Isolation and Characterization of Lactic Acid Bacteria from “Ting” in the
Northern Province of South Africa

by

Julia Mavhungu

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Department of Microbiology and Plant Pathology
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Faculty of Natural and Agricultural Science
University of Pretoria
Pretoria
South Africa

Promoter: Professor T.E. Cloete

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DECLARATION

I declare that this is my own, unaided work. It is being submitted for the degree of Master of Science in the University of Pretoria. It has not been submitted before for any degree in any other university.
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SUMMARY

Isolation and Characterization of Lactic Acid Bacteria from “Ting”

Author: Julia Mavhungu
Supervisor : Prof T.E Cloete
Department: Microbiology and Plant Pathology
Degree: M.Sc (Microbiology)

Traditional fermented food, especially fermented maize and sorghum represents an important part of the diet of peri-urban and rural communities in South Africa. In this study a survey was conducted to determine the popularity and utilization of “Ting” in the Limpopo Province of South Africa. The following areas were selected for the study: Venda, Giyani, Bolobedu and Polokwane. Ting samples were collected from different areas and from different local families. Gram positive, catalase-negative, oxidase negative, non-motile cells were presumptively identified as lactobacilli. Isolates were assigned to a genus on the basis of key characteristics. Growth at 10, 15 and 45°C in MRS broth was evaluated visually after 72h of incubation. Tests for the catalase reaction, production of gas from glucose and growth at 7 and 10% NaCl concentrations were performed. API 50CHL medium and API 50CH strips were used to identify all the isolates to species level. Microorganisms from “Ting” fermented from both sorghum and maize were bacteria, which belong to the genus *Lactobacillus*, *Leuconostoc* and *Pediococcus*. *Lactobacillus pentosaceus*, and *Lactobacillus plantarum*, *Lactobacillus pentosaceus* were dominant in the fermentation of maize, while *Lactobacillus cellobisus*, *Leuconostoc mesenteroides*, *Lactobacillus collinoides*, *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus curvatus* were identified as bacteria from fermented “Ting” sorghum. The use of polyacrylamide gel electrophoresis (PAGE) of total soluble proteins, together with computer analysis was used to analyse the resultant protein profiles. *L. plantarum*, *L. pentosus* and *P. pediococcus* were the most dominant isolates.
CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Fermented foods

In rural and informal settlements of South Africa, where electricity and sanitary facilities are not available, fermented sorghum and maize are frequently used as weaning foods because they are inexpensive, can be stored for long periods at ambient temperature and do not require reheating before consumption (Wood, 1992).

During fermentation, raw materials are converted to food products (by bacteria, yeasts and molds). In a natural fermentation, the conditions are set such that the desired microorganisms grow preferentially and produce metabolic by-products, which give the unique characteristics of the food. In many cases, fermentation contributes to the digestibility and nutritional value of the final product (Board et al., 1995). When the yield is unstable and where the desired microorganisms might not grow, or where pathogenic microorganisms might also grow, a controlled fermentation is used. In a controlled fermentation, the fermentative microorganisms are isolated, characterized and then maintained for further use and termed a starter culture. Starter cultures are added to the raw materials in large numbers and incubated under optimal conditions. In common controlled-fermented products such as sauerkraut and yoghurt, lactic acid is produced by the starter culture bacteria to prevent the growth of undesirable microorganisms in the non-sterile raw materials and these culture bacteria helps to make the products shelf-stable (Yali, 1996; Zulu et al., 1997).

The consumption of fermented foods has increased greatly since 1970. This includes common foods like yoghurt, buttermilk and fermented sausages as well as ethnic foods such as “Kefir”, “Kumiss”, “Togwa”, “Tofu”, “Ogi”, “Mageu”, “Kenkey” and others (Board et al., 1995). One of the reasons for the increase in the consumption of fermented foods is because consumers consider these foods to be healthy and natural. The consumption of live cells of desirable microorganisms and their metabolic products in fermented foods does not cause panic or distress in the safety-
concerned consumers. These foods have been around for thousands of years and therefore have withstood the test of time (Yali, 1996).

Fermentation is an effective method of food preservation. The process of fermentation by lactic acid bacteria (LAB) is capable of lowering the pH to below 4 in food products. Traditional fermented food, especially maize, sorghum and dairy products represent an important part of the diet of peri-urban and rural communities in South Africa and other developing countries. The use of these products manufactured with non-defined cultures or by back slopping practices is however, steadily declining due to improvement in socio-economic status of people in the society that is taking place (Beukes et al., 2001). These beneficial microorganisms, especially the LAB are selected from the natural biodiversity of microorganisms and represent a unique genetic resource for food technology and biotechnology for future development in the region (Beukes et al., 2001).

According to Steinkrause (1995), the traditional fermentation of foods serves several functions:

1. Enrichment of the diet through development of a diversity of flavors, aromas and textures in food substrates
2. Preservation of substantial amounts of foods through lactic acid, alcoholic, acetic acids, and alkaline fermentations
3. Enrichment of food substrates biologically with protein, essential amino acids, essential fatty acids, and vitamins
4. Detoxification during food fermentation processing
5. A decrease in cooking times and fuel requirements.

LAB have been used traditionally for the fermentation of foods and beverages because of their contribution to flavour and aroma development and to spoilage retardation. The preservative effect of LAB during the manufacture and subsequent storage of fermented foods is mainly due to the acidic conditions that they create, converting carbohydrates to organic acids (lactic and acetic acids) in the food during their development (Hiromi et al., 1997).
Cereals have been known to man from the earliest times. Initially they were eaten raw, later they were crushed between two stone surfaces and cooked to make porridge. Around 400 B.C., the porridge was baked into bread, resulting in enhanced taste and improved digestibility and keeping qualities (Steinkraus, 1996). Fermented dough and porridge is prepared from various types of cereal in South Africa. Maize, millet and sorghum are used to produce numerous staple foods and beverages (Steinkrause, 1995; Calderon, et al., 2002). Similar to wheat and rye sourdough, the natural fermentation of African cereal dough is dominated by heterolactic bacteria (Steinkrause, 1996). Acid porridges prepared from cereals are still eaten in varying amounts in different parts of the world, particularly in the developing countries where they may represent the basic diet. Acid porridges are called by different names in different parts of the world. For example: Ting (South Africa), “Ogi” (Nigeria), “Uji” (Kenya), “Togwa” (Tanzania), “Obushera” (Tanzania and Uganda) (Board et al., 1995; Chinyere et al., 1996; Steinkrause, 1996; Kunene et al., 1999; Mugula et al., 2002). Below are examples of some traditional fermented foods.

**Ogi**

Ogi is a porridge prepared from fermented maize, sorghum or millet in West Africa. It is a staple food of that region, and serves as weaning foods for infants. Odunfa (1985), determined that *L. plantarum* was the predominant organism during fermentation and responsible for lactic acid production (Johansson et al., 1995)

**Kenkey**

Kenkey is fermented maize dough, which is consumed in Ghana. Kenkey varieties vary widely throughout Ghana. In northern Ghana, sorghum is sometimes used instead of maize for the preparation of dough. Halm et al. (1993) concluded that a homogenous group of obligatively heterofermentative lactobacilli related to *L. fermentum* and *L. reuteri* play a dominant role during Kenkey production.

**Togwa**

Togwa is widely produced in Tanzanian homes for use directly as a weaning food or diluted for use as refreshment. The bacteria isolated from togwa were *L. plantarum, L. brevis, L. fermentum,*
L. cellobiosus, and Pediococcus. pentosaceus. All of these bacteria were present from the beginning to the end of the fermentation (Mugula et al., 2003).

Obushera

Bushera is the most common traditional beverage prepared in the Western highlands of Uganda (Kabala and Rukungiri districts). Women from low-income families at village level produce Obushera for human consumption and sale. Both young children (1-day fermented Obushera) and adults consumed the product. The LAB isolated was L. brevis, L. fermentum, L. plantarum and L. paracasei (Muyanja et al., 2002).

Indigenous fermented food represents a unique source for future application in food technology. Little or no scientific information on lactic acid bacteria in certain indigenous, fermented sorghum products in South Africa are available. Ting is a prime example of such a product.

1.2 “Ting” (South Africa)

Ting is a traditional, sour, non-alcoholic maize food product, which is commonly used, in rural and informal settlements of South Africa. Fermented sorghum and maize are frequently used as weaning foods, mainly because they are inexpensive, can be stored for a long period of time at ambient temperatures and do not require re-heating before consumption. Ting is normally consumed as a porridge, which has a smooth texture, with a sour taste reminiscent of yoghurt. The color depends on the type of cereal used, i.e. cream for maize and reddish-brown for sorghum. There are various reasons why foods may be fermented. Perhaps the most important one is to produce a variety in flavors in existing foods. Others are to mask some undesirable flavors in existing foods. Another reason is to make some inedible foods edible, and for preservation. The importance of fermented foods in the nutrition of Africa is now better appreciated, and consequently efforts are now being made to industrialize some of the products.
1.3 LAB IN BIOCONTROL AND FERMENTATION

1.3.1 Biological control of foodborne pathogens

Research has focused on the biological approach to the control and eradication of food-borne pathogens. Commensal bacteria (species that have a positive influence on health and the capacity to exclude pathogenic bacteria) that inhabit the gastrointestinal tract of animals and humans, as well as those involved in food fermentation have been investigated (Grasson, 2002). Scientists developed natural antimicrobial products for the biocontrol of pathogens and have exploited LAB for the competitive exclusion of pathogens and delivery of vaccines and bioactive compounds (Grasson, 2002).

1.3.2 LAB in competitive exclusion

The gastrointestinal tract of humans and animals contain a complex bacterial ecosystem. Commensal strains of LAB have a history of use with the intention of enhancing health in the form of probiotics and controlling human pathogens in farm animals. Research has demonstrated the capacity of *Lactobacillus* species (spp) to control a range of human pathogens including *E. coli*, *Campylobacter jejuni* and *Clostridium perfringens* (Grasson, 2002).

1.3.3 LAB as Probiotics

In discussing the importance of *Lactobacillus sp.* in fermented foods one also needs to consider their importance as probiotics. Probiotics have been described as organisms and substances that contribute to intestinal microbial balance. Probiotics was later defined as a live microbial feed supplement, which is beneficial to the host animal through improving its intestinal microbial balance (Steinkrause, 1995). *Lactobacillus* sp. has been used as probiotic organisms. In this case, *L. acidophilus* have been used because it was thought to be the dominant *Lactobacillus* in the intestine. However, a wide range of *Lactobacilli* has been used in probiotic preparations. These include: *L. delbreuckii subsp. bulgaricus*, *L. casei*, *L. brevis*, *L. cellobiosus*, *L. lactis*, *L. fermentum*, *L. plantarum* and *L. reuteri* (Steinkrause, 1995; Vinderola *et al.*, 2002).
1.3.4 LAB as vaccine delivery vehicles

Commensal LAB can be exploited to deliver vaccines and other biologically active material to the gastrointestinal tract. Their use in vaccine delivery is of special value in stimulating mucosal immunity that is protective at the site of pathogen entry. The advantages of LAB delivery include: ease of administration; survival in stomach acid; inherent safety; particulate nature and size for uptake by cells; economic technology in that the bacteria manufacture the vaccine or therapeutic agent (Grasson, 2002).

1.3.5 LAB as beneficial microorganisms

LAB are important commercially in the processing of meats, alcoholic beverages and vegetables. The products include sausages, cured hams, wines, beer, fortified spirits, pickles and saukerkraut (Collins et al., 1976; Sharpe et al., 1981; Jay, 1986, Kandler & Weiss, 1986; Hastings et al., 1987; Schillinger et al., 1987; Jay, 1992). Although LAB have beneficial effects in the food industry, they can sometimes be a nuisance as contaminants by producing off flavours (Kandler et al., 1983, Aguirre et al., 1993; Cai et al., 1998).

*Lactobacillus and Streptococcus faecum* are beneficial microorganisms, which have been proven to replenish essential microflora and decrease the incidence of gastrointestinal disorders. Beneficial bacteria, especially *Lactobacillus spp*. can produce specific anti-microbial substances, which have been observed to inhibit the growth of some pathogenic microorganisms. The addition of type O Lactic culture may be an additional safeguard to established good manufacturing practices and hazard analysis and critical control point (HACCP) programs in the control of growth of *E. coli* 0157: H7 in minas cheese (Saad et al., 2001; Yost et al., 2002). These beneficial microorganisms are most effective during periods of disease or stress and following antibiotic treatment.
1.3.6 Importance of LAB and their effect on human health

Of interest is the role of LAB in the treatment of people suffering with tumours and immuno compromised subjects. The evidence that LAB can stimulate the immune system is remarkable and fascinating in it, and opens many questions about mechanisms and effective utilization. If this potential is supported in practice, then there are many components to conventional therapies. This may include cost effectivity due to their ease of products derived from LAB seem to have relatively low toxicity compared to other treatments (Wood, 1992).

1.3.7 Lactic acid bacteria and other effects on the immune system.

LAB are present in the intestines of most animals. The beneficial role played by this microorganism in humans and animals, including the effect on the immune system has been extensively reported (Perdigon et al., 2001). LAB are present in many foods and are frequently used as probiotics to improve some biological functions in the host. Through different mechanisms they send signals to activate immune cells. Thus the knowledge of the normal intestinal microflora, the contribution of LAB and their role in the numerous functions in the digestive tract as well as the functioning of the mucosal immune system. In the selection of LAB by their immunostimulatory capacity, it helps to know not only the effect which they have on the mucosal immune system, but the specific use to which these oral vaccine vectors are being put (Perdigon et al., 2001).

1.4 Lactic acid bacteria as a starter culture

Lactic acid bacteria frequently termed “Lactis” are basic starter cultures with widespread use in the dairy industry for cheese making, cultured buttermilk, cottage cheese and cultured sour cream (Jay, 1986). Identifying and providing a practical means of using appropriate starter cultures is advantageous due to the competitive role of microorganisms and their metabolites in preventing growth and metabolism of unwanted microorganisms. A strong starter may reduce fermentation
times, minimize dry matter losses, avoid contamination with pathogenic and toxigenic bacteria and molds and minimize the risk of incidental microflora causing off flavor, etc and technology (Kotze et al., 1992; Holzapfel, 2001; Mullan, 2001). Lactic acid bacteria always include organisms that convert lactose to lactic acid, e.g. *L. lactis*, *L. cremoris*, or *L. diacetilactis*. Where flavor and aroma compounds, such as diacetyl are desired, the lactic starter culture will include a heterolactic fermenter such as *Leuconostoc citrovorum*, *L. diacetilactis*, or *L. dextranicum*. Starter cultures may consist of single or mixed strains. The cultures may be produced and preserved by freezing over liquid nitrogen or by freeze-drying. *Lactococci* generally make up 90% of a mixed dairy starter population, and a good starter culture normally converts most of the lactose to lactic acid (Jay, 1992).

Yoghurt is produced with a yoghurt starter culture, which comprises equal cell numbers of *S. thermophilus* and *L. bulgaricus*. The coccus (*S. thermophilus*) grows faster than the rod (*L. bulgaricus*) and is primarily responsible for acid production, while the rod adds flavor and aroma. The associative growth of the two organisms result in lactic acid production at a rate greater than that produced by either when growing separately and more acetaldehyde (the main volatile flavor component of yoghurt) is produced by *L. bulgaricus* when growing in association with *S. thermophilus* (Jay, 1992; Board et al., 1995).

1.5 Need for Additional research

Some advantages of traditional fermentation are that they are labour-intensive, integrated into village life, familiar, utilize locally produced raw materials, inexpensive, have better potential and the subtle variations resulting, tradition to local consumers. From this perspective, research leading to new fermentation technologies should be sensitive to social and economic needs in developing countries. Rapid displacement of traditional foodstuffs in developing countries with technology development in more affluent countries may result in centralized production, distribution, less local involvement in food processing, less employment in some areas, less nutritionally adequate substitutions in raw materials, displacement of traditional arts, loss of unique local know-how, dependence on importation of equipment and materials, initially require the use of outside consultants, and may otherwise not meet local needs as fully as traditional
fermented products. On the other hand, indigenous fermentation may have a number of problems, i.e., they are uncontrolled and often unhygienic, labor intensive, seen as primitive by some people, are normally not integrated into the economic mainstream, difficult to tax, have limited export potential (Wood, 1994) and in some cases, the impact on nutritive value and food safety is not known (Fao, 1999).

Specific microflora involved with indigenous fermentations is, in many cases, not known at this time. Specific information on microflora appears to be lacking for several indigenous fermented cereal products. The microbiology of many of these fermentations is undoubtedly quite complex. Many indigenous cereal fermentations involve the combined action of bacteria, yeast and fungi. Some microflora may co-exist while others may participate in a sequential manner with a changing dominant flora during the course of the fermentation. The specific microflora involved may vary somewhat from village to village and from family to family within the same village. The identification of specific microflora involved is needed to control positive factors such as the excretion of lysine by strains of *Lactobacillus plantarum* (Newman and Sands, 1984) and the metabolic detoxification of mycotoxins by *Rhizopus oryzae* (Nout, 1994), as well as to minimize or prevent negative factors such as growth and metabolism of pathogenic and toxinogenic bacteria, e.g., bongrek acid and toxiflavin formation by *Pseudomonas cocovenenans* (Ko, 1985).

Identifying and providing a means of using appropriate starter culture is advantageous due to the competitive role of microorganisms and their metabolites in preventing growth and metabolism of unwanted microorganisms. A strong starter culture may reduce fermentation times, minimize dry matter losses, avoid contamination with pathogenic and toxigenic bacteria and molds, and minimize the risk of incidental microflora causing off-flavor, etc (Fao, 1999).

With this information in hand, consideration can then be given to use of enzyme supplements and other additives to improve the rate and quality of fermentations.

Traditional fermentation is likely to remain an important part of global food supply; many may evolve into fermentations involving the use of starter cultures, enzyme additives and controlled environmental conditions, and others may benefit from genetic modification of the cereal or starter bacteria.
Traditionally, spontaneous occurring microorganisms perform the fermentation with lactobacilli as the key actor. However, the product quality can differ widely between different batches from different households depending on the rate of lactic acid production and on the composition of the active microflora. Thus, it would be beneficial for the production control to use a starter culture. However, the question is, what type of lactobacilli that is most suited for the task? In order to provide a base for selection of strains for further testing, it would be of interest to know what types of lactobacilli usually dominate in Ting of good quality.

1.6 TAXONOMY OF THE LACTIC ACID BACTERIA

1.6.1 Properties of LAB

Orla – Jensen (1919) classified lactic acid bacteria (LAB) into six genera based on sugar fermentation and growth at specific temperatures. This group is composed of at least eight genera (Sneath et al., 1986). The traditional genera of Lactobacillus, Leuconostoc, Pediococcus, and Streptococcus have been expanded to include Carnobacterium, Enterococcus, Lactococcus and Vagococcus (Jay, 1992). The Carnobacteria were once classified as Lactobacilli, and other three genera (Enterococcus, Lactococcus, Vagococcus) comprised a strain formerly classified as Streptococci. The species once classified as L. hordine and L. xylosus have been transferred to the genus Lactococcus. S. thermophilus, which is important in food was imported in the latter group. S. diacetilactis is classified as a citrate utilizing strain of L. lactis (Jay, 1992). The pioneer work of Orla-Jensen (1919) resulted in the division of four genera namely Lactobacillus (rod-shaped), Streptococcus (homofermentative), facultatively anaerobic cocci, ‘betacoccus’ and ‘tetracoccus,’ remains influential (Campbell et al., 1996). The importance of the LAB rests in their ability to form lactic and other acids from carbohydrates. This lactic fermentation is, by definition more or less, characteristic of the LAB of which the most common and important genera are the Streptococcus, Pediococcus, Leuconostoc and Lactobacillus (Carr et al., 1975; Frank et al., 2002).
LAB are gram positive, typically non-sporulating rod or coccus shaped. They lack catalase and are strictly fermentative, producing either a mixture of lactic acid, carbon dioxide, acetic acid and/or ethanol (heterofermentation) or almost entirely lactic acid ( homofermentation) as the major metabolic end-product (Rogosa et al., 1974; Collins et al., 1984; Jay, 1986; Kandler et al., 1986; Schillinger et al., 1987; Campbell et al., 1996). Kluyver divided LAB into two groups based on the end product of glucose metabolism; those that produce lactic acid as the only product of glucose fermentation are designated homofermentative. The homofermentative pattern is observed when glucose is metabolized but not necessarily when pentose sugars are metabolized, for some, homolactics produce acetic and lactic acids when utilizing pentose. Also the homofermentative character of homolactics may be shifted for some strains by altering cultural conditions such as glucose concentration, pH and nutrient limitation. The homolactics are able to extract twice as much energy from a given quantity of glucose as are the heterolactics. Those lactics that produce equal molar amounts of lactate, carbon dioxide and ethanol from hexoses are designated heterofermentative. All members of the genera Pediococcus, Streptococcus, Lactococcus and Vagococcus are homofermenters, along with some of the Lactobacilli, while all Leuconostoc spp, as well as some Lactobacilli are, heterofermenters. The heterolactics are more important than the homolactics in producing flavour and aroma components such as acetylaldehyde and diacetyl (Sharpe et al., 1979; Jay, 1986; Schillinger et al., 1987).

The end products of homo and heterofermenters may differ when glucose is converted and these form basic genetic and physiological differences. The homolactis posses the enzyme aldolase and hexose isomerase’s but lack a phosphoketolase. They use the Embden-Meyerhof-Parnas (EMP) pathway for the production of 2 lactates/glucose molecules. The heterolactics, on the other hand, have a phosphoketolase but do lack an aldolase and hexose isomerases, and instead of the EMP pathway for glucose degradation, these organisms use the hexose monophosphate or pentose pathway (Jay, 1992).

LAB are typically fastidious and require a variety of amino acids, B vitamins, purine and pyrimidine bases for growth. In many environments LAB obtain these amino acids through proteolytic activity, although compared with bacilli or pseudomonades, LAB are weakly
proteolytic. Proteolysis has been particularly well documented in relation to the growth of *Lactococci* in milk, where they are largely responsible for flavor development during cheese production (Dykes, 1991; Jay, 2000). Although they are mesophilic, some can grow below 5°C and others at temperatures as high as 45°C. Some LAB can grow in acidic pH (3.2) and others in alkaline pH (9.6), with most growing in the pH range 4.0 - 4.5 (Jay, 2000).

Orla-Jensen (1919) recognized that LAB occurs in a variety of habitats, and in his investigation included a selection from sources other than dairy products. When considering plant products, heterofermentative beta-bacteria and *Leuconostoc* appear more common, while *Streptococci* are rare, especially those of group N. In meat products, which are generally refrigerated, atypical streptobacteria predominate with *Streptococci* of group D being the most common whereas thermobacteria are seldom encountered (Carr *et al*., 1975). LAB can be grown and enumerated on agar plates, as long as the agar plates are incubated in an oxygen poor environment. Most LAB grow on MRS or Rogosa agar and form snow white colonies on these media (Theron, 1999). The industrial importance of LAB is related to the production of lactic acid, flavor compounds or bacteriocins (Hiromi *et al*., 1997) and has stimulated the interest of many scientists during the last decades (Cocaing-Bousquet *et al*., 1995).

### 1.6.2 The genus *Lactobacillus*

Following the initial work of Orla-Jensen (1919) the taxonomy of the Lactobacilli progressed slowly despite the economic and environmental importance of this group (Campbell *et al*., 1996). In his studies on the temperature range for growth and mode of fermentation, Orla-Jensen divided Lactobacilli into three subgenera namely betabacterium, streptobacterium and thermobacterium. The streptobacteria (for example, *L. casei* and *L. plantarum*) produce up to 1.5% lactic acid at an optimal growth temperature of 30°C while the thermobacteria (e.g. *L. acidophilus*, *L. bulgaricus*) can produce up to 30% of lactic acid and have an optimal temperature of 40°C. All the heterolactic lactobacilli are betabacterium (Jay, 1992). Recent terminology referring more directly to the end product of metabolism has been suggested, thus the streptobacteria may also be referred to as facultative heterofermentative *Lactobacilli* (Sharpe, 1981; Kandler *et al*., 1986; Campbell *et al*., 1996).
The subgeneric names were no longer used (Weiss and Holzapfel, 1993) and the thermobacteria were now referred to as group 1. This group (1) comprises obligate homofermentative species (*L. acidophilus*, *L. bulgaricus*, *L. delbrueckii*, etc), which do not ferment pentose. The ‘streptobacteria’ which are facultative heterofermentative species (*L. casei*, *L. plantarum*, *L. sake* etc) formed group 2 and they ferment pentoses whereas the ‘betabacteria’ (*L. fermentum*, *L. brevis*, *L. reuteri*, *L. sanfrancisco*, and others) ferment glucose and other hexoses. Group 1 and 2 are both ethanol tolerant and constituted the obligately heterofermentative group 3. Weiss and Holzapfel (1993) similarly considered these three groups for identification purposes in their comprehensive review of these bacteria (Campbell *et al.*, 1996; Jay, 2000). The *Lactobacilli* can produce a pH of 4.0 in foods that contain fermentable carbohydrates, and they can grow at a neutral pH (Jay, 2000).

*Lactobacilli* are important in foods because:

i. they are able to ferment sugars with the concomitant production of considerable amounts of lactic acid. This makes it possible to use *Lactobacilli* in the production of fermented plant and dairy products or to manufacture industrial lactic acid, although this may result in the deterioration of some products such as wine or beer.

ii. they produce gas and other volatile products by heterofermentative species, which may reduce the quality of food as exemplified by *L. fermentum* growing on swiss cheese or *L. hilgardii* / *L. trichodes* in wines.

iii. they are unable to synthesize most of the vitamins they require, a characteristics which makes them unable to grow well in foods that are deficient of the vitamins they require, making them unable to grow well in foods poor in vitamins but useful in assay for the vitamin content of food.

Because of their heat resistance or thermoduric properties, *Lactobacilli*, survive pasteurization or other heating processes, such as in the manufacture of swiss and similar cheese. These *Lactobacilli* are exceptional because of their ability to grow at low temperatures, examples include *L. viridescens* and *L. salinmandus* that both grow in sausages (Frazier *et al.*, 1988).
Lactobacillus has complex nutritional requirements. They require carbohydrates for energy and carbon and in addition a variety of nucleotides, amino acids and vitamins for growth (Kandler & Weiss 1986; Wood, 1992). Based on the observation that mutant strains of Lactobacilli can be obtained and that they have lost their requirements for an exogenous source of amino acids, it is probable that the multiple nutritional requirements of the Lactobacilli existing in nature today reflect a stepwise natural selection of deficient strains of a population with a full component of biosynthetic pathways (Wood, 1992).

1.6.3 The genus Leuconostoc

Leuconostoc was first described by Van Tieghein in 1878. They are heterofermentative cocci that are sometimes oval or even short rods and occur in pairs or short chains. (Campbell et al., 1996). These organisms are gram positive, non-motile, non-spore forming, facultative anaerobes. These microorganisms form small, grey, flat colonies on agar media (Hucker et al., 1957; Collins et al., 1976). The genus is commonly found on the surface of and inside of fruits and vegetables, in dairy products and has been implicated as a cause of diseases in humans (Garvie, 1984; Barreau et al., 1990). The genus plays a prominent role in the fermentation of numerous dairy products, the production of sauerkraut, pickles, and various meats (Kitchell et al., 1975; Whiting, 1975; Garvie, 1984; Schillinger et al., 1987; Singleton et al., 1987; Jay, 1992). They are catalyse-negative, lack cytochrome, does not hydrolyse arginine and milk is usually not acidified and curdled. They are also non proteolytic, indole is not formed and nitrates are not reduced. Growth is dependent on the presence of fermentable carbohydrates and glucose is fermented by a combination of the hexoses-monophosphate and phosphoketolase-pathway (Sneath et al., 1986).

Leuconostoc can be distinguished from most lactobacilli by their inability to produce ammonia from arginine and by forming D (-) rather than DL-lactate from glucose (Sharpe, 1979). Leuconostoc sensustricto, comprises Leuc. mesenteroides and five other Leuconostoc species. The Leuconostoc paramesenteroides group has been reclassified as Weissella and includes a single Leuconostoc species (W. paramensneteroides) and W. confusa (previously Lactobacillus) and some other heterofermentative lactobacilli. Oenococcus oeni, form a single membered taxon. Many of the early workers were more interested in the determinants of flavour formation than in
bacterial taxonomy, and Orla- Jensen and Hammer did not separate *Pediococcus* from *Leuconostoc* (Garvie, 1960). The most definitive study is that of Garvie (1960) who divided the genus into four species; *Leuc. cremoris* (citrovorum), *Leuc. lactis*, *Leuc. dextranicum*, and *Leuc. mesenteroides*, with two further species, *Leuc. paramesenteroides* and *Leuc. oenos*, been added. *Leuc. oenos* is completely different from the other species in that it is acidogenic (grows at a pH of 4.8), is found only in wines and many strains require a growth factor present in tomato juice. The five-nonacidogenic species (*Leuc. cremoris*, *Leuc. lactis*, *Leuc. dextranicum*, *Leuc. mesenteroides* and *Leuc. paramesenteroides*) are phenotypically homogeneous. The *Leuconostoc* were initially reported to be largely of plant and dairy origin, but have subsequently been isolated from a number of other environments including sugar-refining plants. *Leuc. mesenteroides*, *Leuc. paramesenteroides*, *Leuc. lactis* and *Leuc. dextranicum* are found in silage whereas *Leuc. mesenteroides* is a common soil inhabitant and its association with the sugar-cane plant normally has been observed during harvesting (Wood, 1992; Campbell *et al.*, 1996).

*Leuconostoc mesenteroides* has been isolated from fruit mashes and they are also associated with traditional African beverages, such as the fermentation of sauerkraut. Since, the 1930s, *Leuc. mesenteroides* has been important in initiating the fermentation of many vegetables, i.e., turnips, chard and caulifower (Wood, 1992) and is a common fermenting organism in rice, bean flour and wheat flour for the production of “ldli” and sour pumpernickel (Jay, 1992). Other species of the genus *Leuconostoc*, which are important, are *Leuconostoc lactis*, which ferment lactose. The species may not be as widely distributed as documented. The little isolation has mostly been from dairy sources (Sneath *et al.*, 1986). *Leuconostoc oenos* is mainly associated with wine (Campbell *et al.*, 1996; Sneath *et al.*, 1986). *Leuconostoc paramesenteroides*, is salt tolerant compared to other species, particularly those strains isolated from foods containing high levels of salt. Strains of *Leuc. paramesenteroides* were considered to be nondextran-forming variants of *Leuc. mesenteroides*. However, it is difficult to distinguish *Leuc. paramesenteroides* from nondextran forming strains of *Leuc. mesenteroides* by phenotypic tests (Sneath *et al.*, 1986).

There are several characteristics that make *Leuconostoc* species important in foods:

1. Ability to produce diacetyl and other flavouring products,
ii. tolerance of salt concentration for example, in sauerkraut and dill-pickle fermentation, permitting *Leuc. mesenteroides* to carry on the first part of the lactic fermentation,

iii. ability to initiate fermentation in vegetable products more rapidly than other lactis or other competing bacteria producing enough acid to inhibit nonlactics,

iv. tolerance of high sugar concentration (up to 55 to 60 percent for *Leuc. mesenteroides*), permitting the organisms to grow in syrup, liquid cake and ice-cream mixes etc,

v. production of considerable amounts of carbon dioxide from sugar leading to undesirable “opennes” in some cheese, spoilage of foods high in sugar (syrup, mixes, etc) and leavening in home breads,

vi. and heavy slime production in media containing sucrose. This is a desirable characteristic for the production of dextran but a hazard in material high in sucrose, as in the production of sugar-cane or beets. The habitat of this genus is the surface of plants (Fraizer *et al*., 1988).

1.6.4 Genus *Pediococcus*

The genus was first described by Wochnschre F.Balcke in 1884. The *Pediococci* are Gram-positive, nonmotile cocci that divide at right angles in two planes, resulting in tetrad morphology especially when isolates are cultured in a broth medium. These microorganisms lack cytochromes and are catalase negative. The *Pediococcus* require complex nitrogen compounds and therefore, unlike *Bacillus* spp., are unable to use ammonia salts as a sole nitrogen sources or to reduce nitrates to nitrites. They are aerobic to microaerophilic, ferment glucose by the Embden Meyerhof Pathway, and form either DL- or L-lactate without the production of carbon dioxide from glucose. Both *P. pentosaceus* and *P. acidilactici* are aerobic; *P. dextrinicus* is a facultative anaerobe, while others are microaerophilic (Hucker *et al*., 1957; Sneath *et al*., 1986; Raccach, 1987; Schillinger *et al*., 1987; Singleton *et al*., 1987). The *Pediococcus* are relatively easy to differentiate from other closely related genera, such as the *Leuconostoc*, on the basis of morphology, since this genus is the only lactic acid bacteria that divide into two planes. Short chains may be seen but these are formed by pairs of cells in a single plane and not by division (Sneath *et al*., 1986). The close relationship of the *Pediococcus* with *Lactobacilli* has been emphasized by the rRNA studies in which most *Pediococci* form a small cluster with the *L. casei*
group of obligate homofermentative and heterofermentative bacilli. The closest phylogenetic relatives include *L. kefir* and *L. bushneri*. RNA studies have also clarified the boundaries of the genus *Pediococcus* and reveal it as a homogenous taxon, with *P. dextrinicus* as the most distantly related true *Pediococcus* species separated mainly on the basis of tolerance to temperature, pH and sodium chloride (Sneath *et al*., 1986; Dykes, 1991).

All strains are apathogenic to plants and animals. Species of the genus *Pediococcus* were originally thought to be of plant or dairy origin. However, *P. damnosus* is most common in beer and brewing habitats with the exclusion of all other species. Growth in beer results in cloudiness and the undesirable production of diacetyl (Wood, 1992). *Pediococcus damnosus* is undoubtedly the most common and feared *Pediococcus* found in beer. It is particularly interesting that this organism is apparently only found in beer, brewing yeast and wine but not in raw materials or plant materials for brewing (Campbell *et al*., 1996). *P. dextrinicus* has been found in beer and silage but more often occurs in silage. *P. parvulus* and *P. inopinatus* occur in sauerkraut, with the former in silage and the latter in beer. *P. pentosaceus* is widespread on vegetables materials and often occurs together with *P. acidilactici*. These two species may occur in milk and dairy products but are of less importance than other lactic acid bacteria. *P. pentosaceus* is used as a starter culture in fermented sausages (Dykes, 1991). The salt tolerant *P. halophilus* occurs in soy sauce and picking brines. *P. urinaeaequi* has been seldom isolated. The original strains came from horse’s urine but the general distribution is unknown. *P. damnosus* and *P. parvulus pediococcus* are responsible for the souring of uncooked maize grits including *L. krusei* and *S. cerevisiae* yeasts, *Lactobacillus helveticus, L. salvarius, L. brevis, L. viridescens* and *L. plantarum* (Nout, 1991).

### 1.6.5 Genus *Streptococcus, Enterococcus, Lactococcus and Vagococcus*.

The genus *Streptococcus* (*S*) was first described by Rosen Bach in (1884) (Dykes, 1991). Members of the genus *Streptococcus, Enterococcus, Lactococcus and Vagococcus* are all homofermentative cocci, gram-positive cocci and also occur as chains (coccobacilli). These bacteria are widely distributed in raw milk and dairy products, on plant material and in the mouths and intestines of humans and animals (Campbell *et al*., 1996).
Members of the genus *streptococcus* are gram-positive cocci, catalase negative, that form pairs and chains of cells when cultured in liquid media, but form only the L (+) isomers of lactic acid. The *streptococci* are a large group consisting of thirty-seven species (Dykes, 1991; Wood, 1992). This group may be classified serologically by a precipitation reaction into lancefield groups with capital letter designations (A, B, C, D etc.) but ordinarily the *Streptococci* that are important in foods are divided into four groups, the *Pyogenic, Viridans, Lactics* and *Enterococcus* group (Frazier et al., 1988). These namely group ferment glucose primarily via the Embden-Meyerhof pathway (Carr et al., 1975).

The Pyogenic (pus-producing) groups have been found in raw milk and include species of pathogenic *Streptococci*, of which *S. agalactae*, a cause of mastitis in cows and *S. pyogens*, a cause of human septic sore throat, scarlet fever and other diseases, are representatives. The *Pyogenic streptococcus* cannot grow at 10 or 45°C. The *viridans* groups include *S. thermophilus*, a coccus important in cheese made by cooking the curd at high temperatures and in certain fermented milks such as yoghurt, and *S. bovis*, which comes from cow manure and saliva. *S. thermophilus*, is thermoduric and therefore encountered in the plating of pasteurized milk. These species can grow at 45°C but not at 10°C. The lactic acid group contains the important dairy bacteria, *S. lactis* and *S. cremoris*, which can grow at 10 but not at 45°C. These bacteria are used as starter culture for cheese, cultured buttermilk, and some type of butter along with *Leuconostoc* spp. and *S. lactis* is often concerned in the souring of rawmilk. These lactic acid bacteria tolerate not more than 2-4% NaCl and therefore are not involved in the lactic fermentation of brined vegetables. Some sources of these lactis are green plants, feeds, silage and utensils (Frazier et al., 1988).

The members of the genus *Enterococcus* were, until recently, included amongst the *Streptococci* (Schlefer, 1987). They have the same general morphology and growth characteristics as *streptococcus* but differ in their tolerance for sodium chloride (6.5%) and alkaline pH (9.6) when cultured in the laboratory (Wood, 1992). The *Enterococcus* group consists of *S. faecalis* and *S. faecium* and some related subspecies. *S. faecalis* and *S. faecium* resemble each other but can be distinguish by physiological tests. *S. faecalis* is usually the more heat resistant and comes more
from plant sources. *S. faecalis subsp. liguefaciens* is an acid proteolytic variety of *S. faecium*, and *S. faecalis subsp. zymogenes* is a beta hemolytic variety of *S. faecium*. *S. faecium* are commonly found in raw foods. Bacteria of this group can grow at both 10 and 45°C. The *Enterococci* originate in the intestinal tracts of humans and animals and are sometimes used as indicator organisms of faecal contamination of foods and as a measure of plant sanitation. They also survive in dairy products and may contaminate utensils and equipment (Frazier *et al*., 1988).

The *Enterococci* most frequently isolated from foods are *S. faecalis* and *S. faecium*, all of the lancefield group D. *Streptococci* can be considered as *Enterococci*. The group D *Streptococci* includes *S. faecium*, *S. faecium subsp. durans*, *S. bovis*, *S. equinus*, and *S. avium*. The term faecal *Streptococci* is often used in the industry to describe those *Enterococci*, which are used as indicator organisms (Frazier *et al*., 1988).

1.6.6 The genus *Aerococcus*

The genus *Aerococcus* (A) was first described by Williams *et al*., (1953); and Sneath *et al*. (1986). It is a monospecific genus which is related to *Pediococcus* and *Tetragenococcus* and yet another genus, *Globicatela*, has been introduced as cocci from human clinical sources which were related to, but pylogenetically distinct from the *Streptococci* (Campbell *et al*., 1996). The genus consists of gram positive, catalase activity is absent or weak and when present is a non-heme pseudocatalase. This genus is different from other genera of gram-positive cocci primarily by its wide range of negative carbohydrates characteristics that are positive for other genera. These includes their sparse growth on an agar media, particularly anaerobically, their inability to produce acetyl methyl carbinol from glucose or ammonia from arginine and their failure to reduce nitrate. Among their positive features is the greening of blood agar, tetrath formation, and tolerance to several commonly used inhibitory growth conditions (Campbell *et al*., 1996; Sneath *et al*., 1986).
1.6.7 The genus *Bifidobacteria*

Members of the genus *Bifidobacterium* are gram-positive, non-sporing bacilli of varying morphology. Cells can be short and thin, with pointed ends, coccobacillary, rods with bends and protuberances, branching rods, bifurcated rods, club-shaped or spatulate. Description of cell morphology is helpful in identifying some species of *bifidobacteria*. The nutritional requirements of the *bifidobacteria* are complex and growth factors from exogenous sources are required. Most members of the genus can utilise ammonium salts as a sole source of nitrogen. Human or other animal milk stimulates the growth of *Bifidobacterium bifidum* and much efforts has been directed towards elucidating the nature of “*bifidus*” factors in the milk. Several milk components that stimulate the growth of *bifidobacteria* have been detected, ranging from “*bifidus*” factor I (an N-acetylglucosaamine- containing polyoside) and oligopolysaccharides to a hydrolytic product of bovine casein. Glucose is catabolised exclusively by the fructose- 6-phosphate shunt in which fructose -6- phosphoketolase cleaves fructose –6-phosphate into acetylphosphate and erythrose -4-phosphate (Wood, 1992).

End products of fermentation are produced by the sequential action of transaldolase, transketolase, xylulose-5-phosphate phosphoketolase and Embden-Meyerhof pathway enzymes acting on glyceraldehyde -3-phosphate. Acetic and lactic acids are produced in a ratio of 3:2, in addition to minor amounts of formic and succinic acids and ethanol. Fructose-6- phosphoketolase is the key enzyme of “shunt” metabolism exhibited by *Bifidobacteria*. The detection of this enzyme in bacterial extracts is therefore the most direct and reliable way of identifying members of the genus *Bifidobacteria* (Wood, 1992).

1.6.8 The genus *Vagococcus*

There are currently four recognized species in the genus *Vagococcus*. *Vagococcus fluvialis* was the first described species when it was shown that a group of motile lactococcus like bacteria formed a distinct line of descent by 16 rRNA sequencing (Collins *et al.*, 1989). The previous studies have shown that the Vagococcus are phylogenetically and phenotypically similar to the enterococci and are often difficult to differentiate based on conventional physiological testing.
The phenotypic identification scheme used initially subdivided the enterococci and vagococci species into groups based on the combination of results obtained for the three key tests: acid production from mannitol and sorbose, and hygrolysis of arginine. *V. salmoninarum, V. fessus* and *V. carniphilus* were placed in the same group, since they are negative for all three key tests. (Facklam and Jahm, 1995; Facklam et al., 2002).

1.6.9 The genus *Weissella*

The phylogeny of the lactic acid bacteria currently classified and clarified the genus *Weissella* (1990) using both 16S and 23S rRNA sequence data. Maritnex-Murcia et al., (1990) showed that *Leuconostoc paramesenteroides* is phylogenetically distinct from *Leuconostoc mesenteroides* and it groups together with five heterofermentative lactobacilli, *L. confuses, L. halotolerans, L. kandleri, L. minor* and *L. viridescence*. In a study of Leuconostoc-like organisms originating from fermented sausage (Collin et al., 1993) the taxonomy of these species was further assessed. This resulted in the description of the genus *Weissella* comprising the former *Leuconostoc paramesenteroides*. Recently, a novel species, *Weissella thailandensis*, has been described and suggested to belong to this genus (Tanasupawat et al., 2000). Thus there are currently eight species in the genus *Weissella*, namely: *Weissella confusa, W. halotolerans, W. hellenica, W. kandleri, W. minor, W. paramesenteroides, W. thailandensis* and *W. viridescence*. Weissella strains have been isolated from a variety of sources. *W. paramesenteroides* is one of the predominant species in fresh vegetables and it also play an important role in the first phase of silage fermentation (Bjorkroth et al., 2002). *W. hellenica* and *W. videscens* have been commonly associated with meat or meat products (Bjorkroth et al., 2002). The *W. kandleri* type strain originated from a desert spring and desert plants and it was suggested to be the main habitat of this species (Holzapfel et al., 1982).
1.7 Methods used for the identification of LAB

1.7.1 Conventional taxonomy

Traditionally, LAB have been identified and grouped by means of key biochemical, physiological and morphological tests (Sharpe, 1979). Briggs (1953) based an early classification system for LAB on only six physiological tests which included the production of gas from glucose, hydrolysis of arginine, growth and survival at 15, 45, 48, 60 and 65°C and the tolerance of 4, 6 and 8 per cent NaCl. The use of features such as the isomer of lactic acid produced, the comparative DNA base composition and cell wall composition have been included in LAB identification schemes (Sharpe, 1979; Kandler and Weiss, 1986). All the above features have been combined in a number of studies for the identification LAB, which proved useful in identifying LAB isolated from the particular environment under study (Garvie, 1984; Hastings and Holzapfel, 1987; Schillinger and Lucke, 1987). The use of rapid identification systems, such as the API (API systems S.A., La Balme Les Grottes, Motalieu, France), can examine isolates to a wider range of carbohydrates fermentation characteristics, but because of the broad application of these system for examining many different genera many tests may be redundant. A complete phenotypic taxonomic study should include as many characterization tests as is practically possible, together with an objective method to analyse results, such as numerical taxonomy.

1.7.2 Numerical taxonomy

The application of numerical taxonomy as a technique to characterize bacteria is well established and allows the simultaneous examination of large numbers of phenotypic characterization tests in an objective manner (Dykes, 1991). This is achieved by coding the results of individual characteristics tests as either binary or numerical values, the combination of which represents specific profiles characteristic of a particular strain. The principles of numerical taxonomy have been applied to lactic acid bacteria from a number of sources including processed meats (Hastings and Holzapfel, 1987); Wine (Dicks and Van Vuuren, 1988); Scotch whisky distilleries (Priest and Pleasant, 1988) and human sources (Barreau and Wagener, 1990). These methods have proved to be useful in determining the taxonomic status of isolates.
1.7.3 Electrophoresis of total soluble proteins

Many techniques have been developed in an attempt to clarify the taxonomic status of lactic acid bacteria, for example, serology (Sharpe, 1955) DNA-DNA hybridization (Champonnier et al., 1989; Collins et al., 1989), gas chromatography of cellular fatty acids and neutral monosaccharides (Rizo et al., 1987), and plasmid profiling (Tannock et al., 1990) and restriction endonuclease patterns (Stahl et al., 1990) have all been applied to LAB taxonomy with some degree of success (Dykes, 1991).

Since proteins are the direct translation product of DNA, the examination of the total soluble protein profile of bacteria represents a method for the rapid determination of natural molecular relationships (Jackman, 1987). However, these methods are often both labor intensive and costly, requiring complex extraction and labeling procedures, particularly if large numbers of isolates are to be examined (Jackman, 1987).

Analysis of total soluble proteins by polyacrylamide gel electrophoresis PAGE has been successfully applied to taxonomic studies of lactic acid bacteria such as *Lactobacillus* (Dicks and Van Vuuren, 1987), *Carnobacterium* (Venter, 1988) and *Leuconostoc* (Dicks et al., 1990; Barreau and Wagener, 1990; Dykes, 1991). According to Kersters and De Ley (1975) protein electrophoresis can be considered as a genomic “fingerprint” which allows accurate clustering and identification of strains within a species that corresponds to results obtained by DNA-DNA hybridization and even % G+C value (Kersters and De Ley 1975).

1.7.4 DNA based techniques

The analysis of the diversity of natural microbial populations relied on direct extraction, purification and sequencing of 5S rRNA molecules from environmental samples (Theron *et al*., 2000). Determination of the DNA base composition of LAB by reassociation or electrophoresis is a well-known technique (Garvie, 1984). This technique has limited use in bacterial taxonomy since the base composition of two biological unrelated organisms as determined by these methods may be the same. However, examination of bacterial at the molecular level by DNA homology technique is regarded as one of the most reliable indicators of natural relationships
between difference strains (Dykes, 1991; Gerhardt et al., 1994). This technique has widely been used for LAB taxonomy. It has been used in the creation of new genera such as Lactococcus and Carnobacteria (Collins et al., 1987) and the more accurate delineation of relationships within genera such as leuconostoc (Dicks et al., 1990).

1.7.5 DGGE

DGGE is an electrophoretic separation method based on differences in melting behavior of double stranded DNA fragments (Fisher and Lermann, 2003). DGGE exploits the fact that identical DNA molecules, which differ by only one nucleotide within a low melting domain, will have different melting temperatures. When separated by electrophoresis through a gradient of increasing chemical denaturant (usually form amide and urea), the mobility of the molecule is retarded at the concentration at which the DNA strands of low melt domain dissociate (Webster et al., 2002). The branched structure of the single stranded moiety of the molecule becomes entangled in the gel matrix and no further movement occurs. Complete strand separation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This is accomplished during PCR amplification using a PCR primer with a 5' tail consisting of a sequence of 40 GC.

1.7.6 Other techniques

A number of other techniques have been used in the taxonomy the LAB with varying degrees. They are PCR, Nucleic Acid Hybridization, genomic DNA Hybridization and quantitative DNA Hybridization and Polymorphism-Based Procedures.
2. REFERENCES


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CHAPTER TWO

Survey of the popularity and utilization of “Ting” in the Northern Province of South Africa.

2.1. Introduction

Most traditional food processes have stemmed from the need to preserve food for the off seasons or to make the foods safer for consumption. The technologies involved include drying, fermentation and pickling and these have been known to mankind for centuries and passed from one generation to the next. Some practices have been modified and the technologies have evolved to use more sophisticated equipment, while others have been lost along the way (Azam-Ali et al., 2001).

Fermentation is the oldest form of biotechnology; records of barley conversion to beer date back more than 5000 years (Borgstorm, 1986). According to Steinkrause (1995), the traditional fermentation of foods serves several functions:

- “enrichment of the diet through development of a diversity of flavours, aromas, and textures in food substrates,
- preservation of substantial amounts of food through lactic acid, alcoholic, acetic acid, and alkaline fermentations,
- enrichment of food substrates biologically with protein, essential amino acids, essential fatty acids, and vitamins and
- a decrease in cooking times and fuel requirements” (Campbell, 1994).

Within each country, traditional food products are produced from indigenous crops and raw materials and are therefore unique to a certain region or area. Consequently, there are many similarities, especially in areas with similar climate and terrain, but numerous regional specialties are also evident.
Aside from alcoholic fermentations and the production of yoghurt and leavened bread, food fermentation continues to be important primarily in developing countries where lack of resources is a limitation for use of techniques such as vitamin enrichment of foods, and the use of energy and capital-intensive processes for food preservation. The technology of producing many indigenous fermented foods from cereals remains a household art in these countries (Chaven and Kadam, 1989). Prospects for applying advanced technologies to indigenous fermented foods (Wood, 1998) and for the production of value-added additive products, such as colors, flavours, enzymes, antimicrobials and health products during fermentations have been reviewed (Cook, 1994).

Special mention should be made of the microbiological risk factors associated with fermented foods. Major risk factors include the use of contaminated raw materials, lack of pasteurisation, and use of poorly controlled fermentation conditions. On the other hand, non-toxigenic microorganisms can serve to antagonize pathogenic microorganisms and even degrade toxic substances such as mycotoxins in fermented foods (Nakazato et al., 1990; Nout, 1994).

Traditional food products are important because they:

(i) have the potential to reduce poverty, through employment opportunities and income generation in both rural and urban environments;

(ii) can contribute to sustainable livelihoods through household food security and improved home processing and storage and

(iii) add nutrients and variety to the diet. In addition, to being an ideal convenient food for low-income urban people, they have a strong cultural identity therefore associated with traditional customs and beliefs.
2.1.1 Nutritional value of traditional food products

Sorghum beer, a popular traditional drink in South Africa, makes an important nutritional contribution to the diet. The fermentation process greatly enhances riboflavin and nicotinic acid levels of these important B group vitamins. The vitamins are essential in the diet, but even more so in those who consume a high maize diet. Pellagra, a vitamin deficiency disease associated with the consumption of a high maize diet, is relatively uncommon in communities that drink sorghum beer. Even the children benefit from drinking the dregs, which contain very little alcohol but are high in vitamins (Nevman et al., 1984; Chinyere et al., 1996).

2.1.2 Culture well-being

In many communities, celebrations and festivals are closely linked with specific food products. The birth of a child, a marriage, a death, the end of harvest and numerous religious and non-religious festivals are all occasions that call for the preparation of traditional food products.

2.1.3 Traditional foods under threat in South Africa

The trend today, particularly for many developing countries such as South Africa, is towards declining consumption of traditional staples and increasing consumption of exotic foods. The change in diet are largely linked to change in income and wealth, the wider availability of a range of alternative foods, changing perceptions and values of foods, changing demographics and globalisation of trade (The Vha-Venda, Ba-pedi and Vatsonga Community, Limpopo Province).

For people with very low incomes, appeasing hunger is the major factor determining type of food consumed. As income raises so does the desire for variety in the diet, for example meat and vegetables taken with the staples. The demand for variety and quality in the diet increases with economic development and non-staples become increasingly important in the diet. With further growth in income, cereals reach a peak, root crops decline further and non-traditional foods expand (Chinyere et al., 1996).
Ting is a traditional, sour, non-alcoholic maize food product, which is commonly used, in rural and informal settlements of South Africa. Ting is normally consumed as a porridge, which has a smooth texture, with a sour taste reminiscent of yoghurt. Its colour depends on the type of cereal used, i.e. cream for maize and reddish-brown for sorghum. Ting is a lactic acid fermented food that constitutes a significant proportion of people’s diet in the Limpopo province.

The objective of this study was to determine the popularity and utilization of Ting in peri-urban and rural communities.

2.2 Materials and Method

1. Population sample size

Six municipality areas were randomly selected for this study namely: Greater Giyani, Greater Letaba, Greater Tzaneen, Mutale, Thohoyandou and Polokwane (refer to Appendix A). 15 families were selected randomly from each of these selected study areas. Samples were collected on the 06\textsuperscript{th} of January 2003 at 12h00.

A questionnaire was designed to gain information about the popularity and the utilization of the Ting. Families involved in this study were asked a few questions about the materials, methods and cost for Ting production and utilization. The following questions were asked:

1. What materials or ingredients are used to prepare Ting?
2. How do you prepare Ting?
3. How long does it take to ferment?
4. Are there any problems that may be encountered during the process of Ting preparation?
5. How do you know the mixture is ready for use?
6. Are there any costs carried forward in preparation of Ting?
7. How and when do you use Ting?
2.3 Results and Discussion

Random sampling was conducted in all the six municipality areas (Greater Giyani, Greater Letaba, Greater Tzaneen, Mutale, Thohoyandou and Polokwane) to determine the consumption of Ting. The outcome of the survey results varied from area to area and from family to family. In all the abovementioned areas, the type of material used was more or less the same. For example in Venda there are families, which prefer clay pots instead of a plastic bucket, and others used either clay pots or plastic bucket for the fermentation of Ting.
The following were the findings from this survey:

**Table 2.1. Survey Findings**

<table>
<thead>
<tr>
<th>Question</th>
<th>Answers</th>
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<tr>
<td><strong>What are the most common raw materials used for Ting production?</strong></td>
<td>◆ Maize meal,&lt;br&gt;◆ Sorghum,&lt;br&gt;◆ Water.&lt;br&gt;These ingredients were mixed in either:&lt;br&gt;◆ Plastic buckets and/or&lt;br&gt;◆ Clay pots with lids.</td>
</tr>
<tr>
<td><strong>How do you prepare ting?</strong></td>
<td>Maize meal and water was mixed inside the container to make slurry. The mixture was then fermented at room temperature for 2 to 3 days (depending on the weather). After fermenting, a pot of water was brought to boiling and the fermenting mixture was added bit by bit until soft porridge was formed. It was allowed to simmer for 20 minutes. Another maize meal was added, which was not fermented to make it thicker, and it was cooked for another 20 minutes. After that a Ting porridge was produced, which can be served with meat, stew etc.</td>
</tr>
<tr>
<td><strong>How long does it take to ferment?</strong></td>
<td>Summer- 24 hours (hot) and Winter- 48 hours (cold).</td>
</tr>
<tr>
<td><strong>Are there any Problems encountered during the preparation?</strong></td>
<td>Change of weather has either a positive or negative effect on fermentation period of the sample. It takes a long time for the sample to ferment when it is cold than when it is warm or hot.</td>
</tr>
<tr>
<td><strong>How do you know the mixture is ready for use?</strong></td>
<td>People either smell or taste the sample mixture, and when the sample tastes sour, it is believed that it is suitable for the preparation of their meals. This mixture is (in tshivenda called “mutuku”, in sepedi they call it “Tini” and in Xitsonga they call it “Dini”).</td>
</tr>
<tr>
<td><strong>Are there any costs carried forward in preparation of ting?</strong></td>
<td>It is cheap to prepare this sample as the materials used can be easily accessed. The method used is also easy to follow (refer to the second bullet above).</td>
</tr>
<tr>
<td><strong>How is Ting Consumed?</strong></td>
<td>Ting is a substance used to prepare sour porridge, which is served with the main meals. People prepare soft sour porridge from Ting to be served during breakfast and hard porridge either during lunch or supper. Its popularity is amongst all the age groups especially in rural areas. Unavailability of Ting would cause serious food problems to some people. They use Ting to add some taste to their food and not for socio-cultural or medical reasons.</td>
</tr>
</tbody>
</table>
Fermentation Time

It was found that the method used for the preparation of Ting was similar in all households surveyed. The fermentation period in some areas differed slightly because of weather for example, in summer, Thohoyandou, Sibasa and Giyani is extremely hot (+34 to 36°C) as compared to Duiwelskloof (+30°C), which resulted in a fermentation period of approximately 24h. In colder areas where the temperature is +15 °C, the fermentation period was approximately 36h. Temperature was found to be the most important factor in determining the fermentation periods in the different areas.

Taste

The product texture was the same because the ingredients used were similar while the taste differed from one family to the other; because some preferred it more sour and others preferred it less sour. In order to decrease or increase sourness of the taste, families used a different length for the fermentation period depending on their individual preference. Smell and taste were the determining factor used to indicate that Ting was ready for consumption.

Consumption Pattern

It was found that 63.3% of the population sample size used Ting as their main meal of the day. Within the Vha-venda, Ba-pedi and Vatsonga population groups, 94%, 29% and 67% respectively used Ting as their main meal, which is called sour porridge. Ting was popular among all the age groups in rural areas. Ting was found to be consumed in the morning as breakfast (soft porridge), mid-day as lunch (porridge and stew) and evening as supper.

Cost of Production

It was discovered that Ting production is cost-effective; it takes an estimated cost of R1.50/ℓ of Ting. The price of 1kg of maize meal/sorghum was approximately R3.00, and when mixed with 1 ℓ of water, it produced 2 ℓ of Ting. The preparation process was easy and it usually takes two days get it fully fermented.
Problems during Ting production

A view of the traditional fermented foods as they are used in traditional families reveals that the production process is laden with problems. The problems can be considered in the categories of production environment, microbiology in processing, nutritional and toxicological status.

In our traditional settings where the production level is not of subsistence housekeeping, the processing environment is very unpredictable: the equipment used (calabash, clay pots, plastic buckets and other utensils used), the hygiene of handlers, equipment and facilities is not checked. The quality of the water used, especially the edges of streams, fountains and boreholes cannot be used, because the water is not potable. Temperature and humidity was optimum for all fermentation and storage purposes respectively. All these factors affect the quality of final product and ultimately the health of consumers.

A lot of information about the fermentation of Ting was flawed, for example:

- there was no way to assure a consistently uncontaminated environment for the fermentation;
- the microbial composition of the inocula was unknown;
- identification of type, the identity of microbes, the age and purity of each culture and the predominant microbes; and
- pH conditions, temperature and ionic strength.

There is no scientific protocol for the production of traditionally fermented Ting. Fermentation periods were chosen according to human judgment. The quality and quantity of water and substrate to be used was not regulated or standardized and heating processes were not controlled or measured. All the abovementioned factors resulted in inconsistent quality.

The lack of knowledge or information on the effect of fermentation on the nutritional quality of the final product resulted in consumers not being aware of the actual nutritional value of the foods. In addition, the type and degree of health risks posed by the consumption of fermented products were not known.
2.4 Conclusion

Traditionally, Ting fermentation is performed by indigenous microorganisms with lactobacilli as the dominant bacteria. However, the product quality can differ widely between different batches from different houses depending on the rate of lactic acid production and on the composition of the active microflora. Thus, it would be beneficial to use a starter culture. However, the question is what type of lactobacilli would be most suited for the task. In order to provide a base for selection of strains for further testing, it would be of interest to know what type of lactobacilli usually dominates in Ting of good quality. There is a need to educate people especially on the microbiological risk factors associated with fermented foods. Scientists who acquired expertise in the fields of food processing, human nutrition and health science are needed to improve traditional fermented Ting.

However Ting remains a popular staple food, which is consumed among the people of Limpopo province. From the survey conducted during this study, it was found that 63.3% of the population use Ting as their main meal of the day.

2.5 REFERENCES


CHAPTER THREE

3. CONVENTIONAL TAXONOMY OF LACTIC ACID BACTERIA ISOLATED FROM TING

3.1 INTRODUCTION

Traditional fermented foods, especially fermented sorghum, represent an important part of the diet of peri-urban and rural communities in South Africa and other developing countries. These products are manufactured with non-defined cultures or by back slopping practices. However, the production of traditional fermented food is steadily declining due to socio-economic changes that are taking place (Beukes et al., 2001). Some people use ingredients such as Tartaric Acid and/or Vinegar to add the sour taste to their porridge because they don’t need to go through fermentation process, which sometimes is time consuming.

In rural and informal settlements of South Africa, fermented doughs and porridge are frequently used as weaning food because they are inexpensive, can be stored for long periods at ambient temperatures and do not require reheating before consumption. These products are prepared from various types of cereals in South Africa including maize, millet and sorghum (Steinkrause, 1995).

Depending on staple availability, geographical area and cultural preference, acid porridge prepared from cereals are still consumed in varying amounts in different parts of the world. It is called by different names according to locality Ting (South Africa), “Ogi” (Nigeria), “Uji”(Kenya), “Togwa”(Tanzania), “Obushera”(Tanzania and Uganda) (Board et al., 1995; Steinkrause, 1996; Mugula et al., 2002). Although Ting (sorghum or maize) is an important staple food in rural areas of South Africa, there is no documentation of the traditional Ting fermentation process from sorghum and maize.

The provision of starter cultures is an issue commonly raised in relation to improvement in quality and safety of fermented foods in developing countries. The development of specific starter cultures with desirable properties therefore deserves careful consideration (Olasupo, et al., 2001). Potential starter organisms must have desirable technological properties in terms of
adaptation to the food substrate, beneficial metabolic activities and functional properties. The identification of representatives strains isolated from Ting will appeared to be the main organisms involved in the fermentation of Ting. Lactic acid fermented foods have proven their wholesome value for thousands of years and are widely accepted and appreciated by the consumers. The LAB involved in these products possesses numerous metabolic properties, which enable their successful application as starter cultures in various fields of food production (Olasupo et al., 2001).

This study was undertaken to isolate, identify and characterize the lactic acid bacteria present during Ting fermentation. This information can contribute to the development of starter cultures with predictable characteristics, for use in small-scale and commercial production of Ting with consistent quality.
3.2 MATERIALS AND METHODS

Sample preparation
Maize fermentation for the production of Ting

1.25g Maize meal + 2L of water in a closed bucket

Slurry

Ferment in a room temperature for 2 to 3 days depending on the weather

Bring a pot of water to boil and add the fermented mixture bit by bit (until you have soft porridge)

Allow it to simmer + 20 to 25min

Add another maize meal (which is not fermented) to make it thicker

Allow it to simmer for another 20 minutes

Ting (ready to eat with meat, stew, milk etc)

Figure 3.1. Flow sheet: production of Ting (Maize)
Sorghum fermentation for the production of Ting

Four cups of coarse mabele + warm water

Leave in a warm place for 1 to 3 days (depending on the sourness) to ferment

Bring a pot of water to boil and add the fermented mixture bit by bit

Allow it to simmer on low heat for ± 30 minutes (until you have a smooth soft consistency) ready to eat as a soft sour porridge with sugar for breakfast.

(Making Ting). Add some more fermented mixture to the already cooked soft porridge to get a thicker consistency.

Cook for another 20-25 minutes on low heat

“TING (can be served with stew or tripe in place of rice, pap or samp)

Figure 3.2. Flow sheet: production of Ting (sorghum)
3.2.1 Source of organisms

Lactic acid bacteria isolates were obtained from two traditional fermented foods (maize and sorghum) from different geographical regions in Limpopo (Giyani, Venda and Bolobedu) South Africa.

3.2.2 Sample Collection

Fifteen samples of traditionally fermented Ting made from maize and sorghum were obtained from local households of the villages in the Limpopo province and 15 samples were laboratory preparations of Ting.

3.2.3 Laboratory preparation and fermentation of Ting

In the laboratory, Ting made from sorghum was prepared following the recipe from the manufacturer (Fig 3.2.) while the one from maize was prepared according to the traditional procedure (Fig 3.1.). Ting was prepared under sterile laboratory conditions.

3.2.4 Isolation of LAB from Ting

After homogenization, one ml of each sample was pipetted aseptically into 9 ml quarter strength ringer’s solution and mixed thoroughly. Serial dilutions (10⁻¹ to 10⁻⁸) were made and 1ml portions of the appropriate dilutions were plated on De Man, Rogosa and Sharpe (MRS) agar (De Man et al., 1960), incubated anaerobically for 48h at 42°C for the enumeration of thermophilic Lactobacilli and Streptococci. MRS agar plates were also incubated anaerobically for 48 hrs at 35°C for enumeration of mesophillic Lactobacillus and Leuconostoc. Anaerobic jars (Biolab) with gas generating kits (Oxoid BR 038B) were used for anaerobic incubation. All isolates were obtained from the countable plates of MRS agar (incubated at 42°C for thermophillic bacteria and at 35°C for mesophillic bacteria). Isolates were cultivated in MRS broth (Merck) at 25°C. The purity of the isolates was checked by repetitive streaking on fresh MRS agar plates, followed by microscopic examination. Isolates were preserved at –20°C (Beukes et al., 2001).
3.2.5 Scanning electron microscope (SEM)

All the isolates were grown up in MRS broth for 24h at 37°C. Four drops of the culture were fixed in 3ml of 2% gluteraldehyde for 10min at room temperature. Preparations were then filtered through a 0,2μm membrane filter and dehydrated in a series of between 30 to 100% ethanol ascending in 10% stages (Dykes et al., 1991). Filters were critical point dried, coated with gold and then viewed under a JEOL-840 scanning electron microscope at an acceleration voltage of 15 -20 kv.

3.2.6 Identification of the LAB to genus level

Gram positive, catalase-negative, oxidase negative, non-motile cells were presumptively identified as lactobacilli. Isolates were assigned to a genus based on key characteristics, (Table 3.1). Growth at 10, 15 and 45°C in MRS broth were evaluated visually after 72 h of incubation. Tests for the catalase reaction, production of gas from glucose and growth at 7 and 10% NaCl concentrations were performed.

3.2.7 Identification of the LAB to species level.

The API 50CHL medium and API 50CH strips (API systems, Biomerieux, France) were used to identify all the isolates to species level. Bacterial cells were inoculated according to the instructions provided by the manufacturer. The APIs were incubated at 30°C and reactions were observed after 24 and 48h. The API database (Biomerieux SA) and accompanying computer software were used to interpret the results (Johansson et al., 1995).
3.3 RESULTS AND DISCUSSION

Table 3.1. Characteristics used to differentiate lactic acid bacteria isolated in this study.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolates and Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.42</td>
</tr>
<tr>
<td>Cell form</td>
<td>Lr</td>
</tr>
<tr>
<td>Cellular arrangement</td>
<td>Cluster</td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
</tr>
<tr>
<td>10°C</td>
<td>-</td>
</tr>
<tr>
<td>15°C</td>
<td>+</td>
</tr>
<tr>
<td>45°C</td>
<td>+</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>+</td>
</tr>
<tr>
<td>Growth in:</td>
<td></td>
</tr>
<tr>
<td>6.5%NaCl</td>
<td>+</td>
</tr>
<tr>
<td>10%NaCl</td>
<td>+</td>
</tr>
<tr>
<td>Catalase reaction</td>
<td>-</td>
</tr>
</tbody>
</table>

Key:
Lr - long rods
Sr - short rods
- - negative
+ - positive
Figure 3.3. SEM electron micrographs showing cell morphology of different Ting isolates.
Scanning electron microscopy was useful in distinguishing the morphological features like the shape, size and arrangement of the cell (Figure 3.3). Only two basic cell morphologies, namely cocco-bacillus and rods shaped cells were distinguished among the LAB isolates examined in this study (Figure. 3.1.). All the isolates grew at 15 and 45°C and produced gas from glucose and growth at 6.5 and 10% NaCl concentrations. None of the isolates grew at 10°C and all were catalase negative (Table 3.1).

In discussing the characteristics of LAB, attention will be given to the distinguishing sugars, for the purpose of indicating which were more relevant in the identification. All sorghum isolates fermented L-Arabinose, D-Xylose and L-Xylose except for isolate 5.30, which did not ferment L-Arabinose. Isolate 2.35 did not ferment galactose, also 3.42 and 3.30 did not ferment mannose. None of the isolates fermented α-methyl-D-glucoside except for isolates 2.35, and isolates 4.30, 3.30 and 4.35 were the only three isolates fermenting lactose, while the rest of the isolates did not. All the isolates (sorghum) fermented melibiose, sucrose, raffinose, gluconate and 5-Keto-Gluconate except for isolate 5.30 and 2.35 was the only isolate not fermenting gluconate (see Appendix C.).

Isolates from white maize (11.2, 3.2, 10.1, 2.2,) were manitol positive, they also fermented α-methyl D-mannoside, but did not ferment α-methyl D-glucoside (Appendix C).

All the results from API were interpreted by using the identification Biomerieux software (Omnimed). Microorganisms from Ting fermented from both sorghum and maize were found to be lactic acid bacteria, which belong to the genus Lactobacillus, Leuconostoc and Pediococcus. Isolate 2.35, was identified as an excellent identification of Leuconostoc mesenteroides with 99% while 3.30, 3.42 and 4.35 were all identified as Lactobacillus cellulosus with 99.3, 98 and 80.4% respectively. Isolate 1.42, 5.42, 5.35, 4.30 and 5.30 were identified as Lactobacillus collinoides and Lactobacillus curvatus with 60.3 and 72.5% respectively.
Table 3.2. Identification of isolates by API 50CH

<table>
<thead>
<tr>
<th>ISOLATES</th>
<th>NAME OF SPECIES</th>
<th>IDENTIFICATION (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.42(sorghum)</td>
<td><em>Lactobacillus collinoides</em></td>
<td>60.3 (doubtful)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus cellobiosus</em></td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>8.8</td>
</tr>
<tr>
<td>3.42(sorghum)</td>
<td><em>Lactobacillus cellobiosus</em></td>
<td>98 (good identification)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus brevis</em></td>
<td>0.8</td>
</tr>
<tr>
<td>5.42(sorghum)</td>
<td><em>Lactobacillus collinoides</em></td>
<td>60.3 (doubtful)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus cellobiosus</em></td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>8.8</td>
</tr>
<tr>
<td>2.35(sorghum)</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>99.9 (excellent identification)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus fermentum</em></td>
<td>0.1</td>
</tr>
<tr>
<td>4.35(sorghum)</td>
<td><em>Lactobacillus cellobiosus</em></td>
<td>80.4 (doubtful)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus fermentum</em></td>
<td>11.3</td>
</tr>
<tr>
<td>5.35(sorghum)</td>
<td><em>Lactobacillus collinoides</em></td>
<td>60.3 (doubtful)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus cellobiosus</em></td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>8.8</td>
</tr>
<tr>
<td>3.30(sorghum)</td>
<td><em>Lactobacillus cellobiosus</em></td>
<td>99.3 (very good identification)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus brevis</em></td>
<td>0.3</td>
</tr>
<tr>
<td>4.30(sorghum)</td>
<td><em>Lactobacillus collinoides</em></td>
<td>60.3 (doubtful)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus cellobiosus</em></td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>8.8</td>
</tr>
<tr>
<td>5.30(sorghum)</td>
<td><em>Lactobacillus curvatus</em></td>
<td>72.5 (doubtful)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus collinoides</em></td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td><em>Leuconostoc lactis</em></td>
<td>3.1</td>
</tr>
<tr>
<td>11.2</td>
<td><em>Lactobacillus pentosus</em></td>
<td>99.7 (very good identification)</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Lactobacillus brevis</em></td>
<td>0.1</td>
</tr>
<tr>
<td>3.2(white maize)</td>
<td><em>Lactobacillus plantarum</em></td>
<td>98.8 (good identification)</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Lactobacillus pentosus</em></td>
<td>1.1</td>
</tr>
<tr>
<td>10.1(white maize)</td>
<td><em>Lactobacillus plantarum</em></td>
<td>96.3 (good identification to the genus)</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Lactobacillus pentosus</em></td>
<td>3.6</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Lactobacillus brevis</em></td>
<td>0.1</td>
</tr>
<tr>
<td>2.2(white maize)</td>
<td><em>Lactobacillus pentosus</em></td>
<td>65.0 (good identification to the genus)</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Lactobacillus plantarum</em></td>
<td>34.3</td>
</tr>
<tr>
<td>42.11(white maize)</td>
<td><em>Pediococcus pentosaceus</em></td>
<td>99.4 (good identification)</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Leuconostoc lactis</em></td>
<td>0.3</td>
</tr>
<tr>
<td>42.9(white maize)</td>
<td><em>Pediococcus pentosaceus</em></td>
<td>99.9 (excellent identification)</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Leuconostoc lactis</em></td>
<td>0.1</td>
</tr>
<tr>
<td>42.11(white maize)</td>
<td><em>Pediococcus pentosaceus</em></td>
<td>40.8</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus brevis</em></td>
<td>22.8</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>0.1</td>
</tr>
<tr>
<td>42.10(white maize)</td>
<td><em>Pediococcus pentosaceus</em></td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus brevis</em></td>
<td>27.7</td>
</tr>
<tr>
<td>Next choice</td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>0.1</td>
</tr>
</tbody>
</table>
In white maize Ting isolates 11.2, 3.2, 10.1 and 2.2 were identified as *Lactobacillus pentosaceus*, *Lactobacillus plantarum*, and *Lactobacillus pentosaceus* respectively while isolates 42.14, 42.9, 42.11 and 42.10 were identified as *Pediococcus pentosaceus*.

In summary *Lactobacillus cellobiosus*, *Leuconostoc mesenteroides*, *Lactobacillus collinoides*, *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus curvatus* were identified as bacteria from sorghum used for Ting fermentation. *L.cellobiosus*, a difficult microorganisms to differentiate from *L. fermentum* was the dominant organism (Table 3.3). In white maize (Ting) the LAB isolated were *L. plantarum*, *L. pentosaceus*, *L. brevis* and *P. pediococcus*. *L. plantarum* and *P. pediococcus* was the dominant organism (Table 3.3). According to bergy’s manual of determinative bacteriology *L. pentosaceus* is a strain of *L. plantarum*.

Mugula *et al.* (2002) reported that *L. cellobiosus*, *L. fermentum* and *L. brevis* were isolated from several indigenous fermented foods including “Togwa” (fermented sorghum or maize (or mixture)), “Fufu” (fermented cassava) and “Kenkey” or “Ogi” (fermented maize).

It was also reported that *L. brevis*, *L. cellobiosus*, *L. fermentum* and *L. plantarum* were isolated from several indigenous fermented foods including “Fufu” (fermented cassava), “Iru” (fermented African locust bean), “Kenkey” and “Ogi” (fermented maize) “Kukun-zaki” (fermented millet), “Ugba” (fermented African oil bean) and “Wara” (fermented skimmed cow’s milk). The species most commonly isolated was *L. plantarum* was identified as the dominant organisms in the fermentation of maize (Ting) (Adegoke *et al.*, 1988; Johansson *et al.*, 1995; Mugula *et al.*, 1996). *Lactobacillus plantarum* is known to be commonly associated with plants. Thus in studies on the occurrence of lactic acid bacteria, *L. plantarum* constituted the highest number of lactobacillus species isolated from fermented plant material (Olukoya *et al.*, 1993; Olasupo *et al.*, 1997).

**3.4 CONCLUSION**

This study was undertaken to characterize the microorganisms responsible for Ting fermentation and provide the necessary information for development of starter cultures with predictable characteristics that could be used commercially for the production of Ting.
Lactobacillus plantarum and Pediococcus pentosaceus were the dominant microorganisms in fermented white maize Ting, whereas Lactobacillus cellobiosus, Leuconostoc mesenteroides, L. fermentum and L. brevis were the dominant organisms in fermented sorghum.

3.5 REFERENCE


CHAPTER FOUR

4. TAXONOMY OF LACTIC ACID BACTERIA FROM TING BY ANALYSIS OF TOTAL SOLUBLE PROTEINS USING SDS-PAGE

4.1. INTRODUCTION

The major expression of the microbial genome results in the synthesis of about 2000 protein molecules that constitute the microbial cell. These molecules form an information source of immense potential and richness for the characterization of microorganisms.

Many techniques have been developed in an attempt to clarify the taxonomic status of lactic acid bacteria, for example, serology (Sharpe, 1955) DNA-DNA hybridization (Champonnier et al., 1989; Collins et al., 1989), gas chromatography of cellular fatty acids and neutral monosaccharides (Rizo et al., 1987), and plasmid profiling (Tannock et al., 1990) and restriction endonuclease patterns (Stahl et al., 1990) have all been applied to LAB taxonomy with some degree of success (Dykes, 1991).

Since proteins are the direct translation product of DNA, the examination of the total soluble protein profile of bacteria represents a method for the rapid determination of natural molecular relationships (Jackman, 1987). However, these methods are often both labor intensive and costly, requiring complex extraction and labeling procedures, particularly if large numbers of isolates are to be examined (Jackman, 1987).

Many techniques have been developed for the separation and characterization of proteins and one of these is sodium polyacrylamide gel electrophoresis that is a convenient and valuable tool in microbial systematics. Electrophoresis methods of suitable resolution and reproducibility provide a basis for microbial systematics that is objective and rapid. The application of whole cell protein (PAGE) electrophoresis in microbial classification, identification and typing has been reviewed by Kersters & De Ley (1980) and Jackman (1985).
Jackman (1984) proposed a standard technique for microbial systematics, which has been adopted by the National Collection of Type Cultures (NCTC) as the foundation of a system for the computer identification of medically important bacteria (Jackman, 1985). The use of (PAGE) of total soluble protein, together with computer-assisted analysis of the resultant protein profiles, is a well-established procedure in bacterial taxonomy (Kersters and De Ley, 1975; Kersters and De Ley, 1980; Jackman, 1987; Dykes, 1991). The high correlation of PAGE methods to results obtained by DNA-DNA hybridization has been shown (Jackman, 1981; Dicks et al., 1990).

Analysis of total soluble proteins by PAGE has been successfully applied to taxonomic studies of lactic acid bacteria such as *Lactobacillus* (Dicks and Van Vuuren, 1987), Carnobacterium (Venter, 1988) and *Leuconostoc* (Dicks et al., 1990; Barreau and Wagener, 1990; Dykes, 1991). According to Kersters and De Ley (1975) protein electrophoresis can be considered as a genomic “fingerprint” which allows accurate clustering and identification of strains within a species that corresponds to results obtained by DNA-DNA hybridization and even % G+C value (Kersters and De Ley, 1975).

In sodium dodecyl sulphate (SDS) PAGE, bacteria are lysed using ultrasound or enzymes to release protein, which are then solubilised in a suitable buffer. Proteins are then separated using polyacrylamide gel electrophoresis (PAGE) and the resulting pattern after staining with Coomassie blue examined by a numerical taxonomic method (Kersters and Delay, 1980; Jackman, 1987). Numerical analysis of these protein profiles by means of a computer, generating a dendrogram. The objective of this study was to determine the similarity or variations of protein profiles between different LAB isolates, obtained from “Ting”.

### 4.2. Materials and Method

The same cultures used previously as described in Chapter 3 were used in this study.
4.2.1. Preparation of bacterial protein samples

Each isolate together with the reference strain was grown in 50 ml MRS broth (Oxoid) at 37°C 24h. They were subsequently centrifuged at 10 000rpm for 9min to sediment the bacteria. The bacterial pellet was resuspended in 10 ml of phosphate buffer (pH 6.8), washed twice in 10ml phosphate buffer and finally resuspended in 1ml phosphate buffer using Eppendorf tubes and centrifuged for 10min at 10 000rpm. The supernatant was discarded and the mass of the sediments was measured to determine the quantity of buffer to be added. 100μl of 20% SDS was added to 900μl of sample treatment buffer (STB). The Eppendorf tubes were then heated for 3min at 96°C. The cells were broken by sonification and 120μl of SDS-STB mixture was added again. It was centrifuged for 10min at 10 000rpm. The clear supernatant was placed into clean Eppendorf tubes for electrophoresis (Kersters, 1990).

4.2.2. Electrophoresis of bacterial protein samples

Electrophoresis of protein extracts was carried out using the discontinuous buffer system of Laemmli (1970). Protein was separated on gels (1.5mm thick and 125mm long) run in a Hoefer SE600 dual cooled vertical slab unit. Separation gel (12%) and stacking gel (5%) were prepared from Bis-acrylamide stock solution (Appendix C). Electrophoresis was performed at a constant current of 30mA through the separation gel at 10°C. After electrophoresis gels were stained for 1h in a Coomasie Blue solution (Appendix C). After staining, gels were destained overnight with agitation. The background of the gels becomes colorless and leaves protein bands colored blue-purple.

4.2.3. Analysis of protein patterns.

Gels with the protein were analyzed with a Hoefer GS300 densitometer. Data obtained were directly stored on a computer and analyzed with the GelCompar II programme (Applied Maths, Kortrijk, Belgium). The programme calculated the Pearson product moment correlation coefficient (r) between the samples, and clustered the samples using the unweighted pair group method of arithmetic averages (UPGMA). Psychrobacter immobilis (LMG 1125) was used as
reference strain on each gel. Reproducibility of electrophoresis was determined by comparing these tracks with a *Psychrobacter immobilis* protein profile selected in the GelCompar II program as standards. Pure bacterial cultures previously isolated or associated with the fermentation of traditional fermented foods were used as reference strains. They were *L. plantarum* (NCFB 1752), *L. collinoides* (DSM 20515), *L. mesenteroides* (VRRL 1355), *L. curvatus* (DSM 20010), *L. fermentum* (DSM 20052) and *L. mesenteroides* (DSM 20193).

### 4.3 Results and Discussion

All the strains were related at the 40% similarity level. The protein fingerprints of the reference strains were used to determine if the Ting samples clustered with a specific pure culture. This would have indicated pre-dominance. The dendrogram was divided into 3 clusters.

**CLUSTER 1**

Cluster 1 was divided into sub-clusters 1a, 1b, and 1c. Members of cluster one are composed of reference strains only, which show a correlation of 60 to 95%. This cluster included isolates 6.2 with 70% correlation.

**CLUSTER 2**

This is the biggest cluster which comprised of the following sub-clusters: 2a, 2b, 2c, 2d, 2e and 2f. This cluster consisted of 34 isolates and reference strains. Sub-cluster 2a consisted of 13 isolates of which eleven isolates were previously identified as *L. plantarum*, *L. pentosus* and *P. pentosaceus* on the basis of phenotypic data, the ability to ferment ribose, no gas production from glucose and the fermentation of α-methyl-D-glucoside and melezitose (Chapter 3). *L. plantarum* and *L. pentosus* are both homofermentative where lactic acid is the major product from glucose and they also displayed a typical rod- shape structure. Sub-cluster 2b consisted of 3 isolates and no reference strain grouped in these cluster. Isolate 8.1 was found to be 90% similar to *P. pentosaceus*, which was previously identified in chapter 3. Isolate 7.3 was also 80% similar to isolate 8.1.

The resulting protein profile as indicated in cluster 2 with many sub-clusters showed that the most dominant organism was *L. plantarum* which is always grouped with *L. pentosus*, *P.
*Pediococcus*, *L. cellobriosus* and *L. collinoides*. Other organisms like *L. curvatus*, *L. mesenteroides* and *L. rhamnosus* were there, but they were not dominant. Cluster 3 could be further divided into two sub-clusters (3a and 3b). In cluster 3, isolate 42.8 was grouped with *L. sali* 20054 with similarities of about 90%. From the literature it was found that *L. plantarum* – a homofermenter produces high acidity in all vegetable fermentations. Sulma *et al.* (1991) reported that *L. brevis*, *L. confusus*, *Lactobacillus* spp. and *P. pentosaceus* were observed in the fermentation of kisra (which is a sorghum fermented product produced in Sudan) (Sulma *et al.*, 1991).

**4.4. Conclusion**

SDS-PAGE was useful in showing the most dominant organisms from Ting. The clustering of reference strains by means of protein profile was an advantage since it allowed improved comparisons between strains of known and unknown identity. The resulting protein profile shows the most dominant organisms but no correlation was found between protein bands of reference strains and pure cultures of Ting. It was discussed that *L. pentosus* is a biotype of *L. plantarum* (Chapter 3) and Figure 4.1 also show them by grouping the two organisms together. All the samples were not collected from the same geographical area and samples varied from family to family. Future studies should include the use of several molecular techniques for the identification of the main bacterial population isolated from Ting, such as hybridisation with rRNA probes, restriction fragment length polymorphism analysis of the 16S rRNA gene, randomly amplified polymorphic DNA-PCR analysis, PCR-temperature gradient gel electrophoresis.
Figure 4.1. Dendrogram showing similarities and variations of protein profile between different isolates.
4.5. REFERENCES


CHAPTER FIVE: CONCLUSIONS

General Conclusions

- This study was carried out to investigate the use of traditional fermented Ting in rural-urban areas of the Limpopo. The survey, investigated the popularity and utilization of Ting. By interviewing families that consume Ting, information was gathered which, indicated how Ting is produced and consumed traditionally. It was found that 63% of the population sample size used “Ting” to prepare their main meals of the day.

- Lactic acid bacteria are the predominant microorganisms and are the major producers of acid and flavor compounds in fermented cereals. Due to the fermentation process, Ting can be stored for a few days at room temperature, because LAB produce a considerable amount of lactic acid which can preserve the quality of Ting at a pH of about 3.7 to 4.5.

- PAGE analyses of the isolates were done in order to study the taxonomy of the isolated pure cultures. The dominant isolates obtained from Ting were gram positive, catalase – negative and non-motile. All the sorghum and maize isolates were able to grow at 10, 15 and 45°C. The morphology of the Ting isolates was curved cells, coco-bacilli and rod shaped. API 50CHL conducted on pure cultures isolated from Ting, from both sorghum and maize belonged to the genus Lactobacillus, Leuconostoc and Pediococcus and more specifically L. plantarum, L. fermentum, L. brevis, L. cellobiosus, L. collinoides, L. pentosus, Leuc. mesenteroides, Leuc. lactis and P. pentosaceus. L. plantarum and P. pentosaceus were the dominant microorganisms in fermented white maize Ting, whereas Lactobacillus cellobiosus, Leuconostoc mesenteroides, L. fermentum and L. brevis were the dominant organisms in fermented sorghum. It appears that most of the lactic acid bacteria predominant on traditional fermented cereals are of the mesophillic hetero-fermentative type and most strains are of the genus Lactobacillus, as also found in Ogi, Uji and Poto-poto. Several strains of L.
*plantarum* and *L. pentosus* clustered together, indicating that the protein profile was unable to differentiate between the two species. According to Bergey’s Manual of Determinative Bacteriology, *L. pentosus* is a biotype of *L. plantarum*. *L. plantarum* was the most dominant isolate.

- The production cost of Ting was R1.50 per litre. Therefore, Ting offers an opportunity for industrial scale-up, to market the food (or beverage) to a much wider population. The results of this study can facilitate this industrialization and lead to a more standardized product using the starter cultures isolated in this study with characteristics such as an improved texture, better storage properties, and enhanced nutritional value.

**Future perspective**

- Future studies should include the use of molecular techniques to confirm identification of the dominant microorganisms isolated from Ting in this study. These techniques could include rRNA hybridization, randomly amplified polymorphic DNA-PCR analysis and PCR-temperature gradient gel electrophoresis.
Appendix A
Study Areas for "Ting" Consumption Survey

University of Pretoria, Mavhungu N. J. (2006)
## Appendix B

### Isolates and Results

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| Methylumbelliferyl- 
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| Methylumbelliferyl- 
  glucuronide                       | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | -   | -    | -   | -   | -      | -    | -     | -     | -    |
| Methylumbelliferyl- 
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| Methylumbelliferyl- 
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| Methylumbelliferyl- 
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| Methylumbelliferyl- 
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Biochemical characterization of LAB species level using the API 50CH systems: (-) negative, (+) positive
Appendix C

Bis –acrylamide stock solution (Monomer solution)

Acrylamide 29.2g
N’N-Methylenediacrylamide 0.8g
This was made up to 100 ml volumetrically with double distilled water, Stored in a schott bottle covered with foil in the fridge at 4°C.

Tris –glycine buffers
Tank or running buffer for lower buffer reservoir (the solution should not be older than 7 days)
2-Amino-2-hydroxymethyl-1,3-propanediol (Tris)BDH 12g
Glycine (BDH) 57.5g
SDS (BDH) 4g
Distilled water up to 4000ml
The conductivity is 624 microsiemens (µs) at 19°C. store at room temperature.

Tank or running buffer for upper buffer reservoir (make up fresh on the day you run).
Tris (BDH) 1.5g
Glycine (BDH) 7.2g
SDS 10% (BDH) 5ml
Distilled water up to 500ml
Fresh buffer was prepared just before starting an electrophoresis. The conductivity is 624µS, and the pH is 8.59 at 19°C.

Sodium dodecyl Sulphate (SDS) 10%
SDS (BDH) 10g
Made up to 100 mls with distilled water.

Ammonium Persulphate 10%
Ammonium Persulphate 0.1g
Made up fresh to 1ml with distilled water.

**Separating gel 12%**

- Double distilled water 26,8ml
- Separation gel buffer 20ml
- Acrylamide-Bis monomer 32ml
- 10% SDS 0,8ml
- 10% Ammonium Persulphate 0,28ml
- TEMED 40µl

**Stacking gel 5%**

- Double distilled water 11,4ml
- Stacking gel buffer 5ml
- Acrylamide –Bis monomer 3,4ml
- 10% SDS 200µl
- 10% Ammonium Persulphate 100µl
- TEMED 25µl

**Sample buffer**

- Tris 3,75g
- Double distilled water 200-250 ml
- Mercapto-ethanol (Merck) 25 ml
- Glycerol (Merck) 50 ml
- 1,72N HCl 17,25 ml
- Conductivity 3,87 ms/cm
- Store in a freezer (-20°C) in a schott bottle.

**Stain Stock Solution 2%**

- Comassie brilliant blue R250 10g
- Double distilled water to 500ml filter
Store at room temperature

**Gel stain solution (0.3%)**

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**Gel distaining solution**

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