microbiol.*57, 1523-1527.

83. Halami,P.M., A.Chandrasekar. and K.Nand. (1999). *Lactobacillus farcimnis* MD, a newer strain with potential for bacteriocin and antibiotic assay.*Lett.Appl.Microbiol*,30, 197-202.

84. Hammes,W.P. and R.F.Vogel. (1995). The genus *Lactobacillus* . In: B.J.B-Wood and W.H.Holzapfel (Editors), The Lactic Acid Bacteria, The genera of Lactic Acid Bacteria, Vol.2, Blackie Academic.London.pp.19-54.

85. Hannan,R.S. *(1956). Science and Technology of Food preservation by ionizing radiations. Chemical Pub.Co.New York.

86. Hano, J.* (1936). Uber das pharmakologische verhalt des Diazetyls. *Bull. Int. Acad. Pol. Sci. Lett. Cl. Med.*, 555-562.

87. Hardy.K.G. (1982). Microbe-microbe interactions, bacteriocins..In R.G.Burns and J.H.Slater (ed). Experimental micrbial ecology. Balckwell Scientific Publications, St.Louis, 368-378.

88. Hayner,G.A. and B.E.Proctor. (1953). Investigations relating to the possible use of atomic fission products for food sterilization. *Food.Technol.7*, 6-10.

89. Hechard,Y., M.Dherbomez., Y.Cenatiempo. and F.Letellier. (1990). Antagonism of lactic acid bacteria from goats milk against pathogenic strains assessed by sandwich method. *Lett.Appl.Microbiol.*11,185-188.

90. Heinitz,M.L., R.D.Ruble., D.E.Wagner. and S.R.Tatini. (2000). Incidence of *Salmonella* in fish and seafood.*J.Food.Prot.*63,(5),579-592.

91. Herbert.R.A., M.S.Henderie., D.M.Gibson. and J.M.Shewan. (1971). Bacteria active in the spoilage of certain seafoods.*J.Appl.Bact.*34, 41.

92. Hilmarsdottir,E. and E.Karmas. (1984). Microbial stability of a fermented intermediate moisture fish product.*Lebensm-wiss-Technol.*17, 328-330.

93. Hitchener, B.J., A.F.Egan. and P.J.Rogers. (1982). Characteristics of Lactic Acid Bacteria isolated from vacuum-packaged beef. *J. Appl. Bacteriol.* 52, 31-37.

94. Hiu,S.F., R.A.Holt., N.Sriranganathan., R.J.Seilder. and J.L.Fryer. (1984). *Lactobacillus piscicola*, a new species from Salmonid Fish. *Int. J. Syst.Bacteriol.*, 34, 393-400.

95. Hobbs, G. (1983). Microbial spoilage of Fish. In Food Microbiololgy: Advances and Prospects ed. Roberts, T.A. and Skinner, F.A. pp.217-229. London . Academic Press.

96. Hobbs,G.(1976). *Clostridium botulinum* and its importance in fishery products. Advances in Food Research. 22,135-185.

97. Hobbs.G. (1977). *Clostridium botulinum* in irradiated fish. *Fd.Irrad.Int.*7, 39-54.

98. Holzapfel,W.H., R.Geisen. and U.Schillinger. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins, and food grade enzymes. *Int.J.Food.Microbiol.*24,343-362.

99. Horsley, R.W.* (1973). The bacterial flora of Atlantic salmon (*Salmo salar*) in relation to its environment. *J. Appl. Bact.* 36, 377-386.

100. Horsley, R.W.*(1977).*J.Fish.Biol.*10, 529.Quoted from Shewan,J.M. (1961)- Fish as Food.Vol.I.

101. Hoshina, T. (1956). An epidemic disease affecting rainbow trout in Japan. *J. Tokyo Univ. Fish.* 42,35-46.

102. Huss, H.H., A.Pedersen. and D.C.Cann. (1974). The incidence of *C. botulinum* in Danish trout farms I. Distribution in fish and their environment. *J. Food. Technol.* 9, 443- 450.

103. ICMSF (International Commission on Microbiological Specifications for Foods) (1980). Microbiological Ecology of foods.Vol.I. Factors affecting life and death of microorganisms. London: Academic Press Inc (London) ltd.

104. Ijong,F.G. and Ohta-Yoshiyuki.(1995). Microflora and chemical assessment of an Indonesian traditional fermented fish sauce "bakasang"*Appl.Biol.Sci.Seibutsu-Seisangaku-Kenkyu.*34,95-100.

105. Itoh,T., Y. Fujimoto., Y.Kawai., T.Toba. and T.Saito. (1995). Inhibition of food-borne pathogenic bacteria by bacteriocins from *Lactobacillus gasseri*. *Lett.Appl.Microbiol.* 21, 137-141.

106. Iyer, T.S.G. and K.P. Srivastava. (1989). Incidence and low temperature survival of *Salmonella* in fishery products. *Fish. Technol.* 26, 131.

107. Iyer,T.S.G., S.P.Damle., D.K.Garg., V.N.Nambiar. and N.M.Vasu. (1986). Quality of certain ready to eat fish products of the domestic trade.*Fish.Technol.*23,78-81.

108. Jacob.S.S., K.M.Iyer., M.R.Nair. and V.K.Pillai.*(1962). *Indian J. Fish.*9:97. Quoted from Thampuran.N. (1987). Ph.D.Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology. Cochin-16.

109. Jadhav, M.G. and N.G.Magar *(1970). Preservation of fish by freezing and glazing I. Bacteriology of fresh, frozen and glazed fish. *Fish Technol.* 7, 86.

110. Jamias-Apilado, R.B. and R.C.Mabesa. (1992). Influence of commercial starter cultures on the rate of rice-fish fermentation. Papers presented at the Eighth session of the Indo-Pacific Fishery Commission. Working party on Fish technology and Marketing, Yogjakarta, Indonesia,24-27 September,1991.FAO Indopacific fisheries commission,Bangkok, Thailand,Rome,Italy. FAO 1992.no.470,Suppl.

111. Jeyasekaran.G., I.Karunasagar., and I.Karunasagar. (1996). Incidence of *Listeria* spp. in tropical seafoods. *Int.J.Food.Microbiol.* 31,333-340.

112. Jimnez-Diaz, R., R.M.Rios-Sanchez., M.Desmazend. and J.L.Ruiz-Barba. (1993). Plantaricus S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPC 010 isolated from a green olive fermentation. *Appl. Environ. Microbiol.* 59, 1416-1424.

113. Jin, L.Z., Y.W.Ho., M.Ali., N.Abdullah., B.K.Ong. and S.Jalaludin.(1996). Adhesion of *Lactobacillus* isolates to intestinal epithelial cells of chicken. *Lett. Appl. Microbiol.* 22, 229 - 232.

114. Jones,D.*(1978). Composition and differentiation of the genus Streptococcu.In:F.A.Skinner and L.B.Quesnel (edt) Streptococci.Academic Press,London,pp 1-49.

115. Jonsfon, E. and P. Conway. (1992). Probiotics for Pigs. In: Fuller, R. (Ed.), Probiotics. The scientific basis. Chapman & Hall, London, pp. 259-316.

116. Kamat, A.S. and P.M.Nair. (1994). Incidence of *Listeria* species in Indian seafoods and meat. *J. Food Safety*. 14, 117-130.

117. Kandler, O. and N.Weiss. (1986). Regular nonsporing Gram positive rods. In Bergey's Manual of Systematic Bacteriology Vol.2 pp 1208 - 1234. Baltimore Williams and Wilkins.

118. Karthiayani, T.C. and K.M.Iyer (1971). Seasonal variations of bacterial flora of fresh oil sardines (*Sardinella longiceps*). *Fish. Technol.* 8, 69-79.

119. Karthiayani,T.C. and K.M.Iyer. (1967). Quantitative and Qualitative studies on the bacterial flora of fresh sardines. *Fish. Technol.*, 4, 89-97.

120. Karthiyani, T.C. and K.M.Iyer. (1975) Bacterial flora of certain marine fishes and prawns in Cochin waters in relation to their environs. *J.Mar. Biol.Assocn.*, 17, 96-100.

121. Kawabata,T., T.Mizukami., R.Ohara.and J.Shinohara. *(1975).*Bull.Japan.Soc.Sci.Fish.*41, 667. Quoted from Shewan.J.M. (1961)-Fish as Food.Vol.I.

122. Kawai, Y., T.Saito.,T. Toba., S.K.Samant. and T.Itoh. (1994). Isolation and characterization of a highly hydrophobic new bacteriocin (Gassericin A) from *Lactobacillus gasseri* LA 39. *Biosci.Biotech.Biochem.* 58, 1218-1221.

123. Keddie, R.M. *(1959). The properties and classification of lactobacilli isolated from grass and silage. *J. Appl. bacteriol.* 22, 403-416.

124. Kiser.J.S. and T.D. Beckwith. *(1942). The effect of fast freezing on bacterial flora of mackerel. *Food Research*, I, 255.

125. Klaenhammer, T.R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie.* 70, 337-349.

126. Knochel, S. (1981). Mikrobiel fermentering of fisk v. by a natrualight foskommende Lactobaciller stud. from thesis, Technical University Lungby, Denmark.

127. Kodama,R.(1952). Studies on Lactic acid bacteria. Part II.Lactolin, a new antibiotic substances produced by lactic acid bacteria.*J.Antibiot.*5, 72-74.

128. Konisky,J.(1978). The bacteriocins. In.I.C.Gunsalus, L.N.Ornston. and J.R.Sokatch (ed.), The bacteria, a treatise on structure and function.Vol.6.Bacterial diversity, Academic press,Inc.,New York. pp71-136

129. Kraus, H. (1961) Kurze Mittelilung uber das Vor Kommen von Lactobazillen anf frischen Heringen. *Arch. Lebensmittelhygeiene*, 12, 101 - 102.

130. Kusuda, R. (1992). Bacterial fish diseases in mariculture in Japan with special emphasis on streptococciosis. *Bamidgeh*, 44, 140.

131. Lakshmanan, P.T., M.Cyriac., P.R.G. Verma. and T.S.G.Iyer. (1984). Studies on the quality of frozen fish in retail cold stores. *Fish Technol.* 21, 98.

132. Lalitha, K.V. and K.M. Iyer. (1990). Isolation of *C. botulinum* from tropical fish. *Lett. Appl. Microbiol.* 11. 179-181.

133. Lalitha.K.V. and K.Gopakumar. (1996). Microbial and biochemical changes during the fish sauce fermentation. Proceeding of the 10th session of APFIC of the FAO Fish Report.563, 43-54.

134. Lancefield,R.C.*(1933). A serological differentiation of human and other groups of hemolytic *Streptococci.J.Exp.Med.*,57, 571-595.

135. Lannelongue, M., M.O.Hanna., G.Finne., R. Nickelson. and C.Vanderzant. (1982). Storage characteristics of finfish fillets (*Archosargus probatocephalus*) packaged in modified gas atmospheres containing carbon dioxide. *J.Food.Prot.* 45, 440-449.

136. Lee, J.S. and D.K.Pfiefer. (1975). Microbial characteristics of Dungeness crab (*Cancer magister*). *Appl. Microbiol.* 30, 72-8.

137. Lee,J.V., P.Shread., A.L. Furniss. and T.Bryant. (1981). Taxonomy and description of *V.fulvialis* sp.nov; (Synonym group F vibrios, Group EF6). *J.Appl.Bacteriol.*50, 73-94.

138. Lekshmy,A., T.K.Govindan., A.Mathew. and V.K.Pillai. *(1962). *Indo Pacific.Fish.Coun.Occ.*Paper 63/2. Quoted from Thampuran,N. (1987). Ph.D.Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology, Cochin-16.

139. Lennon,D., B.Lewis., C. Mantell., D.Becroft., B.Dove., K.Farmer., S.Tonkin., N.Yeats., R.Stamp. and K.Mickelson. (1984). Epidemic perinatal listeriosis.*Pediatr.Infect.Dis.*3, 30-34.

140. Lima Dos Santos, C.A.M.* (1981). The storage of tropical fish in ice – a review. *Trop.Sci.*23, 97- 127.

141. Lindgren, S. and G.Clenstrom. (1978). Antibacterial activity of lactic acid bacteria 2. Activity in vegetable silages. Indonesian fermented foods and starter cultures. *Swedish J. Agric. Res.* 8, 67-73.

142. Liston, J. *(1956). Quantitative variations in the bacterial flora of flat fish. *J. Gen. Microbiol.* 15, 305-314.

143. Liston, J. *(1957). The occurrence and distribution of bacterial types on flat fish. *J. Gen. Microbiol.* 16, 205-216.

144. Liston, J.* (1955). A quantitative and qualitative study of the bacterial flora of skate and lemon sole trawled in the North sea. Ph.D. Thesis. Aberdeen University. Aberdeen, Scotland, Quoted by Shewan (1961).

145. Liston, J.* (1980). Microbiology in Fishery Science. In advances in fish science and technology. Papers presented at the Jubilee Conference of the Torry Research Station, Aberdeen, Scotland, 23-27, July 1979, edited by J.J. Connel *et al.* Farnham, surrey, Fishing News Books Ltd., pp. 138-57.

146. Liston.J. *(1976). *Vibrio parahaemoliticus.* In. Food Microbiology:Public health and spoilage aspects. Eds.M.P.Defigurredo and D.F.Splitt Stoesser.Avi.publ.Co. West port, Connecticut,USA.

147. Liston.J. and J.M.Shewan. *(1958). Bacteria brought into brines on fish. In the microbiology of fish and meat curing brines. International Symposium on Food microbiolgy. Cambridge B.P. Eddy (ed), Her majesty's stationary office, London. p.299.

148. Liston.J. and J.R.Matches.* (1968). Single and multiple doses in the radiation pasteurization of seafoods. *Fd. Technol.*,22, 893-896.

149. Magnusson, H. and K.Traudottir. (1982). The microbial flora of vacuum packed smoked herring fillets. *J. Food Technol.* 17, 695 - 702.

150. Matrozza, M.A., M.F.Leverone. and D.P.Boudreaux. (1988). Method for inhibiting psychrotrophic bacteria in cream or milk based products using a pediococcus. *U.S. patent* 4, 790-994.

151. Maugin, S. and G.Novel. (1994). Characterization of Lactic Acid Bacteria isolated from seafood. *J. Appl. Bacteriol.* 76, 616 - 625.

152. McKay, L.L. and K.A.Baldwin. (1990). Applications for Biotechnology: present and future improvements in lactic acid bacteria. *FEMS. Microbiol. Rev.* 87, 3-14.

153. Messina, M.C., H.A. Ahamad., J.A.Marckello., C.P.Gerba. and M.W.Paquette. (1988). Effect of liquid smoke on *L. monocytogenes*. *J.Food.Prot. 51*, 629-631.

154. Miller,A., R.A.Scanlan., J.S.Lee., and L.M.Libbey. (1973). Volatile compounds produced in sterile fish muscle (*Sebaster melanops*) by *Pseudomonas putrefaciens*, *Pseudomonas fluorescens* and an *Achromobacter* species.*Appl.Microbiol.*25, 257.

155. Mohamed Hatha,A.A. and P.Lakshmanaperumalsamy. (1977). Prevalence of *Salmonella* in fish and crustaceans from markets in Coimbatore, South India. *Food Microbiol.*, 14, 111-116.

156. Molin,G., S.Inga-Maj. and A.Ternstrom. (1983). The microbial flora of herring fillets after storage in carbondioxide, nitrogen or air at 2°C. *J. Appl. Bacteriol.* 55, 49-56.

157. Montes, A.J. and D.G. Pugh. (1993). The use of probiotics in food animal practice. *Vet. Med.* 88, 282-288.

158. Moon.N.J., L.R.Beuchat., D.T.Kinkaid. and E.R.Hays. (1982). Evaluation of Lactic acid bacteria for extending the shelf life of Shrimp.*J.Food.Sci.*47,897.

159. Morishita.K., W.Otokasaka., K.Yamazaki., Y.Kawai., N.Inoue. and H.Shinano. (1994). Chemical and microbiological characteristics of commercial "Shiokara". *Bull.Fac.Fish.Hokkaido.Univ.* 45,100-107.

160. Morishita.K., W.Otokasaka.. K.Yamazaki., Y.Kawai., N.Inoue. and H.Shinano. (1995). Isolation and characteristics of lactic acid bacteria in commercial "ika-shiokara".*Fish.Sci.*61, 371-372.

161. Mortojudo, J.W., E.G.Siagian., F.Suhadi., B.Q.Ward. and W.M.S.Ward. (1973). The presence of *C. botulinum* in Indonesian waters. *J.Appl. Bacteriol.* 36, 437-440.

162. Mugula,J.K. and M.H.Lyimo.(1992). Microbiological quality of traditional market cured fish in Tanzania.*J.Food.Safety.*13, 33-41.

163. Nambiar, N.M. and P.K.Surendran .(1999). Incidence of enterotoxigenic *Bacillus* in fish. Presented in the National Symposium on Sustainable development of Fisheries towards 2020 AD. Opportunities & Challenges, conducted by School of Industrial Fisheries, Cochin University of Science and Technology, Cochin-16 from 21-23 April 1999,24.

164. Nambiar, V.N. and K.M.Iyer. (1991). Distribution of *Salmonella* serotypes in fish in retail trade in Kochi. *Fish. Technol.* 28, 33-37.

165. Nambiar, V.N. and P.K.Surendran. (1998). Factors affecting the growth and survival of *Salmonella* serotypes in fish. 346-351. In: M.S.Hameed and B.M.Kurup (Eds.) Technological Advancements in Fisheries. Publn. No.1. School of Industrial Fisheries, Cochin University of Science and Technology, Cochin.

166. Nambiar,V.N. and K.M.Iyer. (1973). Bacteriological investigations of prawn canneries: II-Incidence of *Clostridium perfringens*. *Fish. Technology*. 10, 6-14.

167. Nambiar,V.N. and K.M.Iyer. (1990). Microbial quality of fish in retail trade in Cochin.*Fish.Technol.*27,51-59.

168. Neufeld,N. *(1971). Influence of bacteriological standards on the quality of inspected fisheries products. In: Fish Inspection and quality control Ed K.Kreuzer.Fishing News (Books) Ltd.London.234-240.

169. Novak.A.F.* (1973). In Microbial safety of Fishery products C.O.Chichester and H.D.Graham (ed.),p-59.Academic Press,London. Quoted from Liston, J.* (1980). Microbiology in Fishery Science. In advances in fish science and technology. Papers presented at the Jubilee Conference of the Torry Research Station, Aberdeen, Scotland, 23-27, July 1979, edited by J.J. Connel *et al.* Farnham, surrey, Fishing News Books Ltd., pp. 138-57.

170. Oberlender, V., M.O.Hanna., R.Miget., C.Vanderzant., and G.Finne. (1983). Storage characteristics of fresh sword fish steaks stored in CO_2 enriched controlled (flow through) atmospheres. *J. Food Prot.*, 46, 434-440.

171. Okabe,S. (1974). Statistical review of food poisoning in Japan especially by *Vibrio parahaemolyticus*. In: International symposium on *Vibrio parahaemolyticus* , Eds T.Fugino, G.Sakaguchi,R.Sakazaki and Y.Takeda. Saikon Publ.Co.Tokyo,Japan.

172. Okafor, N. and B.C.Nzeako. (1985). Microbial flora of fresh and smoked fish from Nigerian fresh water. *Food. Microbiol.*, 2, 71-75.

173. Oliver, J.D., R.A.Warner and D.R. Clevland. (1983). Distribution of *Vibrio vulnificus* and other lactose fermenting marine vibrios in the coastal waters of southern United States. *Appl Environ. Microbiol*. 44, 1404-1414.

174. Olympia.M., H.Ono., A.Shinmoyo. and M.Takano. (1992).Lactic acid bacteria in a fermented fishery product "Burong bangus" *J.Ferment.Bioengg*.73,193-197.

175. Orberg,P.K. and W.E.Sandine. (1984). Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc* spp. *Appl.Environ.Microbiol.* 48, 1129-1133.

176. Orla-jensen.* (1919). The Lactic Acid Bacteria. Fred Host and Son. Copenhagen.

177. Ostergaard.A., P.K.B.Embarek., C.Wedell-Neergaard., H.H.Huss. and L.Gram. (1998). Characterization of antilisterial lactic acid bacteria isolated from Thai fermented fish products. *Food.Microbiol*.15, 223-233.

178. Pawar.S.S and N.G.Magar.* (1966). Bacterial flora of marine fish Doma (Sciaenoids) (small spp.) from Bombay coast. *Fish Technol*.3, 26.

179. Phillips,S and A.Wallbridge. (1977). The micoflora associated with dry salted tropical fish in *Handling, Processing and Marketing of tropical fish*, (Eds Penelope Sutcliffe and J.Disney). Tropical Products Institute,London.353-356.

180. Pilet, M., X. Dousset., R. Barre., G. Novel., M. Desmazeaud. and Jean-Christophe Piard (1995). Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. *J.Food. Prot*. 58, 256-262.

181. Pillai,V.K., A.Lekshmy, T.K.Govindan, C.Mathen. and H.Krishna Iyer. (1965). Survey of the microbiological quality of commercial frozen prawn products. *Fish. Technol.*, 2, 115-125.

182. Pillai,V.K.. P.V.K.Sastri. and M.R.Nair. *(1961).*Indian J.Fish.*8.430.Quoted from Thampuran,N. (1987).Ph.D.Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology, Cochin-16.

183. Pivnick,H.*(1949). Bacteriological and biochemical studies of the rate of decomposition of frozen and defrosted cod muscle, Ph.D. Thesis.Dalhousie University.Halifax.Nova Scotia.quoted by Shewan,(1962).

184. Raccah, M., R.C.Baker., J.M. Regenstein.. and E.D.Munix. (1979). Potential application of microbial antagonism to extend storage stability of flesh type food. *J. Food. Sci.* 44, 43-46.

185. Raj,H. and J.Liston. (1961). Survival of bacteria of public health importance in frozen seafoods. *Food Technol.* (Champaigh), 15,429.

186. Ray, B.R. and W.E.Sandine. (1992). Acetic, propionic and lactic acids of starter culture bacteria as biopreservatives. In B.Ray and M.Daeschel(ed.) Food biopreservatives of microbial origin. CRC. Press, Inc. Boca Raton. FL. p.103-136

187. Reay, G.A. and J.M.Shewan. *(1949). The spoilage of fish and its preservation by chilling. *Advances in food Res.* 11, 343-398.

188. Reeves, P. (1972). The Bacteriocins. Molecular Biology, Biochemistry and Biophysics. Springer, New York. N.Y. Vol.II.

189. Reeves, P.(1965). The bacteriocins.*Bacteriol.Rev.*29, 24-45.

190. Ringoe, E. and F.J.Gatesoupe. (1998). Lactic acid bacteria in fish: A review. *Aquaculture* 160, 177 - 203.

191. Rosenbach.F.J*. (1884).Mikro-Organismen bei den Wund-In-fektions-Kranstein des Menschen.J.F.Bergman,Wiesbaden.

192. Ross, A.J. and R.J.Toth. (1974). *Lactobacillus* - a new fish pathogen? Progressive *Fish- Culturist*. 36, 191.

193. Ross, D. (1981) The inhibition of growth of spoilage microorganisms in milk by *Streptococcus lactis subsp. diacetylactis*, *Leuconostoc cremoris* and *L. dextranicum*. *Aust. J. Dairy. Technol.* 36, 147-152.

194. Rucker, R.R., B.J.Earp. and E.J.Ordal. (1953). Infectious diseases of pacific salmon. *Am. Fish. Soc.* 83, 297-312.

195. Rudrasetty. (1985). Postmortem changes and bacteriology of fish. In Symposium on harvest and post harvest technology of fish. Society of Fisheries Technologists, Cochin.pp-401.

196. Ruello,J.N.*(1974). *Food.Technol.*(Aust).26,180. Quoted from Thampuran,N. (1987). Ph.D. Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology, Cochin-16.

197. Russel, J.B. (1992). Another explanation of the toxicity of fermentation acids at low pH: Anion accumulation versus uncoupling. *J. Appl. Bacteriol.* 73: 363-370.

198. Saisithi,P., B.O.Kasemsarn., J.Liston. and A.M.Dollar.(1966). Microbiology and chemistry of fermented fish.*J.Food.Sci.*31, 105-110.

199. Sakaguchi, G. (1979). Botulism. In Food borne infections and Intoxication's, 2nd edn. ed: Riemann, H. & Bryan, F.L. pp. 390-442. New York: Academic Press.

200. Sakazaki,R. and T.Shimada. (1986). *Vibrio* species as causative agent of food borne infection. In Developments in Food Microbiology. Ed. Robinson, P.k. pp.123-149. London: Elsevier Applied Science Publishers.

201. Saleh, M.A. and Z.J.Ordal. (1955). Studies on growth and toxin production of *Clostridium botulinum* in a precooked frozen food. *Food Res.* 20, 340-350.

202. Salle,A.J. (1954). Laboratory Manual on Fundamental principles of Bacteriology. Mc Graw-Hill book Company.Inc.p 1094.

203. Sandine,W.E., P.C.Radich. and P.R.Elliker.(1972). Ecology of lactic streptococci. A review.*J.Milk.Food.Technol*,35,176-184.

204. Sanjeev, S. and P.K.Surendran. (1992). Evaluation of Reversed Passive Latex Agglutination Test Kits for the Retention of Staphylococcal Enterotoxin A, B, C and D in Fishery Products. *J. Fd. Sci. Technol.* 29, 311-312.

205. Sarkar, P.K. and S.Banerjee. (1996). Antibacterial activity of lactic acid bacterial isolates obtained from natural habitats. *J. Food. Sci. Technol.* 33, 231 - 233.

206. Schillinger, V. and F.K.Lucke. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol*, 55, 1901.

207. Schleifer,K.H. (1987) Recent changes in the taxonomy of Lactic acid bacteria.*FEMS Microbiol. Rev.*46,201-203.

208. Schleifer,K.H., J.Kraus., C.Dvorak., R.Klipper-Balz., Collins,M.D. and Fischer,W. (1985). Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen.nov.*Syst.Appl.Microbiol.*6, 183-195.

209. Schroder, K., E.Clausen., A.M.Sandberg. and J.Raa. (1979). Psychrotrophic *Lactobacillus plantarum* from fish and its ability to produce antibiotic substances.In Advances in Fish Science and Technology. Ed. Connel.J.J. Farnham, England. Fishing newsbook Ltd. pp.480-483.

210. Scopes ,R.K.(1982). Protein Purification, Principles and Practice. Ed. Charles R.Canter. Springer-Verlag, new York, 10010, U.S.A. pp.279.

211. Seeliger, H.P.R.* (1961). Listeriosis. Hafner Publishing Co., New York. Quoted from Shewan.J.M. (1961). Fish as Food.Vol.I.

212. Shaikmahmud, F. and N.G.Magar. *(1956). Effect of washing on the nutritional characteristics of three marine fish flesh. The first Indian Fisheries forum proceedings, p.149.

213. Sharp, M.E. (1981). The genus lactobacillus. In: Starr, M.P., Stolp, H., Truper, H.G., Balows, A., Schlegel, H.G. (Eds.) The Prokaryotes. A handbook on Habitat, isolation and identification of bacteria, Vol.I. Springer, Berlin, pp. 1653-1679.

214. Sharpe. M.E., T.F.Fryer. and D.G.Smith. (1979). Identification of Lactic Acid Bacteria. In: Gibbs,E.M.,Skinner,F.A.(eds). Identification Methods for Microbiolgists. Academic Press, London.

215. Shaw,B.G. and C.D.Harding. (1984). A numerical taxonomic study of Lactic acid bacteria from vacuum packaged beef, pork, lamb and bacon.*J.Appl.Bacteriol.*56,25-40.

216. Sherman,J.M.* (1937).The *Streptococci.Bacteriol.Rev.*I. 3-97.

217. Shewan, J.M. *(1949). Some bacteriological aspects of handling, processing and distribution of fish. *J. Roy. Saint. Inst.* 59, 394-421. Quoted by Shewan (1961).

218. Shewan, J.M. *(1961). The microbiology of sea water fish. In "Fish as food" Vol. I. pp. 487-560. (Ed.) G.Borgstrom. Academic Press, New :York and London.

219. Shewan, J.M. *(1977). The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. In *Handling, Processing and Marketing of Tropical Fish.* (Eds. Penelope Sutcliff and J.Disney) pp. 51 - 57. Tropical Products Institute, London.

220. Shewan, J.M.* (1962). The bacteriology of fresh and spoiling fish and some related chemical changes. In "Recent advances in food Science". pp.167-193. (Eds). Hawthron and Miel Leitch, Butterworth, London.

221. Shewan,J.M. and G.Hobbs.(1967). In progress in Industrial Microbiology D.J.D.Hockenhull (ed.), p.169, Iliffe Books, London.

222. Shewan,J.M. and J. Liston. (1955). A review of food poisoning caused by fish and fishery products.*J.Appl.Bacteriol*,18, 522-534.

223. Shewan,J.M.* (1971). The microbiology of fish and fishery products- a progress report. *J.Appl.Bacteriol*.34, 299-315.

224. Shewan.J.M.* (1938). The salt curing of herring. In:Report of the Director of Food Investigation for the year 1938.HMSO, London.115-117.

225. Shih, D.-Yang-Chih, Lai-Chao-Ling, Chen-Chao-Rong, Wang-Jan-Yi. (1996). Occurrence of *Vibrio parahaemolyticus* in imported aquatic foods from Mainland China. *J. Food-Drug-Anal*. 4, 239-346.

226. Sigma Chemical Company.(1993).Biochemicals organic compounds for research and diagnostic reagents.Sigma Chemical Co.St.Louis,MO.

227. Simidu, V., E.Kaneko and K.Aiso *(1969). Microflora of fresh and stored flat fish.*Kareins bicoloratus*. *Bull. Jap. Soc. Sci. Fish.*. 35,77-82.

228. Singh.B.R., M.P.Saxena., S.B.Kulsheshtra. and K.N.Kapoor. (1996). A note on prevalence of vibios in marine fish and shell fishes. *Ind.J.Fisheries,* 43,407-410.

229. Sissons, J.W. (1989). Potential of probiotic organisms to prevent diarrhoea and promote digestion in farm animals. A review. *J.Sci. Food Agric.*, 49, 1-13.

230. Sneath, P.H.A., N.S.Mair., M.E.Sharpe. and J.G. Holt. (1986). Bergey's Manual of Systematic Bacteriology. Vol.2. Williams and Wilkins, Baltimore.

231. Stiles,M.E. and W.H.Holzapfel.(1997) Lactic acid bacteria of foods and their current taxonomy.*Int.J.Food.Microbiol.*36,1-29.

232. Stiles,M.E.(1989). Less recognised or presumptive food-borne pathogenic bacteria.In MP.Doyle (editor).Food-borne bacterial pathogens, Marcel Dekker, NewYorl.pp.673-733.

233. Suhadi, F., Thayib, S.S. and Sumarsono, N. (1981). Distribution of *C. botulinum* around fishing areas of the Western part of Indonesian waters. *Appl.Environ Microbiol.* 41, 1468-1471.

234. Surendran P.K. and K.Gopakumar. (1982). Bacterial flora of EDTA treated oil sardine (*Sardinella longiceps*), Indian mackerel (*Rastralliger kanagurta*) and prawn (*Metapenaeus dobsoni*) in iced storage. *Fish. Technol.* 19, 33-39.

235. Surendran. P.K. (1980). Chemical preservatives in relation to control of microbial changes in fishery products. Ph.D. thesis, University of Kerala, Trivandrum. India.

236. Surendran, P.K. and Gopakumar, K. (1991). Microbiology of cultured fish in: Aquaculture Productivity. ed. by V.R.P.Sinha and H.C.Srivastava, New Delhi. Oxford and IBH Publishing Co. Pvt. Ltd. 863.

237. Surendran, P.K. and K.Gopakumar. (1981). Selection of microbial flora in CTC-treated oil sardine (*Sardinella longiceps*) Indian mackerel (*Rastralliger kanagurta*) and prawn (*Metapenaeus dobsoni*) held in iced storage. *Fish. Technol.* 18: 133-141.

238. Surendran. P.K. and K.M.Iyer *(1976). Bacterial flora of fresh and iced Indian mackerel (*Rastralliger kanagurta*) and its response to chlorotetracycline (CTC) treatment. *Fish Technol.* 13: 139.

239. Surendran, P.K., J.Joseph., A.V.Shenoy., P.A.Perigreen., K.M.Iyer. and K.Gopakumar. (1989). Studies on spoilage of commercially important tropical fishes under iced storage. *Fisheries Research*, 7, 1-9.

240. Surendran,P.K. and K.M.Iyer. (1973). Experimental preservation of sardines (*Sardinella longiceps*) using chlortetracycline. *Fish.Technol.*10.110-115.

241. Surendran,P.K. and K.M.Iyer. (1980). The bacterial flora of pearlspot *(Etroplus suratensis*) caught from Cochin backwaters. Presented in the International Symposium on coastal aquaculture, held at Cochin, from 12 to 18 January,1980 under the auspices of the Marine Biological Association of India.

242. Tagg,J.R., A.S.Dajani. and L.W.WannaMaker. (1976). Bacteriocins of gram-positive bacteria. *Bacteriol Rev.* 40, 722 - 756.

243. Tanaka, N., L.Meske., M.P.Doyle., E.Traisman., D.W.Thayer. and R.W.Johnston. (1985). Plant trials of bacon made with lactic acid bacteria, sucrose and lowered sodium nitrate. *J. Food.Prot.* 48: 679-686.

244. Tanikawa,E. (1965). Marine products in Japan. Hokkaido University, Hokaido, Japan.

245. Tannock, G.W. (1988). The normal microflora: a new concepts in health promotion. *Microbiol.* Sci. 5, 4-8.

246. Tannock, G.W., O.Szylit., Y.Diwal. and P.Raiband. (1982). Colonization of tissue surface in the gastrointestinal tract of gonobiotic animals by *Lactobacillus* strains. *Can. J. Microbiol.* 28, 1196-1198.

247. Taylor, L.Y., D.D.Cann. and B.J.Welch. (1990). Antibotulinal properties of nisin in fresh fish packaged in an atmosphere of carbondioxide. *J. Food. Prot.* 53, 953-957.

248. Teuber,M., A.Geis. and H.Neve.(1991). The geunus *Lactococcus*. In:A.Balows, H.G.Truper, M.Dworkin, Harder,W.and K.H.Schleifer (editors). The Prokaryotes, Vol.II.2nd edition Sprnger-Verlag. NewYork.pp 1482-1501.

249. Thampuran, N. and P.K.Surendran.(1998). Incidence of motile aeromonads in marine environment, fishes and processed fishery products.. In: M.S.Hameed and B.M.Kurup (Eds.) Technological Advancements in Fisheries. Publn. No.1-School Indl. Fish. Cochin University of Science and Technology, Cochin. 352-358

250. Thampuran, N. and P.K.Surendran. (1996). Effect of chemical agents on swarming of *Bacillus* species. *J. of Appl. Bacteriol.* 80, 296-302.

251. Thampuran,N. (1987). Ph.D. Thesis. Quantitative and Qualitative studies on the bacteriology of frozen fishes and prawns. Cochin University of Science and Technology, Cochin-16.

252. Thampuran,N. and K.M.Iyer. (1985). Evaluation of certain factors affecting the total plate count of frozen seafoods. Harvest and Post Harvest Technology of Fish. Soc. Fish. Technol., India. 156-159.

253. Thampuran,N. and P.K.Surendran. (1998). Occurrence and distribution of *Vibrio vulnificus* in tropical fish and shellfish from Cochin (India). *Lett.Appl.Microbiol.* 26, 110-112.

254. Thatcher, F.S. *(1960) Food in Canada, 20, 24. Quoted from Shewan.J.M. (1961)- Fish as Food.Vol.I.

255. Thornley,M.J.* (1957). Observations on the microflora of mince chicken. Meat irradiated with 4 mev cathode rays. *J.Appl.Bacteriol.*20, 286-298.

256. Toba, T., S.K.Samant. and T.Itoh. (1991). Assay System for detecting bacteriocin in microdilution wells. *Lett. Appl. Microbiol.* 70, 473-478.

257. Tramer, J. *(1966). Inhibitory effect of *Lactobacillus acidophilus*. *Nature*, 211, 204-205.

258. Tressler.D.K. and C.F.Evers. *(1957). In the freezing preservation of food.Vol.I.p.1067. The AVI Publishing Company,Inc., Westport,Conn.

259. Upreti, G.C. and R.D.Hindsill. (1973). Isolation and characterization of a bacteriocin from a homofermentative lactobacillus. Antimicrobial Agents and Chemotherapy. 4, 487-494.

260. USFDA (1995). Bacteriological Analytical Manual (Food and Drug Administration) Publ.AOAC International Maryland, 20877,USA.

261. Valdimarsson.G. and B.Gudbjornsdottir. (1984). The microbiology of stock fish during the drying process. *J.Appl.Bacteriol.* 57, 413-421.

262. Van Laack,R.L.J.M., U.Schillinger. and W.H.Holzapfel .(1992). Characterization and partial purification of bacteriocin produced by *Leuconostoc carnosum* LA 44A. *Int.J.Food. Microbiol.* 16, 183-195.

263. Vandamme,P., B.Pot, M.Gillis., P.deVos., K.Kersters., and J.Swings.(1996) Polyphasic taxonomy a consensus approach to bacterial systematics. *Microbiol Rev.*60, 407-438.

264. Vandenbergh, P.A. (1993). Lactic acid bacteria, their metabollic products and interference with microbial growth. *FEMS Microbiol Rev.* 12, 221-238.

265. Vanderzant,C., B.F.Cobb. and R.Nickelson. (1974). Role of microorganisms in shrimp quality. A research summary.pp.45.Texas A and M University, SeaGrant College,Texas.

266. Velazquez,L., C.del., M.E.Escudero. and A.M.S.de.Guzman. (1996). Prevalence of *Yersinia enterocolitica* in Lake (*Merluccius hubbsi*) fillets. *J. Food. Prot.* 59, 781-783.

267. Venkataraman, R. and A. Sreenivasan. *(1953). A preliminary investigation of the bacterial flora of the mackerels of the west coast. *J. Med.Res.* 40, 529-533.

268. Walker, P., D. Cann. and J.M. Shewan.* (1970). The bacteriology of "scampi" (*Nephrops norvegicus*). I. Preliminary bacteriological, chemical and sensory studies. *J. Fd. Technol.* 5, 375-385.

269. Wang, M.Y. and D.M.Ogrydziak. (1986). Residual effect of storage in an elevated carbon dioxide atmosphere on the microbial flora of rock cod (*Sebastes* spp.). *Appl.Environ. Microbiol.* 52, 727-732.

G8 2 27

270. Weagant, S.D., P.Nisado., K.G.Colburn., J.D.Torkelson., F.A.Stanley., M.H.Krane., S.C.Shields. and C.F.Thayer. (1988). The incidence of *Listeria* species in frozen seafood products. *J. Food. Prot.* 51, 655-657.

271. West,C.A and P.J.Warner. (1988). Plantacin B, a bacteriocin is produced by *Lactobacillus plantarum* NCDO 1193, *FEMS Microbiol Letters*. 49: 163-165.

272. West,P.A., C.J. Knoweles. and J.V. Lee. (1980). Ecology of *Vibrio* spp including *V.cholerae* in water in Kent,U.K. *Soc.Gen.Microbiol.*Quart.7,80.

273. Williams, O.B., L.L.Campbell. and H.B.Rees. (1952). *Texas J. Sci.*, 4, 53. Quoted from Shewan.J.M. (1961)-Fish as Food.Vol.I.

274. Williams,A.M., J.L.Fryer. and M.D.Collins. (1990). *Lactobacillus piscicum sp.nov.*a new *Lactococcus* species from salmonid fish. *FEMS.Microbiol.Lett.*68, 109-114.

275. Wong-Hin-dung, Chen-Li-Li. Yu-Chung-Ming (1995). Occurrence of vibrios in frozen seafoods and survival of psychrotrophic *Vibrio cholerae* in broth and shrimp homogenate at low temperatures. *J. Food. Prot.* 58, 263-267.

276. Wood, E.J.F. *(1953). Heterotrophic bacteria in marine environments of E. Australia. *Austr. J. Mar. Fresh. Res.* 4, 160-200.