STUDIES ON LACTIC ACID PRODUCING MICROFLORA IN *MURSIK* AND *KULE NAOTO*, TRADITIONAL FERMENTED MILKS FROM NANDI AND MASAI COMMUNITIES IN KENYA.

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DECLARATION

This is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my beloved parents Mr. and Mrs. Stanley Mathara Muchui whose guidance and support is worthy emulating.

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List of abbreviations used in the text.

LAB: Lactic acid bacteria

TA: Titratable acidity

GRAS: Generally Regarded As Safe

Abstract

The following study gives information on identity and some characteristics of fermentative lactic acid producing microflora isolated from *Mursik* and *Kule naoto,* traditional fermented milk products from Nandi and Masai communities respectively. Both products were assessed for their chemical and microbial composition. The study showed that the fermented milk products had high ash content compared to the raw or commercial processed milk. Protein content in *Mursik* was higher than that of fresh milk possibly due to addition of boiled blood before fermentation, and concentration through whey removal. Both products had no coliforms; an indication of either good hygienic processing practices or antimicrobial activity against the coliforms.

Several species of lactic acid bacteria together with some yeasts and molds were isolated from the products, and their physiological and biochemical characteristics determined. The study showed that *Lactobacillus* and *Lactococcus* species are the dominant lactic acid bacteria in *Mursik* and *Kule naoto* respectively. *Saccharomyces* yeasts and *Ospora lactis* molds were found to be the dominant fungal microflora in both products. The yeasts and molds increased, while the lactic acid bacteria decreased with storage.

Isolated dominant lactic acid bacteria showed varying functional properties namely, acid production, proteolytic and anti-microbial activities. Investigations on the fermentation characteristics of the isolates under controlled laboratory conditions showed that several isolated strains of *Lactobacillus* species of bacteria and *Ospora lactis* mold could be used in the commercial development of fermented milk product, with good quality attributes and possibly longer shelf

life. Strains of *Lactobacillus plantarum* and *Lactobacillus confusus* species isolated exhibited good proteolytic and anti-microbial activities.

1. Introduction

Fermented milk is basically milk cultured using microbes in order to impart it with appropriate consumption properties. They are either sour milk products, such as yogurt, acidophilus milk or cultured butter milk, due to lactic fermentation by lactic acid bacteria, or alcoholic fermentation such as in Kefir or Koumis, involving fermentation by yeast in addition to lactic fermentation (Nakazawa and Hosono, 1992; Hui 1993b; Verman and Sutherland 1994, and Wood and Holzapfel, 1995).

Fermented milk can be prepared from whole milk, or skimmed milk using specific cultures; which may remain alive until consumption (IDF, 1988). The specific physical-chemical changes in milk associated with the growth of starter bacteria are essentially the most significant factors in determining the sensory attributes of the product, such as appearance, viscosity and flavor (Puhan and Zambrini, 1990 and Wango *et al.,* 1992).

Pastoral people of savannah region, extending from Eastern to Central Africa and predominantly rearing indigenous cattle, and the pastoral people of the oasis in the desert of North Africa, who mainly rely on goats and camel, have made fermented milk and cheese from ancient times (Shalo and Hansen 1973, IDF, 1988). Among the milk products made include *Zabady* in Egypt, *Nono* in Nigeria, *Aoules* and *Takammart* in Algeria, *Kule naoto* by the Masai in Kenya and Tanzania and *Mursik* by the Kalenjin Community in Kenya (Atanda and Ikenebomeh, 1991; Dick *et al.*, 1993; Nakazawa and Hosono , 1992; Isono *et al.*, 1994; Miyamoto *et al.*, 1996, and UNDP/SU/TCDC, 1996). These products made produced using traditional practices, and their starter culture microorganisms have not been adequately studied (Nakazawa and Hosono, 1992; Dick *et al.*, 1993; and Mathara *et al.*, 1996).

Some studies carried out on the microbial flora of traditional fermented milk products in Africa and Eastern Europe indicate a significant potential, for their development into starter cultures for utilization in industrial production of these products (Morita *et al.*, 1991; Morita *et al.*, 1992; Jøse-leis Montelengo *et al.*, 1993; Isono *et al.*, 1994; and Nakamura *et al.*, 1996).

Kenya produced a total of 1.826 billion tons of milk in 1992 and an estimated 1.976 billion tons of milk in 1996 (Development plan 1994-1996). Milk production is carried out in high to medium potential part of the country, covering 10 million hectares (17.6%) out of the total land area of 56.9 million hectares. About 60 % of this land is devoted for both crop and milk production. 42.1 Million Hectares (80 % of the total land area) is classified as low potential and is used for livestock production. This portion of the country supports over 25 % of total human population and slightly more than 50 % of total livestock population. There are several constraints in social and economic development of this region. One of the constraints is technological in nature because of lack of know how in economic utilization of available livestock and crop resources (Development plan 1994-1996).

Kule naoto production has remained a cultural and traditional practice among the Masai community over many centuries. This traditional fermented milk product is prepared in special treated gourds by all Masai families. The product, with blood sometimes added to raw milk, is fermented spontaneously for five days (Miyamoto *et al.*, 1986). Another common traditional fermented milk product in Kenya is *Mursik* produced by the Nandi Community. *Mursik* is a high acid, low moisture content product, which has historically been a daily diet for the Kalenjin communities in general (Mathara *et al.*, 1996). No studies on the lactic acid producing microflora involved in fermentation of these traditional fermented milk products have been

undertaken. Such studies are necessary for improvement and control of fermentation processes of these products. Spontaneous fermentation in such products is difficult to control. Functional properties of lactic acid bacteria involved, such as flavor compounds production, texture formation, and antimicrobial properties are especially important to the dairy industries because of their applicability to a large variety of fermented dairy milk products (Fuller, 1992, Nakazawa and Hosono, 1992, and Dick *et al.*, 1993).

Most fermented milk products previously processed through spontaneous fermentation, are today fermented via controlled processes using pure cultures. Such cultures impart the characteristic desired to the products. Accordingly, research aimed at developing appropriate cultures that impart the necessary traditional preference qualities on the traditional products is worth undertaking. It's also worthwhile exploring on the unique properties that may be associated with the undescribed microflora in these traditional fermented products.

In this study, information on the characteristics of dominant lactic acid producing microflora isolated from the Masai and Kalenjin traditional fermented milk products *Kule naoto* and *Mursik* respectively is given. This project was therefore undertaken with the main objective of isolation and characterisation of the microflora responsible in fermentation of *Kule naoto* and *Mursik* milk products in Masai and Nandi communities respectively.

The specific objectives are:

1. To describe the production processes *Mursik* and *Kule naoto.*
2. To determine and characterize the dominant microflora involved in the fermentation of the products.
3. To investigate the functions of the isolates namely: acid and aroma production, , protein hydrolysis and antimicrobial activity .

2. Literature Review

2.1. Traditional fermented milk production in Kenya.

Food processing in African societies are deeply rooted in rural traditional environments. Even though societies used the same scientific principles as now, the mode of application may be different (Sefa Dedeh, 1993).

Fermented foods are defined by Campbell (1987) as foods which have been subjected to the action of microorganisms or enzymes to bring about the desirable biochemical changes in the foods. The art of milk fermentation as part of traditional food processing is practiced extensively in Kenya. Pastrolist communities among them the Kalenjin and the Masai still practice this art. The traditional fermented milk products from the two communities are commonly known as *Mursik* and *Kule naoto* respectively. Fermented milk products form a substantial part of their diet and are seen, among other factors, as a means of introducing variety into the consumption of staples, milk, and other livestock products (Mathara *et al.,* 1996; Nakamura *et al.,* 1996; and Miyamoto *et al.*, 1986). Fermented products are known to have a better nutritional value than the raw material from which they are made.(Nakazawa and Hosono, 1992 and Fuller, 1992).

Many traditional fermented milk products have been made and consumed in Africa, Asia, Middle East and European countries. The microbiological characteristics of several of these products have been studied. Reddy *et al*,. (1986) described the isolation and identification of lactic acid bacteria from fermented products in Eastern Europe. Isono *et al*,. (1994) reported on identification and characteristics of lactic acid bacteria isolated from Masai fermented milk products in Northern Tanzania.

Dick *et al.*,(1993) gave information on isolation, screening and identification of lactic acid bacteria from traditional fermentation processes and culture collections. Lactic acid bacteria isolated from traditional fermented milk are reported to bind to several kinds of mutagenic amino acid pyrolysates (Nishioka *et al.*, Isono *et al;* 1994 and Fuller, 1993). These properties of lactic acid bacteria may contribute to the health benefits attributed to the traditional cultured dairy products in Kenya (Mathara *et al.*, 1996 and Isono *et al.*, 1994).

A study carried out on traditional fermented milk produced by the Masai in Kenya led to isolation and identification of a unique strain named *Streptococcus lactis KM* (Miyamoto *et al*;, 1986). Further investigations on metabolic characteristics of the isolated bacteria showed that the strain produced L type of lactic acid isomer and had a strong antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Miyamoto *et al,*. 1986). Use of this strain has resulted in successful intergeneric transfer of lactose fermenting ability to *Pediococcus acidolactici* via conjugation (Morita *et al,*. 1991). The transconjugant, 25-EM-KM, was noted to posses the best fermentative properties such as milk coagulation and flavor production, when compared to several other commercial strains. This strain could be used in commercial production of fermented milk products with improved quality (Morita *et al.*, 1991,1992).

Viable cell counts and over-souring in fermented milks during cold storage is an important quality aspect of a fermented milk. The strain 25-EM KM had a suitable titratable acidity (approximately 0.9 %) and viable cell count of 10^6 cfu/ml in fermented milk samples after storage at 4°C for more than 60 days (Morita *et al,*. 1992). This strain also exhibited salt tolerance of 8-9% NaCl and can thus be used in making some types of cheeses.

2.2 Classification of Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram positive bacteria typified by production of lactic acid as an end product. They are catalase negative, facultative anaerobes, and non motile bacteria. This group comprises of at least eight genera namely, *Lactobacillus, Leuconostoc, Pediococcus, Streptococcus, Carnobacterium, Enterococcus, Lactococcus,* and *Vagococcus* (Sneath *et al.*, 1986).

The species once classified as *Lactobacillus hordniae* and *Lactobacillus xylosus* have been transferred to the genus *Lactococcus*. With the enterococci and lactococci having been removed from the genus *Streptococcus,* the only left lactic acid bacteria of importance in food fermentation is *Streptococcus thermophilus* renamed *Streptococcus sarivarius var. thermophilus. Streptococcus diacetylactis* has also been re-classified as a strain of *Lactococcus lactis* subspecies *lactis*. Although *Lactococcus cremoris* has been re-classified as a subspecies of *Lactococcus lactis,* this biovar is important in cheddar cheese production (Hui 1993b, Varnam and Sutherlands, 1994).

While the LAB group is loosely defined with no precise boundaries, all members share the property of producing lactic acid from hexoses (Wood and Holzapfel, 1995, and Hui, 1993b). They can be divided into two groups based on the end products of glucose metabolism. Those that produce lactic acid as a major or sole product of glucose metabolism are designated as homofermentative. The homofermentative pattern is observed when only glucose is metabolized but not necessarily when pentoses are metabolized, since some homofermentative LAB produce acetic acids when utilizing pentoses. Also the homofermentative character of

LAB may be altered for some strains by changing cultural conditions such as glucose concentration, pH, and nutrient limitation (Nakazawa and Hosono 1992).

The homofermentative LAB are able to extract about twice as much energy from a given quantity of glucose than the heterofermentative ones. LAB that produce equal molar amounts of lactate, carbon dioxide, and ethanol from hexose are designated heterofermentatives. All genera of *Pediococcus*, *Lactococcus, Vagococcus* and some lactobacilli are homofermentative, while *Leuconostoc* species and some lactobacilli are heterofermenters (Wood and Holzapfel,1995). The heterofermentative LAB are more important than the homofermentative ones in producing flavor and aroma components such as acetyladehyde and diacetyl (Carr, 1983; Wood and Holzapfel,1995; Gasson and de Vos, 1994).

The difference in end product between homofermenters and heterofermenters when glucose is utilized are as a result of basic genetic and physiological differences (Valerie and Barry, 1984). The homofermentative LAB possess the enzyme aldolase and hexose isomerase but not lactose phosphoketolase. They use the Embeden-Meyerhof -Parnas (EMP) pathway to produce two lactate molecules. The heterofermentative LAB on the other hand, have phosphoketolase but do not possess aldolase and hexose isomerase, and instead of EMP pathway they use the hexose monophosphate or pentose pathway for glucose degradation (Wood and Holzapfel,1995; Nakazawa and Hosono 1992; and Gasson and de Vos, 1994).

The genus *Lactobacillus* has been subdivided into three subgenera: *Betabacterium, Streptobacterium* and *Thermobacterium* (Wood and Holzapfel, 1995) The *Streptobacterium* such as *Lb. casei* and L*b. plantarum* produce up to 1.5 % lactic acid with optimal growth temperature of 30°C, while *thermobacteria* such as *Lb. acidophilus* and *Lb. bulgaricus* can produce up to 3 % lactic acid, and have

optimal growth temperature of 40 °C (Hui, 1993b, and Tamine and Robinson, 1986). Lactic acid bacteria require certain amino acids, B vitamins, purine and pyrimidine bases for growth and are used in microbiological assay for these compounds (Hui, 1993b;Valerie and Barry, 1984). Although they are mesophilic, some lactic acid bacteria can grow at temperatures below 5°C and others as high as 45°C.

Most LAB can grow in the pH range 4.0 to 4.5, while some can grow at pH 3.2 and others at pH 9.6. The lactic acid bacteria are only weakly proteolytic and lipolytic (Valerie and Barry, 1984; Wood and Holzapfel, 1995).

2.3 Lactic acid microflora in fermented milk products.

2.3.1 Bacterial microflora in fermented milk

The dominant microflora in traditional fermented milk are the lactic acid bacteria. However, fungi and yeast have been isolated in some traditional fermented milk products (Isono *et al.*, 1994, and Miyamoto *et al.*, 1986). Food fermentations which have traditionally been via spontaneous fermentation are today carefully controlled microbial processes, for which selected pure cultures have been developed. These starter cultures have been formulated by using microorganisms which impart the special desired characteristics to the fermented product.

Most of lactic acid bacteria used in dairy industry belong to the genera, *Streptococcus*, *Lactococcus*, *Leuconostoc* and *Lactobacillus*. Attention has now been focused on use of new isolates of genus *Streptococcus* with unique characteristics. Several Lactic acid bacteria strains have been isolated and identified, from traditional fermented milk products in Kenya and Tanzania, and some of their properties studied (Nakamura *et al.*, 1996; Isono *et al.*, 1994 and Miyamoto *et al.*, 1986).

Fermentation is defined as a metabolic process in which carbohydrates and related compounds are oxidized with the release of energy in the absence of any external electron acceptors. The final electron acceptors are the organic compounds produced directly from the breakdown of the carbohydrates. Consequently, only partial oxidation of the parent compound occurs, and only a small amount of energy is released during the process, As fermenting organisms, lactic acid bacteria lack a heme-linked electron transport systems or cytochromes, and they obtain their energy by substrate-level phosphorylation while oxidizing carbohydrates. They do not have a Krebs cycle (Wood and Holzapfel, 1995 and Nakazawa and Hosono, 1992).

In the food industry, lactic acid bacteria are used for the production of various fermented milk products such as cheese, yoghurt, cultured butter milk *etc* (Hui, 1993b; Varnam and Sutherland, 1994; Robinson and Tamine, 1990). They act to preserve foods by virtue of their acidification of the product. In addition they impart certain desirable organoleptic characteristics due to acidity and flavor (Hui, 1993b).

Furthermore, certain health benefits have been attributed to certain probiotic lactic acid bacteria, e.g by *Lactobacillus* and *Bifidobacterium* (Fuller, 1992). These benefits include alleviation of abdominal and intestinal disorders, reduction of dental caries, and anti-tumor activity (Fuller, 1992 and Masaki *et al.*, 1998).

Ingestion of yoghurt has been reported to aid in lactose absorption in lactase deficient people, by facilitating intraintestinal lactose cleavage by β-galactosidase released by yoghurt- borne lactic acid bacteria (Wood and Holzapfel 1995; Nakazawa and Hosono 1992, and Hui 1993b). In addition lactic acid bacteria are used purposely as probiotics and as silage innoculants (Wan-Yin fu *et al,*. 1995 and Fuller, 1992).

2.3.2 Fungal microflora in fermented milk

Information on the role played by the fungal microflora in traditional fermented milk products is quite limited. (Mathara *et al,*.1996). *Saccharomyces* and *Candida* species of yeast were isolated from traditional fermented milk product samples prepared by the Masai community in northern Tanzania (Isono *et al,*.1994). *Saccharomyces* species of yeast are currently widely used in production of alcoholic beverages and in baking industry (Amoa-Awua, 1996). They are also involved in spontaneous fermentation of several vegetable products (Miyamoto *et al*., 1986)

Some mold species of *Penicillium* have been developed and are in being used in cheese processing (Samson *et al*., 1981). They are also recommended inoculum for some fermented meat products (Samson *et al*,.1981). *Saccharomyces cerevisiae* has been isolated from stracchino cheese and kefir (Hui, 1993b and Samson *et al*., 1981). *Klyveromyces maxianus* var. *lactis* presently known as *Klyveromyces lactis* is commonly associated with yoghurt, and has been isolated from milk, Italian cheese, buttermilk and cream. *Klyveromyces maxianus var. maxianus* presently known as *K. fragilis* is associated with manufacture of Kefir and Kumis.

One of the most important functions of the fungal microflora in food fermentation is synthesis of enzymes (Samson *et al*., 1981). These enzymes generally decompose complex compounds such as proteins, carbohydrates and fats into smaller molecules. Other compounds may be synthesized from the food substrate. *Saccharomyces* species do not utilize lactose but can utilize glucose and galactose (MIRCEN UNESCO, 1998).

Some molds, for example species of *Rhizopus oligosporus* used in tempe fermentation are reported to reduce the aflatoxin content of a substrate by 40% (Samson *et al.*, 1981). *Rhizopus oligosporus* has also been reported to inhibit growth, sporulation and aflatoxin production by the *Aspergillus flavus,* (MIRCEN UNESCO, 1998.)

2.4 Metabolic functions of lactic acid bacteria

2.4.1 Lactose utilization.

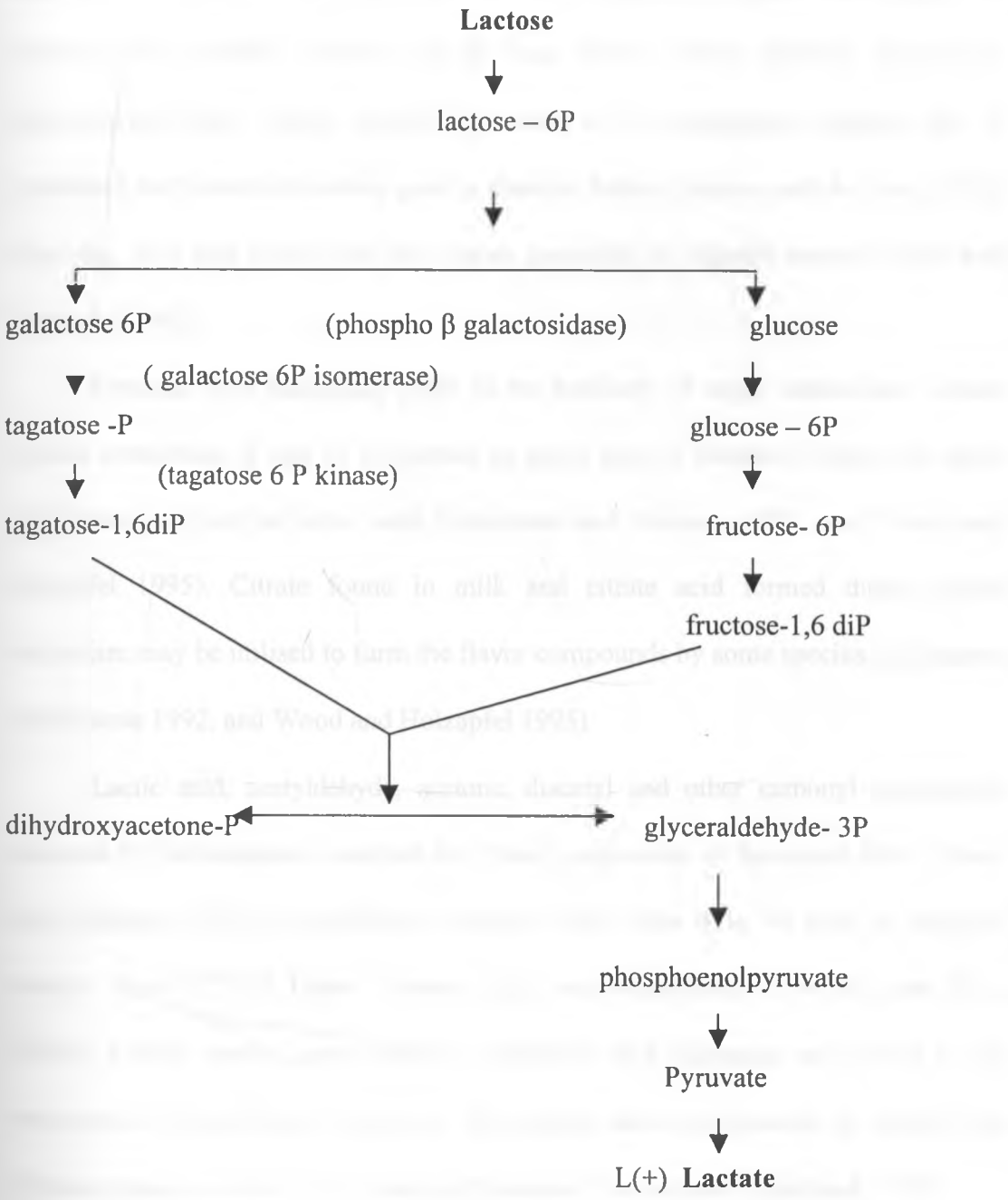
The metabolism of carbohydrates by lactic acid bacteria can be classified as homofermentative or heterofermentative. Homofermentative lactic acid bacteria follow the familiar Embden Meyerhof Panas (EMP) pathway for glycolysis and unlike animal muscle, may either form D(-) or L(+) lactic acid or a racemic mixture of the two isomers (Wood and Holzapfel, 1995).

There are major differences in lactose transportation and metabolism between different LAB bacteria. *Lactococcus* transport lactose into the cell using the phosphoenol pyruvate phosphotransferase system which involves simultaneous formation of lactose phosphate in *Lactobacillus*, *Leuconostoc* and *Streptococcus Salivarious* subsp. *thermophilus* (Nakazawa and Hosono, 1992). Lactose enters the cell intact via permease system, and is subsequently hydrolysed to glucose and galactose by ß-galactosidase (Varnam and Sutherland, 1994). The glucose is then metabolised via glycolytic pathway in *Lactobacillus* and *Streptococcus salivarius* subsp. *thermophilus,* but through phosphoketolase pathway in *Leuconostoc* (Varnam and Sutherland, 1994).

Galactose is usually metabolised via glucose 1- phosphate through the Leloir pathway by *Lactobacillus delbrueckii* and most strains of *Streptococcus salivarius*

species (Varnam and Sutherland, 1994). Some strains of *Lactobacillus delbrueckii* subsp. *lactis* are unable to metabolise galactose which is excreted, (Varnam and Sutherland, 1994). Figure 1 illustrates common pathways of lactose utilisation by a typical *Lactococcus lactis* strain.

Figure 1: Pathway for lactose utilization by typical *Lactococcus lactis* strain.



2.4.2. Flavor compounds production.

Production of flavor compounds during fermentation is of great importance in fermented milk products. Diacetyl (from citrate fermentation) and acetaldehyde are major flavor compounds. Fermentation of citrate by lactic acid bacteria has long been known to be unstable (Gasson and de Vos, 1994). Citrate negative mutants of *Streptococcus lactis* subsp. *diacetylactis* lack a 5.5 megadalton plasmid and is postulated that citrate utilization gene is plasmid linked (Gasson and de Vos, 1994). However, it is also likely that only citrate permease is plasmid coded (Wood and Holzapfel, 1995).

Pyruvate is a branching point in the pathway of sugar catabolism. Under various conditions, it can be converted to acetic acid or butane-2,3-diol and other compounds, as well as lactic acid (Nakazawa and Hosono, 1992, and Wood and Holzapfel 1995). Citrate found in milk and citrate acid formed during sugar catabolism may be utilised to form the flavor compounds by some species (Nakazawa and Hosono 1992, and Wood and Holzapfel 1995).

Lactic acid, acetyldehyde, acetone, diacetyl and other carbonyl compounds produced by fermentation constitute key flavor compounds in fermented milk (Wood and Holzapfel, 1995). Acetyldehyde content varies from 4 to 60 ppm in yoghurt, diacetyl from 0.1 to 0.3 ppm whereas acetic acid varies from 50 to 200 ppm (Hui, 1993b). Certain amino acids such as methionine and threonine are known to be precursors of acetaldehyde formation. The amino acids are believed to modify the enzyme system involved in aceyldehyde formation (Varnam and Sutherland, 1995).

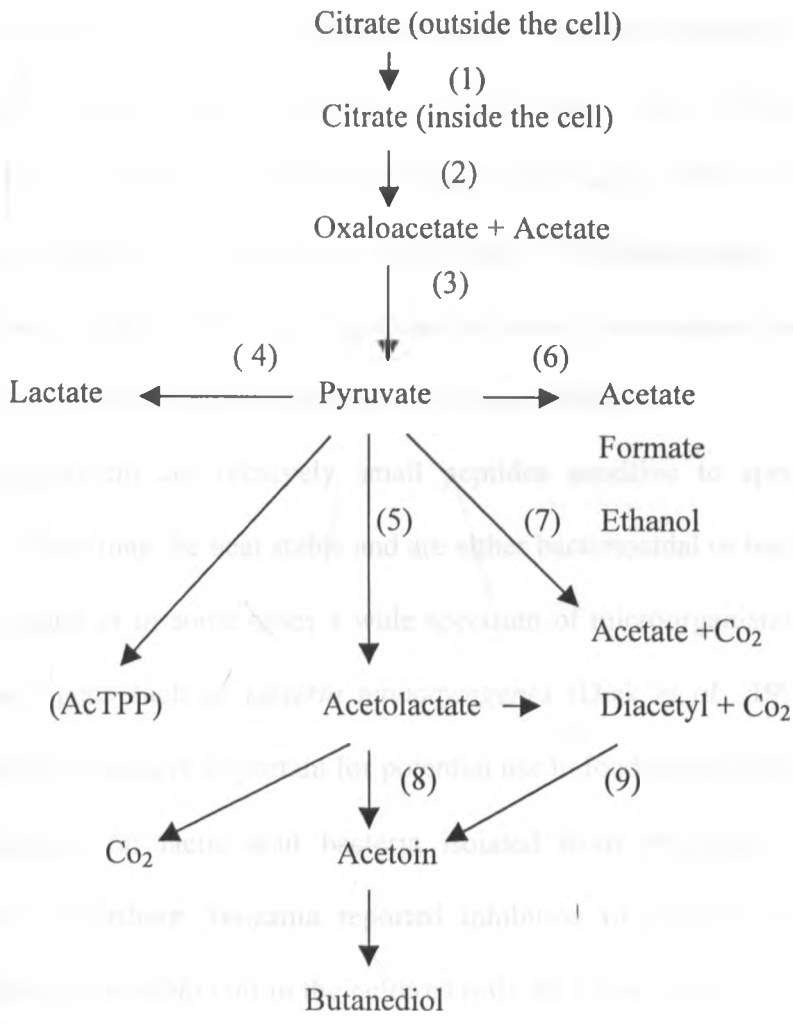
A common citrate metabolism in lactic acid bacteria is illustrated in Figure 2 using *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis.* As shown in Figure 2, citrate metabolism involves four pathways; First, lactate production via enzyme

lactate dehydrogenase, secondly, formate and acetate or ethanol production via enzyme pyruvate formate lyase, thirdly, acetate and Co_2 production via enzyme pyruvate dehydrogenase, and lastly, α-Acetolactate production via enzyme acetolactate synthase and subsequent acetoin and butanediol production by enzymes acetolactate decaboxylase and acetoin reductase.

Production of diacetyl is by spontaneous chemical disintegration of α-acetolactate. The enzyme citrate permease is coded on plasmids genes, whereas citrate lyase and oxaloacetate decarboxylase are coded on the chromosome. The resulting diacetyl concentration in sour milk, cream, and butter are between 1.5 and 2.5 mg/kg (Wood and Holzapfel, 1995,).

Figure 2: Pathway for Citrate metabolism in *Lactococcus lactis* subsp. *lactis*

biovar ***diacetylactis*** **(Wood and Holzapfel,1995)**



Key: Names of enzymes involved:

(1): Citrate permease, (2): Citrate lyase (3): Oxaloacetate decarboxylase (4): Lactate dehydrogenase (5): Acetolactate synthase (6): Pyruvate formate lyase (7): Pyruvate dehydrogenase (8): Acetolactate decarboxylase (9): Acetoin reductase

2.4.3. Antimicrobial activity

The lactic acid bacteria are known to produce and excrete compounds with antimicrobial activity such as bacteriocin, and Nicin (Mbugua and Njenga, 1991; Nakazawa and Hosono, 1992; Fuller, 1992; and Wood and Holzapfel, 1995). Nisin is produced by many strains of *Lactococcus lactis* subsp. *lactis*. Nicin is permitted as food additive in at least 47 countries (Wood and Holzapfel, 1995). Nisin is primarily useful in inhibition of Clostridium species like *C. botulinum* and *C. tryobutyricum* (Wood and holzapfel, 1995). *Lactococcus* produces a bacteriocin called lactococcins which may be heat stable (Nakazawa and Hosono, 1992).

Bacteriocin are relatively small peptides sensitive to specific proteolytic enzymes. They may be heat stable and are either bacteriocidal or bacteristatic against closely related or in some cases a wide spectrum of microorganisms including food borne pathogens such as *Listeria monocytogenes* (Dick *et al,*. 1993). This makes bacteriocin particularly important for potential use in food preservation.

Studies on lactic acid bacteria isolated from traditional fermented milk products in Northern Tanzania reported inhibition of growth of *Staphylococcus aureus* and *Escherichia coli* in the cultured milk by *Lactococcus lactis* ssp. *lactis* and *Lactobacillus confusus* (Isono *et al,*. 1994). Miyamoto *et al*., (1986), reported that a *Streptococcus lactis* strain isolated from Masai fermented milk product in Kenya showed antimicrobial activity against growth of *Eschelichia coli* and *Staphylococcus aureus* strains of bacteria. Gram negative bacteria inhibition by lactic acid bacteria has also been reported by Chang *and* Hearsberger (1994).

Dick *et al,* (1993), reported production of nisin-like bacteriocin from eight *Lactococcus* strains isolated from traditional food fermentation processes. More

bacteriocins have been studied at molecular level and are being categorised on the basis of size, stability to heat and the presence of modified amino acids (Gasson and de Vos, 1994). Increased understanding of bacteriocins in lactic acid bacteria is leading to new biological preservatives for use in food industry (Gasson and de Vos ,1994, and Nakazawa and Hosono, 1992, and Wood and Holzapfel, 1995). Antimicrobial compounds produced may ensure a safe product and extension of products shelf life (Gasson and de Vos, 1994; Kazushi *et al.*, 1994). Consumption of the fermented milk products is also thought to have some therapeutic and probiotic activity (Fuller, 1992)

2.4.4 Proteolytic activity

The proteolytic activity by most lactic acid bacteria is not only a prerequisite for growth, but also affects product texture and flavor in dairy products (Dick *et al.*, 1993). Proteolytic activity encompasses proteinases that degrade proteins such as casein, into relatively small fragments, and peptidases that break down protein fragments into small peptides and amino acids (Thomas and Pritchard, 1987).

All lactic acid bacteria either require or are stimulated by amino acids for growth yet free amino acid concentration in milk is not sufficiently high to permit commercially useful growth, and acid production (Wood and Holzapfel, 1995). The dairy lactic acid bacteria overcome this nutritional problem using a complex combination of proteinases, peptidases and a transport system to make bound amino acids available for growth (Gasson and de Vos 1994). Like other bacteria, the lactic acid bacteria can actively transport amino acid and peptides across the cell membrane into the cell against a concentration gradient.

There are three times as much peptide in cow's milk compared to free amino acids. Free amino acids and peptides are important sources of nitrogen for the growth of lactic acid bacteria (Nakazawa and Hosono, 1992). The proteolytic ability which the lactic acid bacteria have in order to acquire essential amino acids from cows milk contribute to flavors and characteristics of fermented milk products (Nakazawa and Hosono, 1992). Amino acid and peptide uptake in *Lactobacillus*, *Leuconostoc* and group N Streptococci are mediated by separate systems. *Streptococcus* have also distinct dipeptide and oligopeptide transport system (Dick *et al.,* 1993, Varnam and Sutherland, 1994). Studies during the last decade have given an insight into the complexity of the proteolytic ability of the lactic acid bacteria. This complexity is seen not only in the numbers and types of different proteases and peptidases but also their sub cellular distribution (Gasson and de Vos, 1994).

Studies of protein utilization have not been sufficiently systematic to allow for the building up of a complete picture of the inter-relationships between proteolytic and transport systems. However, complete understanding of the physiology of lactic acid bacteria is helpful in arriving at a rational decisions as to the best starter strain for a particular product, and the best conditions under which to culture them (Gasson and de Vos, 1994). Proteolytic activity of lactic acid bacteria is important in fermented milk products and especially in sensory and kinesthetic quality attributes (Hui, 1993b). Information on proteolytic activity is also a useful tool in characterization of lactic acid bacteria and in efforts towards culture development for use in dairy industries (Nakazawa and Hosono,1992, and Varnam and Sutherland, 1994).

2.4.5. Antimutagenic activity

Amino acid pyrolysates are commonly found in food and exhibit mutagenic properties. Lactic acid bacteria isolated from traditional fermented foods have been shown to exhibit ability to bind to several amino acid pyrolysates. A high binding ability of amino acid pylorysate to Try-P-1 and Try-P-2 respectively has been reported for lactic acid bacteria and yeasts isolated from traditional food products (Isono *et al.*, 1994; Masaki *et al.,* 1998 and Nishioka *et al.*, 1988). In addition lyophilized cells from microorganisms fed to rats prevented absorption of Trp-P-1 and Try-P-2 in the small intestines (Fuller, 1992). These observations suggest that the consumption of cultured dairy products could reduce the risk of cancer, because such amino acid pyrolysates are commonly formed in foods (Fuller, 1992, and Isono *et al*; 1994).

According to epidemiological studies, close to 90% of all cancer is associated with environmental factors including food, while 30 to 40 % of cancer in men and 60% in women is thought to be food related. A cancer controlling action by lactic acid bacteria and fermented milk has been described in details (Nakazawa and Hosono 1992, and Fuller, 1992,).

Investigation on the antitumor activity by *Lactobacillus* species through sarcoma 180 tumors transplanted in mice has been carried out (Nakazawa and Hosono, 1992). Results of the investigation showed that nearly all the lactobacilli had a suppressing action on sarcoma 180, but the degree of suppression differs among species and strains (Nakazawa and Hosono, 1992).

Table 1 shows the anticancer effect of lactobacilli on Sarcoma 180. The following conditions were used in that study: one million Sarcoma cell per mouse

transplanted sub cutaneously intravenous injection of lactobacilli for 5 successive days (total 50mg/kg); weight of cancer cell measured after 21 days, and % tumor suppression calculated (Nakazawa and Hosono, 1992)

Table 1 The Anti cancer effect of lactobacilli on Sarcoma 180.

(Nakazawa and Hosono, 1992)

Species	Strain number	Tumor suppression %
Lactobacillus casei	YIT 9018	82.7
	YIT 0078	79.4
	YIT 0105	57.9
	YIT 0151	59.5
	YIT 0075	72.7
Lactobacillus acidophilus	YIT 0075	72.1
	YIT 0163	18.3
	YIT 0168	43.5
Lactobacillus fermentum	YIT 0082	14.9
	YIT0159	-10.2
Lactobacillus salivarius	YIT 0104	40.9
	YIT 0155	19.7
	YIT 0089	21.5
	YIT0153	63.7
Lactobacillus plantarum	YIT 0102	-17.2
	YIT 0158	48.4
Lactobacillus bulgaricus	YIT 0046	64.7
Lactobacillus jugurti	YIT 0085	17.7
Lactobacillus helveticus	YIT 0083	29.0
Lactobacillus lactis	YIT 0086	12.8
Lactobacillus brevis	YIT 0076	5.3
Lactobacillus jensenii	YIT 0084	38.4
Lactobacillus buchneri	YIT 0077	30.5

2.4.6 Exopolysaccharide (EPS) production

Exopolysaccharide (EPS) production is a property exhibited by a large variety of microorganisms including lactic acid bacteria (Dick *et al.*, 1993). EPS is thought to play a role in protection against desiccation and phagocytosis as well as against predation by protozoa through interference in microbial adhesion to the surfaces of protozoa and bacteria recognition by protozoa.

In a large variety of foods, EPS is used as a thickener and/or as stabilizing agent. Xanthan gum is widely used EPS mainly because of its unique rheological properties in food, (Varnam and Sutherland, 1994). However, xanthan gum is produced by the plant pathogen *Xathomonas campestris* and addition of xanthan gum to food products have to be labelled (Dick *et al.,* 1993). Therefore, food industry is interested in EPS produced by Generally Regarded as Safe (GRAS) organisms such as Lactic acid bacteria. A number of such lactic acid bacteria have been isolated from traditional food systems and deposited in culture collections (Dick *et al.*, 1993). Some *Lactobacillus* species (*Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helviticus*, *Lb. hilgadii* and *Lb. kefiranofaciens*) have been reported to produce EPS (Cerning, 1990).

2.4.7 Lowering of blood Cholesterol levels.

Studies carried out with Finland's traditional ropy fermented milk, *Viili*, in rats, showed lowering serum cholesterol levels. A high serum high density lipoprotein (HDL) to low density lipoprotein (LDH) ratio was reported on rats fed on *Viili* (Nakajima *et al.*, 1992). After several studies carried out among the Masai men, it has been reported that those men who drank large amounts (3 to 5 litres per day) of *Kule*

naoto fermented with wild strains of *Lactobacillus* species had very low values of blood cholesterol (Nakazawa and Hosono, 1992).

2.5 Isolation and identification of lactic acid bacteria

Isolation and screening of microorganisms from naturally occurring fermentation processes have always been the most powerful means of obtaining cultures for scientific and commercial use. This is true for lactic acid bacteria, which plays an important role in a large number of various traditional food fermentation. Isolation involves obtaining of either mixed or pure culture followed by assessment to determine which of the isolated strain(s) carries out the desired reaction to produce a particular quality attribute (Wood and Holzapfel 1995, Dick *et al*;, 1993, and Pulusani *et al*;,1979).

Traditional fermentation of dairy products create a specialized environment highly selective to bacteria strains. The resulting strains can be important in commercial application, where their functions are optimized for superior quality products. Secondly, the unique strains can be used for genetic improvement of the existing commercial strains (Gasson and de Vos, 1994).

Classical methods for microbial identification are widely used in lactic acid bacteria studies (Wood and Holzapfel, 1995). These are based on morphology and biochemical characteristics of culture under standard conditions. Factors such as the size of the innoculum used, incubation temperature, length of incubation, type and composition of the supportive growth media are significant. Developed chemical, enzymatic and physiological techniques are used for identification of isolated strains (Wood and Holzapfel, 1995).

RNA and DNA homology are being used increasingly for lactic acid bacteria classification. RNA techniques classifies bacteria into larger groups while DNA techniques classifies organisms to species level. The data on composition of DNA, expressed as % guanine + cytosine (mol % G+C) is well established for most species (Wood and Holzapfel, 1995 and Gasson and de Vos, 1994). The mol % G +C for different strains and natural genera fall within narrow range, varying only by few percentages but the exact range is however not yet established (Garvie, 1984). Components of cell walls and cell membranes are also important tools in classification of lactic acid bacteria (Wood and Holzapfel, 1995). The *Streptococcus* have a variety of patterns suggesting a heterogeneous genus, but the groupings do not correspond with those from rRNA/DNA hybridization studies (Wood and Holzapfel, 1995).

Physiochemical tests are also used in classification of lactic acid bacteria. These tests are largely based on the ability of lactic acid bacteria to carry out certain chemical changes. This includes acid production from different sugars, hydrolysis of arginine, growth under different conditions (temperature and NaCl), survival at 60 °C for 30 minutes, and utilization of citrate among others (Wood and Holzapfel, 1995, Sneath *et al.*, 1986 and Lilian, 1977). Lactic acid bacteria can be divided into two groups depending on whether their main pathway for glucose fermentation is the Embden-Myerhof (EM) glycolytic pathway or a combination of the hexose monophosphate (HMP), followed by the phosphoketolase pathways (Wood and Holzapfel, 1995, and Sneath *et al.*, 1986).

The production of acid (formic, lactic or acetic acid) from carbohydrates is an indispensable property used in the identification and differentiation of *Lactococcus* species (Wood and Holzapfel, 1995, Nakazawa and Hosono,1992, and Jung Chang *et*

al, 1993 , 1994). Using an API 50 CH kit system, all lactococci are known to produce acid from glucose, fructose, mannose and N- acetyl glucosamine. Acid is not, however, produced from D arabinose, arabitol, adonotol, 2-keto-gluconate, 5-ketogluconate, dulcitol, erythritol, d-fucose, L-fucose, glyceral, glycogen, inositol, D-lyxose, Mythyl-D-mannoside, β-methyloxide, rhamnose, Xylose and Xilitol (Sneath *et al.*, 1986).

Understanding the metabolic pathways clarifies the taxonomy of bacteria and also helps to explain their importance in fermentation. In lactic acid bacteria the pathway for glucose fermentation can be ascertained by the end product formed, namely carbon dioxide, acetate, ethanol and lactic acid. Fermentation route can also be established by detecting key enzymes. The presence of fructose- 1 diphosphate (FDP) aldolase shows that strains have EM pathway, while glucose-6-phosphate dehydrogenase (G-6-PDH) indicates heterofermentative species (Wood and Holzapfel, 1995). The enzymes involved help to separate species with same fermentation pathways. Lactate dehydrogenase (LDH) enzymes are found in all lactic acid bacteria, and catalyse the final steps of their energy metabolism. There are three types of Nucleotide adenine Dinucleotide dependent LDH, forming D(-) lactate, L (+) lactate and L(+) lactate but requiring activation by FDP. It is therefore important to determine the isomer of lactic acid produced.

3. Materials and methods

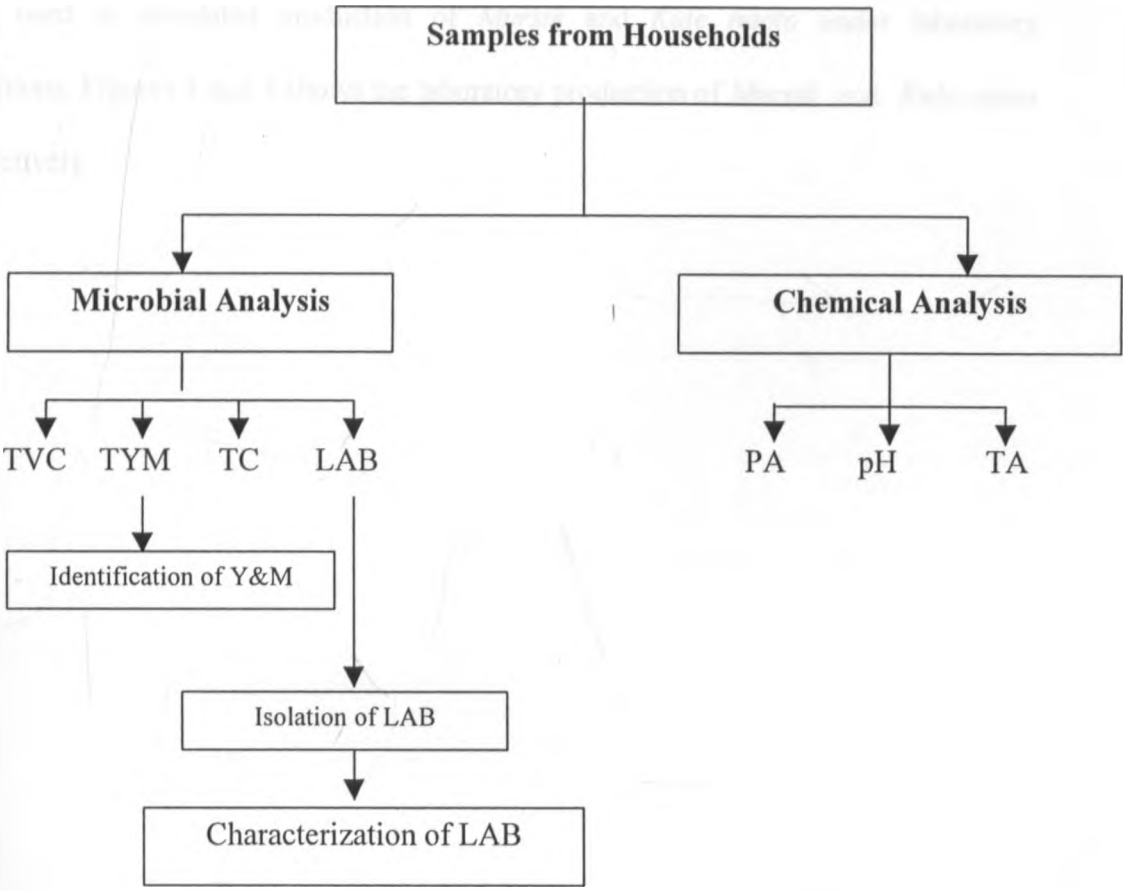
3.1 Materials

3.1.1. Study and Experimentation design.

3.1.1.1 Fermented milk samples

Traditional fermented milk samples were obtained from the two study regions, that is Kajiando and Nandi Districts. The fermented milk products, *Kule naoto* and *Mursik* were prepared by the Masai and Nandi communities respectively using traditional practices. The fermented milk product samples were obtained at random from several homes, mixed and a representative sample, amounting to about half a litre drawn from the mixture. The mixture was transported in their fermentation vessels, namely gourds to the laboratory for analysis. Figure 3 represents analytical work on traditional fermented milk *Kule naoto* and *Mursik.*

Figure 3: Flow chart of analytical work on traditional fermented milk *Kule naoto* and *Mursik*.

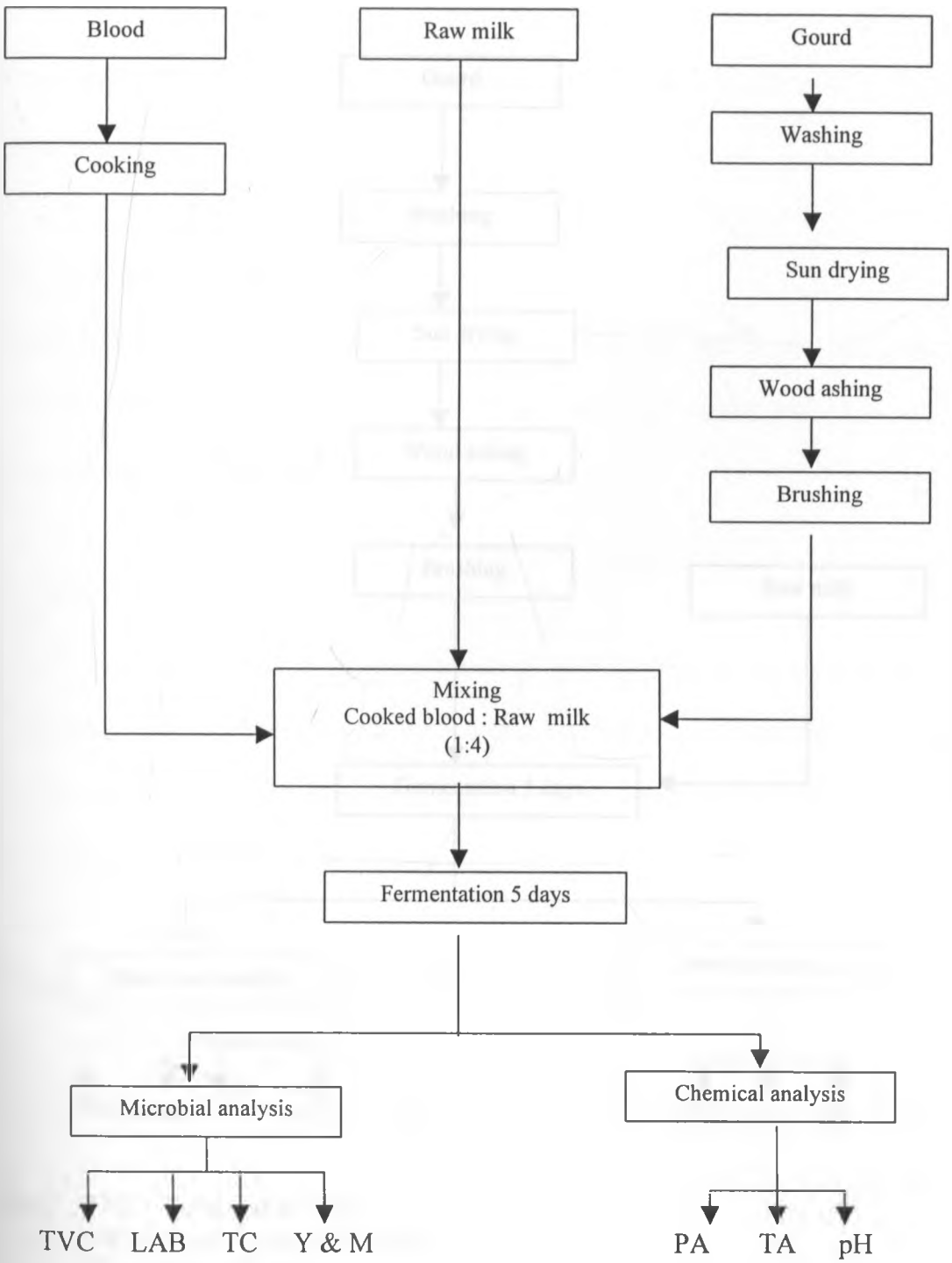


Key:
TVC: Total viable count
TYM: Total Yeast and Mold
TC: Total Coliform
LAB: lactic acid bacteria
TA: Titratable acidity
PA: Proximate analysis
Y&M: Yeast and Mold

3.1.1.2 Laboratory fermentation trials

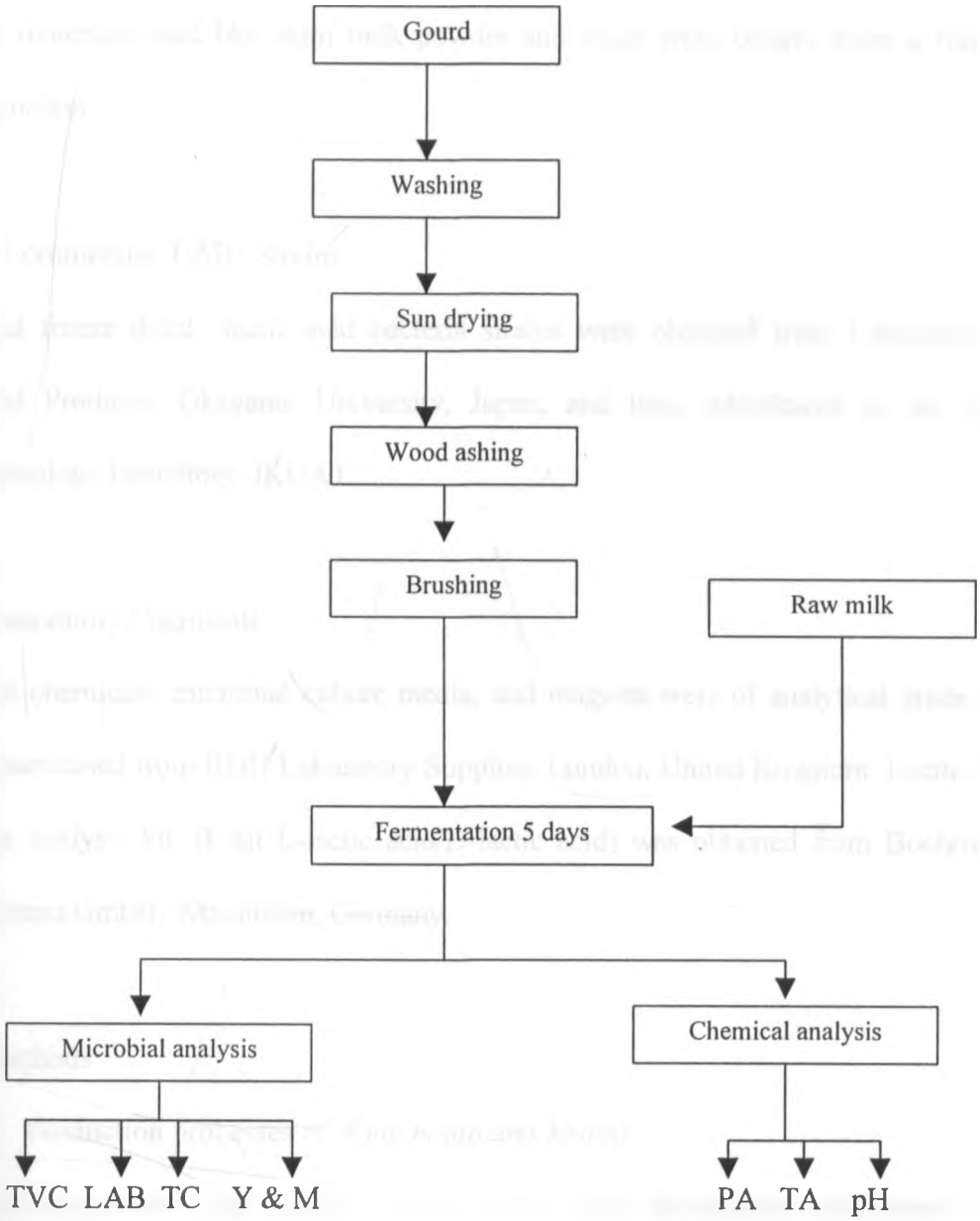
Gourds, smoking wood and cleaning brushes obtained from some Masai and Nandi homes were used for investigations with laboratory fermentation trials. These materials were used in simulated production of *Mursik* and *Kule naoto* under laboratory conditions. Figures 4 and 5 shows the laboratory production of *Mursik* and *Kule naoto* respectively.

Figure 4 Laboratory production of *Mursik*



Key: TVC: Total viable count
Y&M: Total Yeast and Mold
TC: Total Coliform
LAB: Lactic acid bacteria
TA: Titratable acidity
PA: Proximate analysis

Figure 5. Laboratory production of *Kule naoto*



Key: TVC : Total viable count
Y&M: Total Yeast and Mold
TC: Total Coliform
LAB: Lactic acid bacteria
TA: Titratable acidity
PA: Proximate analysis

3.1.2 Fresh milk.

The raw milk for fermentation was obtained from the JKUAT University farm in Juja. Other materials used like skim milk powder and sugar were bought from a Nairobi supermarket.

3.1.3 Commercial LAB Strains

Several freeze dried lactic acid bacteria strains were obtained from Laboratory of Animal Products, Okayama University, Japan, and then subcultured in the Food Microbiology laboratory JKUAT.

3.2. Laboratory Chemicals

All the chemicals, microbial culture media, and reagents were of analytical grade and were purchased from BDH Laboratory Supplies, London, United Kingdom. Lactic acid isomer analysis kit, (F-kit L-lactic acid/D-lactic acid) was obtained from Boehringer Mannheim GmbH, Mannheim, Germany.

3.3. Methods

3.3.1 Production processes of *Kule naoto* and *Mursik.*

Specific information with regard to type of milk used, fermentation equipment, milk handling before fermentation, fermentation conditions and storage conditions was obtained with the help of a questionnaire (Appendix 1), and personal examination. Samples were then collected as in 3.1.1.1 for analysis.

3.3.2 Proximate Analysis.

3.3.2.1 Moisture content

Moisture content of the fermented product was determined according to methods of analysis of AOAC (1984). Two grams of the sample were dried for 12 hours in an air oven, cooled to room temperature in an airtight dessicator and weighed.

3.3.2.2 Fat content.

Fat content was determined by the method recommended by Gerber using Gerber butyrometer (Klupsch, 1976). 10 ml of Sulphuric acid was pipetted into the milk butyrometer. 11 ml of mixed sample was also pipetted into the butyrometer and 1 ml of amyl alcohol then added. Butyrometer was placed in a water bath at 65° C for 10 minutes. It was then transferred to a centrifuge at 1200 rpm for 5 minutes and after centrifuging the butyrometer was placed in a water bath at 65° C for 5 minutes before the reading was taken

3.3.2.3 Crude protein Content

Percent total Nitrogen was determined by Kjeldahl method according to methods of analysis of AOAC, (1984). About 1 g of sample was weighed into the digestion flask. 0.5 g of $CuSO_4$ and 5.0g K_2SO_4 and 15 ml of H_2SO_4 was added to the sample before digesting it for one hour. Digested sample was distilled using Parnas Wagner's apparatus and then titrated with 0.02 N HCL solution. A factor of 6.38 was used to convert percent total nitrogen to percent crude protein.

3.3.2.4 Total ash Content

Ash content was determined by the thermogravimetric method according to methods of analysis of AOAC (1984). Two grams of the sample were weighed in porcelain ashing dishes previously dried in an air oven at 100 °C, cooled and tarred. Dishes were then held in a muffle furnace at approximately 550 °C for 12 hours, cooled to room temperature in a dessicator and weighed. The weight of the residue was converted to percent total ash.

3.3.2.5. Total carbohydrates content

Total carbohydrates were calculated by differences while crude fibre was assumed to be zero. {Total carbohydrates = %Total solids – (% Protein + % Ash + %Fat)}

3.3.3 Titratable acidity (TA)

Titratable acidity was determined by titration to pH 8.3 with 0.1N NaOH potentiometrically (IDF, 1991b) using pH meter model Metrohn 691 (The acidity was expressed as percent lactic acid.

3.3.4 pH

pH was measured using a Metrohn 691 pH meter.

3.3.5. Microbiological analysis

3.3.5.1 Total Viable Count (TVC)

Total viable count was determined using standard method agar (Nissui) according to Harrigan and McCance (1976). Decimal dilutions were made with 0.1 percent peptone water. 0.1 ml of each of the 10^{-5}, 10^{-6} and 10^{-7} dilutions was pour plated with

plate count agar. Plates were incubated aerobically at 37 °C for 48 hours and the counts on colony made.

3.3.5.2 Total Yeast and Molds (Y & M).

Yeast and mold counts were determined according to a method described by the International Dairy Federation standard 94B, (IDF, 1990) except that modified Potato dextrose agar (Difco) acidified to pH 3.5 using tartaric acid was used. Decimal dilutions were made with 0.1 percent peptone water. 0.1 ml of each of the 10^{-5}, 10^{-6} and 10^{-7} dilutions was pour plated and incubated aerobically at 25° C for 5 days.

3.3.5.3 Total coliforms (TC)

The method recommended by Harrigan and McCance (1976) was followed. Most Probable Number was determined using three tube system. 1 ml of the prepared dilutions was aseptically pipetted into Lauryl sulphate tryptose broth. Tubes were incubated at 30° C and examined after 24 and 48 hours for growth accompanied by the gas production.

3.3.5.4 Lactic acid bacteria (LAB)

Total lactic acid bacteria count was determined using standard plate count agar with bromo cresol purple (Nissui), according to Miyamoto *et al.*, (1986). Decimal dilutions were prepared using 0.1 percent tryptone water . 0.1 ml of each of the 10^{-5}, 10^{-6} and 10^{-7} dilutions was pour plated and incubated at 35 °C for 72 hours and colonies surrounded by yellow halo were counted.

3.3.6 Isolation of lactic acid bacteria from the samples.

One or more loopful of innoculum was added into culture tubes containing sterile Litmus milk supplemented with 0.5% glucose. Culture tubes were incubated at three different temperatures as follows: 20°C for Psychrotrophic strains, 37°C for mesophilic strains and 45°C for thermophilic strains. Incubation was done for 1 to 3 days until red color and/or coagulation developed.

Serial dilutions of 10^{-5}, 10^{-6} and 10^{-7} were then prepared from the culture tubes, using 0.85 % NaCl solution. Duplicate pour plates of 10^{-6} and 10^{-7} dilutions of the selective plate count agar containing Bromo Cresol Purple (Difco) and MRS agar (Difco) were used. Incubation was done for 2 to 3 days. Single colonies that developed on the above plates with colony forming unit (cfu) below 30 colonies per ml were picked according to Miyamoto *et al.*, (1986). Several streaking on Plate count agar was done for individual colonies to obtain a pure colony. After this stage gram staining reaction and colony characteristics of pure colonies of lactic acid bacteria were checked. Colonies picked were then transferred into Peptone Yeast (PY) broth consisting of 1 % tryptone (Difco), 0.5 % yeast extract (Difco), 0.5 % lactose (Difco), 0.5% glucose (Difco), 0.1 Tween 80 (Difco) and 0.01 % L-cysteine at pH 6.8. Cultures obtained after several transfers were characterised and identified. Pure lactic strains were maintained at 4°C on litmus milk supplemented with 0.5 % glucose through weekly transfers.

3.3.7 Identification and Characterisation of the LAB

A set of tests for identification of lactic acid bacteria according to Bergeys manual of systematic bacteriology (Sneath *et al,* 1986), were used on pure LAB strains. The tests included influence on growth by various temperatures and pH, and NaCl

tolerance together with morphology and Gram reaction according to methods described by Harrigan and McCance (1986). Ability to utilize a set of sugars and type of lactic acid isomer produced were assessed.

3.3.8 Identification of Yeasts and Molds

Morphological and biochemical characteristics of yeasts and molds isolated were used to identify the dominant fungal microflora according to Samson *et al.*, (1981).Pure colonies of Yeast and Mold colonies were first isolated after several streaking steps on modified Potato dextrose agar. Pure colonies were physically and microscopically examined.

3.3.9 Acid production ability

The acid production ability by the lactic acid bacteria isolated was determined potentiometrically by titration to pH 8.3 with 0.1N NaOH after incubation at 37°C for 5 days as in 3.3.3.

3.3.10 Aroma production.

Aroma compounds, diacetyl and acetoin, produced by the isolated strains were estimated according to Hammer (1935). 1 % innocuated Sterilised 10 % reconstituted skim milk was innoculated with 1% active culture. After incubation at 37 C for 5days, aroma production was tested. 5mg of creatine was added to 2.5ml of skim milk culture. 2.5 ml of 40% NaOH was added and mixed after which results were taken. Results were scored depending on degree of color change to pink after 5 days incubation as shown below

- \- no color change, (less than 0.5 mg% diacetyl/acetoin)
- \+ slightly pale pink, (0.5 to 3 mg% diacetyl/acetoin)

++ pale pink, (3 to 10 mg% diacetyl/acetoin)

+++ red, (10 to 30 mg% diacetyl/acetoin)

++++ dark red (above 30 mg% diacetyl/acetoin)

3.3.11 Assay for antimicrobial activity

Skim milk fermented by various isolated lactic cultures was tested for antimicrobial activity by agar diffusion technique, according to the method described by Pulusani *et al.,* (1979). *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* were used as test organisms. Melted nutrient agar was inoculated with 1 % of an 18 to 24 hour old broth culture of the test organism. Eight millilitres of this seeded agar were poured into sterile petri dishes and allowed to solidify. A sterile filter paper disc of 12.7mm diameter was dipped into the fermented milk and the disc was then placed on the seeded agar surface. The plates were left at room temperature for 1 hour to allow the lactic culture diffuse into the agar, then incubated at 37°C for 24 hr. The plates were then examined for zones of growth inhibition of the test organism around the discs. The degree of inhibition was measured by indicating +++ (strong inhibition; zones of 25 mm diameter and above), ++ (moderate inhibition; zones of 21-25 mm diameter) and + (weak inhibition; zones of 15 – 20 mm diameter).

3.3.12 Proteolytic activity

Qualitative determination of proteolytic activity was carried out according to a method described by Dick *et al.*, (1993). 3 µl of an overnight grown lactic acid bacteria culture was spotted on the MRS-ma agar and the plates incubated anaerobically using Anarocult A (Merck; Darmstadt, Germany) in a closed jar for 16 hours at 30 °C. The radius of the precipitation zone was used as a qualitative measure

of proteolytic activity. Proteolytic activity was expressed as the radius (r) in mm of the precipitation zone. Based on these results a division into 4 groups with increased proteolytic activity was made as shown below

r = 0mm, negative

$0 < r < 3$ mm, low

$3 < r < 5$ mm, average

$r > 5$mm, high

3.3.13 Hipurate Test

1% Sodium Hippurate in Tryptone Yeast extract Lactose Glucose (TYLG) broth was inoculated with a loopful of 24 hour culture and incubated for 10 days at 37C. The cultures were then centrifuged at 3000 rpm for 30min after which 1ml of supernatant was taken. 1ml of 50% H_2SO_4 was mixed with the filtrate and placed in a shaker for 30 minutes. Crystal formation was observed as an indication of positive results for hippurate hydrolysis.

3.3.14 Litmus milk test.

0.1 ml of 24 hour old pure culture was inoculated into litmus milk broth containing 10 % reconstituted skim milk and incubated at 37 % for 24 to 48 hours. Gas production and nature of the curd formed (coagulum) and color changes were recorded.

4. Results and Discussion

4.1. Preparation of *Mursik* by the Nandi community

4.1.1. Equipment and materials

Fermentation gourds:

Gourd is obtained from the plant *Lageria siceraria.* The green ripe gourd is first cut at the top. The freshy portion inside is removed by adding ash to aid its removal. The gourd is then sun dried until it turns brown in color. The dried gourd is then decorated with beads and shells. Handling straps and a cover made from cow's hide are fitted. The interior of the gourd is then rubbed with the burning end of a special herb *Cassia marylandica.* The gourd is then cleaned using warm water. Sometimes ash is added to the warm water. After washing, it is sun dried for two days. It is after this sun drying that hot 'ashing' is done. 'The ashing' process involves burning a branch of the tree red hot and immediately rubbing it against the walls of the gourd thus emitting smoke and burnt pieces of wood inside the gourd. This process is repeated three times.

A special brush *'osek'* is used to remove loose and broken pieces of charcoal. Hot ash is also applied to a special gourd cover made of cow's hide before tightly fixing it to the mouth of the gourd. Fermentation gourds are of various sizes capable of holding between one and five litres of milk. The communities prefer using old gourds, some of which are inherited from the grand parents and some are over twenty years old.

Type of milk used.

Raw unpasteurised whole milk is normally used. Milk which is not heated or boiled is thought to produce high quality product in terms of flavor, consistency and taste by the community.

Blood procurement and preparation.

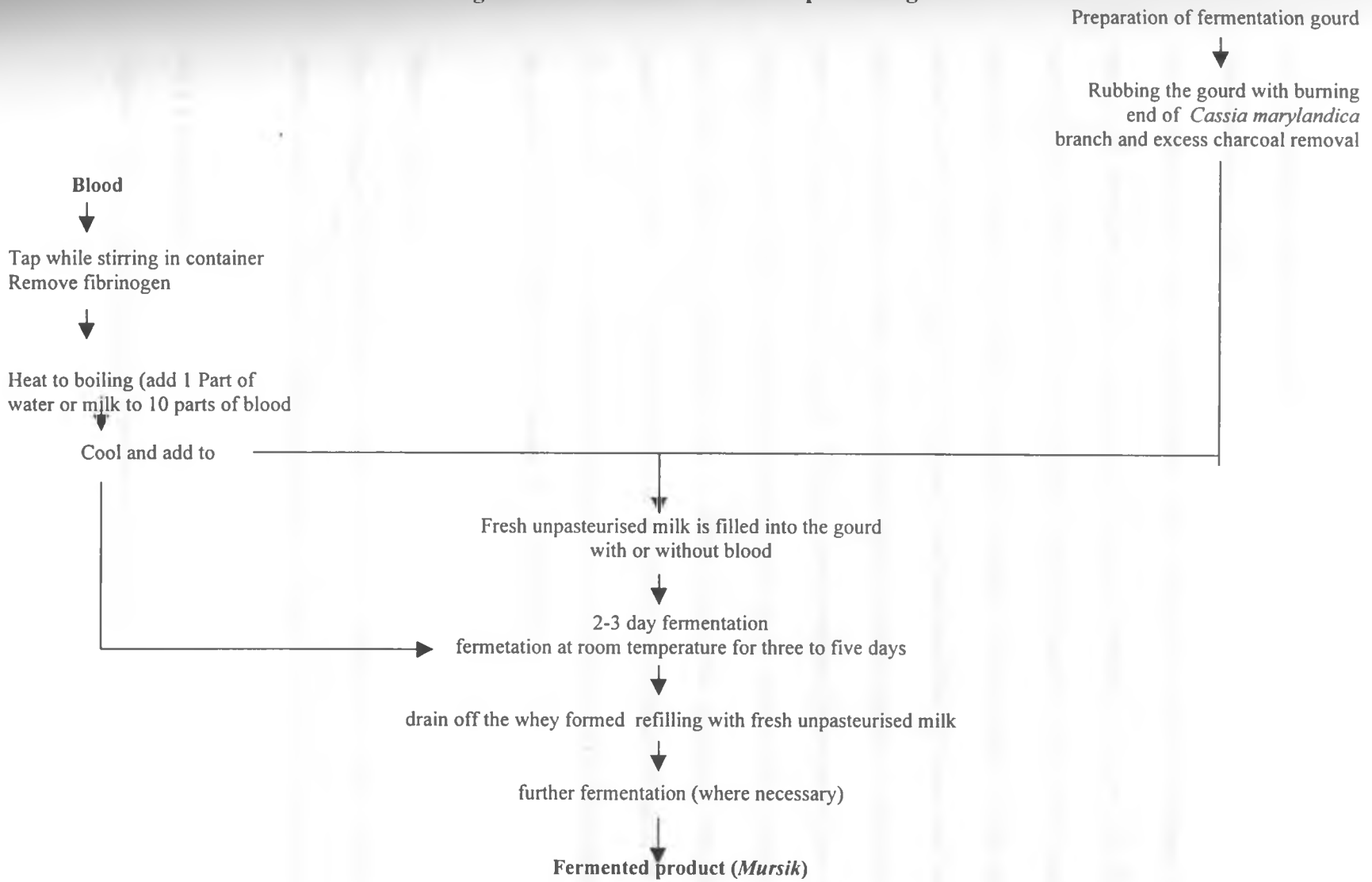
Mursik can be produced with or without blood. Blood is obtained fresh from an artery of a heifer. Blood tapping is normally done by an experienced man either in the morning or evening. About two litres of blood is tapped from a single heifer. Some cow dung is smeared at the point of tapping thereafter. During tapping, blood is thoroughly stirred to prevent clotting. Upon stirring fibrinogen forms and it is removed using a small tree twig locally known as *'Kimolwet'*. Blood is then boiled while still stirring. Some milk is added during heating. Some people add some water to prevent formation of large globules of blood clots. About 1 part of milk or water is added into 10 parts of blood. Heating of the mixture is done until boiling point is reached. Boiled blood and milk mixture is then allowed to cool to room temperature.

4.1.2 Fermentation.

There are two ways in which blood may be incorporated into the milk; either to the already fermented milk or to the fresh milk before fermentation. In the former case, milk is first fermented for two to three days in a gourd. For every four parts of fermented milk, one part of prepared blood is added. Further fermentation of the mixture is then carried out for between 3 to 5 days after which it is ready for consumption. Fermented product can be stored for future consumption in the same container for up to three or more months.

In the second case, milk is mixed with blood in a ratio of about 4:1 in a gourd, and the mixture gently shaken. Fermentation is then carried out for the next five or more days at ambient temperature (22 ± 3°C). This can be allowed to continue for an extra five days, during which whey that forms is drained off and more fresh raw milk is used to refill the gourd. After further fermentation, The top layer that forms on the surface called '*sumbarariet*' is first removed before mixing it using a special stirrer locally known as *burmet* from a local tree *Sosiot*. The product is then ready for consumption. Figure 6 shows a flow chart for *Mursik* processing.

Figure 6. Flow chart for *Mursik* processing



4.2 Preparation of *Kule naoto* by the Masai community in Kajiado District.

4.2.1. Equipment and materials

Fermentation gourds.

Fermentation gourd known as *Enkukuri* is used. The gourd which is obtained from species of the plant *Lageria siceraria* is thoroughly cleaned to remove the fleshy portion inside. Warm water mixed with ash is commonly used to aid in fleshy portion removal. It is then let to sun dry outside the *Manyatta.* The dry gourd is then gently rubbed with the hot burning end of chopped stick from the *Olea africana* tree locally known as *Enkidogoe,* letting charcoal break inside. Broken pieces of charcoal are removed by gently shaking the gourd. This practice is repeated three times. Raw milk (unpasteurised) is then filled into the gourd. A gourd cover made of skin or hide and previously rubbed with hot burning stick is tightly fixed at the opening of the treated gourd. The material is kept inside the house at ambient temperature of about 25 ± 3 °C, for up to 5 days for spontaneous fermentation to take place. After fermentation, the product is gently shaken and is ready for consumption.

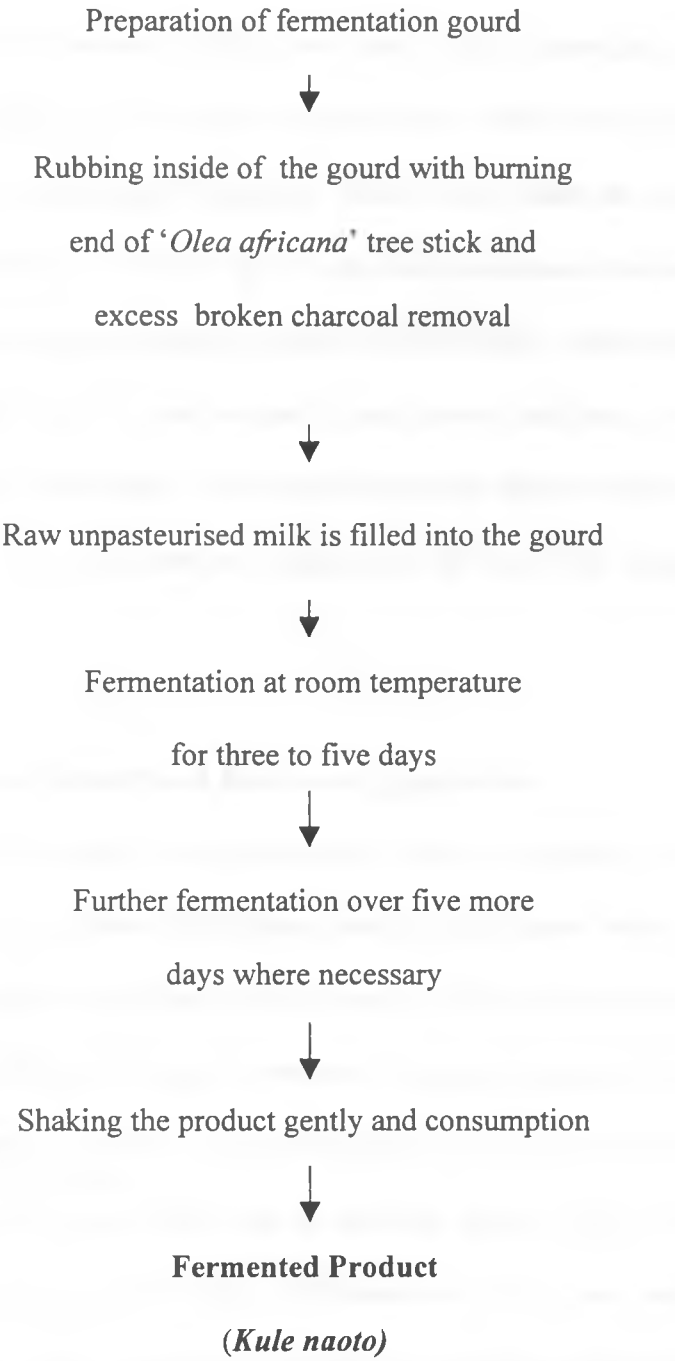
Type of milk used.

Raw unpasteurised whole milk is used. The milk is obtained by hand milking of several indigenous *Zebu* cows which produce on average 2 to 5 litres of milk per day.

4.2.2 Fermentation

Figure 7 shows a flow diagram of *Kule naoto* processing.

Figure 7. Flow diagram of *Kule naoto* processing



The spontaneously fermented milk is known as *Kule naoto*. The Raw milk (unpasteurised) is filled into the well treated gourd. Fermentation gourds of various sizes can be filled with between half and two litres of milk depending on their size.

A gourd cover made of skin or hide and previously rubbed with hot burnt stick is tightly fixed over the opening of the gourd. Milk is kept inside the house at ambient temperature of about 25 ± 3 °C, for up to 5 days for spontaneous fermentation to take place. After fermentation, the product is gently shaken before consumption.

Processing of *Kule naoto* is synchronised in such a way that there is ready to drink product every day by the family. All Masai community families prepare this product on a daily basis. Adults consume on average over 500 ml of the fermented product daily.

4.2.3 Comparison of *Mursik* and *Kule naoto* preparation.

From the field studies carried out on production of the two products it was noted that some Nandi families produce the product using cooked blood while others omit it altogether. It was noted that Masai families used to add blood in preparation of *Kule naoto* but the practice is no longer common. However, the current practice of the *Kule naoto* production is almost similar to production of *Mursik* where blood is not used. However, it was noted that whey is removed during *Mursik* processing thus making the product much more thicker. Different tree species are used for smoking the gourds although room temperatures are used for incubation for both products. The taste of both products without blood was described as 'good'.

4.3. Chemical composition of *Kule naoto* and *Mursik*

Kule naoto and *Mursik* were analyzed for protein, fat content, ash content, total carbohydrates, water content, titratable acidity and pH. Results of the analysis are shown in Table 2.

Mursik had a higher protein content of 6.39 percent compared to *Kule naoto* with3.76 percent (Table 2). The protein percentage content of *Mursik* was higher than that reported for both fresh milk and yoghurt of 3.29 percent (Nakazawa and Hosono 1992, Hui 1993b, and Varman and Sutherland, 1994).

Both products had a high ash content when compared to average ash content of whole fresh milk. On average normal milk has ash content of 0.76% (Hui, 1993a). Commercial yoghurt has average ash content ranging from 0.9 to 0.7% when prepared from skim milk and full fat milk respectively (Nakazawa and Hosono, 1992).

Both *Mursik* and *Kule naoto* had almost similar values of fat content of 5.42 and 4.65 percent respectively. However, both products had slightly higher total solid content of 16.84 and 13.58 percent respectively when compared to average total solid content values of 12 % of fresh milk reported (Hui 1993b).

Mursik used in this study had blood added during fermentation process. Its pH value of 4.1 to 4.3 was lower than that one of *Kule naoto* of 4.6 to 4.8 (Table 2). Of importance in chemical attributes, is the higher values of total titratable acidity recorded for the *Mursik* than those recorded in *Kule Naoto*. The pH values for *Kule Naoto* were above the iso-electric point of casein (pH 4.6) ranging from 4.6 to 4.8.

Table 2. Proximate composition, pH and Titratable acidity of *Mursik* and *Kule naoto*

Constituent	*Mursik*		*Kule naoto*	
	Mean values	**SD**	**Mean values**	**SD**
Total Titratable acidity (as % lactic acid)	1.51a*	0.21	0.96a	0.35
pH range	4.1- 4.3		4.6 – 4.8	
Total solids (%)	16.84 a	0.12	13.58a	0.13
Fat content (%)	5.42 a	0.14	4.65a	0.27
Protein content (%)	6.39a	0.20	3.76 b	0.17
Ash content (%)	1.77a	0.00	0.94 a	0.45
Total carbohydrates (%)	3.95a	0.11	4.20a	0.01

Key: SD- Standard deviation n = 3

* Same letters indicate no significance difference at 5% level amongst the two products for each parameter .

The percent total titratable acidity (as lactic acid) of *Mursik* was about 1.5%, an indication that much of the acid was in undissociated form. This is not common in commercial mesophilic product like *mala* which has a total titratable acidity value of 1% and pH 4.5 .

4.4 Microbiological characteristics of *Mursik* and *Kule naoto*.

Total count, lactic acid bacteria, yeast and mold and coliforms were enumerated in ready to drink fermented milk products and in the same product after seven days storage at 15 ± 2°C (Table 3).

The initial average lactic acid bacteria count for *Mursik* and *Kule naoto* was 1.1 $x10^8$ and 7.0 $x10^8$ respectively. However, the count decreased after seven days storage to 4.5 $x10^7$ and 4.2 $x10^7$ respectively. The average initial yeast and mold count for both *Mursik* and *Kule naoto* of 2.1 $x10^6$ and 2.1 $x10^7$ was lower but recorded a substantial increase after storage. This results indicates that the acidity of the fermented product did not inhibit growth of yeasts and molds. However the yeasts and molds count remained higher than the lactic acid bacteria count in both *Mursik* and *Kule naoto*. No coliforms were detected in the processed product and after its storage.

Table 3. Microbial characteristics of *Mursik* and *Kule naoto* after production and after seven days storage.

Counts* cfu/ml	Initial		After 7 days	
	Mursik	*Kule naoto*	*Mursik*	*Kule naoto*
Total viable count	7.2×10^7a$^{\$}$	7.0×10^8a	5.2×10^6b	4.3×10^7b
	(0.0)**	(0.2)	(0.3)	(0.4)
Lactic acid bacteria	1.1×10^8a	7.0×10^8a	4.5×10^7b	4.2×10^7b
	(0.3)	(0.3)	(0.5)	(0.7)
Yeast and mold count	2.1×10^6a	2.1×10^7a	3.2×10^8b	1.9×10^8b
	(0.3)	(0.1)	(0.2)	(0.3)
Coliforms count	<1	<1	<1	<1

Key: * Mean value, n = 3

( )** Standard deviation.

$^{\$}$: same letters for the specific counts indicates no significance difference at 5% level for each product when compared before and after storage.

4.5 Laboratory production of *Mursik* and *Kule naoto.*

Results on microbial analysis after a laboratory production and after 7 days storage (Table 4) indicated similar trends on TVC, LAB, yeast and molds counts for both *Mursik* and *Kule naoto* and those for the respective samples from the field (see Table 3). A general decrease in lactic acid bacteria count and an increase in yeast and molds counts were observed for both *Mursik* and *Kule naoto* samples. No coliforms were presumptively detected. The results indicated that microbial characteristics of *Mursik* and *Kule naoto* could be reproduced in the laboratory, and compared well with those of traditional products.

Proximate composition of the samples indicated high total ash in both products and high crude protein content in *Mursik* (see Table 5). The high protein content in *Mursik* could be attributed to the cooked blood added, while the residual ash introduced during 'hot rubbing' could account for the high ash content in both products. The Fat content was high for both products and this was due to high fat content of the milk used namely 4.5 % (Table 6). Acidity and pH of the simulated products was similar to that obtained in field samples (see Table 2). *Mursik* had much lower pH than *Kule naoto* as well as more titratable acidity. This could be due to blood added although more investigation is needed to investigate the cause of this difference.

Table 4. Microbial characteristics of *Mursik* and *Kule naoto* produced under simulated laboratory conditions and after seven days storage.

Counts* cfu/ml	Initial		After 7 days	
	Mursik	*Kule naoto*	*Mursik*	*Kule naoto*
Total viable count	9.3×10^7 a ***	6.3×10^8b	7.2×10^7a	3.4×10^7b
	(0.0)	(0.2)**	(0.4)	(0.4)
Lactic acid bacteria	7.8×10^7a	6.0×10^8b	6.5×10^6a	3.4×10^7b
	(0.1)	(0.3)	(0.4)	(0.1)
Yeast and mold count	4.6×10^6a	5.2×10^7b	8.2×10^7b	7.9×10^7b
	(0.3)	(0.1)	(0.4)	(0.1)
Coliforms count	<0.3a	<0.3a	<0.3 a	<0.3a

Key: * Mean value, n = 3; Values in the bracket with double star ( )**, indicates the Standard deviation. ***, similar letters in the same column for ever parameter indicate no significance difference at 5% level.

Table 5. Proximate composition, pH and Titratable acidity of laboratory produced *Mursik* and *Kule naoto*

Constituent	*Mursik*		*Kule naoto*	
	Mean values	**SD**	**Mean values**	**SD**
Total Titratable acidity (% lactic acid)	1.5a*	0.2	1.1a	0.3
pH range	4.1- 4.3a		4.4 – 4.6a	
Total solids (%)	16.7a	0.3	14.5a	0.1
Fat content (%)	5.5a	0.5	5.4a	0.4
Protein content (%)	6.3 a	0.2	3.9b	0.3
Ash content (%)	1.8 a	0.0	0.9b	0.0
Total carbohydrates (%)	4.9a	0.1	4.3a	0.2

Key: SD- Standard deviation n = 3

* Same letters for each parameter indicate no significance difference at 5% level among the products .

Table 6. Proximate composition of raw milk used in laboratory produced *Mursik* and *Kule naoto*

Constituent	Raw milk	
	Mean values	SD
Total solids (%)	13.6	0.1
Fat content (%)	4.5	0.1
Protein content (%)	3.8	0.2
Ash content (%)	0.7	0.0
Total Carbohydrates (%)	4.9	0.1

Key: SD- Standard deviation n = 3

4.6 Lactic acid bacteria isolated from *Mursik*

Table 7 shows the biochemical characteristics of cocci shaped strains of lactic acid bacteria isolated from *Mursik*. Three microbes coded 11, 12 and 13 were able to release ammonia from L-arginine. These three microbes were also able to grow at 45°C. To distinguish Enterococci strains, 6.5 % NaCl salt solution and broth adjusted to pH 9.6 were used. The three microbes also produced L(+) isomer of lactic acid.

Microbes coded 131, 132 and 133 produced gas from glucose and were therefore hetero-fermentative. The other nine isolated cocci coded; 11, 12, 13, 21, 22, 23, 31,32 and 33 did not produce gas from glucose hence were homo-fermentative.

All cocci were catalase negative, produced acid and coagulated litmus milk but did not hydrolyze hippurate. Microbes coded 31, 32, and 33 had lobate irregular medium sized colonies. The rest of the isolated strains had circular raised small colonies with entire margins.

Table 7. Biochemical characteristics of cocci microbes isolated from *Mursik*

Characteristics	Strain Codes											
	11	12	13	21	22	23	31	32	33	131	132	133
Gram staining		+			+			+			+	
catalase test		-			-			-			-	
Gas from glucose		-			-			-			+	
Ammonia from Arginine		+			-			-			-	
Hippurate hydrolysis		-			-			-			-	
Growth at 10°C		-			±			±			±	
Growth at 15 °C		-			+			±			+	
Growth at 37°C		+			+			+			+	
Growth at 45 °C		+			-			-			-	
Growth at 3.0 % NaCl		+			+			+			+	
Growth at 4.0 %NaCl		+			+			+			+	
Growth in 6.5 % NaCl		±			-			-			-	
Growth at pH 9.6		-			-			-			-	
Litmus test												
Acid		+			+			+			+	
Coagulation		+			+			+			+	
Colony features												
Margins		Entire			Entire			Lobate			Entire	
Size		Small			Small			Medium			Small	
Form		Circular			Circular			Irregular			Circular	
Elevation		Raised			Raised			Raised			Raised	

Key: + Positive, - Negative , ± Weak positive

Traditional fermented milk product *Mursik* was dominated by strains of the L*actobacillus* genus of lactic acid bacteria. Most of the *Lactobacillus* strains were homofermentative and were unable to utilize arginine. The rest of the lactic acid bacteria strains were those of *Lactococcus* genus (Table 8).

Low pH values are known to favour survival of *Lactobacillus* strains much more than the *Lactococcus* strains (Nakazawa and Hosono, 1992 and Wood and Holzapfel, 1995). *Mursik* had low pH value and high lactic acid concentration (see Table 3). This condition also favours growth of yeasts and molds (Samson *et al.*, 1981). Results of isolation of different lactic microflora from *Mursik* is consistent with several other studies carried out on traditional fermented food systems which highlights presence of a complex lactic acid producing microflora, (Dick *et al.*, 1993, Nakazawa and Hosono, 1992, Puhan and Zambrini,1990, and Isono *et al.*, 1994).

Table 8 indicates the bacteriological characteristics of twenty four rod shaped microbes from *Mursik.* The microbes were examined for various identification aspects according to Bergey's manual of systematic bacteriology (Sneath *et al.*, 1986).

Table 8. Biochemical characteristics of some rods isolated from *Mursik*

Characteristics	Strain codes							
	4	5	8	9	11	14	16	18
Gram staining	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-
Gas from glucose	-	-	+	-	-	-	-	-
Ammonia from Arginine	-	+	-	-	-	-	-	-
Hippurate hydrolysis	-	-	-	-	-	-	-	-
Growth at 10 °C	+	+	+	±	+	±	±	±
Growth at 15 °C	+	+	+	+	-	+	+	+
Growth at 37°C	+	+	+	+	+	±	±	±
Growth at 45 °C	-	-	-	-	-	-	-	-
Growth at 3.0 % NaCl	+	+	+	+	+	+	+	+
Growth at 4.0% NaCl	±	±	+	±	±	+	+	+
Growth in 6.5 % NaCl	-	-	+	-	-	±	±	±
Growth at pH 9.6	+	-	±	-	-	-	-	-
Litmus test								
Acid	+	+	+	+	+	+	+	+
Coagulation	-	+	+	+	+	+	+	+
Colony features								
Margins	L	E	E	E	E	E	E	E
Size	M	S	M	S	M	S	S	S
Form	I	C	I	C	C	C	C	C
Elevation	R	F	R	R	R	R	R	R

Key: L- Lobate, M -Medium, I- Irregular, E- entire R- raised, C- Circular, S-Small,

+ Positive, - Negative, ± Weak positive

* Three strains were tested under each code.

Results in Table 8 indicate that only three similar strains coded 8 produced gas from glucose and therefore were heterofermentative lactic acid bacteria. The rest were homofermentative.

Three stains coded 5 were able to release ammonia from arginine. The rest of strains tested did not release ammonia from arginine. None of the tested strain hydrolysed hippurate. The results also indicate that all the isolated and tested rods were mesophilic. None of them grew at 45 °C.

Growth characteristics at various salt concentrations showed that all strains tested grew at 3 and 4 % NaCl concentration. However, three strains coded 8 grew well at 6 % NaCl where as nine strains coded 14, 16 and 18 showed weak growth at 6% NaCl.

All strains tested except three coded 4 formed acid curd as the other three formed rennet curd. Litmus milk forms an excellent differential medium in which microorganisms can metabolise milk substrates depending on their enzymatic complement (Cappuccino and Sherman, 1992).

4.7 Biochemical characteristic of some lactic acid bacteria isolates from *Mursik*

The most frequent occurring lactic acid bacteria strains were considered as dominant lactic acid bacteria in *Mursik* and they were tested for their ability to ferment various sugars. Three of the isolated cocci coded 11, 12, and 13 were identified as *Enterococcus faecium*, six isolates coded 21, 22, 23, 31, 32, and 33 were identified as *Lactococcus lactis* and three others coded 131, 132, and 133 as *Leuconostoc mesenteroides* (Table 9)

A diverse reaction of the isolated strains was noted. Lactose was fermented by isolated *Leuconostoc mesenteroides* though its transport system into the cell is little known (Table 9). Its not known if Leloir pathway exist in *Leuconostoc* species (Wood and Holzapfel, 1995). Fructose is fermented by all *Leuconostoc* species except *Leuc. mesenteroides* subsp. *cremoris* and some strains of *Leuc. argentinu* (Wood and Holzapfel, 1995). All heterofermentative lactic acid bacteria have phosphoketolase and are thus theoritically able to ferment pentoses to lactate and acetate (Sneath *et al.*, 1986).

Enterococcus feacium can derive energy from arginine (Table 9). *Enterococcus feacium* is dominant in raw milk and several types of milk products (Wood and Holzapfel, 1995). *Ent. feacium* has received much attention as a possible means to improve growth and feed conversion of farm animals and to prevent or to cure disease in animals and humans. Similar studies on traditional fermented milk products have reported presence of *Ent. feacium* (Isono *et al*,. 1994, and Dick *et al.*, 1993).

Table 9. Biochemical characteristics and identification of cocci isolated from *Mursik*.

Characteristics	Strain codes											
	11	12	13	21	22	23	31	32	33	131	132	133
L+ Arabinose		-			+			+			+	
D Mannitol		-			±			+			+	
Sucrose		±			-			+			+	
Cellobiose		±			±			+			+	
D- Fructose		±			+			+			+	
D+ Melezitose		-			±			+			+	
D+ Arabitol		-			±			+			+	
Salicin		-			±			+			+	
Lactose		±			+			+			+	
Xylose		±			-			+			-	
D+ Melibiose		-			+			+			+	
D+ Raffinose		±			-			+			+	
D+ Glucose		±			±			+			+	
Galactose		-			±			+			+	
Sorbitol		±			-			+			±	
Mannose		-			+			+			+	
Maltose		-			-			+			+	
Trehalose		±			-			±			±	
Dulcitol		+			±			-			-	
Ribose		+			+			+			+	
Identification	*Enterococcus faecium*			*Lactococcus lactis*			*Lactococcus lactis*			*Leuconostoc mesenteroidis*		

Table 10. Biochemical characteristics and identification of rods isolated from *Mursik*.

Characteristics	Strain codes							
	9	4	5	8	11	14	16	18
D+ Raffinose	+	-	±	±	±	+	±	±
D+ Glucose	+	+	+	+	+	+	+	+
Galactose	+	+	±	±	±	+	±	±
Sorbitol	+	+	-	-	-	+	±	-
Mannose	+	+	+	+	+	+	+	+
Cellobiose	+	-	+	-	+	+	-	±
Maltose	+	+	±	±	+	+	±	±
Trehalose	±	+	+	-	+	+	-	-
Dulcitol	+	-	±	-	±	±	-	-
Ribose	+	+	+	+	+	+	+	+
L+Arabinose	+	+	+	+	±	±	+	+
D- Mannitol	+	+	+	-	-	±	-	-
Sucrose	+	+	+	-	+	+	-	-
D – Fructose	+	+	+	+	+	+	+	+
D+ Melezitose	+	±	±	-	±	±	-	-
D+ Arabitol	-	-	±	±	±	±	-	±
Salicin	+	+	+	±	+	+	±	±
Lactose	+	+	+	±	+	+	±	±
Xylose	-	+	+	±	±	±	±	±
Identification	*Lb.plantarum*	*Lb. casei*	*Lb. plantarum* *Lb. pentosus*	*Lb. confusus*	*Lb curcatus* *Lb. murinus*	*Lb. plantarum* *Lb. pentosus*	*Lb. plantarum* *Lb. pentosus*	*Lb. plantarum* *Lb. pentosus*

Key: + positive, - negative, ± weak positive

Cultural, Physical chemical and biochemical characteristics of the isolated lactic acid producing bacteria were used to identify the isolated strains. Results showed that *Mursik* contained Lactic acid bacteria as the dominant bacteria microflora. Lactic acid bacteria composition in the product ranges from 8.5 x 10^{7} to 1.3x 10^{8} cfu/ml (see Table 3).

This characteristic identity of lactic acid producing microflora indicates mutual participation of several lactic acid cultures in the fermentation process of the traditional product. Mixed culture fermentation systems are quite complex and they are self controlled in a traditional system (Nakazawa and Hosono, 1992 and Mathara *et al.*, 1996).

– The dominant *Lactobacillus* strains isolated from *Mursik* were *Lb. plantarum, Lb. confusus* and *Lb. pentosus* (see Table 10). *Leuconostoc mesenteroides* species were also isolated and identified in a few samples.

Lb. plantarum is common lactic acid producing bacteria in several vegetable fermentation systems and it has been reported in most traditional fermented milk products in Africa and Europe (Nakazawa and Hosono, 1992, and Miyamoto *et al.*, 1986).

Most of the microbes involved in *Mursik* fermentation were of the homofermentative and mesophilic *Lactobacillus* genera (see Table 10). The high occurrence of the *Lactobacillus* strains in *Mursik* could be attributed to its low pH (see Table 2) which favours the survival of *Lactobacillus* species and inhibits growth and survival of *Streptococcus* (Hui, 1993b, Nakazawa and Hosono, 1992). The pre-treatment practice of rubbing gourd with hot burnt end of a special tree and addition of blood could also be playing some role in microbial composition of the fermented milk product.

The dominant lactobacilli strain isolates from product *Mursik* was *Lb. plantarum* (see Table 10). *Lb. plantarum* is commonly used in fermentation of meat and vegetables, and is a lactic acid bacteria possessing citrate fermenting ability (Nakamura *et al.*, 1991). Since pyruvate accumulated during citrate fermentation is converted to diacetyl and acetoin which are flavor compounds, the citrate fermenting ability of this bacteria strain plays an important role in enhancing flavor of the fermented milk products. These homofermentative rods produce DL isomer of lactic acid. *Lb. confusus* strains were also isolated and identified from the product.

Lactobacillus plantarum are also known to have unused metabolic potential for example they may reduce nitrate while some strains exhibit catalase activity (Wood and Holzapfel, 1995). *Lb. plantarum* and *Lb. pentosus* are phylogenetically closely related e.g. their 16s RNA sequence has a similarity of over 95 % and the two have Diamino pimelic acid in their peptidoglycan (Sneath *et al.*, 1986 and Nakazawa and Hosono, 1992).

4.8 Lactic acid bacteria isolated from *Kule naoto*

Table 11 and 12 shows the biochemical characteristics of some of the isolated cocci and rods respectively. All rod strains tested were gram positive, catalase negative and were nitrate reduction negative (Table 12). Two isolates under each of code tested M1, M3, M4, M13 and M14, were heterofermentative whereas two isolates under code M2 were homofermentative and had short rods.

Forty coccus shaped isolates of lactic acid bacteria were isolated from *Kule naoto*. Out of these, twenty four isolates, were randomly selected and tested for their cultural and physical chemical characteristics. All isolates were Gram positive, catalase negative and homofermentative (Table 11). The isolates also produced acid and coagulated litmus milk. They had a proteolytic action based on litmus milk test results.

Table 11. Biochemical characteristics of cocci isolated from *Kule naoto*

Characteristics	Strain codes											
	M7	M9	M11	M12	T1	T2	T3	T4	T8	T10	T12	T14
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-
Lactic acid isomer	nd	DL	nd	nd	DL	DL	L	L	nd	nd	nd	nd
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-
Ammonia from Arginine	±	+	±	±	+	+	+	+	+	nd	nd	nd
Hippurate hydrolysis	-	-	-	-	-	-	-	-	nd	-	-	-
Growth at 10°C	±	±	±	±	-	-	-	-	-	-	-	-
Growth at 15 °C	+	±	±	±	-	-	-	-	-	-	-	-
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 45 °C	-	-	-	-	±	±	+	+	+	+	+	+
Growth in 3.0 % NaCl	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 4.0 %NaCl	±	±	±	±	-	-	-	-	-	-	-	±
Growth in 6.5 % NaCl	-	-	-	±	-	-	-	-	-	-	-	-
Growth at pH 9.6	-	-	-	-	-	-	-	-	-	-	-	-
Litmus test												
Acid	+	+	+	+	+	+	+	+	+	+	+	+
Coagulation	+	+	+	+	+	+	+	+	+	+	+	+
Colony features												
Margins	E	L	E	E	E	E	E	E	E	E	E	E
Size	M	S	S	S	M	M	S	S	S	S	S	S
Form	C	C	C	C	C	C	C	C	C	C	C	C
Elevation	R	R	R	R	R	R	R	R	R	R	R	R

Key: E-entire, C – circular, L- lobate, R- raised, M – medium, + positive, _ negative, ± weak positive, nd- not done. * Two isolates were tested under each strain code
nd - not done

Table 12. Bacteriological characteristics of rods isolated from *Kule naoto*

Characteristics	Strain code*					
	M1	M2	M3	M4	M13	M14
Gram staining	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-
Lactic acid isomer	DL	DL	DL	nd	DL	DL
Gas from glucose	+	-	+	+	+	+
Ammonia from Arginine	-	-	-	-	-	-
Hippurate hydrolysis	-	-	-	-	-	-
Growth at 10°C	-	-	-	-	-	-
Growth at 15 °C	+	±	±	±	-	-
Growth at 37°C	+	+	+	+	+	+
Growth at 45 °C	-	-	-	-	±	±
Growth in 3.0 % NaCl	+	+	+	+	+	+
Growth in 4.0 %NaCl	±	±	±	±	-	-
Growth in 6.5 % NaCl	-	-	-	±	-	-
Growth at pH 9.6	-	-	-	-	-	-
Litmus test						
Acid	+	+	+	+	+	+
Coagulation	+	+	+	+	+	+
Colony features						
Margins	Entire	Entire	Entire	Entire	Entire	Entire
Size	Small	Small	Medium	Small	Small	Small
Form	Circular	Circular	Circular	Circular	Circular	Circular
Elevation	Raised	Raised	Raised	Raised	Raised	Raised

Key: + positive, - negative, ± weak positive, nd not done, * two isolates were tested under each strain code.

The fermented milk product *Kule naoto* had more cocci strains than rods most of the isolated strains being cocci (see Table 11 and 12). Isono *et al*,. (1994), reported a proportion of *Streptococcus* to *Lactococcus* of 10^7 to 10^9/g in Masai traditional fermented milk product in Northern Tanzania. All *Streptococcus* isolates were homofermentative, while most *Lactobacillus* strains were heterofermentative (see Tables 8 and 9). Similar findings have been reported by Isono *et al*,. (1994). All *Lactobacillus* strains studied produced DL isomer of lactic acid (see Table 12). Growth of most tested isolates was inhibited by higher NaCl (4.0 and 6.5 %) and pH (9.6) conditions in growth medium (see Tables 11 and 12).

4.9 Biochemical characteristics of lactic acid bacteria isolated from *Kule naoto.*

The most frequently occurring strains were considered dominant strains and were tested on their ability to ferment various sugars. Their results together with those in Tables 11 and 12 were used for identification.

Kule naoto lactic microflora comprises of mixed strains of *Lactococcus, Leuconostoc*, *Pediococcus* (Table 13) and *Lactobacillus* genera (Table 14). *Leuconostoc* species have an ability to convert citrate to diacetyl and acetoin at low pH, a characteristic which could be responsible for aroma in the traditional fermented milk product. They also play an important role in improving the viscosity of the fermented product through their slime production. *Lactoccocus* strains are actively involved in acid production from lactose.

Table 13: Biochemical characteristics and identification of Lactic acid cocci isolated from *Kule naoto*.

Characteristics	Strain codes				
	T1	T2	T3	T4	M9
Arabinose	+	+	+	+	+
Mannose	-	-	-	-	-
Trehalose	-	-	-	-	-
Arabitol	-	-	-	-	-
Lactose	+	+	+	+	+
Mannitol	-	-	-	-	-
Sucrose	+	-	-	-	-
Melibiose	+	+	+	+	-
Ribose	+	+	-	+	+
Maltose	+	+	-	+	+
Fructose	+	+	+	+	+
Xylose	+	+	+	+	+
Raffinose	±	±	±	±	-
Glucose	+	+	+	+	+
Galactose	+	+	+	+	+
Ribose	+	+	+	+	+
Rhamnose	-	-	-	-	-
Salicin	-	-	-	-	±
Saccharose	-	-	-	-	±
Cellobiose	+	+	+	+	+
Identification	*Leu. mesenteroides*	*Leu. mesenteroides*	*Str. salivarius*	*Str. salivarius*	*P. pentosus*

Table 14: Biochemical characteristics of some Lactic acid rods isolated from *Kule naoto*.

Characteristics	Strain codes				
	M1	M2	M3	M13	M14
Arabinose	+	+	+	±	±
Mannose	-	+	+	+	+
Trehalose	-	-	-	-	-
Arabitol	-	-	-	-	-
Lactose	+	+	+	+	+
Mannitol	-	-	±	-	-
Sucrose	-	-	+	-	-
Melibiose	-	±	+	-	-
Ribose	+	+	+	+	+
Maltose	+	+	+	+	+
Fructose	+	+	+	+	+
Xylose	+	±	+	+	+
Raffinose	+	+	+	±	±
Glucose	±	+	+	+	+
Galactose	+	+	+	+	+
Ribose	+	+	+	±	+
Rhamnose	±	±	±	-	-
Salicin	+	+	+	-	-
Saccharose	+	+	+	±	+
Cellobiose	+	+	+	+	+
Identification	*Lb. brevis*	*Lb. plantarum*	*Lb. confusus*	*Lb. plantarum*	*Lb. plantarum*

4.10 Isolation and identification of yeast and mold from *Mursik* and *Kule naoto*

Among the 27 isolates from *Mursik* tested, 21 strains were identified as *Saccharomyces* species because they reproduced by multilateral budding, formed pseudohyphae and asci containing one to four globose ascospores (Table 15).

They fermented glucose, galactose, maltose lactose and fructose but did not assimilate sucrose (Table17). The other 6 isolates were identified as mold *Ospora lactis*. This is a white coloured mold which does not assimilate maltose, sucrose and lactose, but can ferment glucose, fructose and galactose. The asci developed in the mycelium at branches of the hyphae. *Saccharomyces* species dominated the fungal microflora in *Mursik* (Table 15).

Kule naoto had four of the isolates identified as *Ospora lactis* mold. Two isolates were identified as *Saccharomyces* yeast based on their morphological features as described in Samson *et al*., 1981and Harigan and MacCance 1976 (Table 16). Two other isolates from *Kule naoto* were identified as *Aspergillus* mold species.

Information on the functions of fungal strains in the fermented milk product is quite limited. *Ospora lactis* (*Endomyces lactis*) also known as *Geotrichum candidum* featured prominently in the two traditional fermented milk products. *Ospora lactis* mold whose colonies are white becomes butyrous when they grow older. The species fall under the genus *Endomyces* (Samson *et al*,. 1981).

Table 15. Morphological characteristics and identification of some yeasts and molds isolated from *Mursik* and their presumptive identification.

Isolates code and Number*	Colony characteristics	Identification*
Y1 (6)	White dry surface mold. The asci developed in the mycelium at branches of the hyphae.	*Ospora lactis* species
Y2 (21)	Cream moist surface yeast. They reproduced by multilateral budding, formed pseudohyphae and asci containing one to four globose ascospore.	*Saccharomyces* species

*** Number of strains isolated shown in the bracket**

Table 16. Morphological characteristics of some yeasts and molds isolated from *Kule naoto* and their presumptive identification

Isolates code and nunber*	Colony characteristics	Identification
KJ2 (4)	White dry surface mold. The asci developed in the mycelium at branches of the hyphae.	*Ospora lactis* species
KJ 1 (2)	Cream moist surface yeast They reproduced by multilateral budding, formed pseudohyphae and asci containing one to four globose ascospore.	*Saccharomyces species*
KJ3 (2)	Black colonies consisting of conidiophores phialidae borne directly on the vesicle	*Aspergillus* species

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*** No of isolated strains shown on the bracket**

Table 17. Utilisation of sugars by isolated yeasts and molds from *Mursik* and *Kule naoto*

			Sugars					
Sourse	Codes	Identification	Glucose	fructose	Galactose	Maltose	Sucrose	Lact
Mursik	KJ2	*Ospora lactis*	±	+	±	-	-	-
	KJ1	*Saccharomyces* species	+	+	+	+	-	+
	KJ3	*Aspergillus* species	±	±	+	±	±	±
Kule	Y1	*Ospora lactis*	±	+	±	-	-	-
naoto	Y2	*Saccharomyces* species	+	+	+	+	-	+

Key:

+ positive action
- negative action
± weak action

Action of *Ospora lactis* in litmus milk showed that the strains isolated were highly proteolytic, a characteristic that is quite beneficial in dairy lactic acid fermentation due to low free amino acid levels in fresh milk. The property is also important in flavor development of the fermented product (Hui, 1993b). However, the *Ospora lactis* strains isolated were not able to ferment lactose: a possible reason why they might have utilized protein for energy and nutritional requirements (Table 17). By means of proteolytic enzymes, the mold hydrolyses milk protein casein into amino acids a process which results in evolution of large quantities of ammonia causing litmus milk to turn deep purple in the upper portion of the culture tube (Cappuccino and Sharman, 1992).

Saccharomyces species have been isolated and identified in several traditional fermented milk products. Isono *et al*,. (1994), reported that out of 19 strains of yeast isolated from *Kule naoto* produced by the Masai community in Northern Tanzania, 10 strains were of *Saccharomyces* species . In this study, the viable yeast and mold count in the product ranged from 10^6 to 10^8 in all samples tested but the count increased on storage (see Tables 3).

In this study *Aspergillus* species were isolated and identified from the traditional fermented milk product. *Aspergillus* species e.g. *Asp. oryzae* and *Asp. soyae* are widely used in fermentation of traditional Japanese foods *Shoyu, Miso* and *Sake* (Samson *et al*,. 1981). Detailed studies on the isolated species of *Aspergillus* from the traditional fermented milk is necessary to establish their functional role in the production of the product.

One of the most important functions of molds in food fermentation is synthesis of enzymes. The enzymes generally decompose complex compounds including proteins, carbohydrates and fats into smaller molecules (Samson *et al*,. 1981). Several studies

have shown a protective role of fungi used in food fermentation especially in inhibition of toxin production by both bacteria and fungi (Samson *et al,*. 1981).

4.11.1 Functional characterisation of some of the isolated lactic acid bacteria from *Kule naoto*.

Selected single strains were tested for their ability to produce lactic acid (section 3.3.9) and flavor compounds (section 3.3.10). The resulting pH of the fermented milk was also determined. Results of the pH, total titratable acidity as % lactic acid and flavor production by selected isolated microbes from *Kule naoto* is recorded in Table 19. *Lactobacillus* cultures produced more acid (up to 0.81) than both *Streptococcus* and *Leuconostoc* cultures. It was noted that commercial strains produced less acid compared to *Lb. plantarum, Lb. brevis* and *Lb. pentosus* isolates. Commercial culture containing *Lb. bulgaricus* had the highest acid production among the commercial cultures. In terms of flavour production, none of the isolated cultures produced flavor as high as that produced by the commercial *Str. diacetylactis* which produced an average of above 30 mg % diacetyl (Table 19). *Lactobacillus brevis* and *Leuc. mesenteroides* cultures produced average of 10 to 30 mg% diacetyl.

4.11.2 Functional characterisation of some of the isolated lactic acid bacteria from *Mursik*.

Table 19 shows the flavor and acid production by the isolated LAB from *Mursik*. Culture containing *Lb. plantarum* coded 5 produced the highest lactic acid (0.6 %).

Table 18. Flavor and acid production by commercial* and LAB isolated from *Kule naoto* .

Strain code	Name	Characteristics		
		pH	Titratable acidity (% lactic acid)	Creatine test Flavor scores
M1	*Lactobacillus brevis*	5.0	0.67	+3
M2	*Lactobacillus pentosus*	5.3	0.53	+2
M3	*Lactobacillus confusus*	5.1	0.69	+2
M9	*Pediococcus pentosus*	4.7	0.75	+2
M13	*Lactobacillus plantarum*	4.6	0.81	+3
M14	*Lactobacillus plantarum*	4.6	0.79	+3
T1	*Leuconostoc mesenteroides*	5.6	0.48	+2
T2	*Leuconostoc mesenteroides*	6.0	0.33	+3
T3	*Streptococcus salivarius*	5.9	0.34	+2
T4	*Streptococcus salivarius*	5.6	0.46	+3
Type 1	*Lactobacillus casei* subsp. *casei*	5.3	0.41	+1
Type 2	*Streptococcus salivarius*	4.7	0.5	+3
Type 3	*Streptococcus diacetylactis*	4.8	0.52	+4
Type 4	*Lactobacillus bulgaricus*	4.5	0.73	+1

Key: nd -not done

* Commercial cultures are coded Type 1, 2, 3, and 4

Table 19. Flavor and acid production by commercial* and some of the isolated lactic acid bacteria strains from *Mursik* .

Code	Name	Characteristics		
		pH	Titratable acidity* (% lactic acid)	Creatine test flavor scores
2	*Lactococcus lactis*	5.2	0.43	+1
5	*Lactobacillus plantarum*	4.8	0.61	+2
18	*Lactobacillus plantarum*	6.2	0.28	+2
9	*Lactobacillus plantarum*	5.4	0.39	+1
13	*Leuconostoc mesenteroides*	6.1	0.26	+1
8	*Lactobacillus confusus*	5.2	0.53	+1
Type1	*Lactobacillus casei* subsp. *casei*	5.3	0.41	+1
Type 2	*Streptococcus salivarius*	4.7	0.50	+3
Type 3	*Streptococcus diacetylactis*	4.8	0.53	+4
Type 4	*Lactobacillus delbruenkii bulgaricus*	4.5	0.73	+1
Blank		6.8	0.18	nd

Key: nd; not done,;

*** commercial cultures coded Type 1, 2, 3, and 4**

The other isolated cultures produced low lactic acid compared to the commercial cultures (see Table 19). In terms of flavor production, *Lb. plantarum* cultures produced on average 3 to 10 mg % diacetyl. Other isolated cultures produced an average of between 0.5 and 3 mg % diacetyl. They produced less flavor compared to the commercial cultures. Results of the activity of mixed strains in acid production is relatively low as compared to single strains tested. This action may explain the complex functionality of the lactic acid producing microflora in the traditional fermented milk products.

Lb. plantarum and *Lb. confusus* isolated from the fermented milk produced more lactic acid compared to those isolated from fermented vegetable products (Wood and Holzapfel, 1995). Wood and Holzapfel (1995), reported that the organoleptic and keeping quality of fermented milks improves when fermented by leuconostocs.

Four commercial cultures of *Lactobacillus casei* subsp. *casei*, *Streptococcus salivarius, Streptococcus diacetylactis* and *Lactobacillus delbrueckii* subsp *bulgaricus* were used for comparison purposes and were tested for their ability to produce lactic acid and flavor compound (section3.3.10). The results (see Table 18 and 19) showed varying abilities to produce lactic acid with *Lactobacillus delbrueckii* subsp. *bulgaricus* producing the highest amount of lactic acid 0.72%, where as *Lactococcus casei subsp. casei* produced an average of 0.41 % (see Table 17). The four commercial cultures had comparatively similar trend as isolated microbes from both *Mursik* and *Kule naoto* with regard to lactic acid and flavor production.

The results indicate that microbes coded M13 and M14 namely *Lactobacillus plantarum* produced the highest lactic acid compared to other isolated strains and commercial cultures tested. The single strains tested showed that there are some strains that have a much higher ability to convert lactose to lactic acid than others. The *Lactobacillus* strains tested produced the highest amounts of lactic acid as compared to *Lactococcus* strains tested (see Tables 18 and 19). All microbes except *Lb. plantarum,* coded M13 and M14 did not attain the iso-electric point pH value of lactose after five days of fermentation (see Table 18).

All the selected strains tested were able to produce the flavor compounds but at different intensities (see Tables 18 and 19). However none of the tested isolated strain could attain the flavor intensity produced by commercial cultures *Streptococcus diacetylactis* which recorded the highest flavor score of 4 (see Table 17).

Lactic acid bacteria metabolise citrate to acetoin, diacetyl, 2,3- Butyleneglycol and CO_2. The reaction in which these compounds are formed relative to one another is dependent on the pH of the medium, (Wood and Holzapfel, 1995). At low pH, acetoin and small quantities of diacetyl are produced (Cogan *et al*,. 1981). It's expected therefore that the citrate metabolising microbes present in *Mursik* produced more of acetoin than the diacetyl. Many strains of *Lb. casei* and *Lb. plantarum* use citrate to produce flavor compounds in milk, (Gel-Gendy *et al*,.1983). These results indicate that *Mursik* and *Kule naoto* has strains with potentially high ability to produce flavor compounds.

4.12 Lactic acid production by mixed strains system

The ability by selected mixed LAB from both *Mursik* and *Kule naoto* to produce lactic acid was tested in 10 % reconstituted skim milk supplemented with 0.5 %

glucose. The results of the findings are shown in Table 20. The results indicate that there was a general decline in acid production as reflected by low percentages of lactic acid and pH with mixed strain system. The maximum acid recorded was when three species isolated from *Mursik* samples were used (Table 20). LAB species from both *Kule naoto* and *Mursik* recorded a decline in acid production. It was noted that dominant species from both products produced more lactic acid when tested as single cultures than when tested as mixed cultures (see Tables 18 and 19). There seems to be antagonistic rather than synergistic factors that are responsible for this observation. This observation needs further investigation since the common industrial practice is use of mixed selected species. The testing medium used in this investigation was reconstituted 10 % skim milk which may have influenced the growth rate of one or all species used in the test.

The dominant bacterial species of lactic acid bacteria and fungi from both *Mursik* and *Kule naoto* were selected and used in laboratory fermentation trials. The product characteristics are shown in Table 21.

Table 20. Acid production by mixed lactic acid bacteria cultures from *Mursik* and *Kule naoto*

Combination	Species name and (code)*	Source	pH	Mean Titratable acidity (% lactic acid) ± SD
1	*Leuc. mesenteroides* (13) + *Lb. plantarum* (18)	*Kule naoto* *Kule naoto*	6.3	0.15 ±0.00
2	*Lc. Lactis* (3) + *Lb. plantarum* (18)	*Kule naoto* *Kule naoto*	6.4	0.13 ±0.01
3	*Lb. confusus* (8) + *Leuc. mesenteroides* (13)	*Kule naoto* *Kule naoto*	5.1	0.44 ±0.05
4	*Leuc. mesenteroides* (13) + *Lc. lactis* (3) + *Lb. confusus* (8)	*Kule naoto* *Kule naoto* *Kule naoto*	5.1	0.46 ±0.01
5	*Lb. plantarum* (M13) + *Lb. brevis* (M1)	*Mursik* *Mursik*	5.5	0.32 ±0.02
6	*Lb. planterum* (M13) + *Lb. pentosus* (M2)	*Mursik* *Mursik*	5.5	0.30 ±0.00
7	*Lb. planterum* (M13) + *Lb. confusus* (M3)	*Mursik* *Mursik*	5.6	0.30 ±0.04
8	*Lb. brevis* (M1) + *Lb. pentosus* (M2)	*Mursik* *Mursik*	5.7	0.25 ±0.00
9	*Lb. brevis* (M1) + *Lb. confusus* (M3)	*Mursik* *Mursik*	5.8	0.23 ±0.02

Key: Sd standard deviation n =3

* ( ) Species code

Table 21: Product characteristics in 10 % reconstituted skim milk using selected isolated cultures

Product quality	Isolated Pure Cultures												
Attribute	**M14 +YI-1**	**M14**	**CTY 3**	**KJ2-1 +M3**	**KJ2-3 +M14 +S1**	**M13 + M14**	**KJ2-2 + M3**	**KJ2-2**	**KJ2-1+ M13+ M2**	**KJ2-1**	**M3**	**M2**	**M13**
% TA	0.52	0.66	0.46	0.50	0.41	0.66	0.52	0.21	0.80	0.30	0.61	0.53	0.80
PH	4.6	4.6	5.0	4.9	5.0	4.9	5.0	6.2	4.5	6.3	5.2	5.0	4.6
Flavor score	+++	+++	++	++	++	++	++	-	+++	-	++	+++	+++
Coagulum texture	F	F	F	F	F	F	F	NC	VF	NC	F	F	F

Key: **Name of strains**: M2, *Lb plantarum*; M3, *Lb. confusus*; M13, *Lb plantarum;* M14, *Lb plantarum*; Y1-1 *Ospora lactis*; KJ2-1 *Ospora lactis*; KJ2-2 *Ospora lactis*; KJ2-3 *Ospora lactis;* CTY 3, commercial strain Str. *Diacetylactis* and S1 Saccharomyces species.

Flavor index: -, less that 0.5 mg% diacetyl/acetoin; +, 0.5 to 3 mg% diacetyl/acetoin; ++, 3 to 10 mg% diacetyl/acetoin; +++, 10 to 30 mg% diacetyl/acetoin

Coagulum texture: NC, no coagulum formed; F, firm coagulum; VF, very firm coagulum.

Others: TA, percent titratable acidity as lactic acid.

The acidity produced in milk in which *Ospora lactis* mold was used together with lactic acid bacteria was not as high as when the lactic acid bacteria cultures were used alone. The pH of the mold fermented milk was above 4.6, the iso eletric point of casein. It was also noted that when mold culture alone was used it did not cause coagulation of milk and pH drop was minimal. The acidity of the resulting fermented milk product obtained when *Ospora lactis* mold was used remained higher than when it was omitted.

Presence of *Saccharomyces* species of yeast in the fermentation culture ensured a relatively low acidity in the product. More studies on use of isolated *Ospora lactis* and *Saccharomyces* species together with lactic acid bacteria in the fermentation of milk are suggested. Flavor development was higher in cultures containing *Lb. plantarum* and *Ospora lactis*. A mixed culture containing *Lb. plantarum* M2 and M13 and *Ospora lactis* KJ2-1 produced a fermented product with very firm coagulum, high lactic acid (0.80 %) and high flavour production (estimated as 10-30 mg % diacetyl), (see Table 21). Use of molds in mesophilic milk production has not been investigated in detail especially microbes isolated from traditional fermentation systems. However, these studies indicate a possibility of using a mixed culture system to produce a fermented milk product with acceptable quality attributes.

4.13 Proteolytic activity of the isolated lactic acid bacteria cultures

Results for proteolytic activity of selected strains of lactic acid bacteria from *Kule naoto* and *Mursik* are shown in Tables 22 and 23 respectively.

Table 22. Proteolytic activity by LAB cultures isolated from *Kule naoto*.

Strain name and code	*Radius mm (R) of the clear zone	Rating
Lb. brevis (M1)	3.84	Average
Lb. plantarum (M2)	3.17	Average
Lb. confusus (M3)	5.15	High
Leuc. mesenteroides (T1)	6.65	High
Leuc. mesenteroides (T2)	5.00	Average
Str. salivarius (T3)	5.30	High
Str. salivarius (T4)	5.65	High
Lb. plantarum (M13)	3.00	Low
Lb. plantarum (M14)	3.65	Average

Key: Proteolytic activity rating: $R \leq 3$ mm = low; $3 < R < 5$ mm = Average; $R \geq 5$mm = High

Table 23. Proteolytic activity by some lactic acid bacteria cultures isolated from *Mursik*.

Strain name and code	Radius (R) mm of clear zone	Rating
Lc. lactis (3)	4.00	Average
Lb. plantarum (14)	5.15	High
Lc. lactis (2)	5.50	High
Lb. plantarum (18)	3.84	Average
Lb. murinus (11)	4.50	Average
Leuc. mesenteroides (13)	3.75	Average
Lb. plantarum (5)	4.50	Average
Lb. casei (4)	5.00	Average
Lb. plantarum (9)	6.15	High
Lb. confusus (8)	4.50	Average

Key: Proteolytic activity rating: $R \leq 3$ mm = low; $3 < R < 5$ mm = Average; and $R \geq 5$mm = High

Nineteen isolated LAB were selected and tested for their proteolytic activity qualitatively as described by Dick *et al,*.(1993). *Lactococcus* recorded a high proteolytic activity among the isolates tested from the *Kule Naoto* (Table 22). *Lactobacillus* species tested had an 'average' proteolytic activity. In this study we established that *Leuc. mesenteroides* species isolated from *Kule naoto* had a 'high' proteolytic activity. Species of *Lb. plantarum* from *Kule naoto* had 'low' to 'average' proteolytic activity (see Table 22). *Streptococcus. salivarius* culture tested recorded 'high' proteolytic activity. From these studies it was also noted that that the dominant lactic acid bacteria in *Kule naoto* had 'low' to 'average proteolytic activity. *Leuc. mesenteroides* subsp. *dextranicum and Leuc. mesenteroides* subsp. *mesenteroides* cultures have been isolated from *Kule naoto* from Narok District in Kenya though their proteolytic activity had not been established (Miyamoto *et al.*, 1986). The proteolytic system of LAB is used by the bacteria to acquire essential amino acids from cows milk can contribute to the distinctive flavor characteristic of fermented milk products (Nakazawa and Hosono, 1992, Hui, 1993b, and Dick *et al,*. 1993).

Lactic acid bacteria isolated from *Mursik* samples had 'average' proteolytic activity by seven cultures with three cultures recorded 'high' proteolytic activity (see Table 23). *Lb. plantarum* species showed a 'high' proteolytic activity. The present data indicate isolates with high potential as starter cultures in fermented milk and cheese processing. In their studies Dick *et al.*, (1993), reported the proteolytic activity of 607 lactic acid bacteria isolated from traditional fermented food systems and culture collections. They reported four subdivisions of the isolates with increasing proteolytic activity as follows, 'negative' 131, 'low' 236, 'average' 234 and 'high' 16.

Proteolytic activity of lactic acid bacteria is associated with flavor development in cheese. Observations made in this study on isolates from both *Kule naoto* and *Mursik* samples indicate some potential of several isolated bacteria as cheese ripening cultures. Proteolytic activity in lactic acid bacteria is plasmid genes encoded and is as a result highly unstable (Gasson and de Vos, 1993).Therefore the property could be lost in subsequent cell division thus reducing the activity of cultures.

4.14 Antimicrobial activity of isolated lactic acid bacteria cultures

The antimicrobial activity of dominant species from *Mursik* and *Kule naoto* against *Eschelichia coli, Staphylococcus aureus and Bacillus cereus* is given in Table 24.

There was a great antagonistic effect against *S. aureus*. Two *Lb. plantarum* cultures had no growth inhibition against growth of *E. coli*. The highest anti-microbial effect was recorded by *Lb. plantarum* M13 and M14 cultures isolated from *Kule naoto. Str. salivarius* and *Lb. confusus* isolates from the same product had moderate inhibition. The dominant lactic microflora from *Mursik, Lactobacillus plantarum* showed moderate antimicrobial activity against *S. aureus* strain.General observations made on tested isolates from both *Mursik* and *Kule naoto* indicated great antimicrobial effect against *E. coli* and *S. aureus*.

The LAB isolated however showed no antimicrobial activity against *B. subtilis*.

Table 24: Antimicrobial activity of selected LAB cultures against *E. coli and S. aureus.*

Lactic acid bacteria name and (code)	Source	Inhibition zone* (mm) ± SD	
		E. coli	*S. aureus*
Lb. *plantarum* (5)	*Mursik*	16 ± 0.0	24 ± 0.0
Lb. plantarum (14)	*Mursik*	-	23 ± 0.2
Lb. plantarum (9)	*Mursik*	-	19 ± 0.1
Leuc. mesenteroides (13)	*Mursik*	-	20 ± 0.0
Lb. confusus (8)	*Mursik*	16 ± 0.1	16 ± 0.4
Lb. plantarum (M13)	*Kule naoto*	24 ± 0.7	26 ± 0.2
Lb. plantarum (M14)	*Kule naoto*	23 ± 0. 0	25 ± 0.2
Lb. confusus (M3)	*Kule naoto*	24 ± 0.0	22 ± 0.0
P. pentosus (M9)	*Kule naoto*	-	24 ± 0.0
Leuc. mesenteroides (T2)	*Kule naoto*	-	17 ± 0. 0
Str. salivarius (T3)	*Kule naoto*	22 ± 0.2	16 ± 0.2

* Mean diameter: greater than 25 mm: strong inhibition,
21 to 25 mm: moderate inhibition
15 to 20 mm: weak inhibition.

The lactic acid bacteria are known to produce and excrete compounds with antimicrobial principles other than acids, such as hydrogen peroxide and antibiotics such as nisin, diplococcin, lactocidin, acidophilin and lactolin (Cook, R.D. 1985; Mbugua and Njenga, 1991; Nakazawa and Hosono, 1992; Fuller, 1992; and Wood and Holzapfel, 1995). Nisin is produced by many strains of *Lactococcus lactis* and is permitted as food additive in at least 47 countries (Wood and Holzapfel, 1995). Nisin is primarily useful to inhibit Clostridium species like *botulinum* and *tryobutyricum* (Wood and holzapfel, 1995). *Lactococcus* produces a bacteriocin called lactococcins which may be heat stable (Nakazawa and Hosono, 1992).

Bacteriocins are relatively small peptides sensitive to specific proteolytic enzymes. They may be heat stable, and have antimicrobial activity either bacteriocidal or bacteriostatic against closely related or in some cases a wide spectrum of microorganisms including food borne pathogens such as *Listeria monocytogenes,* (Dick *et al.,* 1993). Dick *et al.,* (1993) reported that out of the 573 LAB tested for bacteriocin production, 8 strains of *Lactococcus lactis* showed anti-microbial activity against indicator microorganism, namely *Lb.plantarum, Lb. fermentum, Lb. salivarius, Lb. pentosaceus Listeria innocua* and *Cl. tyrobutyricum.* The bacteriocin elaborated by the strains of *Lactococcus lactis* were subsequently determined to be protein in nature. The protein was heat stable with no loss of activity after heating for 2 hours at 100°C. Further characterisation showed that the bacteriocins produced were nisin-like (Dick *et al.*, 1993). This makes *Lc. lactis* particularly important for potential use in food preservation.

Natural antibiotics like nisin and bulgarin are produced by *Streptococcus lactis* and *Lactobacillus bulgaricus* respectively, while lactobrevin and acidophilin are produced by *Lb. brevis* and *Lb. acidophilus* respectively (Ayebo, 1986).

Bacteriocins from *Lb. lactis* and *Pediococcus pentosaceus* have been shown to have a broad spectrum activity against Gram positive bacteria such as *Bacillus cereus, Clostridium perfrigens, S. aureus,* and *Listeria* species (Spelhang and Harlander, 1989).

Studies on lactic acid bacteria isolated from traditional fermented milk in Northern Tanzania reported growth inhibition for *Staphylococcus aureus* and *Escherichia coli* in milk cultured by *Lactococcus lactis ssp. lactis* and *Lactobacillus confusus* (Isono *et al,.* 1994). The growth of *S. aureus* and *E. coli* was restricted by rapid acidification of the milk and a bacteriocin produced by the starter cultures. Miyamoto *et al.* (1986) reported that a *Streptococcus lactis* strain isolated from Masais' fermented milk in Kenya showed anti-microbial activity against growth of *Eschelishia coli* and *Staphylococus aureus* species of bacteria. Inhibition of Gram negative bacteria by lactic acid bacteria has also been reported by Chang *and* Hearsberger (1994).

Studies on culture filtrate from yoghurt cultures namely *Lactobacillus bulgaricus* and *Streptococcus thermophilus* showed a pronounced inhibition of test organisms *S. aureus* , *E. coli*, *Pseudomonas, fragilis* and *Micrococcus flavus.* It was also noted that there was greater inhibition by the yoghurt cultures cultured in Buffalo milk than in cow's milk (Jasjit *et al.*. 1979). Pulusani et al. (1979) showed that the compounds were of low molecular weight after partial purification and characterisation of the antimicrobial compounds produced by *Str. thermophilus*.

5. Conclusions and Recommendations

The study established the traditional process of *Mursik* and *Kule naoto* production by Kalenjin and Masai communities respectively. The process involves use of gourd pretreated by rubbing with specific a hot burnt tip of specific sticks. The products are processed from raw milk, and depend on spontaneous fermentation. The study showed that cooked blood is added to the raw milk used in *Mursik* processing by some families unlike in *Kule naoto* where the practice of adding cooked blood has been abandoned. It was noted that consumption of fermented milk products is a daily practice where both young and old people consume on average 500 mls of the fermented product daily per person. Both products which took on average 5 days of spontaneous fermentation at ambient temperatures could be kept for extended periods of time without getting spoilt. Additional work is needed to establish the influence of the extended storage on chemical and microbial characteristics of the fermented milk product.

The composition of the fermented milk products was investigated. *Mursik* showed high percent total ash content possibly due to the residue ash introduced during pre-treatment of fermentation vessels with burning wood. The percent fat content and crude protein was generally higher than that of fresh milk. This could be attributed to the cooked blood added during product preparation. It was also noted that *Mursik* developed lower pH values on fermentation ranging from 4.1 to 4.3, and high titratable acidity (1.507 %). The proximate composition of *Mursik* could have an influence on development, type and composition of microbial flora present in the product.

Kule naoto's proximate composition showed a high total ash content greater than the average of 0.7 % reported for fresh milk (Hui, 1993b). The percent crude protein and fat in *Kule naoto* were the same as the average in the commercial mesophilic cultured milk products. (Nakamura *et al.,* 1996). The pH was generally higher where as titratable acidity was lower in *Kule naoto* but similar to most other fermented milk products (Nakamura *et al.*, 1996). The proximate composition data of both fermented milk products indicated a much richer product with respect to minerals and protein content. The influence of extra levels of minerals in *Mursik* and *Kule naoto* on the relative growth of lactic acid bacteria, yeast and molds needs further investigation.

Identification studies carried out on the isolates from *Mursik* showed that *Lb. plantarum*, *Lb. confusus*, and *Lb. casei* were the dominant *Lactobacillus* species in *Mursik*. Cocci species were dominated by *Lc. Lactis*, *Leuconostoc mesenteroides* and *Enterococcus feacium* species. Studies on their proteolytic activity indicated that *Lb. plantarum* strains had 'average' to 'high' proteolytic activity while species of *Lb. confusus* and *Leuc. mesenteroides* had an 'average' proteolytic activity. The anti-microbial activity of the dominant microbes isolated from *Mursik* recorded moderate inhibition against *Staphylococcus aureus* whereas one isolate of *Lb. plantarum* had weak inhibition against *Escherichia coli*. Selected strains tested had a higher pH value and lower final percent acid concentration after 5 days fermentation in controlled conditions. A study on any relationship between the antimicrobial factors and shelf life of the fermented milk product should be carried out.

Microbes isolated from *Kule naoto* showed that the dominant lactic acid bacteria was the *Lactococcus* species. The *Lactoccoccus* isolated were homo fermentative while most of the *Lactobacillus* species isolated from the product were

heterofermentative. *Lb. plantarum*, *Lb confusus* and *Lb brevis* were the dominant *Lactobacillus* species of lactic acid bacteria in *Kule naoto.* These *Lactobacillus* species had 'average' to 'high' proteolytic activity. These isolated species also showed antagonistic effect against growth of *E. coli* and *S. aureus* and produced higher acid and flavor in mixed culture system than with single strains.

Among the fungal microflora isolated and identified from both *Mursik* and *Kule naoto* was the *Saccharomyces* species and *Ospora lactis* mold species. Mixed culture system containing fungal strains isolated from the fermented milk products recorded an increase in acid and flavor production in the fermented product under controlled conditions. Presence of *Saccharomyces* strains has been reported in *Kule naoto* though their specific role is little known. We suggest further investigations in the role of *Ospora lactis* and *Saccharomyces* strains in mixed culture system used in dairy fermented products.

Some of the isolated *lactobacillus* species for example *Lb. plantarum* and *Lb. confusus* which had demonstrated a high lactic acid production ability and 'high' proteolytic activity could be studied for their genetic make up for possible development into commercial dairy cultures.

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APPENDIX I:

Traditional fermented milk production study questionnaire.

Introduction:

Fermentation of milk is a biochemical process with primary aim of improving the quality and acceptability of dairy products. Traditional fermentation of milk is widely used in to achieve this purpose with added cultural value to the art of production and consumption of the fermented product. The aim of this questionnaire is find details of the art and practice of traditional fermented milk production in your community in a study whose objective is to isolate identify and characterise lactic acid producing microflora involved in the fermentation of the product.

With regard to traditional fermented milk production, Please answer the following questions:

1. What type of milk do your community use ? (cow, goat, sheep, or mixed)
2. What type of equipment do you use?.
3. How are the above equipment obtained and prepared for use?
4. What kind of milk and equipment pretreatment is done before fermentation?.
5. How is the entire process of fermentation carried out, e.g temperature used, duration of fermentation storage conditions?
6. To what extent is traditional fermented milk produced and consumed in your community?
7. Is the traditional product commercialised.?
8. Is there any cultural belief associated with consumption of the fermented milk product.

THANKS FOR PARTICIPATING.