1. INTRODUCTION

Nearly every civilisation has consumed fermented milk of one type or another. The consumption of fermented milk may be as old as that of fresh milk (Souane, 1997). In South Africa fermented milk was traditionally made in clay pots and calabashes and milk was periodically added to these containers where bacteria, attached on the inner surface of the container, served as a starter culture for the fermented milk (Keller & Jordaan, 1990). By these empirical approaches beneficial micro-organisms were favoured by selection parameters while spoilage and other deleterious microbes were inhibited (Holzapfel, Geisen & Schillinger, 1995).

Fermented milk is of great significance because it preserves and supplies energy, high quality protein, vitamins and minerals, which enrich the diet. Cultured dairy products have been reported to possess therapeutic value (Shahani & Chandan, 1979) and may play a role in alleviating the problem of lactose intolerance. In many cultures, fermented foods are of high social value with certain beliefs linked to the consumption of the product. Under given circumstances traditional fermented milks may also serve as a source of income for entrepreneurs.

Traditional fermented milk in South Africa has successfully been upgraded to large-scale industrial production in the form of Maas and Inkomasi (Keller & Jordaan, 1990). The market share of Maas is estimated at 69 million litres/annum (BMI Food Pack, 1994 cited by Packaging Review, 1995). This market values up to R 180 million per annum. This figure only relates to the established commercial sector and does not consider home production which is still common in rural regions (Hughson, 1995). Fermented milks therefore seem to have a great economic potential in the dairy industry. In the last 20 to 30 years fermented milks have become very popular in South Africa – there is however a need in the market for the introduction of new (or different) types of fermented milks (Keller & Jordaan, 1990).

Salama, Sandine & Giovannoni (1993) stated that the scope of starter cultures used for production of fermented milk products narrowed considerably, particularly with respect to acidification, proteolysis, anti-microbial activities and flavour production. The need

for new products requires the use of other strains with novel properties. Therefore the genetic pool of strains of lactic acid bacteria present in environments not yet contaminated with industrial strains should be preserved (Weerkamp, Klijn, Neeter & Smit, 1996). The bacteria found in traditional fermented milks represent a unique genetic resource for innovative food biotechnology, which should be maintained for the future.

There is no scientific information available on traditional fermented milks in South Africa concerning the beneficial health properties, food safety, positive or negative properties of the microbial strains, microbiology or the bio-diversity of the bacteria in these products. Modern socio-economic changes mean that some traditional technologies for producing fermented foods will eventually be lost together with the associated micro-organisms. It is therefore imperative that traditional, indigenous fermented milk products as well as the preservation and exploitation of the associated fermentative micro-organisms be scientifically investigated.

1.1 Objectives

The objectives of this study were:

- To collect indigenous fermented milk samples from different rural areas.
- To determine the predominant microbial groups in each sample.
- To isolate and identify the lactic acid bacteria in the fermented milks.
- To determine certain technological important properties of the isolates (i.e. acid production, gas production, citrate fermentation and production of flavour compounds).
- To select strains with promising technologically important properties and to evaluate their suitability as starter cultures for cultured milk products.

2. LITERATURE REVIEW

2.1 Classification of fermented milks

Few people know more than five or 10 of the several hundred specific fermented milk products that could be described (Kroger, Kurmann & Rasic, 1992). The world's first encyclopedia and inventory of fermented milk was published in 1992 and describes some 200 traditional fermented milks and several hundred non-traditional ones. Traditional fermented milk products have a substantial historic record. They were made before the industrial era using non-defined cultures. Yogurt and Kefir are examples. Non-traditional products have been made approximately since 1900, using cultures with a defined microflora. Acidophilus milk and bifidus milk are representative examples (Kroger *et al.*, 1992; Weerkamp *et al.*, 1996). Traditional fermented milks can further be categorized according to: Type of fermenting organisms, the pre-and/or post-fermenting processes and the type of milk used (Y. Byaruhanga, MSc student, University of Pretoria, 1996 - personal communication).

2.1.1 Type of fermenting organisms

According to Kurmann (1984), traditional fermented milks in regions with a cold and temperate climate contain mostly mesophilic bacteria (incubation temperature of 10 - 30 °C), while in regions with a hot and temperate or subtropical or tropical climate they contain mostly thermophilic bacteria (incubation temperature of 35 - 45 °C). The cooler climate of the Scandinavian countries encouraged use of mesophilic streptococci and hot countries to the south and east of the Mediterranean were using thermophilic streptococci and lactobacilli (Marshall, 1986).

Mesophilic bacteria such as *Lactococcus lactis* subsp. *lactis (Lc. lactis)*, *Lactococcus lactis* subsp. *cremoris (Lc. cremoris)*, *Leuconostoc mesenteroides* subsp. *cremoris (Leuc. cremoris)* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis (Lc. diacetylactis)* are used for the production of cultured buttermilk. Yogurt is probably the most popular product fermented with thermophilic bacteria, namely *Streptococcus*

salivarius subsp. *thermophilus (Str. thermophilus)* and *Lactobacillus delbrueckii* subsp. *bulgaricus (Lb. bulgaricus)*. Laban, a traditional fermented product from the Middle East (Egypt, Lebanon, Iraq, etc.) and similar to yogurt may in addition also contain *Lactobacillus acidophilus (Lb. acidophilus)* , *Leuconostoc lactis (Leuc. lactis), Kluyveromyces marxianus* subsp. *marxianus* and *Saccharomyces cerevisiae* (Kurmann, Rasic & Kroger, 1992). Kefir and Koumiss are also produced with a mixture of organisms such as lactic acid bacteria, yeasts and acetic acid bacteria. The development of probiotic fermented milk products can probably be traced back to contamination of milk with bacteria from the gastro-intestinal tract of animals e.g. dipping pieces of calf's and sheep's stomachs in milk (Kurmann, 1984). The term "probiotic" has been coined to describe some of the health benefits associated with lactic acid bacteria originating from the gastro-intestinal tract (Buttriss, 1997).

2.1.2 Pre- and/or post-fermenting processes

Differences in the steps of the manufacturing processes such as heat treatment of the milk, churning or agitation, addition/removal of fat, whey drainage, concentration of the product by straining in cloth bags etc. led to various products. Reportedly, a wide range of containers may be used to make traditional fermented milk. This include fresh bamboo tubes capped with banana leaves in Indonesia (Hosono, Wardojo & Otani, 1989), smoked gourds in Tanzania, Ethiopia and Kenya (Shalo & Hansen, 1973; Kassaye, Simpson, Smith & O'Connor,1991; Isono, Shingu & Shimizu, 1994) and clay pots in South Africa, Zimbabwe and Morocco (Tantaoui-Elaraki & El-Marrakchi, 1987; Feresu & Muzondo, 1989; Keller & Jordaan, 1990). Use of specific containers e.g. animal skins instead of earthenware pots, open versus closed vessels and practices such as cleaning of the fermentation vessels with leaves all influenced the type of organisms present and subsequently the characteristics of the product (Marshall, 1986). Herdsman of the Northern-Sotho in South Africa added the juice of a bitter apple (*Citrullus lanatus*) or sour plum (*Ximenia caffra*) to milk in order to accelerate the souring process (S. Moifatswane, Research Assistant, National Cultural History Museum, 1995 - personal communication).

2.1.3 The type of milk used

Kroger *et al.* (1992) mentioned that the milk from eight species of domesticated animals (cow, buffalo, sheep, goat, horse, camel, yak and zebu) has been used to make traditional fermented milk products throughout the world. According to Kurmann *et al.* (1992) Koumiss-like products can also be made from asses' and reindeer milk. In most African countries, cow's milk is the most common (Y. Byaruhanga, MSc student, University of Pretoria, 1996 - personal communication). The Masai of southern Kenya and northern Tanzania prefer a diet of only milk from Zebu cattle (Isono *et al.*, 1994). Zebu cattle farmers are also found in Cameroon (Jiwoua & Milliere, 1990). Camel milk is used in parts of Kenya and Somalia (Farah, Streiff & Bachmann, 1990), while Moroccan traditional fermented dairy products were made from cow, goat, ewe or camel milk (Tantaoui-Elaraki & El-Marrakchi, 1987). In Egypt, buffalo milk is usually used to produce Zabady, though it can be made from cow's milk or just as well from a mixture of both milks (El-Samragy, 1988). The composition of different types of milk varies and this contributes to the different characteristics of fermented products made from different types of milk (Y. Byaruhanga, MSc student, University of Pretoria, 1996 - personal communication). In their study Isono *et al.* (1994) suggested that the chemical composition of Zebu milk may contribute to the specific microflora of the Masai fermented milk being unusual. Compared with cow's milk, the consistency of fermented milk from camels is thin. One type of milk may be preferred to another. Consumers of Susa in Kenya found that mixing camel's with cow's milk affected the typical camel's milk taste and that the taste of the mixture was undesired (Farah *et al.*, 1990). Among the Sotho of South Africa goat's and sheep's milk was used, but neither was liked as much as cow's milk (Ashton, 1939). The Venda preferred goat's milk to cow's milk in making fermented milk in a clay pot (Stayt, 1931).

2.2 SIGNIFICANCE OF FERMENTED MILK

2.2.1 Nutritional value and role in the diet

In terms of overall composition fermented milk is similar to the milk from which it was made and is thus an excellent source of high quality protein, calcium, phosphorus,

magnesium, zinc and the B-vitamins riboflavin, B12 and niacin (Marshall, 1986; Buttriss, 1997). The fermentation process has little effect on the mineral content of milk (Buttriss, 1997). While many lactic acid producing organisms require B-vitamins for growth, several species are capable of synthesizing certain vitamins (Shahani & Chandan, 1979). Reportedly, losses in vitamin B2 and B12 can be attributed to bacterial metabolism, particularly if *Lb. bulgaricus* is present (Rasic & Kurmann, 1978; Hartman & Dryden, 1965 cited by Marshall, 1986 and Food Industries of South Africa, 1996). "Supplementary cultures", such as *Leuconostoc* and *Propionibacterium shermanii* are capable of synthesizing significant amounts of vitamin B12 in milk (Karlin, 1965 and Cerna & Hrabova, 1977 cited by the International Dairy Federation, 1983). According to Rao, Reddy, Pulusani & Cornwell (1984) some species of *Lc. lactis, Lc. cremoris, Str. thermophilus* and *Lb. acidophilus* are able to increase folic acid and vitamin B2 during fermentation. In addition to the specific species, the B-vitamin content of cultured milk is also affected by the amount of microbial inoculum, incubation conditions and the duration of storage (Hartman & Dryden, 1965 cited by Food Industries of South Africa, 1996).

In a study that aimed at analyzing the nutrient intakes of South Africans (Food Industries of South Africa, 1996) it was *inter alia* concluded that adult women from the black, coloured and Indian populations had very low calcium intakes (39 - 46 % of RDA). Many of the black and Indian groups questioned in the study had low intakes of thiamin, riboflavin and niacin. According to the guidelines of the Department of Health (cited by Langenhoven, Wolmarans, Jooste, Dhansay & Benade, 1995) a balanced, healthy diet for adult South Africans requires daily consumption of at least 400 ml of milk equivalent (including all kinds of dairy products) in order to meet calcium needs. The investigation showed that only 13 % of respondents indicated an adequate milk intake of 400 ml or more a day. Inadequate milk consumption was especially noticeable amongst the Asian and black respondents. Complementing the staple food with micronutrient-rich foods is a culturally acceptable practice in black population groups and needs to be encouraged (Langenhoven *et al.*, 1995).

2.2.2 Therapeutic qualities

In addition to their high nutritional properties, cultured dairy products have been reported to possess therapeutic value (Shahani & Chandan, 1979). Stories abound of the longevity of inhabitants of certain regions of Central Europe, frequently associated with their consumption of various forms of fermented milks (Milk Industries, 1994). As early as 1908, Metchnikoff suggested that the longevity of Bulgarians was in part due to their ingesting large quantities of Bulgaricus milk (Shahani & Chandan, 1979). From 1978 to 1981 researchers have focussed on Abkhasia where most of the elderly people are not weak from old age. Lactic acid bacteria isolated from the gut of healthy elderly people in Abkhasia were mixed with ordinary yogurt cultures and this mixture was given the registered name of Causido. Isolates best able to combat potential pathogenic strains were selected (Hougard, 1993; Milk Industries, 1994). Buttriss (1997) listed some of the numerous health benefits attributed to fermented milk:

- Improved lactose tolerance
- Protection against gastro-intestinal infections
- Effective treatment for specific types of diarrhoea
- Relief of constipation
- Improved immunity
- Cholesterol reduction
- Protection against cancer/antimutagenic effect
- Improved mineral absorption

For some of these e.g. improved lactose tolerance, a considerable amount of evidence has been amassed, but others are far from well established from a scientific point of view. Traditional fermented milk products were probably contaminated with bacteria from the gastro-intestinal tract by any of the following methods: (1) infection when milking takes place, (2) adding and conserving milk in the stomachs of young slaughtered animals, (3) pieces of calf's and sheep's, etc. stomachs dipped in milk, (4) giving young animals milk before slaughtering them and then adding the contents of the stomach to the prepared milk in order to ferment it (Kurmann, 1984).

2.2.3 Alleviation of lactose intolerance

Most of the world's people lose their ability to digest lactose in adult age in the same way as most mammals do. Lactose maldigestion is common all over the world but the frequency varies considerably between different races and ethnic groups. The lowest frequency (1 to 5 %) is found among Northern European healthy white individuals such as in Scandinavia. Futhermore, lactose maldigestion is high among Asiatic people (80 - 100 %) (Abbott, 1973; Fernandes, Chanfan & Shahani,1992; Alm, 1993). In Africa the picture is confusing since some tribes consume milk in considerable quantities without ill effects, whereas their fairly near neighbours may have a high incidence of intolerance (Abbott, 1973). Some workers concluded that the cattle-keeping tribes show a high incidence of tolerance but the crop farmers are predominantly intolerant (Cook & Kajubi, 1966 cited by Abbott, 1973; Simoons, 1969, 1970). In South Africa some of the tribes were cattle herders and milk drinkers while others were predominantly agriculturists (Segal, 1983). From the study of Segal (1983) it was evident that most South African Blacks are lactase deficient, despite the fact that two of the largest groups (Zulu and Xhosa) were cattle herders and milk drinkers. The reason given by Segal (1983) for the lactase deficiency is that South African Blacks originated in the West and Central African zone where milk was not produced. They migrated into Southern Africa in relatively recent historical times and took up dairy and milk consumption fairly recently and have not had enough time for genetic selection for lactase availability through life.

Fermented milk products may play a role in alleviating the problem of lactose intolerance since the lactose content is lowered during fermentation. Some species of lactic acid bacteria also contribute ß–galactosidase (lactase) activity that might substitute for the lack of endogenous lactase (Marshall, 1986; Khedkar, Mantri & Khedkar, 1994). Obtaining adequate calcium without consumption of dairy foods is difficult. In analyzing nutritional data in South Africa it was noted that adult women from the black, coloured and Indian populations had very low calcium intakes (39 - 46 % of RDA) (Food Industries of South Africa, 1996). A considerable number of authors confirmed that milk consumption remained one of the most valuable weapons in the fight against malnutrition, particularly protein malnutrition (Abbott, 1973). It

consumed whole, with the maggots included. It seems that the word "preservation" does not have the same meaning in all countries.

2.2.5 Social values

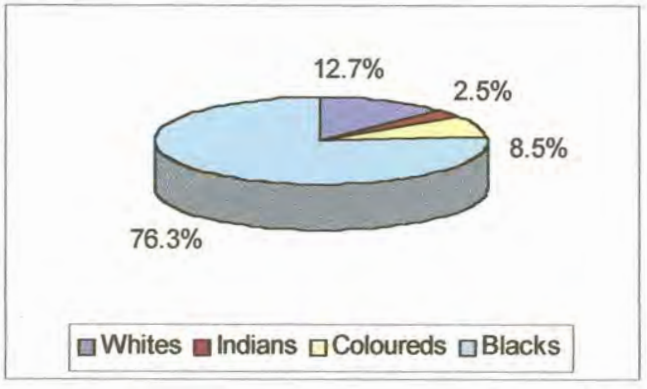

Fig. 2.1: Population percentages in South Africa

(RSA Statistics in brief, 1997)

Blacks make up more than three-quarters of the South African population (76.3 %, Fig. 2.1). The Zulu (22.4 %) and Xhosa (17.5 %) are the two largest tribal groups in terms of languages spoken (RSA Statistics in brief, 1997). In earlier times the peoples of South Africa fermented their milk in milk sacks, calabashes, clay pots, stone jars and baskets (skillfully plaited from a fine kind of reed grass) (Fox, 1939; Quinn, 1959; Bryant, 1967; Fehr, 1968; Bohme, 1976; S. Moifatswane, Research Assistant, National Cultural History Museum, 1995 - personal communication). The Zulus kept their Amasi (fermented milk) in skin sacks before Shaka introduced gourds upon his return from Mtetwaland, along the coast, where gourds had always been used (Bryant, 1967). This may be one of the earliest examples of technology transfer for fermented food production in South Africa! Cereals have long been the staple food for many African peoples but meat and milk have been highly prized foods in the diet (Hunter, 1936; Shalo & Hansen, 1973). Among the Zulu and Xhosa peoples milk was a staple food that vied with grain in importance (Sansom, 1974 cited by Segal, 1983). Milk was not a staple food for the other tribes. Zulu people were (and probably still are) particularly partial to sour milk and said it made "a man strong and desired!" (Elliott, 1978).

can be concluded that sufferers from lactose intolerance must not necessarily avoid dairy products, but can manage the problem by, *inter alia*, consuming fermented milk products.

2.2.4 Preservation

Milk is nutritious, supplying high-quality protein in the diet, but it is also nutritious to micro-organisms and thus highly perishable (Campbell-Platt, 1994). Modern preservation techniques such as refrigeration, freezing, canning or modified atmosphere packaging are expensive, particularly in communities with low levels of disposable income (Reilly & Westby, 1997). Under such conditions lactic acid bacteria play an essential role in the preservation of wholesome foods. They are generally fastidious on artificial media, but grow readily in most food substrates and lower the pH rapidly to a point where competing organisms are no longer able to grow (Steinkraus, 1992). According to Reilly & Westby (1997) the ability of lactic acid bacteria to suppress growth of other micro-organisms can result from organic acid production, hydrogen peroxide production, carbon dioxide production, nutrient depletion or production of antibiotic-like substances.

Additional processing steps such as smoking of gourds (Ashenafi, 1996), boiling before fermentation (Iwuohu & Eke, 1996), addition of salt, storage in olive oil (Ibrahim, Al Khatib, Al-Haik, Daguri, 1996), pickling in brine (Kurmann *et al.*, 1992) and drying of curd balls (Damir, Salama, Mohamed, 1992) can complement lactic fermentation in ensuring safety and extending the shelf-life of the product. Clay pot-fermented milk in Zimbabwe had a shelf-life of 3 days at ambient temperature (Feresu, 1992). Removal of mould growth, smoking of the container and rubbing the lid of the container with leaves of *Ocimum basilicum* contributed to the approximately two months shelf-life of Ititu (Kassaye *et al.*, 1991). For longer-term storage, yogurt-cheese (Labneh) is shaped into balls, soaked in olive oil and stored at room temperature for up to a year (Ibrahim *et al.*, 1996). Dirar (1992) described some fermented foods from Sudan and mentioned that ripening fermented unchurned milk for up to 10 years (!) produced Biruni, also called Leben-gedim. A related product, but not ripened, is mish, which is made by prolonged fermentation to the extent that maggots thrive in it. The product is

Many taboos and beliefs existed among the tribes regarding sour milk. Among the Zulu-Xhosa peoples Amasi acquired a symbolic value in family transactions and rites. Amasi and umcuba - a grain dish - were considered as specially family foods, to be shared by no outsider (Turner, 1909). The exchange of cattle at marriage formed a living bond between the two families in question. The drinking of milk might have contracted a somewhat similar tie. Milk taboos prohibited, among other things, a man from drinking sour milk in any household but his own, or that of the paternal or maternal relatives. Among the Zulu, Pondo (Turner, 1909) and Xhosa (Soga, 1932) the drinking of milk with a member of another clan was equal to pledging blood-brotherhood with him and prevented marriage in that clan. Most of the taboos of the clan were tied to rules that necessitated an abstention from the taking of sour milk (Elliott, 1978). No one with "umlaza" (ritual impurity) may have drunk milk. Common occurrences like someone's contact with the dead was regarded as sources of contamination and the person concerned had "umlaza". Meat of an animal that had died, pork, and honey, infected those who ate them with umlaza until they had washed. People with umlaza had to be purified by various procedures, including abstention from Amasi (Hunter, 1936; Elliott, 1978).

One of the first performances of the chief of the Herero-tribe from Namibia was to consecrate the milk by tasting it after it had become sour. This showed in a symbolic way that he, as living and visible representative of the ancestral line, took a patriarchal and also a despotic view of the chieftainship (Hahn, 1928). Although traditional fermented products are still being produced in some rural communities in South Africa, the traditional containers have been replaced with commercially available ones, especially plastic (Coetzee, Gordeuk, Barnard, Stassen & De Kock, 1996). This may be due to availability and not preference (Dirar, 1997). In 1968 Holt (according to Bohme, 1976) did not see a calabash in use among the Tshezi. "They spoke off it with a sigh, as only a pleasant memory". It can be expected that westernisation has an influence on the food choices of the Black African consumer, but according to Melville Radebe (senior product manager at Clover SA) "Traditional products still enjoy a loyal following" (Hughson, 1995).

2.2.6 *Improved flavour, variety and acceptability*

The fermentation process has the advantage of imparting flavour, variety and acceptability to milk products (Reilly & Westby, 1997). As previously indicated (2.1), a number of factors contribute to the variety of traditional fermented milks that exists, including the type of fermenting organisms. Micro-organisms can produce numerous new compounds, including aldehydes, amines, alcohols, ketones etc. In most African fermented foods spontaneous fermentation involve a number of microbial species and one would expect a welter of chemicals to be produced. The fermentation process and accommodating procedures may also lead to changes in the texture and colour of the substrate (Dirar, 1997). Acid coagulation and whey drainage leads to an increase in viscosity of the milk (Mutukumira, 1996). In Kenya glowing embers are pressed against the inner wall of the gourd using a stalk. Most of the powdered charcoal is poured out, but some remained and is mixed with the milk, resulting in a smooth bluish fermented milk (Shalo & Hansen, 1973). The local people like traditional fermented foods, even though foreigners might find some of them extremely repugnant (Dirar, 1997). Fermentation produces enjoyable foods that impart variety to bland starchy diets (Cook, 1994; Dirar, 1997).

2.2.7 *Source of income*

In some areas in Africa, people in urban centres still demand traditional foods (Reilly & Westby, 1997). Traditional fermented milk products are sold at open markets in Cairo (El-Sadek, Naguib & Negm, 1972), traditional dairy shops in Morocco (Hamama & Bayi, 1991) or by hawkers who patronize different processors in Nigeria (Bankole & Okagbue, 1992). Hamama (1997) mentioned the existence of urban dairy shops that specialised in Jben making. In Zimbabwe the product "Lacto" is produced on an industrial scale and it aims to fill a gap in the urban market created by the absence of traditionally fermented milk (Feresu & Muzondo, 1989; Mutukumira, 1996). The production of traditional fermented milk products may serve as a source of income for rural households.

There is also a need for affordable dairy products in rural communities (Jordaan, Keller & Uys, 1998). In Nigeria indigenous fermented foods are produced in homes, villages and small-scale cottage industries. These products are sold to the rural populace who buy them for food and social ceremonies (Iwuohu & Eke, 1996). The production of fermented milks may satisfy a need of small farmer communities to add value to the primary products in South Africa. Additional spin-offs would be job and wealth creation and a more balanced nutritional diet in rural communities (Nout, 1992; Jordaan *et al.*, 1998). Dairy products are excellent sources of income and the manufacturing of dairy products may stimulate farming activities in an area (H. Keller, head of the Irene Dairy Education Centre, ARC-Animal Nutrition and Animal Products Institute, 1998 – personal communication). South African farmers are encouraged by the authorities to process as much of their products as possible on the farm in order to create work opportunities and thereby counteract urbanization. Deregulation of the Dairy Industry in South Africa has made it easier for milk producers to sell their products directly to consumers (Bester, 1991).

2.3 THE MANUFACTURING PROCESSES OF TRADITIONAL FERMENTED MILKS

The nature of the fermented milk product may vary depending on factors such as the microflora, removal of fat and/or whey, addition of fat, water, salt and spices. The microflora responsible for the fermentation process may originate from the raw milk (spontaneous fermentation), sessile bacteria attached on the walls of containers or from a portion of previously prepared fermented milk (back-slopping). Examples of products resulting from spontaneous fermentations are Ergo in Ethiopia (Ashenafi, 1993; Ashenafi, 1996) and Raib in Morocco (Hamama, 1992). With successive spontaneous fermentations bacteria establish themselves in a biofilm on the walls of containers. This leads to accelerated souring of subsequent fermentations. According to Shalo & Hansen (1973) the gourd for making Maziwa lala (Kenya) was used twice before the next cleaning. It was known that milk from the second use of the gourd coagulated faster and produced a good flavour quicker than that from the first use. In the production of Ititu (Kenya) fresh milk is naturally inoculated with bacteria from the

walls of the container. The smoked pear shaped vessel is rinsed with sour milk before adding fresh milk (Anonymous, 1990). Zabady is produced by inoculation with 2 to 3 % of previously made product (El-Gendy, 1983).

Fermented whole-milk products include Ergo (Ethiopia), Maziwa lala, Iria ri Matii (Kenya) and Zabady (Egypt) (Shalo & Hansen, 1973; Miyamoto, Gichuru, Akimoto & Nakae, 1986; Kimonye & Robinson, 1991; Kurmann *et al.*, 1992; Ashenafi, 1993; Ashenafi, 1996). Other steps in the manufacturing processes of traditional fermented milk include fat removal and/or whey drainage (Table 2.1).

In some products the fat is removed after fermentation by churning or agitation in a pot (Ayib), gourd/calabash (Nono), goatskin, earthenware jar (Lben) or earthenware pots (Laban rayeb) (El-Gendy, 1983; Hamama, 1992; Mogessie, 1992; Iwuohu & Eke, 1996). The fat was usually converted into butter (e.g. Manshanu) for eating purposes, but was also used by the South Sotho of South Africa to make soap or used as a body lotion by itself or when mixed with ochre (Ashton, 1939). The remaining sour buttermilk was consumed as such or processed further by whey drainage (Laban Zeer) or heat treatment followed by whey drainage (Ayib) (El-Gendy, 1983; Mogessie, 1992). In the production of Ititu (Anonymous, 1990; Kassaye *et al.*, 1991; Beyene & Abrahamsen, 1997), Jben (Hamama, 1992) and Amasi (Fox, 1939; Bryant, 1967; Keller & Jordaan, 1990) from whole milk the whey is removed using different methods.

Cream (Keller & Jordaan, 1990) or naturally fermented cream (Mutukumira, 1995) are sometimes added to the final fermented product in South Africa and Zimbabwe and contributes to a full texture and a rich creamy taste. Whey is removed from traditionally fermented milk products by innovative methods, for example by using a wooden pipette (Ititu), draining through a muslin cloth (Ayib), percolating through the porous walls of an earthenware jar (Laban Zeer) or draining through a small hole in the bottom of a gourd or clay pot (Amasi, Mafi) (Fox, 1939; Quinn, 1959; Bryant, 1967; Anonymous, 1990; Kassaye *et al.*, 1991; Mogessie, 1992).

Table 2.1: Countries, product names and manufacturing steps of some of the traditional fermented milk products (and by-products) found in Africa

Country	Product	Fermenting vessel	Fermentation	Fat removal	Defatted milk	Whey drainage	Reference
Southern Ethiopia and northern Kenya (Borana people)	Ititu	Pear shaped vessel ("Gorfa") smoked (with *Acacia nilotica*) and rinsed with sour milk.	Allowed standing until milk coagulates at ambient temperatures (24-48 h).			Whey removed with a wooden pipette. Fresh milk added and process repeated. Ititu kept for up to 2 months. Mould growth removed from surface.	Anonymous (1990). Kassaye *et al.* (1991).
Ethiopia	Ayib (Cottage cheese, whey-drained buttermilk, heat-treated)	Clay pot	Spontaneous souring at about 30 °C for 24 to 48 h	Churning by slowly shaking the contents of the pot. Butter removed and kneaded with water.	Skim milk heated to about 50 °C until a distinct curd form. Ayib contains about 1 % fat.	Curd ladled out or filtered through a muslin cloth. This product is called Ayib.	Mogessie (1992). Kurmann *et al.* (1992).
Ethiopia	Ergo (Traditional yoghurt-like product)	Kettle previously treated by inverting over a piece of smouldering olivewood.	Room temperature for 2 days (Preferably consumed after 24 h souring).				Kurmann *et al.* (1992). Ashenafi (1993). Ashenafi (1996).

Table 2.1: (continued)

Country	Product	Fermenting vessel	Fermentation	Fat removal	Defatted milk	Whey drainage	Reference
Kenya	Maziwalala	Gourds rubbed with glowing embers from a particular tree known as Mutamayio.	Spontaneous fermentation (1-5 days). Some of the charcoal remains to give a smooth bluish fermented milk.				Shalo & Hansen (1973). Miyamoto, Gichuru, Akimoto & Nakae (1986).
Kenya (Masais, Turkanas, Kalenjins, Somalis, Merus)	Iria ri Matii	Gourd (dried fruit of *Lagenaria leucantha*), smoked with glowing splints (*Olea africana*)	Boiled or unboiled milk fermented for 3 or 4 days at room temperature.				Kimonye & Robinson (1991).
Nigeria	Nono (Yoghurt-type fermented milk) Manshanu butter	Calabash	Fresh raw cow's milk is sometimes boiled for 3 h before fermentation. Fermented for 24 h at room temperature.	Fat removal with a wooden spoon. Curd scooped into a gourd. Churning by shaking vigorously for 30 min. Mixture returned to calabash. Buttery pellets skimmed off. Butter is called manshanu.	Remaining colloidal mixture in the calabash constitutes Nono. Variable quantities of water and a local spice called kuka (cream of tartar from the baobab fruit) are usually added.		Iwuohu & Eke (1996). Bankole & Okagbue (1992).

Table 2.1: (continued)

Country	Product	Fermenting vessel	Fermentation	Fat removal	Defatted milk	Whey drainage	Reference
Egypt	Zabady (The national type of yogurt in Egypt)	Porcelain containers called solttaneia	Milk boiled for about 30-60 min, cooled to about 45 °C and inoculated with 2 to 3 % Zabady. The mixture is poured into containers and incubated at 45 °C for 2 to 3 h until the milk curdles.				El-Gendy (1983).
Lower Egypt	Laban rayeb	Earthenware pots	Fresh milk allowed to ferment for one or several days at 20 to 25 °C.	Cream layer is removed and whipped by hand to butter.	The sour milk (Laban rayeb) is consumed as it is or after conversion to a soft acid cheese "Karish cheese".		Kurmann *et al.* (1992). El-Gendy (1983).

Table 2.1: (continued)

Country	Product	Fermenting vessel	Fermentation	Fat removal	Defatted milk	Whey drainage	Reference
Upper Egypt	Laban Khad/Laban Karbah Laban Zeer	Goats' pelts (karbah) Earthenware jar (Zeer)	Milk collected in goats' pelts ("karbah"). Churned when acidity is suitable.	Butter granules removed.	Sour buttermilk remains (Laban Khad or Laban Kerbah). During cold weather Laban Khad is usually used for making Karish cheese. In hot weather it is used for making Laban Zeer.	During storage of sour buttermilk in an earthenware jar (Zeer) the whey percolates through the porous walls of Zeer. The Laban Zeer becomes quite thick. Salt is added.	El-Gendy (1983).
Morocco	Raib (coagulated milk). Lben (buttermilk). Zabda (butterfat). Jben (whey-drained fermented milk).		Raw milk ferments spontaneously at 15 to 25 °C for 1 to 3 days. Coagulated milk is called Raib. Can be consumed as such or churned.	Churning in a goatskin or earthenware jar separates the liquid phase (Lben) from the fat (Zabda).		Jben is prepared by placing the coagulated milk in a cloth at room temperature and draining the whey. Salt is added to Jben in northern Morocco.	Tantaoui-Elaraki & El-Marrakchi (1987). Hamama (1992).

Table 2.1: (continued)

Country	Product	Fermenting vessel	Fermentation	Fat removal	Defatted milk	Whey drainage	Reference
South Africa	Amasi (Xhosa and Zulu)	Calabashes	New gourds are firstly "seasoned" or "ripened" by successive fermentations and removal of whey. Added fresh milk ferments within 2 or 3 h.			Whey is drained through a small hole in the bottom of the gourd that was securely plugged. Because of lipases present in raw milk, a certain degree of rancidity is always present.	Fox (1939). Bryant (1967). Keller & Jordaan (1990).
Zimbabwe	Amasi or Mukaka wakakodzek-wa	Earthenware pot	Fermentation takes 1-2 days depending on the ambient temperature. Fermentation may be speeded by adding fresh milk to a pot containing the remains of a previous batch of sour milk.			The whey is removed by decanting the coagulated milk.	Feresu & Muzondo (1989). Mutukumira (1995).

Traditional fermented milk is sometimes mixed with cereals in the preparation of milk/cereal based foods, such as Fura-nono (fermented milk mixed with fermented cereal millet) (Umoh, Adesiyum & Gomwalk, 1990; Olasupo, 1992) and Kishk (mixture of concentrated sour buttermilk and boiled ground wheat kernels) (El-Gendy, 1983). In South Africa and Zimbabwe fermented milk is often consumed with porridge (Quinn, 1959; Mutukumira, 1995).

2.4 CHARACTERISTICS OF TRADITIONAL FERMENTED MILKS

2.4.1 Chemical characteristics

Table 2.2 gives the chemical composition of a few African traditional fermented milks. The chemical composition of the final product is influenced by the type of raw milk (camel, goat, cow, etc.), concentration practices such as whey drainage, duration of the fermentation period, addition/removal of cream, addition of water as well as the microbial population. The influence of whey drainage can be seen in the total solids content of Ititu (20.87 %), Jben (37.5 %) and to some extent in Amasi (16.53 %) (Kassaye *et al.*, 1991; Hamama & Bayi, 1991; Mutukumira, 1995). The average total solids values for South African full cream cottage cheese, plain yogurt and Maas are respectively 24.84, 13.64 and 12.25 % (Smit, Smith, Schönfeldt & Heinze, 1998).

Large variations in the chemical composition of different batches of traditional fermented milks may be due to the lack of standardization of the raw milk as well as processing procedures. The fat content of Ititu e.g. varied between 5.0 and 13.0 % (Kassaye *et al.*, 1991) and that of Amasi from 2.6 to 9.10 % (Mutukumira, 1995). Addition of cream, degree of product concentration and fat content of the raw milk were indicated as causative factors in this regard (Mutukumira, 1995). The data for total solids, crude protein, fat and ash are quite variable for Nono and the values are low when compared with the corresponding values for plain yogurts. Some elements were reported to be considerably lower in Nono than in milk, for example the calcium content varied between 0.013 and 0.028 % versus 0.12 % in milk. The practice of adding variable quantities of water to the product before sale by the processors was

Table 2.2: Chemical composition of some traditional African fermented milks

Product	pH	Percentage (w/v)					Number of samples	Reference
		Lactic acid	Fat	Protein	Total solids	Ash		
Ititu	3.65[a] (3.5-3.9)[b]	1.92 (1.5-2.6)	9.1 (5.0-13.0)	7.17 (5.4-9.2)	20.87 (15-27)	0.74 (0.6-0.8)	20	Kassaye *et al.* (1991)
Nono	4.3 (4.1-4.3)	0.65 (0.5-0.9)	0.83 (0.2-1.5)	2.55 (1.2-5.7)	6.084 (2.8-13)	0.41 (0.2-0.8)	100	Bankole & Okagbue (1992)
Zabady	3.7 (3.0-4.4)	1.01 (0.6-1.5)	3.62 (1.1-6.8)	4.17 (3.1-5.6)	14.32 (11-19)	0.65 (0.4-1.4)	50	El-Sadek *et al.* (1972)
Raib Jben	4.2 4.1	0.67 1.04	2.22 16.47	3.1 15.8	10.7 37.5	0.54 1.26	42 42	Hamama & Bayi (1991)
Amasi	3.98 (3.5-4.3)	0.97 (0.9-1.4)	5.89 (2.6-9.1)	4.57 (3.3-7.6)	16.53 (13-21)	ND	10	Mutukumira (1995)

[a] Average
[b] Range
ND = No data

thought to be responsible for these low values (Bankole & Okagbue, 1992). Different whey draining periods of 2 to 10 days may account for the high variation (± 6.75 %) of total solids in Jben (Hamama & Bayi, 1991). In Ititu, the extended fermentation (up to 2 months) leads to a higher lactic acid content (1.9 %) compared with other fermented milk products (Kassaye *et al.*, 1991).

2.4.2 Microbiological characteristics

Table 2.3 gives a summary of the microbiological composition of some African fermented milk products. In the production of Iria ri Matii, the preparation of the gourd has a selective influence on the fermenting microflora. Treatment with charcoal suppresses yeasts, moulds and bacterial contaminants in the raw milk and allows the streptococci embedded in the wall of the gourd to develop strongly. According to Kimonye & Robinson (1991) *Str. thermophilus* is the predominant organism in this product. In addition to the geographical influence of climate on the constituent microflora, seasonal variations are also evident. According to El-Gendy (1983) and Marshall (1986) streptococci dominated in cold season samples of Laban rayeb while the lactobacilli predominated in the hot season, on the other hand, the numbers of coliform bacteria were normally high in winter (pH 5.4–6.1) while no coliform bacteria were found in the markedly acid samples produced in the hot season (pH 3.8). Natural fermention of milk may have a successive nature, as seen in the production of Jben from Raib. Lactococci (dominating in Raib) and other bacteria, less tolerant to lactic acid, give way to the more acid tolerant lactobacilli (dominating in Jben). The draining period in the manufacturing of Jben allows for development of lactobacilli which are known to be more resistant to acidity than lactococci.

High lactic acid bacterial counts encountered in fermented milk products may be the result of extended fermentation time (e.g. in Ititu), back-slopping or the re-use of calabashes (e.g. in Nono) and other equipment that could contribute at least partially to the final microbial count (Kassaye *et al.*, 1991; Bankole & Okagbue, 1992). According to Kroger *et al.* (1992) there is still much confusion over the microbial identity of most of the traditional fermented milk products in the world since some have never been studied in depth and some are very variable from batch to batch. Regulatory

Table 2.3: Microbiological information on some African traditional fermented milks

Country	Product	Microbiological information
Southern Ethiopia and northern Kenya	Ititu	Samples investigated were collected from individual households in Southern Ethiopia. The high counts of lactic acid bacteria (2.7×10^{12} cfu/g) can be explained by the extended fermentation time of up to 2 months. *Lb. casei* and *Lb. plantarum* were isolated on MRS-agar. Lactobacilli were prevalent in the end product (Kassaye *et al.*, 1991).
Ethiopia	Ayib	Samples of Ayib purchased from local markets were highly contaminated with various micro-organisms (aerobic mesophiles, yeasts and enterococci). The majority of the samples had mould and lactic acid bacterial counts of 10^5 cfu/g or higher (Mogessie, 1992).
Ethiopia	Ergo	Yogurt-like product; microbial state is not well defined (Vogel & Gobezie, 1977 cited by Kurmann *et al.*, 1992).
Kenya	Maziwa lala	The lactic acid bacterial counts from 5 samples of Maziwa lala varied between 3.4×10^6 and 1.2×10^8/g. Lactobacilli (24 %), streptococci (33 %) and leuconostocs (43 %) were identified. Species isolated included *Lb. curvatus, Lb. plantarum, Lc. cremoris, E. faecium, Lc. lactis, Leuc. dextranicum and Leuc. mesenteroides* (Miyamoto *et al.*, 1986).
Kenya	Iria ri Matii	Streptococci were found at levels of 57×10^6 to 32×10^8/g. *Streptococcus thermophilus* was the dominant organism. No lactobacilli were isolated (Kimonye & Robinson, 1991).
Nigeria	Nono	High lactic acid bacterial counts ($1.1 \times 10^8 - 9.5 \times 10^{10}$/g) were encountered by Bankole & Okagbue (1992). Non-hemolytic streptococci and *Lactobacillus* spp. were abundant (Eka & Ohaba, 1977 cited by Odunfa, 1985 and Kurmann *et al.*, 1992).
Egypt	Zabady	According to El-Sadek *et al.* (1972) counts on Rogosa agar averaged 6.96×10^8/g in Zabady samples collected from the open market at Cairo. *Str. thermophilus* (168 isolates) and *Lb. bulgaricus* (102 isolates) were predominant. Other genera found included micrococci, microbacteria and staphyloccocci.

Lb. = *Lactobacillus* *E.* = *Enterococcus* *Str.* = *Streptococcus*
Lc. = *Lactococcus* *Leuc.* = *Leuconostoc*

Table 2.3: (continued)

Country	Product	Microbiological information
Lower Egypt	Laban rayeb	Streptococci dominated in cold season samples, whereas lactobacilli dominated in the hot season. Species included *Lc. lactis, Leuc. dextranicum, Leuc cremoris, Lb. casei, Lb. plantarum* and *Lb. brevis* (Abd-el-Malek & Demerdash, 1970; cited by El-Gendy, 1983). *Lactococcus lactis, Kluyveromyces marxianus* subsp. *marxianus* and coliforms predominated (Demedash, 1960; cited by Kurmann *et al.*, 1992)
Upper Egypt	Laban Zeer	Lactococci (mainly *Lc. lactis*), *Leuconostoc* spp. and mesophilic lactobacilli such as *Lb.casei, Lb. plantarum* and *Lb. brevis* dominate in the product (Abou-Donia, 1984; cited by Kurmann *et al.*, 1992)
Morocco	Raib	Lactococci predominated, 1.4 x 10^8/g versus 2.6 x 10^6/g for lactobacilli and 2.8 x 10^6/g for leuconostocs (Hamama & Bayi, 1991). *Lc. lactis* and *Lc. diacetylactis* were the principal species found in Lben, Raib and Zabda (Hamama, 1992)
	Jben	Lactococci (5.1 x 10^8/g), lactobacilli (3.2 x 10^8/g) and leuconostocs (2.6 x 10^8/g) were found at almost the same average levels. *Lc. lactis, Lb. casei* subsp. *casei* and *Leuc. lactis* were the main species recovered in Jben (Hamama & Bayi, 1991; Hamama, 1992).
South Africa	Amasi	Undefined microflora, spontaneous fermentation (Feer, 1937; cited by Kurmann *et al.*, 1992). According to unpublished data of Keller (cited by Keller & Jordaan, 1990) hetero- and homofermentative lactobacilli, streptococci, leuconostocs and yeasts were present.
Zimbabwe	Amasi	Species isolated from Amasi made in clay pots included *Lb. helveticus, Lb. plantarum, Lb. delbrueckii* subsp. *lactis* and *Lb. casei* subsp. *casei* (Feresu & Muzondo, 1990). Naturally fermented milk produced in churns (Mutukumira, 1996), contained between 1.9 x 10^8 and 7.5 x 10^9 cfu/g lactic acid bacteria. Species isolated included *Lc. lactis, Lc. diacetylactis, Lb. plantarum, Lb. casei* subsp. *casei, Lb. acidophilus, Leuc. mesenteroides, Enterococcus faecium* and *Enterococcus faecalis* (Mutukumira, 1996).

Lb. = *Lactobacillus* *E.* = *Enterococcus* *Str.* = *Streptococcus*
Lc. = *Lactococcus* *Leuc.* = *Leuconostoc*

authorities in some countries have properly defined yogurt while other fermented milk products are usually loosely defined (Kroger *et al.*, 1992).

Yeasts are important contaminants in yogurt and sour milk because the low pH offers a selective environment for their growth. Typical defects are gas production, yeasty off-flavour and loss of texture quality (Rohm, Eliskases-Lechner & Bräuer, 1992). However, yeasts are apparently indigenous to specific traditional fermented milk such as Kefir, Koumiss, Laban rayeb and Labaneh (Kurmann *et al.*, 1992; Yamani & Abu-Jaber, 1994). Species isolated from Labaneh included *Saccharomyces cerevisiae, Kluyveromyces marxianus* and *Pichia farinosa* (Yamani & Abu-Jaber, 1994). Traditional fermented milks are good potential sources for the isolation of uncultured strains (Kurmann, 1984). These organisms may provide important resources for food technology and biotechnology (Cook, 1994).

2.5 FOOD SAFETY

Food safety is an important issue in developing countries (Reilly & Westby, 1997). The methods of production of the various traditional foods are usually primitive, compared to modern ways of food preparation (Dirar, 1997). According to Hamama (1992) Moroccan traditional fermented dairy products like Lben and Jben showed high counts of indicator micro-organisms (e.g. coliforms, enterococci) and pathogens such as *Salmonella* spp., *Yersinia enterocolitica*, *Listeria monocytogenes* and enterotoxigenic *Staphylococcus aureus* (*S. aureus*). Hamama (1992) also suggested that these high bacterial counts may be ascribed to poor hygienic conditions in the preparation of these products and/or poor bacteriological quality of the raw milk. A number of micro-organisms have been isolated and identified as contaminating microbes in Nono, as a result of the improper handling of the product. These organisms included, *inter alia*, *Escherichia coli* (*E. coli*), *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., *S. aureus*, *Clostridium welchii*, *Bacillus megatherium* and *Candida* spp. (Olasupo, 1992). Ayib purchased from markets in Ethiopia were reportedly highly contaminated with various micro-organisms (yeasts, enterococci, sporeformers, psychrotrophs, coliforms etc.) probably due to low curd cooking temperatures and the

addition of various plant materials to the finished product or packaging of Ayib with Musa leaves (Mogessie, 1992).

Concern regarding food safety is also implicated by research studies that investigated the fate of pathogens in fermented milk products, for example the fate of *Salmonella enteritidis* and *Salmonella typhimurium* during the fermentation of Ergo (Ashenafi, 1993) and the study on the survival of *Listeria monocytogenes* in three Zimbabwean fermented milk products (Dalu & Feresu, 1996). Major risk enhancing factors are the use of contaminated raw materials, lack of pasteurisation, use of poorly controlled natural fermentations and inadequate storage and maturation conditions (Nout, 1994).

Various factors contribute to the safety of fermented milks. In some cases the smoking of containers may be helpful in inhibiting the proliferation of coliforms (Ashenafi, 1996). The formation of acid is one of the greatest advantages of the fermentation process. Spoilage bacteria such as pseudomonads, *E. coli* or *Salmonella* will not tolerate the acidic conditions (Marshall, 1986). The increased concentration of lactic acid (1.92 %) of Ititu, had the beneficial effect of inhibiting the coliform count which was considerably less in the fermented product than in the raw milk used for its preparation (Kassaye *et al.*, 1991). No coliforms were found in the markedly acid samples of Laban rayeb produced during the warm season (pH 3.8), while coliform counts were high in cold season samples with pH 6.1 to 5.4 (El-Gendy, 1983).

Organic acids are not the only inhibiting factors presented by lactic acid bacteria. During fermentation inhibition of pathogens was also noted between pH 6.2 and 5.7. Therefore, the onset of inhibition of pathogens cannot be attributed only to the organic acids (Northolt, 1984; Bester, 1991). Northolt (1984) concluded that the decrease in growth of pathogens at the beginning of fermention is probably due to anti-microbial components, peroxide and a decrease in redox-potential. Low pH, lactic acid, lower fatty acids and perhaps diacetyl contribute to inhibition in the later stages. Tatini (1981), cited by Umoh *et al.* (1990) stated that staphylococcal counts decline in fermented foods whereas their enterotoxin is stable at low pH (4.0 – 4.5). Therefore fermented foods may contain enterotoxins without any viable staphylococci. Dirar (1997) stated that even when lactic acid bacteria dominated the microflora involved in

the fermentation and produced their compliment of anti-microbial substances such as acids, alcohols, hydrogen peroxide and antibiotic-like secondary metabolites, the food was not completely safe. Additional processing steps can complement lactic acid fermentation in ensuring safety (Champagne, 1997).

Pathogens must be exposed for a certain period of time to the acidity of the product so that their complete deactivation may occur (Champagne, 1997). *Salmonella enteritidis* and *Salmonella typhimurium* were not completely inhibited after 24 h in Ergo. The use of 3 day-old Ergo inoculated in boiled milk was recommended to ensure the microbiological safety of this product (Ashenafi, 1993). Corrective procedures need to be implemented to reduce risks. The use of heat-treated milk instead of raw milk and the use of selected cultures will contribute to food safety (Hamama, 1992). Furthermore, fermentation temperatures around 37 °C should be avoided since it is favourable to pathogenic bacteria (Champagne, 1997). The hygienic production of a food is much more easily monitored when modern ways of production are followed (Dirar, 1997).

2.6 MODERNISATION AND INDUSTRIALISATION OF AFRICAN FERMENTED MILKS

In addition to the production of safer foods, industrialisation has advantages such as standardised products (Reilly & Westby, 1997), reduced processing times, increased production of traditional dairy products, better distribution and marketing (Hamama, 1992). The traditional knowledge behind the production of local foods is beginning to dwindle and in time it will be lost forever. Modernisation and industrialisation would be one way to salvage these foods (Dirar, 1997). Ideas for new products may come as a development from existing ones or from re-discovering old ones (Marshall, 1986).

The provision of starter cultures is an issue commonly raised in relation to fermented foods in developing countries (Reilly & Westby, 1997). The question could be asked whether the creation of African starter culture suppliers is warranted. There might be a need for cultures specific to the African products (Champagne, 1997). According to Nout (1992) the use of pure starter cultures is not always a realistic solution because

they are expensive and require sterile processing conditions. In some cases a more feasible approach would be inoculum enrichment or back-slopping (Nout, 1992). Atanda & Ikenebomeh (1989) suggested the use of 48 h starters for the production of Nono, since a more appetizing and more acidic Nono could be produced. The use of 3 day-old Ergo as a starter for boiled milk was recommended by Ashenafi (1993). Back-slopping may not always give the best results as succession of bacterial species during the fermentation process usually results in the rapid domination of bacteria which should normally only be dominant during the final stages of fermentation (Nout, 1992).

2.7 STARTER FUNCTION

In fermented milks the processing steps and aids (enzymes, salting, cooking, whey expulsion) are minimal. In these products the starter culture is the most important factor dictating the type and character of the final product. Each of the constituent genera offers its own contribution to the character of the final product (Sanders, 1991). In 1919 three independent groups of workers in Holland, Denmark and the United States established that lactic cultures that showed good flavour development were in fact a mixture of two different types of lactic acid bacteria. One of these bacterial groups is responsible predominantly for acid production (*Lc. lactis* and *Lc. cremoris*) and the other for flavour production (*Leuconostoc* spp. and *Lc. diacetylactis*) (Heap & Lawrence, 1988).

2.7.1 Acid production

The production of lactic acid is a primary requirement of starter cultures. In most milk fermentations lactococci, or the closely related lactobacilli are responsible for the production of lactic acid (Heap & Lawrence, 1988). Lactic acid is a principal flavour component of cultured milk products. It is an odourless, non-volatile acid that creates the typical acid sensation of fermented dairy products (Margalith, 1981). It imparts a fresh flavour to fermented milks (Heap & Lawrence, 1988). Lactic acid is also needed for coagulation of milk, which takes place at pH 4.6 to 4.7. Although milk is usually considered as a rich medium for the growth of bacteria, the relatively low content of free amino acids (0.01 %) makes it rather sub-optimal for the lactic acid bacteria that

are unable to synthesize many amino acids (Reiter & Oram, 1962 cited by Margalith, 1981). Rapid growth and acid production in milk requires efficient systems for the degradation of milk protein as well as for lactose fermentation (Thomas, 1985; Heap & Lawrence, 1988; Holler & Steele, 1995). The lactococci are generally only weakly proteolytic and lypolytic. Heap & Lawrence (1988) also made the interesting remark that use of bacteria with greater biochemical diversity would almost invariably give products with undesirable organoleptic characteristics. The lactococci are better equipped for growth in milk than the leuconostocs. Leuconostocs do not grow well in milk, unless a stimulant such as yeast extract is present (Marshall, 1986). Lactococci have a proteinase associated with their cell walls so that peptides and amino acids can be readily transported into the cell. Leuconostocs, however, may grow slowly in milk because they lack this protein hydrolysis mechanism (Cogan, 1984).

According to Marshall (1987a) micro-organisms constantly respond to environmental change by either changing their genome or by phenotypic adaptation. Many lactic acid bacteria are variants which are adapted for growth in milk. Their metabolism provides a good example of the selective pressures of a natural environment. *Lc. lactis* and *Lc. cremoris* have the usual uptake mechanisms for glucose (phosphotransferase system, PTS) and galactose (permease), but also have an efficient (plasmid-encoded) lactose PTS uptake system. In addition, they have the ability to metabolize the two component sugars of lactose concurrently. Marshall (1987a) also stated that it may be significant that proteinase is also a plasmid-encoded character with the consequence that C and N metabolism is nicely "poised" for rapid growth in milk. However, it seems that the rate of casein breakdown by the proteolytic systems of the lactococci is not high enough for the cells to grow at maximal rate (Hugenholtz, 1986).

The rate at which the pH is lowered and the minimum pH obtained are the main criteria for selection of the right culture (Laulund, 1993). A predictable and controllable acid production has become an essential property of starter cultures (Laulund, 1993; Davidson & Hillier, 1995). According to Holler & Steele (1995) the ideal starter culture rapidly and dependably produces lactic acid during growth in milk. Marshall (1986) stated that organisms are selected and combined depending on the requirements of the specific fermented product. Fast acid-producing, thermophilic organisms are

used for yogurt manufacture, while buttermilk requires a slower rate of acidification. Apparently, acid production in buttermilk and cottage cheese is still more rapid than for Cheddar cheese where the steady acid production rate is an asset (Kosikowski & Mistry, 1997). Margalith (1981) stated that slow acid producers (48 h for milk coagulation at 21 °C versus 18 h for fast strains) usually gave a more delicate, pleasing flavour often preferred by consumers of cultured buttermilk.

The minimum pH of the final product has gained importance as market trends indicate that products with a high pH (>4.1) and no post-acidification are requested (Laulund, 1993). With cultures such as *Lb. bulgaricus* and *Lb. acidophilus* high values of titratable acidity (2 % and above) may be achieved. The desired acidity of traditional yogurt varies between 0.85 and 0.90 % lactic acid (Margalith, 1981). The final acidity of the product is monitored principally in relation to consumer preference and the end-point will vary from country to country. In the Netherlands, Bulgarian yoghurt may have an acidity of up to 1.48 % lactic acid, while other types were usually sold with a maximum of 1.17 % lactic acid (Tamime & Robinson, 1985). Over-acidification was less pronounced in cultured milk than in yogurt (Sinha, Modler & Emmons, 1989). According to Vedamuthu (1982) a top-quality buttermilk has a titratable acidity of 0.75 to 0.85 % lactic acid. Most of the homofermentative lactics produce abundant lactic acid (0.8 to 1.0 %), even more than is necessary for milk coagulation (Margalith, 1981). *Lc. diacetylactis* grows well in milk and is able to ferment lactose and accumulate up to 0.4 to 0.65 % lactic acid in 24 h at 30 °C (Vedamuthu, 1982). The leuconostocs grow poorly in milk and they produce very little, if any, acid from lactose in milk (Vedamuthu, 1982).

2.7.2 Gas production

Carbon dioxide provides the effervescence and "lift" to cultured buttermilk (Vedamuthu, 1994). Cogan (1985) reported that CO_2 may as well be involved in flavour perception when one considers the dramatic effect that it has on the taste of carbonated drinks. The aroma intensity and sensory impression of a cultured milk product results from a balance between various aroma compounds and CO_2, which implies that CO_2 also contributes to the acceptability of such a product (Kneifel,

Kaufman, Fleischer & Ulbreth, 1992). Carbon dioxide production from lactose by leuconostocs in milk may be as important as CO_2 production from citrate (Cogan, 1985). Gel disruption may occur from excess CO_2 production giving rise to the phenomenon of "curd floating" (Marshall & Law, 1984). In certain products a relatively high concentration of microbiologically generated CO_2 is desirable, for example for the formation of the open texture of some cheese varieties. In cultured milks too much CO_2 may cause texture problems and package swellings (Kneifel & Gretner, 1992). According to Sanders (1991) *Lc. diacetylactis* produces abundant CO_2 and may be the choice for certain applications. In the work of Sandine, Elliker & Hays (1962) only strains of *Lc. diacetylactis* (16 strains tested) were capable of producing high amounts of CO_2 (more than 450 µl) in single strain milk cultures. Twenty-three leuconostoc strains were found to be low gas producers (less than 50 µl). Kosikowski (1977) stated that this was unfortunate because *Lc. diacetylactis* strains, when producing higher than normal rates of carbon dioxide, also produced a good aroma. Excessive CO_2 production may be avoided by correct balance of cultures (Marshall & Law, 1984). Several methods to determine CO_2 generated by micro-organisms have been developed. Bellengier, Foucaud & Hemme (1993) described an enzymatic method to measure total CO_2 produced from citrate and glucose in cell suspensions of leuconostoc strains. Carbon dioxide production by different types of bacteria could be routinely determined by this quantitative method. Sandine, Elliker & Anderson (1957) developed a simple, rapid laboratory test involving an easily constructed gasometer for testing starter cultures for gas production. They classified starter cultures into low (less than 100 µl), intermediate (100 to 200 µl) and high (more than 200 µl of CO_2 produced) gas producers.

2.7.3 Flavour production

2.7.3.1 Compounds involved in flavour production

In cultured milks and cultured butter the main flavour compounds are usually assumed to be diacetyl (Van Niel *et al.*, 1929 cited by Keenan & Bills, 1968; Cogan, 1985; Marshall, 1987b; Vedamuthu, 1994) and acetaldehyde with the acid background

provided by lactic acid bacteria (Urbach, 1995). Yogurt is expected to have a buttery (diacetyl) and nutty (acetaldehyde) aroma (Marshall, 1987b), while buttermilk may have a flat or insipid flavour if diacetyl is not produced (Marshall & Law, 1984). At low concentrations diacetyl (2,3-butanedione, $CH_3COCOCH_3$) is pleasing and definitely suggests the aroma of butter cultures, but at high concentrations it is pungent and rather objectionable (Hammer & Babel, 1943). Generally, 1.6 to 4.0 ppm of diacetyl is needed to give a good "nut meat" flavour in cultured dairy products (Parker & Elliker, 1953). Acetaldehyde (CH_3CHO, ethanal) has a distinct pungent odour, which at suitable low concentrations imparts a characteristic flavour, often described as yogurt flavour (Margalith, 1981). Formisano (1974) reported values of 4 to 17.5 ppm for acetaldehyde in yogurt (cited by Margalith, 1981). Keenan, Lindsay, Morgan & Day (1966) have shown that even the standard acid producers (*Lc. lactis* and *Lc. cremoris*) will produce some acetaldehyde (5 to 8 ppm). *Lc. diacetylactis* produced 11 ppm acetaldehyde within 24 h incubation. Acetaldehyde will thus also play a role in the flavour formation in non-yogurt cultured milk products (Margalith, 1981). According to Cogan & Jordan (1994) mesophilic cultures generally produce traces (3 to 8 µg/ml) of acetaldehyde. Lindsay (1967, cited by Margalith, 1981) suggested an acetaldehyde concentration of 0.2 mg/kg and a diacetyl content of 0.5 to 2.0 ppm for a synthetic flavour for cultured butter. Acetoin production is always much greater than that of diacetyl. The ratio has been reported to be as high as 43:1 (Cogan, 1985). The reduced products of diacetyl (acetoin and 2,3-butylene glycol) are flavourless (Cogan, 1985), and at concentrations found in dairy products, probably has no effect on the taste of such products (Margalith, 1981).

The homofermentative lactics produce at least 85 % lactic acid and other metabolites (Margalith, 1981). Among the volatile acids, acetic acid (0.01 to 0.015 %) (Hammer & Sherwood, 1923 cited by Keenan & Bills, 1968) and to some extent propionic acid are also produced by the homofermentative lactics such as *Lc. lactis* and *Lc. cremoris* (Margalith, 1981). According to Chou (1962, cited by Margalith, 1981) the volatile acids such as formic, propionic, butyric and valeric acids found in cultured milk products will not play a role in flavour formation because of their minute quantities. Kosikowski & Mistry (1997) stated that with citric acid fermentation, a distinct flavour results from a fine balance between diacetyl, propionic acid, acetic acid, and other

related compounds. Overbalance of any of these essential components results in a coarse flavour.

Imhof, Glattli & Bosset (1994; 1995) investigated volatile organic aroma compounds produced by single and mixed strain dairy starter cultures. Special interest was laid on the so-called minor constituent compounds that were present in the low µg/kg range. They found that compounds that were present in low concentrations could have an influence on the aroma when their perception threshold value was very low. In mesophilic single strain starter cultures 2,3-butanedione (diacetyl), 2,3-pentanedione, and to a lesser extent dimethyl sulfide and benzaldehyde are supposed to have an impact on flavour of the end-product (Imhof *et al.*, 1995). In mixed strain dairy starter cultures 2,3-butanedione, 2,2-pentanedione, limonene, undecanal together with ethanal (acetaldehyde) are considered to be important in the resulting aroma (Imhof *et al.*, 1994).

2.7.3.2 Factors determining and influencing diacetyl production and destruction

Strains of lactic acid bacteria have different capabilities for diacetyl formation, with *Leuc. cremoris* and *Lc. diacetylactis* producing the highest levels of diacetyl in dairy products (Escamilla-Hurtado, Tomasini-Campocosio, Valdes-Martinez & Soriano-Santos, 1996). According to Marshall (1987a) the two important organisms for aroma (diacetyl) production in milk fermentations are *Leuc. cremoris* and *Leuc. lactis*. According to Cogan & Jordan (1994) the number of flavour producers in starter cultures are usually between 1 and 10 %, but the exact species of leuconostoc found was not known with any degree of certainty. *Leuconostoc cremoris*, *Leuconostoc mesenteroides* subsp. *dextranicum (Leuc. dextranicum)* and *Leuc. lactis* were likely to be involved. In the characterisation of leuconostoc isolates from commercial mixed strain mesophilic starter cultures Johansen & Kibenich (1992) identified *Leuc. cremoris* and *Leuc. lactis*. Bellengier, Hemme & Foucaud (1994) investigated citrate metabolism in leuconostoc strains (two *Leuconostoc paramesenteroides*, two *Leuc. lactis*, nine *Leuconostoc mesenteroides* subsp. *mesenteroides* and seven *Leuc. dextranicum* species). Only four leuconostoc strains (one *Leuc. paramesenteroides* and three *Leuc. mesenteroides* subsp. *mesenteroides*) produced low levels of acetoin and

diacetyl. Keenan & Bills (1968) also mentioned that some strains of *Leuc. dextranicum* produced significant amounts of diacetyl. *Lb. bulgaricus*, *Lb. helveticus*, *Str. thermophilus* and some stains of *Lc. cremoris* were indicated as important species in diacetyl production (Escamilla-Hurtado *et al.*, 1996). According to Collins & Bruhn, 1970 (cited by Ono, Goto & Okonogi, 1992) only *Lb. casei* and *Lb. plantarum* (among the lactobacilli) can metabolize citric acid like *Lc. diacetylactis*, producing lactic acid, acetic acid, acetoin and diacetyl. Seitz, Sandine, Elliker & Day (1963) showed that *Lb. casei* possessed diacetyl-metabolizing enzymes and supplied a buttery aroma to milk fermented with this organism.

Growing cultures of *Lc. diacetylactis* and leuconostocs behave completely different in the production of diacetyl and acetoin. *Lc. diacetylactis* produce diacetyl and acetoin as soon as growth begins (Cogan, 1985) while the leuconostocs need an acidic environment for the production of these compounds (Vedamuthu, 1994). The normal pH of milk is between 6.4 and 6.6. Citrate uptake by leuconostocs is active only below pH 6.0; therefore sufficient acid production by lactococci is needed to depress the pH below 6.0 (Vedamuthu, 1994). *Leuconostoc* species metabolize citrate without producing diacetyl or acetoin at neutral pH. Thus, one needs to ensure that the leuconostocs present in mixed cultures will not grow sufficiently rapidly to utilise all the citrate before a low pH is reached (Cogan, 1985).

The associative growth of lactic acid bacteria is a frequent phenomenon in the dairy industry. Many flavour nuances of dairy products, as well as unwanted deviations from the desired flavour profile, may be related to fluctuations in the associative growth of these organisms (Margalith, 1981). Hucker & Pederson, 1930 (cited by Vedamuthu, 1994) stated that the types of leuconostocs found in milk products are not in their true habitat and pure cultures are inert in milk. Lactococci produce stimulatory substances necessary for the growth of leuconostocs. The selection of compatible strains of the two groups is necessary. Only a small number of the combinations of a series of lactococci and a series of leuconostocs can be expected to prove satisfactory (Foster *et al.*, 1957 cited by Vedamuthu, 1994). According to Levata-Jovanovic & Sandine (1996) the production of diacetyl and acetoin by leuconostoc strains in combination with different lactococci strains was not reproducible in all experimental replications.

Inconsistent results reflect the complexity of diacetyl production by leuconostocs in milk in the presence of lactococci and the complicated interrelationship (symbiosis) between growth and citrate metabolism in mixed cultures (Levata-Jovanovic & Sandine, 1996). Cogan (1985) reported that, although it has been accepted for a considerable time that a symbiosis exists between the lactococci and the leuconostocs in mixed cultures, the nature of the interaction has never been determined. B and BD cultures were initially haphazard but today stable mixtures are in use that have evolved through continual use (Cogan, 1985).

The temperature of incubation will also influence flavour production. The leuconostocs must be permitted to reach a high population before the pH drops to a low level, so that there will be enough leuconostoc cells to carry through citrate metabolism. A balanced growth of lactococci and leuconostocs occurs during incubation between 21 and 25 °C. Above 25 °C the metabolically more active lactococci are favoured (Vedamuthu, 1994). At 22 °C the average generation time (GT) for the leuconostocs (3.5 h), is more than double that of *Lc. cremoris* (1.5 h) in complex broth. Larger differences in GT would probably exist in milk because of the inability of the leuconostocs to grow well in it (Cogan, 1985). Such differences in GT could easily lead to domination of the leuconostocs by the lactococci in mixed cultures. The fact that mixed cultures are quite stable in commercial practice, indicates that there are other factors involved (Cogan, 1985). Pack, Vedamuthu, Sandine, Elliker & Leesment (1968) found a greater number of flavour producers in B and D cultures when incubated at 30 °C than when incubated at 21 °C, but greater amounts of diacetyl were produced at 21 °C. This may be due to decreased activity of diacetyl reductase at 21 °C or to slower growth of the flavour producers and, at least in the case of the B culture, the presence of more citrate to act as precursor at low pH. Narvhus, Østeraas, Mutukumira & Abrahamsen (1998) analysed the volatile compounds produced by a malty compound-producing strain of *Lc. diacetylactis* in fermented milk. Diacetyl, acetoin, acetaldehyde and the malty aldehydes were more rapidly produced in products fermented at 37 °C and their reduction began earlier. For the products incubated at 22 °C, a decline in the concentration of acetaldehyde and diacetyl did not occur until during refrigerated storage.

The concentration of diacetyl in dairy fermentations neither accumulates indefinitely nor remains unchanged (Vedamuthu, 1994). The lactic acid bacteria that produce acetoin and diacetyl are also able to reduce these compounds to butanediol (Hugenholtz, 1993). Diacetyl reductase (EC 1.1.1.5) is the enzyme responsible for the irreversible reduction of diacetyl to acetoin and the further reduction to 2,3-butanediol. Diacetyl reductase (DR) is widely distributed among bacteria (Seitz *et al.*, 1963). Citrate represses the syntheses of diacetyl reductase and acetoin reductase (AR). With the completion of citrate metabolism, increased synthesis of DR and AR occurs resulting in decreased levels of diacetyl and acetoin (Cogan, 1985). Escamilla-Hurtado *et al.* (1996) stated that a stage of increase of diacetyl production has been observed in several foods lasting 12 to 24 h to be followed by a slow decrease even if the microbial growth continues, in contrast with the rate of acetaldehyde formation which is closely related with the microbial growth. Levata-Jovanovic & Sandine (1996) found large differences in DR activities observed among strains of *Leuc. cremoris*. DR is present at much higher levels in some coliform and pseudomonads than in lactic acid bacteria (Cogan, 1985). Contamination by these bacteria may lead to a lack in flavour. Immediate cooling of cultured products to a temperature below 7 °C arrests the destruction of diacetyl by retarding diacetyl reductase activity (Vedamuthu, 1994).

Citrate is the most important substrate for the production of diacetyl and CO_2 and its concentration in milk may vary throughout the dairying season. The failure of diacetyl flavour development or CO_2 production in cultured dairy products may be due to an insufficient concentration of citrate in milk. *Lc. diacetylactis* mutants that produce diacetyl and CO_2, independent of the citrate content of milk, might be obtained by isolating variants deficient in lactic dehydrogenase. The effect would be a natural accumulation of pyruvate, independent of citrate concentration (McKay & Baldwin, 1990). Milk usually contains 0.15 to 0.18 % citric acid. Addition of 0.3 to 0.38 % citric acid enhances aroma production (Margalith, 1981; Escamilla-Hurtado *et al.*, 1996). Diacetyl formation is strongly related to oxygen availability (Branen & Keenan, 1970) and the incorporation of air by agitation in actual commercial production yielded better flavour (Vedamuthu, 1994; Escamilla-Hurtado *et al.*, 1996). The manganese level in the milk also influences diacetyl levels. Lower diacetyl levels during spring are a direct result of low manganese levels in milk and the consequent inability of the

leuconostocs to grow adequately (Cogan, 1985; Cogan & Jordan, 1994). The genetics of the lactic acid bacretia may also influence flavour production because, like the other industrially important parameters of starter cultures (e.g. lactose and protein metabolism), citrate metabolism is also encoded by plasmid DNA. In many strains plasmid DNA is easily lost and such strains cannot metabolize the compound for which that plasmid coded nor can they regain the ability to do so except by gene transfer (Cogan, 1985).

2.7.3.3 Metabolism problems in relation to culture use and balance of cultures

Green or yogurtlike off-flavours in fermented products are caused by over-production of acetaldehyde in relation to diacetyl. The diacetyl to acetaldehyde ratios and flavours encountered by Lindsay, Day & Sandine (1965); Margalith (1981) and Vedamuthu (1994) were:

13:1	↔	5:1	↔	3:1	↔	0,4:1
Harsh		Good		Green		

Cogan (1985) was of the opinion that the times of peak production for diacetyl and acetaldehyde are different and that the optimum ratio of 4:1 found by Lindsay *et al.* (1965) was not an absolute figure and should only be used as a guideline. Green flavours in fermented products caused by excessive acetaldehyde production may be controlled with starter strains high in alcohol dehydrogenase (aldehyde reductase) activity or by ensuring that high populations of leuconostocs are present (Sandine, Daly, Elliker & Vedamuthu, 1972). All the dairy leuconostoc species studied by Keenan & Lindsay (1966) were capable to remove acetaldehyde from milk cultures, with *Leuc. cremoris* showing the highest activity.

Varying percentages regarding the content of leuconostocs and other aroma bacteria in starter cultures have been reported in the literature. According to Keenan *et al.* (1966) the green flavour defect could be overcome by incorporating 25 to 50 % leuconostocs in lactic starter cultures. Metabolism of pyruvate to compounds other than lactate gives rise to CO_2. Correct balance of culture is thus important to avoid excess CO_2

production. Pettersen (1975) (cited by Marshall & Law, 1984) investigated the balance for Scandinavian buttermilk and suggested a 20 % proportion of aroma bacteria. In composite commercial culture concentrates the proportion of leuconostocs varied between 5 and 10 % of the culture (Farr, 1969) (cited by Vedamuthu, 1994). Products made with such cultures often lacked full aroma and flavour because of the lower viable cell numbers of the aroma bacteria per unit volume or weight of cell concentrate relative to those of the lactococci (Vedamuthu, 1994). Cogan & Jordan (1994) reported that the number of flavour producers in starter cultures varied between 1 and 10 %, but that the exact species of leuconostoc found was not known. Aroma bacteria were in general less than 10 %, in certain cases only 1 to 2 %, of the lactic microflora (Margalith, 1981).

3. MATERIALS AND METHODS

3.1 Collection of samples

Indigenous fermented milk samples were obtained from individual households in rural areas indicated in Table 3.1. After collection, the samples were kept on ice and transported to the laboratory to be analysed within 36 to 48 h. On receipt the pH of the samples was measured with a Radiometer pH meter (pH meter 29, Copenhagen, Denmark) with a combined glass/reference electrode (GK 2401 C, Radiometer, Denmark). The samples were then stored under refrigeration at 4 - 7 °C, those in clay pots and calabashes were covered with aluminium foil.

Table 3.1: Traditional fermented milk samples collected in South Africa and Namibia

Sample number	Collection point	Type of container
1	Badplaas	Plastic container
2	Trichardtsdal	Clay pot
3	Gazankulu	Clay pot
4	Gazankulu	Clay pot
5	Gazankulu	Clay pot
6	Tzaneen	Clay pot
7	Tzaneen	Clay pot
8	Tzaneen	Calabash
9	Tzaneen	Calabash
10	Nelspruit	Clay pot
11	Eerstehoek	Calabash
12	Ovamboland	Plastic container
13	Kaokoveld	Calabash
14	Swartbank Kuseb	Calabash
15	Soutrivier Kuseb	Calabash

3.2 Microbiological analysis

3.2.1 *Enumeration of micro-organisms*

Total aerobic plate counts, lactic acid bacterial counts, coliform counts (including *E. coli*) and yeast and mould counts were performed on each fermented milk sample. Proteolytic bacteria and citrate fermenting organisms were also enumerated. Ten millilitres of the fermented milk were pipetted aseptically into 90 ml of quarter strength Ringer's solution and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-8}) were made and 1 ml portions of the appropriate dilutions were pour-plated on the media indicated in Table 3.2. The spread plate technique was used for determination of the yeast counts. Anaerobic jars (Biolab and Oxoid) with gas generating kits (Oxoid BR 38B) were used for anaerobic incubation. Plates having between 30 and 300 colony forming units (cfu) were counted. Plates of violet red bile agar with 4-methylumbelliferyl-B-D-glucuronide (MUG) were examined under long wave UV light (366 nm) for the detection of presumptive *E. coli*.

3.2.2 *Isolation, cultivation and preservation of lactic acid bacteria*

Ten isolates each were isolated from the countable plates of respectively Rogosa agar, M17-agar, MRS agar (incubated at 35 and 42 °C). Pakes' enumerating disc (Harrigan & McCance, 1966) was used to ensure random selection of colonies. The isolates were cultivated in MRS broth (Oxoid CM359) at 25 °C and their purity checked by streaking on MRS agar. Isolates were preserved by a modification of the method described by Joubert & Britz (1987) and stored at –20 °C.

3.2.3 *Identification of lactic acid bacteria to genus level*

Gram-positive, catalase-negative isolates were assigned to a genus on the basis of key characteristics and tests indicated in Table 3.3. Microscopic appearances of day-old cultures were judged using Gram-stained preparations (Gerhardt, Murray, Costilow, Nester, Wood, Krieg & Phillips, 1981). Growth at 10, 15 and 45 °C was assessed in tubes with MRS broth inoculated with 24 to 48-h old cultures and incubated in waterbaths. Growth was evaluated visually after 3 d incubation. Tests for presence of catalase, production of ammonia from arginine and production of CO_2 from Gibson's medium

Table 3.2: Methodology for microbiological analysis of indigenous fermented milks

Microbiological count	Growth medium	Incubation			Reference
		Time (h)	Temp (°C)	Atmosphere	
Total aerobic plate count	Plate count agar (Oxoid M325)	72	30	Aerobic	International Dairy Federation, 1991
Lactic acid bacteria count:					
Thermophilic lactobacilli and streptococci	MRS agar (Oxoid CM361)	48	42	Anaerobic	De Man, Rogosa & Sharpe (1960)
Mesophilic lactobacilli and leuconostocs	MRS agar (Oxoid CM361)	48	35	Anaerobic	De Man *et al.* (1960)
Lactococci	M17-agar (Oxoid CM 785)	48	30	Aerobic	Terzaghi & Sandine (1975)
Lactobacilli	Rogosa agar (Merck)	48	35	Anaerobic	Rogosa, Mitchell & Wiseman (1951)
Coliform count (including *E.coli*)	Violet red bile agar (Oxoid CM107 adding supplement BRO 71 E)	24	37	Aerobically	International Dairy Federation, 1985
Yeasts	Yeast malt extract agar	96	25	Aerobic	Wickerham (1951)
Proteolytic bacteria	Milk agar	24-48	25	Aerobic	Harrigan & McCance (1976)
Citrate-fermenting bacteria	Modified Nickels and Leesment medium	48	25	Aerobic	Vogensen *et al.* (1987)

Table 3.3: Differential characteristics of lactic acid bacteria (Harrigan & McCance, 1976; Garvie, 1984; Hammes, Weiss & Holzapfel, 1992; Holzapfel & Schillinger, 1992; Teuber, Geis & Neve, 1992; Weiss, 1992; Axelsson, 1993)

Characteristics	Leuconostocs	Streptococci			Enterococci	Pediococci	Lactobacilli		
		Pyo-genes	Viri-dans	Lactic			Strepto[a]	Thermo[b]	Beta[c]
Cell form	Spherical but often lenticular	Spherical or ovoid			Spherical to ovoid	Spherical	Rods/Coccobacilli		
Cellular arrangement	Pairs and chains	Chains and pairs			Mainly in pairs short chains	Pairs, tetrads, clusters, single cells are rare no chains	Chain formation common		
Growth at 10 °C	+	-	-	+	+	±	ND	ND	ND
15 °C	+	ND	ND	ND	ND	ND	+	-	±
45 °C	-	-	+	-	+	±	±	+	±
NH_3 from arginine	-	+	-	±	+	±	-	±	±
Gas from glucose	+	-	-	-	-	-	-	-	+
Growth in 6.5 % NaCl	±	-	-	-	+	±	±	±	±
Reaction in litmus milk	Comparatively inactive Few strains capable of producing acid Very few strains capable of clotting the milk No strains giving reduction	No reduction of litmus before clotting of milk		ARC	ARC	Comparatively inactive Rarely produce sufficient acid to cause clotting	Various reactions depending on the species		

[a] Streptobacterium
[b] Thermobacterium
[c] Betabacterium

+ = Positive
- = Negative
± = Response varies between species

ARC = Acid, reduction, clot
ND = No data

were carried out as described by Harrigan & McCance (1976). The salt tolerance test was done using MRS broth, containing 6.5 % (m/v) NaCl and incubated for 4 d at 37 °C. All isolates were also tested for their action on chalk litmus milk (Oxoid CM45 with added calcium carbonate) (Harrigan & McCance, 1976). Kanamycin aesculin azide agar (Oxoid CM591) was used for the presumptive identification of enterococci. Nickels and Leesment medium as modified by Vogensen, Karst, Larsen, Kringelum, Ellekaer & Waagner Nielsen (1987) were used to differentiate between citrate fermenters and non-citrate fermenters. The insoluble calcium-citrate is hydrolyzed by the citrate fermenters, which form a clear zone around the colony.

3.2.4 Identification of the lactic acid bacteria to species level

3.2.4.1 Lactococci

Arginine tetrazolium agar (Harrigan & McCance, 1976) was used to differentiate between *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*. *Lactococcus lactis* subsp. *lactis* gives rise to pink/red coloured colonies while *Lc. lactis* subsp. *cremoris* gives rise to white coloured colonies. The colony change of *Lc. lactis* subsp. *lactis* is due to its ability to produce ammonia from arginine (Turner, Sandine, Elliker & Day, 1963).

3.2.4.2 Leuconostocs

The scheme given by Villani, Moschetti, Blaiotta & Coppola (1997) (Fig. 3.1) was used for the presumptive identification of the leuconostoc species. The test for the production of dextran from sucrose on Sucrose agar (Garvie, 1984) was used to divide all the leuconostoc isolates into two groups namely dextran-producing and non-dextran-producing isolates. Subsequent tests done on these two groups differ. Dextran producing isolates were tested for fermentation of arabinose, maltose, raffinose, galactose and for growth at 37 °C. Non-dextran producing isolates were tested for fermentation of sucrose, fructose, galactose and trehalose. Growth at 15 °C and growth in MRS broth containing 6.5 % (m/v) NaCl were also tested. The basal medium given by Garvie (1984) was used for all the carbohydrate fermentation tests. The individual sugars were prepared as 2 % (m/v) solutions and 0.5 ml of the sterile filtrate was added to 5 ml of basal medium.

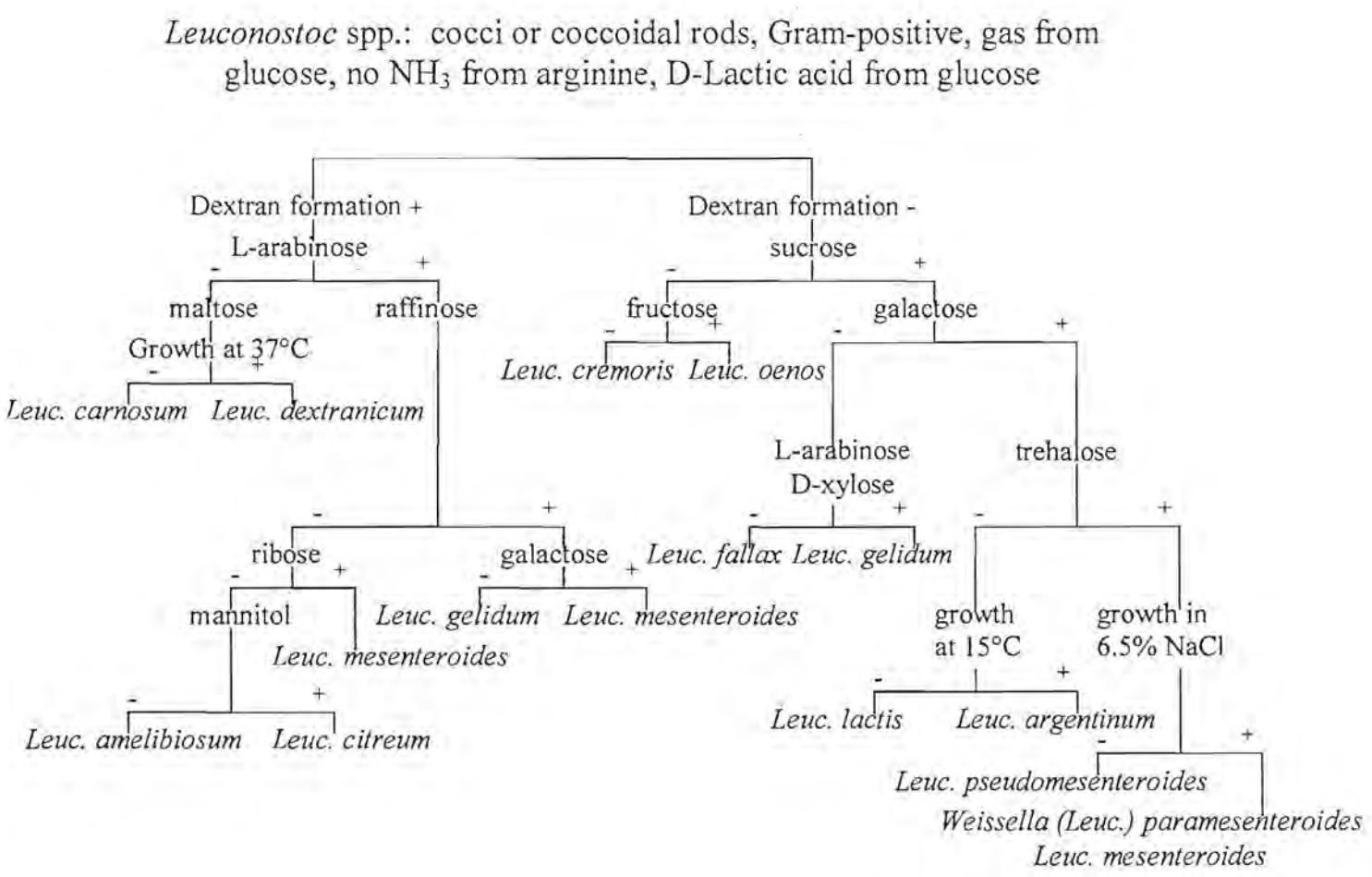

Fig.3.1: Scheme for presumptive identification of *Leuconostoc* spp. (Villani, Moschetti, Blaiotta & Coppola, 1997)

Growth studies at 15 °C and 37 °C were done in MRS broth.

3.2.4.3 Identification using the API 50 CH system

Ten representative strains were selected for identification to species level using the API 50 CH identification system (BioMérieux sa). Seven strains presumptively identified as lactobacilli, one leunonostoc and two strains from the *Leuconostoc*/Betabacterium group were selected. Bacterial cells were inoculated into tubules of the API galleries according to the manufacturer's instructions. The strips were incubated at 30 °C and changes in colour were observed after 24 and 48 h. The APILAB PLUS database (BioMérieux) was used to interpret the results.

3.2.5 Detection of pathogens

All samples were tested by the accredited laboratory at the ARC-Animal Nutrition and Animal Products Institute, Irene for the presence of *Salmonella* species, *Staphylococcus aureus* and *Listeria monocytogenes*. The following methods were used:

Salmonella species: The reference method of the IDF (International Dairy Federation, 1995a), which utilises Brilliant Green agar, Bismuth Sulphite agar and XLD (Xylose Lysine Desoxycholate) medium as a selective solid medium. was used for the detection of *Salmonella* species.

Staphylococcus aureus: The IDF reference method was used (International Dairy Federation, 1990).

Listeria monocytogenes: Listeria selective medium (Oxford formulation) as well as PALCAM Listeria selective agar were used for isolation and presumptive identification of *Listeria monocytogenes* (International Dairy Federation, 1995b).

3.3 Evaluation of technologically important properties of lactic acid bacteria

3.3.1 Acid production

The acid production of the lactococci was evaluated by monitoring their ability to coagulate sterile 10 % reconstituted non-fat milk (NFM) within 16 h at 22 °C using a 1 %

(v/v) freshly coagulated inoculum (Huggins & Sandine, 1984). Freshly coagulated cultures were prepared by inoculating 0.1 ml of MRS broth cultures into 10 ml of sterilised reconstituted NFM (10 % m/v). Cultures that coagulated within 16 h at 22 °C were used to re-inoculate reconstituted NFM in triplicate using a 1 % inoculum. After an incubation period of 16 h at 22 °C the pH and titratable acidities of the replicates were measured. A PHM 83-autocal-pH meter (Radiometer, Denmark) was used to measure the pH. Cultures were incubated in waterbaths controlled at 22 °C.

Automatic end-point titrations were performed using a TTT 80 titrator, ABU 80 Auto burette and a PHM 83-autocal-pH meter (Radiometer, Denmark). Nine grams of coagulated milk were weighed into conical plastic flasks and titrated with 0.1 N NaOH while stirring with an automatic stirrer. A pH of 8.4 was used as the end-point for the titrations. A number of titrations were also done by hand (Dixon, 1973) for comparison with the automatic titrations. Commercial cultures (CH-Normal 22 and R-707 phage control, Chr. Hansen's, Denmark) were included in the activity tests for comparative purposes.

3.3.2 Gas production

Gas production by the leuconostoc isolates was determined by inoculating the strains into sterile reconstituted NFM separately, and in combination with a commercial mesophilic homofermentative starter culture containing only acid producers (R-707 phage control, Chr. Hansen's, Denmark). Inocula of 1 % were used and the test tubes were sealed with 1 to 2 cm layers of 1.2 % (m/v) bacteriological agar (Biolab) and incubated at 22 °C. Gas production was evaluated visually by observing the disruption of the agar plug after 16 and 48 h. These tests were also duplicated in reconstituted NFM with 3 % (m/v) added sodium citrate (Parmelee, 1967). The commercial starter culture was cultured in 10 % (m/v) reconstituted NFM and the isolates in MRS broth.

3.3.3 Production of volatile organic compounds by selected strains

3.3.3.1 Preparation of the milk samples for determination of volatile organic compounds

The following lactic acid bacterial cultures were used for determination of volatile organic compounds:

(i) Four strains isolated from traditional fermented milk and identified with the API identification system.

(ii) A mesophilic aroma producing heterofermentative culture (CH-Normal 22, Chr. Hansen's).

(iii) A strain of *Lc. diacetylactis* (culture collection of the ARC-Animal Nutrition and Animal Products Institute, originally from Chr. Hansen's laboratory).

Table 3.4: Preparation of samples for the determination of volatile organic compounds

Strain/culture description	Culture designation	Inoculum
Leuc. lactis	A2	10 % of a freshly coagulated NFM milk culture
Leuc. lactis	A4	Concentrated cells obtained from MRS broth cultures
Lb. plantarum	A6	Concentrated cells obtained from MRS broth cultures
Lb. plantarum	A7	Concentrated cells obtained from MRS broth cultures
Commercial culture	CHN 22	Concentrated cells obtained from MRS broth cultures
Lc. diacetylactis	M15	1 % of a freshly coagulated culture

The cultures were inoculated (Table 3.4) into 100 ml reconstituted non-fat dry milk (NFM) (10 % m/v), either from freshly coagulated NFM or concentrated cells obtained from MRS broth cultures. Cells were concentrated by centrifuging for 10 min at 2500 r/min. The flasks with the inoculated NFM were incubated at 22 °C for 24 or 48 h. At the time of coagulation the cell numbers in the NFM samples, designated for determination of volatile organic compounds, were determined by plating on MRS agar. Dilutions were prepared as described in 3.2.1. and the plates incubated at 30 °C for 48 h. The pH values of the six samples were measured using a PHM 83-autocal-pH meter (Radiometer, Denmark).

3.3.3.2 Determination of volatile organic compounds

The volatile organic compounds were determined by the accredited laboratory of the Food Science and Technology Division of the Council for Scientific and Industrial Research (Pretoria). Equilibrium headspace gas chromatography applying a polyethylene glycol column was used. The temperature setting was programmed to increase from 50 to 80 °C at a rate of 5 °C/min. A flame ionisation detector was employed. Quantification was based on recorded peak areas as no calibration was done.

3.3.4 Growth characteristics of selected strains in reconstituted non-fat milk

The growth characteristics of nine selected strains (No.'s 1, 2, 4, 5, 6, 7, 8, 9 and 10) in NFM were evaluated by monitoring the time needed for coagulation. A primary culture (mother culture) of each individual strain was prepared by inoculating NFM with concentrated cells harvested from MRS broth cultures. The time needed for these primary cultures to coagulate was documented. The primary culture was used to make subsequent inoculations in NFM (10 % m/v) in 100 ml quantities at increasing inoculation levels (1, 5, 10 and 20 %). The cultures were incubated in waterbaths at controlled temperatures of 22 and 30 °C and visually evaluated for coagulation after 24, 48 and 72 h.

3.4 Evaluation of selected strains for their suitability as starter cultures for fermented milk products

3.4.1 Preliminary evaluation of selected strains using "bench-top" sensory evaluation

Six selected strains, previously isolated from naturally soured milk, were used as starter organisms for the manufacturing of fermented milk products. One strain of *Lc. diacetylactis* (M15) was included in this investigation principally for its role as a flavour producer. The seven selected strains (Table 3.5) were used individually and in specific combinations to manufacture fermented milk products.

Table 3.5: Preparation of fermented milk products with seven selected strains for evaluation of flavour acceptability

Number of product	Strain	Strain designation or description	Percentage inoculum
1	*Leuc. lactis*	A2	25
2	*Leuc. dextranicum*	A10	10
3	*Leuc. paramesenteroides*	Mixture of all the strains assigned to this species	10
4	*Lb. plantarum*	A7	25
5	*Lc. lactis*	S6M3	10
6	*Lc. lactis*	S6R5	10
7	*Lc. lactis; Leuc. lactis*	S6M3; A2	5; 5 *
8	*Lc. diacetylactis*	M15	25
9	*Lc. lactis; Leuc. lactis; Lc. diacetylactis*	S6M3; A2; M15	5; 5; 5 *
10	*Lc. lactis; Leuc. dextranicum*	S6M3; A10	5; 5 *

* 5% of each strain used

The aim of this experiment was to select strains that were most suitable for inclusion in starter cultures for fermented milk products. A primary culture of each strain was prepared as indicated in 3.3.4. Full-fat milk (FFM) with a fat percentage of *ca.* 3 % was used in the preparation of the fermented milk products designated for sensory evaluation. Quantities of 100 ml milk in Schott bottles were pasteurised in a waterbath at 85 °C for 30 min. The bottles with milk were immediately cooled in ice before inoculation with the primary cultures (Table 3.5). The bottles with milk were incubated in a waterbath at 22 °C for 16 h and cooled in ice before evaluation for sensory acceptability. An untrained panel consisting of members of the dairy laboratory staff was asked to characterise the aroma of the products after fermentation. The samples were assessed at *ca.* 5 °C. Comments on each product were documented. The pH of the 10 fermented milk products was determined at *ca.* 25 °C. On account of these results three strains were selected for further investigation.

3.4.2 Evaluation of three selected strains for their suitability as starter cultures for fermented milk products

3.4.2.1 Obtaining commercial "Maas" products

Commercial Maas products of 4 different manufacturers (two containers of 2 litres each) were purchased from two separate retail stores in the local marketplace. All samples were obtained on the day before the products were used for vocabulary development by the panel of judges. The expiry dates of all the products were beyond the date of completion of the sensory analysis. The fermented milks were stored at 5 °C.

3.4.2.2 Training and vocabulary development for sensory evaluation using four commercial products

The sensory panel consisted of six adult women who were staff members of the ARC-Animal Nutrition and Animal Products Institute at Irene. All panel members were trained in evaluating the primary tastes. Training was done in two two-hour sessions on two consecutive days. In the first session the panel was briefed on the nature of the product. They tasted all four commercial products and a list of descriptors was developed during a

panel discussion. In the second session a preliminary score card was used for evaluation of three of the commercial products. Reference standards (Table 3.6) were provided to help in reaching consensus on the definition of a few terms. Panellists rated the descriptors for each commercial product using a 6-point intensity scale (1=none, 2=slightly, 3=moderately, 4=fairly, 5=very, 6=extremely). After the two days of training a sensory score card for sensory evaluation as well as a list of the definitions of terms was finalised and documented (Appendix A).

Table 3.6: Reference standards for selected descriptors

Term		Reference standard
Aroma:	Sourness	Sour cream
Flavour:	Sourness	Sour cream
Aroma:	Cottage cheese	Cottage cheese
Flavour:	Cottage cheese	Cottage cheese
Mouth-feel:	Creamy	Fresh cream

3.4.2.3 Manufacturing of four fermented milk products using three selected strains of lactic acid bacteria

Primary cultures were prepared by inoculating sterile NFM (10 % m/v) and Marlac-medium (8.9 % m/v) with concentrated cells of each individual strain, harvested from MRS broth by centrifugation at 2500 r/min for 10 min. The NFM-cultures were incubated at 22 °C for 16 h while the cultures cultivated in Marlac bulk starter medium (Seravac Division of Prochem (Pty) Ltd) were incubated on magnetic stirrers in a temperature-controlled room at *ca.* 30 °C for 16 h. Full-fat milk was used for the manufacturing of the final fermented milk products. The milk (3-liter quantities in Erlenmeyer flasks) was pasteurised at 85 °C for 30 min in a waterbath. After the milk had been cooled in ice it was inoculated with primary cultures which have been propagated in NFM or Marlac-medium (Table 3.7). The fermented milk products were incubated in

Table 3.7: Composition and percentage inoculum of starter cultures used in the manufacturing of four fermented milk products

Propagation medium	**Marlac**		**NFM**	
Product code	E1	E2	E3	E4
Strains:				
Lc. lactis S6M3	0.06%	0.06%	1%	1%
Leuc. lactis A2	0.06%	0.06%	1%	1%
Lc. diacetylactis M15	0.2%	0.4%	10%	20%

waterbaths at 22 °C for 16 h.

3.4.2.4 Sensory evaluation of four commercial and four experimental fermented milk products

The six panel members became accustomed to tasting fermented milk products during the preliminary sessions in which the vocabulary was developed. Sensory evaluation was conducted within the sensory laboratory at the ARC-Animal Nutrition and Animal Products Institute at Irene. The four commercial products were evaluated on the first three days. Samples were presented in two sets, each consisting of two samples. Panellists rested for a while between sets to avoid or minimise fatigue. Each session lasted 10 or 15 min depending on the panellist. The fermented milk products were shaken 10 times in their original containers before transferring a sample of 50 ml into 100 ml glass beakers. Samples were coded with three-digit random numbers on the tinfoil lid covering each beaker. The serving temperature was approximately 5 °C. Instructions for evaluation of appearance, aroma, visual texture, mouth-feel, flavour and aftertaste were given on the sensory score card. Panellists were instructed to eat a piece of carrot and rinse their mouths with water between samples to clean the pallet. The manufactured milk products were evaluated in the same manner on the last three days.

3.4.2.5 Determination of pH, viscosity and flow properties of the fermented milk products

The pH of each fermented milk product was measured on every day of the sensory evaluation of the products. A PHM 83-autocal-pH meter (Radiometer, Denmark) was used at room temperature. The Rapid Visco Analyser (Newport Scientific) was used for the objective measurement of viscosity. Thirty grams of each sample was weighed into a canister. The sample was initially mixed at a speed of 400 r/min (minimum for mixing) for 10 s. The samples were stirred for another 5 min at 160 r/min at 25 °C. The viscosity in mPa.s after 5 min was documented and used for comparison. Measurements were done in duplicate or in triplicate where necessary. The line-spread test (Campbell, Porter Penfield & Griswold, 1987) was used to measure the consistency of the fermented milk products in terms of the distance that it spread on a glass plate within 2 min.

3.4.2.6 Statistical analysis

The data for each attribute were analysed by using a SAS computer program (SAS, 1985) for the ANOVA model. Main effects analysed were panellist, product, day and serving position of the sample. Mean, standard deviation, minimum and maximum values for every variable were calculated for each product. The average of the mean values for every variable was also calculated for the four commercial products and the four manufactured fermented milk products, respectively. For further statistical analysis the results of the first three days (evaluation of commercial products) and that of the last three days (evaluation of the experimental fermented milk products) were treated separately. Correlation analysis (Pearson correlation coefficients) was based on estimated mean values that compensated for differences caused by the fact that the commercial and fermented milk products were evaluated on different days. Principal component analysis (PCA) was applied based on the correlation matrix. Further simplification was achieved by varimax rotation of the significant principle components. Analysis of variance, correlation and principle component analyses were conducted using SAS statistical package (SAS Institute, Cary, NC). Statistical significance was accepted if the probability values were $p \leq 0.05$ for the ANOVA and correlation.

4. RESULTS

4.1 Collection of samples

A total of 15 naturally soured milk samples were collected in South Africa and Namibia. Detailed information of the areas from which the samples were obtained, the types of containers in which fermentation took place as well as the pH of the samples when received is given in Table 4.1.

Table 4.1: Traditional fermented milk samples collected in South Africa and Namibia

Sample no.	Collection point	Type of container	pH
1	Badplaas	Plastic container	4.9
2	Trichardtsdal	Clay pot	5.4
3	Gazankulu	Clay pot	4.1
4	Gazankulu	Clay pot	4.45
5	Gazankulu	Clay pot	4.0
6	Tzaneen	Clay pot	4.9
7	Tzaneen	Clay pot	5.0
8	Tzaneen	Calabash	4.45
9	Tzaneen	Calabash	4.51
10	Nelspruit	Clay pot	4.8
11	Eerstehoek	Calabash	4.1
12	Ovamboland	Plastic container	ND
13	Kaokoveld	Calabash	ND
14	Swartbank Kuseb	Calabash	ND
15	Soutrivier Kuseb	Calabash	ND

ND = Not determined

4.2 Microbiological analysis

4.2.1 Enumeration of micro-organisms

All the microbiological counts obtained on the different media are fully expanded in Table 4.2. Counts on MRS agar (35°C) and M17-agar were on average higher than the total plate count, indicating the predominance of the lactic acid bacteria. These counts also exceeded counts obtained from Rogosa agar. Thermophilic counts on MRS agar (42 °C) were noticeably less than the mesophilic counts (35 °C). The mean value for the counts of lactic acid bacteria obtained on MRS and M17-agar was 7.38×10^8 cfu/ml, but when counts on Rogosa agar were also included, the mean count was 5.15×10^8 cfu/ml. The counts for citrate fermenting organisms varied between 1.35×10^6 and 8.4×10^8 cfu/ml (samples 1-11) while the yeast counts ($7 \times 10^3 - 2.1 \times 10^7$: mean value = 4.1×10^6 /ml) were noticeably lower than the lactic acid bacteria counts. The numbers of caseolytic organisms were also relatively high and contributed substantially to the composition of the total bacterial population. Coliform counts varied between <1 and *ca.* 1.5×10^7 /ml (mean value = 1.55×10^6 /ml). The coliform counts from samples 6 and 7 (clay pots, CP) were respectively *ca.* 3 million and 15 million per ml. An important finding was the presence of *E. coli* in samples 3, 4 and 5 with counts of 190×10^2, 0.7×10^2 and 0.4×10^2 /ml respectively. The mean microbial counts on all media, except for Yeast malt extract agar, were higher for samples in clay pots than for samples in calabashes (CB) (Table 4.3).

4.2.2 Identification of the lactic acid bacteria to genus level

Four-hundred-and-twenty-eight isolates from samples 2 to 10 were subjected to primary identification tests. Most of the isolates were Gram-positive and catalase-negative. A total of 366 isolates could be identified and were divided into 5 genera: *Lactococcus, Leuconostoc, Lactobacillus*, *Enterococcus* and *Streptococcus*. Identifications were based on the secondary phenotypic characteristics summarised in Table 4.4. The total numbers of isolates within each genus as well as the number of positive and negative isolates for specific tests are also indicated. Some heterofermentative lactobacilli grow as

Table 4.2: Microbiological counts (per ml) obtained from traditional fermented milk samples

Sample no.	Culture media								
	Plate count agar	MRS agar incubated at 42°C	MRS agar incubated at 35°C	M17-agar	Rogosa agar	Violet red bile agar	Yeast malt extract agar	Milk agar	Nickels and Leesment medium
PC 1	133×10^7	1.01×10^6	14.2×10^8	14×10^8	11.1×10^7	1.25×10^4	7.4×10^5	1500×10^6	840×10^6
CP 2	ND	1270×10^6	11.7×10^8	12.6×10^8	12.8×10^7	ND	0.07×10^5	720×10^6	102×10^6
CP 3	11.8×10^7	28×10^6	6.5×10^8	5.2×10^8	11.8×10^7	2.7×10^4	15.4×10^5	0.36×10^6	22.8×10^6
CP 4	156×10^7	1230×10^6	20.3×10^8	18.6×10^8	50×10^7	0.018×10^4	9.2×10^5	95×10^6	310×10^6
CP 5	111×10^7	1190×10^6	17.4×10^8	18.7×10^8	51×10^7	0.0006×10^4	1.21×10^5	121×10^6	470×10^6
CP 6	136×10^7	780×10^6	15.6×10^8	12.3×10^8	19.1×10^7	320×10^4	8.6×10^5	900×10^6	98×10^6
CP 7	148×10^7	840×10^6	17.4×10^8	15.9×10^8	20.2×10^7	1510×10^4	14.1×10^5	690×10^6	19×10^6
CB 8	24×10^7	0.47×10^6	2.4×10^8	4.1×10^8	24.5×10^7	<1	5.2×10^5	ND	206×10^6
CB 9	47×10^7	10.2×10^6	6.1×10^8	4.6×10^8	45×10^7	33×10^4	45×10^5	12×10^6	236×10^6
CP 10	2.92×10^7	19.1×10^6	0.61×10^8	0.78×10^8	8.1×10^7	0.02×10^4	8×10^5	9.8×10^6	13.5×10^6
CB 11	1×10^7	1.95×10^6	2.3×10^8	0.153×10^8	20.9×10^7	0.0010×10^4	210×10^5	2.18×10^6	1.35×10^6
PC 12	75×10^7	4.8×10^6	1.04×10^8	0.098×10^8	0.032×10^7	<1	88×10^5	1.24×10^6	<1
CB 13	0.086×10^7	0.66×10^6	0.108×10^8	0.0141×10^8	0.121×10^7	<1	22.4×10^5	0.03×10^6	<1
CB 14	18×10^7	25.3×10^6	4.7×10^8	3.4×10^8	1.33×10^7	1.8×10^4	131×10^5	67×10^6	ND
CB 15	4.8×10^7	7.2×10^6	0.065×10^8	0.094×10^8	0.099×10^7	<1	30×10^5	2.1×10^6	ND

PC = Plastic container; CP = Clay pot; CB = Calabash; ND = Not determined

Table 4.3: Average microbiological counts of products manufactured in clay pots and calabashes

Media	Mean counts (per ml)		
	Clay pots (n=7)	Calabashes (n=6)	Clay pots and calabashes (n=13)
Total plate count agar	9.43×10^8 (n=6)	1.58×10^8	5.5×10^8 (n=12)
MRS agar (42 °C)	7.65×10^8	7.6×10^6	3.86×10^8
MRS agar (35 °C)	1.28×10^9	2.6×10^8	7.7×10^8
M17-agar	1.2×10^9	2.1×10^8	7.05×10^8
Rogosa agar	2.47×10^8	1.53×10^8	2.0×10^8
Violet red bile agar	3.05×10^6 (n=6)	5.8×10^4	1.55×10^6 (n=12)
Yeast malt extract agar	8.1×10^5	7.39×10^6	4.1×10^6
Milk agar	3.62×10^8	1.67×10^7 (n=5)	1.89×10^8 (n=12)
Nickels and Leesment	1.47×10^8	1.12×10^8 (n=4)	1.29×10^8 (n=11)

coccobacilli and are not easily distinguished from leuconostocs. Twenty-nine isolates were assigned to the *Leuconostoc*/Betabacterium group, which means that they may either belong to the genus *Leuconostoc* or *Lactobacillus.* Eighty-four of the isolates were unmistakably rod-shaped and could easily be identified as Streptobacterium (57 isolates) or Betabacterium (27 isolates). The ability to produce gas from Gibson's milk (Garvie, 1984) was an important characteristic for distinguishing the leuconostocs. One-hundred and thirty isolates belonged to the genus *Leuconostoc*, 103 to *Lactococcus*, 13 to *Streptococcus* and 7 to *Enterococcus*. Members of the genus *Lactococcus* dominated in clay pot samples 6 and 7, while *Leuconostoc* species prevailed in calabash samples 8 and 9. Table 4.5 gives a comparative view of the distribution of the genera in clay pots and calabashes.

Table 4.4: Summary of the results of the secondary phenotypical tests

Genus	Morphology	Reaction in chalk litmus milk	CO_2[a]	Hydro-lysis of arginine	Growth				Citrate utiliza-tion[b]	Total no. of isolates
					10°C	15°C	45°C	NaCl		
Lactobacillus:										
Streptobacterium	Rods in chains and single cells	Varied from very active (ARC) to little activity (acid formation but no reduction or coagulation)	-	-	+	+	+ (41) - (16)	+ (50) - (7)	+ (19) - (38)	57
Betabacterium	Rods in single cells, pairs and less frequently in short chains	Varied from strong ARC, gas formation and syneresis to acid formation with little reduction and no coagulation	+	+ (10) - (17)	+ (24) - (3)	+	+ (2) - (25)	+ (13) - (14)	+ (12) - (15)	27
Leuconostoc/ Betabacterium	Coccobacilli in pairs, single cells and short chains	Varied from acid production, coagulation, no/little reduction, excessive gas production with syneresis to little acid formation with no coagulation and reduction	+	+ (1) - (28)	+	+	-	+ (2) - (27)	+ (21) - (8)	29

ARC = Acid, reduction, clot
( ) = Number of isolates
+ = Positive
- = Negative
[a] CO_2 production from glucose
[b] Citrate utilization on Nickels and Leesment medium (Vogensen *et al.*, 1987)

Table 4.4: Summary of the results of the secondary phenotypical tests (continue)

Genus	Morphology	Reaction in chalk litmus milk	CO_2 [a]	Hydrolysis of arginine	Growth				Citrate utilization[b]	Total no. of isolates
					10°C	15°C	45°C	NaCl		
Lactococcus	Cocci in strings, pairs and single cells	ARC	-	+	+	ND	-	-	-	103
Leuconostoc	Cocci in curved chains	Acid formation Little/no reduction Little/no coagulation Gas formation	+	-	+	ND	-	-	+ (78) - (52)	130
Enterococcus	Large spherical and ovoid cells chiefly in pairs and short chains. Some single cells present	Varied from ARC to acid formation with no reduction and coagulation	-	+	+	ND	+	+	-	7
Pyogenic streptococci	Cocci in relatively long chains	Acid formation and reduction were common. A less firm coagulum formed.	-	+	-	ND	-	-	-	13
Total isolates										366

ARC = Acid, reduction, clot
( ) = Number of isolates
ND = Not determined

+ = Positive
- = Negative

[a] CO_2 production from glucose
[b] Citrate utilization on Nickels and Leesment medium (Vogensen *et al.*, 1987)

Table 4.5: Distribution of genera in products manufactured in clay pots and calabashes

Genera	Number of isolates	
	Clay pots (n=7)	Calabashes (n=2)
Leuconostoc	45	85
Lactococcus	89	14
Lactobacillus:		
Streptobacterium	57	0
Betabacterium	29	0
Leuconostoc/Betabacterium	28	1
Streptococcus	13	0
Enterococcus	0	7

4.2.3 Identification of the lactic acid bacteria to species level

4.2.3.1 Lactococci

All the *Lactococcus* strains belonged to the species *Lc. lactis*. Neither *Lc. cremoris* nor *Lc. diacetylactis* were identified from the total of 103 *Lactococcus* strains.

4.2.3.2 Leuconostocs

Eighty-six strains from a total of 130 *Leuconostoc* strains produced dextran from sucrose. With the exception of two isolates, all the strains grew at 37 °C and fermented maltose and galactose but not arabinose and raffinose. *Leuconostoc mesenteroides* subsp. *dextranicum* (*Leuc. dextranicum*) and *Leuconostoc carnosum* are the only two species that form dextran from sucrose and do not form acid from arabinose. Most strains of *Leuconostoc carnosum,* however, do not grow at 37 °C (Dellaglio, Dicks & Torriani,

1995). The 86 strains were presumptively identified as *Leuc. dextranicum*. Eighteen of the *Leuconostoc* isolates were non-dextran-producing strains. All of these strains formed acid from sucrose, fructose, galactose and trehalose and were able to grow at 15 ° C and in the presence of 6.5 % NaCl. According to the identification scheme these isolates could either be *Leuconostoc paramesenteroides* or *Leuconostoc mesenteroides* subsp. *mesenteroides*.

4.2.3.3 Identification using the API 50 CH system

To confirm the identity of certain strains, especially those isolates identified as *Leuconostoc* and *Lactobacillus*, 10 previously identified strains were selected and characterized with the API 50 CH identification system (Table 4.6). From the 10 isolates identified, two strains (no. 1 and 9) belonged to *Leuconostoc citreum* (*Leuc. citreum*), two strains (no. 2 and 4) to *Leuc. lactis*, one strain (no. 5) to *Lactobacillus delbrueckii* subsp. *lactis (Lb. lactis)*, and one strain (no. 10) to *Leuc. dextranicum*. Three strains (no. 6, 7 and 8) belonged to the species *Lactobacillus plantarum (Lb. plantarum)*. Strain number 3 could not be identified with the API system.

4.2.4 Detection of pathogens

The only pathogen detected was *Staphylococcus aureus*, which was detected in 10 ml of samples 1, 4 and 11. *E. coli* was also encountered, but it is uncertain whether it was of pathogenic nature. *Salmonella* and *Listeria monocytogenes* were not detected in 25 ml of any of the samples.

4.3 Evaluation of technologically important properties of lactic acid bacteria

4.3.1 Acid production

The activity tests indicated that 55 out of 103 strains of *Lactococcus lactis* subsp. *lactis* coagulated reconstituted skim milk within 16 h. The average titratable acidity of the replications varied between 0.62 and 0.82 % lactic acid and the pH between 4.58 and

Table 4.6: Results of the biochemical reactions of 10 selected lactic acid bacterial strains tested with the API 50 CH identification system

API number	1	2	3	4	5	6	7	8	9	10
Isolate designation	S_4 M_{17} 4	S_6 R 1	S_5 M 7	S_{10} M 7	S_{10} M 8	S_4 T 6	S_2 R 1	S_4 R 1	S_5 R 7	S_9 M_{17} 8
L-Arabinose	+	-	-	+	-	+	+	-	+	-
Ribose	-	-	+	-	-	+	+	+	-	-
D-Xylose	+	-	+	-	-	-	-	-	+	+
Galactose	-	-	+	+	-	+	+	+	-	+
D-Mannose	+	+	-	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	+	+	+	-	-
Sorbitol	-	-	-	-	-	+	+	+	-	-
Alpha methyl-D-mannoside	-	-	-	-	-	+	+	+	-	-
Alpha methyl-D-glucoside	+	-	+	-	-	-	-	-	+	+
Amygdalin	+	-	-	-	-	+	+	+	-	-
Arbutin	-	-	-	-	-	+	+	+	+	-
Esculine	+	-	-	-	-	+	+	+	+	-
Salicine	+	-	-	-	-	+	+	+	+	-
Cellobiose	+	-	-	-	-	+	+	+	-	-
Lactose	-	+	+	+	+	+	+	+	-	-
Melibiose	-	+	+	+	-	+	+	+	-	-
Trehalose	+	-	+	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	+	+	-	-	-
D-Raffinose	-	-	+	+	-	+	+	+	-	-
Amidon	-	-	+	-	-	-	+	-	-	-
Glycogen	-	-	-	-	-	-	+	-	-	-
ß Gentobiose	-	-	-	-	-	+	+	+	-	-
D-Turanose	+	-	+	-	-	+	-	+	+	+
D-Arabitol	-	-	-	-	-	+	+	-	-	-
Gluconate	+	-	-	-	-	+	+	+	+	-
Identified as:	*Leuc. citreum*	*Leuc. lactis*	Unidentified	*Leuc. lactis*	*Lb. delb.lactis*	*Lb. plant*	*Lb. plant*	*Lb. plantarum*	*Leuc.citreum*	*Leuc. dextr.*

Notes: $S_2 - S_{10}$ refers to the samples of traditional fermented milks. M17 (M17-agar), M (MRS agar) and R (Rogosa agar) refer to the media from which the strain was isolated. The last number in the combination refers to the isolate number. Positive and negative reactions are indicated by (+) and (-) respectively. All strains produced acid from D-glucose, D-fructose, N-acetyl-glucosamine, maltose and saccharose. None of the isolates produced acid form glycerol, erythritol, D-arabinose, L-xylose, adonitol, ß-methyl-xyloside, L-sorbose, rhamnose, dulcitol, inositol, inuline, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, 2 keto-gluconate and 5 keto-gluconate. *Lb. delb. lactis* = *Lactobacillus delbrueckii* subsp. *lactis*, *Lb. plant* = *Lactobacillus plantarum*.

5.04. Titration values with the automatic end-point titration system where on average 0.08 % lower than for titrations done manually. Commercial cultures (CH-Normal 22 and R-707 phage control, Chr. Hansen's) were included in the activity tests for comparative purposes. CH-Normal 22 consisted of the species *Lc. lactis*, *Lc. cremoris*, *Leuc. cremoris* and *Lc. diacetylactis*. R-707 contained only the homofermentative species of *Lc. lactis* and *Lc. cremoris*. Fermentation with the commercial culture CH-Normal 22 gave an average titratable acidity of 0.85 % lactic acid and a pH of 4.76. The corresponding values for R-707 were 0.82 % lactic acid and a pH of 4.53. Two strains of *Lc. lactis* that produced the highest percentage of lactic acid were selected for evaluation for use as starter organisms in fermented milk products.

4.3.2 Gas production

The results of gas production by the *Leuconostoc* strains are given in Table 4.7.

4.3.3 Production of volatile organic compounds by selected strains

Peak areas were used as a quantitative measurement of the production of specific volatile organic compounds from fermentative cultures (Table 4.8). Only trace amounts of diacetyl were detected and formed by only one strain isolated from traditional fermented milk namely *Leuc. lactis* (A2). Two strains of *Lb. plantarum* produced acetoin, but no diacetyl was detected. *Lc. diacetylactis,* however, produced diacetyl and acetoin as well as the highest quantity of acetaldehyde.

4.3.4 Growth characteristics of selected strains in reconstituted non-fat milk

Five *Leuconostoc* and four *Lactobacillus* strains were selected and their growth characteristics determined in skim milk (Table 4.9). *Leuconostoc lactis* (A2) coagulated NFM at both 22 and 30 °C within 24 h at inoculate levels of 1, 10 and 20 %. *Leuconostoc lactis* (A4) did not show the same potential for growth in reconstituted skim milk. The two strains of *Leuconostoc citreum* were unable to grow in skim milk. The strains of *Lb. plantarum* did not coagulate milk at 22°C.

Table 4.7: Gas production by 110 *Leuconostoc* strains in skim milk, with and without added sodium citrate, after 16, 48 and 144 h at 22 °C.

Time	No. of leuconostocs producing gas in sterile skim milk		No. of leuconostocs producing gas in sterile skim milk in association with acid-producers	
	With sodium citrate	Without sodium citrate	With sodium citrate	Without sodium citrate
16 h	42	3	55	0
48 h	40	31	26	5
144 h	23	32	14	5

Table 4.8: The production of volatile organic compounds by some lactic acid bacteria in skim milk after 16 h at 22 °C

Strain no.	Strain/culture	Peak areas (mV.s)[a]				
		Diacetyl	Acetoin	Acetaldehyde	Acetone	Ethanol
A 2	*Leuc. lactis* (103x10^7/ml; pH 4.38)[b]	trace	0	trace	1103	44023
A 4	*Leuc. lactis* (66x10^7/ml; pH 4.58)[b]	0	0	11311	838	954960
A 6	*Lb. plantarum* (104x10^7/ml; pH 4.48)[b]	0	1023	trace	2549	509
A 7	*Lb. plantarum* (65x10^7/ml; pH 4.16)[b]	0	644	568	1687	trace
CHN-22[c]	*Lc. diacetylactis*, *Leuc. cremoris* *Lc. cremoris*, *Lc. lactis* (16x10^7/ml; pH 4.21)[b]	2916	5106	1789	1005	7689
M 15	*Lc. diacetylactis* (102x10^7/ml; pH 4.16)[b]	1646	5907	1699	1218	16269

[a] mV.s = milliVolts.second
[b] Cell counts and pH after incubation at 22 °C for 16 h
[c] CH-Normal 22 (Commercial starter culture)

Table 4.9: The ability of nine selected lactic acid bacterial strains to coagulate milk at 22 and 30 °C using 1, 10 and 20 % (v/v) inoculum sizes

API no.	Strain	Coagulation time (h) at:					
		22 °C			30 °C		
		1 %	10 %	20 %	1 %	10 %	20 %
1	*Leuc. citreum*	—	—	—	—	—	—
2	*Leuc. lactis*	24 h	24 h	24 h	24 h	24 h	24 h
4	*Leuc. lactis*	—	—	48 h	—	—	—
5	*Lb. delb.* sp. *lactis*	—	—	48 h	—	48 h	24 h
6	*Lb. plantarum*	—	—	—	—	48 h	48 h
7	*Lb. plantarum*	—	—	—	—	24 h	24 h
8	*Lb. plantarum*	—	—	—	—	24 h	24 h
9	*Leuc. citreum*	—	—	—	—	—	—
10	*Leuc. dextranicum*	—	—	—	—	—	—

— = No coagulation of milk within 72 h

4.4 Evaluation of the sensory and objective characteristics of fermented milk products

4.4.1 Sensory evaluation of products made with selected strains of lactic acid bacteria

Experimental products 1, 3 and 8 which were made with single strains of *Leuc. lactis*, *Leuc. paramesenteroides* and *Lc. diacetylactis* received positive comments (Table 4.10) from by the panellists. Product 8 had a typical diacetyl flavour. In milk fermented with *Leuc. dextranicum* a taint was observed that was not typical of an acceptable mesophilic fermented milk product. Fermentation with *Lb. plantarum* resulted in a lack of flavour. Both strains of *Lc. lactis* produced a malty flavour that was considered a defect. Of the four products fermented with mixed strain cultures, product 9 received the most favourable evaluation. It was concluded that the combination of *Lc. lactis, Leuc. lactis* and *Lc. diacetylactis* showed the best potential as a starter culture for fermented milk products.

4.4.2 Sensory evaluation of four commercial and four experimental fermented milk products using a trained sensory panel

The mean sensory scores for all the attributes were the same for the commercial and the experimental fermented milk products (Table 4.11). It can, however, be concluded from the comments given on the sensory score-card that the majority of the panellists evaluated the experimental fermented milk products less favourably. Comments on the experimental fermented milk products mentioned a mealy after-taste, strong Cheddar cheese flavour, bad taste, bitter taste and insufficient acid production. There were several significant correlations between the descriptors for both commercial products and experimental fermented milk products (Table 4.12). Statistical significance was accepted if the probability values were $p \leq 0.05$ for the correlation. Significant positive correlations were found between sour aroma and cottage cheese aroma, the presence of lumps and viscosity, viscosity and creamy mouth-feel and between creamy mouth-feel and coating aftertaste for the commercial fermented milk products. For the experimental fermented milk products positive correlations were also found between cottage cheese aroma and sour flavour as well as fresh aroma and cottage cheese flavour. Common

Table 4.10: Sensory evaluation and pH of ten experimental fermented milk products manufactured with selected lactic acid bacterial strains

Product number	Strains	Comments	pH[a]
1	*Leuc. lactis*	Acceptable clean aroma and flavour. Typical effervescens observed due to the presence of CO_2.	4.82
2	*Leuc. dextranicum*	Less agreeable flavour with an atypical taint.	4.74
3	*Leuc. paramesenteroides*	Acceptable clean aroma and flavour with effervescens caused by CO_2.	4.82
4	*Lb. plantarum*	Little flavour development. No flavour observed that was typical for fermented milk products. No foreign or strange flavour either.	4.39
5	*Lc. lactis*	Disagreeable flavour with the presence of a malty taint.	3.98
6	*Lc. lactis*	Presence of a slight malty flavour. Detection of a lactic acid flavour.	4.03
7	*Lc. lactis* and *Leuc. lactis*	Agreeable taste. Acceptable clean aroma. Slight atypical flavour.	4.10
8	*Lc. diacetylactis*	Typical diacetyl flavour. Pleasant taste.	4.45
9	*Lc. lactis, Lc. diacetylactis* and *Leuc. lactis*	Typical clean flavour of fermented milk. Balance of culture not perfect. Very pleasant taste.	4.07
10	*Lc. lactis* and *Leuc. dextranicum*	Acceptable clean flavour. Slight malty flavour.	4.09

[a] pH of the product after fermentation

Table 4.11: Average sensory scores for 19 attributes describing four commercial (C1, C2, C3, C4) and four experimental fermented milk products (E1, E2, E3 and E4)

Sensory attribute	Commercial fermented milk products				Mean score	Experimental fermented milk products				Mean score
	C1	C2	C3	C4		E1	E2	E3	E4	
Aroma:										
Sourness	2.44	2.58	2.51	2.51	**2.51**	2.54	2.52	2.52	2.53	**2.52**
Cottage cheese	2.25	2.38	2.31	2.31	**2.31**	2.33	2.32	2.32	2.32	**2.32**
Fresh	3.22	3.25	3.24	3.24	**3.24**	3.24	3.24	3.24	3.24	**3.24**
Musty	2.53	2.46	2.49	2.49	**2.49**	2.48	2.49	2.49	2.49	**2.49**
Visual texture:										
Air bubbles	4.31	4.46	4.38	4.38	**4.38**	4.41	4.39	4.39	4.40	**4.40**
Smooth	4.47	4.46	4.47	4.47	**4.47**	4.46	4.46	4.46	4.46	**4.46**
Lumpiness	1.33	1.33	1.33	1.33	**1.33**	1.33	1.33	1.33	1.33	**1.33**
Flocculated	1.58	1.54	1.56	1.56	**1.56**	1.56	1.56	1.56	1.56	**1.56**
Viscosity	2.31	2.38	2.34	2.34	**2.34**	2.35	2.34	2.34	2.35	**2.35**
Mouth-feel:										
Watery	4.06	4.08	4.07	4.07	**4.07**	4.07	4.07	4.07	4.07	**4.07**
Creamy	2.47	2.46	2.47	2.47	**2.47**	2.46	2.46	2.46	2.46	**2.46**
Sour	3.31	3.38	3.34	3.34	**3.34**	3.35	3.34	3.34	3.35	**3.35**
Astringent	2.22	2.25	2.24	2.24	**2.24**	2.24	2.24	2.24	2.24	**2.24**
Buttery	1.56	1.5	1.53	1.53	**1.53**	1.52	1.53	1.52	1.52	**1.52**
Cottage	2.56	2.67	2.61	2.61	**2.61**	2.63	2.62	2.62	2.62	**2.62**
Aftertaste:										
Mouth-coating	2.56	2.5	2.53	2.53	**2.53**	2.52	2.53	2.52	2.52	**2.52**
Rancid	1	1	1	1	**1**	1	1	1	1	**1**
Astringent	2.14	2.21	2.17	2.17	**2.17**	2.19	2.18	2.18	2.18	**2.18**
Bitter	1.14	1.13	1.13	1.13	**1.13**	1.13	1.13	1.13	1.13	**1.13**

Table 4.12: Correlation values and probability values of selected attributes for commercial and experimental fermented milk products

Attributes	Correlation value	*p*-value
Commercial fermented milk products:		
Sour aroma and cottage cheese aroma	0.63	0.0280
Lumps and viscosity	0.84	0.0006
Viscosity and creamy mouth-feel	0.74	0.0057
Creamy mouth-feel and coating aftertaste	0.76	0.0041
Fresh aroma and musty aroma	-0.63	0.0292
Lumps and smooth	-0.90	0.0001
Lumps and watery mouth-feel	-0.79	0.002
Flocculated texture and smooth texture	-0.97	0.0001
Viscosity and watery mouth-feel	-0.79	0.0023
Watery mouth-feel and creamy mouth-feel	-0.84	0.0006
Watery mouth-feel and coating	-0.81	0.0014
Buttery flavour and cottage cheese	-0.89	0.0001
Experimental fermented milk products:		
Cottage cheese aroma and sour flavour	0.59	0.0424
Fresh aroma and cottage cheese flavour	0.89	0.0001
Lumps and viscosity	0.62	0.0301
Viscosity and creamy mouth-feel	0.63	0.0305
Coating and astringent aftertaste	0.86	0.0004
Fresh aroma and musty aroma	-0.91	0.0001
Musty aroma and sour flavour	-0.62	0.0304
Musty aroma and cottage cheese flavour	-0.73	0.0075
Flocculated texture and watery mouth-feel	-0.76	0.0042
Watery mouth-feel and creamy mouth-feel	-0.66	0.02

negative significant correlations for the two groups of fermented products were between fresh aroma and musty aroma and between watery and creamy mouth-feel.

4.4.3 Objective physical characteristics of the fermented milk products

Mean values for the physical characteristics (pH, viscosity and flow properties) of the products are shown in Table 4.13. The experimental fermented milk products showed more post-acidification than the commercial products. The commercial products were more viscous than the experimental fermented milk products. The viscosity as measured with the Rapid Visco Analyser correlated positively with: the viscosity evaluated by the trained panellists; flocculated appearance; presence of lumps; and creamy mouth-feel (Table 4.14). The viscosities measured objectively were negatively correlated with sensory attributes such as the presence of air bubbles, smooth visual texture and watery mouth-feel. There was a significant correlation ($p = 0.026$) between pH and sour aroma for the experimental fermented milk products.

Table 4.13: Mean values for the physical characteristics of both commercial and experimental fermented milk products

Fermented milk products	pH	Viscosity (mPa.s) [a]	Spreading capacity (cm)
Commercial: C1	4.62	140.37	18
C2	4.53	196.67	16
C3	4.55	273.33	15
C4	4.58	134.2	18
Experimental: E1	4.58	116.0	18
E2	4.59	70.0	19
E3	4.41	58.0	18
E4	4.39	61.33	17

[a] mPa.s = milliPascal.second

Table 4.14: Correlation values and probability values of selected sensory attributes and objective physical characteristics

Attributes	Correlation value	*p*-value
Commercial fermented milk products:		
Viscosity measured objectively and sensorial	0.91	0.0001
Viscosity measured objectively and flocculated appearance	0.73	0.0073
Viscosity measured objectively and presence of lumps	0.85	0.0005
Viscosity measured objectively and creamy mouth-feel	0.77	0.0037
Spreadability and creamy mouth-feel	0.58	0.0487
Viscosity measured objectively and presence of air bubbles	-0.70	0.0115
Viscosity measured objectively and smooth visual texture	-0.75	0.0049
Viscosity measured objectively and watery mouth-feel	-0.77	0.0033
Experimental fermented milk products:		
pH and sour aroma	0.64	0.0260
pH and spreadability	0.69	0.0130
Spreadability and presence of air bubbles	0.59	0.0134
Viscosity measured objectively and presence of air bubbles	-0.69	0.0392
Viscosity measured objectively and spreadability	-0.60	0.0442

5. DISCUSSION

The lactic acid bacteria predominated the microbiological population with mean values of *ca.* 10^8 cfu/ml on MRS, M17- and Rogosa agars. The counts compared favourably with findings of similar studies on fermented milks by other workers. According to Stadhouders (1975) the numbers of lactococci may easily reach 10^9 viable units per gram of cheese or sour milk. The mean total viable cell count for 13 samples in the present study was 5.5 x 10^8 cfu/ml. The mean counts for mesophilic lactic acid bacteria obtained on MRS agar (35 °C) and M17-agar was 7.4 x 10^8 cfu/ml. Miyamoto *et al.* (1986) used Plate count agar with bromcresol purple to isolate lactic acid bacteria from five samples of Maziwa lala. Their results varied from 3.3 x 10^6 to 1.2 x 10^8 cfu/ml. The above-mentioned results indicate the superior growth of lactic acid bacteria on elective media such as MRS and M17-agar. The fact that counts on MRS agar (incubated at 35 °C) and M17-agar exceeded counts on Rogosa agar may be explained by the fact that MRS and M17-agars are elective media while Rogosa agar is selective (Reuter, 1985). According to Reuter (1985) the term "elective medium" for lactic acid bacteria describes highly nutrient media enabling good growth of nearly all or special groups of the lactic acid bacteria. The term "selective medium" is applied to a medium, which enables good growth of a selected group of lactic acid bacteria out of a complex microflora.

The mean thermophilic counts on MRS agar ranged from 7.6 x 10^6 (calabashes) to 7.65 x 10^8 cfu/ml (clay pots), while corresponding mesophilic counts varied between 2.6 x 10^8 (calabashes) and 1.28 x 10^9 cfu/ml (clay pots). Twelve of the fifteen samples were collected in the cooler months of May, July and August. The ambient temperatures at which the natural fermentation of the tested samples took place probably favoured proliferation of mesophilic bacteria leading to their dominance in the microflora. From previous studies it is evident that lactic acid bacterial counts from whey-drained products are higher than that of unconcentrated products. The cell count on MRS agar for Jben, a whey-drained fermented milk, was 3.2 x 10^8 cfu/ml compared to 2.6 x 10^6 cfu/ml for Raib, a coagulated milk product (Hamama & Bayi, 1991). Whey-drained Amasi examined in the study of Mutukumira (1996) contained on average 1.86 x 10^9 cells per ml

(enumerated on MRS agar at 30 °C). Bankole & Okagbue (1992) encountered high lactic acid bacterial counts ($1.1 \times 10^8 - 9.5 \times 10^{10}$ /ml) in Nono, a fermented milk, "heavy-bodied liquid" sold on farms, open-air markets and by hawkers in Nigeria. These authors attributed the high aerobic plate counts ($1.6 \times 10^9 - 4.9 \times 10^{11}$ /ml) to some extent to the levels of sanitation practised by the processors. The use of an inoculum or re-use of calabashes and other equipment could contribute to the high counts observed in their study. In the present study the mean bacterial counts on MRS (35 °C) and M17-agars were 7.7×10^8 and 7.05×10^8 cfu/ml respectively. Unfortunately it was not clear whether the samples tested in this study were all whey-drained products or not. The relatively high counts encountered may indicate a concentrating step or a lengthened fermentation period.

The number of citrate fermenting organisms varied between 1.35×10^6 and 8.4×10^8 cfu/ml (mean value = 1.29×10^8 /ml). Noteworthy, however, was the apparent absence of citrate fermenting organisms in samples PC 12 (plastic container) and CB 13. No satisfactory explanation could be given for these results. The fact that no *Lc. diacetylactis* spp. could be identified was disappointing, from both an ecological and practical point of view. Picking citrate fermenting colonies from Nickels and Leesment's medium probably would have increased the likelihood of isolating *Lc. diacetylactis*. The prevalence of proteolytic organisms, capable of hydrolysing casein, in high numbers (mean value = 1.89×10^8 /ml), contributed substantially to the total microbial population of the samples tested. Qualitatively, the identity of the predominant microbial populations established in this study compared favourably with results reported by other authors (Miyamoto *et al.*, 1986; Hamama & Bayi, 1991; Hamama, 1992; Mutukumira, 1996).

The high coliform count in some of the samples was alarming when considering South African health regulations that state that "no person shall sell for consumption raw milk that has become sour which contains more than 50 coliform bacteria /ml of the product" (South Africa, 1997). Coliform counts varied between <1 and *ca.* 15×10^6 /ml, with a mean value of 1.55×10^6 /ml for all the samples analysed. Undesirable bacteria were also detected in other fermented milks in Africa (El-Sadek *et al.*, 1972; Hamama & Bayi,

1991; Ashenafi, 1992; Hamama, 1997). Coliforms were detected in 28 of 50 Zabady samples, a product from pasteurised or boiled milk (El-Sadek *et al.*, 1972). The counts varied from <1 to a maximum of 24 x 10^5 /ml, with an average of 152 x 10^3 /ml and the coliform organisms were identified as *E. coli* (228 isolates) and *Aerobacter cloacae* (58 isolates). El-Sadek *et al.* (1972) attributed the presence of these organisms to inadequate heat treatment or more likely to post-heating contamination during manufacturing and handling. High average counts of coliforms were also found in both Raib (1.7 x 10^5 cfu/ml) and Jben (4.3 x 10^5 cfu/ml). In the present study *Staphylococcus aureus* was detected in 10 ml of samples PC 1, CP 4 and CB 11. Ashenafi (1992) also isolated this organism from 23 % of Ayib samples, but at very low numbers (10^2 to 10^3 cfu/ml). *Staphylococcus aureus* was reportedly recovered at levels between 10 and 100 /ml in 22 Raib and 17 % of Jben samples (Hamama & Bayi, 1991). Only one sample of Raib contained more than 10^4 *S. aureus* cfu/ml. All Raib and Jben samples were TNase-negative, indicating the probable absence of staphylococcal enterotoxins in these samples (Hamama & Bayi, 1991). Hamama (1997) cited the following results regarding the detection of pathogens in Jben: *Salmonella* spp. were detected in 10 % of Jben samples tested by Hamama (1989), *Yersinia enterocolitica* in 4.1 % of samples examined by Hamama, El Marrakchi & El Othmani (1992) and *Listeria monocytogenes* in 18.1 % of Jben samples examined by El Marrakchi, Hamama & El Othmani (1993). Twenty-seven samples from one hundred samples of Ayib contained fecal coliform loads of more than 10^2 /ml. *Listeria* spp., however, were not detected in any of the samples tested (Ashenafi, 1992).

Neither *Lc. lactis* subsp. *cremoris* nor *Lc. diacetylactis* were isolated from the traditional fermented milk samples analysed in this study. In 1975 Mostert isolated 32 isolates of *Lc. diacetylactis*, 11 of *Lc. lactis* and 2 of *Lc. cremoris* from 7 raw cream samples. Twenty-five strains belonging to *Lc. diacetylactis* were also isolated by him from cauliflower. Miyamoto *et al.* (1986) found that in Maziwa lala the incidences of *Lactobacillus*, *Lactococcus* and *Leuconostoc* genera were 24, 33 and 43 %, respectively. Both *Lc. lactis* and *Lc. cremoris* were among the species identified. From Dadih, an Indonesian fermented milk, Hosono *et al.* (1990) isolated a total of 36 isolates, 4 of which were

strains of *Lc. cremoris*, 3 were strains of *Lc. diacetylactis* and one was a strain of *Lc. lactis*. The principle species found in Lben, Raib and Zabda were *Lc. lactis* and *Lc. diacetylactis* (Hamama, 1992). Lactococcal isolates from 40 Dahi and 20 buttermilk samples from India included 13 of *Lc. lactis*, 4 of *Lc. cremoris* and 2 of *Lc. diacetylactis* (Padmanabha-Reddy, Habibulla-Khad & Purushothaman, 1994). In an ecological study of lactic acid bacteria done by Salama, Musafija-Jeknic, Sandine & Giovannoni (1995), *Lc. lactis* occurred in potato, cucumber, sweet pea, bean, cantaloupe, corn, body and tail of cows, colostrum, raw milk from goats and cows, Cottage cheese and cream. *Lc. diacetylactis* was isolated from cow's raw milk from Morocco and from goats' raw milk and Cottage cheese from Yugoslavia. *Lc. cremoris* was isolated from raw milk originating from Morocco, China and Ukraine and from Cottage cheese and raw milk from Yugoslavia. In the study of Moreno & Busani (1990) *Lc. lactis* dominated in raw milk samples while *Lc. cremoris* was more prevalent in commercial starters. The percentage distribution of lactococci isolated from raw milk was 93 % for *Lc. lactis*, 2 % for *Lc. diacetylactis* and 5 % for *Lc. cremoris*. The distribution in commercial starter cultures were 34 % for *Lc. lactis*, 6 % for *Lc. diacetylactis* and 60 % for *Lc. cremoris* (Moreno & Busani, 1990).

According to Holler & Steele (1995) *Lactococcus lactis* subsp. *cremoris* was isolated only rarely from natural sources. Centeno, Cepeda & Rodriguez-Otero (1996) and other workers have found that *Lc. lactis* was the *Lactococcus* species most frequently isolated from raw milk cheeses. Weerkamp *et al.* (1996) and Crow, Coolbear, Holland, Pritchard & Martley (1993) also reported that lactococci isolated from natural sources were usually identified as *Lc. lactis*, whereas the phenotype *Lc. cremoris*, which is common in industrial mixed-starter cultures, was isolated only rarely. Salama *et al.* (1995) stated that the natural habitat of *Lc. cremoris* remained uncertain and is normally restricted to environments where starters are used. The range of organisms used in starter cultures has probably narrowed due to the selection for better industrial performance, resulting in special varieties of *Lc. cremoris* which have lost properties that are important for their survival outside a dairy environment (Klijn, Weerkamp & De Vos, 1995). The relatively high proportion of *Lc. diacetylactis* (60 % of all *Lactococcus* spp. isolated) in Idiazabal

cheese is one of the main differences between this cheese and other Spanish cheeses made with ewes' milk (Rua, Olivares, Romero & Aldamiz-Echebarria, 1993). From 21 isolates identified from Amazi, a fermented milk in Zimbabwe, Mutukumira (1996) found that five isolates belonged to *Lc. lactis* and four to *Lc. diacetylactis*. The fact that no *Lc. diacetylactis* could be detected in the present study does not suggest that strains of this species were not present in the fermented milks, especially if the relatively high counts of citrate fermenting organisms on Nickels and Leesment's medium (mean = 1.29×10^8 cfu/ml) are taken into consideration. This finding may also be explained by the fact that only phenotypical tests were used for identification. Furthermore, the characteristic of citrate metabolism is encoded on plasmid DNA, which may be lost in some strains (Cogan, 1985). Plasmid linkage of phenotypic properties (such as lactose fermentation, protease activity, citrate utilization, or bacteriophage resistance) in lactococci has been demonstrated by McKay (1983). *Leuconostoc mesenteroides* subsp. *cremoris* was also not encountered in this study. Studies on 182 representative strains of lactic acid bacteria associated with raw milk in Brazil showed *Leuc. mesenteroides* subsp. *cremoris* as a minor group, representing only 1.1 % of the total population (Antunes & De Oliveira, 1986 cited by Holzapfel & Schillinger, 1992). The apparent absence of the industrially important species of *Lc. cremoris*, *Leuc. cremoris* and *Lc. diacetylactis*, may also be ascribed to the isolation procedures that were followed during the initial selection and isolation of colonies from MRS, Rogosa and M17-agars.

Previous studies on traditional fermented milks reported *inter alia* the presence of *Lb. plantarum*, *Lb. lactis*, *Leuc. lactis* and *Leuc. dextranicum*. Thus, from 21 isolates identified from naturally fermented milk in Zimbabwe (Mutukumira, 1996), three were identified as *Lb. plantarum*. In the present study, strains A6, A7 and A8 were assigned to *Lb. plantarum*. Four *Lb. plantarum* strains were identified (from a total of 100 strains) from Masai fermented milk in Northern Tanzania (Isono *et al.*, 1994) and 47 (n=426) from cultured milk in Cameroon (Jiwoua & Milliere, 1990). Feresu & Muzondo (1990) identified one strain of *Lb. plantarum* and two strains of *Lb. lactis* from traditional fermented milk in Zimbabwe. The prevalent lactic acid bacteria isolated from Ititu, a whey-drained product from east Africa were identified as *Lb. casei* and/or *Lb. plantarum*

(Kassaye *et al.*, 1991). In studies on the occurrence of lactic acid bacteria, *Lb. plantarum* constituted the highest number of *Lactobacillus* species isolated from fermented plant materials (Olukoya, Ebigwei, Adebanwo & Osiyemi, 1993; Olasupa, Olukoya & Odunfa, 1997). According to Daeschel *et al.* (1987) (cited by Olasupo *et al.*, 1997), *Lb. plantarum* is known to be commonly associated with plants. *Leuc. lactis* was one of the main species recovered from Jben, a traditional soft cheese from Morocco (Hamama, 1992). Strains A2 and A4 were identified as *Leuc. lactis* in this study. From the total of 72 leuconostoc isolates obtained from a raw cows' milk cheese in Spain (Arzúa cheese) 31 were identified as *Leuc. mesenteroides*, 18 as *Leuc. dextranicum*, 22 as *Leuc. paramesenteroides* and one as *Leuc. lactis* (Centeno *et al.*, 1996). The isolation of *Leuc. citreum* from dairy products was not reported frequently by researchers. It was, however, isolated from Afuega'l Pita cheese (Cuesta, Fernandez-Garcia, Gonzalez-de-Llano, Montilla & Rodriguez, 1996). In the present study two of the 10 strains, which were characterized by the API 50 CH system, were *Leuc. citreum*.

The average counts for yeasts obtained from the calabash samples were higher (7.39×10^6 cfu/ml) than for clay pot samples (8.1×10^5 cfu/ml). In a parallel study on the same traditional fermented milk samples fifty colonies representative of each sour milk sample were isolated and identified to species level by Loretan (1999). *Torulaspora delbrueckii* (40%), *Debaryomyces hansenii* (22%) and *Kluyveromyces marxianus* (18%) represented the highest percentage of the overall yeast population. Other species encountered were *Saccharomyces cerevisiae* (6%), *Yarrowia lipolytica* (4%), *Dekkera anomala* (2%), *Pichia membranaefaciens* (2%), *Rhodotorula glutinis* (2%), *Trichosporon beigelii* (2%) and *Galactomyces geotrichum* (2%). *Geotrichum* species were prevalent in all the samples with counts ranging from 10^1 to 10^2 cfu/ml. The Finnish buttermilk (Villi) is similar to Swedish buttermilk (Filmjolk) but the starter of *Lc. lactis*, *Lc. cremoris*, *Lc. diacetylactis* and *Leuc. cremoris* is enriched with the mould *Geotrichium candidum* which gives the product a slightly different taste and appearance (Marshall, 1986). Researchers investigating African fermented milk products reported varying amounts of yeasts and moulds present in products (El-Sadek *et al.*, 1972; Hosono *et al.*, 1989; Jiwoua &

Millière, 1990; Kimonye & Robinson, 1991; Bankole & Okagbue, 1992 and Isono *et al.*, 1994).

The presence of yeasts is influenced by containers and processing methods used. In Iria ri Matii evidence suggested that components of the smoke that is used in the charcoal treatment suppressed naturally-occurring yeasts and moulds brought in by the raw milk. Kimonye & Robinson (1991) reported counts of <10 cfu/ml yeasts and moulds at pH 4.5 in these products. Yeasts were present in relatively small numbers compared to bacteria in Zabady collected from the open market in Cairo (El-Sadek *et al.*, 1972). They were detected in 84 % of the samples with a range between <1 and 11 x 10^6 /ml, and an average of 6.88 x 10^5 cfu/ml. The majority of the yeasts isolated belonged to the genus *Candida* (*Cand.*) and were identified as *Cand. mesenterica* (34 strains), *Cand. parapsilosis* var. *intermedia* (28 strains), *Cand. krusei* (23 strains), *Cand. parapsilosis* (21 strains), *Cand. pseudotropicalis* (15 strains) and *Candida tropicalis* (10 strains). A few strains belonged to the genus *Torulopsis* and eight were identified as *T. fomata*. The presence of yeasts in Zabady might well have been due to contamination (El-Sadek *et al.*, 1972). More than 75 samples from a total of one hundred samples of Ayib purchased at the Awassa open-air market in Ethiopia had yeast counts of 10^7 cfu/ml or higher. The majority of the samples had mould counts of 10^5 cfu/ml and higher. Yeasts, which can grow at lower pH values, may affect the flavour and keeping quality of Ayib (Ashenafi, 1992). Dadih is a fermented milk product made by pouring buffalo milk into fresh bamboo tubes and capping it with banana leaves. The milk is fermented by naturally occuring micro-organisms derived from the milk, banana leaves and the bamboo tube at room temperature for two or three days. Hosono *et al.* (1989) also found an appreciable number of yeasts (1.1 x 10^7 /ml) in samples of Dadih, which were *inter alia* identified as *Endomyces lactis*. The involvement of yeasts in Dadih might be the feature differentiating it from other fermented milk such as yoghurt. Yeast counts of 10^6 to 10^8 /ml were, reportedly, found in seven samples of fermented Zebu milk prepared by pastoral Masai in smoked gourds. Ten strains were identified as *Saccharomyces* spp. and nine belonged to the genus *Candida* (Isono *et al.*, 1994). Lactic acid bacteria and yeasts were also the predominant groups of micro-organisms in Nono. The yeast numbers ranged from 1.5 x 10^7 to 9 x 10^8

/ml in all the samples of Nono examined by Bankole & Okagbue (1992). Yeasts were also reported to have an adverse effect on physiochemical composition, texture and organoleptic properties of a fermented milk product (Pindidam) from northern Cameroon (Jiwoua & Millière, 1990).

The most commonly used activity test for measuring acid production consists of inoculating a young 1 % starter culture into milk, previously heated (for instance 80 °C for 30 min) and measuring the acidity after 6 h incubation at 30 °C (Mocquot & Hurel, 1970). In the study of Cogan, Barbosa, Beuvier, Bianchi-Salvadori, Cocconcelli, Fernandes, Gomez, Gomez, Kalantzopoulos, Ledda, Medina, Rea & Rodriguez (1997) only 8 % of the 1582 *Lactococcus* isolates and 2 % of the 482 isolates of mesophilic lactobacilli tested produced sufficient acid to lower the pH of milk to <5.3 in 6 h at 30 °C. Referring to Farrow (1980), Cogan *et al.* (1997) explained this by stating that lactococci that ferment lactose slowly, contain both β-galactosidase (EC 3.2.1.23) and phospho-β-galactosidase (EC 3.2.1.85), while those that ferment it rapidly contain only phospho-β-galactosidase. The activity test used in this study more closely resembled the incubation period and temperature for the production of Maas. Lactic acid producing strains are considered as "fast" strains if they coagulate autoclaved reconstituted non-fat milk within 16 h at 21 °C from a 1 % freshly coagulated inoculum (Porubcan & Sellars, 1979 and Sandine, 1979; cited by Huggins & Sandine, 1984). Fifty three percent of the *Lc. lactis* strains evaluated in this study were fast-acid producing strains.

In their study Holler & Steele (1995) considered a pH between 4.7 and 5.2 to be desired. The titratable acidity of a top-quality buttermilk varies between 0.75 to 0.85 % lactic acid (Vedamuthu, 1982). The best performing strain of *Lc. lactis* produced 0.82 % lactic acid with a corresponding pH of 4.58 and thus showed potential for incorporation into a starter culture for fermented milks. From the 120 lactococcal strains chosen for further testing in the study of Salama *et al.* (1995), 64 were fast-acid producers which coagulated milk within 17 to 18 h at 21 °C or after being streaked on fast-slow differential agar to screen for fast-acid producing cells. According to, *inter alia*, titratable acidity (0.595 – 0.860 % lactic acid) and milk curdling time (6 – 12 h) Gupta & Batish (1990) isolated lactococci

strains that were found to be quite promising as starter cultures. In the study of Mutukumira (1996) only one out of four strains of *Lc. diacetylactis* coagulated reconstituted non-fat dry milk within 12 h and only one out of five strains of *Lc. lactis* coagulated the milk in 18 h.

Mocquot & Hurel (1970) stated that certain strains of lactic acid bacteria are not very sensitive to the conditions in which milk has been heated before being inoculated, while others are much more sensitive. They suggested that this sensitivity must be judged in the course of selection by cultivating the strains in milk which has been submitted to a moderate and well defined heat treatment, and not only to cultivate them in milk which has been autoclaved or heated to 100 °C. It might be expected that combinations of *Lc. lactis* and *Lc. cremoris* might produce higher quantities of lactic acid. Dahiya & Speck (1963) observed mutualism between a particular strain of *Lc. lactis* and *Lc. cremoris*. The *Lc. lactis* strain produced a growth factor (adenine) that stimulated the growth of both lactococci strains. The activity of the mixed culture was higher than the activity of both strains grown in separate pure cultures.

The evaluation of gas production by the leuconostoc strains in the present study showed that added sodium citrate contributed to gas production by leuconostocs, grown separately and in combination with acid producers. The selection of compatible strains of the leuconostocs and acid producers is an intricate task, involving factors such as inoculum size of each group to be considered. Overall, the *Leuconostoc* strains varied in their gas production capability. Kneifel *et al.* (1992) investigated the CO_2 production properties of 16 mesophilic starter cultures. Four out of 16 cultures showed a high velocity of CO_2 production and three starter cultures could be classified as yielding a high gas-producing intensity. Bellengier, Foucaud & Hemme (1993) used the following species to examine total CO_2 production from citrate and glucose by *Leuconostoc* strains: *two Leuc. paramesenteroides*, two *Leuc. lactis*, ten *Leuc. mesenteroides* and nine *Leuc. dextranicum* strains. The production of CO_2 by the 23 strains varied to a large extent. In one strain of *Leuc. mesenteroides* and two strains of *Leuc. dextranicum* no CO_2 production was detected. Strains belonging to *Leuc. mesenteroides* were the best CO_2 producers with a

mean value of 16.1 mEq/l compared to 11.7 for *Leuc. dextranicum*. Parmelee (1967) determined gas production by lactic cultures by incubating the cultures in sterile skim milk, containing 3 % sodium citrate, sealed in test tubes with 1 to 3 cm layers of Plate count agar. Cultures containing *Lc. diacetylactis* produced 2 to 10 ml of gas in 16 to 20 h at 32 °C, whereas cultures containing *Leuc. cremoris* produced gas only after 36 to 48 h incubation at 32 °C.

Evaluation of a few strains for the vital property of flavour production revealed an absence of strains with the ability to produce diacetyl. It would probably have been helpful to evaluate firstly the growth of a number of strains in milk (other than lactococci) and then subjecting promising strains to analysis of flavour production. Only trace amounts of diacetyl were detected in one strain of *Leuc. lactis* (A2) isolated from traditional fermented milk. Usually organisms produce much more acetoin than diacetyl (Collins, 1972). The absence of diacetyl in the present study may be related to the fact that its production is species and strain dependent. The detection of volatile organic compounds in fermented milk may also be a function of time because the concentration of flavour compounds changes during the course of the fermentation process. Reduction of diacetyl to acetoin and of acetoin to 2,3-butylene glycol, as well as the volatilisation of diacetyl, are responsible for the decrease in amounts of diacetyl and acetoin that normally occur when the incubation of cultures is continued after the amounts of diacetyl and acetoin are maximal (Collins, 1972). The latter author also stated that while several micro-organisms produce large amounts of acetoin, no detectable diacetyl is produced. This may be true for the two strains of *Lb. plantarum* that showed peak areas of 1023 and 644 mV.s for acetoin, but zero for diacetyl. *Lc. diacetylactis* produced diacetyl, acetoin and acetaldehyde in quantities comparable to that of the commercial culture CH-Normal 22. Bellengier *et al.* (1992) investigated citrate metabolism in 20 *Leuconostoc* strains (two *Leuc. paramesenteroides*, two *Leuc. lactis*, nine *Leuc. mesenteroides* subsp. *mesenteroides* and seven *Leuc. dextranicum* species) and found that only four *Leuconostoc* strains (one *Leuc. paramesenteroides* and three *Leuc. mesenteroides* subsp. *mesenteroides*) produced low levels of acetoin and diacetyl. In the study of Cogan (1985) acetoin reductase was present in both *Lc. diacetylactis* and *Leuconostoc* species, though it

was not apparent why it was more active in leuconostocs. Cultures containing *Lc. diacetylactis* and *Leuconostoc* species differed with regard to rate and extent of acetoin and diacetyl production. The higher rate of production and larger quantities of diacetyl produced reflects the more active growth of *Lc. diacetylactis* (Cogan, 1985).

The amount of acetaldehyde produced by *Lc. diacetylactis* (peak area of 1699 mV.s) was not more than that produced by the commercial starter culture with a peak area of 1789 mV.s. It is known that overgrowth of *Lc. diacetylactis* may lead to undesirable high values of acetaldehyde in cultured buttermilk and sour cream (Margalith, 1981). This defect may be controlled with starter strains high in alcohol dehydrogenase or by ensuring that a high population of leuconostoc is present (Sandine *et al.*, 1972). Higher quantities of ethanol were also detected for the two strains of *Leuc. lactis* and *Lc. diacetylactis* than for the commercial culture. Ethanol is an ubiquitous metabolite in micro-organisms including lactic acid bacteria. It is formed by the reduction of acetaldehyde, which is catalysed by alcohol dehydrogenases (Lees & Jago, 1978). *Leuconostoc* species also produce 1 mol each of CO_2, lactate and ethanol per mol of lactose metabolised (Cogan, 1985). High levels of ethanol have been incriminated in the development of a fruity flavour defect (Keenan & Bills, 1968). The levels of acetone normally encountered in cultures and cultured products are so far below the threshold of this compound that it would have no effect on flavour (Keenan & Bills, 1968). Yuguchi, Hiramatsu, Doi, Ida & Okonogi (1989) established that the concentration of acetone, ethanol and acetic acid in fermented milk correlated negatively with good flavour.

The two strains of *Leuc. citreum* (A1 and A9) failed to grow in reconstituted NFM. According to Schillinger, Holzapfel & Kandler (1989) *Leuconostoc amelibiosum* did not ferment lactose. *Leuc. amelibiosum* is a later subjective synonym for *Leuc. citreum* (Takahashi, Okada, Uchimura & Kozaki, 1992). The strain of *Leuc. dextranicum* (A10) isolated from traditional fermented milk also failed to coagulate NFM. According to Garvie (1984) *Leuc. mesenteriodes* subsp. *dextranicum* showed variable reactions in the fermentation of lactose. Wisby (supplier of starter cultures) uses a specific strain of *Leuc. dextranicum* as a supplement which is added to the starter culture. Depending on the

strain, *Leuc. dextranicum* forms high quantities of gas, but hardly any acid (I. Dreyer, Dairy Technologist, Area Sales Manager North America, Wisby Starter Cultures and Media, 1997 - personal communication). The only strain that showed acceptable growth in milk was *Leuc. lactis* (A2), which coagulated reconstituted NFM within 24 h. Heap & Lawrence (1988) stated that leuconostocs, with the exception of *Leuc. lactis,* grow poorly in milk. *Leuc. lactis* is adapted to live in milk and ferments lactose more readily than other species (Garvie, 1984). Most leuconostocs grew at 30°C and normally not at 37°C. She also stated that *Leuc. cremoris* and *Leuc. oenos* preferred 22°C, which was probably a better general temperature for the whole genus, although growth would be slower than at 30°C for many strains. *Lb. plantarum* does ferment lactose, galactose and glucose (Kandler & Weiss, 1986). These authors also reported that the growth temperature of the genus *Lactobacillus* ranged between 2 and 53°C; the optimum generally being between 30 and 40°C. In the study of Mutukumira (1996) the three *Lb. plantarum* strains took 38 h to coagulate NFM at 25 °C.

In the initial sensory evaluation (untrained panellists) of strains for utilisation in fermented milk, the two strains of *Lc. lactis* produced a malty flavour. Citrate negative strains of *Lc. lactis* which produce malty compounds in milk are common in nature, and are regarded as highly undesirable in the dairy industry (Narvhus *et al.*, 1998). Salama *et al.* (1995) reported the isolation of a malty compound-producing strain of *Lc. lactis* from Moroccan milk. Mutukumira (1996) evaluated the suitability of lactic acid bacterial strains, isolated from naturally fermented milk in Zimbabwe, for inclusion in starter cultures for fermented milk products. Full-fat fermented milks inoculated respectively with strains of *Lb. casei* (2 strains), *Lb. plantarum* (3 strains), *Lb. acidophilus* (1 strain), *Leuc. mesenteroides* (2 strains), *Lc. lactis* (5 strains), *Lc. diacetylactis* (4 strains), *E. faecium* (3 strains) and *E. faecalis* (1 strain) were evaluated by two sensory panels. Milk fermented by one of the *Lc. diacetylactis* strains was characterised by a slight malty flavour but the flavour of the product was highly acceptable during sensory evaluation. The sensory panel did not accept the flavour of the products inoculated with the other strains, as they were watery and not firmly coagulated. The strain of *Lc. diacetylactis* produced amounts of diacetyl and other desirable flavour compounds similar to those

found in commercial mesophilic fermented milk products. The compound 3-methylbutanal, and to a limited extent 2-methylpropanal, 3-methyl-1-butanal and 2-methyl-1-propanol, were reported to be responsible for the malty flavour defect (Jackson & Morgan, 1954; Morgan, 1976; Mutukumira, 1996).

With regard to the sensory evaluation of the commercial and experimental products it can be concluded that the panellists were unable to describe the differences between the two groups of products using the available descriptive terms on the sensory score-card. The fact that only commercial products were used for vocabulary development may account for the inadequate list of descriptive terms and ineffective score-card. The commercial products did not provide a broad enough spectrum of characteristics in order to develop an effective score-card. A more extensive training period may also be recommended with more reference standards included and exposure to the relevant flavours and tastes. In other studies involving sensory evaluation the panellists were trained, for example, over five 1.5 h sessions (Guinard, Zoumas-Morse, Mori, Uatoni, Panyam, Kilara, 1997), 14 training sessions (Barnes, Harper, Bodyfeldt & McDaniel, 1991) and 34 training sessions over 3 months (Lederer, Bodyfelt & McDaniel, 1991). The pH of all products used for sensory evaluation by the trained panel was within the acceptable range (pH>4.1) according to Laulund (1993). Differences in starter-strain characteristics may explain the lower viscosity of the experimental products compared with the commercial products.

6. GENERAL CONCLUSIONS

In the present study on traditional fermented milk in South Africa, it was found that the method of preparing cultured milk in traditional containers such as calabashes and clay pots has diminished and is nowadays probably only practised in the most remote areas of the country. Modern containers that are readily available are replacing the calabashes and clay pots of which the fabrication and preparation is a dying art. The availability of milk is also determining the production of fermented milk products.

Lactic acid bacteria dominated the microflora of the traditional fermented milk samples, especially the genera *Leuconostoc* (35%), *Lactococcus* (28%) and *Lactobacilli* (23%). Eighty-three percent of the leuconostocs isolates were *Leuc. dextranicum*. Other species identified included *Lc. lactis*, *Leuc. citreum*, *Leuc. lactis*, *Lb. lactis* and *Lb. plantarum*. The lactic acid bacteria isolated were mainly of mesophilic nature and coincided with that of commercial mesophilic starter cultures with regard to the dominance of lactococci and leuconostocs. However, the industrially important species of *Lc. cremoris*, *Leuc. cremoris* and *Lc. diacetylactis* were not encountered in these natural products, as was the case in other similar studies (Antunes & De Oliveira, 1986 cited by Holzapfel & Schillinger, 1992; Crow *et al.*, 1993; Holler & Steel, 1995; Weerkamp *et al.*, 1996).

According to South African health regulations soured raw milk should not contain pathogenic organisms, any *E. coli* or more than 50 coliform bacteria in 1.0 ml of the product (South Africa, 1997). Only six of the fourteen samples examined for coliform bacteria complied with the regulations for products to be sold. This, as well as the presence of *E. coli* and *S. aureus* in some of the products may raise concern regarding the safety of traditional fermented milk products.

Selected isolates of lactic acid bacteria were subjected to screening tests for technological important properties. Acidity development tests showed that 53 % of the lactococci strains coagulated reconstituted non-fat milk within 16 h after incubation at 22 °C. Only one strain namely *Leuc. lactis* grew well in milk and had the potential to be used in starter

cultures. *Leuc. citreum* and *Leuc. dextranicum* were unable to ferment lactose and grow in milk. Two strains of *Lactobacillus plantarum* coagulated non-fat milk within 48 h. Evaluation of a few strains for the vital property of flavour production revealed an absence of strains with the ability to produce diacetyl. *Leuc. citreum, Leuc. dextranicum* and *Lb. plantarum* isolated from these traditional products did not divulge any potential for utilisation in dairy starter cultures. Demands put forward by any producer of fermented dairy products require starters of reliable performance. In this regard commercial cultures have the advantage of having established credibility.

The development of starter cultures is an intricate task with several obstacles and complicating factors:

- The high cost involved in identifying bacterial isolates to the species level influences starter culture research.
- A large number of strains have to be screened for relevant properties in order to find useful strains for starter cultures. According to G. Stanley (Technical Manager, Rhodia Texel Ltd, United Kingdom, 1997 - personal communication) the success rate is only 5 % for finding suitable strains for a specific application.
- The lack of simple, inexpensive methods for the determination of flavour compounds may limit research efforts especially in developing countries.
- The complex metabolic interrelations between micro-organisms to be included in multi-species/strain starters is a main problem of their selection and work of this nature is difficult and laborious, particularly in such a composite medium as milk (Semenikhina, 1986).
- Starter cultures need to deliver consistent technological performance. Investigation on the stability of the properties of strains may be hampered by the fact that the most important industrial parameters (lactose, protein and citrate metabolism) are encoded by plasmid DNA, which is easily lost in many strains.

Future research objectives in the field of mesophilic fermented milk products may be the optimisation of probiotic activity within these widely consumed products.