Taxonomic studies on *Lactobacillus* and *Pediococcus* isolates from fermented tef *(Eragrostis tef)* and kocho *(Ensete ventricosum)* and microbiological safety of the baked products

Ayele Nigatu

Addis Ababa University
School of Graduate Studies

Addis Ababa
June, 1998.

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Systematics of *Lactobacillus* and *Pediococcus* isolates from fermented tef (*Eragrostis tef*) and kocho (*Ensete ventricosum*) and microbiological status of the baked products

by

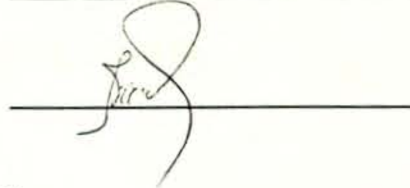
Ayele Nigatu

Department of Biology, Faculty of Science

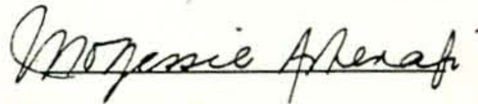
Approved by the Examining Board:

Prof. S. Feresu
External examiner

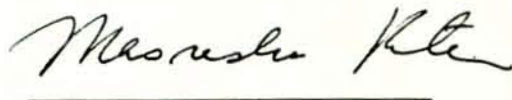
Dr. Fekadu Beyene
Internal examiner



Prof. Mogessie Ashenafi
Advisor



Dr. Masresha Fetene
Head, Department of Biology



Addis Ababa University
School of Graduate Studies

Taxonomic studies on *Lactobacillus* and *Pediococcus* isolates from fermented tef *(Eragrostis tef)* and kocho *(Ensete ventricosum)* and microbiological safety of the baked products

Ayele Nigatu

(A dissertation submitted to the Department of Biology in partial fulfillment for the Ph.D. degree in Biology)

Addis Ababa
June, 1998.

አቤቱ፡ እንደቃልህ፡ ለባሪያህ፡ መልካም፡ አደረግህ፡
በትእዛዛትህ፡ ታምኛለሁና፡ መልካምነትን፡ ምግባርና፡
ዕውቀትን፡ አስተምረኝ።

መዝ 118(119)፥65-66

***"...the sure and definite determination (of species of bacteria) requires so much time, so much acumenof eye and judgment, so much of perseverance and patience that there is hardly anything else so difficult-*Mueller"** (From the cover page of the *Int. J. Syst. Bacteriol.*)

Dedicated to:

Meseret Taddesse, for your encouragement, stamina in shouldering all the burdens and love; Hilena, Ruth and Dawit, for your patience and love; Yeshiwork Taddesse, for your genuine compassion and support.

ACKNOWLEDGEMENTS

It is absolutely essential at this juncture to witness that the entire work accomplished in this thesis is neither an out put nor due to a mere effort of a single individual. Thus it would be fair to breathout due thanks to all of you who in one way or the other enabled me arrive at the present state of this work.

I would however like to mention few names:

My supervisor Assoc. Prof. Göran Molin, it is a pleasure for me to tell you that I am truly impressed by your stimulating encouragement, guidance, critical views, reading the thesis and manuscripts patiently and showing me the way to microbial taxonomy. My co-supervisor Assoc. Prof. Siv Ahrné, it is really nice to work with you; thanks for showing me the road to microbial genetics. When ever I remember the beginning of my study I can't forget my former supervisor, Prof. Berhanu A. Gashe, who from the start coined this project, found such appropriate supervisors and paved the layout of my study. I am very grateful to you for your unlimited encouragement to grow in microbial systematics. May I thank the support of Dr. Demissu Gemeda during the first phase of my study period and Prof. Tewodros Solomon for your encouragement of my study.

Many thanks should also go to my recent supervisor Prof. Mogessie Ashenafi, for your help towards finalizing my work.

Special thanks should also go to my friends Diya Adawi, Behzad Kasravi, Åsa Eneroth, Birgitta Sorenby, Martin Antonsson, Anita Sandberg, Jose de Cruiz, Mikel Quednau, Kristina Lodiate, Stalin Santacruz, Fredrik Tufesson, Wang Mei, Anders, Ingmar, Markus, Marie-Louis Johansson, Ann-Marie Lindberg, Crister and Rafal for your unlimited support compassion and kindness. It is very hard to forget the support of my friends Teketel Abebe, Nigussie Megersa, Kelemu Lemma, Kelbessa Urga, Habtamu Fufa, Tassew Dabba, Chernet Aboye, Drs. Aberra Geyid and Almaz Abebe, Senaiet Zewdie, Drs. Seyfu Ketema and Abebe Demissie, Almaz Negash, Gashaw Mamo, Dr. Fiseha Mekuria and Eleni for your support. Whenever I remember Friday dinner the very special "Asreson of Lund" it would be nice to acknowledge the energy I got from the delicious chicken supplemented with your entertainment Amare Gessesse, Girma

Akalu, Yirgalem Yigzaw and Tigist Girma. Thank you so much for you social and scientific input to my work. My brothers and sisters Negash Gebre-Tsadik and Etfwork Asnake, Fekade Gebre-Mikael and Zewdnesh Dubale, Solomon Gebre-Mariam and Mulatua Tilahun, Tagegn and Arne Almqvist, Azaria, Haregewoin and Gebre, Azeb and Rasmus Witzel, Hiruy Kidane and Tsehay Haile, Mekdes, Sihin and Dr. Tarkegn Adebo, Simegn and Tesfaye, thank you so much for your support during my stay in Sweden and valuable discussions. Due thanks to my sisters and brothers, Amsale Zenebe, Genet Zenebe, Mulumebet Zenebe, Bisrat Berhanu, Nega Wubayehu, Efrem Girma, Wondwossen Taddesse, Abebe Bekele and Tamiru Kassa. It would eventually be unwise if I forget to thank my friends Genet Emeshaw and Alem, Zewdinesh, Ato. Teshome Teferi and Tewodaj Meaza, Almaz Asseffa, Bekelu Teka, Aberru Nurga, Alem Abele, Senaiet, Astawesegn and Simret and all those who I did not mention your names here for your encouragement and support.

At last I should witness that the entire work would not have been a reality without the sole support of my beloved wife Meseret Taddesse, my sister Yeshiwork Taddesse and the love of my children. Many thanks should also go to my father Ato Nigatu Chernet for your love, unlimited encouragement and concern throughout my life.

I am also grateful to Mrs. Kerstin Johnson (SAREC) and Assoc. Prof. Malin Akerblom (IPICS, ISP) for enabling me print the thesis. The project was financially supported by SAREC-Sida through the School of Graduate Studies, Addis Ababa University.

ABSTRACT

Tef dough and *kocho* are products of typical lactic acid food fermentations. Both foods are the main dietary sources in Ethiopia. Lactic acid bacteria (LAB) belonging to *Lactobacillus*, *Pediococcus* and *Leuconstoc* are amongst the main fermentative microflora in these foods. Molecular taxonomic studies using RAPD, DNA:DNA hybridization, 16S rRNA sequencing and API 50 CH fermentation profiles were carried out on food isolates together with type and reference strains of *Lactobacillus* and *Pediococcus* species. The results revealed the presence of a diverse range of species in *tef* and *kocho*. *Lactobacillus* and *Pediococcus* species were abundant in both foods while *Weissella* species were identified among *kocho* isolates. The use of RAPD was valuable for grouping lactobacilli and pediococci isolates from *kocho* and *tef*. The method enabled discrimination between phenotypically inseparable pediococci, *Ped. acidilactici* and *Ped. pentosaceus* and among type strains of *Lactobacillus* species. Its use might however, be limited in large number of lactobacilli isolates. DNA homology and sequence data were found to be supplementary and essential for efficient identification of an isolate. API 50 CH utilization profiles were influenced by incubation temperature in many strains of *Lactobacillus* species. Diversity and higher test vigours of isolates while utilizing different carbohydrates showed pertinent metabolic roles in fermenting *tef* dough and *kocho*. The inability of different pathogenic and spoilage bacteria to survive the fermentation processes and baking heat implied the high safety levels of the products.

TABLE OF CONTENTS

PAGE

ACKNOWLEDGEMENTS 4
ABSTRACT 6

CHAPTER ONE

INTRODUCTION 10
1.1. Food fermentations 10
1.2. Lactic Acid Bacteria (LAB) 12
1.3. Taxonomy of lactic acid bacteria 16
- 1.3.1. Phenotypic methods 17
  - 1.3.1.1. Common phenotypic analyses 17
  - 1.3.1.2. Automated systems 18
  - 1.3.1.3. Cell wall composition 18
  - 1.3.1.4. Whole-cell protein analysis 19
  - 1.3.1.5. Lactate dehydrogenase 19
- 1.3.2. Genomic methods 20
  - 1.3.2.1. Moles per cent G+C 20
  - 1.3.2.2. DNA-DNA reassociation studies 21
  - 1.3.2.3. rRNA homology studies 22
    - Ribotyping 23
  - 1.3.2.4. DNA-based typing methods 23
    - (a) Restriction Endonuclease Analysis (REA)
    - (b) Restriction Fragment Length Polymorphism (RFLP)
    - (c) Plasmid DNA analysis
    - (d) Randomly Amplified Polymorphic DNA (RAPD) and other PCR-based DNA typing methods
- 1.3.3. The polyphasic approach 26
- 1.3.4. The genus *Lactobacillus* 29
  - Grouping of lactobacilli 30
- 1.3.5. The genus *Pediococcus* 35
- 1.3.6. The genus *Weissella* 37

1.4. The role of LAB in food fermentations 38
1.5. Ecology of LAB and their applications 43
- 1.5.1. Antagonistic properties of LAB against pathogens 46
- 1.5.2. LAB as probiotics 48

1.6. Status and features of lactic acid fermented foods in Ethiopia 51
1.7. Tef dough fermentation 53
1.8. Kocho fermentation 56
1.9. Why the present study? 59

1.10. Aims of the study 61
1.10.1. General 61
1.10.2. Specific 61

CHAPTER TWO
MATERIALS AND METHODS 62

2.1. Food samples, sampling and sampling sites 62
2.1.1. *Tef* 62
2.1.2. *Kocho* 64
2.2. The effect of baking heat on the antimicrobial properties of tef dough, kocho, injera, aradisame and fate of pathogens and contaminants 64
2.2.1. Test bacteria and culture media 64
2.2.2. Preparation of extracts, heat treatment and assaying 65
2.3. Taxonomic studies 66
2.3.1. Genomic identification 68
2.3.1.1. Randomly Amplified Polymorphic DNA (RAPD) 68
2.3.1.2. DNA-DNA Hybridization 70
2.3.1.3. 16S rRNA sequencing 71
2.3.2. Phenotypic studies using API 50 CH 71

CHAPTER THREE
RESULTS 73

3.1. The effect of baking heat on the antimicrobial properties of tef dough, kocho, injera, aradisame and fate of pathogens and contaminanats 73
3.2. RAPD for discrimination of Pediococcus pentosaceus and Ped. acidilactici and for strain typing in pediococci 76
3.3. Identification of pediococci from kocho and tef 78
3.4. Identification of lactobacilli from kocho and tef 84
3.5. RAPD for distinction of Lactobacillus type strains 92
3.6. Effect of incubation temperature on API 50 CH metabolic profiles of Lactobacillus species and related taxa 95

CHAPTER FOUR
DISCUSSION 101

4.1. The effect of baking heat on the antimicrobial

properties of tef dough, kocho, injera, aradisame and fate of pathogens and contaminanats 101

4.2. RAPD for discrimination of Pediococcus pentosaceus and Ped. acidilactici and for strain typing in pediococci 103

4.3. Identification of pediococci from kocho and tef 104

4.4. Identification of lactobacilli from kocho and tef 107

4.5. RAPD for distinction of Lactobacillus type strains 110

4.6. Effect of incubation temperature on API 50 CH metabolic profiles of Lactobacillus species and related taxa 112

CONCLUSIONS 116

REFERENCES 117

CHAPTER ONE

INTRODUCTION

1.1. Food Fermentations

Food fermentation, is the oldest means of biotechnology, which has been used by man for many thousands of years. It was one of the methods (including drying and salting) of preservation developed from need. As recorded history tells us, the Egyptians, Sumarians, Babylonians and Assyrians were skilled in producing alcoholic beverages from barley. Early Christian and Sanskrit records also cite the use of fermented dairy products. The Romans were processing fish and used fish sauce around AD 200 in Europe and North Africa along with fermented milk such as sweet and savory products (Campbell-Platt, 1987). As clearly presented by Pederson (1979), Campbell-Platt (1987) and Steinkraus (1994), alcoholic fermentation of barley to beer and grapes to wine represented early evidences. Babylonians brewed beer which was supposed to have been produced through both lactic and alcoholic fermentations and exported it to Egypt around 300 BC. 'Leavening' of sour dough, the use of 'dough starter', wine making, production of alcoholic beverages, and references in relation to fermented products were cited in many places in the holy bible, both in the Old Testament and the New Testament (e.g. Luke 13:21). Food fermentation has been believed to have evolved from "natural" processes in which nutrient bioavailability and environmental selective pressures resulted in specific microbial species and strains by the use of starter cultures and possibly strain improvement.

Fermentation is a process by which raw substrates are biochemically converted by activated endogenous enzymes or from actions of microorganisms into more nutritious, energy-rich and acceptable products (Pederson, 1979). These significant changes involve development of new aroma, flavour, taste, and texture therein increasing the sensory quality, palatability and acceptability of the product (Campbell-Platt, 1987; Steinkraus, 1994). Fermented foods are known to play a crucial role particularly in the daily diet of people living in tropical developing countries where the fermentation of cereal and root crops remarkably contributes to the chemical and microbiological safety of the products (Cooke, *et al.*, 1987). It is also evident that sour-fermented

cereal products are able to arrest proliferation of diarrhoegenic bacteria, a serious and primary cause of infant and child mortality in the tropics (Mensah, *et al.*, 1991; Simango and Rukure, 1992; Svanberg, *et al.*, 1992). Besides this, fermentation is relatively a low-energy preservation which increases the shelf life of products (Cooke, *et al.*, 1987).

The process of fermentation not only improves the keeping quality and palatability but also the digestibility and nutritional value of the end product (food or feed). This is by increasing nutrient availability and modifying or reducing antinutritional food components such as tannins, phytates, polyphenols, glucosides (such as linamarins, lantamarins), cellulose, hemicellulose and polygalacturonic and glucuronic acids (Campbell-Platt, 1987; Steinkraus, 1994). Breakdowns of these compounds may result in improved bioavailability of mineral and trace elements (Steinkraus, 1994; Kalantzopoulos, 1997). Additional nutrients such as vitamins, amino acids and proteins may be synthesized during fermentation. Animal products, including dairy, meat, fish, and related substrates are fermented resulting in products of superior quality than the raw materials (Steinkraus, 1994). In foods of plant origin, the chemical composition and biophysical properties of the raw foods differ. Roots and tubers such as potatoes, yam, cassava and enset as well as cereals and tree-crops, such as bread fruit, are known to possess high content of starch, whereas legumes and oil seeds have a higher protein content. Green vegetables, carrot, beet, tomato, olive, cucumbers, okra and forage crops for animal feed silages are known to have a high moisture content; fruits contain high concentrations of reducing sugars (Nout and Rombouts, 1992; Steinkraus, 1994; Gebre-Mariam and Schmidt, 1996). Each type of raw food substrate has its own unique combination of physical structure, chemical composition and associated natural microflora which influence the succession of microbial development and action of endogenous enzymes. However, the availability of fermentable substrate is the key factor for fermentation to take place. Cereal grains and milled products contain only 0.5-2.5% of freely available carbohydrates as mono- and di-saccharides (Becker and Hanners, 1991). Autoamylolysis at ambient temperatures and moisture, may occur by endogenous hydrolases such as alpha- and ß-amylases (Odunfa and Adeyele, 1987). The fermentative preservation of vegetables and cereals is mainly as a result of the action of lactic acid bacteria (LAB) in co-operation with fermentative yeasts. Other bacteria, such as *Bacillus* spp., or filamentous fungi such as *Rhizopus* and

Aspergillus spp. are unequivocally essential for the fermentation of some legumes and oil seeds (Campbell-Platt, 1987).

1.2. Lactic Acid Bacteria (LAB)

The name Lactic Acid Bacteria (LAB) refers to a functional group and not a taxonomic one. LAB comprise a diverse group of Gram-positive, non-spore-forming cocci, cocco-bacilli or rods which are generally non-pathogenic to man and animals. In most cases they are anaerobic, microaerophilic or aero-tolerant in their oxygen demands. They are generally catalase- and oxidase-negative although some may produce pseudocatalase when grown at low sugar concentrations. Most LAB grow between 10°C and 45°C although there may, in some cases, be growth at lower or higher temperature ranges. They are chemo-organotrophic and grow in complex media. They need fermentable carbohydrates for growth and produce lactic acid as a sole, or main product from the energy-yielding fermentation of sugars. Accordingly they may be grouped as homofermentative those mainly producing lactate from glucose or heterofermentative those that produce lactate, CO_2 and ethanol (Kandler and Weiss, 1986). Homofermenters are further divided into obligate homofermenters and facultative heterofermenters based on their end products from hexoses. Facultative heterofermenters utilize hexoses and produce almost entirely lactic acid via the Embden-Meyerhof-Parnas (EMP) route. The obligate heterofermenters instead ferment hexoses using the phosphogluconate pathway and produce lactate, ethanol (acetic acid) and CO_2 (Wood and Holzapfel, 1995).

The homolactic fermentation follows the EMP pathway of glycolysis. The enzyme aldolase plays a pivotal role in the EMP metaboilc route. The heterolactic fermentation instead follows two routes where one is limited to bifidobacteria, which are not LAB, hence called the Bifidus pathway and the other the 6-phospho-gluconate pathway that is common to LAB. Both of these later pathways are also called the phosphoketolase or heterofermentative pathways and the enzyme phosphoketolase plays a key role (see Figure 1). Glucose, fructose and sucrose are the primary substrates in plant material that are fermented by LAB. Pentoses, citric acid and malic acid are also present in most plant material though in small quantities and can also be fermented

by LAB. Mannitol is usually formed during heterolactic fermentation by the reduction of fructose and serves for heterofermenters to dominate later. Free pentoses are not normally available in intact plants but are liberated after harvest as a result of hydrolysis of hemicellulose. Hexose metabolism is presented in Figures 1.

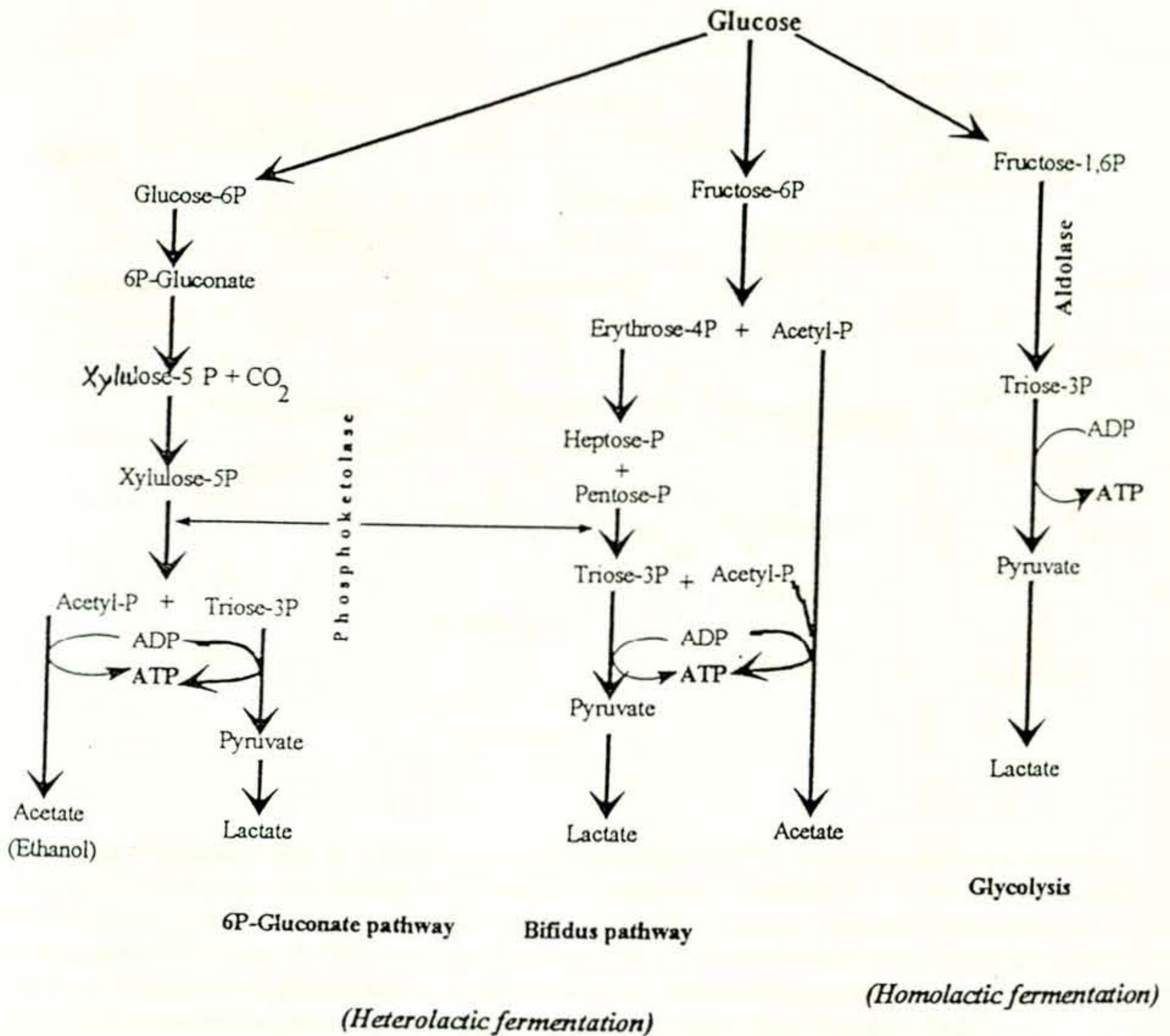
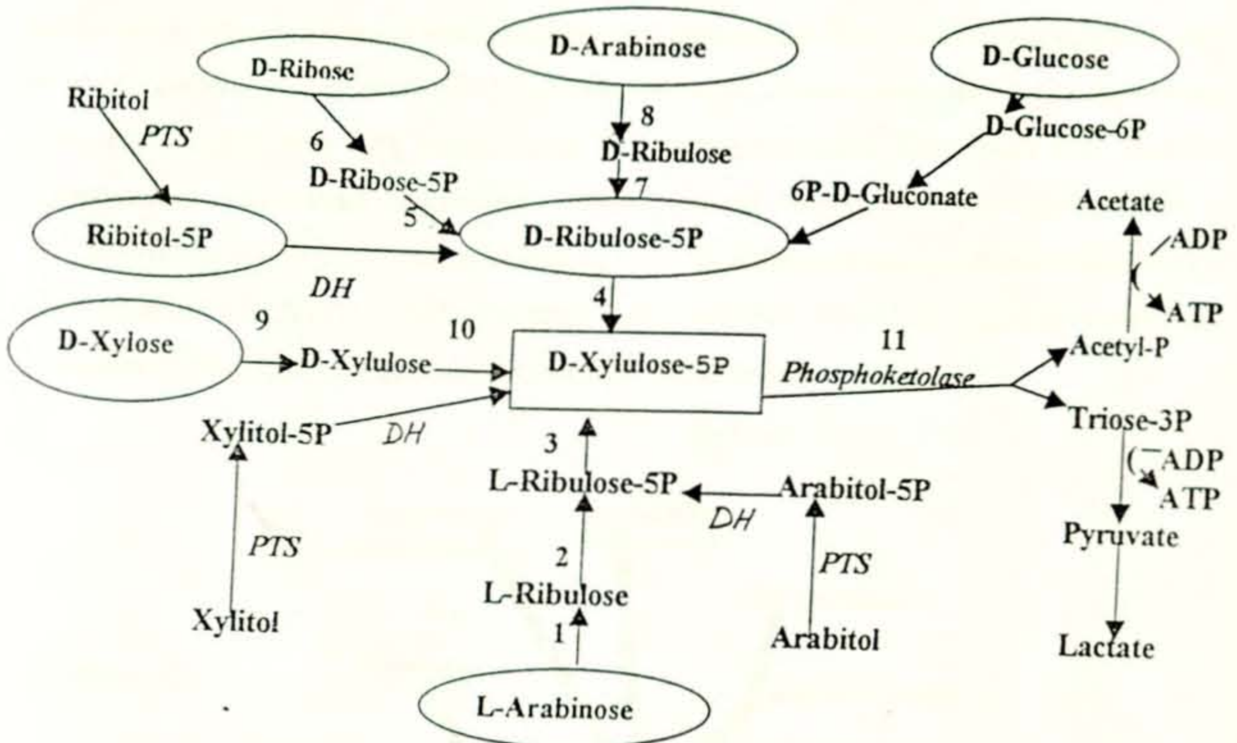


Figure 1. Hexose fermentation in lactic acid bacteria *(Adapted from Kandler, 1983).*

Most LAB frequently isolated from fermenting plant materials are either obligate or facultative heterofermenters hence they make use of their phosphoketolase for the dissimilation of

pentoses. On the other hand, the strictly homofermentative lactobacilli are rarely isolated from plant fermentations because they lack this enzyme (Daeschel, *et al.*, 1987). Heterofermentative LAB ferment pentoses via the pentose phosphate pathway where equimolar amounts of lactate and acetate are produced through catalysis by phosphoketolase (Figure 2).



The enzymes involved include:*1, L-Arabinose ketol-isomerase; 2, ATP:L-ribulose 5-phosphotransferase; 3, L-ribulose 5-phosphate 4-epimerase; 4, D-ribulose 5-phosphate 3-epimerase; 5, D-ribose 5-phosphate ketol-isomerase; 6, ATP:D-ribose 5-phosphotransferase; 7, ATP:D-ribulose 5-phosphotransferase; 8, D-arabinose ketol-isomerase; 9, D-xylose ketol-isomerase; 10, ATP:D-xylulose 5-phosphotransferase; 11, D-xylulose 5-phosphate D-glyceraldehyde 3-phosphate lyase; DH, dehydrogenase.*

Figure 2. Pentose metabolism in lactic acid bacteria ***(Adapted from Kandler, 1983).***

A variety of other carbohydrates and sugar alcohols are also metabolized by LAB as energy sources. Previously these fermentation properties were used as a basis for LAB taxonomy (Kandler

and Weiss, 1986). Nevertheless, apart from the genuine LAB, other taxa were also found to follow such metabolic routes. The term lactic acid bacteria still refers to a functional group and not a taxonomic one. Recently the delineation and assignment of taxa has thus been shifted from metabolic relationships and now is based on phylogenetic, hence those related taxa producing lactic acid are excluded from the group (Wood and Holzapfel, 1995). According to this recent taxonomic rearrangement based on 16S and 23S rRNA sequence data, the Gram-positive bacteria are known to have two lines of descent (Figure 3); those with a DNA base composition less than 50 mol% guanine plus cytosine (G+C) form the so-called *Clostridium* branch, while those with G+C mol% higher than 50% belong to the Actinomycetes branch. The typical LAB such as *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus thermophilus*, have a G+C content below 50 mol% and hence belong to the *Clostridium* branch (Schleifer and Ludwig, 1995). Due to its higher G+C mol%, the genus *Bifidobacterium,* which was originally regarded as a member of the LAB, is assigned into the Actinomycetes branch.

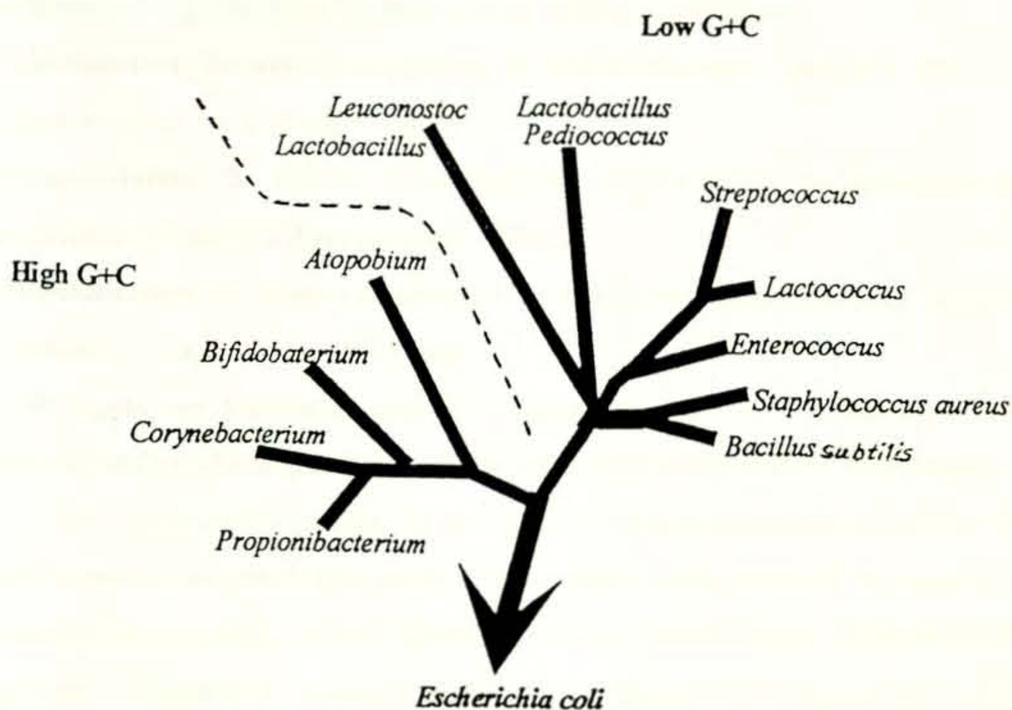


Figure 3. Phylogenetic relationships of Gram-positive bacteria based on 16S rRNA sequence similarities ***(Modified from Schleifer and Ludwig, 1995).***

True lactic acid bacteria belong to the following genera: *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella.* LAB are distributed in various habitats and are thus frequently found in foods (dairy products, fermented meat, sourdough, fermented vegetables, silage, beverages), on plants, in sewage and in the genital, intestinal and respiratory tracts of man and animals (Marklinder and Lönner, 1992; Hammes and Vogel, 1995). LAB associated to food and feed are very important and responsible for the desirable changes in the final products.

1.3. Taxonomy of Lactic Acid Bacteria

Bacterial systematics is the scientific study of organismal diversity and interrelationships with an eventual goal of characterizing and arranging them in an orderly manner (Ludwig and Schleifer, 1995). Taxonomy is empirically understood as a synonym of systematics or biosystematics and traditionally consists of the following components.

(i) **Classification**: the orderly arrangement of defined taxonomic units (e.g. species) into groups (e.g. genus) on the basis of relationships;

(ii) **Nomenclature**: the labeling of units defined in (i) according to the International Code of Nomenclature of Bacteria (Lapage, *et al.*, 1975);

(iii) **Identification**: the process of determining whether an organism belongs to one of the units defined in (i) and labelled in (ii); and

(iv) **Phylogeny and population genetics**: Vandamme, *et al.* (1996) further moved on addition of phylogeny and population genetics for a complete definition of modern biosystematics.

If a bacterial classification has to reflect the natural relationships between bacteria, then various methods are needed to confirm the presence and extent of relatedness. As a result extensive information is utilized describing various features of an organism. Comparison of organismal characteristics serves to affiliate the organism with a group with which it shares common properties. The determination of at least part of the most typical properties would thus be essential in order to evoke reliable identification.

Previously classification was based on phenotypic expression and therefore it did not

reflect phylogeny. Thus it was difficult to draw up phylogenetic trees because bacteria being unicellular did not have any fossil records. The breakthrough was then the application of 16S rRNA analysis, and as a result there were major revisions in the classification of most prokaryotic taxa (Woese, 1987). The information obtained during classification is used to define prokaryotes into their basic taxonomic units, i.e., the species (Schleifer and Stackebrandt, 1983, Starr, *et al.*, 1986). The different methods are thus used to confirm the presence and extent of relatedness, i.e., identification.

Different types of information may be used for this purpose but as a group they should all serve to identify, classify and characterize the isolate. The major phenotypic and genetic methods employed for the taxonomy of lactic acid bacteria are briefly described below.

1.3.1. Phenotypic methods:

Phenotypic methods include all procedures that do not make use of DNA or RNA. These could be categorized as follows.

1.3.1.1. Common phenotypic analyses:

Phenotypic traits include those common features such as morphological, physiological and biochemical properties used in any laboratory for identification and association of an isolate to a given taxon. Morphological features could be cell shape; endospore formation; presence or absence of flagella and inclusion bodies; Gram's reaction and colour, form and dimension of colonies. Properties of physiological and biochemical traits are quantified from data on growth at different temperatures, pH values, concentrations of salt, atmospheric conditions as well as growth patterns in the presence of substances like antibiotics; possession and activity of different enzymes; and assimilation and production of specific compounds. Historically data on these properties served as the basis for LAB taxonomy before the use of chemotaxonomy and the recent advent of nucleic acid-based phylogenetic analyses (Axelsson, 1993; Schleifer and Ludwig, 1995). Phenotypic traits were, however, be effectively used in standardized procedures to get reproducible results. Based on these properties LAB were characterized phenotypically as Gram-

positive, catalase- and oxidase-negative, non-spore-forming, cocci, cocco-bacilli or rods. LAB need fermentable carbohydrates for growth and ferment glucose to produce mainly lactic acid (homofermentatives) or in addition CO_2, ethanol and/or acetic acid (heterofermentatives) (Axelsson, 1993). This definition has however been found ambiguous since other genera unrelated to the true lactic acid bacteria could also display similar characteristics (Holzapfel and Wood, 1995; Schleifer and Ludwig, 1995) and is therefore inexhaustive for LAB identification.

1.3.1.2. Automated Systems:

Commercial kits containing dehydrated substrates such as API 20 and API 50 CH fermentation systems have been used for a long time for many genera including LAB. These kits contain carbohydrates, polyols and glucosides whose assimilability could be detected by the change in colour after incubation for 1, 2 and 7 days. The API strips are not strictly automated systems as they could either be read by eye or be automated.

1.3.1.3. Cell wall composition:

The distribution of the different teichoic acid and murein types in lactobacilli is well reviewed by Hammes and Vogel (1995). Cell wall composition is a useful taxonomic character in Gram-positive bacteria as it is specific to a genus or a species (Schleifer and Kandler, 1972) but the analysis is time consuming. Despite this cumbersome analysis, lactobacilli could be grouped using cell wall composition as follows (see details in section 1.3.4.):

Homofermentative and heterofermentative lactobacilli from the *Lactobacillus delbrueckii* group (Groups **Aa** and **Ba**, respectively) are known to possess the Lys-D-Asp-type. The obligate heterofermentatives belonging to the *Leuconostoc* group (group **Cc**) all have the Lys (monoamino, monocarbonyl) amino acid type (Lys-L-Ser-L-Ala_2 or Lys-L-Ala_2) (Dellaglio, *et al.*, 1995). Representatives from *Lb. casei-Pediococcus* group (homofermentative [**Ab**], facultatively heterofermentative [**Bb**], and obligately heterofermentative [**Cb**]) have either the Lys-D-Asp type or the diaminopimelic-direct type (Vandamme, *et al.*, 1996). However, some exceptions have been found such as, *Lb. fermentum* and *Lb. vaginalis*, where lysine is replaced by the chemically

similar ornithine (Orn-D-Asp type); the Lys-Ala type is found in *Lb. sanfrancisco* and likewise the Orn-D-Asp type in *Lb. pontis* (Vogel, *et al.*, 1994; Vandamme, *et al.*, 1996). Furthermore, Simpson and Taguchi (1995) have elaborated the distinct cell wall composition of pediococci stressing their possession of interpeptide bridges of the L-Lys-L-Asp type between alanine and lysine residues. In addition they are known to contain D-Asp linkages between positions three and four of the two peptide bridges. In *Ped. urinae-equii* however, like in *Aerococcus viridans*, the presence of only one type of peptidoglycan polypeptide without any interpeptide bridge has been demonstrated. In these organisms therefore the D-Ala carboxyl residue was found to bind to the amino group of the adjacent L-Lys. In all pediococci, however, teichoic acids were not found in the cell walls (Garvie, 1986).

1.3.1.4. Whole-cell protein analysis:

This method is being utilized as a rigorous procedure for resolving uncertainties in identification between different LAB as it particularly helps distinguishing strains at the species and subspecies levels (Van den Berg, *et al.*, 1993; Descheemaeker, *et al.*, 1994; Patarata, *et al.*, 1994; Tsakalidou, *et al.*, 1994; Devriese, *et al.*, 1995). The method has been employed for lactococci (Descheemaeker, *et al.*, 1994), vagococci (Pot, *et al.*,1994), lactobacilli (Du Plessis and Dicks, *et al.*, 1995; Dicks, *et al.*,1987), *Lb. acidophilus* (Pot, *et al.*, 1993) and *Leuconostoc* spp. (Barreau and Wagner, 1990; Dicks and Van Vuuren, 1990; Villani, *et al.*, 1997). It was also used for examining relatedness of heterofermentative lactobacilli (Dicks, *et al.*, 1987) and to differentiate subspecies of *Lb. curvatus* and *Lb. sake* (Torriani, *et al.*, 1996). Recently Tsakalidou, *et al.* (1997) have employed SDS-PAGE for a taxonomic study of unidentified *Weissella* species. With the creation of the database for all studied LAB species, the use of the SDS-PAGE method has been found productive and sensitive (Pot, *et al.*, 1993; Vandamme, *et al.*, 1996).

1.3.1.5. Lactate dehydrogenase:

The electrophoretic mobility of lactate dehydrogenases (LDH) in lactic acid bacteria has been studied originally using starch gels (Gasser, 1970) and later on with SDS-PAGE (Hensel, *et*

al., 1977). The method had clearly distinguished species such as *Lb. acidophilus*, *Lb. crispatus*, *Lb. gallinarum*, *Lb. gasseri* and *Lb. johnsonii* which were indistinguishable by other phenotypic tests (Fujisawa, *et al.*, 1992). NAD-dependent D-LDH patterns have also been used for *Leuconostoc* species (Dicks and Van Vuuren, 1990). It has been used in combination with quantitative immunological techniques as a phylogenetic marker along with other enzymes such as fructose-1, 6-biphosphate aldolase, malic enzymes and glyceraldehyde-3-phosphate dehydrogenase (London and Kline, 1973).

1.3.2. Genomic methods:

Genomic information is generated from the nucleic acids (DNA and RNA) of the bacterial cell. Various methods are available amongst which those in common use are mentioned below:

1.3.2.1. Moles per cent G+C:

This is one classical genotypic method employed in taxonomic studies of almost all bacteria (Johnson, 1986). Generally the moles percent G+C does not exceed 3% within a well defined species and 10% within a well defined genus. The overall moles percent G+C ratio is 24-76% across all bacteria (Ludwig and Schleifer, 1995).

As a general rule the DNA base composition of LAB is less than 50 mol% G+C; but in the genus *Lactobacillus*, because of the apparently higher genetic diversity of the different species, there is a variation between 32 to 54 mol% (Hammes and Vogel, 1995). The highest value was recorded for *Lb. pontis* (53-55 mol% G+C) followed by *Lb. fermentum* (52-54 mol % G+C) and *Lb. sharpae* (53 mol% G+C) and the minimum for *Lb. mali* (32-34 mol% G+C) (Vogel, *et al.*, 1994; Hammes and Vogel, 1995). This span is actually about twice as large as that normally accepted for a well defined genus (Schleifer and Stackebrandt, 1983). This is the major reason for the strong need to reorganize the genus into small groups (Hammes and Vogel, 1995; Schleifer and Ludwig, 1995).

1.3.2.2. DNA-DNA reassociation studies:

This procedure relies on the determination of the degree of hybridization between heteroduplexes and the decrease in the thermal stability of the hybrid. Stackebrandt and Goebel (1994) have explicitly explained the greater value of DNA reassociation as the best applicable procedure at the present time. They further emphasized the very reason why DNA reassociation is used as a gold standard for species delineation based on data generated from several studies showing strong agreement between DNA relatedness and chemotaxonomic, genomic, serological and numerical phenetic similarity. The species is microbiologically defined in the polyphasic approach as it would generally include strains "...approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m " and "Phenotypic characteristics should agree with this definition..." (Stackebrandt and Goebel, 1994).

Various protocols are available for hybridization studies each with its own peculiarity. Those previously in common use were the hydroxyapatite method, the optical renaturation rates method and the S1 nuclease method. These methods have been, however, found to be time-consuming and need large quantities of DNA. As a result new, rapid procedures requiring less DNA and time are being devised (Vandamme, *et al.*, 1996).

DNA-DNA hybridization has been applied to the *Lactobacillus* and *Pediococcus* species (Dellaglio, *et al.*, 1973; Dellaglio, *et al.*, 1975; Back, 1978a,b; Dellaglio, *et al.*, 1981; Johansson, *et al.*, 1995c; Hamad, *et al.*, 1997). Mauguin and Novel (1994) have efficiently used data from the slot-blot hybridization for clear separation of phenotypically indistinguishable LAB isolates from sea food. The major constraint in using such hybridization results for assigning an isolate is there might be cross hybridization and it is sometimes difficult to quantify the degree of hybridization. A reverse dot blot procedure has also been developed for rapid and simultaneous direct identification of LAB in fermented foods (Ehrmann, *et al.*, 1994). This method has proved to be of major significance in describing new species and deciding the extent of relationships between related species and strains (Curk, *et al.*, 1996; Weise, *et al.*, 1996). Despite the use of these new methods, however, evidence is still strong that no other method including 16S rRNA sequence analysis should replace DNA reassociation for the delineation of species and measurement of interspecific relationships (Stackebrandt and Goebel (1994),.

1.3.2.3. rRNA homology studies:

The use of the ribosomal RNA (rRNA) for taxonomic purposes in bacteria is an important tool for phylogenetic decisions. This is because of its apparent universality in all bacteria, functional constancy and possession of both highly conserved and more variable domains (Olsen, *et al.*, 1991; Collins, *et al.*, 1993; Olsen and Woese, 1993; Ludwig and Schleifer, 1995; Vandamme, *et al.*, 1996). Consequently, at present there is a general consensus to use it extensively as the best molecular chronometer or phylogenetic marker. As a result the 16S rRNA and, to a lesser extent, the 23S rRNAs are utilized. Both of these macromolecules have been found to fulfill the necessary prerequisites of an ideal phylogenetic marker molecule, i.e., universal distribution, structural and functional conservation and sufficient size (Ludwig and Schleifer, 1995; Schleifer, *et al.*, 1995). Indirect comparisons using hybridizations or rRNA cataloging of RNase T_1-resistant oligonucleotides of 16S rRNA have also been utilized (Collins, *et al.*, 1993).

The gradual emergence of sequencing possibilities has brought about the availability of databases first for the 5S rRNA and later on for the 16S rRNA. Today the use of direct sequencing of partial or total 16S rRNA molecules is possible by employing PCR procedures along with appropriate primers. Sequencing of 16S rRNA by reverse transcriptase has been considered as the most rapid and powerful technique for elucidating the natural relationships of microorganisms as it gives long stretches of sequence (ca. 95% of the total sequence), that enable precise determination of phylogenetic relationships (Wallbanks, *et al.*, 1990). These sequence data, therefore, serve as a backbone for modern microbial phylogenetic taxonomy as they retain larger conserved elements which are likely to possess a wealth of information that would lead to more reliable identification/classification (Vandamme, *et al.*, 1996).

Martinez-Murcia and Collins (1990) used 16S rRNA reverse transcriptase sequences for phylogenetic analysis of the genus *Leuconostoc*. Later on such sequence analyses were employed for evaluation of similarities and description of new genera and species of LAB (Collins, *et al.*, 1991; Collins and Wallbanks, 1992; Collins, *et al.*, 1993; Ludwig and Schleifer, 1995; Schleifer and Ludwig, 1995; Mori, *et al.*, 1997). Recently Mora, *et al.* (1997) employed sequence data together with D-LDH gene-targeted multiplex PCR anlaysis for rapid and accurate discrimination

of *Ped. acidilactici* and *Ped. pentosaceus*.

The use of 16S rRNA sequences has split the various genera of lactic acid bacteria and evoked the establishment of new genera such as *Atopobium, Carnobacterium, Oenococcus, Tetragenococcus, Vagococcus* and *Weissella* (Collins, *et al.*, 1991; Collins and Wallbanks, 1992; Collins, *et al.*, 1993). The morphologically distinct genera *Lactobacillus, Leuconostoc* and *Pediococcus,* however, presumably became phylogenetically intermixed based on phylogenetic data from their 16S RNA sequences. In a similar fashion there is no good correlation between the physiologically-based subdivision of lactobacilli and their phylogenetic relatedness (Collins, *et al.*, 1991; Schleifer and Ludwig, 1995). As a result, Collins, *et al.*, (1991) proposed the subdivision of *Lactobacillus* and related genera into three phylogenetic groups, and Hammes and Vogel (1995) have very well reconciled these groupings with three corresponding physiological groupings.

Ribotyping:

In this procedure complex DNA patterns generated by restriction enzyme digestion are blotted onto a membrane and hybridized with a labeled probe which enables us to visualize the hybridized fragments. There are different protocols of ribotyping but in common they use rRNA as a probe. There might also be variations in the labelling technique and sequences used as well as utility of 16S or 23S rRNA or both, with or without the spacer region, or conserved oligonucleotide portion of the rRNA (Bingen, *et al.*, 1994). Johansson, *et al.* (1995a) have used ribotyping for *Lb. plantarum* and recommended it for species recognition but not for strain typing within this taxon.

1.3.2.4. DNA-based typing methods:

Numerous nucleic acid-based techniques have been developed in the last few years that enabled categorization of related strains of a given species into subgroups. These include restriction endonuclease analysis, restriction fragment length polymorphism, plasmid DNA analysis, ribotyping, randomly amplified polymorphic DNA and other PCR-based DNA typing

methods.

(a) Restriction Endonuclease Analysis (REA):

The use of restriction endonucleases for cutting pure DNA into different fragments of varying length has been used as an important tool for species and subspecies differentiation. Ståhl, *et al.* (1990) and Ståhl and Molin (1994) have employed it for species allocation in lactobacilli and analysis of *Lb. reuteri* strains, respectively. The procedure previously employed, although coupled with computerized numerical analysis, was time-consuming. Johansson, *et al.* (1995b) changed the protocol and as a result, the method can now be performed in a relatively shorter time. The method is reproducible and can be employed to discriminate between similar strains of the same species, to group strains with a species, and to allocate strains to a related *Lactobacillus* species. However, the produced bands are too many and the patterns are not that easy to interpret (Johansson, 1995). Quednau, *et al.* (1998) have also used REA for separation of *Enterococcus faecium* strains and have found it a powerful method for typing strains in this species.

(b) Restriction Fragment Length Polymorphism (RFLP):

The procedure employs restriction endonucleases to cut pure DNA into fragments of varying sizes which are then resolved by agarose gel electrophoresis. Developed bands are then blotted on membrane filters and hybridized to rDNA labeled probes. RFLPs of rDNA from *Lactobacillus* species were studied by Ståhl, *et al.* (1994) for *Lb. reuteri* and that of the 16S rRNA gene of chromosomal DNA by Johansson, *et al.* (1995a) for *Lb. plantarum*. Complex fragments are, however, difficult to compare.

(c) Plasmid DNA analysis:

Plasmids are common in LAB (Ahrné and Molin 1991; Kumar, *et al.*, 1994; Curk, *et al.*, 1996; Davidson, *et al.*, 1996; Langella, *et al.*, 1996) and most phenotypic traits such as carbohydrate fermentation are plasmid-mediated (Ahrné, *et al.*, 1989). Plasmids in LAB are more or less stable where they are found uniformly in most species.

Plasmid profiling has been found to be a good method for classifying lactobacilli but is only useful for those strains that carry the plasmids. For example plasmids are less frequently isolated from most intestinal lactobacilli, lactobacilli from other sources and pediococci (Davidson, *et al.*, 1996). It is possible to use RFLPs of plasmids and because of the reduced number of DNA fragment bands for comparison RFLPs of plasmids are more dependable and have a better discriminatory power than other nucleic acid-based methods (Vandamme, *et al.*, 1996). Today the procedure has become an effective discriminatory DNA-based typing method when combined with either the pulsed-field gel electrophoresis or the low frequency restriction fragment analysis technique (Gordillo, *et al.*, 1993; Johansson, 1995; Tenover, *et al.*, 1995).

(d) Randomly Amplified Polymorphic DNA (RAPD) and other PCR-based DNA typing methods:

These are methods of universal utility, simple and fast. Arbitrary primers with short sequences varying from 5 to 20 base oligonucleotides have been used in different studies (Welsh and McClelland, 1990; Williams, *et al.*, 1990; Cateno-Anolles, *et al.*, 1991). The use of RAPD with 9-mer primers has proved advantageous in lactobacilli (Johansson, *et al.*, 1995c). Du Plessis and Dicks (1995) and Van Reenen and Dicks (1996) have successfully employed 10-mer primers while Hamad, *et al.* (1997) have used 21-mer oligonucleotide primers, all for the differentiation of *Lactobacillus* species. RAPD-PCR was also effectively used to separate strains within the subspecies of *Lb. curvatus* and *Lb. sake* (Torriani, *et al.*, 1996). Genomic DNA, tRNA and rRNA have been utilized as primers in different studies (Welsh and McClelland, 1990).

Other studies have applied PCR-based DNA typing procedures together with REA. This has resulted in the development of the amplified-rDNA restriction analysis (ARDRA) procedure (Gurtler, *et al.*, 1991; Jayaro, *et al.*, 1991) thus making use of the rRNA genes that are highly conserved. Very recently this ARDRA (PCR) procedure was employed for typing in *Leuconostoc* species (Villani, *et al.*, 1997). A new selective amplification procedure for restriction fragments using a combination of restriction enzyme methods and PCR products has become another powerful tool for simultaneous use for the identification and typing of LAB. One of the methods

which makes use of only fragments that completely match the primer sequence to be amplified is termed amplified fragment length polymorphism (AFLP) (Huys, *et al.*, 1996). Furthermore, it was also proposed that the tRNA gene profile could also be used in the low-molecular-weight RNA profiling method (Höfle, 1988). In this method both the 5S rRNA and the total tRNA pool are used and the former fingerprints help discriminate among the major eubacterial groups, whereas the tRNA fraction reveals more specific taxonomic information (Höfle, 1988; 1990).

1.3.3. The Polyphasic Approach:

The taxonomy of lactic acid bacteria has remained volatile for a long time. The original group whose definition was based on their production of lactic acid during food and feed fermentation, has now been re-classified using current methods. On the basis of chemotaxonomic and genomic studies, the LAB now only include members of the genera *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997). The major genera of LAB found in most fermented foods are *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus thermophilus* and *Weissella*. *Oenococcus* is important in wine fermentations. As a consequence of using these phylogenetic data based on rRNA homology between the different species belonging to the major genera *Lactobacillus, Pediococcus, Leuconostoc, Streptococcus thermophilus* and *Weissella,* shifts and additions have been made (Wood and Holzapfel, 1995; Stiles and Holzapfel, 1997).

Polyphasic approach in bacterial taxonomy is the use of multiple taxonomic methods (both phenotypic and genotypic) for the proper classification, description of taxa or assignment of a new isolate into a new group. The term *polyphasic taxonomy* was first coined by Colwell (1970) and later adopted by Murray, *et al.* (1990) an approach well elaborated by Vandamme, *et al.* (1996). The later group of authors has further extended the concept to *polyphasic classification* and *polyphasic identification* which, in both cases, could be used with the same context as it is used for the delineation of taxa at all levels.

The recent strategy to bacterial taxonomy, particularly at the species level, is thus based on the coherent use of multiple data generated using the different methods, which has proven to give a

stable system (Stackebrandt and Goebel, 1994). However, several taxonomic studies on LAB have reached at an unequivocal conclusion using such a polyphasic strategy with some of the methods producing different classifications (Collins, *et al.*, 1993; Pot, *et al.*, 1993; Dicks, 1995; Du Plessis and Dicks, 1995; Dicks, *et al.*, 1996; Van Reenen and Dicks, 1996; Villani, *et al.*, 1997). Meanwhile continued effort to further clarify the status and relationships of isolates and the different taxa, various methods have been developed and their sensitivities refined. These different methods and cellular components in common use for microbial taxonomy are grouped according to their basis and are schematically presented in Figure 4 below.

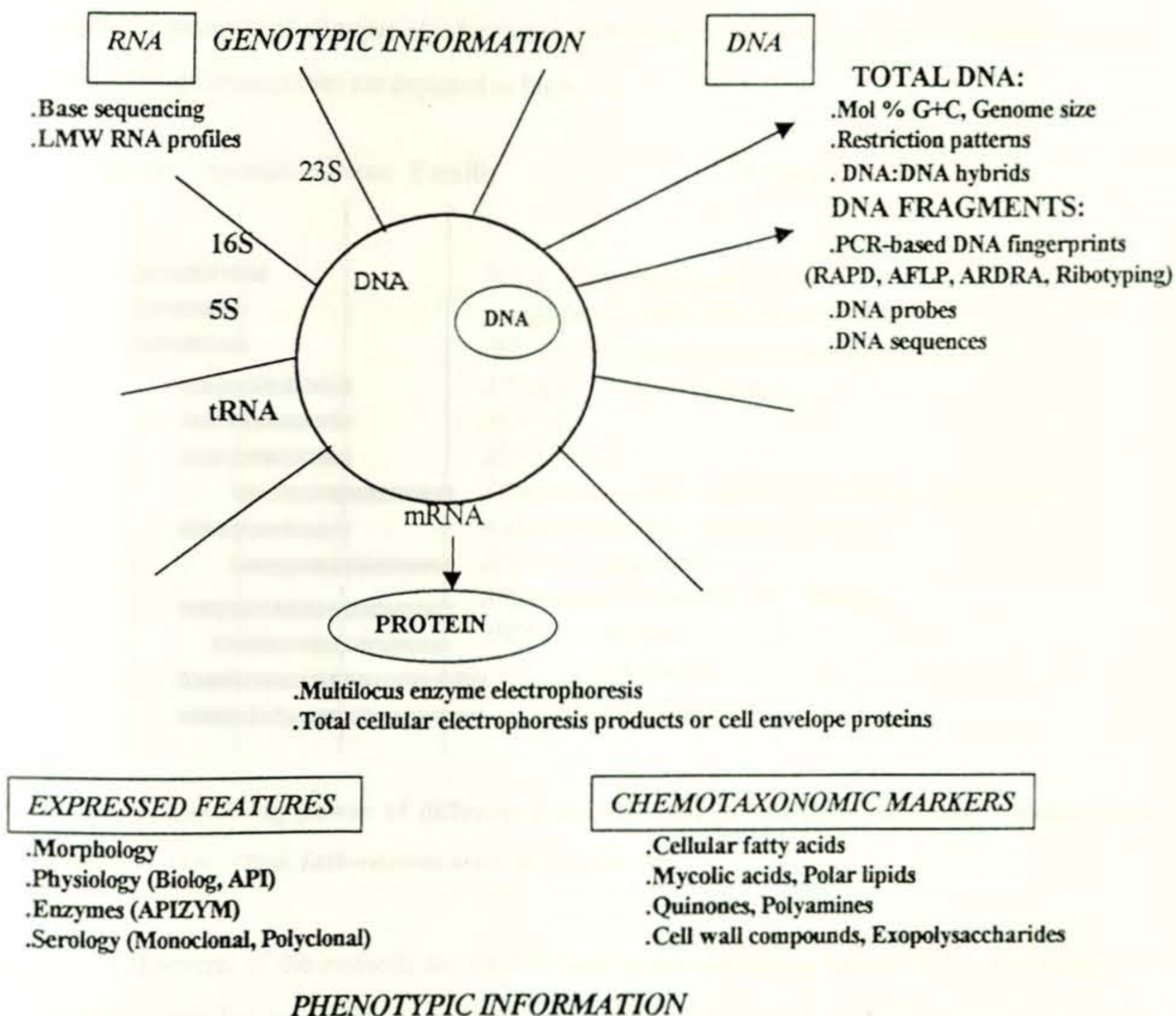


Figure 4. Phenotypic and genotypic methods and cellular components employed in bacterial taxonomy *(Adapted from Vandamme, et al., 1996).(Abbreviations are as used in the text).*

And it has now become vivid that the use of phenotypic procedures alone has long shadowed uncertainties leading to wrong affiliation of an isolate. With the advent of genetic markers for use in taxonomy this has later been resolved, but still the identity of an isolate could not be clearly known by making use of a single genetic method alone. This arises because of the varying levels of sensitivities each method possesses in revealing relationships and differences in the taxonomic hierarchy. Some of the methods are sensitive at the species and sub-species levels, while others at species and genus levels and some others from species to family levels. Thus the resolving potential of all types of information differs across all methods. The diverse methods and their inherent sensitivities are depicted in Figure 5.

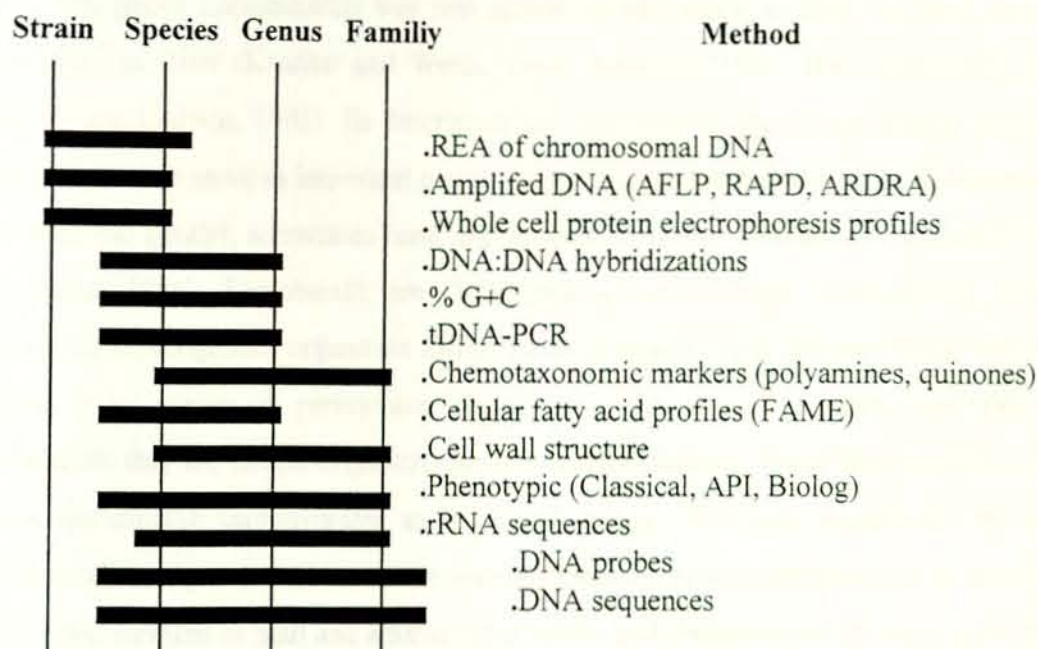


Figure 5. Resolving power of different methods used in bacterial taxonomy ***(Modified from Vandamme, et al., 1996). (Abbreviations are as used in the text).***

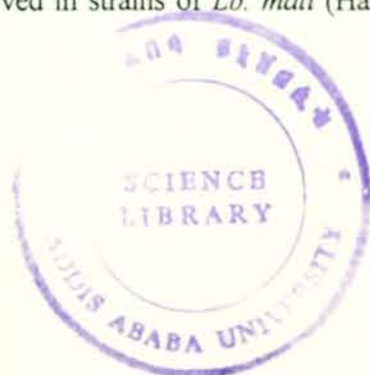
If several of the methods are used the results are sometimes equivocal (i.e. inconclusive). Therefore, one has to choose the right combination of tests for this purpose. For instance DNA-DNA hybridizations could be used for sifting reference strains for rRNA sequencing and could

again aid to see inter- and intra-specific relatedness that could not at all be quantified by the rRNA sequence analysis (Dykes and von Holy, 1994; Vandamme, *et al.*, 1996). But there is variation even between the hybridization procedures used in different laboratories. Since no single method has been found to be effective and complete in lactic acid bacterial taxonomy several methods which complement one another are now used. It has therefore become important to reevaluate phenotypic information together with phylogenetic data used for the identification and description of different LAB taxa (Collins, *et al.*, 1989; Vogel, *et al.*, 1994; Dicks, *et al.*, 1995; Du Plessis and Dicks, 1995; Ferrero, *et al.*, 1996; Van Reenen and Dicks, 1996).

1.3.4. The Genus Lactobacillus:

The genus *Lactobacillus* was first coined by Beijerinck in 1901 and later described by Orla-Jensen in 1919 (Kandler and Weiss, 1986; Axelsson, 1993; Hammes and Vogel, 1995; Schleifer and Ludwig, 1995). Its description is still based on physiological and morphological traits as these data serve as important preliminary identification features. Cells of lactobacilli vary from long and slender, sometimes bent, rods to short and often coryneform coccobacilli (Kandler and Weiss, 1986). Lactobacilli are Gram-positive aero-tolerant or anaerobic, aciduric or acidophilic, asporogenous organisms that often form chains. Most are non-motile and if present motility is by means of peritrichous flagella (e.g. *Lb. agilis*) (Kandler and Weiss, 1986). Nutritionally they are chemo-organotrophic and mostly fastidious since they need rich media and require fermentable carbohydrates as source of energy. Therefore, lactobacilli are known to inhabit nutritionally rich habitats where carbohydrate-containing substrates are in excess such as the mucosal surfaces of man and animals, oral cavity and fermenting or spoiling food (Axelsson, 1993; Hammes and Vogel, 1995; Stiles and Holzapfel, 1997).

They have a strictly fermentative metabolism. The homofermentative lactobacilli are known to produce more than 85% lactate while the heterofermentatives, in addition, produce acetate, ethanol and carbon dioxide (Axelsson, 1993). They lack heme-dependent activities because they do not synthesize porphyrinoids. Some strains of few species may perhaps use porphyrinoids from the environment and show catalase activity, nitrate reduction or even have cytochromes. Consequently, pseudo-catalase has been observed in strains of *Lb. mali* (Hammes


SCIENCE
LIBRARY

and Vogel, 1995). Increased amounts of acetate might be produced in the presence of oxygen or other oxidants at the expense of lactate or ethanol whereby an extra mole of ATP is gained via the acetate kinase reaction. As a result, variations in the metabolic end products may happen. In addition to glucose, several other compounds such as citrate, malate, tartarate, quinolate, nitrate, nitrite, etc., may be used as energy sources by building up a proton motive force or electron acceptors.

Currently the genus *Lactobacillus* consists of more than 60 species based on 16S rRNA phylogenetic relatedness and that these are not yet stable since there is still re-shuffling of the already known species and discovery of new species (Stiles and Holzapfel, 1997).

Grouping of lactobacilli

The currently recognized species of the genus *Lactobacillus* and their grouping are presented in Tables 1 and 2. The list covers lactobacilli which appeared in the approved list and which are validly published and is based on the latest grouping provided by Hammes and Vogel (1995), Vandamme, *et al.* (1996) and Stiles and Holzapfel (1997) with incorporation from various sources of recently appearing taxa in valid publications where, in all cases, authentic data both from phylogenetic and sugar metabolism were utilized coherently. The genus *Lactobacillus* can be divided on the basis of sugar fermentation which can be either hexose or pentose fermentation or both as well as phylogenetic data. Based on the sugar fermentation patterns, *Lactobacillus* and related genera have been recently categorized into the following three groups (see Hammes and Vogel, 1995; Stiles and Holzapfel, 1997):

■**Group A:Obligately Homofermentative.** Almost exclusively (>85%) ferment hexoses to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway. The organisms have fructose-1,6-bisphosphate-aldolase but lack phosphoketolase; thus they do not ferment both gluconate and pentoses. This category includes 18 species of *Lactobacillus* and four species of *Pediococcus*.

■**Group B:Facultatively Heterofermentative.** Ferment hexoses almost exclusively to lactic acid by the EMP pathway. They possess both aldolase and phosphoketolase and thus can ferment pentoses (and often gluconate). The phosphogluconate pathway is known to be repressed in the

presence of glucose. In this group were placed 19 *Lactobacillus* and one *Pediococcus* species.

■**Group C:Obligately Heterofermentative.** Ferment hexoses via the phosphogluconate pathway yielding equimolar amounts of lactate, ethanol (acetic acid) and CO_2. Pentoses may also be fermented by this pathway. This group is comprised of 18 species of *Lactobacillus*, one species of *Pediococcus*, all species of *Weissella* and of *Leuconostoc* as well as *Oenococcus oeni*.

Members of the genus *Lactobacillus* have also been divided into three groups based on phylogenetic data as follows (Kandler and Weiss, 1986; Collins, *et al.*, 1991) (see Table 2):

■Group **a:*Lactobacillus delbrueckii* group.** Encompasses 14 species of *Lactobacillus*.

■Group **b:*Lb. casei-Pediococcus* group.** Includes 41 *Lactobacillus* and six *Pediococcus* species.

■Group **c:*Leuconostoc* group.** A group which contains one species of *Lactobacillus*, eight *Weissella*, eight *Leuconostoc* and *Oenococcus oeni*.

When the sugar based and the phylogenetic groupings are combined, the resulting groups are designated with two letters (a higher and lower case). For example "**Aa**" is meant to define a species as belonging to the obligately homofermentative lactobacilli affiliated to the *Lb. delbrueckii* group, "**Bb**" a facultatively heterofermentative species affiliated to the *Lb. casei-Pediococcus* phylogenetic group, and so forth.

All of these taxonomic revisions and rearrangements are results of extensive studies on rRNA sequencing or DNA-DNA hybridizations (Kandler and Weiss, 1986; Collins, *et al.*, 1991; Hammes and Vogel, 1995; Schleifer and Ludwig, 1995; Vandamme, *et al.*, 1996). Furthermore, whole cell protein profiles (Dicks, 1995; Du Plessis and Dicks, 1995) have also helped to resolve several confusions in LAB taxonomy. The relevance of using phenotypic and genetic data in consensus has, therefore, been demonstrated in Table 2 below where both have interphases although genetic data is more profound. As a result of this fact, thus, the three phylogenetic groups fell into the three different sugar fermentation (metabolic) categories.

Table 1. Current list of recognized *Lactobacillus* species

Organism	Source of isolation	Group*	Mol % G+C
Lactobacillus acetotolerans	Fermented vinegar broth	Ba	35-36.5
Lb. acidophilus	Intestinal tract of man & animals, human mouth, vagina	Aa	34-37
Lb. agilis	Municipal sewage, habitat unknown	Bb	43-44
Lb. alimentarius	Marinated fish, meat products, sourdough	Bb	36-37
Lb. amylophilus	Swine waste-corn fermentations	Aa	44-46
Lb. amylovorus	Cattle waste-corn fermentations	Aa	40-41
Lb. arizonae[a]	-	-	-
Lb. aviarius subsp. *araffinosus*	Chicken duodenum	Ab	39-43
Lb. aviarius subsp. *aviarius*	Chicken & duck alimentary tract, faeces	Ab	39-43
Lb. bifermentans	Spoiled Edam & Gouda cheeses	Bb	45
Lb. brevis	Milk, cheese, sauerkraut, silage, sourdough, cow dung, intestines of humans & rats	Cb	44-47
Lb. buchneri	Milk, cheese, fermenting plant material, human mouth	Cb	44-46
Lb. casei subsp. *alactosus*	Dairy products, sourdough, cow dung, silage, human intestine, mouth & vagina, sewage	Bb	45-47
Lb. casei subsp. *casei*	" " " "	Bb	45-47
Lb. casei subsp. *pseudoplantarum*	" " "	Bb	45-47
Lb. casei subsp. *tolerans*	" " " "	Bb	45-47
Lb. collinoides	Cider	Cb	46
Lb. coryniformis subsp. *coryniformis*	Silage, cow dung, dairy barn air, sewage	Bb	45
Lb. coryniformis subsp. *torquens*	" " " "	Bb	45
Lb. crispatus	Human faeces, vagina, buccal cavities, crops & caeca of chicken, in patients with purulent pleurisy, leucorrhoea and urinary tract infection	Aa	35-38
Lb. curvatus subsp. *curvatus*[a]	Cow dung, milk, silage, sauerkraut, sourdough, meat	Bb	42-44
Lb. curvatus subsp. *melibiosus*[a]	" " "	Bb	42-44
Lb. delbrueckii subsp. *bulgaricus*	Yoghurt, cheese	Aa	49-51
Lb. delbrueckii subsp. *delbrueckii*	Plant material fermented at 40-50°C	Aa	49-51
Lb. delbrueckii subsp. *lactis*	Milk, cheese, yeast & grain mash	Aa	49-51
Lb. farciminis	Fermented dry sausages, sourdough	Ab	34-36
Lb. fermentum	Yeast, milk products, sourdough, fermenting plant material, manure, sewage, human mouth, faeces	Cb	52-54
Lb. fructivorans	Vinegar preserves, salad dressings, spoiled sake, dessert wines, aperitifs	Cb	38-41
Lb. fructosus	Flowers	Cc	47
Lb. gallinarum	Chicken intestine	Aa	36-37
Lb. gasseri	Mouth, intestine, faeces, vagina	Aa	33-35
Lb. graminis	Grass silage	Bb	41-43
Lb. hamsteri	Intestine of rats & mice	Ba	33-35
Lb. helveticus	Sour milk, cheese	Aa	38-40
Lb. hilgardii	California table wines	Cb	39-41

Lb. homohiochii	Spoiled sake	Bb	35-38
Lb. intestinalis	Intestine of rats & mice	Bb	33-35
Lb. jensenii	Human vaginal discharge & blood clot	Aa	35-37
Lb. johnsonii	Faeces of chicken, mice, calves & pigs	Aa	33-35
Lb. kefir	Kefir grains, drink kefir	Cb	41-42
Lb. kefiranofaciens	Kefir grains	Aa	34-35
Lb. kefirgranum[a]	Kefir grains	Aa	34.3-38.6
Lb. mali	Fresh apple juice, cider, wine must	Ab	32-34
Lb. malefermentans	Beer	Cb	41-42
Lb. murinus	Digestive tract of mice and rats	Bb	43-44
Lb. oris	Human saliva	Cb	49-51
Lb. panis	Long fermented rye sourdough	Cb	48-48.3
Lb. parabuchneri	Human saliva, cheese, contaminated brewery yeasts	Cb	44
Lb. paracasei subsp. *paracasei*	Dairy products, sewage, silage, humans & clinical sources	Bb	45-47
Lb. paracasei subsp. *tolerans*	Dairy products	Bb	45-47
Lb. parakefir[a]	Kefir grains	Cb	41.4-42
Lb. paraplantarum[a]	Beer, human faeces	Bb	44
Lb. pentosus	Corn silage	Bb	46-47
Lb. plantarum	Sourdough, silage, sauerkraut, cow dung, human mouth, intestinal tract, stools, sewage, dairy products	Bb	44-46
Lb. pontis	Rye sourdough	Cb	53-55
Lb. reuteri	Faeces of humans & animals, from meat, sourdough	Cb	40-42
Lb. rhamnosus	Dairy products, sewage, humans & clinical sources	Bb	45-47
Lb. ruminis	Bovine rumen, sewage	Ab	44-47
Lb. sake subsp. *carnosus*	Sake, sauerkraut, fermented plant material, meat products, sourdough, herbage, silage, vacuum packed meat	Bb	42-44
Lb. sake subsp. *sake*	" " " "	Bb	42-44
Lb. salivarius subsp. *salicinius*	Mouth & intestine of man & hamster	Ab	34-36
Lb. salivarius subsp. *salivarius*	" " "	Ab	34-36
Lb. sanfrancisco	Wheat & rye sourdough	Cb	36-38
Lb. sharpae	Municipal sewage	Ab	53
Lb. suebicus	Stored apple, pear mashes	Cb	40
Lb. vaccinostercus	Cow dung	Cb	36
Lb. vaginalis	Vagina of a patient with trichomoniasis, sorghum dough	Cb	38-41
Lb. zeae[a,b]	Corn steep liquor	Bb	48-49

(Compiled from Kandler and Weiss, 1986; Collins, *et al.*, 1991 ,Hammes and Vogel, 1995; Vandamme, *et al.*, 1996).

[*]Phylogenetic grouping is based on 16S rRNA sequence data similarities (Collins, *et al.*, 1990).

[a]New species.

[b]*Lactobacillus zeae* was proposed by Dicks, *et al.*, (1996) and further studied by Mori, *et al.* (1997); it is related to the *Lb. casei* group.

Table 2. Grouping of lactobacilli and related taxa based on their physiologic and phylogenetic affiliations

Phylogenetic group	Species in fermentation group		
	A (Obligately homofermentative)	B (Facultatively heterofermentative)	C(Obligately heterofermentative)
a (*Lb. delbrueckii* group)	*Lb. acidophilus, Lb. amylophilus, Lb. amylovorus, Lb. crispatus, Lb. delbrueckii* subsp. *bulgaricus*, subsp. *delbrueckii* and *lactis, Lb. gallinarum, Lb. gasseri, Lb. helveticus, Lb. jensenii, Lb. johnsonii, Lb. kefiranofaciens, Lb. kefirgranum,*	*Lb. acetotolerans, Lb. hamsteri*	
b (*Lb. casei-Pediococcus* group)	*Lb. aviarius* subsp. *araffinosus* and *aviarius, Lb. farciminis, Lb. mali, Lb. ruminis, Lb. salivarius* subsp. *salicinius* and *salivarius, Lb. sharpae, Pediococcus damnosus, Ped. dextrinicus, Ped. parvulus .*	*Lb. agilis, Lb. alimentarius, Lb. bifermentans, Lb. casei, Lb. coryniformis* subsp. *coryniformis* and *torquens, Lb. curvatus* subsp. *curvatus* and *melibiosus, Lb. graminis, Lb. homohiochii, Lb. intestinalis, Lb. murinus, Lb. paracasei subsp. paracasei* and *tolerans, Lb. pentosus, Lb. plantarum, Lb. paraplantarum, Lb. rhamnosus, Lb. sake* subsp. *carnosus and sake, Lb. zeae, Ped. acidilactici, Ped. inopinatus*	*Ped. pentosaceus, Lb. brevis, Lb. buchneri, Lb. collinoides, Lb. fermentum, Lb. fructivorans, Lb. hilgardii, Lb. kefir, Lb. malefermentans, Lb. oris, Lb. parabuchneri, Lb. panis, Lb. parakefir, Lb. pontis, Lb. reuteri, Lb. sanfrancisco, Lb. suebicus, Lb. vaccinostercus, Lb. vaginalis*
c (*Leuconostoc* group)			*Lb. fructosus, W. confusa (Lb. confusus), W. (Lb.) viridescens, W. (Lb.) halotolerans, W. (Lb.) hilgardii, W. (Lb.) kandleri, W. (Lb.) minor, W. (Lb.) hellenica, W. (Leu.) paramesenteroides, Leu.. amelibiosum, Leu. argentinum, Leu. lactis, Leu. mesenteroides, Leu. pseudomesenteroides, Leu. carnosum, Leu. gelidum, Leu. fallax, Oenococus oeni*
Other lactobacilli[a]	*Lb. catenaformis, Lb. vitulinus, Lb. rogosae*[b], *Atopobium minutium, Atopobium rimae, Atopobium uli, Carnobacterium divergens, C. piscicola, Lactococus lactis*		

Lb. kefirgranum, Lb. parakefir and *Lb. arizonae* were not included in 16S rRNA sequence analysis.

[a]Species in this group are no longer members of the genus *Lactobacillus.*

[b]*Lb. rogosae* was described to have a DNA base composition of 59 mol% G+C, a value outside the range for the genus *Lactobacillus.*

(Based on Vandamme, *et al.*, 1996 and Stiles and Holzapfel, 1997).

1.3.5. The genus Pediococcus:

The name *Pediococcus* was first used for sarcina-like lactic acid producing organisms causing spoilage of beer("beer sickness") by Balcke in 1884 (Pederson, 1949). The genus was described by Claussen in 1903 (Garvie, 1986). The ability of the different species of *Pediococcus* to bring about desirable fermentation products was not, however, recognized until 1926 when Henneberg associated them with those fermentations carried by *Lactobacillus* and *Streptococcus* (Pederson, 1949). Cells of pediococci occur in pairs or tetrads, single cells are rare and chains are never formed at all. Division is in two perpendicular directions in a single plane and this is why cells occur in tetrads (Simpson, 1994). They are non-motile, facultative anaerobes which tolerate oxygen. They grow optimally between 25-40°C (Garvie, 1986). Previously all pediococci were presumed to be homofermentative, producing lactate from glucose and no gas, but recently it was shown that one species is facultatively and another obligately heterofermentative (Stiles and Holzapfel, 1997). Members of this genus have mol% G+C range of 35-42. Previously species of pediococci were separated by their tolerance to temperature, pH and salt. The former basis of their classification was totally phenotypic which was later changed when 16S rRNA and DNA:DNA reassociation data was available (Back, 1978 a,b; Dellaglio, *et al.*, 1981; Garvie, 1986; Collins, *et al.*, 1991).

Pediococci are non-pathogenic to plants and animals, but have been extensively studied because they are important in food fermentations where they spoil beverages but can also be used as protective cultures because of their ability to produce bacteriocins (Simpson and Taguchi, 1995). Most species are ubiquitous contaminants in breweries, some being associated with malt and beer, and in other products such as wine and cider they produce diacetyl and acetoin causing off-flavours. *Pediococcus damnosus* is known to be resistant to hop acids. They are also found in carbonated soft drinks but have so far not been associated with spoilage. They are however common and desirable in fermenting foods like soya sauce and miso, cheese, meat and fish products (Simpson and Taguchi, 1995).

The genus currently consists of six species. The validly published species include: *Pediococcus acidilactici, Ped. damnosus, Ped. dextrinicus, Ped. parvulus, Ped. pentosaceus* and

Ped. inopinatus. *'Ped. urinae-equii'*, was proposed to be moved for its rather strong affiliation with *Aerococcus*. Consequently, this species has, then, been renamed as *Aerococcus urinae* (Collins, *et al.*, 1992; Collins, *et al.*, 1993). Simpson and Taguchi (1995) and Stiles and Holzapfel (1997) have also stressed this clearly distant relation of this species with pediococci. In this thesis the six species of *Pediococcus* will be regarded as the recognized and validly published members of the genus while the *Ped. urinae-equii* will be considered as an ex-member of the taxon.

Pediococci belong to the phylogenetic group *Lb. casei-Pediococcus* (Collins, *et al.* (1990, 1991, 1992). It is very difficult to distinguish these species phenotypically (Garvie, 1986; Tanasupawat, *et al.*, 1993). As a result, molecular genetic studies have been employed such as the 16S rRNA sequencing and the use of multiplex PCR analysis using rRNA probes (Mora, *et al.*, 1997). Schleifer and Ludwig (1995) have pointed out the intermixing of pediococci with lactobacilli as shown by Collins, *et al.* (1991) where *Pediococcus* as a genus has been undifferentiated from the lactobacilli. As a consequence of the unusually high levels of sequence homologies, however, it was even hard to discriminate between species like *Ped. acidilactici* and *Ped. pentosaceus* where they shared 98.3% 16S rRNA base sequences.
Ped. dextrinicus had clustered with lactobacilli while the other four pediococci made their own distinct clade (Collins, *et al.*, 1991; Tanasupawat, *et al.*, 1993; Mora, *et al.*, 1997). *Ped. inopinatus* was very close to the clade while *Ped. dextrinicus* showed a closer relationship with lactobacilli (Collins, *et al.*, 1991). The taxonomy of *Pediococcus* species has, therefore, still remained unclear. The ecological, physiological and phylogenetic relationships of these organisms are mentioned in Table 3.

Table 3. Phenotypic and phylogenetic affiliation of the species of *Pediococcus*

Species	Original source of isolation	Group	Mol% G+C
Pediococcus acidilactici	Fermenting plant material, silage, malt, dry leaves, salami	**Bb**	38-44
Pediococcus damnosus	Brewer's yeast & products, fermenting wort, beer	**Ab**	37-42
Pediococcus dextrinicus	Silage, brewer's spent grains, beer	**Ab**	40-41
Pediococcus inopinatus	Sauerkraut, beer, hops, wine, fermented beans	**Ab**	39-40
Pediococcus parvulus	Sauerkraut, fermented vegetables/beans, beer, cider, wine	**Ab**	40.5-41.6
Pediococcus pentosaceus	Various plant materials, malt, hops	**Cb**	35-39
Aerococcus urinae[a]	Horse urine, rabbit dung, phak-gard-dong (Thai pickled vegetables)	***Aerococcus***	39.6-39.7

[a]Formerly *Pediococcus urinae-equii* **(After Collins, *et al.*, 1991; Simpson and Taguchi, 1995; and Stiles and Holzapfel, 1997).**

1.3.6. The genus Weissella:

The genus *Weissella* was named after the German microbiologist Norbert Weiss. *Weissella* is a newly organized genus of the lactic acid bacteria proposed by Collins, *et al.* (1993) and validly published in 1994 (Stiles and Holzapfel, 1997). Cells are generally short rods with rounded to tapered ends or coccoid and occur singly, in pairs or in short chains. Other descriptions of LAB also hold true for this taxon. The genus consists of organisms with complex nutritional requirements and which are acidoduric nature growing at 15°C (Shah and Jelen, 1990). With the exception of some strains of *Weissella confusa* (formerly *Lactobacillus confusus*) they do not grow at 45°C. Their guanine plus cytosine content of DNA ranges between 37-47 mol%.

Initially this taxon was described to retain *Leuconostoc paramesenteroides* based on the 16S rRNA phylogenetic distinctness of the species for the need to separate it from other leuconostocs. Later on it further absorbed divergent *Lactobacillus* species as well. Currently it comprises *W. confusa (Lb. confusus), W. halotolerans (Lb. halotolerans), W. kandleri (Lb. kandleri), W. minor (Lb. minor), W. paramesenteroides, W. viridescens (Lb. viridescens)* and *W. hellenica* (Collins, *et al.*, 1993; Vandamme, *et al.*, 1996). Stiles and Holzapfel (1995) have also

considered *Lb. hilgardii* as it has been already moved to this genus and renamed it *W. hilgardii*. Consequently, this genus included the previous heterofermentative lactobacilli of unusual interpeptide bridges in the peptidoglycan. Morphological and physiological features of *Weissella* do not directly favour this grouping which now contains both species producing D(-)- and DL-lactate (Collins, *et al.*, 1993; Vandamme, *et al.*, 1996).

1.4. The Role of LAB in Food Fermentations

Most LAB, with the exception of *Lb. amylovorus*, some strains of *Lb. plantarum* and pediococci, are not able to hydrolyze starch. Ability to generate fermentable carbohydrate is a vital precondition to initiate lactic acid fermentations in food of plant origin. It is therefore common practice to add malted cereal flour to raw flour to initiate and encourage development of lactic acid bacteria in such fermentations (Back, 1978a; Nout and Rombouts, 1992; Johansson, 1995; Gobbetti and Corsetti, 1997)

Natural fermentation, takes place by the interaction of a mixed culture. In the natural succession of cereal fermentation for instance, microorganisms other than LAB, like the Enterobacteriaceae, *Bacillus* spp. and fermentative yeasts initiate the production of reducing sugars for the LAB to follow (Pederson, 1979; Daeschel, *et al.*, 1987). Fresh vegetables have fructose, glucose and saccharose as major sugars, totally ca. 1-4%, depending upon the level of maturity (Nout and Rombouts, 1992). In vegetables the sugar content is sufficient enough to promote LAB fermentation processes. Fermentable sugars are made available to the associated microflora due to physical disruption of cellular structures as in shredding of leafy vegetables, chopping of roots and tubers, milling of cereal grains coupled with, in some cases, salting to avail the sap from cells and the matrix. Legumes, oil seeds and sugary fruits may be soaked or crushed since such processes also promote availability of fermenting matter. Other substances such as lignocellulosics, pectins and organic acids could also serve as carbon sources for organisms like *Bacillus* spp. In many cases, however, pectolysis is undesirable in foods as it causes them to loss firmness (Daeschel, *et al.*, 1987).

Regarding nitrogen sources, most plant materials possess organic nitrogen which promotes fermentation. But the availability of nitrogen might be limited by the presence of protein- or alpha-amino-complexing substances such as phytic acid, tannins and phenolic compounds (Nout and Rombouts, 1992).

Mineral bioavailability is an important factor for microbial development. A variety of mineral ions, such as Ca^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, P^{3+} and Zn^{2+} are known to regulate microbial proliferation. Each one of them is known to be involved in specific functions in the microbial cell. For example, Mn (II) is an essential component of the defense system against endogenous O_2^- in plant associated *Lactobacillus, Pediococcus* and *Leuconostoc* species (Archibald, 1986; Daeschel, *et al.*, 1987). Zn^{2+} stimulates α-amino N-assimilation (Nout and Rombouts, 1992). Natural fermentation of cereal batters involves the action of grain phytases along with the generation of organic acids and some ß-complex vitamins and it is supposed to have some potential to improve native zinc availability. Fermentation as a whole is perhaps known to increase bioavailability of both zinc and iron (Agte, *et al.*, 1997). Antinutritional factors, such as phytic acid not only affect nitrogen availability but also chelate mineral ions thus minimize the bioavailability of the minerals to the microflora. LAB fermentations reduce such antinutrients as phytate and tannins in the fermented products as shown in *tef* dough fermentation (Urga, *et al.*, 1997a). Some lactobacilli, such as *Lb. amylovorus* have been found to produce phytases (Sreeramula, *et al.*, 1996). Studies by Urga, *et al.* (1997a,b) have revealed the effect of natural LAB fermentation in reducing the level of antinutrients, such as phytates, tannins and trypsin inhibitor, simultaneously improving the availability of proteins and free amino acids.

Foods contain aerobic and anaerobic epiphytic flora amongst which species of *Bacillus*, *Flavobacterium, Pseudomonas* and moulds are strictly aerobic. Enterobacteriaceae, lactic acid bacteria and yeasts are facultative anaerobes. In a mixed microflora, succession and dominance is dependent on microbial properties such as energy metabolism, lag time, maximum specific growth rate and adherence or penetration capacity into substrate. Physico-chemical properties of the food, such as pH and water activity are also important. Besides these, plant materials and fruits have antimicrobial constituents but LAB, particularly heterofermentative lactobacilli such as *Lb.*

collionides and *Lb. brevis*, are known to hydrolyze such compounds as chlorogenic acid to caffeic and quinic acids both of which are stimulatory for their growth (Stead, 1994). In most natural fermentations of plant foods without pre-heat treatment epiphytic microflora will initiate and direct the fermentation. In natural cereal fermentations members of the family *Enterobacteriaceae* (such as species of *Enterobacter*, *Klebsiella*) proliferate during the early periods of fermentation at neutral pH values. However, their growth is halted when the pH is reduced to about 5.5 due to their mixed acid fermentation products such as acetic acid, formic acid and CO_2. Thus the acid-tolerant LAB will take over as they will be favoured by the availability of fermentable substrates, the reduced pH and the anaerobiosis so created. They in turn will further produce various products and make the microenvironment hostile for other microorganisms.

Obligate homofermentative species such as *Lb. acidophilus*, *Ped. dextrinicus*, *Ped. damnosus* and *Ped. parvulus* are known to produce lactic acid from all assimilable carbohydrates. Obligate heterofermentative LAB on the other hand such as *Lb. brevis*, *W. confusa*, *Lb. fermentum*, *Leuconostoc dextranicum* and *Leu. mesenteroides* produce many products other than lactic acid. These products include acetic acid, formic acid, butyric acid, diacetyl, H_2O_2, CO_2 and alcohol (Axelsson, 1993; Berggren, 1996). Facultative heterofermenters such as *Lb. plantarum*, *Lb. casei*, *Lb. pentosus* and *Ped. acidilactici* ferment glucose into different products including acetic acid, ethanol and CO_2. Therefore, the heterofermentative LAB contribute mostly to the aroma development and leavening of fermented products while the homofermentatives mainly improve the taste by lowering the pH thus making them sour which may desired in some non-leavened products.

Most epiphytic yeasts common in foods, such as *Aerobasidium*, *Cryptococcus*, *Rhodotorulla* and *Sporobolomyces* spp. do not ferment sugars. Instead they have pectolytic properties as a means of extracting carbon from the food. Oxidative yeasts then degrade the produced organic acids thereby increasing the pH. This allows growth of spoilage-causing, acid-sensitive organisms (Nout and Rombouts, 1992).

The commensal relationship between fermentative yeasts and LAB in most cereal lactic acid fermented foods has been found to generate paramount benefits for the fastidious LAB

(Lönner, 1988; Nout, 1991; Salovaara, 1993; Gobbetti and Corsetti, 1997). Another commensal association is found in sour dough between maltose-fermenting lactobacilli such as *Lb. sanfrancisco* (Gobbetti and Corsetti, 1997), and maltose non-fermenting yeasts. The lactobacilli ferment maltose make simpler sugars available to the yeast and in return the lactobacilli are stimulated by yeast peptides (Sugihara, 1985). Furthermore, *Lb. sanfrancisco* has been found to have a positive impact on yeast leavening and CO_2 generation (Gobbetti, *et al.*, 1995). Lactobacilli and pediococci are common in dough preparations such as in tef (Gashe, 1985; Nigatu, 1992) and in most instances the latter are maltose fermentative (Garvie, 1986).

The composition and action of the lactic acid microflora may differ from food to food or in different geographical settings. Nevertheless, their main task is to ferment the food together with the other microorganisms in the mixed culture so that the resultant fermented product becomes more energy-rich, acceptable and well ripened or palatable. *Lb. sanfrancisco, Lb. reuteri* and *Lb. fermentum* are unique among the lactobacilli because they phosphorylate maltose (Vogel, *et al.*, 1994). The enzyme maltose phosphorylase may be regarded as a key enzyme for the predominance of lactobacilli during sourdough fermentation. Another pertinent attribute of Lab in the ecological interaction with other fermentative flora in lactic acid fermentations is their possession of a vast array of proteases and peptidases which help in flavour development and supply of low molecular weight nitrogen metabolites (Collar, *et al.*, 1992).

In traditional LAB-fermented foods it is very common to use controlled natural fermentation processes. This is usually achieved by using inocula from a previous batch of the product (back-slopping). By so doing the inocula will have been naturally selected during the fermentation. Environmental factors such as partial or total anaerobiosis, temperature and water activity are then adjusted in order to obtain the desired product. This practice is well developed in rye and wheat sourdough (Lönner, 1988), lactic acid fermentations of cereal-legume mixtures (Nout, 1991), sorghum dough for *kisra* (Hamad, *et al.*, 1997) and *tef* dough for *injera* and in *enset* fermentations (Gashe, 1985, 1987). This back-slopping practice or the use of undefined mixed starter is a common technique both in temperate and tropical regions (Steinkraus, 1994). The process encourages the dominance of LAB and leads to the desired pattern of fermentation. The

practice is used both in animal- and plant-based foods such as curdled milk or yoghurt production, and the sourdough process. The process sometimes fails if the portion used for back-slopping is kept for a long time as this allows for the inactivation of desired organisms or groups of organisms, or death of the LAB due to shortage of nutrients or accumulation of toxic metabolites and waste, change in composition, and mutation of the LAB. Cold keeping could help reduce some of these effects but is not affordable in tropical climates. As a result in most uncontrolled natural fermentation processes the desired products may not always be satisfactory or in some cases are not reproducible. The use of pure culture fermentation where single or defined mixed cultures are added to raw substrates to achieve dominance over endogenous epiphytic microflora is, therefore, an alternative. Such a practice is common in silages and in vegetable fermentations like cabbage, carrots, cassava and okra (Nout and Rombouts, 1992). Mixed starter cultures consisting of LAB and fermentative yeasts are usually recommended as they assimilate all available carbohydrates (Daeschel, *et al.*, 1987).

The fermentative preservation is a result of microbial metabolites produced during the process including short-chain organic acids (SCFA), diacetyl, H_2O_2, CO_2, ethanol and bactericidal peptides such as bacteriocins and competitive dominance by the LAB. The dominance occurs as a result of the low pH, anaerobic conditions and accumulated metabolites. In such circumstances the LAB are able to use the accumulated metabolites which might be toxic to other microorganisms and are not affected by the acidity. Later on, however, oxidative yeasts may take over as observed in over-fermented *tef* flour in the liquid accumulated on top of the dough causing spoilage (Gashe, 1985; Nigatu and Gashe, 1994b). It is therefore, it would be essential to avoid excessive fermentation and by not letting spoilage flora oxidize the fermentation acids. This can be achieved by creating anaerobic conditions during the preparation of substrates for lactic acid fermentation such as using covered tanks or pits, thus allowing for the predominance of LAB. These conditions when created at room temperature would also raise the internal temperature so that metabolic activities and acid production might be enhanced.

Although anaerobiosis inhibits potential spoilage flora (bacteria, oxidative yeasts and moulds), complete absence of O_2 is not always desirable as some facultative anaerobes might need

oxygen for better metabolism. In some fermentations, aeration may speed up the process and improve the product (Vahvaselkä, *et al.*, 1990). Addition of salt, reduction of water activity and rise in fermentation temperature to the mesophilic range have been shown to accelerate the rate of acidification (Tanasupawat and Komagata, 1995).

1.5. Ecology of LAB and Their Applications

Lactic acid bacteria are widely utilized to produce fermented foods and contribute to flavour development and preservation of quality of the food. Their importance is associated with their safe metabolic activities while growing in foods in which they selectively assimilate available sugar for the production of organic acids and other metabolites. Their presence and desired use in fermented foods coupled with their use without harmful effects shows their natural acceptance as GRAS (Generally Recognized As Safe) by the human body (Aguirre and Collins, 1993). In this metabolic relationship, thus, LAB essentially provide a protective function to the safety of the food against growth and dominance by pathogenic and spoilage microflora. They achieve this through production of large quantities of inhibitory substances and specific peptide antibiotics which help in the preservation of food (Holzapfel, *et al.*, 1995; Stiles, 1996).

LAB are known to be ubiquitous in most environments. They are common in milk and dairy products, in fermented foods from meats, vegetables, cereals, fruits, on intact and rotting vegetable materials, silage, intestinal and urogenital tracts and mucous membranes of man and animals (Mikelsaar and Mäander, 1993; Salovaara, 1993). This colonization of such diverse habitats and particularly the positive associations they have with animals and humans has drawn the attention of researchers since the turn of the century. Although the exact mechanisms are not clear, many ecological benefits the LAB endow to the host are well documented (Gorbach, 1990; Fuller, 1992; Wood and Holzapfel, 1995; Salminen, *et al.*, 1996). Their distribution is primarily determined by concentrations of carbohydrate, protein breakdown products, vitamins and low oxygen tension. In addition to their saccharolytic properties, LAB are equipped with proteolytic enzymes which are particularly important in cereal flour dough fermentations where they interact

with yeasts (Gobbetti and Corsetti, 1997). Lactococci are apparently the most useful starter organisms for cheese production, and proteolysis is the most important process in cheese maturation (Gobbetti, *et al*., 1995; Kunji, *et al*., 1996). Consequently, there has been more interest and more data generated on lactococci proteolysis than on lactobacilli. Lactococci have also been studied more extensively and elaborately than any other LAB (Law and Haandrikman, 1997). *Lactobacillus acidophilus, Lb. helveticus, Lb. casei* and *Lb. delbrueckii* subsp. *bulgaricus* are perhaps the major lactobacilli of significance in milk fermentation and as a result more knowledge has been accumulated regarding their proteolysis. Lactic acid bacteria from plant sources are thought to be less proteolytic (Daeschel, *et al*., 1987), but available data, however, indicate their critical proteolytic importance in cereal fermentations. Their presence is essential for acidification and proteolysis, good texture, flavour and shelf-life of wheat sourdough breads (Gobbetti and Corsetti, 1997). Their role in *tef* fermentation has also been suggested from their predominance in the fermented dough and the resulting nutritional improvement of the fermented product when compared with that of the raw substrate (Nigatu, 1992; Urga, *et al*., 1997a). Studies have revealed that increased proteolysis during sourdough fermentation is stimulatory for the growth of yeasts. This helps sustain the commensal relationship between the yeasts and the LAB in the sour dough (Martinez-Anya, *et al*., 1990). Free amino acids contribute directly or serve as precursors for flavour development during wheat sourdough fermentation and baking. The proteolysis of gluten is also believed to influence texture and staling of wheat bread. About a third of proteolysis in sourdough is as a result of proteases from the flour with LAB contributing the rest of the proteolysis activity of the fermentation process (Collar, *et al*., 1992; Gobbetti, *et al*., 1994; Gobbetti and Corsetti, 1997). Change in volatile fatty acids composition impart desired flavours during the fermentation and baking of *tef* dough. In all cases LAB are responsible for the partial or entire production of organoleptic properties of such fermented products and for the preservation of a vast array of foods (Holzapfel, *et al*., 1995). Some representative lactic acid-fermented products from different geographical regions of the world are shown in Table 4. Besides their uses in foods, LAB such as *Lb. delbrueckii* and *Lb. helveticus* are industrially utilized for lactic acid production while *Leu. mesenteroides* is used for dextran production and *L. lactis* subsp. *lactis* for nisin production.

Table 4. Some common lactic acid fermented foods and the major LAB taxa involved

Food/Product	Raw ingredients	Taxon/Taxa
Dairy products:		
Acidophilus milk	Milk	*Lb. acidophilus*
Bulgarian butter milk	Milk	*Lb. delbrueckii* subsp. *bulgaricus*
Ripened cheeses	Milk	*Lactobacillus, Lactococcus*
Kefir	Milk	*Lb.kefir, Lb.kefiranofaciens, Lb.delbrueckii* subsp.*bulgaricus, Lb. casei, Lb. acidophilus*
Kumiss	Mare's milk	*Lb. delbrueckii* subsp. *bulgaricus*, other lactobacilli
Taette	Milk	*Lactococcus lactis*
Yogurt	Milk	*St. thermophilus, Lb. bulgaricus*
Ergo	Milk	*Lactobacillus, Lactococcus & other LAB*
Ititu	Milk	*Lactobacillus, Lactococcus & other LAB*
Meat and fish products:		
Dry sausages	Pork, Beef	*Pediococcus, Lb. plantarum, Lb. brevis*
Semi-dry sausages	Beef	*Pediococcus*
Buroung dalag	Fish, Rice	*Leu. mesenteroides, Lb. plantarum*
Izushi	Fish, Rice, Vegetables	*Lactobacillus*
Pla-som	Fish	*Lactobacillus, Pediococcus*
Som-fak	Fish	*Lactobacillus, Pediococcus*
Nham	Pork, Beef	*Lactobacillus, Pediococcus*
Plant products:		
Kenkey	Corn	*Lactobacillus*
Ogi	Corn	*Lb. plantarum, L. lactis*
Olives	Green olives	*Lb. plantarum, Pediococcus, Lb. brevis, Leu. mesenteroides*
Gari	Cassava	*Lb. plantarum, Leuconostoc, Lactococcus*
Bulla	Enset	*Lactobacillus, Pediococcus, Leuconostoc*
Kocho	Enset	*Lactobacillus, Pediococcus, Leuconostoc*
Pickles	Cucumber	*Pediococcus, Lb. plantarum*
Sauerkraut	Cabbage	*Leu. mesenteroides, Lb. plantarum*
Soy sauce	Soy beans	*Lb. delbrueckii, Pediococcus*
Breads, beverages and others:		
Sanfrancisco sourdough	Wheat	*Lb. sanfrancisco*
Sourdough	Rye	*Lb. pontis*
Sour Pumpernickel	Wheat flour	*Leu. mesenteroides*
Idli	Wheat flour	*Leu. mesenteroides*
Injera	Tef, Wheat, Barley, Sorghum	*Lactobacillus, Pediococcus, Leuconostoc*
Kisra	Sorghum	*Lb. vaginalis, Lb. helveticus, L. lactis, Lb. fermentum, Lb. reuteri*
Siljo	Beans, chickpeas, safflower, Ginger, Garlic	*Lactobacillus, Lactococcus*
Wine	Grapes	*Oenococcus oeni*
Sake	Rice	*Lb. sake*
Borde	Corn	*Lactobacillus* & other LAB
Shamita	Barley	*Lactobacillus* & other LAB
Tella	Barley, maize, sorghum, millets, wheat	*Lactobacillus* & other LAB

(Compiled from Campbell-Platt, 1987; Aguirre and Collins, 1993; Tanasupawat and Komagata, 1995; Hamad, *et al.*, 1997; Zewdie, *et al.*, 1995; Patidar and Prajapati, 1997; and Nigatu, unpublished data).

1.5.1. Antagonistic Properties of LAB Against Pathogens:

LAB are known to produce various primary and secondary metabolites during the process of fermentation. These include low molecular mass compounds such as organic acids, alcohols, CO_2, diacetyl, H_2O_2 and bacteriocins. Most of these substances have a broad spectra of antimicrobial activities and their production depends on the composition of the environment (food matrix), pH, temperature and redox potential, presence of competing organisms (Helander, *et al.*, 1997). The presence of more than one of them sometimes has additive effects. The non-peptide inhibitors such as H_2O_2, diacetyl, organic acids are major preservative components in LAB-fermented foods. Amongst the peptide/protein inhibitors (bacteriocins) it is only nisin which has been in use as a commercial food-grade preservative since the 1950's (Davidson and Hoover, 1993). New preservatives from LAB such as reuterin and bioprofit have also been developed recently (Helander, *et al.*, 1997). Some of the different antimicrobial products from LAB in common use and those developed recently are shown in Table 5.

Table 5. Primary and secondary metabolites produced by LAB and their common targets

Metabolite	Producer organisms	Target organisms
Acids:		
Lactic acid	All LAB	All microorganisms
Acetic acid	Heterofermentative LAB	All microorganisms; pH-dependent
Alcohols	Heterofermentative LAB	All microorganisms
Carbon dioxide	Heterofermentative LAB	Most microorganisms; pH-dependent
Diacetyl	*Lactococcus* spp.	Yeasts, Gram-negative bacteria >200 ppm, Gram-positive bacteria >300 ppm (butter flavour: 2-4)
Hydrogen peroxide	All LAB	All microorganisms
Reuterin	*Lactobacillus reuteri*	Broad spectrum: Gram-positive and Gram-negative bacteria, fungi
Benzoic acid, mevalonic acid lactone, methylhydantoin	*Lactobacillus plantarum*	Gram-negative bacteria, fungi
Bioprofit®	*Lactobacillus rhamnosus*	Broad spectrum: fungi, yeasts, heterofermentative LAB, *Bacillus* spp. (Gram-positive bacteria)
Nisin	*Lactococcus lactis* subsp. *lactis*	Gram-positive bacteria (LAB, streptococci, bacilli and clostridia

(Modified from Helander, *et al.*, 1997).

SCIENCE LIBRARY ABABA UNIVERSITY

As shown in Table 5, Helander, *et al.* (1997) have identified the inhibitory compounds produced by *Lb. plantarum* E76 as a mixture of low molecular mass compounds among which were benzoic acid, mevalonic acid lactone and methylhydantoin all in low concentrations but exerting strong effects due to their synergistic action. *In situ* production of such metabolites in the food matrix is an advantage to the consumer for the fermented product will be safe from pathogens and have a better shelf-life (Nout, 1994). Nevertheless, there is yet not much progress on the commercial use of these metabolites. In general, bacteriocins have a limited inhibition spectrum and are usually species-specific in their action. Thus, recent views seem to focus on the synergistic use of several metabolites instead of a single bacteriocin (Helander, *et al.*, 1997). With respect to the production of bacteriocins, the genera *Lactococcus, Lactobacillus, Leuconostoc* and *Pediococcus* are amongst those extensively studied. *Lactobacillus, Leuconostoc* and *Pediococcus* are very often isolated from LAB-fermented plant foods. They are also known to produce antimicrobial substances of low molecular mass components which have caught research attention revealing potential application in the food industry (Davidson and Hoover, 1993; Helander, *et al.*, 1997). Organic acids, such as lactic and acetic acids, are well known to be antimicrobial in nature and their inhibitory effect in fermented food products has been explicitly shown (Adams and Hall, 1988).

As we look into traditional LAB-fermented foods, the fact that the foods arrest the survival and growth of pathogens is more related to the *in situ* action of LAB. Such inhibition has been demonstrated in fermented corn flour (Simango and Rukure, 1992), cereal gruels (Svanberg, *et al.*, 1992; Lorri and Svanberg, 1994; Kingamkono, *et al.*, 1995) and in *tef* dough and *kocho* fermentations (Nigatu and Gashe, 1994a,b). Nevertheless very little is known on the mechanism of the combined actions of the inhibitory substances elaborated by LAB (Helander, *et al.*, 1997). Defined starter culture may be intentionally added into food or feed or preconditions may alternatively be set for them to develop and dominate. Some studies have shown that homofermentative LAB are more inhibitory to coliforms than heterofermentatives (Adams and Hall, 1988). It seems, however, an advantage to employ mixed cultures in order to make use of a wider range of metabolites since their combined actions tend to impose better effects on spoilage

and pathogenic microflora (Helander, *et al.*, 1997).

1.5.2. LAB as Probiotics:

Lactic acid bacteria are essential components of the animal microecology. For instance, humans are known to harbour a normal microflora population of 10^{13}-10^{14} cells on body surfaces and organs (a number which exceeds the total number of body cells). The microflora is varied and is composed of over 500 different species (Mikelsaar and Mäander, 1993). The gastrointestinal (GI) and the genital tracts and the skin microbial ecosystems harbour the highest population of microflora in humans. Research has focused on the analysis of the role of most microbial genera and species in these varied microhabitats. In the GI tract alone, for instance, a kilo of microflora consists of over 400 species out of which members of the genera *Lactobacillus* and *Bifidobacterium* are known to play a beneficial role (Gibson and Roberfroid, 1995).

In a recent study Tannock (1997) has investigated the pattern of faecal lactobacilli and bifidobacteria on ten healthy human subjects and showed that each human had their unique collection of strains. Nevertheless, despite this variation there is an overall need for the LAB to be an essential component of the GI system.

The faecal lactobacilli count from humans is normally between 10^{8}-10^{10} cfu/g (Gibson and Roberfroid, 1995; Mital and Garg, 1995). However, the faecal count is just an approximate value as there are masking reasons besides the non culturability of most inhabitants of the GI tract in synthetic media. In most cases the consensus on the lactoflora of the human GI tract shows the prevalence of various species, subspecies and biotypes of homo- and hetero-fermentative LAB. The most frequent lactobacilli found include *Lb. acidophilus*-like, *Lb. salivarius*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. reuteri*, *Lb. plantarum*, *Lb. fermentum* and *Lb. brevis* in various combinations (Mikelsaar and Mäandar, 1993; Molin, *et al.*, 1993; Ahrné, *et al.*, 1998). Lactobacilli are also the predominant flora isolated from the vagina of healthy women with a population range of 10^{8}-10^{9} cfu/ml (Mikelsaar and Mäandar, 1993).

LAB are apparently supplied to the alimentary tract from the non-heated lactic-fermented foods consumed. In cases where the intestinal lactobacilli are diminished it was found essential, therefore, to maintain their prevalence by external intentional supply of the LAB as probiotics. Live

LAB have been used as probiotics both in humans and animals. Foods intentionally supplemented with such live LAB for human gut reconditioning are termed functional foods and their claimed benefits include reduction of cholesterol or prevention of its synthesis in the human body (Gilliland, 1989; Ebringer, *et al.*, 1995), antitumor and immunostimulatory effects (Ebringer, *et al.*, 1995; Mital and Garg, 1995; Patidar and Prajapati, 1997), neutralization of enterotoxins and competitive inhibition of pathogens (Mital and Garg, 1995; Helander, *et al.*, 1997; Kalantzopoulos, 1997; Tannock, 1997) are sought.

The probiotic concept was initially founded on the fermentative properties of the LAB as observed by Metchnikoff in early 1900 where he curiously asked "..If a lactic fermentation prevented the putrefaction of milk, would it not have the same effect in the digestive tract if appropriate microorganisms were used?" (Tannock, 1997). As a result nowadays, though extensive data is still required, a wide number of LAB amongst which lactobacilli, bifidobacteria and *Streptococcus thermophilus* are the major organisms used in most probiotic preparations (Fuller, 1992; Mital and Garg, 1995; Salminen, *et al.*, 1996; Kalantzopoulos, 1997; Tannock, 1997). Table 6 presents the most commonly utilized probiotic strains of LAB for humans and animals.

Table 6. Probiotic LAB and bifidobacteria commonly supplemented into foods and feeds.

For humans	For farm animals
Lactobacillus acidophilus	*Lb. acidophilus*
Lb. casei Shirota strain	*Lb. casei*
Lb. delbrueckii subsp. *bulgaricus*	*Lb. delbrueckii* subsp. *bulgaricus*
Lb. johnsonii	*Lb. plantarum*
Lb. plantarum	*Lb. reuteri*
Lb. reuteri	*Bf. bifidum*
Lb. rhamnosus	*Enterococcus faecium*
Bifidobacterium adolescentis	*St. thermophilus*
Bf. bifidum, Bf. breve	*Pediococcus pentosaceus*
Bf. longum, Bf. infantis	
Streptococcus thermophilus	

(Compiled from Molin, 1995; Salminen, *et al.*, 1996 and Tannock, 1997).

The LAB used as probiotics are predominantly lactobacilli and as they are ecologically

more related to mucosal surfaces and the intestinal epithelium of warm blooded animals (Table 6). Among the major benefits they endow to the gut are: their contribution to the control of pathogens in the gut by competition for nutrients and adhesion, production of inhibitory metabolites, serving as barriers preventing bacterial translocation into the circulatory system and other organs, immunostimulation and immunomodulation and supply of SCFA (Short Chain Fatty Acids) for epithelial cells in the colon. Sullivan, *et al.,* (1988) demonstrated the *in vitro* inhibition of *Clostridium botulinum* by intestinal microflora isolated from infants while Nousiainen and Setälä (1993) showed inhibition of *Helicobacter (Campylobacter) pylori* by LAB. The preventive potential of intestinal lactobacilli against such serious bacterial pathogens as *Helicobacter pylori, in vivo,* has also been demonstrated (Kabir, *et al.*, 1997).

Nousiainen and Setälä (1993) have also further documented evidence supporting the strong antagonistic properties of LAB on harmful gut bacteria in piglets, pigs, calves and gnotobiotic mice. They have shown the suppression of coliforms and the neutralization of their toxins as direct effects rendered by the LAB although the exact mechanism of inhibition is not clearly known. The authors have also monitored the beneficial shifts in microbial or host metabolism by feeding LAB probiotics mainly *Lb. acidophilus* and *Lb. delbrueckii* subsp. *bulgaricus* to humans and animals. The associated benefits observed include decreased serum cholesterol, formation of inhibitory bile acids, decreased amine production, decreased production of carcinogenic N compounds, availability of hydrolytic enzymes and increased activity of brush-border enzymes. Other authors have demonstrated the improvement of gastrointestinal health when *Lb. plantarum* and *Lb. rhamnosus* were added to an oatmeal-based diet including the commercial product Proviva (Johansson, *et al.*, 1993; Molin, 1995). Consequently, intestinal strains of lactobacilli have got priority in most human probiotic preparations and are used as therapeutic agents in disturbed gut microecology. Apart from this, data confirming accumulation of anticarcinogenic metabolites in fermented foods points to the need for more studies on the potential use of lactobacilli to reduce or eliminate procarcinogens or carcinogens in the alimentary canal (Mital and Garg, 1995). This has, in turn, added value to lactobacilli for use as human probiotics (Mital and Garg, 1995; Kalantzopoulos, 1997). In animals this growth promotion by controlling pathogens is of prime

importance (Tannock, 1997).

Concerns have been raised on whether it is safe to use LAB as probiotics. Aguirre and Collins (1993) have indicated a possible association of LAB with human clinical while some reports consider LAB as opportunistic pathogens. Enterococci, leuconostocs and lactococci have been associated with infections but lactobacilli and pediococci have been rarely incriminated (Aguirre and Collins, 1993). Enterococci have been found to be of great clinical significance in relation to plasmid-mediated antibiotic resistance (Quednau, 1998). This has, therefore, casted doubt on the use of LAB or as human probiotics. Reassessment of the clinical significance of LAB is thus important.

1.6. Status and Features of Lactic Acid Fermented Foods in Ethiopia

Traditional fermented foods are commonly eaten in Ethiopia where the main source of the daily diet is based on household food production. Nutritional diversity is one important means by which a society sustainably uses the energy flow. In Ethiopia plant foods such as cereals, legumes, roots and tubers, oil seeds, fruits and vegetables make a major contribution to the dietary needs of the human population. Animal products, to a lesser extent are also included in the daily diet. These include dairy products, poultry, eggs, meat and meat products. Fish consumption is, by and large, limited to areas where they are available. The use of traditional fermentation as a means of food processing and preservation is an old household practice practiced in all agro-ecological zones. In rural Ethiopia, traditional beverages including *tella* supplement the daily diet. Soaking seeds for malt, for softening and for producing sprouts is a common practice. In this way dry chick peas, faba beans, peas, maize seeds are rehydrated. Fermenting cereal flours prepared from raw or roasted cereals and/or grains mixed with ground dry malt, *gesho* and roasted and ground barley, wheat, sorghum, maize or millets are an essential part of the household *tella, derekot* and *korefie* brewing technologies (Sahle and Gashe, 1991). Flour from cereals and grains is also used in making local spirits. Dough is mainly fermented for *injera* and *anebabero* from cereals such as wheat or barley, tef, sorghum or composite flour from these. Alternatively *dabo* and *ambasha* may also be made from wheat. Fermentation of legume flour is not very common except in the production of a side

dish called *siljo*. *Siljo* is very commonly eaten during the fasting season amongst the Orthodox Christians. It is prepared from legumes such as chick peas or faba beans made into flour and fermented together with safflower extracts, crushed mustard seeds, garlic, bishop's weed, spices like rue and ginger and table salt so as to develop a strongly acidic side dish served uncooked usually with legume sauce or stew (Mehari and Ashenafi, 1995; Zewdie, *et al.*, 1995). Fermented low-alcohol beverages like *borde* and *shamita*, which are made from barley and maize flours, respectively, constitute the main breakfast meal in southern Ethiopia and even in some towns and cities including the central highlands (Ashenafi and Mehari, 1995). Amongst the root crops *Enset (Ensete ventricosum)* is fermented mainly to produce *bulla* and *kocho*. Whereas fruits such as grapes may be fermented for wine. Honey is used as a substrate in *tej* production, a famous traditional fermented alcoholic beverage, honey wine.

Fermented milk and milk products and butter are produced in almost every household owning milking cows, particularly in rural Ethiopia (Ashenafi, 1990; Urga, *et al.*, 1992; Beyene, *et al.*, 1998). Fermented milk is also produced as a small-scale household product in cities. Fermentation basically serves as a major means for keeping large amounts of milk produced in areas where people own large herds or during the fasting seasons when consumption of dairy products is abstained from or reduced.

Traditionally milk containers are smoked with olea *(Olea africana)* splinters together with the leaves. Raw milk is added every day to the containers and allowed to undergo natural fermentation at ambient temperature. The coagulated (curdled) milk called *ergo* and *ititu* with their characteristic aromas and flavours are commonly eaten alone, as part of the meal or might also be churned for butter production (Urga, *et al.*, 1992). Ethiopian cheese, *ayib*, is another peculiar product produced by boiling *arera* (defatted milk) after churning at a high temperature (Ashenafi, 1990; Ashenafi, 1992). Aroma, flavour and taste are developed by keeping the cheese in smoked containers, into which fresh leaves of *koseret* or *kessie* and aromatic herbs are added. This process imparts the typical aroma characteristics of these spicy plants. The *ayib* is kept under airtight conditions at room temperature. Taste improves as the pH is lowered and additional desirable flavour and aroma compounds are developed later on, within a couple of days to weeks, apparently

from coupled lactic acid fermentation and proteolysis. A different procedure may be followed where *ayib* is still used as a substrate but a variety of spices, including hot pepper, are added and fermentation is carried out for months to years to produce a peculiar dehydrated, delicious product called *metata* which probably undergoes ripening by moulds. This product is perhaps typical to Gojjam.

Based on available data and personal observations, lactic acid fermented foods are quite rich, deep rooted to the cultural practices and well distributed in almost all parts of in Ethiopia. Mould-fermented foods are not common and have not been recorded as desirable. Therefore, most Ethiopian fermented foods and non-alcoholic beverages are either primarily or usually blended with lactic acid fermentation while alcoholic fermentation is employed for producing alcoholic beverages. These two fermentation systems seemingly constitute the major methods of producing fermented foods and beverages both in the rural and urban settings.

1.7. Tef Dough Fermentation

Injera is one of the major foods in Ethiopia. It contributes the largest share in the daily meal in most parts of the country. *Injera* is normally prepared from cereals like tef, wheat, barley, sorghum, maize and millets or a composite flour from them. However, *injera* from tef is the most preferred. Tef *(Eragrostis tef)* is a seasonal cereal of fine grains from the grass family, *Poaceae*, used for food only in Ethiopia. It grows in the altitude ranges between sea level up to 2800 meters above sea level (Ketema, 1991). The nutritional composition of *tef* flour is usually improved through fermentation into dough so that the resultant *injera* produced by baking has better nutritional quality (Nigatu, 1992; Urga, *et al.*, 1997a). Thus *tef* dough fermentation serves to get an improved food product.

Injera is a wide, flat bread baked from partially or well fermented dough. In making the dough, *tef* flour is thoroughly mixed with sufficient quantity of water and with *irsho* (a leftover batter from previous dough) and then allowed to ferment at room temperature (18-21°C). The steps and inherent events during the fermentation process are schematically presented in Figure 6. The procedures followed in different regions are variable. For instance, in Gojjam and Gonder regions,

it is very common to use hot water during mixing of flour with water. They mix the fermented dough with cold water after the first dough rising and then bake it directly. In other cases, instead of boiling portion of the dough for the thin paste *absit*, it is customary to add hot water and mix the dough before baking in order to facilitate the secondary dough rising. Therefore, there is no standard procedure universally followed in *injera* making. Partially fermented *injera* (for less than 24 hours), *aflegna*, has a pH of about 5.5 and is particularly preferred for its less acidity by people with irritated bowel due to gastritis. A fully fermented *tef* dough is that usually left to ferment for 48-96 hours or even longer (Gifawossen and Besrat, 1982; Gashe, 1985; Nigatu, 1992). During the process of *tef* dough fermentation, a number of biochemical transformations occur besides the sequential and successive drops in pH from near-neutral (pH 6.7) to below pH 4.0 (Gashe, *et al.*, 1982; Gashe, 1985; Umeta and Faulks, 1989; Nigatu, 1992; Urga, *et al.*, 1997a). The nature and role of *irsho* have been described by Ashenafi (1994). Species of bacteria belonging to different genera are involved along with yeasts, actinomycetes and moulds (Gashe, *et al.*, 1982; Gifawossen and Besrat, 1982; Gashe, 1985; Nigatu, 1992; Nigatu and Gashe, 1997). The bacteria are important in the primary fermentation periods while the yeasts are responsible for the secondary dough rising. The pattern of *tef* dough fermentation is a typical lactic acid fermentation that resembles the sourdough process (Lönner, 1988). In *tef*, LAB belonging to the genera *Lactobacillus, Pediococcus, Leuconostoc* and *Enterococcus* have been frequently isolated (Gashe, *et al.*, 1982; Gashe, 1985; Nigatu, 1992; Nigatu and Gashe, 1994b). Phenotypic taxonomy by Gashe (1985) showed the aciduric heterofermenters *Lb. plantarum, Lb. brevis, Lb. fermentum* and *Ped. cerevisiae (Ped. pentosaceus)* to be the predominant LAB at lower pH values. The identity and role of *Bacillus* species from these two foods have been described by Nigatu and Gashe (1997). The moulds and yeasts were found to develop later and act as spoilage organisms (Gifawossen and Besrat, 1982; Gashe, 1985; Nigatu, 1992).

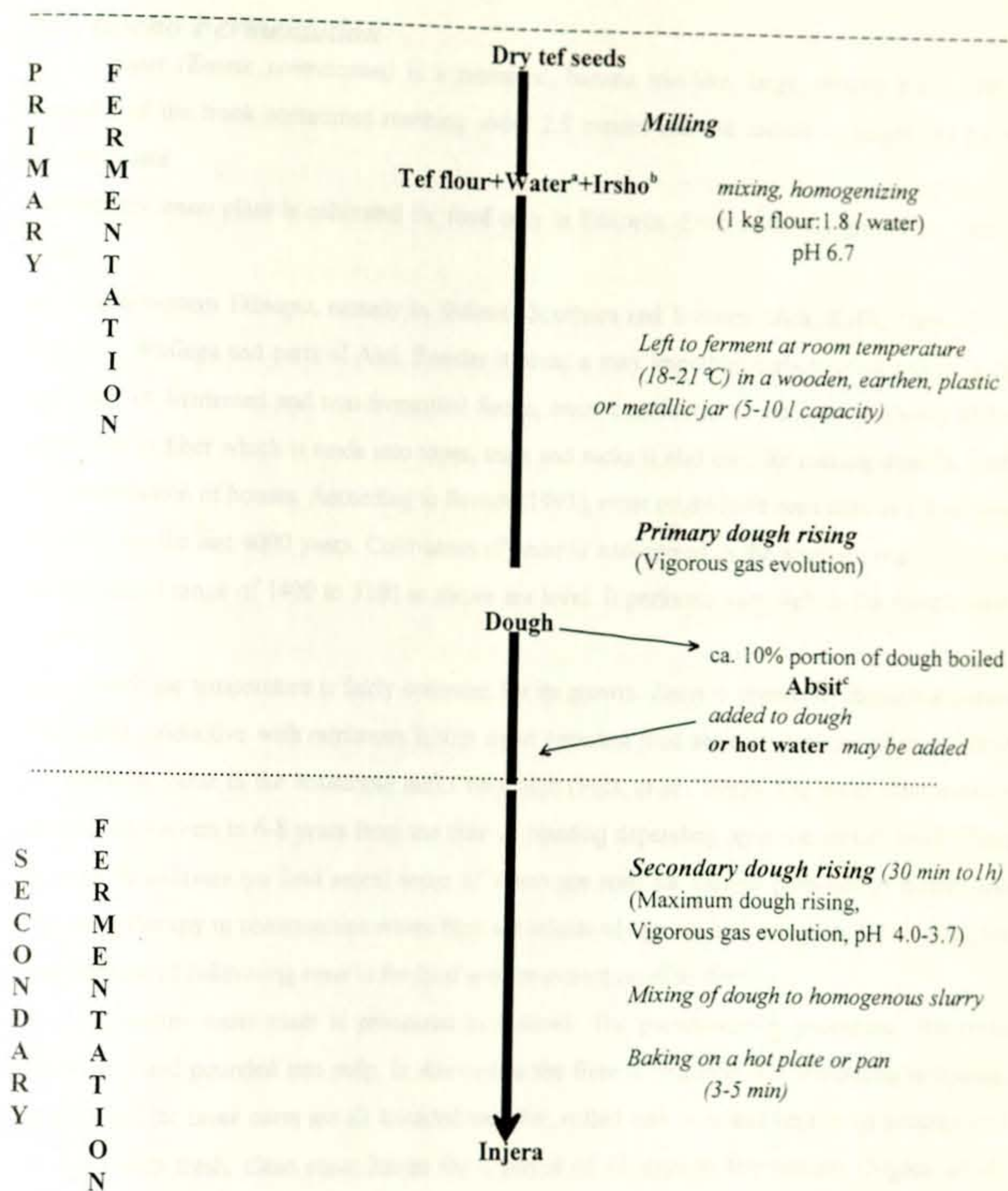


Figure 6. Schematic overview of *tef* dough fermentation for *injera* (fermentation lasts between 2-4 days). ([a] Flour may alternatively be mixed well with hot water; [b]*irsho* is a portion of previous batter used as a starter; [c]*absit* is a dough enhancer, but only used in cities and central regions of Ethiopia; pH values are given only for the initial and final average measurements since in the intermediate steps values were variable).

1.8. Kocho Fermentation

Enset (Ensete ventricosum) is a perennial, banana tree-like, large, starchy plant with a diameter of the trunk sometimes reaching about 2.5 meters and 6-8 meters in height. As far as available data

indicate, the *enset* plant is cultivated for food only in Ethiopia. *Enset* is mainly grown for food in South

and South-western Ethiopia, namely in Sidamo, Southern and Western Shoa, Kaffa, Gamo-Gofa, Illubabor, Wollega and parts of Arsi. Besides it being a very important carbohydrate source in the daily diet in fermented and non-fermented forms, *enset* contributes to the main economy of the family for its fiber which is made into ropes, mats and sacks is also used for making utensils, roofs and construction of houses. According to Brandt (1993), enset might have been used as a food crop probably for the last 4000 years. Cultivation of *enset* is widespread in the southern regions within the altitudinal range of 1400 to 3100 m above sea level. It performs very well in the middle land, *woyna*

dega, where the temperature is fairly optimum for its growth. *Enset* is apparently drought-resistant and highly productive with minimum labour input ensuring food security in times of crop failure and hence its value in the household ranks very high (Pijls, *et al.*, 1995). The *enset* plant matures and reaches harvest in 6-8 years from the time of planting depending upon the variety used. There are over 70 cultivars (or land races) some of which are used for various purposes of dietary and nutritional therapy in communities where they are selectively grown and maintained. However, the main purpose of cultivating *enset* is for food and for extraction of its fibre.

A mature *enset* plant is processed as follows. The pseudostem is pulverized, the corm decorticated and pounded into pulp. In due course the fibre is removed. The remaining scrapping, the pulp and the inner corm are all kneaded together, rolled into balls and kept in an underground silo lined with fresh, clean *enset* leaves for a period of 15 days to few months (Nigatu, *et al.*, unpublished data). *Kocho* and *bulla* are two major fermented products from *enset*. *Kocho* is produced from the fermentation of the whole mashed mass whereas *bulla* is fermented from the residual soluble starch oozing out from the ruptured tissues. The duration of fermentation depends

on environmental temperature and the needs of the household. A completely fermented *kocho* has a pH of about 4.2 (Nigatu, 1992). In cases of urgent food needs of the family however, it is very common to boil the fresh pseudostem (pseudocorm) called *amicho* for immediate consumption at home, but in other times the *enset* plant is fermented in underground pits for years where fermentation serves both as a means of processing and preservation (storage). That is why the *enset* plant is considered as a food security plant and hence is named *workie* ("my gold").

The process of *enset* fermentation is schematically presented in Figure 7. The steps and names for the different components and processes are slightly variable in the different *enset* growing regions. The flow chart depicted in Figure 7 is adopted from a procedure followed during traditional *enset* processing in Sidama area. *Shigido* is a "starter" material traditionally used to inoculate a portion of *amicho* (pseudocorm). This then anaerobically as well as aerobically decomposes due to the action of both autolytic and elaborated enzymes from the associated microflora. It develops into a soft mucilaginous mass, called *gamancho*, in about a week time. *Dessa* is the fresh scrapped mass including other *enset* parts prepared for fermentation. When mashed *gamancho* is added to it is called *abicho*, which is a mass ready for the main course of fermentation. When this material is anaerobically digested for a few weeks there is a remarkable drop in pH from about 6.13 to around 4.7. This gives rise to a mass with dough-like consistency locally termed *Wassa*, which is further fermented to produce the final product *kocho*. *Wassa* and *kocho* are interchangeably used as synonyms for the partially- or entirely-fermented product. *Bulla* has a relatively brighter colour; it is a dehydrated starch and the yield is very small as compared with the bulk of *kocho* produced from the same plant.

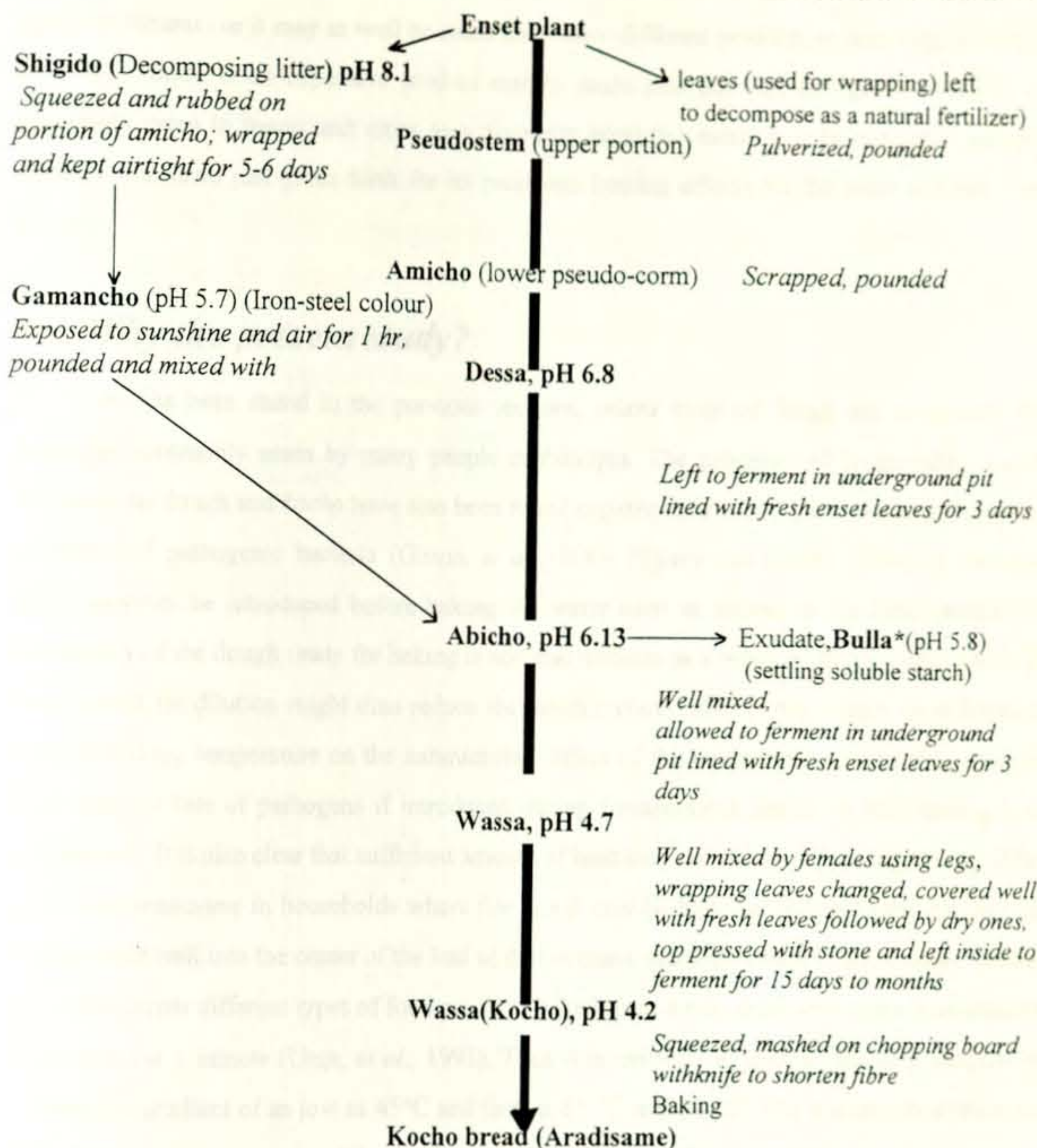


Figure 7. Flow chart showing the steps in *enset* processing and fermentation for *kocho* and *bulla* at 18°C, 1800 meters above sea level (Nigatu, Umeta and Urga, Fermentation studies on *Enset* at Aleta-Wondo, Sidamo, Ethiopia, 1993, unpublished data). (pH values are means for 5 *enset* cultivars; *Aradisame* is a local name for baked *kocho* bread in Sidama area).(**Bulla* is fermented kept beside of *wassa* and its pH also drops to around 4.0).

Kocho is mainly baked into bread locally called in different names (for instance in Sidama area it is called *aradisame*) or it may as well be made into many different products as described by Urga, *et al.* (1993). *Bulla* is an expensive product mainly made into porridge and gruel, which is very commonly eaten in towns and cities as a favourite breakfast meal. It is in particular served for mothers who have just given birth for its presumed healing effects for the wear and tear during labour.

1.9. Why the present study?

As has been stated in the previous sections, *injera* from *tef* dough and *aradisame* from *kocho* are commonly eaten by many people in Ethiopia. The processes of fermentation and the fermented *tef* dough and *kocho* have also been found capable of arresting the survival and growth of spoilage and pathogenic bacteria (Girma, *et al.*, 1989; Nigatu and Gashe, 1994a,b). Pathogens might however be introduced before baking via water used as diluent or the food handler. The consistency of the dough ready for baking is not also uniform as a result of dilution with water. The water added for dilution might thus reduce the antimicrobial effect of the dough. In addition, the effect of baking temperature on the antimicrobial effect of the fermentation products in these two foods and the fate of pathogens if introduced during fermentation, before or after baking is not documented. It is also clear that sufficient amount of heat may not be applied during baking of both *injera* and *aradisame* in households where fire wood may be limiting. In *aradisame* the heat may not penetrate well into the center of the loaf so that in many cases the center is white mass. We have also come across different types of food products baked from *kocho* even with slight heat-treatment as short as for 1 minute (Urga, *et al.*, 1993). Thus it is essential to see the fate of pathogens at a temperature gradient of as low as 45°C and then at 61 °C and 80 °C. The lowest temperature may simulate least heat treatment while the rest two slightly better baking situations.

Regarding identification of *Lactobacillus* and *Pediococcus* isolates from *tef* and *kocho*, previous phenotypic studies have shown the prevalence of some species (Gashe, 1985; 1987). Thus it has been found pertinent to use both phenotypic and genetic data for such purposes. Randomly

Amplified Polymorphic DNA (RAPD) has been found appropriate and fast in differentiating *Lactobacillus* isolates. And thus it was supposed that this procedure might be appropriate if used for *tef* and *kocho* isolates belonging to *Lactobacillus* and *Pediococcus* and related taxa.

API 50 CH fermentation results are used for taxonomic purposes in lactobacilli. The usual recommended incubation temperature for lactobacilli is both 30° and 37°C. Assessment of metabolic profiles using this method for taxonomic purposes in type strains of *Lactobacillus* and related taxa has, however, shown inconsistency between 30 and 37°C of incubation. The disagreement in the fermentation profiles has thus caused misidentification (misplacement) of the known species. This has therefore caused suspicion in that there might be suitable temperature for a given species. As a result, we have found it essential to reevaluate the appropriate temperature of incubation for a specific species in *Lactobacillus* and related taxa. The results of the assessment would also serve as a ground for the use of API 50 CH fermentation system as a reliable tool for identification of lactobacilli isolates from *tef* dough and *kocho*.

1.10. Aims of the present study

1.10.1. General:

The current research is aimed at identifying *Lactobacillus* and *Pediococcus* species frequently isolated from fermented *kocho* and *tef* dough using reliable and reproducible methods such as molecular genetic techniques. It was also found essential to see the fate of some pathogenic and spoilage microflora of both non-spore forming and spore forming, Gram-positive and Gram-negative nature including epiphytic contaminants as affected by fermentation and the baking heat.

1.10.2. Specific:

1. Determine the effect of heat treatment on the antimicrobial properties of primary and secondary metabolites elaborated during the fermentation processes in *tef* dough and in *kocho* and assess the fate of pathogens during baking of *tef* dough and *kocho* to *injera* and *aradisame*, respectively.
2. Employ RAPD analyses and use developed band patterns for grouping and identification of *Lactobacillus* and *Pediococcus* isolates from *kocho* and fermented *tef* dough into known species.
3. Assess the suitability of numerical analyses of RAPD electrophoretic profiles as a discriminatory tool for type and reference strains within the genus *Lactobacillus*.
4. Verify the genetic identity of lactobacilli and pediococci isolates using multiple molecular methods: RAPD and/or DNA-DNA hybridization and 16S rRNA sequencing, respectively.
5. Assess metabolic profiles of food isolates of lactobacilli and pediococci using the API 50 CH system.
6. Evaluate numerical analyses of API 50 CH fermentation data as an identification tool for lactobacilli and pediococci isolates from traditional fermented foods and see its agreement and complementarity with genetic tools of taxonomy.
7. Evaluate the consistency of API 50 CH fermentation profiles of type and reference strains of *Lactobacillus* species as affected by incubation temperature, 30°C vis-à-vis 37°C.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Food samples, sampling and sampling sites

2.1.1. Tef

For the study on the effect of baking heat on the antimicrobial properties of *tef* dough, *kocho*, *injera* and *aradisame* and fate of test organisms, *tef* grain was purchased from an open air market in Addis Ababa and ground using a local flour mill. For the taxonomic studies isolations, one hundred different *tef* flour samples collected from 25 households in Addis Ababa at two months intervals for a period of eight to ten subsequent months making sure a new batch of *tef* seeds was used to prepare the flour for each sample. This was done in order to randomize samples to be representatives of *tef* grown in different regions which is supplied to markets in Addis Ababa. In each case about 200 g of *tef* flour was collected in a new plastic bag. The bag was sealed and stored at room temperature in boxes in a dry condition until used. From the sample lots 100 g portions were then made into dough, well mixed with tap water (without addition of starter) in the laboratory at the Addis Ababa University, Ethiopia, and later at Lund University, Sweden. Fermentation was carried out in 600 ml glass beakers following previously described procedures (Gashe, 1985; Nigatu, 1992) and incubated at room temperature (18-21°C) for 72 hours. The fermentation process followed and sampling points of *tef* dough employed in the study are shown in the following flow chart.

Dry tef seeds

Milling

Tef flour+Water *mixing, homogenizing*
(1 kg flour:1.8 *l* water)
pH 6.7
***Inoculation of test bactera**

Left to ferment at room temperature (18-21 ℃) in a plastic/glass beaker (600ml capacity) for 48-72 h

Vigorous gas evolution)
(pH 4.0-3.7)

Dough

***Sampling for taxonomic study**
***Inoculation of test bacteria**
Baking in a sealed flask (3-5 min)
***Sampling for counting survivors of baking heat**

Injera

SCIENCE LIBRARY
ADDIS ABABA UNIVERSITY

2.1.2. Kocho

In the challenge study mentioned in section 2.2.2, *kocho* was bought from retail sellers in Addis Ababa. For the taxonomic study isolations, 125 fermented *kocho* samples were purchased from markets in Arsi, Sidamo, Gamo-Gofa, Southern Shoa, Western Shoa, Kaffa and Illubabor regions. Samples of 250-500 g were obtained from five different producer-sellers in 25 markets. Each sample represented a separate geographical locality. The origin of the sample was recorded in all cases to avoid multiple sampling from a single locality. Samples were sealed airtight, labelled, stored in cold and transported to Addis Ababa for further microbiological analyses and pH measurement.

2.2. The effect of baking heat on the antimicrobial properties of tef dough, kocho, injera, aradisame and fate of pathogens and contaminants

2.2.1. Test bacteria and culture media

The test bacteria used in the study were clinical isolates obtained from patients presenting samples to the public health laboratory, Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa. These included *Bacillus cereus, Klebsiella* spp., *Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus*. Their purity and identity were checked using standard tests. The rest of the test flora comprised of epiphytic spore-forming bacterial contaminants, yeasts and moulds indigenous to the food samples. To minimize the interference effects on the population of the indigenous bacterial contaminants in the foods on the survival studies of the clinical isolates, the initial microbial load of each food was determined.

The test bacteria were maintained in Nutrient broth (Oxoid, CM 1). Tests for antimicrobial inhibition were carried out in an assay broth (AB) described by Nigatu and Gashe (1994a,b). After assaying the survivors were selectively isolated from the AB using Egg-yolk agar (Oxoid, SR 47) for *Bacillus cereus*; Mannitol salt agar (Oxoid, CM 85) for *Staphylococcus aureus*; *Pseudomonas* isolation agar (Difco) for *Pseudomonas aeruginosa*; *Salmonella-Shigella* agar (Oxoid, CM 99) for *Salmonella* and *Shigella* spp. and MacConkey agar (Oxoid, CM 115) for *Klebsiella* spp. Yeasts and

moulds were harvested using Potato Dextrose Agar (Oxoid, CM 139).

2.2.2. Preparation of extracts, heat treatment and assaying

a. ***Fate of different pathogens in different concentrations of extracts heated at 45°, 61° and 80°C***

Clear supernatant liquids were obtained by centrifugation from 30 h fermenting *tef* dough or from fermented *kocho;* the later was diluted with one-half its volume of sterile water (2:1, *kocho*:water). Supernatants of both *tef* dough and *kocho* were collected aseptically in sterile containers and heat treated at 45°, 61° or 80°C for 5 min which were later supplemented into 5 ml Assay Broth (AB) to give graded concentrations.

b. ***Fate of the different pathogens in different concentrations of extracts of injera and aradisame:***

One hundred gram samples from fermented *tef* dough (30 h) or *kocho* were baked at 100°C for 3-5 min in a sterile screw-capped flask and cooled to room temperature. Portions of the baked foods were broken into small pieces, soaked in an equal volume of sterile distilled water for 5 min and then mashed aseptically. The mixture was centrifuged and clear supernatant transferred into sterile containers for later use in the assay. For comparison purposes, home made *injera* was also used and extract produced in the same way.

c. ***Survival of the test bacteria inoculated into tef flour and kocho:***

Into tubes containing graded volumes of extracts (20-120 μl/ml or 100-600 μl/5 ml assay broth) were inoculated with 3-4x10^3 cfu/ml broth of 24 h cultures of each of the test bacteria and incubated at 32°C for 24 h. Survival and growth of the test organisms after fermentation was determined spectrophotometrically at OD 540 nm.

d. ***Survival of the test organisms and indigenous flora to baking heat:***

The survival of the inoculated bacteria and also the epiphytic flora subjected to baking heat

was monitored as follows:3-4x10^3 cfu/g dry wt of food of 24 hr culture of each of the test bacteria were inoculated into *tef* flour-water or *kocho*-water mixture and allowed to ferment at room temperature for 30 h. The doughs were then baked in sterile screw-capped flasks at 100°C for 5 min and cooled to room temperature. Twenty-five gram aliquots from each baked food were diluted with 225 ml sterile 0.1% peptone water diluent and then 1 ml portion from each of the dilute foods were plated onto an appropriate selective medium for the respective test organism. For *B. cereus, Ps. aeruginosa, S. aureus*, yeasts and moulds, incubation was carried at 32°C while for *Klebsiella, Salmonella* and *Shigella* spp. at 37°C. In all cases incubation was performed for 2-3 days.

e. Determination of the populations of indigenous yeasts, moulds and Bacillus cereus in tef dough, kocho, injera and aradisame:

The populations of yeasts, moulds and *Bacillus cereus* in *tef* dough (30 h), *kocho* (immediately after purchase) and *injera* and *aradisame* were determined by plating from serially diluted portions of the doughs or baked foods in sterile diluent peptone. The *tef* dough at 30 h of fermentation was selected as it is the earliest possible time of baking in cases when completely fermented dough is not needed, or when a partially fermented one *'aflegna'* is required. The other underlying reason for selecting this time point is that this is the optimum period of *tef* dough fermentation found out in our previous studies containing the maximum and strong antimicrobial potency with high nutrient content (Nigatu and Gashe, 1994a,b). The differences in populations between *tef* flour and *injera* and raw *kocho* as purchased and *aradisame (kocho* bread*)* served to determine changes in the bacterial, yeast and mould flora as affected by fermentation and/or baking heat. Mean values of four food samples in triplicate experiments were recorded.

2.3. Taxonomic studies

Isolation of *Lactobacillus* and *Pediococcus* species from *tef* dough and *kocho*:

Throughout all taxonomic studies mentioned in the thesis, 25 g (ml) aliquot from fermented

tef dough (48 h, pH 4.3) or fermented *kocho (wassa)* (pH 4.2) were diluted with 225 ml of 0.1% sterile peptone water which were further serially diluted for proper population sampling. One ml sample from appropriate dilution was pour-plated onto Rogosa agar and incubated anaerobically using a BBL GasPak Anaerobic system (GasPak, Anaerobic system, Becton Dickinson Microbiological Systems, Cockeysville, Maryland, USA) at 30°C for 24-72 h. From developed individual colonies, 6-8 representatives were picked and subcultured in *Lactobacillus* carrying medium, LCM (Efthymiou and Hansen, 1962) supplemented with 2% glucose. All Gram-positive, catalase-negative rods, cocco-bacilli and cocci were presumptively taken as LAB. Those isolates possessing the former two cell shapes were taken as *Lactobacillus* and those of the latter with cells existing in pairs or in tetrads as *Pediococcus* species. Further microscopic observations were coupled with successive transfers of individual colonies on LCM broth, LCM agar, MRS broth and MRS agar 6-9 times to ensure purity of strains. Purity of the cultures was confirmed by microscopy by looking for a homogenous morphology of cells from each culture. Several hundreds of different isolates were obtained out of which four hundred, i.e., two hundred isolates each from *Lactobacillus* and *Pediococcus*, were selected for further taxonomic studies.

Type and reference strains used in the study:

Lactobacilli: Type and reference strains of 56 validly published species of *Lactobacillus* obtained from different culture collection centres were compared with 196 food isolates based on their RAPD band patterns. Later on 22 of the type and reference strains and three type strains of three species of *Weissella* were further studied. In the study on the use of RAPD for *Lactobacillus* type, 41 type and reference strains were used. Whereas in the API 50 CH fermentation study, 44 *Lactobacillus*, one *Atopobium* and three *Weissella* strains were studied. The strains used are listed in Table 13. Species identities and purities were regularly checked.

Pediococci: The seven validly published species of *Pediococcus* were represented by their type strains and compared with 202 food isolates were compared. The type strains used were *Pediococcus acidilactici* CCUG 32235^T, *Ped. damnosus* CCUG 32251^T, *Ped. dextrinicus* CCUG 18834^T, *Ped. inopinatus* CCUG 38496^T, *Ped. parvulus* CCUG 28439^T, *Ped. pentosaceus* CCUG

32205^T and *Ped. urinae-equii* CCUG 28094^T. All of these type strains were obtained from the Culture Collection University of Gothenburg, CCUG, Sweden. The strains were sub-cultured twice in LCM broth and then subjected to PCR analysis together with the field strains.
For convenience *Ped. urinae-equii* was included and treated as a *Pediococcus* although it has been reclassified as an *Aerococcus* species.

Taxonomic methods employed:

Both phenotypic and nucleic acid-based tests were utilized. Growth characteristics, such as ability to grow on Rogosa agar, LCM and MRS; colony and cellular morphologies, cell arrangement, colony characteristics, Gram's and catalase reactions served for preliminary screening of the LAB. DNA-based genetic procedures were used for tracing relatedness within and between isolates and type strains using numerical analyses supplemented with statistical softwares. For this purpose then the random amplified polymorphic DNA fingerprints served for grouping while DNA:DNA reassociation results helped to confirm relatedness. Subsequent phenotypic tests based on carbohydrate fermentation and production of acid were all used for characterization purposes. Furthermore, those *Pediococcus* isolates which did not show relatedness to known species with RAPD were compared with the type strains based on their 16S rRNA sequences.

2.3.1. Genomic identification

2.3.1.1. Randomly Amplified Polymorphic DNA (RAPD)

RAPD was used for discrimination and grouping of type strains and food isolates of lactobacilli and pediococci. This nucleic acid-based detection method employs comparison of the electrophoreses fragments of polymorphic DNA randomly amplified using short oligonucleotides of 9-mer bases as described by Johansson, *et al.* (1995c).

Preparation of crude cell extract:

Overnight cultures grown in LCM at 30°C in eppendorf tubes were centrifuged at 14,000 g for 5 min and washed twice with 1 ml sterile double distilled water (SAQ). Into each tube were added 0.25 ml SAQ and about 8 sterile glass beads (0.2 mm diameter). Cells were disintegrated by

thorough cold shaking for 30-45 min at 4°C using an Eppendorf Mixer (Model 5432, Eppendorf, Hamburg, Germany). The tubes were then centrifuged for 5 min and clear supernatants were transferred into sterile eppendorf tubes and kept at
-20°C until used.

PCR Processing:

Into a reaction tube were added 34.5 µl SAQ, 5 µl of 10x PCR reaction buffer (Boehringer Mannheim Scandinavia, Bromma, Sweden), 1 µl each from the dNTPs (Perkin Elmer, Branchburg, NJ, USA), 5 µl Primer 73 (a 9-mer with a base sequence of 5'-ACGCGCCCT-3', Symbicon AB, Umeå, Sweden), 1 µl of crude genomic DNA (crude cell extract) and 0.5 µl of Taq polymerase (Boehringer Mannheim Scandinavia, Bromma, Sweden). The buffer already contained $MgCl_2$. The reaction mixture was overlaid with mineral oil to avoid evaporation while running the PCR steps and effect thermal conduction. The amplification was done in a Perkin Elmer Cetus DNA thermal cycler (Model 480, Perkin Elmer, Norwalk, USA). The cycles followed in the PCR had the temperature profile: 94°C, 45s; 30°C, 120s; 72°C, 60s for four cycles followed by 94°C, 5s; 36°C, 30s; 72°C, 30s for 26 cycles; the extension step was increased by 1s for every new cycle. The PCR amplification reaction was terminated at 75°C for 10 min after which samples were cooled to 4°C.

Gel electrophoresis and photography:

Submerged horizontal slab gels with 1.5% agarose (Type III:High EEO, Sigma, St. Louis, USA) in TB buffer (89 mM boric acid, 23 mM H_3PO_4, 2.5 mM EDTA, pH 8.3) as described by Johansson, *et al*, (1995), were prepared. Twenty µl aliquot from each sample was mixed with 5 µl of dye; 3 µl DNA molecular weight marker VI (Boehringer Mannheim Scandinavia, Bromma, Sweden) was added to 5 µl dye plus 17 µl distilled water. The molecular weight marker mixture was loaded on the gel along with the samples serving as a standard and electrophoresis was run at 100 V for 2.5 h in a TB electrophoresis buffer without cooling. In all cases fresh buffer was used for every gel electrophoresis run. Gels were stained in dilute ethidium bromide (0.2 µg ml^{-1}) for 5 min and then washed in distilled water for 5 min. Dewetted gels were visualized, at 302 nm on a

UV transilluminator board (UVP Inc., San Gabriel, CA, USA) and photographed with a Polaroid camera loaded with polaroid film (Polaroid, Number 665, Polaroid Corporation, Cambridge, MA, U.S.A.) and developed as recommended by the manufacturer.

Numerical Analyses

Image preparation, comparison and analyses:

Developed bands on the photo or negative were scanned with a flatbed scanner (UMAX UC630 Max Color) at a resolution of 200 dots per inch. The length of each lane finally tracked was the length corresponding to the length of the lane for DNA molecular weight marker VI. All scanning, tracking, normalization, comparison and analyses of gel images were carried out using GelCompar Version 4.0 program (Applied Maths, Kortrijk, Belgium). The percentage similarity was based on the compared zones, i.e., bands within the length of the DNA molecular weight marker (0.154 and 2.176 kbp). Similarity matrix and cluster analysis of the matrix of similarity values were calculated using the Pearson product moment correlation coefficient (r) with the unweighted pair group method using arithmetic averages (UPGMA) clustering algorithm. Resultant dendrograms were produced and major clusters defined at a 45% similarity (relatedness) value as a cut-off point for a single cluster.

Reproducibility of the RAPD procedure:

To test for reproducibility of the RAPD procedure, cell lysates were prepared in duplicate and in some cases in triplicate from pure cultures of the type and reference strains and few field isolates and were then amplified independently following the same procedure. Gels were run, scanned and analyzed using the same statistical software.

2.3.1.2. DNA:DNA Hybridization

Pure DNA was prepared from *Lb. plantarum* ATCC 14917^{T} following the procedure of Ståhl and Molin (1994) as modified by Johansson, *et al.* (1995b). In preparing crude chromosomal DNA from the isolates and the negative control, *Lb. reuteri* DSM 20016^{T}, washed pellets of

overnight cultures were lysed following the procedure described in the RAPD study. In the pre-hybridization step, chromosomal DNA obtained from the isolates was blotted on positively charged nylon membrane (Boehringer Mannheim, GmbH, Mannheim, Germany) on a slot-blot apparatus (SlotBlot, Model PR648, Hoefer Scientific Instruments, San Francisco, CA, USA). Hybridization was performed with a vertically-rolling hybridizer (Hybridizer HB-2D, Techne Cambridge Ltd, UK) using DIG-labelled hybridization procedure described by Johansson, *et al.* (1995b). Images were developed on a Kodak X-omatic film in an X-ray developer and cassette (Kodak, Eastman, Rochester, NY, USA) as recommended by the manufacturers. Results obtained on the X-ray film were then visually compared against the intensity of the pure homologous DNA for the type strain.

2.3.1.3. 16S rRNA sequencing

The same procedure of preparing crude cell lysates as used in the RAPD study was used on type strains and isolates of *Pediococcus*. Two different primers, primer 593 and 621B, were used. The sequences and characteristics of these primers were described by Pettersson (1997). The procedure used for agarose gel electrophoresis and photography in the RAPD study was followed to produce gel pictures of the 16S rRNA PCR products. A direct solid-phase DNA sequencing procedure described by Pettersson (1997) was employed for a partial sequence of the 16S rRNA samples from on about 1500 nucleotides was generated and compared with available data. In this study all of the seven type strains of *Pediococcus* species together with 15 representative food isolates from the major RAPD clusters described in Figure 17 were subjected to genomic sequence analyses and their resultant stretches were compared.

2.3.2. Phenotypic studies using API 50 Carbohydrate fermentation

Preparation of cultures and assaying for carbohydrate metabolism:

Overnight cultures of *Lactobacillus* or *Pediococcus* isolates or type strains grown in 10 ml MRS broth at 30°C were washed twice with sterile physiological saline (0.9% sodium chloride) and the pellets were resuspended in API 50 CHL medium (API systems, BioMéreux, SA, France) by vortex mixing. Using sterile pasteur pipettes homogenized suspensions of the cells in the medium,

were transferred into each of the 50 wells on the API 50 CH strips. All wells were overlaid with sterile paraffin oil (Merck, Darmstadt, Germany) to effect anaerobiosis. Strips were moistened and covered as recommended by the manufacturer and incubated at 30°C. Changes in colour from violet to yellow were monitored after 1, 2, and 7 days. Results of each of the 49 strips were graded from 0-5, with "5" standing for complete change to yellow and "0" for no change at all. The first strip served as a control well. Esculine hydrolysis (revealed by change to darker or black colour) was represented by a positive sign (+) while a negative sign (-) was designated for no change.

Evaluation of API 50 CH fermentation profiles:

Those grades of fermentation results ranging from 3-5 were interpreted as positive (+) whereas zero, 1 and 2 were redefined as negative (-) for computer analysis, the data was scored as "one" for positive and "zero" for negative for all strips. Percentages of positive results on the profiles recorded for the 49 carbohydrates were calculated and test vigor values generated; values below 20% were taken as low. The fermentation patterns were evaluated for relationships (similarities and differences) between the type and field strains employing cluster analyses and dendrograms produced. These fermentation patterns were also used to characterize the metabolic properties of the organism. The fermentation profiles of type and reference strains of *Lactobacillus* and related genera, in particular those related to food and the human microenvironments, were evaluated for their patterns of API 50 carbohydrate utilization when incubated at 30°C and 37°C.

Data analyses:

Key characteristics of carbohydrate metabolism from the Bergey's manual (Kandler and Weiss, 1986) and Hammes and Vogel (1995) were used for comparison of each strain for its differential physiological traits with records for type strains. Consistency and discripancy of test scores based on the relationships between the results obtained in the two incubation temperatures were evaluated. In order to check the reproducibility of the results, *Lb. plantarum* and *Lb. salivarius* subsp. *salivarius* type strains were checked in duplicate at both temperatures.

CHAPTER THREE

RESULTS

3.1. The effect of baking heat on the antimicrobial properties of tef dough, kocho, injera, aradisame and fate of pathogens and contaminants

Fermented *tef* dough or *kocho (wassa)* contains a wide spectrum of aerobic, facultative and anaerobic bacteria, yeasts and moulds. The predominant microflora in both foods, however, belong to the lactic acid bacteria (LAB) followed by *Bacillus* spp., yeasts and moulds. The LAB belong to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Streptococcus* (Table 7). Members of the family *Enterobacteriaceae* were scanty in *kocho* but were isolated in higher numbers from *tef* dough.

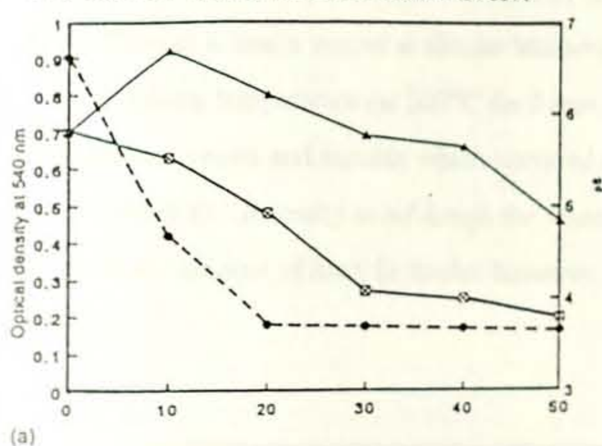
Table 7. Population of LAB and related organisms in *tef* flour and spontaneously fermenting 30 h dough.

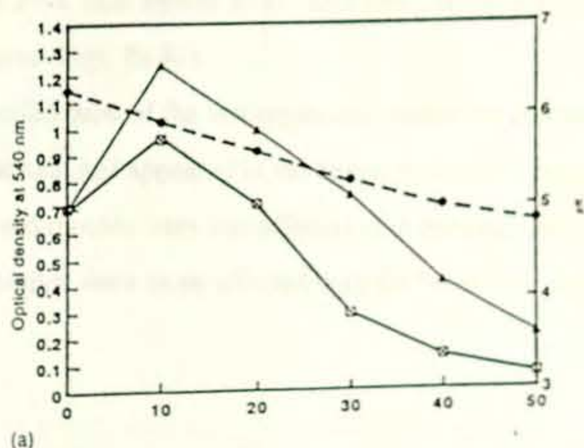
Food item	pH	*Microbial load (cfu/g dry wt of flour or fermented *tef* dough)							
		Entb.	***Lb.***	***Ped.***	***Leu.***	***Entc.***	***Bac.***	**Y & M**	**Tot. count**
Flour	6.3	$8.2x10^1$	$2.2x10^1$	$6.8x10^1$	$1.5x10^1$	<10	<10	$6.3x10^2$	$8.2x10^2$
Dough	4.7	$1.9x10^7$	$3.0x10^8$	$6.5x10^8$	$3.4x10^8$	$4.3x10^6$	$6x10^4$	$6x10^7$	$3.7x10^8$

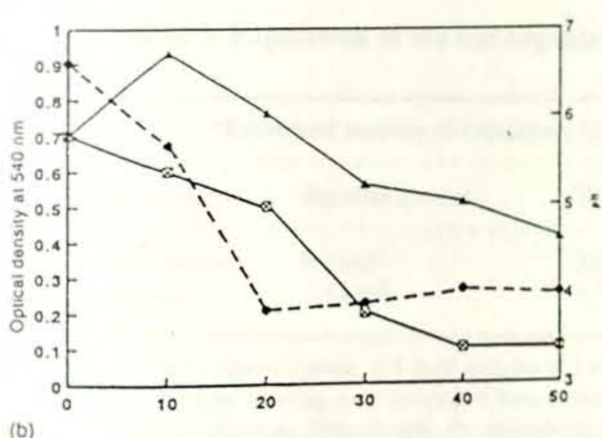
* Average values of 4 food samples in 3 determinations are presented.
Entb.=Enterobacteriaceae; Lb.=Lactobacillus; Ped.=Pediococcus; Leu.=Leuconostoc; Entc.=Enterococcus; Bac.=Bacillus; Y & M=Yeasts and moulds.

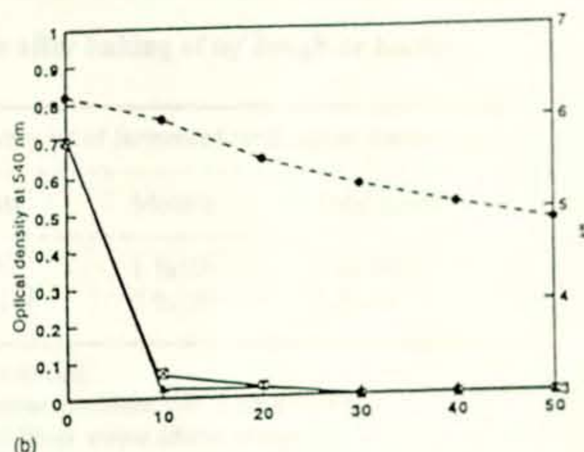
Heat-treated extracts from fermented *tef* dough (30 h) showed inhibitory activities against both to Gram-negative and Gram-positive bacteria including sporeformers, the effects being much more pronounced with increasing temperatures of heat treatment (Figs.8a-8c). The extract heat-

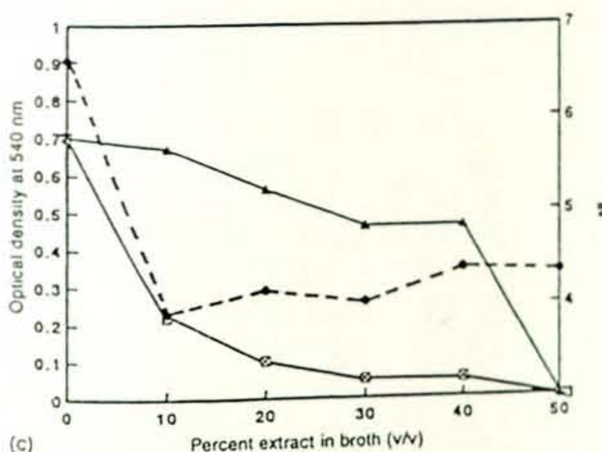
treated at 80°C inhibited *Ps. aeruginosa* much more severely than extracts heat-treated at 45°C or 61°C. A similar trend of antagonism was observed on *B. cereus* for extracts heated at 80°C where like *Ps. aeruginosa* total inactivation of vegetative cells was achieved at 50% extract volume in broth. pH did not appear to be the only inhibiting factor as the pH of this proportion of extract, with the greatest efficacy (80°C treated), was above 4.3 while extracts heated at 61°C and 45°C the other two had pH values 4.1-3.65, respectively.











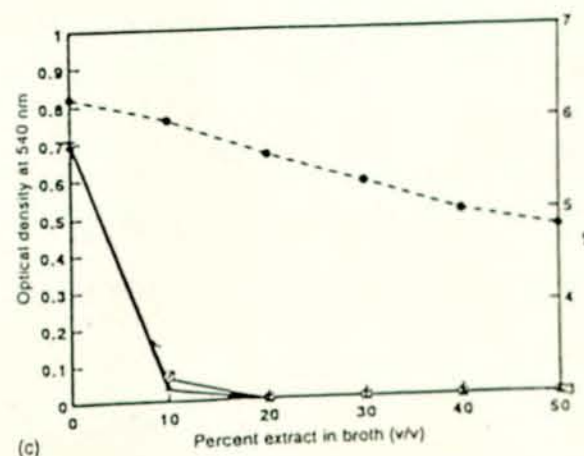


Figure 8. (a) Survival and growth of test bacteria in broth containing heat-treated (45 °C) 30 h *tef* dough extract. (b) Survival and growth of test bacteria in broth containing heat-treated (61 °C) 30 h *tef* dough extract. (c) Survival and growth of test bacteria in broth containing heat-treated (80 °C) 30 h *tef* dough extract. ●, pH; ⊠, *Pseudomonas aeruginosa*; ▲, *Bacillus cereus*.

Figure 9. (a) Survival and growth of test bacteria in broth containing heat-treated (45 °C) *Kocho* extract. (b) Survival and growth of test bacteria in broth containing heat-treated (61 °C) *Kocho* extract. (c) Survival and growth of test bacteria in broth containing heat-treated (80 °C) *kocho* extract. ●, pH; ⊠, *Pseudomonas aeruginosa*; ▲, *Bacillus cereus*.

Inhibitory properties of *kocho* extracts also showed similar patterns of increasing efficacy in their antimicrobial activities as the temperature of heat treatment was raised. This was despite the observations that the final pH values for all of the three extracts treated at the three different temperatures were above 4.8. *Kocho* extracts were more inhibitory than *tef* dough extracts as *kocho* extracts heat-treated at 61° and 80°C inactivated all of the test bacteria immediately at lower proportions to the assay broth (at less than 31 and 20%, heat treated at 61° and 80°C, respectively) than *tef* dough extracts treated at similar temperatures (Figs. 8a-8c).

Baking temperature (at 100°C for 5 min) killed most of the test organisms except the spores of *B. cereus*, yeasts and moulds which survived the heat and appeared in the baked products of both foods (Table 8). Generally in *tef* dough the yeasts and moulds were less affected than bacterial cells, by the same amount of heat. In *kocho*, however, moulds were more affected than the bacteria or the yeasts.

Table 8. Population of the test organisms after baking of *tef* dough or *kocho*.

	*Estimated number of organisms (cfu/dry wt of fermented *tef* dough or *kocho*)			
Food item	*Bacillus cereus*	Yeasts	Moulds	Total count
Tef dough	0.5×10^1	3×10^3	1.5×10^2	3.2×10^3
Kocho	1.5×10^3	1.7×10^3	7.5×10^1	3.2×10^3

* Each value is a mean of 4 food samples in 3 experiments.
No colony forming units developed from culture tubes inoculated with *Klebsiella* spp., *Salmonella* spp., *Shigella* spp., *Ps. aeruginosa* and *Staph. aureus* after challenge.

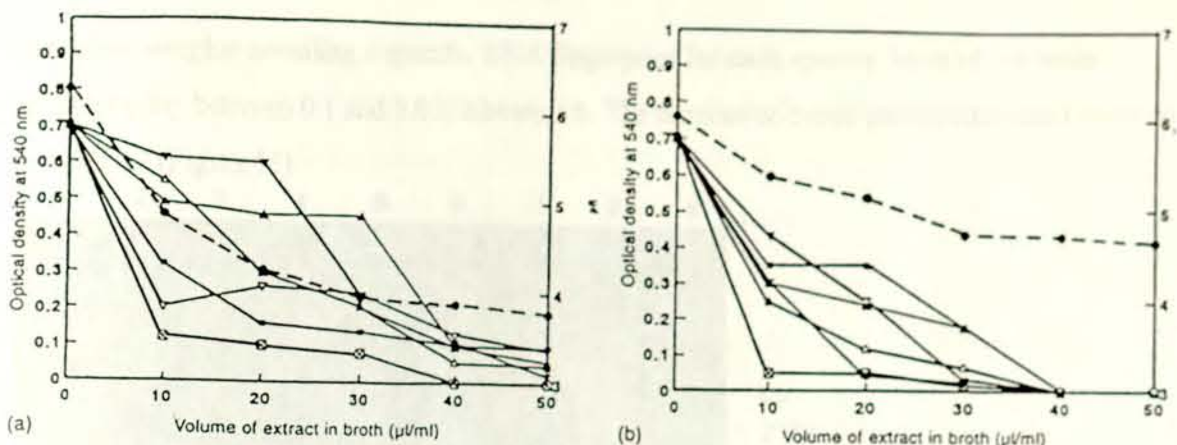


Figure 10. (a) Survival and growth of test bacteria in broth supplemented with extract from *tef injera*
(b) Survival and growth of test bacteria in broth supplemented with extract from *aradisame*
●, pH; ⊠, *Pseudomonas aeruginosa*; ▲, *Bacillus cereus*; △, *Klebsiella* spp.; ▽, *Staphylococcus aureus*; ■, *Salmonella* spp.; ▼, *Shigella* spp.

Different concentrations of aqueous extracts from *injera* or inhibited the growth of all of the six test bacteria from as low as 10 µl/ml of extract in broth (Figs. 10a & 10b). The fall in pH as the result of increasing the volume of *injera* extract in proportion with the broth was very sharp (i.e., from pH 6.21 to 3.77) whereas in *aradisame* it was gentle with the drop in pH from 5.8-4.7. The effects exerted by the later food were, however, much more dramatic as it caused complete inhibition of growth of all test bacteria right after addition of 40 µl of the extracts/ml of broth. The effects of the extracts from both foods were higher and more severe on *Ps. aeruginosa* than on *B. cereus*.

3.2. RAPD for discrimination of Pediococcus pentosaceus and Ped. acidilactici and for strain typing in pediococci

Agarose gel electrophoresis band profiles of the crude DNA extracts from the type strains of *Pediococcus* are shown in Figure 11.

Band patterns developed for a type or a field strain used in duplicate experiments were stable and consistently the same. Each of the seven species showed distinct bands of differing

molecular weights revealing a specific DNA fingerprint for each species. Most of the bands developed lay between 0.1 and 3.0 kilobases, kb. The number of bands per species varied between two and 12 (Figure 11).

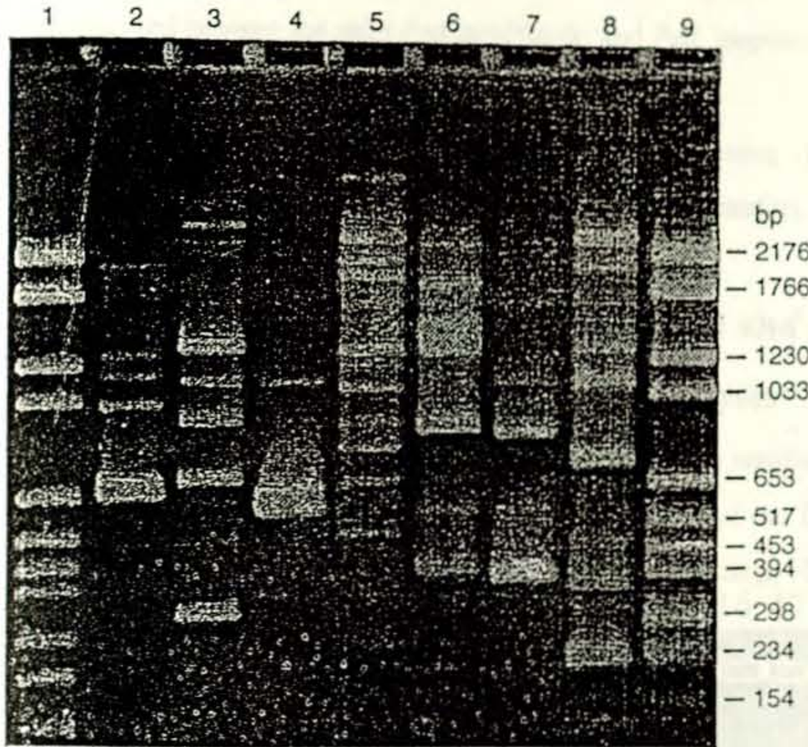


Fig. 13.RAPD agarose gel electrophoresis band profiles with ethidium bromide of type strains of *Pediococcus* species. Lanes 1 and 9 represent the reference DNA molecular weight (bp) marker VI and lanes 2–8 are bands for *Ped. acidilactici* CCUG 32235^T, *Ped. pentosaceus* CCUG 32205^T, *Ped. inopinatus* CCUG 38496^T, *Ped. parvulus* CCUG 28439^T, *Ped. damnosus* CCUG 32251^T, *Ped. dextrinicus* CCUG 18834^T and *Ped. urinaeequi* CCUG 28094^T, respectively

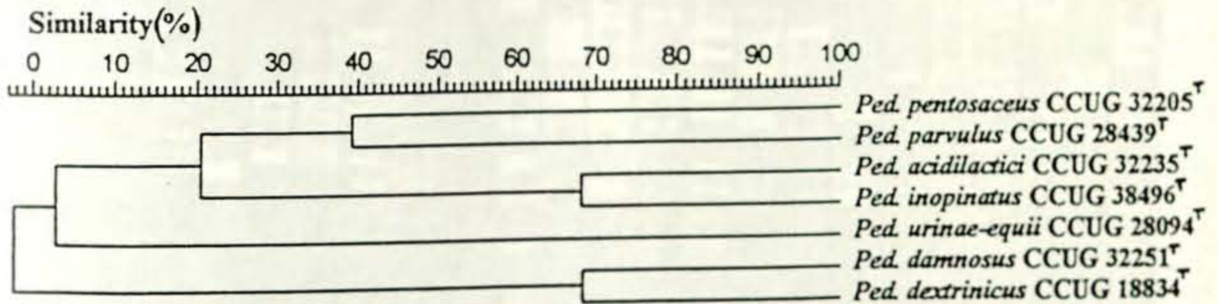


Figure 12. Dendrogram showing clustering patterns of type strains of *Pediococcus* species using the GelCompar program.

The Pearson product moment correlation coefficient (r) with UPGMA clustering algorithm produced the best association and enabled us to define the relatedness at a higher percentage by comparison with the Ward's method of cluster analysis (data not shown). As a result we have got clear differences between the different *Pediococcus* species and were also able to define clustering

levels for the field isolates with the known strains. The degree of relatedness between the seven type strains of pediococci was above 89% S_J and individual values of similarities have been calculated in a matrix (data not shown). This similarity was very high and the maximum relatedness was observed between the pairs *Ped. acidilactici* and *Ped. inopinatus*, and *Ped. damnosus* and *Ped. dextrinicus* in each case the similarity being 96%.

Another observation was the superior advantage of using a photo negative over the positive. The clarity of the bands also enables visual comparison of banding.

3.3. Identification of pediococci from kocho and tef

PCR amplification products and statistical analyses

Agarose gel electrophoresis of *Pediococcus* strains resulted in clear bands which in most cases were located within the range of the 11 fragments of the DNA molecular weight marker. The band profiles of the type strains and isolates are depicted in Figure 13.

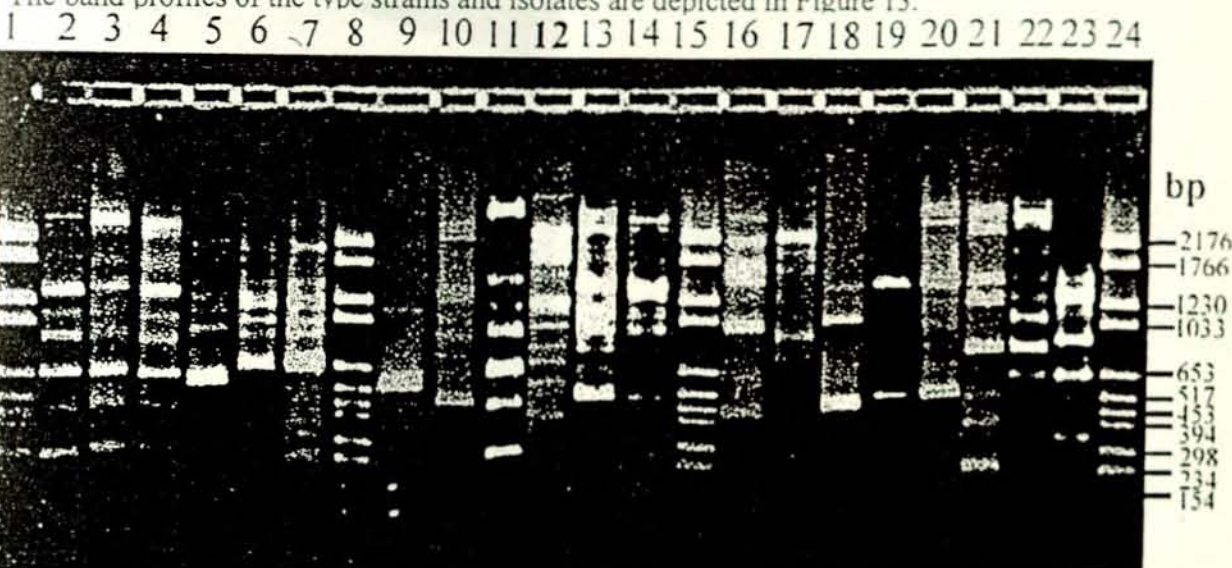


Figure 13. RAPD Agarose gel electrophoresis band profiles with ethidium bromide of type strains and food isolates of *Pediococcus* species from fermented *tef* dough and *kocho*. Lanes 1, 8, 15 and 24 were for the reference DNA molecular weight marker VI. The other lanes represented *Ped. pentosaceus* CCUG 32205^T (lane 2), *Ped. pentosaceus* (lanes 3 and 4), *Ped. acidilactici* CCUG 32235^T (lane 5), *Ped. acidilactici* (lanes 6 and 7), *Ped. inopinatus* CCUG 38496^T (lane 9), *Ped. inopinatus* (lane 10), unidentified isolate (lane 11), *Ped. parvulus* CCUG 28439^T (lane 12), *Ped. parvulus* (lanes 13 and 14), *Ped. damnosus* CCUG 32251^T (lane 16), *Ped. damnosus* (lane 17), *Ped. dextrinicus* CCUG 18834^T (lane 18), *Ped. dextrinicus* (lane 19), unidentified isolate (lane 20), *Ped. urinae-equii* CCUG 28094^T.(lane 21), *Ped. urinae-equii* (lanes 22 and 23). Lanes 3, 4, 6, 7, 10, 11, 13, 14, 17, 19, 20, 23 and 24 were food isolates.

Most of the bands had molecular weight ranges of 154-2176 base pairs, bp. Comparison of the developed bands gave clear relationships between isolates and type strains at various similarity levels. Food isolates most closely related to the type strains have grouped together revealing high degrees of relatedness.

Comparison of the band profiles using UPGMA clustering analysis had better and definite results as compared to the Ward's method particularly in delineating the outlier groups from the clustering isolates.

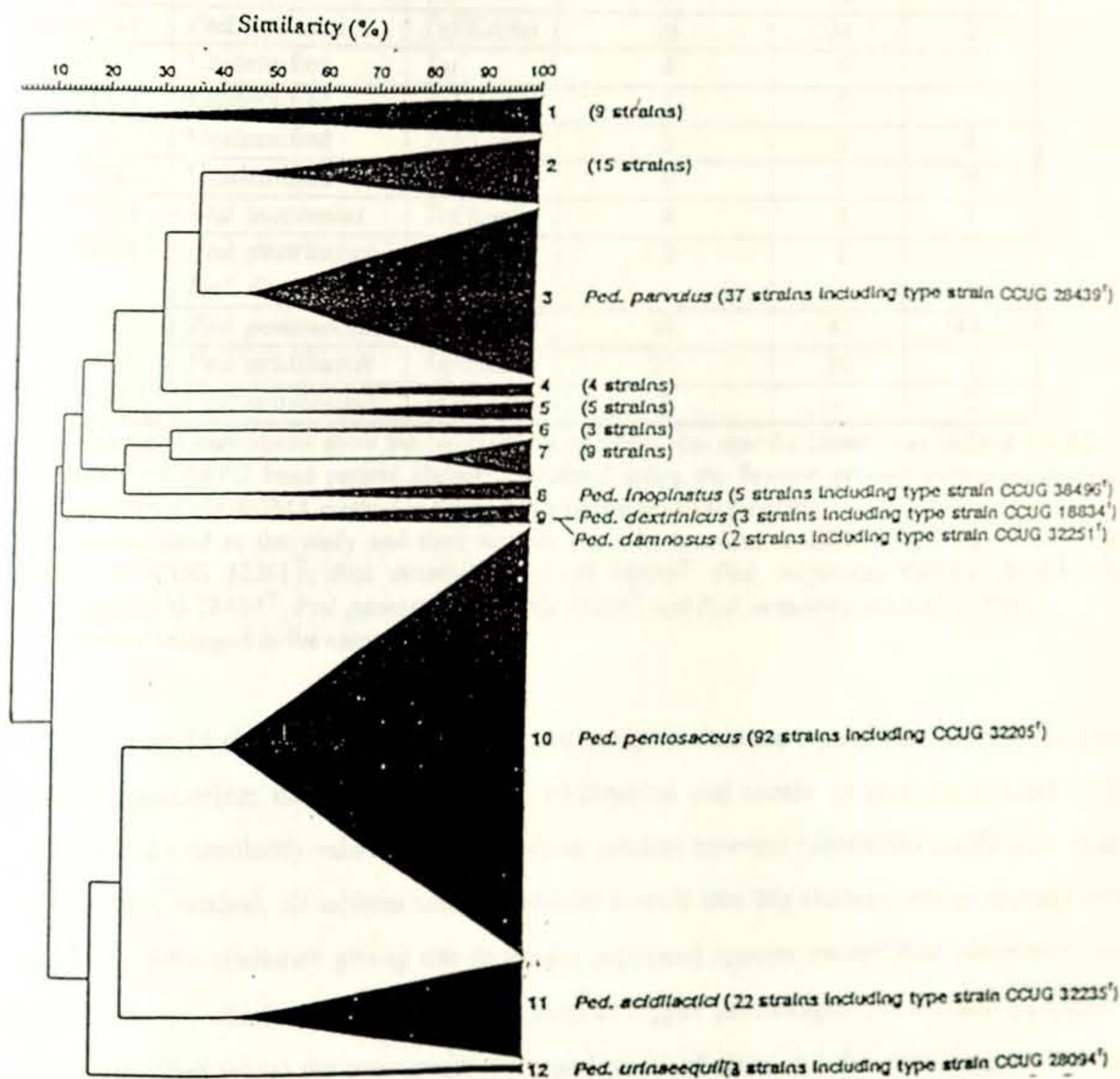


Figure 14. Simplified dendrogram showing clustering patterns of food isolates with type strains of *Pediococcus* species using the Pearson product moment correlation coefficient (r) and UPGMA clustering algorithm with the GelCompar version 4.0 program. Clusters 1, 2, 4, 5, 6 and 7 were straggler groups.

Table 9. Clustering and distribution of food isolates from *tef* and *kocho* in the different species of *Pediococcus*.

*Cluster	Species	Source	Number of affiliated isolates	Distribution of isolates	
				Tef dough	*Kocho*
1(9%)	Unidentified	*Tef/Kocho*	9	7	2
2(41%)	Unidentified	*Tef*	15	15	-
3(44%)	*Ped. parvulus*	*Tef/Kocho*	36	34	2
4(48%)	Unidentified	*Tef*	4	4	-
5(52%)	Unidentified	*Tef*	5	5	-
6(41%)	Unidentified	*Tef/Kocho*	3	2	1
7(78%)	Unidentified	*Kocho*	9	-	9
8(38%)	*Ped. inopinatus*	*Tef/Kocho*	4	3	1
9(32%)	*Ped. dextrinicus*	*Tef*	2	2	-
	Ped. damnosus	*Kocho*	1	-	1
10(42%)	*Ped. pentosaceus*	*Tef/Kocho*	91	47	44
11(44%)	*Ped. acidilactici*	*Tef/Kocho*	21	20	1
12(39%)	*Ped. urinae-equii*	*Tef/Kocho*	2	-	2

*Percentages in parenthesis show the cut-off value at which that specific cluster was defined. Clustering was based on RAPD band pattern clusters calculated using the Pearson product moment correlation coefficient (r) and UPGMA method in the GelCompar version 4.0 program.
Type strains used in the study and their sources were *Pediococcus acidilactici* CCUG 32235^T, *Ped. damnosus* CCUG 32251^T, *Ped. dextrinicus* CCUG 18834^T, *Ped. inopinatus* CCUG 38496^T, *Ped. parvulus* CCUG 28439^T, *Ped. pentosaceus* CCUG 32205^T and *Ped. urinae-equii* CCUG 28094^T.
-, No isolate belonged to the category.

Figure 14 shows the overall trend of clustering between the type strains and food isolates. Table 9 summarizes the clustering pattern, distribution and source of pediococci used in the study. At 12% similarity value using the Pearson product moment correlation coefficient (r) and the UPGMA method, all isolates and type strains formed two big clusters. Major clusters were defined at 40% similarity giving rise to clearly separated species except *Ped. dextrinicus* and *Ped. damnosus* which aggregated together even at higher percentages. As a result 12 clusters were established where the type strains belonged to six of them and the remaining six clusters comprised unidentified isolates. A description of the composition of the different clusters

follows.

Cluster 1. This was a group consisting of nine unidentified isolates (seven from *tef* and two from *kocho*) which only showed a low relatedness (<10%) to the rest of the clusters.

Cluster 2. Is defined at 41% similarity level, and consisted of 15 *tef* strains. This was the largest cluster of unidentified isolates.

Cluster 3. *Ped. parvulus* cluster: Strains in this cluster showed overall similarity at 44% and the cluster was composed of 37 strains including the type strain. This was the second dominant cluster accommodating about 23% of the identified isolates and comprised 34 isolates from *tef* and two from *kocho*.

Cluster 4. Defined at 48% similarity value, this straggler cluster contained four strains all isolated from *tef*.

Cluster 5. This cluster of five unidentified *tef* isolates was established at 52% similarity level.

Cluster 6. Defined at 41% similarity level this straggler cluster consisted of two *tef* and one *kocho* isolates forming the smallest group of unidentified strains.

Cluster 7. Defined at 78% similarity value, this cluster was composed of nine closely related *kocho* isolates forming a homogenous straggler group.

Cluster 8. *Ped. inopinatus* cluster: At 38% similarity level, four isolates (three from *tef* and one from *kocho*) clustered with the type strain.

Cluster 9. *Ped. dextrinicus/Ped. damnosus* cluster: Defined at 42% similarity level, this cluster consisted of two isolates and the two type strains. The *tef* isolate was related to *Ped. dextrinicus* while the *kocho* isolate was closely related to *Ped. damnosus*.

Cluster 10. *Ped. pentosaceus* cluster: Defined at 42% cut-off value, this cluster contained 91 isolates and the type strain. Forty-seven of the strains were isolated from *tef* and 44 from *kocho*. This cluster had relatively homogenous sub-clusters each with high degree of relatedness. This was the largest group of identified strains.

Cluster 11. *Ped. acidilactici* cluster: With an overall 44% similarity level, this cluster consisted 21 strains (20 from *tef* and one from *kocho*) which clustered with the type strain.

Cluster 12. *Ped. urinae-equii* cluster: When defined at 40%, this cluster had two related *kocho*

strains.

As a result of this numerical analysis of clustering, therefore, the following overall pattern was observed: 156 pediococci isolates (77%) were identified while 46 isolates (23%) remained unaffiliated. Isolates related to *Ped. pentosaceus* were found to be the most abundant followed by those related to *Ped. parvulus* and *Ped. acidilactici* a relatively small number of isolates belonged to the rest of the species. An almost equal number of isolates from the two foods were related to *Ped. pentosaceus* while only those isolates from tef were related to *Ped. parvulus, Ped. acidilactici*, *Ped. urinae-equii* and *Ped. inopinatus*. Sixty-seven percent of the identified strains had been isolated from tef with the remaining 33% coming from *kocho*. Of the 139 *tef* isolates, 105 (76%) clustered with five type strains, while 36 (24%) remained unidentified, while 51 (82%) the 63 isolates from *kocho*, clustered with six of the type strains. Most of the isolates from *tef* (101) belonged to *Ped. pentosaceus, Ped. parvulus* and *Ped. acidilactici*, while the rest were members of minor species. From *kocho* isolates, 44 (86%) were related to *Ped. pentosaceus*, making it the single dominant species. *Ped. damnosus, Ped. dextrinicus, Ped. inopinatus* and *Ped. urinae-equii* were represented to a minor extent.

Carbohydrate fermentation

Table 10 shows the fermentation profiles of the representative strains from the RAPD clusters presented in Figure 16. The highest test vigour (ability to assimilate many substrates) recorded was 71% and the minimum 33% both of which were from isolates belonging to the *Ped. pentosaceus* cluster. These results show peculiarities of the isolates in this species.

Table 10. API 50 CH fermentation profiles of *Pediococcus* type strains and food isolates.

Substrate	Strain/Isolate																													
	1	2	3	4	5	6	7	400 a	131 a	22 8a	63 3a	96 a	585 a	347 b	447 b	64 3a	48 1a	17 9b	35 7b	39 8b	437 b	27 5b	34 5b	346 b	362 b	38 0b	406 b	43 1b	475 b	51 4b
Glycerol	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	+	+	+	+	-	+	-	+	-	-	-	+	+	-	-	+	+	+	+	+	+	-	+	-	-	+	+	+
Ribose	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	+	+	+	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	+	-	-	+	-	+	-	-	-	-	-	+	+	-	-	+	-	-	+	+	+	-	+	-	-	+	+	+
Mannitol	-	-	+	-	-	+	-	+	-	+	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Sorbitol	-	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-D-Mannoside	-	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-MD-Glucoside	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NA-Glucosamine	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	+	+	+	+	-	+	-	+	-	-	+	-	-	-	+	-	+	+	-	+	-	+	-	-	+	+	-	+
Saccharose	+	+	+	+	+	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+
Melezitose	-	-	+	-	-	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	+	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+
Amidon	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Turanose	-	-	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
D-Tagatose	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabitol	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-
Gluconate	-	+	+	+	-	+	-	+	-	+	-	-	-	+	+	-	-	+	+	-	-	+	-	-	+	-	-	+	-	+
Test vigour (%)	31	37	57	43	33	55	27	55	33	57	33	35	37	47	39	33	35	39	41	37	37	49	37	71	43	33	37	51	37	49

Erythritol, D-Arabinose, L-Xylose, Adonitol, β-Methyl-D-Xyloside, L- Sorbose, Dulicitol, Inositol, Glycogene, Xylitol, D-Lyxose, D-Fucose, L-Fucose, L-Arabitol, and 2-K-Gluconate were not fermented by any one of the type strains or the food isolates. All isolates and type strains fermented D-Fructose, D-Glucose, D-Mannose, Esculine, Maltose and Trehalose. Except *Ped. parvulus* CCUG 28439^T, all type strains and isolates assimilated Amygdaline, Arbutine, Galactose, Salicine, and Cellobiose. Only *Ped. dextrinicus* CCUG 18834^T hydrolyzed starch and strain 228a assimilated 5-k-gluconate. The first 1-7 strains were *Ped. inopinatus* CCUG 38496^T, *Ped. dextrinicus* CCUG 18834^T, *Ped. urinae-equii* CCUG 28094^T, *Ped. pentosaceus* CCUG 32205^T, *Ped. parvulus* CCUG 28439^T, *Ped. acidilactici* CCUG 32235^T and *Ped. damnosus* CCUG 32251^T, respectively.

16S rRNA sequence data

Results of the 16S rRNA products for 10 representative isolates, from Clusters 1 and 2 (Figure 14), revealed that all of them had high levels of sequence similarities with *Ped. pentosaceus*. The isolates formed two groups where in the first group three of them were very closely related to each other (group A) and in the second six isolates showed close resemblance (group B). Group A differed from group B in nucleotide position 298 where Thymine was replaced by Cytosine and in position 1299 where Guanine was replaced by Adenine. One isolate from group A was different from all other isolates at position 230 in having Cytosine instead of Thymine. The remaining one isolate was intermediate between the two groups and Cytosine in position 298 and Adenine in position 1299 (data not shown).

3.4. Identification of lactobacilli from kocho and tef

Numerical analyses and clustering

Amongst 56 type and reference strains of *Lactobacillus* and *Weissella* species employed those which were closely related to field strains are listed in Table 11. The Table shows the number of food isolates related to each type and reference strain. Figures 15 and 16 show band patterns of some type and reference strains together with some unidentified food isolates.

RAPD clustering analysis of the 220 strains gave 14 distinct clusters and other small ones when defined at 65% using UPGMA. This cut-off value was used because clearly defined the *Lactobacillus plantarum* cluster with known reference strains. Consequently, the following major clusters were observed (see also Annex).

Cluster 1. This cluster is composed of 31 interrelated *kocho* isolates. More than one-half of the isolates in this cluster were very short-to-very long rods, occurring singly or as chains and aggregates. This was the only single cluster accommodating a larger proportion of the isolates.

Cluster 2. Lb. brevis: One *tef* isolate clustered with the type strain at 74% similarity level.

Cluster 3. *Lb. graminis:* Which was defined at 67%, two *kocho* isolates along with the type strain made this group. The isolates were coccobacillary-to-very long rods, occurring singly, in pairs and in chains.

Cluster 4. *Lb. acetotolerans:* Two *kocho* isolates formed this small cluster together with the type strain at 67% similarity value, were short-to-long rods occurring in chains.

Cluster 5. *Weissella minor:* Defined at 70% similarity level, contained 19 *kocho* isolates and the type strain. The isolates were short-to-long rods, existing singly, in pairs or in chains.

Cluster 6. *W. confusa*: One *kocho* isolate made up of very short-to-very long rods, occurring singly or in pairs, together with the type strain formed this small cluster.

Cluster 7. *Lb. casei* subsp. *tolerans:* Three homogenous *kocho* isolates and the type strain formed this cluster at 70% similarity value. They were short-to-long rods appearing in single, in pairs or in aggregates.

Cluster 8. *(*Mostly *Lb. plantarum):* This cluster was composed of eight isolates (six from *kocho* and two from *tef*). At 63%, all of these isolates were related to *Lb. suebicus.* Most of the isolates were short-to-long rods, existing singly, in pairs or in chains.

Cluster 9. *Lb. plantarum:* Two *tef* isolates, two reference and the type strain clustered at 67% similarity level. The isolates were short-to-long rods, existing singly, in pairs or in chains.

Cluster 10. *(Lb. plantarum*): Composed of 16 *kocho* isolates unaffiliated to any one of the type strains, this cluster contained seven isolates which hybridized with *Lb. plantarum* very well. Four of them had similar API 50 CH fermentation profiles with this type strain. The isolates were short-to-long rods, existing singly, in pairs or in chains.

Cluster 11. *(Lb. plantarum*): Five *tef* isolates, short-to-long rods, existing singly, in pairs or in chains, were included together with the type strain of *Lb. amylovorus*.

Cluster 12. *(Lb. plantarum*): Four *tef* and one *kocho* isolates were related to the type strain of *Lb. homohiochi* at and above 66% similarity. The isolates had very short-to-long rods existing in single, in pairs or in chains.

Cluster 13. *Lb. vaccinostercus:* The cluster was defined at 68% similarity accommodating two *kocho* isolates, short-to-very long rods appearing singly or in pairs, and the type strain.

Cluster 14. *W. kandleri*: Defined at 73% similarity value, this cluster contained five highly related *kocho* isolates and the type strain. The isolates were medium-to-long rods, existing in pairs and in chains.

Table 11. Type and reference strains of *Lactobacillus* and *Weissella* species and food isolates used in the RAPD study and their distribution in the different clusters at 65% similarity level.

RAPD Cluster	Type and reference strains	Affiliated isolates		
		Total	Source	
			tef dough	*kocho*
1	Unidentified	31	-	31
2	*Lb. brevis* CCUG 30670 T	1	1	-
3	*Lb. graminis* CCUG 32238 T	2	-	2
4	*Lb. acetotolerans* CCUG 32229 T	2	-	2
5	*Weissella minor* CCUG 30668 T	19	-	19
6	*W. confusa* CCUG 30113 T	1	-	1
7	*Lb. casei* subsp. *tolerans* DSM 2258 T	3	-	3
8	Unidentified	8	2	6
9	*Lb plantarum* ATCC 14917 T *Lb plantarum* DSM 9843 *Lb. plantarum* DSM 6595	2	2	-
10	Unidentified	16	-	16
11	*Lb. amylovorus* DSM 20531 T	5	5	-
12	*Lb. homohiochi* CCUG 32247 T	5	4	1
13	*Lb. vaccinostercus* CCUG 30723 T	3	-	3
14	*W. kandleri* CCUG 32237 T	5	-	5

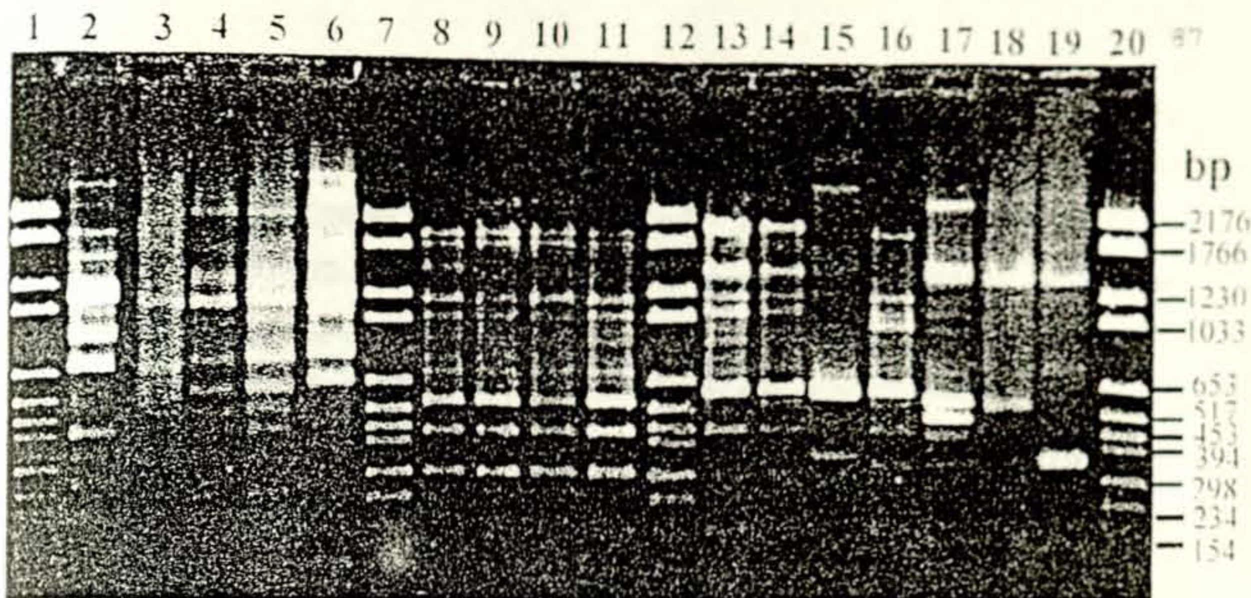


Figure. 15. RAPD band profiles of representative type strains and field isolates. Lanes 1, 7, 12 and 20 show bands of the reference DNA molecular weight marker VI. The others were *Lb. paraplantarum* CCUG 35983ᵀ (lane 2), *Lb. amylovorus* (lanes 4 and 5), *Lb. amylovorus* DSM 20531ᵀ (lane 6), *Weissella kandleri* (lanes 13, 14 and 16), *W. kandleri* CCUG 32237ᵀ (lane 15), *Lb. brevis* CCUG 30670 ᵀ (lane 17), unidentified food isolates (lanes 3, 8-11, 18 and 19). Lanes 3-5, 8-11, 13, 14, 16, 18 and 19 were food isolates.

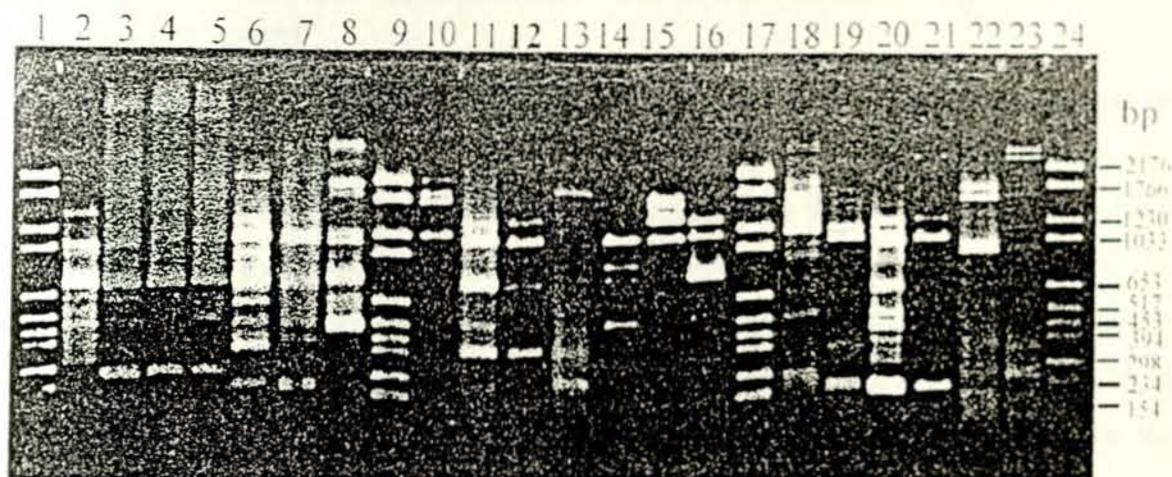


Figure 16. RAPD band patterns of some type and reference strains of *Lactobacillus* species and food isolates used in the study. Lanes 1, 9 ,17 and 24 represent bands for the reference DNA molecular weight marker VI. The rest lanes were *Lb. fermentum* ATCC14931ᵀ (lane 2), *Lb. plantarum* ATCC 14917ᵀ(lane 6), *Lb. plantarum* (lane 7), *Lb. casei* subsp. *casei* ATCC 334 (lane 8), *Lb. homohiochi* CCUG 32247ᵀ (lane 11), *Lb. acetotolerans* CCUG 32229ᵀ (lane 13), *Lb. farciminis* (lane 15), *Lb. suebicus* (lane 13), *Lb. parabuchneri* CCUG 32261ᵀ (lane 18), *Lb. suebicus* CCUG 32233 ᵀ (lane 19), *Lb. paracasei* subsp. *paracasei* NCFB 151ᵀ (lane 22), *Lb. salivarius* subsp *salivarius* CCUG 31453 ᵀ (lane 23) and unidentified food isolates (lanes 3-5, 10, 12, 14, 16, 20 and 21). Lanes 3-5, 7, 10, 12, 14, 16, 20 and 21 were food isolates.

From the RAPD clustering other type and reference strains including *Lb. farciminis* CCUG 30671^T, *Lb casei* subsp. *casei* ATCC 334, *Lb parabuchneri* CCUG 32261^T, *Lb. casei* subsp. *pseudoplantarum* DSM 20008^T, *Lb. jensenii* DSM 20557^T, *Lb. fermentum* ATCC 14931^T, *Lb. pentosus* ATCC 8041^T, *Lb murinus* CCUG 20452^T, *Lb. rhamnosus* CCUG 21452^T, *Lb. suebicus* CCUG 32233^T, *Lb. paraplantarum* CCUG 35983^T, *Lb. paracasei* subsp. *paracasei* NCFB 151^T, *Lb. salivarius* subsp. *salivarius* CCUG 31453^T, *Lb. alimentarius* DSM 20249^T, *Lb. johnsonii* CCUG 30725^T shoed less than 65% similarity to the isolates. The remainder of the isolates formed small clusters with two or three members which did not include a type strain.

DNA:DNA Hybridization study

The results of the DNA:DNA reassociation study are presented in Table 12. All of the isolates from the *Lb. plantarum* RAPD clusters, hybridized with the type strain. Twenty isolates and a negative control type strain, *Lb. reuteri* DSM 20016^T, which showed distant relationship to the *Lb. plantarum* cluster in the RAPD dendrogram did not hybridize with the target type strain *Lb. plantarum* ATCC 14917^T.

Isolates related to *Lb. plantarum* were concentrated around clusters 8, 9, 10, 11 and 12 as supported by both DNA:DNA reassociation data and their API 50 CH fermentation profiles. The DNA homology data thus resolved the uncertainties created in the RAPD results in the following clusters. One of the *kocho* isolates from cluster 8 had an API 50 CH profile similar to that of *Lb. plantarum* and three *kocho* and one *tef* isolate showed very high DNA:DNA homologies with *Lb. plantarum*. The DNA:DNA reassociation data also confirmed the close relatedness of isolates to *Lb. plantarum* than *Lb. suebicus*. DNA:DNA reassociation data was further used to confirm the high homology between one isolate in cluster 9 and seven isolates from cluster 10 and the type strain of *Lb. plantarum*. In cluster 11 four isolates had higher DNA:DNA homologies and two of these further retained similar API 50 CH fermentation patterns with *Lb. plantarum*. In cluster 12, two of the *tef* strains had high degree of relatedness to *Lb. plantarum* as revealed both by DNA:DNA reassociation and one strain by its API 50 CH fermentation properties

Table 12. DNA:DNA hybridization of type strains and field isolates with DNA probe from *Lactobacillus plantarum* ATCC 14917^T.

RAPD cluster	Species/ Isolate	Strain/ Source	Reaction with *Lb. plantarum* probe	RAPD cluster	Species/ Isolate	Strain/ Source	Reaction with *Lb. plantarum* probe
8	22a	Kocho	+	--	*Lb. reuteri*	DSM20016^T	-
8	48a	Kocho	+	--	505b	Tef	-
8	52a	Kocho	+	5	427a	Kocho	-
8	485b	Tef	+	5	238a	Kocho	-
9	*Lb. plantarum*	ATCC 14917^T	+	5	258a	Kocho	-
9	163b	Tef	+	--	351a	Kocho	-
10	372a	Kocho	+	--	356a	Kocho	-
10	384a	Kocho	+	5	146a	Kocho	-
10	433a	Kocho	+	--	476b	Tef	-
10	489a	Kocho	+	--	176a	Kocho	-
10	497a	Kocho	+	5	487a	Kocho	-
10	517a	Kocho	+	5	79a	Kocho	-
10	521a	Kocho	+	5	490a	Kocho	-
11	430b	Tef	+	1	227a	Kocho	-
11	478b	Tef	+	--	66a	Kocho	-
11	480b	Tef	+	8	391a	Kocho	-
11	490b	Tef	+	5	417a	Kocho	-
12	2b	Tef	+	--	127a	Kocho	-
--	486b	Tef	+	6	216a	Kocho	-
--	484b	Tef	+	--	555a	Kocho	-
--	488b	Tef	+	1	235a	Kocho	-
				--	140a	Kocho	-

Carbohydrate fermentation profiles

API 50 CH fermentation patterns of the tested isolates demonstrated that all isolates fermented galactose, D-glucose, D-fructose and maltose while none of them assimilated erythritol, xylose, β-methyl xyloside, xylitol, D-fucose and 2-K-gluconate. Ten isolates readily hydrolyzed starch and five isolates metabolized glycerol and two others glycogene. Many isolates fermented gluconate. Except two isolates, all the rest have metabolized arabinose and/or ribose (Table 13).

Table 11. API 50 CH fermentation profiles of *Lactobacillus* isolates from *tef* dough and *kocho*.

Substrate*	Strain/Isolate																								
	2a	140a	55a	94a	74a	376a	79a	122a	99a	108a	115a	137a	167a	267a	227a	238a	276a	289a	250a	308a	317a	338a	350a	380a	405a
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	+	-	+	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	+	+	+	-	+	-	-	+	-	-	+	+	+	+	-	+	-	+	-	+	+	+	+
Ribose	+	+	-	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
D-Mannose	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
Dulicitol	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Inositol	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	-	+	-	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+
Sorbitol	+	+	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-	-
α-D-Mannoside	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
α-MD-Glucoside	+	+	+	-	+	-	-	+	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-
NA-Glucosamine	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
Amygdaline	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
Arbutine	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
Esculine	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
Salicine	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
Cellobiose	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
Lactose	+	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-
Melibiose	-	-	+	+	+	-	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+
Saccharose	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Trehalose	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-
Inuline	+	+	-	-	+	-	-	+	-	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-
Melezitose	+	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+	-	+	+	+	-	-	+	-	+
D-Raffinose	-	-	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	-	+	-	+	+
Amidon	+	+	+	-	+	-	-	-	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	-	-
Glycogene	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-
D-Turanose	+	+	+	-	+	-	-	+	-	-	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	+	+	+	-	+	+	+	+	-	-	+	+	+	-	+	+	-	-	+	-	+	+	-	-	-
L-Fucose	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	+	+	+	-	+	-	-	-	+	-	+	+	+	+	-	-	+	-	+	-	-	-	-	-	+
5-K-Gluconate	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	+	+	+

*None of the isolates fermented Erythritol, L-Xylose, β-Methyl-D-Xyloside, Xylitol, D-Fucose and 2-K-Gluconate

**All of the isolates assimilated Galactose, D-Glucose, D-Fucose and Maltose.

Table 11., continued

Substrate*	Strain/Isolate																
	218a	433a	449a	487a	489a	497a	508a	521a	539a	560a	477b	480b	481b	484b	486b	490b	488b
Glycerol	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+	-	+	+	-	-	+	-	+	+	-	-	+	+	+	-	+
Ribose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+
D-Mannose	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
Rhamnose	-	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-
Mannitol	+	+	+	-	+	+	+	+	-	+	-	+	-	+	+	+	+
Sorbitol	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
α-D-Mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
α-MD-Glucoside	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
NA-Glucosamine	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Arbutine	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
Esculine	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
Salicine	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Saccharose	+	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+
Trehalose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Melezitose	+	-	+	-	-	-	+	-	+	+	-	-	-	+	+	-	+
D-Raffinose	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+
β-Gentiobiose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
D-Turanose	+	-	-	-	-	+	-	+	-	-	-	+	+	+	+	+	+
D-Tagatose	+	-	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-
D-Arabitol	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Gluconate	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	+
5-K-Glucohate	+	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	+

*None of the isolates fermented Erythritol, D-Arabinose, L-Xylose, Adonitol, α-Methyl-D-Xyloside, β-Methyl-D-Xyloside, L-Sorbose, Glycogene, D-Lyxose, D-Fucose, L-Fucose, L-Arabitol, 2-K-Gluconate and Xylitol.

Only isolate 218a fermented dulicitol, Inositol, Inuline and Amydon.

All of the isolates assimilated Galactose, D-Glucose, D-Fucose, D-Mannose and Maltose.

3.5. *RAPD for distinction of Lactobacillus type strains*

Agarose gel electrophoresis results of the 45 type and reference strains of *Lactobacillus* showed a distinct band pattern for each one of the strains. The number of bands obtained for the strains ranged from 2-10 and the molecular weight fragments were relying between 154-2176 bp.

Figures 17 and 18 show the band patterns developed for representative type strains in relation to the DNA molecular weight marker. Further analyses of the extent of relatedness and differences between these profiles were carried out using the statistical software and revealed the clear distinctness of each type strain as shown in Figure 19. At 72% similarity level, all strains were clearly discriminated. The three *Lb. plantarum* strains were clustered together at 72% (Figure 20).

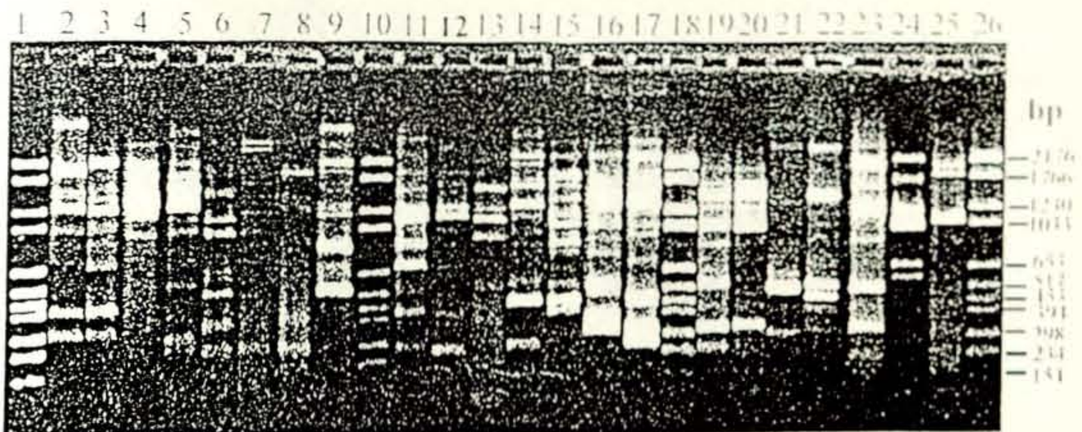


Figure 17. Agarose gel showing RAPD electrophoresis product DNA fragments of type and reference strains of *Lactobacillus* and *Weissella* species. Lanes 1, 10, 18 and 26 represent the molecular weight marker VI (154-2176 bp); lane 2, *Lb. fructivorans* CCUG 32260^T; lane 3, *Lb. pentosus* ATCC 8041^T; lane 4, *Lb. farciminis* CCUG 30671^T; lane 5, *Lb. parabuchneri* CCUG 32261^T; lane 6, *Lb. mali* CCUG 32228^T; lane 7, *Lb. salivarius* subsp. *salivarius* CCUG 31453^T; lane 8, *Lb. acetotolerans* CCUG 32229^T; lane 9, *Lb. casei* subsp. *casei* ATCC 334; lane 11, *Lb. paraplantarum* CCUG 35983^T; lane 12, *Lb. suebicus* CCUG 32233^T; lane 13, *Lb. intestinalis* CCUG 30727^T; lane 14, *Lb. animalis* NCFB 2425^T; lane 15, *Lb. reuteri* DSM 20016^T; lane 16, *Lb. agilis* DSM 20509^T; lane 17, *Lb. delbrueckii* subsp. *lactis* DSM 20072^T; lane 19, *Lb. murinus* CCUG 20452^T; lane 20, *Lb. rhamnosus* CCUG 21452^T; lane 21, *W. kandleri* CCUG 32237^T; lane 22, *Lb. brevis* CCUG 30670^T; lane 23, *Lb. oris* NCFB 2160^T; lane 24, *Lb. graminis* CCUG 32238^T; and lane 25, *Lb. paracasei* subsp. *paracasei* NCFB 151^T.

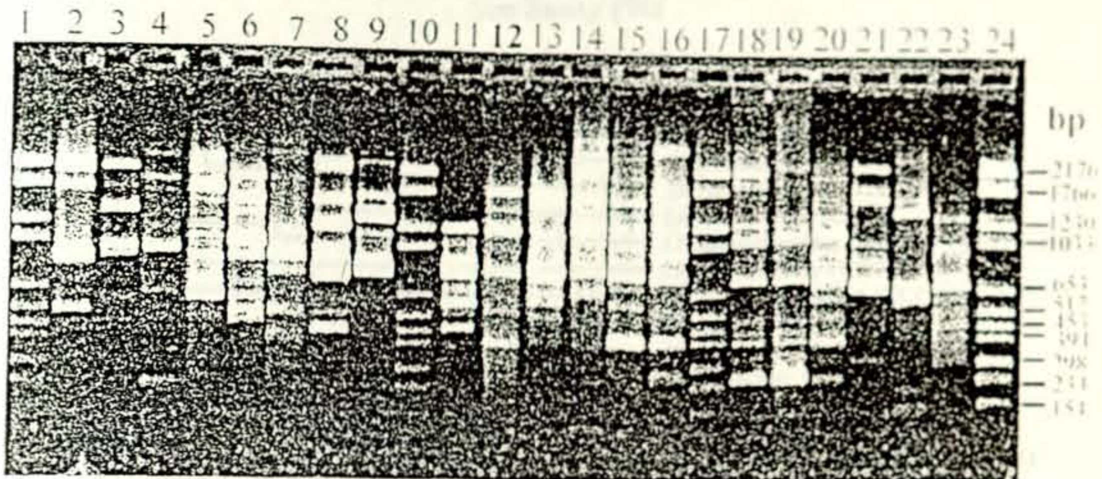


Figure 18. RAPD electrophoresis band patterns of type and reference strains of *Lactobacillus* and *Weissella* species. Lanes 1, 10, 17 and 24 represent the molecular weight marker VI (154-2176 bp); lane 2, *Lb. kefiranofaciens* CCUG 32248^{T}; lane 3, *Lb. gasseri* DSM 20243^{T}; lane 4, *Lb. acidophilus* CCUG 5917^{T}; lane 5, *Lb. vaginalis* CCUG 31452^{T}; lane 6, *Lb. coryniformis* CCUG 30666^{T}; lane 7, *Lb. sake* CCUG 30501^{T}; lane 8, *Lb. bifermentans* CCUG 32234^{T}; lane 9, *W. confusa* CCUG 30113^{T}; lane 11, *Lb. zeae* DSM 20178^{T}; lane 12, *Lb. johnsonii* CCUG 30725^{T}; lane 13, *Lb. casei* subsp. *pseudoplantarum* DSM 20008^{T}; lane 14, *Lb. amylovorus* DSM 20531^{T}; lane 15, *W. minor* CCUG 30668^{T}; lane 16, *Lb. curvatus* CCUG 30669^{T}; lane 18, *Lb. plantarum* DSM 9843; lane 19, *Lb. plantarum* DSM 6595; lane 20, *Lb. plantarum* ATCC 14917^{T}; lane 21, *Lb. paracasei* subsp. *tolerans* CCUG 34829^{T}; lane 22, *Lb. amylophilus* CCUG 30137^{T}; and lane 23, *Lb. fermentum* ATCC 14931^{T}.

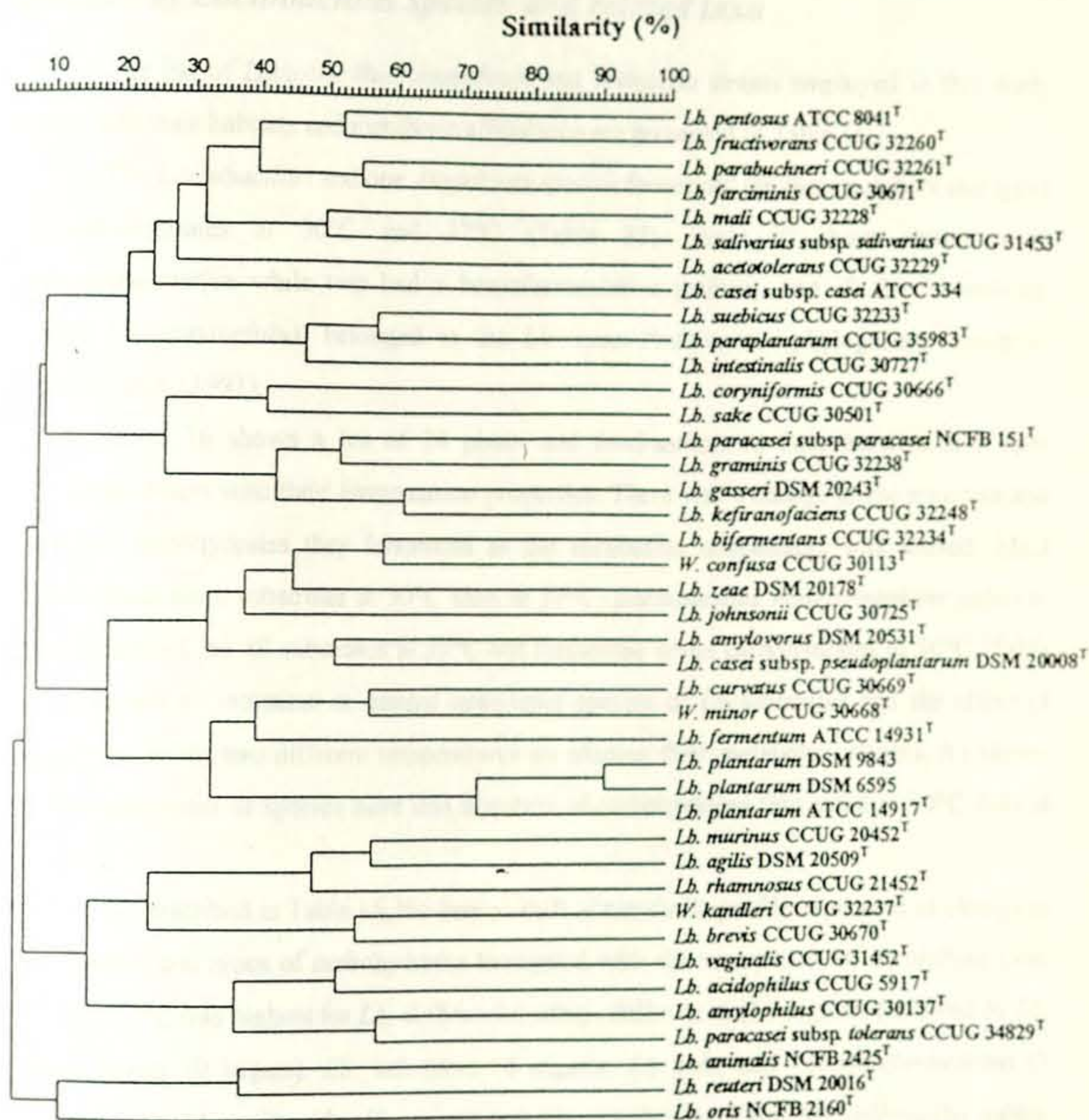


Figure 19. Dendrogram showing normalized agarose gel electrophoresis products for type and reference strains of *Lactobacillus* species developed on a photo and negative analyzed using the GelCompar version 4.0 program with the Pearson product moment correlation coefficient (r) and the UPGMA algorithm.

3.6. Effect of incubation temperature on API 50 CH metabolic profiles of Lactobacillus species and related taxa

The list of *Lactobacillus*, *Atopobium* and *Weissella* strains employed in this study along with their habitats and metabolic affiliations are presented in Table 14.

Ten *Lactobacillus* and one *Atopobium* species feremnted the same numbers and types of carbohydrates at 30°C and 37°C (Table 15). Eight of these strains were heterofermentative while two had a homofermentative pathway and all the lactobacilli, except *Lb. amylophilus,* belonged to the *Lb. casei-Pediococcus* phylogenetic group of Collins, *et al.* (1991).

Table 16 shows a list of 24 plant- and food-associated *Lactobacillus* and three *Weissella* strains with their fermentation properties. There was a change in the numbers and types of carbohydrates they fermented as the incubation temperature was altered. Most metabolized more substrates at 30°C than at 37°C. *Lactobacillus malefermentans* failed to ferment any of the 49 substrates at 37°C but fermented seven carbohydrates at 30°C. Table 17 compares 11 intestinal or animal associated species of *Lactobacillus* and the effect of incubation at the two different temperatures on altering their metabolic patterns. As shown in the table, most of species have less numbers of carbohydrates fermented at 30°C than at 37°C.

As described in Table 16, the loss or shift of metabolic profile expressed as change in the number and types of carbohydrates fermented with the temperature of incubation from 30°C to 37°C was highest for *Lb. delbrueckii* subsp. *delbrueckii* (11 sugars), followed by *Lb. acetotolerans* (9 sugars), *Lb. helveticus* (8 sugars), *Lb. sake* and *Lb. malefermentans* (7 sugars each), *Lb. collinoides* (6 sugars) and *Lb. parabuchneri* and *Lb. delbrueckii* subsp. *bulgaricus* (4 sugars each). Nine of the strains had a change of one carbohydrate. The variations affected 21 of the strains by altering the differential trait recorded for the type strain while five were not affected at all. Nineteen strains maitained their differential traits at 30°C while 11 others preferred 37°C. Seven strains did not show temperature preference

still maintaining their distinct traits although their patterns were altered.

Amongst the animal-associated strains *Lb. jensenii* showed the highest number of differently fermented substrate (7 sugars) while in decreasing order *Lb. oris* (6 sugars), *Lb. intestinalis* (5 sugars), *Lb. johnsonii* and *Lb. crispatus* (4 sugars each) also showed differences in substrate fermentation at the two temperatures. The least effect was observed in *Lb. mali*. The results obtained from duplicate experiments for type strains of *Lb. plantarum* and *Lb. salivarius* subsp. *salivarius* from were consistent.

Table 14. Strains of *Lactobacillus* and related lactic acid bacteria used in the study.

Species	Strain	Habitat	Physiol group[a]	Species	Strain	Habitat	Physiol group[a]
Atopobium uli[a]	CCUG 31116^{T}	Human mouth	OHo	*Lb. intestinalis*	CCUG 30727^{T}	Murine GIT	FHe
Lb. acetotolerans	CCUG 32229^{T}	Plant	FHe	*Lb. jensenii*	DSM 20557^{T}	Human vagina	OHo
Lb. acidophilus	ATCC 5917^{T}	Animal	OHo	*Lb. johnsonii*	CCUG 30725^{T}	Chicken, Pigs	OHo
Lb. agilis	DSM 20509^{T}	Unknown, Sewage	FHe	*Lb. malefermentans*	CCUG 32206^{T}	Beer	OHe
Lb. amylophilus	CCUG 30137^{T}	Plant	OHo	*Lb. mali*	CCUG 30141^{T}	Apple juice	OHo
Lb. amylovorus	DSM 20531^{T}	Plant	OHo	*Lb. murinus*	DSM 20452^{T}	Murine GIT	FHe
Lb. animalis	NCFB 2425^{T}	Animal	FHe	*Lb. oris*	NCFB 2160^{T}	Human saliva	OHe
Lb. bifermentans	CCUG 32234^{T}	Cheese	FHe	*Lb. parabuchneri*	CCUG 32261^{T}	Human saliva, Cheese	OHe
Lb. brevis	DSM 30670^{T}	Animal	OHe	*Lb. paracasei* subsp. *paracasei*	NCFB 151^{T}	Dairy, Humans, Plants	FHe
Lb. casei subsp. *casei*	ATCC 334	Animal, Plant	FHe	*Lb. paracasei* subsp. *tolerans*	CCUG 30669^{T}	Dairy, humans, Plants	FHe
Lb. casei subsp. *tolerans*	CCUG 25599^{T}	Animal, Plant	FHe	*Lb. paraplantarum*	CCUG 35983^{T}	Beer, Humans	FHe
Lb. collinoides	CCUG 32229^{T}	Cider	OHe	*Lb. pentosus*	CCUG 34829^{T}	Silage	FHe
Lb. coryniformis	CCUG 30666^{T}	Silage,Cow dung	FHe	*Lb. plantarum*	ATCC 8014	Human, Sourdough	FHe
Lb. crispatus	DSM 20584^{T}	Animal	OHe	*Lb. plantarum*	ATCC 14917^{T}	Human, Plant	FHe
Lb. curvatus	CCUG 30669^{T}	Cow dung, Plant	FHe	*Lb. plantarum*	DSM 9843	Sourdough	FHe
Lb. delbrueckii subsp. *bulgaricus*	DSM 20081^{T}	Yogurt, Cheese	FHe	*Lb. reuteri*	DSM 20016^{T}	Animal, Sourdough	FHe
Lb. delbrueckii subsp. *delbrueckii*	ATCC 9649	Plant	OHo	*Lb. rhamnosus*	CCUG 21452^{T}	Animal, Milk, Sewage	OHe
Lb. farciminis	CCUG 30671^{T}	Plant, Sausages	OHo	*Lb. sake*	CCUG 30501^{T}	Sake beer, Plants	FHe
Lb. fermentum	ATCC 14931^{T}	Animal, Plant	OHe	*Lb. salivarius* subsp. *salivarius*	DSM 20555^{T}	Human mouth	OHo
Lb. fructivorans	DSM 32260^{T}	Plant	OHe	*Lb. salivarius* subsp. *salivarius*	CCUG 31453^{T}	Human mouth, GIT	OHo
Lb. gasseri	CCUG 32238^{T}	Animal	OHo	*Lb. suebicus*	CCUG 32233^{T}	Apple	OHe
Lb. graminis	DSM 20243^{T}	Grass silage	FHe	*Lb. vaginalis*	CCUG 31452^{T}	Human vagina	OHe
Lb. helveticus	ATCC 15009^{T}	Milk, Cheese	OHo	*Weissella confusa*[b]	CCUG 30113^{T}	Plants, Milk	OHe
Lb. hilgardii	CCUG 30140^{T}	Wine	OHe	*W. kandleri*[b]	CCUG 32237^{T}	Plants	OHe
				W. minor[b]	CCUG 30668^{T}	Milk sludge	OHe

[a] Fermentation groups are based on Hammes and Vogel (1995), Vandamme, *et al.* (1996) and Stiles and Holzapfel (1997). FHe, facultatively heterofermentative; OHe, Obligately heterofermentative; OHo, Obligately homofermentative. [b] Previously species of *Lactobacillus*.

Table 15. Type strains of *Lactobacillus* and related organisms showing same API 50 CH fermentation profiles when incubated at 30° and 37°C.

Species	Strain	Group[a]	Number of carbohydrates fermented
Atopobium uli	CCUG 31116^T	*Atopobium*	18
Lb. amylophilus	CCUG 30137^T	Aa	7
Lb. casei subsp. *casei*	ATCC 334	Bb	22
Lb. fermentum	ATCC 14931^T	Cb	10
Lb. graminis	CCUG 32238^T	Bb	15
Lb. hilgardii	CCUG 30140^T	Cb	9
Lb. paracasei subsp. *tolerans*	CCUG 30669^T	Bb	6
Lb. paraplantarum	CCUG 35983^T	Bb	20
Lb. pentosus	CCUG 34829 ^{T}	Bb	24
Lb. rhamnosus	CCUG 21452^T	Bb	27
Lb. vaginalis	CCUG 31452^T	Cb	10

[a]Grouping based on Collins, *et al*. (1991). Aa, physiologically an obligate heterofermenter and phylogenetically affiliated to the *Lb. delbrueckii* group; Ab, physiologically an obligate heterofermenter and phylogenetically affiliated to the *Lb. casei-Pediococcus* group; Bb, physiologically
a facultative heterofermenter and phylogenetically related to the *Lb. casei-Pediococcus* group; Cb, physiologically an obligate heterofermenter and phylogenetically related to the *Leuconostoc* group.

Table 16. Type and reference strains associated with plants and foods and ubiquitous *Lactobacillus* species between plants and animals and related organisms with different API 50 CH fermentation profiles when incubated at 30°C and 37°C.

Species	Strain	Group[a]	Total no. of CHOs fermented at 30°C	37°C	Variability[b]	Trend[c]	Differently fermented substrate[d]	Suitable temp°C
Lb. acetotolerans	CCUG32229[T]	Ba	21	19	9	Loss	Rib,Gal,AMDM,Amy,Cel,Mel,Su,Mlz,Gnte	37
Lb. agilis	DSM 20509[T]	Bb	23	25	2	Add	Rha,Amd	30
Lb. amylovorus	DSM 20531[T]	Bb	15	17	2	Add	AMDG,Amy	37
Lb. bifermentans	CCUG 32234[T]	Cb	11	10	1	Loss	DArl	30
Lb. brevis	DSM 30670[T]	Cb	11	11	2	Replace	Lac,Su	30
Lb. casei subsp. *tolerans*	ATCC 25599	Bb	15	17	4	Add	Arb,Mel,Gnte,Rib	30
Lb. collinoides	CCUG 32229[T]	Aa	17	11	6	Loss	Gal,Man,NAG,Lac,Mel,Gnte	30
Lb. coryniformis	CCUG 30666[T]	Bb	9	10	1	Add	Mal	Both
Lb. curvatus	CCUG 30669[T]	Bb	26	24	2	Loss	DRaf,DArl	37
Lb. delbrueckii subsp. *bulgaricus*	DSM 20081[T]	Aa	7	11	4	Add	Gal,Glu,Man,Mal	30
Lb. delbrueckii subsp. *delbrueckii*	ATCC 9649	Aa	15	4	11	Loss	LAra,Rib,DXyl,Gal,Rha,Ino,NAG,Esc,Mal,Tre,LFuc	37
Lb. farciminis	CCUG 30671[T]	Cb	17	15	2	Loss	Amy,DTur	30
Lb. fructivorans	CCUG 32260[T]	Aa	6	6	2	Replace	Mne,Man	30
Lb. helveticus	ATCC 15009[T]	Cb	16	8	8	Loss	DXyl,Rha,Man,Arb,Esc,LFuc,Gnte,5KG	37
Lb. malefermentans	CCUG 32206[T]	Bb	7	None	7	Loss	All	30
Lb. parabuchneri	CCUG 32261[T]	Cb	13	13	4	Replace	Man,Lac,Gen,DTur	Both
Lb. paracasei subsp. *paracasei*	NCFB 151[T]	Bb	21	20	1	Loss	Amy	30
Lb. plantarum	ATCC 8014	Bb	27	26	1	Loss	Rha	Both
Lb. plantarum	ATCC 14917[T]	Bb	25	24	1	Loss	Rha	Both
Lb. plantarum	DSM 9843	Bb	26	25	1	Loss	DRaf	Both
Lb. reuteri	DSM 20016[T]	Cb	9	9	2	Replace	Gal,Man	30
Lb. sake	CCUG 30501[T]	Bb	20	13	7	Loss	Rha,Amy,Esc,Sal,Cel,Tre,Glg	30
Lb. suebicus	CCUG 32233[T]	Cb	11	9	2	Loss	BMDX,Gal	37
Weissella confusa	CCUG 30113[T]	Cc	14	15	1	Add	Gnte	30
W. kandleri	CCUG 32237[T]	Cc	9	8	1	Loss	Gal	30
W. minor	CCUG 30668[T]	Cc	12	11	1	Loss	DAral	Both

[a]Grouping same as indicated in Table 2. Ba, physiologically a facultative heterofermenter and phylogenetically affiliated to the *Lb. delbrueckii* group; Bb, physiologically a facultative heterofermenter and phylogenetically related to the *Lactobacillus casei-Pediococcus* group; Cc, physiologically an obligate heterofermenter and phylogenetically related to the *Leuconostoc* group.[b]Variability, number of carbohydrates the strain shifts pattern to or not to degrade due to the change in the incubation temperature. [c]Add, Able to catabolize additional carbohydrate; Loss, Unable to catabolize a specific carbohydrate degraded at 30°C; Replace, Shift to metabolize another carbohydrate in lieu.[d]Abbreviations for carbohydrates: Gly,Glycerol; DAra,D-Arabinose; LAra,L-Arabinose; Rib,Ribose; DXyl,D-Xylose; LXyl,L-Xylose; BMDX,BMthyl-D-Xyloside; Gal,Galactose; Fru,Fructose; Mne,Mannose; Sbe,Sorbose; Rha,Rhamnose; Ino,Inositol; Man, Mannitol; AMDM,A-Methyl-D-Mannoside; NAG,N-Acetylglucosamine; Amy, Amygdaline; Arb,Arbutin; Esc,Esculin; Cel,Cellobiose; Mal,Maltose; Lac,Lactose; Mel,Melibiose; Sal,Salicine; Sor,Sorbitol; Su,Sucrose; Tre,Trehalose; Inn,Inulin; Mlz,Melezitose; DRaf,Raffinose; Amd,Starch; Glg,Glycogen; Xlt,Xylitol; Gen,Gentiobiose; DTur,D-Turanose; DLyx,D-Lyxose; DTag,D-Tagatose; DFuc,D-Fucose; DArl,D-Arabitol; Gnte,Gluconate; 2KG,2-Ketogluconate; 5KG, 5-Ketogluconate. [d]Suitable temperature, temperature at which the distinctive metabolic properties are retained.

Table 17. Type and reference strains of *Lactobacillus* species from intestinal/animal origin and their variable patterns of API 50 CH fermentation profiles between incubation temperatures 37°C and 30°C.

Species	Strain	Group[b]	Total no. of CHOs fermented at 30°C	37°C	Variability	Trend[d]	Differently fermented substrate[e]	Suitable temp°C[f]
Lb. acidophilus	CCUG 5917 T	Aa	13	13	2	Replace	Arb,Lac,Mel	30
Lb. animalis [a]	NCFB 2425T	Bb	12	14	2	Add	Rib,Esc	37
Lb. crispatus	DSM 20584T	Aa	15	15	4	Replace	Amy,DRaf,Glg,Gen	37
Lb. gasseri	DSM 20243T	Aa	2	5	3	Add	Amy,Gen,DTag	37
Lb. intestinalis	CCUG 30727T	Bb	8	9	5	Add,Replace	Gal,Amy,Esc,Lac,Mel	30
Lb. jensenii	DSM 20557T	Aa	20	15	7	Loss, Replace	LAra,Man,Sor,AMDG,Lac,Mel,Amd	37
Lb. johnsonii	CCUG 30725T	Aa	5	7	4	Add,Replace	NAG,Cel,Mal,Gen	37
Lb. mali	CCUG 32228T	Ab	13	14	1	Add	Mal	30
Lb. murinus	DSM 20452T	Bb	21	18	3	Loss	Dxyl,BMDX,DArl	37
Lb. oris	NCFB 2160T	Cb	17	20	6	Add,Replace	BMDX,DRaf,Gen,DTur,Darl,Gnte	30
Lb. salivarius subsp. *salivarius*	CCUG 31453T	Ab	14	16	2	Loss	Rib,DTur	Both

[a] Included in *Lb. murinus* based on its genetic similarity (Vandamme, *et al.*, 1996).
[b] Grouping based on Collins, *et al.* (1991); same as in Table 16.
[c] Variability, number of carbohydrates the strain showed altered metabolic response to by the change in the incubation temperature.
[d] Add, Able to catabolize additional carbohydrate; Loss, Unable to catabolize a specific carbohydrate degraded at 30°C;Replace, Shift to metabolize another carbohydrate in lieu.
[e] Abbreviations to carbohydrates:Gly,Glycerol; DAra,D-Arabinose; LAra,L-Arabinose; Rib,Ribose; DXyl,D-Xylose; LXyl,L-Xylose;BMDX, BMethyl-D-Xyloside; Gal,Galactose; Fru,Fructose; Mne,Mannose; Sal,Salicine; Sbe,Sorbose; Su,Sucrose; Rha,Rhamnose;Ino,Inositol; Man, Mannitol;Sor,Sorbitol;AMDM,A-Methyl-DMannoside;NAG,N-Acetylglucosamine; Amy, Amygdaline; Arb,Arbutin; Esc,Esculin; Cel,Cellobiose; Mal,Maltose; Lac,Lactose; Mel,Melibiose; Tre,Trehalose; Inn,Inulin; Mlz,Melezitose; DRaf,Raffinose; Amd,Starch; Glg,Glycogen; Xlt,Xylitol; Gen,Gentiobiose; DTur,D-Turanose; DLyx,D-Lyxose; DTag,D-Tagatose; DFuc,D-Fucose; DArl,D-Arabitol; Gnte,Gluconate; 2KG,2-Ketogluconate; 5KG,5-Ketogluconate.
[f] Suitable temperature, temperature at which the distinctive metabolic properties are retained.

CHAPTER FOUR

DISCUSSION

4.1. The effect of baking heat on the antimicrobial properties of tef dough, kocho, injera, aradisame and fate of pathogens and contaminants

Contamination of *tef* seeds or flour or later on while preparing dough using water containing such pathogens and spoilage organisms or of *kocho* from various sources during or after fermentation is inevitable and unavoidable. The overall microbial spectrum and load in *tef* flour and *enset* is wide and very high before the initiation of fermentation (Nigatu, 1992; Nigatu, unpublished data). However, the diversity and populations were reduced by the fermentation processes giving rise to the predominance of LAB. However, *Bacillus* spp., yeasts and moulds were also abundant at lower pH values (Nigatu and Gashe, 1997). The effect of fermentation on spoilage and disease-causing bacteria of asporogenous and spore-bearing, gram-positive and gram-negative nature in these two fermented foods has been previously investigated (Nigatu and Gashe, 1994a,b) while the effect of baking heat was not clear.

In the current study the effect of heat treatment on the antimicrobial properties of these foods was evaluated by monitoring the fate of bacteria, epiphytic yeasts and moulds when introduced in extracts of these foods. The results showed that heat treatment promoted the inhibitory effects of the aqueous extracts from both foods against the test organisms and the impact was highest at 80°C. This effect was very strong from extracts of *kocho* on both *Ps. aeruginosa* and *B. cereus* where growth was totally arrested and survival prevented right from lower concentrations in supernatants heat-treated at 61°C and 80°C. This occurred although the

pH was still high (up to 6.00). The effects of *tef* dough extracts were rather moderate as compared to those of *kocho*. *Pseudomonas aeruginosa* was more affected than *B. cereus*, where the supernatant heat-treated at 80°C caused a severe effect and growth was totally halted at 50% extract:broth ratio survival.

Baking heat reduced the populations of surviving bacteria, yeasts and moulds in both foods lowering the total count in the final baked product from the levels observed in the doughs by 5 log units in *injera* and by 3 log units in *aradisame* (compare Tables 7 and 8). The population of *B. cereus* was reduced likewise by more than 3 log units in *injera* and by 1 log unit in *kocho* and in all cases these levels were far below the infective dose level for this organism ($5x10^5$ cfu/g of food)(Doyle, 1989).

Further looking into the antimicrobial effects of *injera* and *aradisame*, addition of increased volumes of extracts from *injera* reduced the pH of the broth substantially and thus affected all of the test organisms. Most affected were *Ps. aeruginosa* and *Salmonella* spp. than the rest but their population levels all decreased around pH 3.8 where the volume of extract in broth was 40%. In *aradisame* extracts the effects were much amplified that although the pH drop was not so sharp as caused by *injera*, the trend was towards inactivating almost all of the test organisms uniformly. Survival was nearly totally halted at about pH 4.7 where the extract concentration approached 40% by volume. The epiphytic yeasts and moulds were also affected by the baking heat but not entirely eliminated.

From this study, therefore, it was noticed that irrespective of the points of contamination during the preparation of *injera* and *kocho* bread, the multiple effects of fermentation and baking heat would destroy viable asporogenous pathogenic bacteria. Thus baking is complementary to the process of fermentation. Regarding the safety levels of the baked products, most of the microflora were inactivated or reduced to a very low level by the baking heat. *Injera* and *aradisame* also appeared self protective against the proliferation of asporogenous pathogenic and spoilage bacteria, but still contained spore-forming bacteria, yeasts and moulds. The actual baking temperatures for both foods are, higher than those used in this study, therefore, if post-baking contamination is minimized or prevented, the products

appear to be microbiologically safe. However, further studies on aflatoxins are required before we are assured of the safety of *kocho*.

4.2. *RAPD for discrimination of Pediococcus pentosaceus and Ped. acidilactici and for strain typing in pediococci*

The genus *Pediococcus* is so homogenous that species delineation remained difficult by mere phenotypic parameters (Garvie, 1986). Even with the use of genetic methods, such as 16S rRNA sequence data, were used, species such as *Ped. acidilactici* and *Ped. pentosaceus* show a sequence homolgy of 98.3% (Collins, *et al.*, 1990). This percent homologies is above the 97% for a well defined species (Stackebrandt and Goebel, 1994). Such a procedure, thus, could not reveal all the interspecific differences in this genus. As a consequence, more sensitive methods like DNA:DNA hybridization have been suggested (Back, 1978a,b; Dellaglio, *et al.*, 1981; Tanasupawat, *et al.*, 1993) which are however, not rapid and applicable in most laboratories.

In this study, the RAPD procedure developed for *Lb. plantarum* using a 9-mer primer was employed (Johansson, *et al.*, 1995c) to differentiate the species of *Pediococcus*. The results revealed distinct bands that enabled easy discrimination between the taxa. The different species had peculiar bands of certain molecular weight which in most cases were specific to a strain.

As a result, *Ped. acidilactici* and *Ped. pentosaceus* were clearly differentiated from one another.

Numerical analyses of the gel images in the GelCompar program using the Pearson product moment correlation coefficient (r) and UPGMA clustering algorithm clarified the degree of similarity and extent of relatedness between the type strains as shown in Figure 11.

The results could also be used to compare band patterns of field isolates with type or reference strains and thus group related isolates together as shown in section 3.4. The

procedure has been found simple, rapid for grouping of isolates, applicable for all species of pediococci and particularly useful to differentiate between strains of *Ped. pentosaceus* and *Ped. acidilactici*.

4.3. Identification of pediococci from kocho and tef

Pediococci are common in fermenting plant materials such as fermenting vegetables and leaves, silage (Dellaglio, *et al.*, 1981; Simpson and Taguchi, 1995) and legumes such as soya beans and cereals in the form of flour (Salovaara 1993) as well as for fermenting meat. They have been previously identified as the major fermentative LAB in *kocho* and *tef* dough (Gashe 1985, 1987; Nigatu and Gashe 1994a,b) and their metabolic contribution towards acceptable and wholesome products should be of paramount importance. It has also been shown that pediococci isolates from fermented *kocho* and *tef* dough have *in vitro* and *in situ* antagonistic effects against different spoilage and pathogenic bacteria (Nigatu and Gashe 1994a,b).

Based on phenotypic criteria Gashe (1985, 1987) reported the occurrence of *Ped. pentosaceus* both in *tef* and *kocho*. In the current study the numerical analysis results of the RAPD band profiles revealed the prevalence of this taxon and also all of the other known species. The numerical analyses of both RAPD and API 50 CH profiles were also supportive of each other. The 16S rRNA sequence analyses also confirmed that majority of the isolates belonged to this species. From this pattern therefore, the results of genetic identification agree with the phenotypic assignment of isolates from these two foods in that in both foods *Ped. pentosaceus* appears the major taxon.

This study showed the presence of most species of this taxon in these two foods, *tef* providing a relatively better microenvironment as implied by its accommodation of higher populations from the diverse species as opposed to that of *kocho* which was predominated by a single species. The dominant species in both *tef* dough and *kocho* was *Ped. pentosaceus* and similar observations have been reported by Gashe (1985; 1987). There were also a lot of

Ped. acidilactici and *Ped. parvulus* from tef while *Ped. urinae-equii* isolates were restricted to *kocho.*

Earlier reports (Garvie 1986) indicated that most pediococci species are limited to distinct habitats because species like *Ped. pentosaceus, Ped. acidilactici* and *Ped. urinae-equii* were known to have been restricted to microenvironments where pentoses were plenty. In this study it was observed that many isolates from both foods were affiliated to *Ped. pentosaceus* implying its abundance. Only two isolates representing *Ped. urinae-equii* originated from *kocho.*

Concerning the ecological relationships of pediococci, in fermenting *tef* dough and *kocho,* most contributory to the desired properties of the products should be from those species which are more abundant. Thus in *tef, Ped. pentosaceus, Ped. parvulus* and *Ped. acidilactici* appear very abundant. Probably the rapid acidification of the dough in a day or two might also have some relationships particularly with a taxon such as *Ped. acidilactici.* In *kocho* it takes longer for the pH to decrease and the most important changes seem to be amylolysis and other activities. Most other pediococci with the exception of *Ped. pentosaceus* might not be that important since other genera, such as *Lactobacillus,* also predominate (Nigatu, *et al.* unpublished data). Nevertheless, pediococci might have a prominent role regarding flavour, aroma development and safety in both foods although their population could be reduced at later stages of fermentation at lower pH values. This could also be related with the broad metabolic profiles of our pediococci isolates. The metabolism of maltose could be of important ecological attribute for the predominance of lactobacilli in such foods. The higher test vigours observed might also be pivotal to the succession of pediococci in *tef* and *kocho.*

Pediococcus pentosaceus, Ped. acidilactici and *Ped. inopinatus* are commonly found in fermenting vegetables while. The first two species are also used for the fermentation of vegetables, meats and as nonstarter bacteria in cheese ripening (Garvie 1986; Simpson and Taguchi, 1995). The predominance of *Ped. pentosaceus* amongst the total number of isolates

in this study (58%) may be attributed to the ubiquity of this species in fermenting plant-based acidic products where fermentable sugars are available. The lower pH values in both foods might as well favour its proliferation more than *Ped. acidilactici* which is known to thrive best in acidic microenvironments (Garvie 1986). The less occurrence of *Ped. dextrinicus* and *Ped. damnosus* could also be related to their preference to higher pH values than as in the fermented *tef* or *kocho*. *Ped. urinae-equii*, an alkaliphilic species, might have been introduced in to *tef* by animals used for trampling of *tef* seeds (Gashe 1985) and in *kocho* by its exposure to litter and drainage during the fermentation and storage processes in the back yard.

The presence of these diverse species in *kocho* and *tef* dough could be responsible for the prolonged shelf life and safety against food associated pathogens since pediococci are well known for production of inhibitory substances and thereby creating hostile microenvironments to aerobic bacteria (Nigatu and Gashe 1994a,b; Simpson and Taguchi, 1995).

In the present study we have not been able to assign 46 isolates into any one of the known *Pediococcus* species using RAPD. An apparent problem with the taxonomy of pediococci observed in this study was the close relationship of the polymorphic DNA patterns of the type strains *Ped. damnosus* CCUG32251^T and *Ped. dextrinicus* CCUG18834^T than any one of the field isolates. The very high sequence homology shared within the different species as well observed in between *Ped. acidilactici* and *Ped. pentosaceus* (98.3) (Collins *et al.* 1990) is a usual difficulty to delineate a species in pediococci. This close relatedness was also encountered from the higher 16S rRNA sequence similarities even between those isolates discriminated using RAPD. The RAPD procedure was however, discriminative in most of the species as we have observed its differentiation potential coupled with the statistical software UPGMA as applied to the GelCompar version 4.0. So far the data generated in this study support the appropriateness and use of RAPD as a relevant and rapid genetic grouping and identification tool for pediococci at the species level. Other complementary procedures should be used with RAPDs to get conclusive

identification.

Moreover, the presence of unidentified *Pediococcus* isolates with relatively different RAPD-patterns from the type strains of known species, though the methods of correlation and association used were very sensitive, suggests the need for thorough study and description from such tropical traditional fermented foods.

4.4. Identification of lactobacilli from kocho and tef

Previous studies on lactobacilli isolates from fermented *tef* and *kocho* have shown their abundance at lower pH values and their contribution to the desired characteristic fermentation properties of the two indigenous lactic acid fermented foods. Gashe (1985; 1987) phenotypically identified lactobacilli isolates from *tef* and *kocho* thereby showing the predominance of *Lb. plantarum*, *Lb. fermentum* and *Lb. brevis* in *tef* and *Lb. coryniformis* subsp. *coryniformis* followed by *Lb. plantarum* in *kocho*.

In the current study, clustering analysis of the RAPD genetic fingerprints from each of the food isolates along with known type and reference strains resulted in grouping of the isolates around the genetically closely related type strain of *Lactobacillus* and *Weissella* species (Table 12). As a result 87 isolates were grouped in 14 clusters, 11 of which included type strains. Eight clusters contained 38 isolates clearly identified based on the agreement of the API 50 CH profiles, RAPD band pattern relationships and DNA:DNA homologies. Accordingly, those isolates from clusters 8, 9, 10, 11 and 12 were found more related to *Lb. plantarum* although in the RAPD clustering the interference of *Lb. suebicus*, *Lb. amylovorus* and *Lb. homohiochi* masked the clear relatedness of the isolates to this species. The RAPD clustering has tightly grouped 31 isolates in cluster 1, but unaffiliated to any one of the type strains. The DNA:DNA homology data for two of the isolates from this cluster showed relatedness to *Lb. plantarum*. However, this cluster appeared to be distantly related to this

species.

Isolates related to *W. minor* were also tightly related and seven of the 19 members were entirely unrelated to *Lb. plantarum* based on their DNA:DNA reassociation results. One of these isolates, of course, had a related API pattern to *Lb. plantarum* (data not shown). The phenotypic traits could thus be similar, but as long as there is no genetic relatedness between the members of this cluster with *Lb. plantarum* then this cluster could be taken as a clearly defined group containing only isolates belonging to *W. minor*. A similar, but clear, clustering was apparent for cluster 14 where all member isolates were distinct and close to *W. kandleri*. On the other hand, unaffiliated clusters from distantly related groups to *Lb. plantarum* contained genetically unrelated isolates to this species as revealed by both the RAPD and DNA:DNA hybridization results.

Consequently then, using this strategy of grouping unknown lactobacilli isolates with RAPD, and later evaluate the API 50 CH fermentation patterns of representative isolates from each cluster and eventually confirming with DNA:DNA hybridization or more specific genetic tool has been found efficient to assess the suitability of the RAPD procedure. Thus from this study it was observed that RAPD could serve group isolates related to *Lb. plantarum, Weissella minor, W. confusa, W. kandleri* and *Lb. vaccinostercus*. However, the mismatch between those isolates related to *Lb. plantarum with Lb. suebicus, Lb. amylovorus, Lb. homohiochi* and *Lb. paraplantarum* should be treated carefully with more specific methods. Thus in using diverse and large number of field isolates, RAPD could not be taken as a single method of identification in most *Lactobacillus* species.

Regarding species composition of the known isolates *Weissella* species appeared to have been restricted to *kocho* and most were identified as *W. minor* and *W. kandleri*. Isolates related to *Lb. plantarum*, as identified by the three methods, were from both foods. Then, it seems that *Weissella* species might have had prominent roles in *kocho* while *Lb. plantarum* being essential in both foods.

Comparison of the spectra of species in Nigerian Ogi and the Swedish Sourdough (Johansson, *et al*, 1995a) with *tef* and *kocho* revealed that the prevalence of wider spectrum of

lactobacilli in the later two foods (Tables 13). The fermentation profiles of the isolates in the latter two foods have also apparently displayed the presence of strains possessing wider arrays of metabolic activities and also shown by the higher test vigor values (maximum of 63%) which could again be indicative of their important role in the fermentation of these two traditional acidic foods.

Apart from these *Lactobacillus* and *Weissella* species, in another study it has been well demonstrated that different species of pediococci have been isolated from both foods (Nigatu, *et al.*, 1998). All of the lactobacilli and the pediococci tested (Nigatu, *et al.*, unpublished data) have assimilated maltose and majority of them utilized both pentoses and hexoses. Such heterofermentative traits appear particularly important in fermenting foods such as *tef* dough and *kocho* for the desired organoleptic properties. Of 35 lactobacilli isolates from *kocho*, (40% were capable of utilizing starch, out of which 21% used hydrolyzed starch as their sole source of carbon. Amongst the *tef* isolates only two of the seven strains (ca. 29%) slowly degraded starch whereas the rest did not at all. Therefore, the occurrence of such amylolytic strains might be an advantage for the utilization of the starch in *kocho*, in contrast to *tef* dough. Therefore, it appears that this trait endows more chance for lactobacilli towards competitive dominance in *kocho* over other saccharolytic and amylolytic organisms so as to cause rapid acidification and proliferate in the later stages of fermentation. Besides this, lactobacilli isolates from both foods, mainly from *kocho*, substantially hydrolyzed starch.

Apparently, all tested isolates assimilated maltose. The advantage of maltose assimilation in *Lb. sanfrancisco* was well reviewed by Gobbetti and Corsetti (1997) and showed that it is a key property particularly for the dominance of lactobacilli in sourdough. This peculiar property of possessing maltose phosphorylase was found unique to *Lb. sanfrancisco, Lb. reuteri* and *Lb. fermentum* amongst the lactobacilli (Vogel, *et al.*, 1994). This inherent trait of most of our isolates to phosphorylate maltose would, therefore, be of ecological advantage for the essential succession of lactobacilli in the doughs and might contribute to the safety and nutritional aspects of *tef* and *kocho*. The overall test vigours of the

tested isolates were also high implying their wider enzymatic acitivities and importance in the fermentation of the two foods.

Regarding the discriminatory potential of the RAPD procedure, as well shown in the band profiles for the type and reference strains and food isolates (Figures 15 and 16), each of the strains had well amplified distinct bands which makes it identifiable and also related to some strain possessing similar patterns. Apparently we have found it simple and very convenient to first group isolates using this RAPD procedure, then characterize representative strains from each cluster based on metabolic profiles and later on confirm the taxonomic identity with hybridization or other specific genetic methods. This strategy of grouping field isolates along with type strains using RAPD as a basis for further selection and definition of a taxon or taxa, could be of remarkable benefit because it is rapid and effective.

Moreover, from this study we have noticed that the use of RAPD for rapid grouping of a large number of isolates facilitates identification in *Lactobacillus* species. The RAPD procedure employed has also proven its suitability and consistency as we used it repeatedly for the same strains from different cultures in different experiments and obtained the same result. Nevertheless, RAPD might not be a regorous method to use as a single identification procedure particularly with large numbers of field isolates because of the possible ambiguities where some of the *Lactobacillus* spp. might be misallocated. Therefore, the use of polyphasic evaluation including sensitive genetic procedures remains crucial for precise identification of new isolates.

4.5. RAPD for distinction of Lactobacillus type strains

In this study it was demonstrated that RAPD is capable of distinguishing between the type strains of a large proportion of the recognized *Lactobacillus* species. The use of a broad-range molecular weight marker has been found helpful to accommodate developed bands from all of the strains.

SCIENCE LIBRARY ADDIS ABABA

Earlier studies have shown the application of RAPD to discriminate between different strains and species of *Lactobacillus* (Du Plessis and Dicks, 1995; Johansson, *et al.*, 1995c; Van Reenen and Dicks, 1996; Hamad, *et al.*, 1997). In all of these studies relatively fewer numbers of species have been compared.

Kandler and Weiss (1986) have mentioned the difficulty of reliably differentiating between strains of the groups, *Lb. acidophilus*, *Lb. gasseri* and *Lb. amylovorus*; *Lb. delbrueckii* and *Lb. jensenii*; and *Lb. fermentum* and *Lb. reuteri* by simple physiological tests and thus it was essential to perform DNA:DNA hybridization or similar genetic analyses. In this study we demonstrate the clear distinction between the type strains in all of these three groups. Van Reenen and Dicks (1996) have also shown previously the discriminability of *Lb. plantarum*, *Lb. casei*, *Lb. paracasei*, *Lb. sake* and *Lb. pentosus* using RAPD and similar to but right at lower similarity values. An interesting observation from this study was therefore, the clear separation of all type strains right at lower levels of similarity. The higher level of clustering of all of the *Lb. plantarum* strains also explains that this association was based on genetic relatedness. Basically the degree of relatedness has much to do with the number of bands of same molecular weight which related strains share in common. The three species of *Weissella* included still showed more relatedness to the lactobacilli than to each other. This is to be expected as most of the *Weissella* species originally belonged to the genus *Lactobacillus* (Collins, *et al.*, 1993). The subspecies of *Lb. casei* also clustered separately appeared to share more common bands with strains of other species than amongst themselves. From this point of view then it appears very important for this RAPD procedure to be able to separate distinct species and related strains at various levels of similarities. The use of UPGMA coupled with the Pearson product moment correlation coefficient (r) in the GelCompar version 4.0 program has further demonstrated the efficient use of this RAPD procedure for distinction of *Lactobacillus* species. As it has also been utilized for discrimination of *Pediococcus* species by Nigatu, *et al.* (1998), this method could be used as a rapid, efficient and reproducible genetic tool for grouping and

identification between the many species of *Lactobacillus*. Strict use of pure and authentic culture and consistency of the procedure, however, remains indispensable for reproducibility and precision. Furthermore, the use of more strains from each species would be of paramount importance to further elucidate the identification and grouping capacity of the procedure. Moreover, as the inclusion of field strains can muddle the discrimination between species due to variations in band patterns and random similarities occurring within and between field and type strains.

4.6. *Effect of incubation temperature on API 50 CH metabolic profiles of Lactobacillus species and related taxa*

Phenotypic identification in *Lactobacillus* is usually carried out based on carbohydrate fermentation patterns of an isolate as compared to those of type strains. Such a method has been in use since the beginning of microbiology and still remains indispensable. Consequently common established trends of the traits for a given species are presented in the Bergey's manual and other related literature (Kandler and Weiss, 1986). The results of carbohydrate fermentation might be inconsistent in some strains for various reasons amongst which temperature of incubation could be one important factor. Therefore, the carbohydrate utilization patterns of 49 lactobacilli type and reference strains falling within 43 *Lactobacillus*, one *Atopobium* and three *Weissella* species were evaluated. Two temperatures of incubation (30°C and 37°C) were used to see if there was consistency in the fermentation patterns of the 49 carbohydrates, heterosides and polyalcohols in the API 50 CH system by the bacteria.

Amongst the studied lactobacilli and strains of related taxa regarding their habitats, 34 were known to be associated with plants and foods or ubiquitous and 13 to animals. Those strains belonging to *Atopobium uli, Lb. amylophilus, Lb. coryniformis, Lb. graminis, Lb. hilgardii, Lb. paracasei* subsp. *tolerans, Lb. pentosus, Lb. vaginalis* and *Lb. rhamnosus* had unaltered patterns despite the change in the incubation temperature from 30°C to 37°C. All of the strains except *Atopobium uli, Lb. rhamnosus* and *Lb. vaginalis* are related to plant

habitats. The rest 36 strains were affected by the change in incubation temperature. This demonstrated the presence of a strong intra-strain fluctuation in carbohydrate metabolism patterns in *Lactobacillus* species due to thermal effects. These fluctuations were also found to be higher in obligately homofermentative (OHo) species such as *Lb. delbrueckii* subsp. *delbrueckii* and *Lb. jensenii* than any of the other species. The subspecies of *Lb. delbrueckii* vary in their degree of difference in carbohydrate metabolism and temperature preference. For example *Lb. casei* subsp. *casei* and *Lb. casei* subsp. *tolerans* differ in that the former is not affected by the change in the temperature of incubation while the later fermented many carbohydrates at 37°C than at 30°C implying that these subspecies have different mechanisms for adaptations. On the other hand, the variation was minimum in the heterofermentative species. An exception from the obligately heterofermentative group was *Lb. malefermentans* which showed a typical metabolic restriction to 30°C and failure to utilize any one of the sugars at 37°C. This revealed its strong adaptation to the lower temperature. The overall trend in the plant and food associated species showed better ability to utilize more substrates at 30°C than at 37°C. There was a marked difference observed in between plant and animal associated species with regard to temperature preference. Most of the strains from the plant species (21 strains) were metabolized better at 30°C while most animal-associated strains (seven strains) metabolized more sugars at 37°C.

Further analysis of the suitable temperature which enabled retention of maximum spectrum of distinctive metabolic features of a species showed no general trend and it was rather strain specific. In general however, 19 strains had consistent results at 30°C and 11 were strictly preferential to 37°C while the remaining four were equally vigourous without marked deviation at both temperatures although in all cases there were shifts in the types and numbers of substrates fermented at each temperature. Therefore, the overall observation leads to avoidance of generalization for plant or animal associated strains though majority of the plant-associated and those of ubiquitous nature tend to favour 30°C while in contrast most of the animal-associated species tend to stick to 37°C. Then it seems rather essential to

follow a specific incubation temperature particularly for those strains having shown variations. Nevertheless, for those with stable patterns (Table 15) and those with changing profiles but not affecting their typical key metabolic traits (Tables 16 and 17) results obtained from any one of the two temperatures could be dependable.

Another interesting observation was that except two species, all the 15 strains showing preference to 30°C were heterofermenters. Conversely 6 of the 11 strains favouring 37°C were homofermenters while three of the four strains not drastically affected by the change in the temperature were all heterofermentative species (Tables 16 and 17). From this fact then it appears that a heterofermentative type of metabolism seems to have a lower temperature optima for effective regulation and expression of enzymes. In contrast again higher temperature might narrow spectrum of enzymatic activity and hence limit an organism to a homofermentative metabolic profile which also appears to unfavour heterofermentation. It therefore seems very likely that in food- and plant-associated lactobacilli maximum array of metabolic expression might be possible or accelerated at 30°C than at 37°C. And the reverse might be true for those of intestinal and/or animal-origin. In line with this, strains with stable patterns would likely have enzymes active at wider temperature ranges and might also have tolerance to stress.

Ahrné and Molin (1991) have shown that metabolism of raffinose is changed due to spontaneous mutations in *Lb. plantarum* ATCC8014. As a result they concluded that the mutant phenomenon could hinder the use of carbohydrate fermentation profiles for *Lactobacillus* identification. However, concerning application of API 50 CH fermentation profiles has generally been found to be an appropriate, reproducible and reliable taxonomic method for those species of *Lactobacillus* with stable patterns. Such organisms could be drawn from two sources, namely those whose patterns are not affected by the change in temperature (Table 15) and those with few or lower levels of alterations where the changes do not cause marked difference in the characteristic fermentation pattern for the species (Kandler and Weiss, 1986; Hammes and Vogel, 1995) (Table 16). Therefore, *Lb. amylophilus, Lb. casei* subsp. *casei, Lb. fermentum, Lb. graminis, Lb. hilgardii, Lb.*

paracasei subsp. *tolerans, Lb. paraplantarum, Lb. rhamnosus, Lb. pentosus* and *Lb. vaginalis* (Table 15) are not affectedby temperature change. While *Lb. coryniformis, Lb. parabuchneri, Lb. plantarum, Lb. salivarius* subsp. *salivarius* and *W. minor* had a very low level of variation which did not affect their differential characteristics as outlined in the Bergey's manual (Kandler and Weiss, 1986) and presented by Hammes and Vogel (1995). Thus it would seem logical to include them with the first group. However, in all the rest of the studied species it appears highly unlikely to get reliable results since the differences arre high.

As outlined by Kandler and Weiss (1986), all animal-associated lactobacilli (the homofermenters according to their grouping) do not characteristically ferment gluconate, melezitose, ribose and xylose but only do the facultative heterofermenters. Contrary to this however, in our stdy, assimilation of these compounds was observed in plant associated species due to the effect of temperature. Such a differnece, therefore, is in disagreement with the definitive property outlined in the Bergey's manual for a particular species. From this then it could be inferred that majority of the plant related species and some of the animal associated ones need to be compared at 30°C and most intestinal strains at 37°C. Moreover, it appears very essential to stick to a temperature optimum for a particular species.

CONCLUSIONS

From the different studies compiled in this thesis the following ideas were generated. The doughs and freshly baked products of *kocho* and *tef* ,were capable of inactivating asporogenous pathogenic and spoilage bacteria and were considered to be microbiologically safe. However, strict care needs to be taken at the pre- and post-baking stages

The isolation of diverse strains of *Lactobacillus, Pediococcus* and *Weissella* species from fermented *kocho* and *tef* dough has good implications that these foods might possess valuable inherent properties found in LAB fermented foods. The wide metabolic performance of the isolates, such as metabolism of maltose and starch, contribute to the predominance of lactobacilli and eventual production of desired aroma, texture, flavour and preservatives of the products.

The use of RAPD coupled with the GelCompar program has been found quite helpful in screening large numbers of field isolates for relatedness although inherent short comings, such as inability to differentiate between closely related species of *Lactobacillus*, need to be rectified by supplementing with other sensitive genetic tools like DNA:DNA hybridization. The use of DNA:DNA hybridization remained essentially crucial for ultimate identification of an isolate. The use of 16S rRNA sequence data in *Pediococcus* species had less relevance in delineating species as a result of the unavoidable high levels of interspecific sequence homologies.

API 50 CH fermentation profiles are a reliable method for the evaluation of metabolic performance of an isolate in both *Lactobacillus* and *Pediococcus*. However, the fermentation profiles were dependent on the temperature used. There were variations in profiles when the type strains of a lot of the *Lactobacillus* species were tested at 30°C and 37°C.

REFERENCES

Adams, M.R. and Hall, C.J. 1988. Growth inhibition of food borne pathogens by lactic and acetic acids and their mixtures. *Int. J. Food Sci. Technol.* **23**:287-292.

Agte, V.V., Gokhale, M.K. and Chiplonkar, S.A. 1997. Effect of natural fermentation on *in vitro* zinc bioavailability in cereal-legume mixtures. *Int. J. Food Sci. Technol.* **32**:29-32.

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

Ahrné, S. and Molin, G. 1991. Spontaneous mutations changing the raffinose metabolism of *Lactobacillus plantarum. Antonie van Leeuwen. J. Gen. Mol. Microbiol.* **60**:87-93.

Ahrné, S., Molin, G. and Ståhl, M. 1989. Plasmids in *Lactobacillus* strains isolated from meat and meat products. *Syst. Appl. Microbiol.* **11**:320-325.

Ahrné, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A.E. and Molin, G. 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. *J. Appl. Microbiol.* **84**:1-7.

Archibald, F. 1986. Manganese:Its acquisition by and function in lactic acid bacteria. *CRC Critic. Rev. Microbiol.* **13**:63-109.

Ashenafi, M. 1990. Effect of curd cooking temperature on the microbiological quality of Ayib, a traditional Ethiopian cottage cheese. *W. J. Microbiol. Biotechnol.***6**:*159-162.*

Ashenafi, M. 1992. The microbiology of Ethiopian *Ayib*. In *Applications of Biotechnology to Traditional Fermented foods*. National Academic Press, Washington, D.C. pp. 71-74.

Ashenafi, M. 1994. Microbial flora and some chemical properties of *Irsho,* a starter for teff *(Eragrostis tef)* fermentation. *W. J. Microbiol. Biotechnol.* **10**:69-73.

Ashenafi, M. and Mehari, T. 1995. Some microbiological and nutritional properties of borde and shamita, traditional Ethiopian fermented beverages. *Ethiop. J. Health Dev.* **9**:1050-110.

Axelsson, L.T. 1993. Lactic acid bacterial:classification and physiology. In *Lactic acid bacteria* (Salminen, S. and Von Wright, A., eds.) Marcel Dekker, Inc., New York. p. 1-64.

Back, W. 1978a. Phänotypische und genotypische abgrenzung der bisher bekannten arten sowie beschreibung einer neuen bierschädlichen art: *Pediococcus inopinatus*. *Brauwissenschaft*. **31**:237-250.

Back, W. 1978b. Elevation of *Pediococcus cerevisiae* subsp. *dextrinicus* Coster and White to species status [*Pediococcus dextrinicus* (Coster and White) comb. nov.]. *Int. J. Syst. Bacteriol.* **28**:523-527.

Barreau, C. and Wagner, G. 1990. Characterization of *Leuconostoc lactis* strains from human sources. *J. Clin. Microbiol.* **28**:1728-1233.

Becker, R. and Hanners, G.D. 1992. Carbohydrate composition of cereal grains. In *Handbook of cereal science and technology* (Lorenz, K.J. and Kulp, K., eds.), Marcel Dekker, New York. p. 469-496.

Berggren, A. 1996. *Formation, pattern and physiological effects of short-chain fatty acids*. Ph.D. Thesis, Department of Applied Microbiology, Lund University, Sweden.

Bergey, D.H., Harrison, F.C., Breed, R.S., Hammer, B.W. and Huntoon, F.M. (eds.). 1925. In *Bergey's Manual of Determinative Bacteriology*, 2nd edn. The Williams and Wilkins Co., Baltimore, USA.

Bergey, D.H., Breed, R.S., Hammer, B.W., Huntoon, F.M., Murray, E.G.D. and Harrison, F.C. (eds.), 1934. In *Bergey's Manual of Determinative Bacteriology*, 4th edn. The Williams and Wilkins Co., Baltimore, USA. p. 1-664.

Beyene, F., Narvhus, J. and Abrahamsen, R.K. 1998. Evaluation of new isolates of lactic acid bacteria as a starter for cultured milk production. *SINET:Ethiop. J. Sci.* **21**:67-80.

Bingen, E.H., Denamur, E. and Elion, J. 1994. Use of ribotyping in epidemiological surveillance of nosocomial outbreaks. *Clin. Microbiol.* **8**:231-238.

Brandt, S. 1993. In *Enset-based sustainable agriculture in Ethiopia*. Proceedings of the international workshop on enset held in Addis Ababa, Ethiopia (Abate, T., Hiebsch, C.,

Brandt, S. and Gebremariam, S., eds.), 13-20 Dec. , Addis Ababa, Ethiopia.

Campbell-Platt, G. 1987. Fermented foods of the world, a dictionary and guide. Butterworths, London. 291 pp.

Cateno-Annoles, G., Bassam, B.J. and Gresshoff, P.M. 1991. DNA amplification f fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* **9**:553-556.

Collar, C., Mascarós, A.F. and De Barber, C.B. 1992. Amino acid metabolism by yeasts and lactic acid bacteria during bread dough fermentation. *J. Food Sci.* **57**:1423-1427.

Collins, M.D., Phillips, B.A. and Zanoni, P. 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei, Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov. *Int. J. Syst. Bacteriol.* **39**:105-108.

Collins, M.D., Rodriguez, U., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A. Williams, A.M. and Wallbanks, S. 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.

Collins, M.D., Samelis, J., Metaxopoulos, J. and Wallbanks, S. 1993. Taxonomic studies on some leuconostoc-like organisms from fermented sausages:description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group species. *J. Appl. Bacteriol.* **75**:595-603.

Collins, M.D. and Wallbanks, S. 1992. Comparative sequence analysis of the 16S rRNA genes of *Lactobacillus minutus, Lactobacillus rimae*, and *Streptococcus parvulus*: proposal for the creation of a new genus *Atopobium. FEMS Microbiol. Lett.* **95**:235-240.

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

Colwell, R.R. 1970. Polyphasic taxonomy of the genus *Vibrio*: numerical taxonomy of

Vibrio cholerae, Vibrio parahaemolyticus, and related *Vibrio* species. *J. Bacteriol.* **104**:410-433.

Cooke, R.D., Twiddy, D.R. and Reilly, P.J.A. 1987. Lactic acid fermentation as a low-cost means of food preservation in tropical countries. *FEMS Microbiol. Rev.* **46**:369-379.

Curk, M.-C., Hubert, J.-C. and Bringel, F. 1996. *Lactobacillus paraplantarum* sp. nov., a new species related to *Lactobacillus plantarum*. *Int. J. Syst. Bacteriol.* **46**:595-598.

Daeschel, M.A., Andersson, R.E. and Fleming, H.P. 1987. Microbial ecology of fermenting plant materials. *FEMS Microbiol. Rev.* **46**:357-367.

Davidson, P.M. and Hoover, D.G. 1993. Antimicrobial components from lactic acid bacteria. In *Lactic acid bacteria* (Salminen, S. and Von Wright, A., eds.), Marcel Dekker, Inc., New York. p. 161-198.

Davidson, B.E., Kordias, N., Dobos, M. and Hillier, A.J. 1996. Genomic organization of lactic acid bacteria. *Antonie van Leeuwen.* **70**:161-183.

Dellaglio, F., Bottazzi, V. and Trovatteli, L.D. 1973. Deoxyribonucleic acid homology and base composition in some thermophilic lactobacilli. *J. Gen. Microbiol.* **74**:289-297.

Dellaglio, F., Bottazzi, V. and Vescovo, M. 1975. Deoxyribonucleic acid homology among *Lactobacillus* species of the subgenus *Streptobacterium* Orla-Jensen. *Int. J. Syst. Bacteriol.* **25**:160-172.

Dellaglio, F., Torriani, D.L. and Sarra, P.G. 1981. DNA:DNA homology among representative strains of the genus *Pediococcus*. *Zbl. Bakt. Hyg., I. Abt. Orig.* C **2**:140-150.

Dellaglio, F., Dicks, L.M.T., and Torriani, S. 1995. The genus *Leuconostoc*. In *The lactic acid bacteria: The genera of lactic acid bacteria* (Wood, B.J.B. and Holzapfel, W.H., eds.), Blackie Academic & Professional, Glasgow. p. 235-278.

Descheemaeker, P., Pot, B., Ledeboer, A.M., Verrips, T. and Kersters, K. 1994. Comparison of the *Lactococcus lactis* differential medium DCL) and SDS-PAGE of whole-cell protein extracts for the identification of lactococci to subspecies level. *Syst. Appl. Microbiol.* **17**:459-466.

Devriese, L.A., Pot, B., Van Damme, L., Kersters, K. and Haesebrouck, F. 1995. Identification

of *Enterococcus* species isolated from food products of animal origin. *Int. J. Syst. Bacteriol.* **169**:1441-1446.

Dicks, L.M.T. 1995. Relatedness of *Leuconostoc* species of the *Leuconostoc sensu stricto* line of descent, *Leuconostoc oenos* and *Weissella paramesenteroides* revealed by numerical analysis of total soluble cell protein patterns. *System. Appl. Microbiol.* **18**:99-102.

Dicks, L.M.T., Du Plessis, E.M., Dellaglio, F. and Lauer, E. 1996. Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., Designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*. *Int. J. Syst. Bacteriol.* **46**:337-340.

Dicks, L.M.T. and van Vuuren, H.J.J. 1990. Differentiation of *Leuconostoc* species by nicotinamide adenine dinucleotide-dependent D(-)-lactic dehydrogenase profiles. *FEMS Microbiol. Lett.* **67**:9-14.

Dicks, L.M.T., van Vuuren, H.J.J. and Dellaglio, F. 1987. Relatedness of homofermentative *Lactobacillus* species revealed by numerical analysis of total soluble cell protein patterns. *Int. J. Syst. Bacteriol.* **40**:83-91.

Doyle, M.P. 1989. *Bacillus cereus*. In *Bacteria associated with food borne diseases* (Oblinger, J.L., ed.), Institute of Food Technology, Chicago. p.19.

Du Plessis, E.M. and Dicks, L.M.T. 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri*, and *Lactobacillus johnsonii*. *Curr. Microbiol.* **31**:114-118.

Dykes, G. A. and von Holy, A. 1994. Strain typing in the genus *Lactobacillus*. *Lett. Appl. Microbiol.* **19**:63-66.

Ebringer, L., Ferencik, M., Lahitova, N., Kacani, L. and Michalkova. 1995. Anti-mutagenic and immuno-stimulatory properties of lactic acid bacteria. *W. J. Microbiol. Biotechnol.* **11**:294-298.

Efthymiou, C and Hansen, P.A. 1962. An antigenic analysis of *Lactobacillus acidophilus*. *J. Infect. Dis.* **110**:258-267.

Ehrmann, M., Ludwig, W. and Schleifer, K.H. 1994. Reverse dot blot hybridization: a useful method for the direct identification of lactic acid bacteria in fermented food. *FEMS Microbiol. Lett.* **117**:143-150.

Ferrero, M., Cesena, C., Morelli, L., Scolari, G. and Vescovo, M. 1996. Molecular characterization of *Lactobacillus* strains. *FEMS Microbiol. Lett.* **140**:215-219.

Fujisawa, T., Benno, Y., Yaeshima, T. and Mitsuoka, T. 1992. Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonomy of *Lactobacillus acidophilus* group A3 (Johnson, et al., 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura, 1981). *Int. J. Syst. Bacteriol.* **42**:487-491.

Fuller, R. (ed.). 1992. Probiotics: The scientific basis. Chapman and Hall, U.K. 388pp.

Garvie, E.I. 1986. Genus *Pediococcus* Claussen 1903, 68[AL]. In *Bergey's Manual of Systematic Bacteriology.* vol. 2 (Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., eds), Williams & Wilkins, Baltimore. p. 1075-1079,

Gashe, B.A. 1985. Involvement of lactic acid bacteria in the fermentation of TEF *(Eragrostis tef)*, an Ethiopian fermented food. *J. Food Sci.* **50**:800-801.

Gashe, B.A. 1987. *Kocho* fermentation. *J. Appl. Bacteriol.* **62**, 473-477.

Gashe, B.A., Girma, M. and Bisrat, A. 1982. Tef fermentation. I. The role of microorganisms in fermentation and their effect on the nitrogen content of Tef. *SINET:Ethiop. J. Sci.* **5**: 69-76.

Gasser, F. 1970. Electrophoretic characterization of lactic dehydrogenases in the genus *Lactobacillus*. *J. Gen. Microbiol.* **62**:223-239.

Gebre-Mariam, T. and Schmidt, P.C. 1996. Isolation and physico-chemical properties of enset starch. *Starch/Stärche.* **48**:208-214.

Gibson, G.R. and Roberfroid, M.B. 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *J. Nutr.* **125**:1401-1412.

Gifawossen, C. and Bisrat, A. 1982. Yeast flora of fermenting Tef *(Eragrostis tef)*. *SINET:Ethiop. J. Sci.* **5**:21-25.

Gilliland, S.E. 1989. Acidophilus milk products: A review of potential benefits to consumers. *J. Dairy Sci.* **72**:2483-2494.

Girma, M., Gashe, B,A. and Lakew, B. 1989. The effect of fermentation on the growth and survival of *Salmonella typhimurium, Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeruginosa* in fermenting tef (*Eragrostis tef*). *MIRCEN: J. Appl. Microbiol. Biotechnol.* **5**:61-67.

Gobbetti, M., Simonetti, M.S., Rossi, J., Cossignani, L., Corsetti, A. and Damiani, P. 1994. Free D- and L-amino acid evolution during sourdough fermentation and baking. *J. Food Sci.* **59**:881-884.

Gobbetti, M., Simonetti, M.S., Corsetti, A., Santinelli, F., Rossi, J and Damiani, P. 1995. Volatile compound and organic acid productions by mixed wheat sourdough starters: influence of fermentation parameters and dynamics during baking. *Food Microbiol.* **12**:497-507.

Gobbetti, M. and Corsetti, A. 1997. *Lactobacillus sanfrancisco* a key sourdough lactic acid bacterium: a review. *Food Microbiol.* **14**:175-187.

Gorbach, S.L. 1990. Lactic acid bacteria and human health. *Ann. Med.* **22**:37-41.

Gordillo, M.E., Singh, K.V., Baker, C.J. and Murray, B.E. 1993. Typing of group B streptococci: comparison of pulsed-field gel electrophoresis and conventional electrophoresis. *J. Clin. Microbiol.* **31**:1430-1434.,

Gurtler, V., Wilson, V.A. and Mayall, B.C. 1991. Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J. Gen. Microbiol.***137**: 2673-2679.

Hamad, S.H., Deing, M.C., Ehrmann, M.A. and Vogel, R.F. 1997. Characterization of the bacterial flora of Sudanese sorghum sourdough. *J. Appl. Bacteriol.* **83**:764-770.

Hammes, W.P. and Vogel, R.F.1995. The genus *Lactobacillus*. *In* The genera of lactic acid

bacteria. *The lactic acid bacteria* vol. 2. (Wood, B.J. and Holzapfel, W.H., eds.). Blackie Academic & Professional, Glasgow, Scotland. p. 19-54.

Helander, I.M., von Wright, A. and Mattila-Sandholm, T-M. 1997. Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. *Trends Food Sci. Technol.* **8**:146-150.

Hensel, R., Mayr, U., Stetter, O. and Kandler, O. 1977. Comparative studies of lactic acid dehydrogenases in lactic acid bacteria. I. Purification and kinetics of the allosteric L-lactic acid dehydrogenase from *Lactobacillus casei* ssp. *casei* and *Lactobacillus curvatus*. *Arch. Microbiol.* **112**:81-93.

Höfle, M.G. 1988. Identification of bacteria by low molecular weight RNA profiles: a new chemotaxonomic approach. *J. Microbiol. Methods.* **8**:235-248.

Höfle, M.G. 1990. Transfer RNA as genotyping fingerprints of eubacteria. *Arch. Microbiol.* **153**:299-304.

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

Huys, G., Coopman, R., Janssen, P. and Kersters, K. 1996. High resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *Int. J. Syst. Bacteriol.* **46**:572-580.

Jayaro, B.M., Doré, J.J.E., Baumbach, G.A., Matthews, K.R. and Oliver, S.P. 1991. Differentiation of *Streptococcus uberis* from *Steptococcus parauberis* by polymerase chain reaction and restriction fragment polymorphism of 16S ribosomal DNA. *J. Clin. Microbiol.* **29**:2774-2778.

Johansson, M-L., Molin, G., Jeppsson, B., Nobaek, Ahrné, S. and Bengmark, S. 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: *In vivo* colonization of human intestinal mucosa and effect on indigenous flora. *Appl. Environ. Microbiol.* **59**, 15-20.

Johansson, M-L., Molin, G., Pettersson, B., Uhlén, M. and Ahrné, S. 1995a. Characterization of *Lactobacillus plantarum* strains by restriction fragment length polymorphism

(RFLP) of the 16S rRNA gene. *J. Appl. Bacteriol.* **79**:536-541.

Johansson, M.-L., Quednau, M., Ahrné, S. and Molin, G. 1995b. Classification of *Lactobacillus plantarum* by restriction endonuclease analysis (REA) of total chromosomal DNA, using conventional agarose gel electrophoresis. *Int. J. Syst. Bacteriol.* **45**:670-675.

Johansson, M.-L, Quednau, M., Molin, G. and Ahrné, S. 1995c. Randomly amplified polymorphic DNA (RAPD) for rapid typing of *Lactobacillus plantarum* strains. *Lett. Appl. Microbiol.* **21**, 155-159.

Johansson, M-L. 1995. *Systematics and starter culture selection of Lactobacillus for human intestine and Nigerian ogi with special reference to Lactobacillus plantarum.* Ph. D. Thesis, Department of Food Technology, Lund University, Sweden. 185 pp.

Johnson, J.L. 1986. Bacterial Classification III. Nucleic acids in bacterial classification. In *Bergey's manual of Systematic Bacteriology.* vol 2 (Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., eds), Williams & Wilkins, Baltimore. pp. 8-11.

Kabir, A.M.A., Aiba, Y., Takagi, A., Kamiya, S., Miwu, T. and Koga, Y. 1997. Prevention of *Helicobacter pylori* infection by lactobacilli in gnotobiotic murine model. *Gut.* **41**:49-55.

Kalantzopoulos, G. 1997. Fermented products with probiotic qualities. *Anaerobe.* **3**:185-190.

Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuween.* **49**:209-224.

Kandler, O. and Weiss, N. 1986. Genus *Lactobacillus* Beijerinck 1901,212[AL]. In *Bergey's manual of systematic bacteriology*, vol **2** (Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., eds), Williams & Wilkins, Baltimore. pp. 1209-1234.

Ketema, S. 1991. Germplasm evaluation and breeding work on teff (Eragrostis tef) in Ethiopia. In *Plant Genetic Resources of Ethiopia* (Engels, J.M.M., Hawkes, J.G. and Worede, M., eds.), p. 323-328, Cambridge University Press, New York, USA.

Kingamkono, R., Sjörgen, E., Svanberg, U. and Kaijser, B. 1995. Inhibition of different strains

of enteropathogens in a lactic-fermenting cereal gruel. *W. J. Microbiol. Biotechnol.* **11**:299-303.

Kumar, R., Garg, S.K., Singh, D.T., Singh, S.P. and Mital, B.K. 1994. Evidence for the presence of plasmids in four therapeutically important strains of *Lactobacillus acidophilus*. *Lett. Appl. Microbiol.* **19**:188-191.

Kunji, E.R.S., Mierau, I., Hagting, A., Poolman, B. and Konings, W.N. 1996. The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwen.* **70**:187-221.

Langella, P., Zagorec, M., Ehrlich, S.D. and Morel-Deville, F. 1996. Intergeneric and intrageneric conjugal transfer of plasmids pAMB1, pIL205 and pIP501 in *Lactobacillus sake*. *FEMS Microbiol. Lett.* **139**:51-56.

Lapage, E.A., Sneath, P.H.A., Lessel, E.F., Skerman, V.B.D., Seeliger, H.P.R. and Clark, W.A. 1975. International Code of Nomenclature of Bacteria. *American Society for Microbiology*, Washington, D.C.

Law, J. and Haandrikman, A. 1997. Proteolytic enzymes of lactic acid bacteria. *Int. Dairy J.* **7**:1-11.

London, J. and K., Kline. 1973. Aldolases of lactic acid bacteria: a case history in the use of an enzyme as an evolutionary marker. *Bacteriol. Rev.* **37**:453-478.

Lönner, C.1988. *Starter cultures for rye sour doughs, Characteristics and functions of lactic acid bacteria.* Ph.D. Thesis, Department of Applied Microbiology, Lund University, Sweden. 188pp.

Lorri, W. and Svanberg, U. 1994. Lower prevalence of diarrhoea in young children fed lactic acid-fermented cereal gruels. *Food Nutr. Bull.* **15**:57-63.

Ludwig, W. and Schleifer, K.H. 1995. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol. Rev.* **15**:155-173.

Marklinder, I. and Lönner, C. 1992. Fermentation properties of intestinal strains of *Lactobacillus* of sourdough and of a yoghurt starter culture in an oat-based nutritive solution. *Food Microbiol.* **9**:197-206.

Martinez-Anya, M.A., Pitarch, B., Bayarri, P. and Benedito de Barber, C. 1990. Microflora of

the sour doughs of wheat flour bread. X. Interactions between yeasts and lactic acid bacteria in wheat doughs and their effects on bread quality. *Cereal Chem.* **67**:85-91.

Martinez-Murcia, A.J. and Collins, M.D. 1990. A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol. Lett.* **70**:73-84.

Mauguin, S. and Novel, G. 1994. Characterization of lactic acid bacteria isolated from seafood. *J. Appl. Bacteriol.* **76**:616-625.

Mehari, T. and Ashenafi, M. 1995. Microbiology of *siljo*, a traditional Ethiopian fermented legume product. *W. J. Microbiol. Biotechnol.* **11**:338-342.

Mensah, P., Thomkins, A.M., Prasar, B.S. and Harrison, T.J. 1991. Antimicrobial effect of fermented Ghanaian maize dough. *J. Appl. Bacteriol.* **70**:203.

Mikelsaar, M. and Mäander, R. 1993. Development of individual lactic acid microflora in the human microbial ecosystem. In *Lactic acid bacteria* (Salminen, S. and Von Wright, A., eds.), Marcel Dekker, Inc., New York. pp. 237-294.

Mital, B.K. and Garg, S.K. 1995. Anticarcinogenic, hypocholesterolemic and antagonistic activities of *Lactobacillus acidophilus*. *CRC Critic. Rev. Microbiol.* **21**:175-214.

Molin, G. 1995. *Lactobacillus* strains for fermented oatmeal soup in PRO VIVA™. In *Lactic acid bacteria:Which strain? For which markets?* Lactic 94, 7-9, September 1994. pp.213-223. Universitaries de Caen, Adria Normandie Press. Caen.

Molin, G., Jeppsson, B., Johansson, M.-L., Ahrné, S. Nobaek, S., Ståhl, M. and Bengmark, S. 1993. Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. *J. Appl. Microbiol.* **74**:314-323.

Mora, D., Fortina, M.G., Parini, C. and Manachini, P.L. 1997. Identification of *Pediococcus acidilactici* and *Pediococcus pentosaceus* based on 16S rRNA and *ldhD* gene-targeted multiplex PCR analysis. *FEMS Microbiol. Lett.* **151**:231-236.

Mori, K., Yamazaki, K., Ishiyama, T., Katsumata, M. Kabayashi, K., Kawai, Y., Inoue, N. and Shinano, H. 1997. Comparative sequence analyses of the genes coding for 16S rRNA

of *Lactobacillus casei*-related taxa. *Int. J. Syst. Bacteriol.* **47**:54-57.

Murray, R.G.E., Brenner, D.J., Colwell, R.R., De Vos, P., Goodfellow, M., Grimont, P.A.D., Pfennig, N., Stackebrandt, E. and Zavarzin, G.A. 1990. Report of the ad hoc committee on approaches to taxonomy within the *Proteobacteria*. *Int. J. Syst. Bacteriol.* **40**:213-215.

Nigatu, A. 1992. *Lactic acid bacteria of fermenting tef dough and fermented kocho and their inhibitory effect on certain food-borne pathogens or spoilage organisms.* M.Sc. Thesis, Department of Biology, Addis Ababa University, Ethiopia. 62 pp.

Nigatu, A., Ahrné, S., Gashe, B.A. and Molin, G. 1998. Randomly amplified polymorphic DNA (RAPD) for discrimination of *Pediococcus pentosaceus* and *Ped. acidilactici* and rapid grouping of *Pediococcus* isolates. Lett. Appl. Microbiol. **26**:407-411.

Nigatu, A. and Gashe, B.A. 1994a. Survival and growth of selected pathogens in fermented kocho *(Ensete ventricosum)*. *East Afric. Med. J.* **71**:514-518.

Nigatu, A. and Gashe, B.A. 1994b. Inhibition of spoilage and food-borne pathogens by lactic acid bacteria isolated from fermenting tef *(Eragrostis tef)* dough. *Ethiop. Med. J.* **32**:223-229.

Nigatu, A. and Gashe, B.A. 1997. *Bacillus* spp. from fermented tef dough and kocho: Identity and role in the two Ethiopian fermented foods. *SINET:Ethiop. J. Sci.* **20**:101-114.

Nousiainen, J. and Setälä, J. 1993. Lactic acid bacteria as animal probiotics. In *Lactic acid bacteria* (Salminen, S. and Von Wright, A., eds.), Marcel Dekker, Inc., New York. pp. 315-356.

Nout, M.J.R. 1991. Ecology of accelerated natural lactic fermentation of sorghum-based infant formulas. *Int. J. Food Microbiol.* **12**:217-224.

Nout, M.J.R. 1994. Fermented foods and food safety. *Food Res. Int.* **27**:291-298.

Nout, M.J.R. and Rombouts, F.M. 1992. Fermentative preservation of plant foods. *J. Appl. Bacteriol. (Sympos. Suppl.)* **73**:136S-147S.

Odunfa, S.A. and Adeyele, S. 1987. Sugar changes in fermenting sorghum during preparation of 'ogi-baba' gruel. *J. Food Agric.* **1**:95-98.

Olsen, G.J., Larsen, N. and Woese, C.R. 1991. *Nucleic Acids Res.* (Suppl.) **19**:2017-2021.

Olsen, G.J. and Woese, C. 1993. Ribosomal RNA: a key to phylogeny. *FASEB J.* **7**:113-123.

Patarata, L., Pimentel, M.S., Pot, B., Kersters, K. and Mendes-Faia, A. 1994. Identification of lactic acid bacteria isolated from Portuguese wines and musts by SDS-PAGE. *J. Appl. Bacteriol.* **76**:288-293.

Patidar, S.K. and Prajapati, J.B. 1997. Methods for assessing the immunostimulating properties of dietary lactobacilli-A critical appraisal. *J. Food Sci. Technol.* **34**:181-194.

Pederson, C.S. 1949. The genus *Pediococcus. Bacteriol. Rev.* **13**:225-232.

Pederson, C.S. 1979. *Microbiology of food fermentations.* 2nd. edn. AVI, Connecticut. 384 pp.

Pettersson, B. 1997. *Direct solid-phase 16S rRNA sequencing: a tool in bacterial phylogeny.* Ph.D. Thesis, Department of Biochemistry and Biotechnology, Royal Institute of Technology, Stockholm, Sweden, 71pp.

Pijls, L.T.J., Timmer, A.A.M., Woldegebriel, Z. and West, C.E. 1995. Cultivation, preparation and consumption of Ensete *(Ensete ventricosum)* in Ethiopia. *J. Sci. Food Agric.* **67**:1-11.

Pot, B., Hertel, C., Ludwig, W., Descheemaeker, P., Kersters, K and Schleifer, K.- H. 1993. Identification and classification *Lactobacillus acidophilus, L. gasseri* and *L. johnsonii* strains by SDS-PAGE and rRNA-targeted oligonucleotide probe hybridization. *J. Gen. Microbiol.* **139**:513-517.

Pot, B., Vandamme, P. and Kersters, K. 1994. Analysis of electrophoretic whole-organism protein fingerprints. In *Modern microbial methods. Chemical methods in prokaryotic systematics* (Goodfellow, M. and O'Donnel, A.G., eds.), John Wiley & Sons Ltd., Chichester, England. p. 493-521.

Quednau, M. 1998. *RAPD and REA for characterization of Lactobacillus plantarum strains, and Enterococcus spp. with special reference to Enterococcus spp. from meat and humans.* Ph. D. Thesis, Department of Food Technology, Lund University, Sweden. 98pp.

Sahle, S. and Gashe, B.A. 1991. The microbiology of Tella fermentation. *SINET: Ethiop. J. Sci.* **14**:81-92.

Salminen, S., Isolauri, E. and Salminen, E. 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie van Leeuwen.* **70**:347-358.

Salovaara, H. 1993. Lactic acid bacteria in cereal-based products. In *Lactic acid bacteria* (Salminen, S. and Von Wright, A., eds.), Marcel Dekker, Inc., New York. pp.111-126.

Schleifer, K.E., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W. and Amann, R. 1995. Application of molecular methods for the classification and identification of l lactic acid bacteria. *Int. Dairy J.* **5**:1081-1094.

Schleifer, K.H. and Kandler, O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**:407-477.

Schleifer, K.H. and Ludwig, W. 1995. Phylogeny of the Genus *Lactobacillus* and related genera. *System. Appl. Microbiol.* **18**:461-467.

Schleifer, K.H. and Stackebrandt, E. 1983. Molecular systematics of prokaryotes. *Ann. Rev. Microbiol.* **37**:143-187.

Shah, N. and Jelen, P. 1990. Survival of lactic acid bacteria and their lactases under acidic conditions. *J. Food Sci.* **55**:506-509.

Simango, C. and Rukure, H. 1992. Survival of bacterial enteric pathogens in traditional fermented foods. *J. Appl. Bacteriol.* **73**, 37-40.

Simpson, W.J. 1994. Opinion: Comments on the mode of division of *Pediococcus* spp. *Lett. Appl. Microbiol.* **18**:69-70.

Simpson, W.J. and Taguchi, H. 1995. The genus *Pediococcus*, with notes on the genera *Tetragenococcus* and *Aerococcus*. In *The genera of lactic acid bacteria. The lactic acid bacteria,* vol. 2. (Wood, B.J. and Holzapfel, W.H., eds), Blackie Academic & Professional, Glasgow, Scotland. pp. 125-172.

Sreeramula, G., Srinivasa, A.S., Nand, K. and Joseph, R. 1996. *Lactobacillus amylovorus* as a phytase producer in submerged culture. *Lett. Appl. Microbiol.* **23**:385-388.

Stackebrandt, E. and Goebel, B.M. 1994. Taxonomic Note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846-849.

Ståhl, M., Molin, G., Persson, A., Ahrné, S. and Ståhl, S. 1990. Restriction endonuclease patterns and multivariate analysis as a classification tool for *Lactobacillus* spp. *Int. J. Syst. Bacteriol.* **40**:189-193.

Ståhl, M. and Molin, G. 1994. Classification of *Lactobacillus reuteri* by restriction endonuclease analysis of chromosomal DNA. *Int. J. Syst. Bacteriol.* **44**:9-14.

Ståhl, M., Pettersson, B., Molin, G., Uhlén, M. and Ahrné, S. 1994. Restriction fragment length polymorphism of *Lactobacillus reuteri* and *Lactobacillus fermentum*, originating from intestinal mucosa, based on 16S rRNA genes. *Syst. Appl. Bacteriol.* **17**:108-115.

Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G.(eds). 1986. *The Prokaryotes* II. Springer-Verlag, Berlin. p.1711-1742.

Stead, D. 1994. The effect of chlorogenic, gallic and quinic acids on the growth of spoilage strains of *Lactobacillus collinoides* and *Lactobacillus brevis*. *Lett. Appl. Bacteriol.* **18**:112-114.

Steinkraus, K.H. 1994. Nutritional significance of fermented foods. *Food Res. Int.* **27**:259-267.

Stiles, M.E. 1996. Biopreservation by lactic acid bacteria. *Antonie Van Leeuwen.* **70**:331-345.

Stiles, M.E. and Holzapfel, W.H. 1997. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.* **36**:1-29.

Sugihara, T.F. 1985. Microbiology of breadmaking. In *Microbiology of fermented foods.* Vol 1. (Wood, B.J.B, ed.), Elsevier Applied Science, Barking. pp. 249-261.

Sullivan, N.M., Mills, D.C., Riemann, H.P. and Arnon, S.S. 1988. Inhibition of growth of *Clostridium botulinum* by intestinal microflora isolated from healthy infants. *Microb. Ecol. Health Dis.* **1**:179-192.

Svanberg, U., Sjogren, E., Lorri, W. Svennerholm, A-M. and Kaijser, B. 1992. Inhibited

growth of common enteropathogenic bacteria in lactic-fermented cereal gruel. *W. J. Microbiol. Biotechnol.* **8**:601-606.

Tanasupawat, S. and Komagata, K. 1995. Lactic acid bacteria in fermented foods in Thailand. *World J. Microbiol. Biotechnol.* **11**:253-256.

Tanasupawat, S., Okada, S., Kozaki, M. and Komagata, K. 1993. Characterization of *Pediococcus pentosaceus* and *Pediococcus acidilactici* strains and replacement of the type strain of *P. acidilactici* with the proposed neotype DSM 20284. Request for an opinion. *Int. J. Syst. Bacteriol.* **43**:860-863.

Tannock, G.W. 1997. Probiotic properties of lactic-acid bacteria: plenty of scope for fundamental R&D. *Trends in Biotech.* **15**:270-274.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. and Swaminathan, B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33**:2233-2239.

Torriani, S., Van Reenen, C.A., Klein, G., Reuter, G., Dellaglio, F. and Dicks, L.M.T. 1996. *Lactobacillus curvatus* subsp. *curvatus* subsp. nov. and *Lactobacillus curvatus* subsp. *melibiosus* subsp. nov. and *Lactobacillus sake* subsp. *sake* subsp. nov. and *Lactobacillus sake* subsp. nov., new subspecies of *Lactobacillus curvatus* Abo-Elnaga and Kandler 1965 and *Lactobacillus sake* Katagiri, Kitahara, and Fukami 1934 (Klein, *et al.*, 1996, Emended descriptions), respectively. *Int. J. Syst. Bacteriol.* **46**:1158-1163.

Tsakalidou, E., Manolopoulou, G., Kabaraki, E., Zoidou, E., Pot, B, Kersters, K. and Kalantzopoulos, G. 1994. The combined use of whole-cell protein extracts for the identification (SDS-PAGE) and enzyme activity screening of lactic acid bacteria isolated from traditional Greek dairy products. *Syst. Appl. Microbiol.* **17**:444-458.

Tsakalidou, E., Samelis, J. Metaxopoulos, J. and Kalantzopoulos, G. 1997. Atypical *Leuconostoc*-like *Weissella* strains isolated from meat, sharing low phenotypic relatedness with the so far recognized arginine-negative *Weissella* spp. as revealed by SDS-PAGE of whole cell proteins. *Int. Syst. Microbiol.* **20**:659-664.

Umeta, M. and Faulks, R.M. 1989. Lactic and volatile (C_2-C_6) fatty acid production in the fermentation and baking of tef *(Eragrostis tef)*. *J. Cereal. Sci.* **9**:91-95.

Urga, K., Gashe, B.A., Fite, A. and Nigatu, A. 1992. Changes in acidity and lactic acid production during Ititu fermentation. *Ethiop. J. Agric. Sci.* **13**:82-87.

Urga, K., Nigatu, A. and Umeta, M. 1993. Traditional *Enset*-based foods: Survey of processing techniques in Sidama. In *Enset-based sustainable agriculture in Ethiopia* (Abate, T., Hiebsch, C., Brandt, S.A. and Gebremariam, S., eds), *Proceedings of the International Workshop On Enset held in Addis Ababa, Ethiopia*, Dec. 13-20, 1993, Institute of Agricultural Research. pp. 305-310.

Urga, K., Fite, A. and Biratu, E. 1997a. Effect of natural fermentation on nutritional and antinutritional factors of tef *(Eragrostis tef)*. *Ethiop. J. Health Dev.* **11**:61-66.

Urga, K., Fite, A. and Biratu, E. 1997b. Natural fermentation of Enset *(Ensete ventricosum)* for the production of Kocho. *Ethiop. J. Health Dev.* **11**:75-81.

Vahvaselkä, M., Hofman, M. and Linko, P. 1990. Effect of oxygen on growth and lactose metabolism of *Lactobacillus fermentum*. In *Proceedings of the 5th European Congress on Biotechnology*, Vol. 1. (Christiansen, C., Munck, L. and Villadsen, J., eds.), Munksgaard, Copenhagen. pp. 298-301.

Vandamme, P., Pot, B., Gillis, M. De Vos, P., Kersters, K. and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.* **60**:407-438.

Van den Berg, D.J.C., Smits, A., Pot, B., Mels, L., Hoste, B., Dewettinck, D., Vlaes, L., Van Den Borre, C. and Verrips, C.T. 1993. Isolation, screening and identification of lactic acid bacteria from traditional food fermentation processes and culture collections. *Food Biotechnol.* **7**:189-205.

Van Reenen, C.A. and Dicks, L.M.T. 1996. Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*. *Curr. Microbiol.* **32**:183-187.

Villani, F., Moschetti, G., Blaiotta, G. and Coppola, S. 1997. Characterization of strains of

Leuconostoc mesenteroides by analysis of soluble whole-cell protein pattern, DNA fingerprinting and restriction of ribosomal DNA. *J. Appl. Bacteriol.* **82**:578-588.

Vogel, R., Böker, G., Stolz, P., Ehrmann, M., Fanta, D., Ludwig, W., Pot, B., Kersters, K., Schleifer, K.-H. and Hammes, W.P. 1994. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. *Int. J. Syst. Bacteriol.* **44**:223-229.

Wallbanks, S., Martinez-Murcia, A.J., Fryer, J.L., Phillips, B.A. and Collins, M.D. 1990. 16S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmonarium*. *Int. J. Syst. Bacteriol.* **40**:224-230.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nuc. Acids Res.* **18**:7213-7218.

Wiese, B.G., Strohmar, W., Rainey, F.A. and Diekmann, H. 1996. *Lactobacillus panis* sp. nov., from sourdough with long fermentation period. *Int. J. Syst. Bacteriol.* **46**:449-453.

Williams, J.G.K., Kubelic, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nuc. Acids Res.* **18**:6531-6535.

Woese, C.R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221-271.

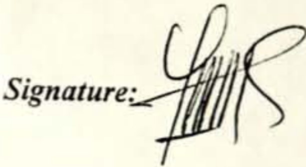
Wood, B.J.B. and Holzapfel, W.H. (eds.). 1995. *The genera of lactic acid bacteria. The lactic acid bacteria* Vol. 2. Blackie Academic and Professional, Glasgow, Scotland. 398 pp.

Zewdie, S., Urga, K. and Nigatu, A. 1995. Microbiology of siljo fermentation. *SINET: Ethiop. J. Sci.* **18**:139-142.

DECLARATION

I, the undersigned, declare that this Ph.D. thesis is my original work, has not been presented for any degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name: Ayele Nigatu

Signature: 

Date: June, 1998