"MICROBIOLOGICAL STUDIES OF *KIRARIO*, AN INDIGENOUS KENYAN FERMENTED PORRIDGE BASED ON GREEN MAIZE AND MILLET."

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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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List of abbreviations used in the text.

LAB: Lactic acid bacteria

Leuc. Leuconostoc

Lc. : Lactococcus

Lb. : Lactobacillus

TA: Titratable acidity

CFU: Colony forming units

MRS: deMan Rogosa Sharpe

TVC: Total viable counts

GIT: Gastrointestinal tract

APHA: American Public Health Association

WCFS: Wageningen Centre for Food Sciences

ABSTRACT

The objective of this study was to isolate, characterize and identify the lactic acid bacteria involved in the fermentation of *Kirario*, a traditional fermented porridge based on green maize, millet and/or sorghum. The traditional production of *Kirario* involves spontaneous fermentation of green maize and millet/sorghum in fermentation vessel (earthware pots, plastic jars and buckets) at ambient temperature for 1-2 days. The traditional art of *Kirario* preparation was studied and documented with the aid of questionnaires. The biochemical and microbial profile changes during fermentation of *Kirario* were monitored for 48 hours. The information obtained was used to characterize and identify the microflora involved in *Kirario* fermentation.

The average total viable counts (TVC), lactic acid bacteria (LAB), lactococci, and yeasts and moulds of samples from various localities in the study region (Meru North District) were 9.30, 9.63, 8.62, and 4.83 $\log_{10}$ cfu/ml respectively. The coliform counts in two of the samples was <1 log cfu/ml while in other samples no coliforms were detected. This is an indication of either good hygienic processing or effect of inhibition of growth of coliforms during fermentation.

The development of pH, titratable acidity and microbial counts were monitored at 6 hourly intervals for 2 days (48 hours). The initial pH of 6.4 dropped to 3.3 at the end of the fermentation, while the titratable acidity increased to 3.15% from an initial value of 1.04%. The TVC, LAB, lactococci, yeasts and molds increased from counts of 8.20, 8.18,

7.20 and 5.86 to 9.64, 9.55, 5.38 and 0.70 $\log_{10}$ cfu/ml respectively at the end of the 48-hour fermentation.

Thirty LAB strains were isolated from samples of *Kirario* and identified using API 50 CHL identification systems. The study showed that the *Lactobacillus, Leuconostoc* and *Lactococcus* genera were the dominant lactic acid bacteria in *Kirario*. The isolated LAB were identified as *Leuconostoc mesenteroides ssp. mesenteroides/detranicum 1 & 2, Leuconostoc citreum, Lactococcus lactis ssp. Lactis 1 & 2, Lactococcus raffinolactis, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus collinoides* and *Lactobacillus coprophilus. Lactobacillus plantarum* 1, was the most predominant LAB [about 47% of total LAB isolates], followed by *Leuconostoc mesenteroides ssp. mesenteroides/detranicum 1* which accounted for 16% of the LAB isolates.

CHAPTER 1. INTRODUCTION

1.1 Background of the study.

Traditional fermentation processes are attracting the attention of many scientific and policy makers as a vital part of food security strategies (Van de Sande, 1997) .In many African countries, cereal based traditional fermentation products are consumed both as beverages and as foods (Lorri, 1993; Steinkraus, 1996). Kenyan *Kirario* is one of the beverages among others increasingly consumed by majority of the communities in Kenya. *Kirario* is a millet/sorghum based non-alcoholic fermented beverage widely consumed in the Eastern and Central provinces of Kenya. Scientific studies of fermentation products in Kenya, refining and promoting utilization of their processes and developing local food processing capabilities can help to alleviate food insecurity if given research priorities (Van de Sande, 1997).

Majority of Kenyan traditional fermented foods have not been documented. However *uji*, a Kenyan thin lactic fermented porridge has been studied and well documented (Mbugua, 1987). About 81-84% of maize, the staple cereal in Kenya is utilised and consumed in form of non-fermented u*gali* (stiff porridge), while 10-12% as fermented porridge called *uji* (Nout, 1987). Natural lactic fermentation is reported to improve the nutritional value of cereals. Mbugua (1987) reported that fermentation of the *uji* slurry from wet-milled maize proved superior to the slurry made from finely sifted maize flour (unga baridi) in terms of improved protein content. Crude protein increased slightly but significantly

during fermentation, which was attributed to concentration of *uji* slurry as result of water lost through evaporation during the fermentation process. Mertz, et al, (1964) reported that fermentation of pearl millet raised its protein digestibility from 74.86% to 85.5%, the same level found in wheat, maize and rice. Steinkraus (1978) showed that vitamins are in many instances increased in a food as a result of microbial growth in it. The microorganisms themselves may possibly contribute their quota of nutritive material.

Quin (1964), a nutritionalist who carried out life long study of Africans, concluded that the recognition and encouragement of the traditional foods and feeding habits of African tribes, could contribute to alleviating and perhaps even solving the problem of malnutrition and disease among the people. Steinkraus (1979) showed that if the world wanted to improve the nutritional status of its poor and hungry, as well as modernize the traditional fermented foods, it ought to look at the processes involved in these indigenous foods in order to achieve its aims. Many of the recent studies on indigenous processes of food fermentation have led to or are aimed at upgrading or modernizing the processes involved in their preparations [Dirar, 1993].

Lactic fermentation involved in traditional fermented porridges is known to inactivate trypsin inhibitors and haemagluttins, and to eliminate alpha-oligosacharides [Reddy et al 1986: WHO 1996]. Lactic fermentation is also reported to improve the digestibility of proteins and starch, bioavalability of micronutrients, palatability of porridges as well as imparting antimicrobial properties in them [WHO 1996 and Lorri 1993]. Fermentation

and cooking are reported to eliminate trypsin inhibitors, haemagluttins and lectins in cereal gruels enriched with Soya bean protein [Mbugua, 1992].

The lactics are currently of major scientific interest due to the discovery of microflora with unique and important probiotic properties and functionalities in food fermentation that have been isolated from traditional fermented foods elsewhere in the world. Some of the probiotic properties associated with some isolated lactic acid bacteria have been of prophylactic and therapeutic value for certain human ailments. Information on appropriate functional properties by the LAB in the relevant fermented food products, has led to the development of suitable starter cultures that can be used in controlled fermentation and quality improvement of traditional fermented food products.

Research should therefore be directed towards identifying the (i) nutritional benefits associated with specific indigenous fermented foods, as well as elucidating the (ii) microorganisms involved and other technological properties in the fermentation process. Also of importance is the (iii) development of starter cultures, from unique microbial strains with special technological properties, and (iv) testing new cereal varieties for their suitability as fermentation substrates.

1.2 Problem statement and study justification

The traditional fermentation systems of products like *Kirario* can lead to microbial evolution of strains with unique technological and other beneficial properties. Fermented foods are consumed in every country of the world and, there is growing scientific evidence that many fermented foods are good for health or contain ingredients that are good for health. Fermented foods that improve or change the intestinal microflora are of particular interest because of our increased knowledge of the role the intestinal microflora play in health and disease resistance.

Kirario has not been studied and documented in previous studies. Research that will be done in this study will help in adding to the pool of knowledge in regard to fermented foods in Africa. This will be achieved by the advancement of knowledge of both microbiology in general and the identification of important human intestinal microflora that may be identified through this study. Fermented foods are becoming even more important in our diets, and the maintenance of health, as scientists continue to identify different microorganisms that can be used in the production of probiotic foods.

Therefore, the traditional fermentations will remain an important part of global food supply, which can be used to alleviate food insecurity and poverty in the developing countries, [Dirar, 1993]. Fermentation of cereals is a technology that is still widely used in Kenya. It plays a crucial role in the nutritional status of many people in the rural communities. Understanding of traditional processing technologies can therefore help in

its standardization and improvement. This study will entail documentation of the process chemically and microbiologically.

Traditional technologies are difficult to incorporate and commercialize in the current industrial set-up. However, they can be upgraded and used in small-scale production of fermented foods [Dirar, 1993]. To improve the nutrition of the many people who consume fermented foods in Africa, indigenous fermented foods should be studied and their processes modernized technologically. This will also help in solving the problem of malnutrition and disease among the vulnerable groups of people in our societies. Studies on traditional fermented products help in establishing the best methods for enrichment by fermentation, the optimum conditions for successful enrichment and changes to be anticipated during the process. Research of traditional fermented foods can help improve the incomes and food security of small-scale farmers by adoption of improved technologies.

1.3 Expected beneficiaries

The documentation and study of *Kirario* 's art of production is of great benefit to the Ameru communities that consume the product as the study will ensure the continuity of the culture and consumption of *Kirario.* This is of importance due to the immerse nutritional and health benefits derived from consumption of fermented foods.

The documentation of the traditional art of preparation, and the identity and functionality of the microbial population involved will open up further research on the fermentation process *Kirario.* The study results will add to the pool of scientific knowledge on the microbial diversity, thus forming a basis for further research and exploitation on any genes with unique functionalities on human health and well being of the communities that consume *Kirario.*

The results of this study will also benefit the rural communities that consume this product by identification of microbial strains with unique technological and nutritional characteristics. The knowledge gained in this project can also be used in research on microbial strain selection and development, with ultimate aim of developing starter cultures for use in small scale commercial processing of *Kirario* by the rural communities. This will benefit the women who prepare *Kirario* by empowering them economically.

The resulting modernization, industrialization and commercialisation of *Kirario* can go a long way in adding value to the raw materials used in *Kirario* production, and alleviation of malnutrition and poverty in the rural communities that consume this fermented food.

1.4 Overall objective

The main objective is microbiological and biochemical characterization of *Kirario.*

1.5 Specific objectives

The specific objectives are:

1. Documentation of the traditional process for *Kirario* production.
2. Investigation of the microbiological and biochemical changes during fermentation of *Kirario.*
3. Characterization and identification of microflora involved in *Kirario* fermentation.

CHAPTER 2: LITERATURE REVIEW

2.1 Sorghum and millet utilization

Sorghum and the millets constitute a major source of energy and protein for millions of people in Asia and Africa [ICRISAT and FAO, 1996]. These grains are of immense nutritional value in the diets of poor people in the semi-arid tropics. Sorghum and pearl millet are the essential grains in regions of Africa where endemic drought causes the frequent failure of other crops such as maize [ICRISAT and FAO, 1996].

Sorghum and millets are currently grown in the semi-arid zones of Western, Nyanza, Rift Valley and Eastern provinces of Kenya. They are traditional crops in the semi-arid areas of Kenya, where they outperform other grains because of their ability to withstand moisture stress [ICRISAT, 1992]. Research on the food and nutritional quality of sorghum and millet based foods is accordingly of major importance to Kenya. This is vital in order to develop methods for adding value to these crops and improve their level of utilisation.

Fermentation as a method of preparing products from millet/sorghum has been found to enhance product storage, maintain consistency, lower pH and hence help in destruction of pathogenic microbes [KARI, 1997]. Malted formulations based on sorghum and cowpeas were found to be better than roller-dried food for weaning [Kari 1997]. Sorghum genotypes with hard endosperm were found to make acceptable *uji* and *ugali* preparations [KARI, 1997]. In view of the growing demand for food grain staples in Kenya, where

maize and wheat production may reach a plateau, exploitation of millets and sorghum as food grains become inevitable. Food products made from sorghum and millets have fairly low protein content hence there has been need to supplement the cereals with legumes, thereby upgrading the overall protein quality of the diet [Khan and Bressani, 1987; Kaul, 1975]

2.1.1 Pearl Millet

Pearl millet accounts for almost half of global millet production. It is the most important species of millet both in terms of cropped area and contributions to food security in regions of Africa and Asia [ICRISAT and FAO, 1996]. In most parts of Africa, millet is grown as a subsistence crop for local consumption. Although millet represents less than 2 percent of world cereal utilization, it is an important staple in a large number of countries in the semi-arid tropics, where low precipitation and poor soils limit the cultivation of other major food crops [ICRISAT and FAO, 1996].

It is estimated that about 80 percent of the world's millet [and over 95 percent in Asia and Africa] is used as food, the remainder being divided between feeds [7 percent], other uses [seed, beer, etc.,] and waste [ICRISAT and FAO, 1996]. Millet is a high-energy, nutritious food, especially recommended for children, convalescing individuals and the elderly. Several food preparations are made from millet, which differ between countries and even between different parts of a country [ICRISAT and FAO, 1996]. In Kenya these

preparations consist primarily of porridges, *ugali*, malt brews and traditional beers. Millet is nutritionally equivalent or superior to other cereals. Protein content in pearl, proso and foxtail millets is comparable to that in wheat, barley and maize. Finger millet has slightly lower protein content, but is in fact nutritionally superior because the protein quality is generally as good as, or better than in other cereals. Finger millet is also high in calcium and iron, and contains fairly high levels of methionine, a major limiting amino acid in many tropical cereals and grain legumes [ICRISAT and FAO, 1996].

Millet will continue to be used primarily for human food, and will remain a major source of calories and vital components of food security in semi-arid areas in the developing countries [ICRISAT and FAO, 1996].

2.1.2 CONSUMPTION

Millet is a staple food in many countries where climatic conditions do not favour growth of other cereals. This is so especially in sub-Saharan countries where it accounts for half of all food consumption. In Kenya, millet is consumed widely in a variety of traditional foods. It is still necessary for women to pound millet in a wooden mortar or grind between two stones since industrial production technology for flours and other products from millet is expensive, is not well developed and does not produce products that are culturally acceptable in production of most indigenous fermented foods. Rooney and Clark (1968), and Freeman and Watson (1969), cited many laboratory trials, mostly in USA, of milling systems for millet and sorghum for human consumption. This increased the potential of sorghum and millet utilization in food products.

Millet is tasty, with a mildly sweet, nut-like flavour and contains many beneficial nutrients. It has nearly 15% protein, with high amounts of fibre, B-complex vitamins including niacin, thiamine, and riboflavin, essential amino acids, lecithin, and some vitamin E. It is particularly high in the minerals like iron, magnesium, phosphorus, and potassium. The grains are also rich in phytochemicals, including phytic acid, believed to lower cholesterol, and phytate, which is associated with reducing cancer, [Hulse et al., 1980; FAO, 1995]

In Kenya many traditional alcoholic and non-alcoholic foods incorporate millet as an ingredient through wet milling of the grains, followed by fermentation. Fermented porridges have been made by a combination of maize or sorghum and millet, which are then fermented.

2.2 Types and nutritional quality of products prepared from sorghum and millets in Africa.

When a grain is processed, some nutrients are removed. The effect of milling on nutrient loss depends on the amount of material removed and the method used in the removal. The removal of nutrients and antinutritional factors should be on balance depending on whether they are beneficial or not, hence must always be analyzed carefully. In many West African countries, sorghum and millet grits are steamed to produce a coarse and uniformly, gelatinized product called *couscous*. Couscous can be consumed fresh or can be dried. In its dried form, it can be stored for more than six months [FAO, 1996].

Porridges are major foods in several African countries. They are either thick or thin in consistency. These porridges carry different local names. Thick or stiff porridges are called **ugali** in Kenya, Tanzania and Uganda, **to** in Burkina faso and the Niger, **tuwo** in Nigeria, **aceda** in Sudan, **bogobe or jwa ting** in Botswana and **sadza** in Zimbabwe. The biological value of sorghum **ugali** was reported to be superior to that of the raw grain [FAO, 1996]. In Mali, parts of Senegal and Guinea, **to** is alkali-treated and has a pH of 8.2. In Burkina Faso, it is acid-treated to a pH of about 4.6. In other regions of Africa, the **to** is neutral. These treatments have implications for the taste and nutrition [FAO, 1996].

Thin porridges are called **uji** in Kenya and Tanzania, **ogi or koko** in Nigeria and Ghana, **edi** in Uganda, **rouye** in the Niger and Senegal, **nasha** in Sudan, **rabri** in India, **bota or mahewu** in Zimbabwe and S.Africa, and **motogo we tiny** in Botswana. Sorghum flour, sorghum malt, pigeon pea and groundnuts are mixed in different proportions to improve the nutritional value of traditional porridges. In Uganda, a sour porridge called **bushera** is prepared by boiling ungerminated millet flour to produce a thick paste. Flour made from freshly germinated millet is then mixed into it. This sweetens the porridge and lowers its viscosity [FAO, 1996].

Fermented porridge is made in several regions of Africa. The differences in the porridges and fermentation result from the activity of microorganisms involved. Fermentation processes have evolved largely as a result of practical needs of the communities consuming the porridges. The palatability and the texture of foods can be changed and their shelf-life can often be improved by fermenting them. In Eastern Africa, a

suspension of maize, millet, sorghum or cassava flour in water is fermented before or after cooking to make thin sour porridges. In Sudan, a thin fermented porridge called **nasha** is prepared from sorghum. Some of the bacteria and moulds identified in **nasha** are also described in a fermented porridge called **ting** from Botswana. **Ogi**, a popular fermented porridge in Nigeria, is prepared using sorghum, millet and maize in various proportions. The **chibuku beer** consumed in southern Africa is a thin alcoholic fermented porridge, usually made from sorghum [FAO, 1996].

2.3 FERMENTATION

Fermentation is defined as a desirable process of biochemical modification of primary food products brought about by microorganisms and their enzymes [Whitaker, 1978]. Fermented foods in Kenya consist mainly of lactic acid fermented cereals (e.g. maize, millet, and sorghum), root crops (e.g. cassava, sweet potatoes), milk, fish, meat and vegetables. Lactic acid fermentation at household level is a natural process brought about by the lactic acid bacteria present in raw foods or those derived from a starter culture [Mbugua, 1984]. Sucrose is the major fermentable sugar in fermented flours and porridge. Lactic acid bacteria have been found to be mainly responsible for the fermentation of various cereals. *Lactobacillus plantarum* is responsible for souring of *uji* although early activity of heterofermentative strains of *L. fermentum*, *L. cellobiosus* and *L. buchneri* during fermentation was evident (Mbugua, 1981).

Fermentation is often part of a sequence of food processing operations, including cleaning, grinding, soaking, cooking, packaging and distribution. Therefore the potential for fermentation to enhance food safety and nutritional value should be assessed in the light of the total manufacturing sequence [Dirar, 1993]. Fermentation is a common traditional household technology in many parts of the world. Regional differences occur in manufacturing practices, consumption habits, quality and level of acceptability of fermented foods. These will depend on such factors like the availability of raw materials, the laborious processing involved and the changing patterns in the society [Dirar, 1993].

2.3.1 FERMENTATION OF CEREALS

Fermented cereals are particularly important as weaning foods for infants and as dietary staples for adults. The advantages of food fermentation have been highlighted by many authors (Van Veen et al., 1968; Whitaker, 1978; Hesseltine and Wang, 1979; Steinkraus, 1996). These advantages may be summarized as follows:

- Fermentation is a method of preservation.
- Fermentation may destroy undesirable factors in raw products.
- The fermented foods may have an enhanced nutritional value and digestibility.
- Fermented foods may have a better flavour than the raw products. Fermented products have varied flavour because of varied ingredients, mixed microorganisms and addition of spices and salt.
- Fermentation may be used to salvage some products that could not otherwise be used for food.

- Fermentation may improve the appearance of some foods.
- Fermentation reduces cooking time.
- Some fermented foods may be safer.
- Fermentation helps solubilize some food components.
- The methods used are inexpensive.
- The techniques are simple and the process involves little waste.
- Products are well established and acceptable.

The preservation of the fermented foods is by action of organic acids and low pH. Hesseltine (1979) has enumerated some of the special merits and features of the fermentations characterizing the fermented food products of Africa. All are self-inoculated fermentations, the microorganisms being typical of those present in or on one of the ingredients. Yeast and bacteria (mainly lactic bacteria) are the only organisms that carry out the fermentation; moulds are present only as chance contaminants. Fermentations are carried out sometimes to produce foods and drinks said to have the ability to quench thirst (Dirar, 1993). Many pointers in thirst-quenching foods and drinks suggest that lactic acid is involved in the physiology of thirst.

In the Ameru community, the Ameru people believe that consumption of fermented foods protect individuals from infections and hence promote health and long life. Hesseltine and Wang (1979) actually supported this idea when they mentioned that many fungi used in oriental food fermentations produced a compound active against Gram-positive bacteria and anaerobic sporeformers hence may limit and control bacterial

infections of people consuming it. The presence of antibiotics has been demonstrated in certain fermented foods. Some microbial species isolated from fermented foods have the ability to produce antibiotics (Hesseltine, 1965, Van Veen and Steinkraus, 1970, Hesseltine 1985).

Fermentation of cereals has been documented in Africa especially in West Africa. In West Africa, the best-documented sour thin porridge is *ogi* porridge prepared from various grains, including sorghum (Dirar, 1993). About three types of sour thin porridges have been reported to exist in South Africa: *mahewu* (*magao*), *leting* and *mdogo* (m*etogo*) (Dirar, 1993). In East Africa, *uji* of Kenya is prepared by diluting, followed by cooking and consumption in liquid form (Mbugua 1987). The African thin porridges are all consumed by healthy adults as daily foods.

Analysis has been done on *medida*, a thin porridge in Sudan. Chemical analysis of *medida* showed that the product contained 95% water, 3.2% starch, 1.3% crude protein, 0.3% crude fibre, 0.4% ash, 0.2% fat and 230 calories per 100g (Monawar and Badi, 1983). This is similar to preliminary studies carried out on *Kirario.*

Fermentation of cereals has been used for a long time in making of porridges and children weaning foods for children. Appropriate weaning foods remain the single most difficult problem facing the developing nations. In Kenya babies are usually weaned on sour paps, or where milk is available on soured milks or fermented porridges (*uji*) (Dirar 1993). In Kigezi, Uganda, millet is germinated, ground and allowed to undergo a slight

fermentation before it is given as gruel to children (Dirar, 1993). In West Africa the sour porridges *ogi* and *koko* are mostly used as weaning foods (Dirar, 1993). The development of an inexpensive baby food recipe that poor families can afford to buy is becoming more and more necessary, hence the need to assess the traditional foods of a country, and to use the information as a launching pad for such an endeavour, and especially those based on fermentation of cereals and legumes.

Mbugua, (1981) showed that fermented (*uji*) cooks more easily, has a lower viscosity and can therefore be made with higher levels of flour solids while maintaining an acceptable consistency. Mensah et al., 1991 observed that fermented foods are a good source of nutrients. Losses however occur during milling and sieving (Wood, 1985). However, Hamad and Fields (1979) reported increases in the protein quality and relative nutritive value of millet by 14% and maize by 13% (determined using Tetrahymena pyriformis). Available lysine also increased significantly.

2.4 TRADITIONAL WET-MILLING PROCESS

Traditionally, wet milling of cereals by the Ameru community is done by the stone quern method. The cereals are usually grounded between two stones and suspended in water. Sieving is carried out to remove the ungrounded maize, which is reground again. Milling of cereals into small range of particles greatly increases the range of culinary preparations that is possible and in particular the ability to absorb water is much enhanced (Uhling and Bhat, 1979). Milled maize like other milled cereals is prepared either as a thin gruel, or a thick porridge or a doughy paste. When the particle size of cereals is small, it is easier to fortify them with a number of vitamins and protein. The blending and fortification can be done during milling.

It is true that slurry produced by traditional mills is much finer and smoother than with the modern mills. Badi and Monawar, (1987) stated for example, that *murhaka ajin,* a wet-milled fermented sorghum dough in Sudan gave a 'very fine dough' compared to that given by modern mills. Burgess (1962) mentioned that the traditional method of grinding between two flat stones was laborious but produced a 'very fine flour'. Mbugua, (1985) actually confirmed this with direct research results on the preparation of the Kenyan *uji.* He found that wet-milled dough proved superior to dry-milled flour in the production of a fine and smooth *uji,* and the slurries prepared from the wet-milled dough cooked more easily.

Badi and Moanwar (1987) reported that grinding by the stone quern led to an increase in the iron content of the flour, probably due to contamination from the ferric iron present in

the stone. Wet milling affect the chemical composition and nutrient content of the product. John and Muller (1973) showed that with the West Africa's traditional milling, nutrient content decreased evenly with the decreasing extraction, while with the modern milling nutrient loss was far greater.

2.5 LACTIC ACID FERMENTATION.

2.5.1 The Lactic Acid Bacteria [LAB]

The lactic acid bacteria (LAB) frequently termed as ''the Lactics'', constitute a diverse group of microorganisms associated with plants [cabbage, corn, barley, mashes, kales, and silage], meat and dairy [Jay, 1986]. The lactics are also important commercially in the processing of dairy products, meats, alcoholic beverages, and vegetables. These products include sausages, cured hams, wines, beer, fortified spirits, pickles, and sauerkraut [Schillinger and Lucke, 1987]. The lactics are highly priced by the food and dairy industries for their ability to produce aromatic and flavour enhancing compounds [Sharpe, 1979].

The lactics have been characterized primarily by their ability to form various isomers of lactic acid from the fermentation of glucose. Lactic acid bacteria elaborate different isomers of lactic acid based on their optical rotatory properties. If the rotation is to the right, it is termed Dextrorotary [D]; if to the left, it is termed Levorotary [L], or, if there is a mixture of both D and L, it is termed racemic [DL], [Jay, 1986; Sharpe, 1979]. Although consisting of a number of diverse genera, the Lactic Acid Bacteria are grouped

as either homofermenters or heterofermenters depending on the proportion of lactic acid elaborated. Those elaborating more than 50% acidity in form of lactic acid are termed homofermentative and those producing less heterofermentative.

The homofermenters produce lactic acid as a major product of fermentation of glucose. The heterofermenters produce a number of products besides lactic acid, including carbon dioxide, acetic acid, and ethanol from the fermentation of glucose. The homofermenters possess the enzyme aldolase and are able to ferment glucose directly to lactic acid unlike the heterofermenters. The heterofermenters use the alternate pentose monophosphate pathway, converting six carbon sugars [hexoses] to five carbon sugars [pentoses] by the enzyme phosphoketolase, thus producing in the process both aldehyde and diacetyl which have highly desirable aromatic and flavour-enhancing substances. The heterofermenters are often used in dairy industry because of these flavour-enhancing substances [Jay, 1986; Sharpe& Fryer, 1965].

The homofermentative Lactics include the genera *Streptococcus* and *Pediococcus*. *Streptococcus* produces L[+] lactate while *Pediococcus* produces DL lactate. The heterofermentative Lactics consist of the genus *Leuconostoc* and a subgroup of the genus *Lactobacillus*, the Betabacteria. *Leuconostocs* produce D[-] lactate while Betabacteria produce DL lactate [Schillinger & Lucke, 1987; Jay, 1986; Sharpe, 1979].

2.5.2 Classification of Lactic Acid Bacteria.

The LAB consists of a number of genera namely: *Lactobacillus, Leuconostoc, Pediococcus, Carnobacterium, Aerococcus, Weisella, Tetragenococcus* and *Lactococcus* [Lactic *Streptococci*]. In general, the LAB may be characterized as Gram-positive, aerobic to facultatively anaerobic, asporogenous rods and cocci which are oxidase, catalase, and benzidine negative, lack cytochromes, do not reduce nitrates to nitrite, are gelatinase negative, and are unable to utilize lactate [Rogosa, 1974; MacFaddin, 1980; Schillinger & Lucke, 1987, and Jay 1986]. Other methods of differentiation are increasingly being used namely mol% G+C of DNA, electrophoretic analysis of cellular proteins, ribosomal DNA sequencing and DNA-DNA hybridization of genomic DNA among others.

2.5.2.1 Lactobacillus.

The genus *Lactobacillus* may be subdivided into three groups: Betabacteria, Streptobacteria, and Thermobacteria [Schillinger & Lucke, 1987; Jay, 1986]. The thermobacteria are capable of growth at 45^0c or higher but are unable to grow at 15^0c. The streptobacteria grow at 15^0c, but most do not grow at 45^0c. The betabacteria also grow at 15^0c. The streptobacteria are able to ferment ribose and other pentoses and form carbon dioxide from gluconate, but not from glucose [Schillinger & Lucke, 1987].

The betabacteria are considered heterofermentative because they produce carbon dioxide from the fermentation of glucose. In contrast to both the streptobacteria and thermobacteria, most betabacteria form carbon dioxide from glucose and hydrolyse

arginine with the formation of ammonia. Almost all the streptobacteria and betabacteria ferment ribose, whereas none of the thermobacteria does. Both the streptobacteria and thermobacteria are homofermentative [Schillinger & Lucke, 1987].

2.5.2.2 Leuconostoc.

The Leuconostoc are Gram-positive cocci that occur in pairs or chains. Their metabolism is heterofermentative, resulting in the formation of carbon dioxide from the fermentation of glucose. The leuconostocs are unable to hydrolyze arginine [Schillinger & Lucke, 1987; Harrigan & MacCance, 1976]. The leuconostocs require a more alkaline environment, of pH≥ 4.5, than the lactobacillus and pediococcus, which are more acid tolerant and survive a pH of 3.6 to 4.0 [Harrigan & MacCance, 1976; Garvie, 1986]. Although MRS agar is suitable for the leuconostocs, Yeast Glucose Phosphate Peptone Broth is recommended [Garvie, 1967].

MRS Broth, with the addition of 0.05% cysteine and a reduced atmosphere, enhances the growth of both *Leuconostoc cremoris* and *leuconostoc oenos* [Garvie, 1986]. Both *L. mesenteroides* ssp. *mesenteroides* and *L. mesenteroides* ssp. *dextranicum* may be recognized when grown on MRS containing 5% sucrose because they produce mucoid capsules [Garvie, 1967]. *L. paramesenteroides* is biochemically similar to *L. mesenteroides* and *L. dextranicum* but does not form a capsule. *L. mesenteroides* also differs from the two species in that it fails to hydrolyze gluconate [Garvie, 1986; MacFaddin, 1980]. *L. cremoris* is one of the few leuconostocs capable of producing

diacetyl from citrate. This process is important in the dairy industry because diacetyl is an important dairy flavour [Garvie, 1986; MacFaddin, 1980].

L.cremoris is unable to ferment trehalose and sucrose but ferments galactose [Garvie, 1984]. *L. cremoris* also produces carbon dioxide from citrate, and is unable to grow in 2% NaCl, or hydrolyze arginine [Harrigan and MacCance, 1976]. *Leuconostoc oenos* is important in the wine process and ferments trehalose but not sucrose [Garvie, 1984, 1986, and 1967].

2.5.2.3 Lactococcus

The lactococci are commonly found in the dairy products and cereals, and may be selectively isolated on Elliker's lactic agar, M17, Arginine Tetrazolium agar or Alsan Medium [MacFaddin, 1980; Elliker et al., 1956]. The Lactococci may be distinguished by their temperature tolerance, presence of Group N Antigen, action in litmus milk, production of carbon dioxide, formation of diacetyl from citrate, fermentation pattern of a variety of carbohydrates as well as their salt tolerance [Harrigan & MacCance, 1976; Elliker et al., 1956]. Both *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *diacetylactis* may be differentiated from other lactococci by their hydrolysis of arginine and by citrate utilization [Harrigan & MacCance, 1976].

Many of the Lactococci produce the Lancefield Group N antigen, grow at 10^0c but not at 45^0c, and grow in broth with 4% NaCL [except *L. lactis* ssp. *cremoris*]. The lactococci

differ with the streptococci by growing at 10^0c whereas the streptococci do not. The lactococci differ from the enterococci by not growing at 45^0c [Schillinger, 1987]. The lactococci may be differentiated by their fermentation patterns for galactose, lactose, maltose, melibiose, melezitose, raffinose, ribose, and arginine hydrolysis [Harringhan & MacCance, 1976]. *Lactococcus lactis* ssp. *lactis* and *lactococcus lactis* ssp. *diacetylactis* differ from other lactococci by fermenting maltose; however, only *lactococcus lactis* ssp. *diacetylactis* forms diacetyl from citrate [Huggins, 1984].

2.5.2.4 Pediococcus

The p*ediococcus*, unlike the other Lactics, have a tetrad arrangement. The pediococci grow on MRS media, and their growth may be enhanced as for the leuconostocs by culturing them under a reduced atmospheric condition [Garvie, 1984 and 1986]. The pediococci are particularly useful as starter cultures for various types of sausages, [Garvie, 1986]. However, in the pickle, wine and beer industries they are unwanted contaminants when they produce off flavours [Jay, 1986; Rogosa & Sharpe, 1959; Harrigan & MacCance, 1976].

2.5.3 Media and biochemical tests

The cultivation of the LAB may be accomplished using enrichment broths and selective or nonselective media, depending on a need to isolate either a particular genus from a mixture of microorganisms or to maintain isolates in culture [Difco 1995]. There are number of sources of information on culture media for culture and isolation of lactics, much of which has been reviewed by Holzapfel, 1992. When processing samples for LAB, they should not be refrozen prior to plating. Chilled 0.1% peptone water should be used for dilution and blender cups should be chilled prior to blending. Phosphate buffer diluents result in lower recovery rates [APHA, 1992].

Most LAB are acid tolerant but have an optimum growth pH: Therefore, a common approach to selective isolation is to adjust the pH for a particular genus or group [Garvie, 1984; APHA, 1992]. A pH of less than 4.5 may be used to differentiate *lactobacillus* and *pediococcus* from *leuconostoc*: The latter requires a pH of 4.5 or greater [Harrigan & MacCance, 1976; Garvie, 1984; and APHA, 1992]. Both *Lactobacillus* and *Pediococus* will grow at pH of less than 4.5 [Harrigan & MacCance, 1976; Difco 1995; Garvie, 1984; and Holzapfel, 1992].

A common approach to LAB isolation is to use a general purpose medium such as APT Agar, Rogosa Agar, or MRS Agar, and then to employ a homofermentative-heterofermentative differential medium [HHD] or a modified MRS Lactobacillus heteroferm Screen Broth [at pH 4.5] for differentiating the heterofermenters from the homofermenters [Garvie, 1984; Holzapfel, 1992;].

The use of MRS broth or agar has gained acceptance as the all-purpose media because of its ability to support a variety of LAB. The MRS name originates from the formula of deMan, Rogosa, and Sharpe (1960) [Harrigan & MacCance, 1976; Difco, 1995; Garvie, 1984; and Holzapfel, 1992]. MRS broth may be used as a general culture medium and also as a basal medium for performing tolerance tests such as temperature, pH, alcohol, salt, and Teepol concentration. Other biochemical tests based on MRS include arginine hydrolysis and acetoin tests [Schillinger and Lucke, 1987; Sharpe & Fryer, 1965; Harrigan & MacCance, 1976]. The glucose normally found in MRS broth may be replaced with 4% sodium gluconate [Modified MRS] for the detection of gas from the fermentation of gluconate [Schillinger and Lucke, 1987; Sharpe & Fryer, 1965; Harrigan & MacCance, 1976].

The media used for isolating and enumerating lactobacilli depend on the type of sample, the specificity required, and to some extent, the characteristics of the particular *lactobacillus* culture. For most lactobacilli, various requirements for essential nutrients are met when the medium contains fermentable carbohydrates, peptone, meat extract, and yeast extract. Supplementation with tomato juice, manganese, acetate and oleic acid esters, especially Tween 80, is stimulatory or essential for most species. A widely used selection medium that includes these compounds is MRS, as mentioned previously (de Man et al, 1960).

Boehringer Mannheim Biochemicals has developed a commercial kit for the detection of the isomeric forms of lactic acid. MRS broth may also be used for the detection of the

type of lactic acid produced during glucose fermentation [Schillinger and Lucke, 1987; Sharpe, 1962; Sharpe & Fryer, 1965; Harrigan & MacCance, 1976].

The traditional method for determining gas from glucose fermentation by heterofermenters has been by either of the two methods: (1) The method of Gibson and Malek [Sharpe & Fryer, 1965; Harrigan & MacCance, 1976; Garvie, 1984] using a semisolid tomato juice skim milk agar, or (2) Using MRS glucose broth with a vaspar overlay [Schillinger and Lucke, 1987; Jay, 1986; Sharpe, 1962; Harrigan & MacCance, 1976; Garvie, 1984]. The use of Durham tubes in MRS broth with 5% glucose is a more practical method for the detection of gas formation [Schillinger and Lucke, 1987; Jay, 1986; Sharpe, 1962; Harrigan & MacCance, 1976; Garvie, 1984].

Arginine hydrolysis is used as a test to separate the heterofermentative LAB [Betabacteria] from arginine negative *Leuconostocs* and other arginine negative Streptobacteria [Harrigan & MacCance]. The test may be performed with MRS base without glucose or meat extract. In addition, the 0.2% sodium citrate normally found as an ingredient in MRS broth must be replaced by ammonium citrate [Schillinger and Lucke, 1987; Harrigan & MacCance, 1976]. Arginine hydrolysis is detected by the addition of Nessler's reagent to 1ml of an arginine broth culture after 2 to 7 days of incubation. This test is used primarily for Streptococcus ssp. or lactobacillus ssp. and detects the presence of free ammonia, with a positive test resulting in an orange to brown colour [Schillinger and Lucke, 1987; Harrigan & MacCance, 1976; Garvie, 1984].

2.5.4 Isolation and identification of Lactic acid bacteria.

Traditional fermented foods present a specialized environment which is very selective to a number of bacteria strains. The resulting strains can be vital in commercial applications, where their functions are optimized for superior quality products. Also, the unique strains can be used for genetic improvement of the existing commercial strains [Gasson and de Vos, 1994]. Isolation of microbes from the lactic fermented foods is always an important step in acquiring cultures for scientific and commercial use. Isolation entails obtaining of either mixed or pure cultures followed by assessment to determine which of the isolated strain(s) carries out the desired reaction to produce a particular product quality [Wood and Holzapfel 1995].

Recent developments in biochemical identification include the API Rapid CH-50: It has been approved for the identification of the lactobacilli by the Compendium of Microbiological Methods [APHA, 1992]. Today, many clinical and industrial laboratories in the U.S. use the kit for the identification of LAB [APHA, 1992, Schillinger et al., 1989]. Biolog Inc. has developed a 96 well microtiter plate method for identifying Bifidobacterium, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, and Weissella. The system is intended to identify 57 LAB species. Each microtiter plate consists of 95 carbon substrate utilization tests [Schillinger et al., 1989 and APHA, 1992].

Ribotyping provides DNA fragments by enzymes; the subsequent fragments are separated by electrophoresis and compared to known sequences of ribosomal RNA.

Ribotyping is a method that is both accurate and applicable for the identification of all the strains of Lactobacillus sp. and is used in epidemiology and taxonomy [Rodtong and Tannock, 1993].

The use of antibiotic susceptibility patterns might be helpful for the identification of the LAB, particularly with the greater incidence in the number of cases reported of involvement of the lactic acid bacteria in clinical infections. The use of Vancomycin discs has been recommended as an aid in the identification of Gram-positive cocci in foods [Aquirre, and Collins, 1993; Green et al., 1990].

Facklam et al., (1995), proposed a disk screening method to presumptively identify clinical strains of Gram-positive, catalase negative cocci using leucine-aminopeptidase [LAPase], pyrrolidonylarylamidase [PYRase], and vancomycin resistance. The Enterococci, Pediococci, Leuconostocs, and some Lactobacillus sp. are resistant to [VRB], Vancomycin 30μg [Facklam et al., 1989]. Many enterococci of medical importance are Vancomysin resistant, PYRase (+), and LAPase (+), while the pediococci are Vancomycin susceptible, LAP (+), and PYRase (-). The leuconostoc are Vancomycin resistant but lack Pyrase and LAPase activity.

The Viridans Streptococcci, Streptococcus bovis strains, Leuconostoc, and the Pediococci are PRYase negative. LAPase is also useful because all Aerococci, Globicatellae, Helcococci, and Leuconostocs are negative. Vancomycin resistance, PYRase (+), and LAPase (+) presumptively identifies an isolate as an Enterococcus, while Vanncomycin resistance, PYRase(-), and LAPase (-) indicates a Leuconostoc, whereas Vancomycin

resistant and LAPase (+) indicates a presumptive Pediococcus [Facklam et al., 1989]. Both convectional biochemical methods and nucleic acid probes for identification are needed when vancomycin-resistant Enterococci are suspected.

CHAPTER 3: EXPERIMANTAL DESIGNS AND METHODOLOGY

3.1 Experimental designs

A preliminary study was carried out in the study region [Meru North] to obtain the specific and relevant information on the traditional preparation processes in the field with the help of the local women. Specific information with regard to the type of raw materials used, fermentation equipment, fermentation time and conditions were acquired with the help of a questionnaire [Appendix 1], and personal examination. The raw materials were supplied to a hundred randomly selected local women experienced in preparing *kirario* in the study regions. They were instructed to prepare the porridge in the traditional way. These women were asked to measure out the required quantities of the raw ingredients using local weight measures and prepare the fermented product. The local weight used were in terms of measuring containers of 1or 2 kilograms's recycled plastic containers.

With the help of data obtained from the traditional process by the women, *Kirario* traditional processing flow diagram was constructed as shown in figure 3.2. This process flow diagram subsequently provided the experimental design, on the basis of which *Kirario* microbiological properties were evaluated and characterised.

The general method followed in the preparation of *Kirario* was used to document this process in form of a flow diagram. Immediately after the women were finished with the preparation of *Kirario*, ten randomly selected samples were immediately taken and

transported in an icebox to the laboratory for analysis. Microbiological enumeration and characterization of *Kirario* was determined at the key operational steps in the process. Based on the findings of the traditional fermentation conditions, a laboratory- controlled simulation of *Kirario* was produced and the product subjected to microbial analyses.

3.2 Materials

3.2.1 Fermented porridge samples

The traditional fermented porridge samples were obtained from the study region, Meru North specifically villages in Tigania West (Uringu and Mbeu locations) and (Athwana and Kimirii locations) Tigania East. The raw materials [green maize and millet] used in preparation of the porridge were bought at a local market in the study region. These materials were given to the randomly selected local women who were instructed to prepare the fermented porridge using the traditional practices. Households with women experienced in the art of *Kirario* preparation were selected and the numbers randomised by the calculator random method. The samples of the fermented porridge were collected in sterile sample containers, cooled to 4-6^{0}c and transported to the laboratory in an insulated cool box containing freeze packs. The samples were analysed within 8 hours of collection, and maintained at 4-6^{0}c during the period between collection and analysis. The raw materials used for the laboratory-simulated product were also obtained from the study region and maintained at 4-6^{0}c prior to the analysis.

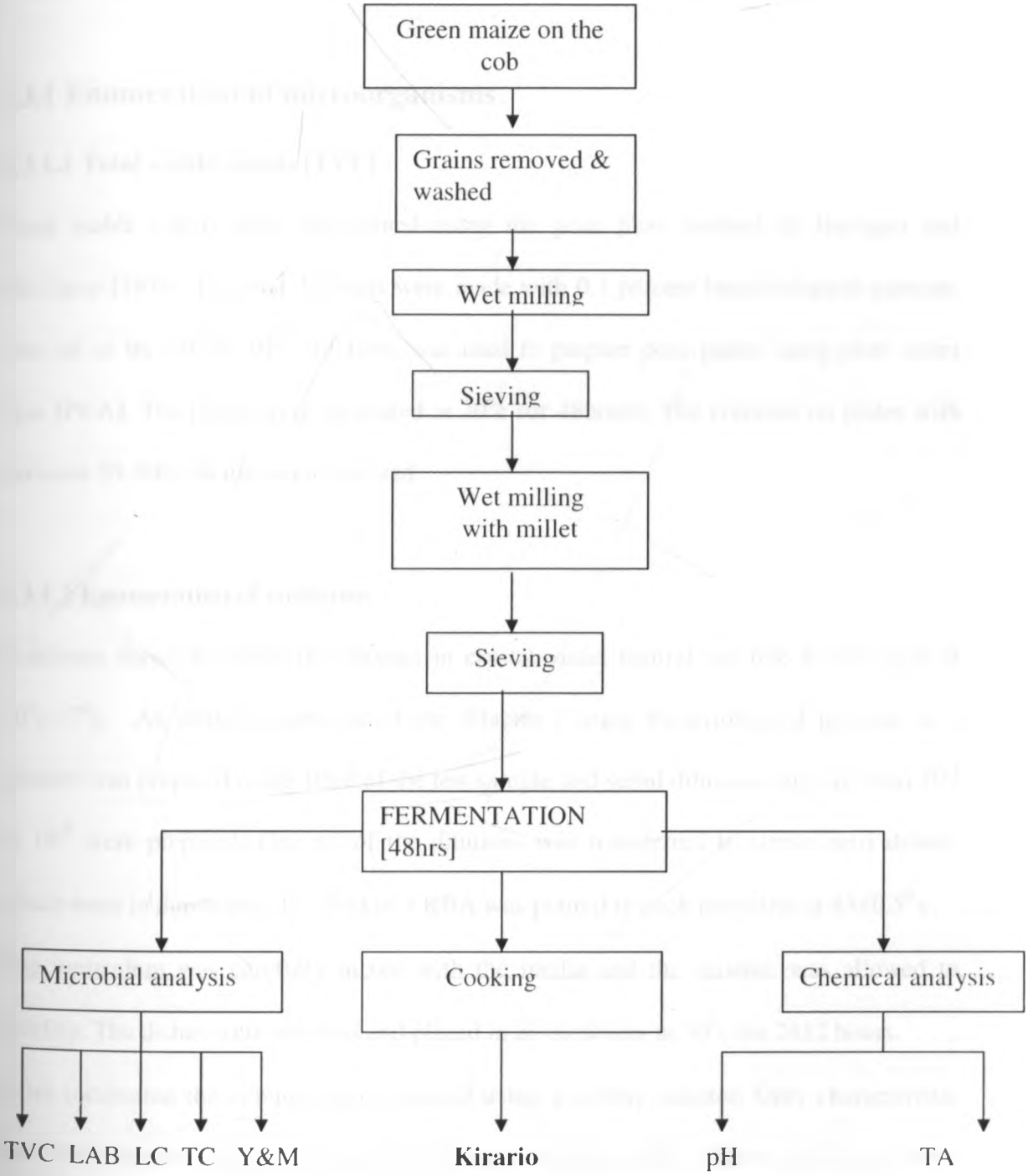
3.2.2 CHEMICALS:

Analytical grade laboratory chemicals and microbiological media used were obtained from MERCK, Germany.

3.2.3 API® MICROBIOLOGICAL IDENTIFICATION KIT

API® microbiological identification kit for lactic acid bacteria was bought from bioMèrieux, France through Hass Scientific and Medical Supplies Limited, Nairobi.

Figure 3.2 Flow diagram of laboratory production of *Kirario*



Key: TVC: Total viable counts
LAB: Lactic acid bacteria
LC: Lactic-acid cocci
TC: Total coliform
Y&M Yeast and mould
TA: Titratable acidity

3.3 ANALYTICAL METHODS

3.3.1 Enumeration of microorganisms

3.3.1.1 Total viable counts [TVC]

Total viable counts were determined using the pour plate method of Harrigan and McCance [1976]. Decimal dilutions were made with 0.1 percent bacteriological peptone. One ml of the 10^{-8} to 10^{-14} dilutions was used to prepare pour plates using plate count agar [PCA]. The plates were incubated at 30^{0}c for 48hours. The colonies on plates with between 30-300 colonies were counted.

3.3.1.2 Enumeration of coliforms

Coliforms form characteristic colonies in crystal violet neutral red bile lactose agar at 30^{0}c-37^{0}c. An initial suspension of the diluents (using bacteriological peptone as a diluent) was prepared using 10ml of the test sample and serial dilutions ranging from 10^{-4} to 10^{-8} were prepared. One ml of the dilutions was transferred to sterile petri dishes, which were in duplicates. 12-15ml of VRBA was poured in each petri dish at 45 ± 0.5^{0}c. The innoculum was carefully mixed with the media and the mixture was allowed to solidify. The dishes were inverted and placed in an incubator at 30^{0}c for 24±2 hours. After incubation the colonies were counted using a colony counter. Only characteristic dark red colonies having a diameter of 0.5mm or greater after 24hour incubation were counted. Counts were made in plates containing not more than 150 colonies.

3.3.1.3 Enumeration of Lactic acid bacteria [LAB]

The LAB count was carried out according to the method outlined by APHA [1976]. Pour plates were prepared from serial decimal dilutions [10^{-4} to 10^{-14}] of the sample, using MRS {deMan Rogosa Sharpe} agar [Merck, Germany]. The media was prepared using distilled water and sterilized by autoclaving for 15 minutes at 118^{0}c according to the manufacturer's directions. One millilitre of the fermented porridge was serially diluted in 9 ml bacteriological peptone and the dilutions were asceptically plated on MRS agar plates. The plates were incubated anaerobically at 30^{0}c for 3 days in GasPak® Oxoid jars. After incubation the colonies that developed were counted. Gram stains of cells from individual colonies were prepared. Individual discrete colonies were examined microscopically and tested for catalase reaction. Gram-positive, and the colonies which reacted catalase negative as cocci or rods were considered as LAB.

3.3.1.4 Enumeration of yeasts and moulds

Enumeration of yeasts and moulds was done using the acidified method outlined by APHA [1976]. Serial dilutions of the sample [10^{-1} to 10^{-10}] were prepared in 0.1% sterile bacteriological peptone water. One ml of the 10^{-4} to 10^{-10} dilutions was used in pour plates with potato dextrose agar, acidified with 10% tartaric acid to pH 3.5. The plates were incubated at 30^{0}c for 3-5 days. Colony counting was done on plates with between 30-300 colonies, first after 3 days and then again after 5 days. The yeast and mould count was reported per ml of sample.

3.3.1.5 Enumeration of lactococci

One ml of the 10^{-4} to 10^{-14} dilutions was used to prepare pour plates using the M17 agar. The plates were incubated at 30^{0}c for 3 days. The colonies on plates with between 30 to 300 colonies were then counted.

3.3.2 Isolation of Lactic acid bacteria

3.3.2.1 Isolation of lactobacilli

The methods described by Harrigan and McCance [1976] were used for isolation of LAB. Characteristic colonies were selected from the MRS agar media and plates with the highest dilutions. These were Gram stained and examined microscopically. Gram-positive rods were sub-cultured into litmus milk and incubated at 30^{0}c for 3 days, after which the cultures were re-examined for morphology, Gram stain and catalase reaction. Gram-positive, catalase negative rods were tentatively considered as lactobacilli, subject to further genus and species identification. Pure cultures for further identification were then obtained from the litmus milk by streaking onto MRS agar. The streak plates were incubated anaerobically in GasPak® Oxoid jars at 30^{0}c for 3 days. The isolated lactobacilli from streak plates were transferred to screw-capped bottles of yeast extract glucose chalk litmus milk, incubated at 30^{0}c for 24 hours and then stored at 4-6^{0}c.

3.3.2.2 Isolation of lactic acid cocci

Pour plates of the 10^{-7} to 10^{-14} dilutions were prepared using the M17 agar. Discrete colonies were selected for microscopic examination and Gram staining after incubating the plates for 3 days at 30^{0}c. Gram-positive cocci were then sub-cultured into litmus milk and incubated at 30^{0}c for 3 days, after which the cultures were re-examined for morphology, Gram stain and catalase reaction. Gram-positive, and catalase negative cocci were tentatively considered presumptively as lactic acid cocci, subject to further genus and species identification. Pure cultures for further identification were then obtained from the litmus milk and streaked onto M17 agar. The streak plates were then incubated at 30^{0}c for 3 days. The isolated cocci from streak plates were transferred to screw-capped bottles of yeast extract glucose chalk litmus milk, incubated at 30^{0}c for 24 hours and then stored at 4-6^{0}c.

3.3.3 Primary classification of LAB isolates

Primary classification of LAB was based on the results of Gram staining, microscopic cell morphology and catalase reaction. Gram-positive, catalase-negative rods and cocci were subjected to further biochemical tests. The methods described by Harrigan and MacCance [1976] were used. Ability to produce carbon dioxide from glucose in Gibson's semi-solid medium was used to indicate whether the organism is homofermentative or heterofermentative. MRS broth was used as the basal medium for further biochemical tests to identify the species/subspecies of the genus *Lactobacillus*. The following tests were carried out: growth at 10^{0}C, 15^{0}C and 45^{0}C, and production of ammonia from arginine in MRS broth containing 2% glucose.

3.3.3.1 Production of ammonia from arginine

Arginine hydrolysis was used as a test to separate the heterofermentative LAB [Betabacteria] from arginine negative *Leuconostocs* and other arginine negative Streptobacteria. MRS broth containing 0.3% arginine monohydrochloride and 2% glucose was used as a medium to test arginine production. The medium was sterilised and dispensed into test tubes in 5ml amounts. A loopful of the young culture in yeast extract glucose chalk litmus milk was asceptically inoculated into the test tubes and incubated at 30^0C for 2-7 days. Arginine hydrolysis was detected by mixing one ml of the culture with one ml of Nessler's reagent in test tubes. A positive reaction was indicated by the development of an orange-brown colour, suggesting the presence of free ammonia. A negative test result was indicated by a pale yellow colour or no colour change suggesting the absence of ammonia.

3.3.3.2 Production of Carbondioxide from glucose

The method for determining gas production from glucose fermentation by heterofermenters as outlined by Gibson and Malek was used [Harringan and MacCance, 1976]. Carbon dioxide production was tested using Gibson's semi-solid tomato juice medium. This medium consisted of 4 parts reconstituted skim milk, 1 part nutrient agar with the addition of 0.25% yeast extract, 5% glucose and 10% tomato juice. The tomato juice provides manganese ions necessary for the growth of LAB. The medium was distributed into test tubes to a depth of 5-6 cm, steam-sterilized and then cooled to 45^0C in a water bath. 0.5 ml of the 24-hour old LAB culture in yeast extract glucose chalk litmus milk was asceptically inoculated into test tubes and mixed gently. The test tubes

were then cooled in tap water and molten nutrient agar at 50^0C was then poured on top of the medium to give a layer 2-3 cm deep above the medium surface. The inoculated tubes were incubated at 30^0C for 3 days. The semi-solid medium and the agar plug trapped any carbon dioxide gas produced and assisted visual detection of gas production through gas bubbles and cracking of the medium.

3.3.3.3 Growth at 10^0C, 15^0 C and 45^0C

MRS broth was used as the basal medium for these tests. MRS broth has gained acceptance as the all-purpose media because of its ability to support a variety of LAB. The MRS broth may be used as a general culture medium and also as a basal medium for performing tolerance tests such as temperature, pH, alcohol, salt, and Teepol concentration {Sharpe and Fryer, 1965; Harrigan and MacCance, 1976}.

The MRS medium was dispensed into tubes in 5ml amounts and sterilised by autoclaving at 121^0C for 15 minutes. A loopful of the young culture in yeast extract glucose chalk litmus milk was inoculated into each of the 3 set tubes of sterile medium under aseptic conditions. One set was incubated at 10^0C, another at 15^0C and the last at 45^oC for 2-7 days. Growth was indicated by the turbidity of the medium.

3.3.3.4 Activity in litmus milk

The activity of the cultures in litmus milk was investigated by inoculating a loopful of 24-hour old isolated cultures in test tubes of steam-sterilized litmus milk. The test tubes were incubated at 30°C for 2-7 days and examined for the following changes in the litmus milk:

- Acid production shown by a colour change in the colour of the litmus from purple to pink and clotting of the milk [acid clot].
- Reduction of the litmus and loss of colour.
- Coagulation of the milk as a result of proteolytic enzyme activity affecting casein, and the litmus colour remaining light purple [sweet clot].
- Hydrolysis of casein as a result of proteolytic enzyme activity causing clearing and loss of opacity in the milk [peptonisation].
- Utilization of citrate in the milk medium resulting in the production of an alkaline medium shown by colour change to a deep purple colour.

3.3.4 Identification of LAB isolates using API ® identification kit

3.3.4.1 Principle of the identification method

API 50 CH is a standardised system, consisting 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. API 50 CH is used in conjunction with API 50 CHL Medium for the identification of *Lactobacilli* and related genera and with API 50 CHB/E Medium for the identification of *Bacillus* and related genera, of the families *Enterobacteriaceae* and *Vibrionaceae*. The API 50 CH strip consists of 50 microtubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives {heterosides, polyalcohols, uronic acids].

The fermentation tests are inoculated with API 50 CHL Medium or API 50 CHB/E Medium, which rehydrates the substrate. During incubation, fermentation is revealed by a colour change in the tube, caused by the anaerobic production of acid and detected by the pH indicator {bromocresol purple indicator} present in the chosen medium. A standardised suspension of the test organism {equivalent to 600 x 10^6 cells/ml} is prepared in the API 50 CHL medium and each tube of the strip is then inoculated. During incubation, carbohydrates are metabolised with formation acids, which results in a decrease in pH observed by a colour change of the bromocresol purple indicator from purple to yellow.

The series of positive and negative results makes up the biochemical profile of the test organism and is used for its identification using the identification software. The first tube,

which does not contain any active ingredient, is used as a negative control. It is worth to note that the API 50 CH strip may be used to test two other pathways i.e. oxidation which is revealed by a colour change in the cupule, caused by the aerobic production of acid and detected by the pH indicator present in the medium used, and assimilation which is revealed by growth of the organism in the cupule when the substrate is used as the only available source of carbon. In this case, the choice of the medium to be used for inoculation, in the strips will depend on the metabolism and nutritional requirements of the microbial group to be tested.

3.3.4.2 Method for LAB identification

The API 50 CH strips were used to identify LAB. The purity of the isolated LAB was checked again by streak plating onto MRS agar and ensuring only single type colonies occurred on the agar plates. A heavy bacterial suspension was prepared by transferring several bacteria colonies from the agar plate to an ampoule containing 2 ml of sterile distilled water, using a sterile swab. Using asceptic techniques, a pre-determined number of drops of the suspension were transferred to an ampoule of 5 ml sterile distilled water in order to obtain a suspension with turbidity equivalent to McFarland Standard no. 2.

The McFarland Standard is a series of standards of barium sulphate suspensions of known different opacities, allowing the estimation of the density of bacterial suspensions. The density of the bacterial suspension is compared to that of a suspension of known opacity contained in an ampoule of the same diameter. The McFarland Standard no. 2 is composed of 9.60×10^{-5} mol/l of barium sulphate. It has a theoretical optical density of

0.50 at 550nm, which is equivalent to a bacterial concentration of 600 x 10^6 cells/ml. The number of drops added to the 5-ml ampoule was noted and twice this number of drops was used to inoculate a 10ml ampoule of API 50 CHL medium.

The medium composition for API 50 CHL is shown in Table 3.3.4.

Table 3.3.4: Composition of API 50 CHL Medium

Component	Quantity
Polypeptone	10g
Yeast extract	5g
Tween 80	1ml
Dipotassium phosphate	2g
Sodium acetate.$3H_2o$	5g
Diammonium citrate	2g
Magnesium sulphate.$7H_2o$	0.2g
Magnesium sulphate.$4H_2o$	0.05g
Bromocresol purple	0.17g
Distilled water	to make 1 litre.

Source: bioMèrieux {2001b}

The medium was mixed gently with a pipette then inoculated into the tubes, which were overlaid with mineral oil to ensure anaerobic conditions. The strips were incubated at 30^0c for 48 hours, after which the results were read. A positive test corresponded to changing of the bromocresol purple indicator, in the medium to yellow due to

acidification on growth. In the case of esculin test, a positive test was indicated by a colour change from purple to black.

3.3.4.3 Identification of lactic acid bacteria.

The biochemical profiles of the microorganisms were recorded on a result sheet and entered into a computer for identification using the identification software with database {v5.0}, APILAB Plus® software (bioMèrieux, France). The APILAB Plus® software database enables the identification of various groups or taxa of microorganisms. The identification of the observed profile is based on calculation of how closely the profile corresponds to the taxon, relative to all other taxa in the database [percentage of identification] and how closely the profile corresponds to the most typical set of reactions for each taxon [T-index]. The T-index is a value ranging between 0 and1 and is inversely proportional to the number of atypical tests.

The taxa are sorted by decreasing values of the percentages of identification [%ID]. For the first four taxa, the ratio of their %ID is calculated to that of the following taxon. The taxon with the highest ratio is selected for identification, as well as the taxa situated before it in the classification, if any. The T-indices are calculated using modal frequencies. If only one taxon is chosen and its % ID is > 80%, it is proposed for identification. If several taxa are chosen and if the sum of the % ID is > 80%, they are proposed for identification, with a comment based on the value of the sum of the % ID and the average of the T-indices.

3.3.5 Chemical analysis

3.3.5.1 Determination of pH

The pH was measured using a Pye Unicam pH meter model 290 Mk 2.

3.3.5.2 Determination of titratable acidity

Titratable acidity was determined according to the methods of AOAC [1984]. The sample [10ml] was pipetted into a conical flask and diluted with 10 ml of distilled water. Drops of phenolphthalein indicator were added and the sample was titrated with 0.1 N NaOH to a persistent pink colour. The titratable acidity was calculated and expressed as % lactic acid equivalent, using the equation:

$$\% \text{ lactic acid equivalent} = \frac{T \times 0.1 \times 9}{\text{Sample volume}} = \frac{0.9T}{25}$$

Where T = titre in ml of 0.1N NaOH

3.4 Data processing

A randomised block design [RBD] was used for data collection, and was analysed using Genstat Statistical Package. The results were presented using the Analysis of Variance [ANOVA]. There were three replicates each for the microbiological counts determinations. Means and standard errors of means [S.E] were compared by ANOVA and tested by least significant difference (Lsd) at 5% level or by unpaired comparison and t-test at 5% significant level.

Descriptive statistics [means and standard deviations] were used to summarize the data on microbial numbers, pH and titratable acidity scores. Data on microbial counts were first transformed to logarithmic [$\log_{10}$] values before computing the mean $\log_{10}$ counts and standard deviations. The independent samples t-test was used to determine whether or not a significant difference existed between the traditional and laboratory- produced *Kirario.*

CHAPTER 4: RESULTS

4.1 Traditional production of *Kirario* in Meru North District

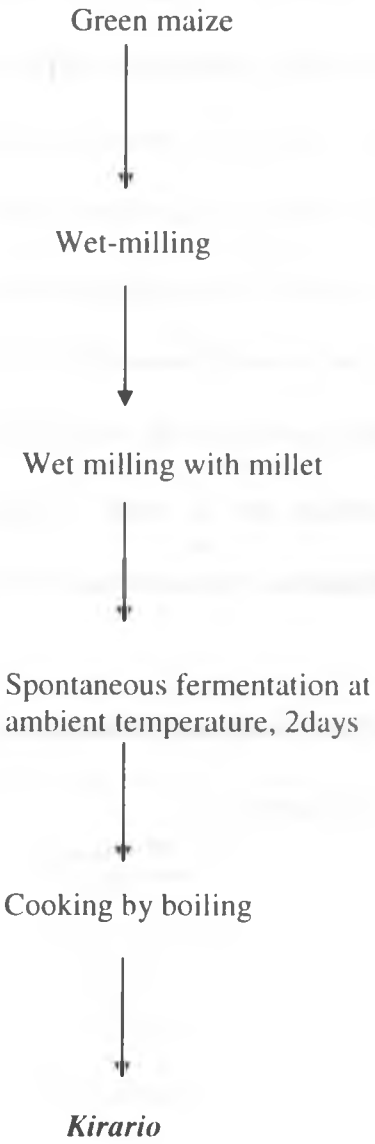
The traditional art of *Kirario* was documented based on information obtained during the field survey and personal observation of the practices through interviews. The flow diagram of traditional processing of *Kirario* is shown in Figure 4.1. The raw materials [green maize and millet] are usually bought from the local markets or harvested in the gardens. Traditionally, older experienced women prepared *Kirario* but nowadays wet milling is mostly the work of young unmarried girls, although women of any age or social status commonly participate depending on the occasion of milling. The green maize is selected carefully to avoid using immature maize. The grains are removed from the cobs, sifted to remove any extraneous matter and washed by rinsing thoroughly with clean water twice. A measured amount [same volume as the grains] of clean water is used to soak the grains for subsequent grinding. Traditionally, a stone quern is used for wet milling. The operator rests on her knees or bends over at a slightly elevated end of the baseplate on which she places a handful of grains. She puts both hands on the roller stone on top of the grain. Wet milling is achieved by moving the roller repeatedly forwards and backwards. The milled grains fall into a receiver at the distal end of the quern. The grains are usually ground between two stones and suspended in water.

The grains are ground till when the mash is milky when one puts their hands in the container being used. Wet milling of millet is then done in a similar manner and mixed with the ground maize. The slurry is then poured in a fermenting container. Traditionally, the slurry was fermented in either earthenware pots or clay pots but the most popular

containers are now plastic buckets and jerricans. The porridge slurry is then covered with a lid or green banana leaves and left to ferment for 2 days. After fermentation the slurry is cooked by boiling into a thick porridge, which is normally consumed in a calabash by use of a spoon or a wooden carved stick. The mixing and turning of *Kirario* during cooking is achieved by employing a special wooden stirrer called *kibiro*. This is a T-shaped tool comprising a long handle and a small piece of wood fixed to its tip. The *Kirario* cooking process requires a great deal of effort and skill in order to produce a product with no defects. Defects such as formation of lumps in porridge, poor thickening of the final product and inadequate cooking are a sign of poor and insufficient cooking of *Kirario*. The amount of heat is frequently adjusted by pulling out burning wood or pushing in fresh firewood, as the situation may call for. The effort put into the process increases as the porridge stiffens and thickens.

The final product usually has a high viscosity, smooth texture and consistency, and a milk-like flavour. *Kirario* is a Bantu name meaning a food that has been kept overnight. The name denotes the sour nature of the product resulting from overnight holding. The product can be stored for one week or longer depending on the storage conditions.

Figure 4.1 Flow diagram of traditional production of *Kirario*.



4.2 Microbial content in *Kirario.*

The total viable microorganisms, lactic acid bacteria, yeasts and moulds, and total coliforms were enumerated in the traditional samples and laboratory –produced *Kirario.* Table 4.2 shows the microbial counts in K*irario.* High total viable counts [>9.51 log cfu/ml] were observed, with the lactic acid bacteria being the predominant microbes. There were no significant difference [$p<0.05$] between total viable counts, LAB, lactic-acid cocci, and yeasts and moulds counts between the traditional and laboratory produced products. The results showed that the microbial contents in *Kirario* produced in the laboratory, were comparable to those of the traditional products. No coliforms were detected in both the traditional and laboratory-simulated *Kirario.*

Table 4.2 Microbiological counts in traditional and laboratory-simulated *Kirario.*

	Log counts [log_{10}cfu/ml]	
	Traditional	Laboratory-simulated
Total viable counts	9.30 (0.14)	9.51 (0.68)
Lactic acid bacteria	9.63 (0.11)	9.40 (0.60)
Lactic-acid cocci	8.62 (0.98)	5.59 (0.98)
Yeasts and molds	4.83 (2.50)	4.20 (2.17)
Coliforms	<1	<1

KEY

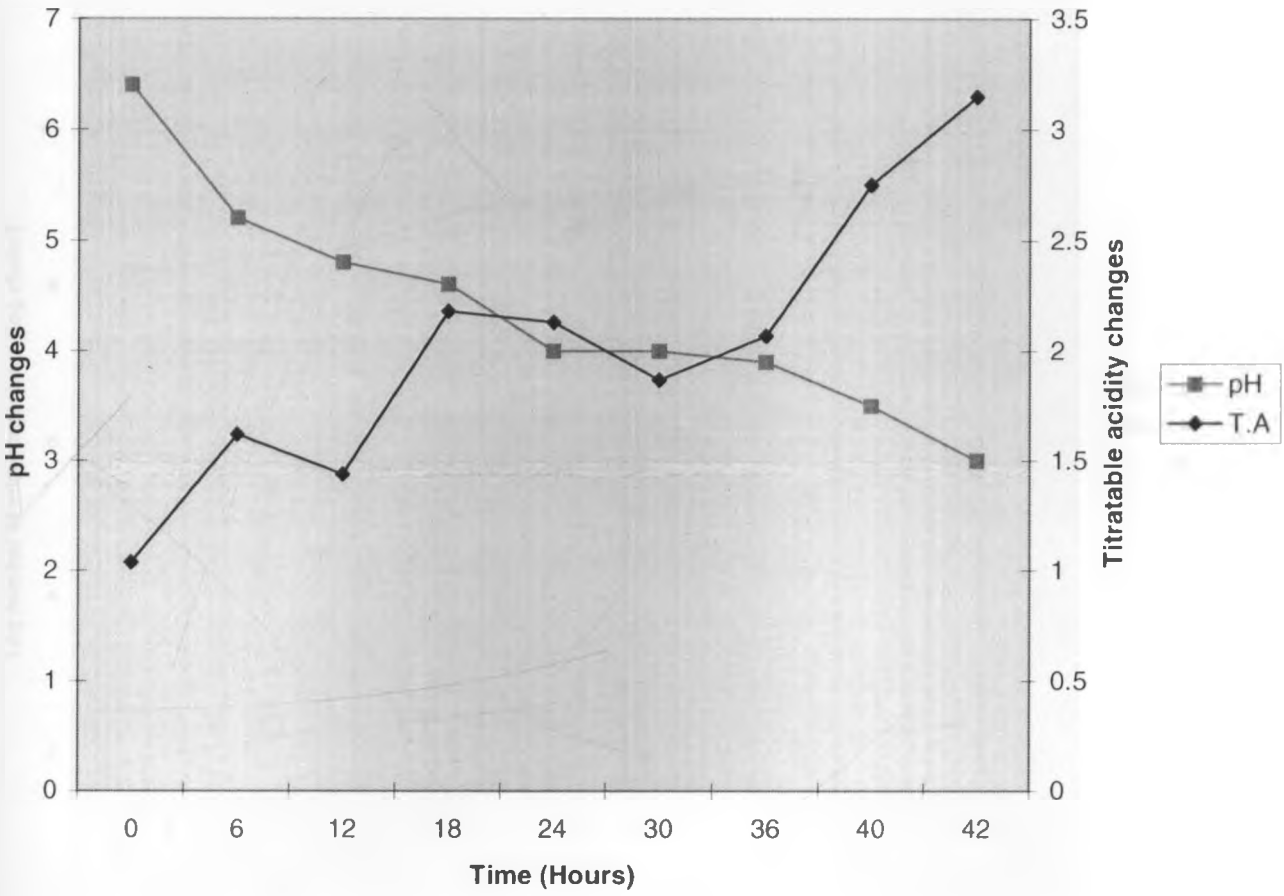
- Standard deviation in parentheses
- Number of replicates = 3

4.3 Biochemical and microbiological changes during *Kirario* fermentation

The microbial profile, pH and titratable acidity changes were monitored during the spontaneous fermentation of *Kirario* at a 6-hour interval for 48 hours. The changes in the microbial counts are shown in Figure 4.3.1. The changes in pH and titratable acidity during fermentation of *Kirario* are shown in Figure 4.3.2. The titratable acidity increased from a value of 1.04 to 3.15 during the spontaneous fermentation of *Kirario*. The pH showed a rapid decline from an initial value of 6.4 to 4.0 within 24-hour fermentation and then a steady decline from a pH of 4.0 to 3.0 in the remaining 24 hours of fermentation. Figure 4.3.3 is a graph of pH versus titratable acidity (%L.A) showing the buffer point of pH in *Kirario*. The variation in the graph was associated with the high initial counts of LAB resulting in high pH and titratable acidity.

The total viable counts were high and continued to increase steadily throughout the fermentation of *Kirario* with the LAB being the predominant microorganism. The lactococci (Lactic *Streptococci*) showed a fairly constant trend during fermentation with values ranging from 5 - 8 log CFU/ml. The yeasts and moulds counts were relatively constant during the fermentation with a rapid decline of 3log cycles towards the end of the fermentation (42^{nd} hour). This decrease was up to about a value of <1 log cfu/ml. There was a steady increase in total viable counts, LAB, and lactococci within the first 24 hours by a value of 1 log cycle.

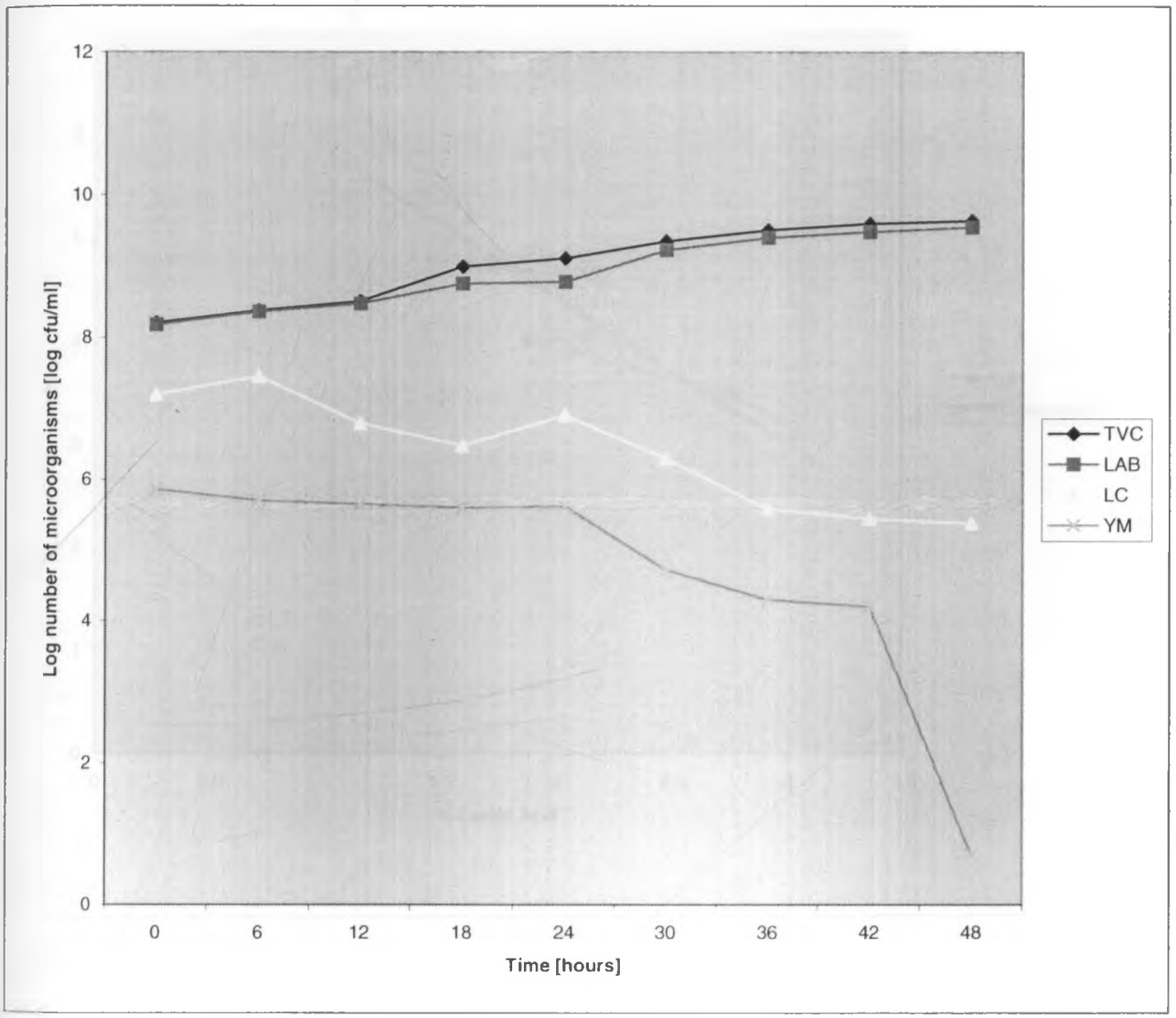
Figure 4.3.1 Changes in pH and titratable acidity during fermentation of *Kirario*



Key:

T.A – Titratable acidity

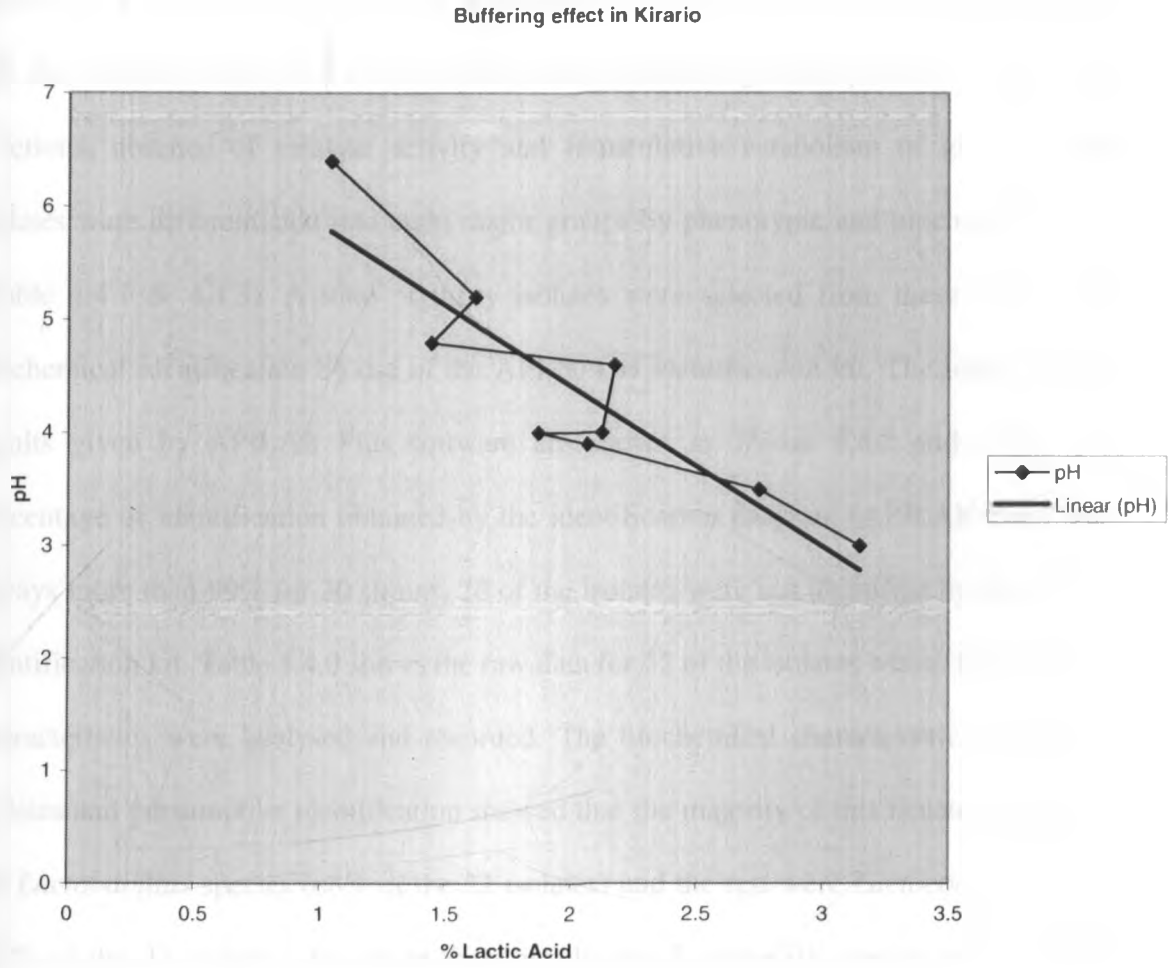
Figure 4.3.2. Changes in the microbial profile during fermentation of *Kirario*.



Key:

TVC – Total viable counts
LAB – Lactic acid bacteria
LC – Lactic acid cocci
YM – Yeasts and moulds

Figure 4.3.3 A Graph showing relationship between pH and Lactic acid roduced during *Kirario* fermentation.



4.4 Characterization of LAB isolates in *Kirario*.

Thirty LAB isolates were characterized from the 10 samples of traditionally fermented household *Kirario* collected from the households and from laboratory-prepared *Kirario*. All the isolates were shown to be lactic acid bacteria by their positive Gram stain reactions, absence of catalase activity and fermentative catabolism of glucose. The isolates were differentiated into eight major groups by phenotypic and biochemical tests (Table 4.4.1 & 4.4.3). A total of thirty isolates were selected from these groups for biochemical identification by use of the API 50 CH identification kit. The identification results given by APILAB Plus software are shown in Tables 4.4.2 and 4.4.4. The percentage of identification obtained by the identification program (APILAB Plus) was always more than 99% for 30 strains. 28 of the isolates were not identified by the API ® identification kit. Table 4.4.0 shows the raw data for 22 of the isolates whose biochemical characteristics were analysed and recorded. The biochemical characteristics of the 22 isolates and presumptive identification showed that the majority of this isolates belong to the *Lactobacillus* species (68% of the 22 isolates) and the rest were *Lactococcus* species (32% of the 22 isolates). Based on these results the *Lactobacilli* species were still the dominant species among the total isolates accounting for 63% of the 52 isolates while the *Lactococcus* species were 27%.

Traditional fermented *Kirario* was more dominated by strains of the *lactobacilli* and were identified more frequently as opposed to the *leuconostocs* and *lactococci*.

Based on the biochemical characteristics, the LAB were separated into four groups namely:

Obligately heterofermentative rods [3 strains]

Facultatively heterofermentative rods [14 strains]

Obligately homofermentative rods [1 strain]

Obligately homofermentative cocci [4 strains]

Obligately heterofermentative cocci [8 strains]

So out of the 30 LAB isolates identified by sugar fementation profiling, 25 (83.3%) and 5 (16.7%) were heterofermentative and homofermentative respectively.

Eight LAB species were identified as *Leuconostoc mesenteroides ssp. mesenteroides/detranicum 1 & 2, Leuconostoc citreum, Lactococcus lactis ssp. lactis 1 & 2, Lactococcus raffinolactis, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus collinoides* and *Lactobacillus coprophilus. Lactobacillus plantarum 1* was the most predominant LAB among all the LAB isolates [about 47% of the LAB] isolates, followed by *Leuconostoc mesenteroides ssp. mesenteroides/detranicum 1* which accounted for 16% of the LAB isolates.

The proportions of the Lactic acid bacteria strains isolated from *kirario* are shown in Figure 4.4 in percentages.

Figure 4.4; Percentages of lactic acid bacteria [LAB] isolated from *Kirario*.

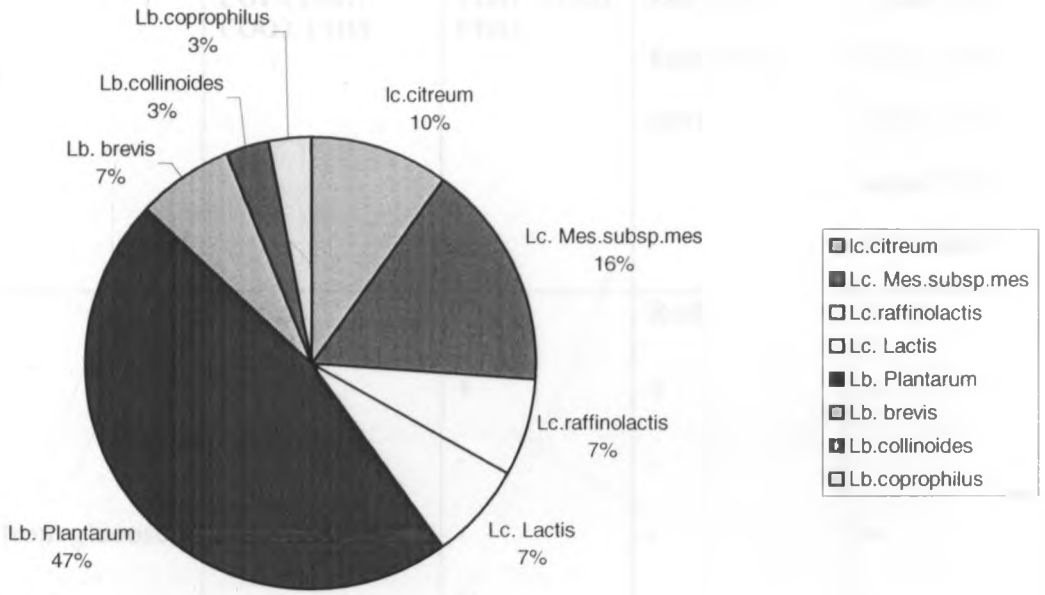


Table 4.4.0 Biochemical characteristics of the 22 isolates not identified by the API® 50 CH test strips.

Characteristics	Homogenous LAB clusters			
	Group 1	**Group 2**	**Group 3**	**Group 4**
	CO13, C007, COO2, CO19	**CO17, COO4, CO16**	**R039, R043, R009, R045, R017**	**R004, R008, R037, R026, R033, R026, R028, R010, R011, R015**
Morphology	Cocci	Cocci	Rods	Rods
Gram stain	+	+	+	+
Catalase test	-	-	-	-
Carbon dioxide from glucose	-	-	-	+
Arginine hydrolyzed	-	V	-	+
Type of lactic acid	L [+]	L [+]	DL- Lactate	DL- Lactate
Growth at 10^0c	+	+	+	+
Growth at 15^0c	+	+	+	+
Growth at 45^0c	-	-	-	+
Litmus test				
Acid	+	+	+	+
Coagulation	+	+	+	+

Key

Coding C – Cocci,

Coding R - Rods

[D] – Dextrorotary

[L] – Levorotary

Positive reaction (+),

negative reaction (-).

4.4.1 Identification and characterization of lactococcus [Lactic Streptococci]

The biochemical characteristics and identification profiles of the isolated lactococci are shown in Table 4.4.1 to 4.4.2. The isolates were tested for their biochemical characteristics before identification by fermentation of sugars using an API carbon source (sugars). These species were generally Gram-positive cocci, catalase-negative, capable of growth at 10^{0}c and 40^{0}c but not 45^{0}c, capable of growth in 4% NaCl and were classified as facultatively anaerobic.

From the API 50 CH strips, all the lactococci strains fermented D-glucose, D-frucose, D-mannose, N-acetylglucosamine, esculin, maltose, sucrose, galactose, trehalose and gentiobiose. The isolates in group 2 (coded as CO37, CO21, CO11, COO1, CO12) and group 1 (CO15, CO14, CO30) were identified as *Leuconostoc mesenteroides* ssp. *mesenteroides/detranicum* and *Leuconostoc citreum* respectively. *Leuconostoc mesenteroides* ssp. *mesenteroides/detranicum* and *Leuconostoc citreum* were identified as

heterofermentative cocci, capable of producing CO_2 from glucose and unable to hydrolyze arginine to form ammonia. They were able to form D [-] lactic acid and showed relative inactivity in litmus milk by not producing enough acid to cause milk coagulation. These two species were able to grow at 10^0c and 45^0c but only *Leuconostoc citreum* was able to grow at 15^0c

The isolates in group 3 (coded as CO28, COO3) and group 4 (COO6, CO1O) in Table 4.4 were identified as *Lactococcus raffinolactis* and *Lactococcus lactis ssp.lactis 1&2.* The biochemical tests showed that these two species were able to produce carbondioxide from glucose, unable to hydrolyze arginine from ammonia, capable of growth at 10^0c and 15^0c but not at 45^0c. They were able to form L [+] lactic acid and activity in litmus milk by production of acid subsequent milk coagulation. In general, the lactococci strains isolated from *kirario* were identified as *Leuconostoc mesenteroides* ssp. *mesenteroides/detranicum 1, Leuconostoc citreum, Lactococcus raffinolactis* and *Lactococcus lactis ssp.lactis 1&2*

Table 4.4.1 Biochemical characteristics of the cocci isolated from *Kirario*.

Characteristics	Homogenous LAB clusters			
	Group 1	**Group 2**	**Group 3**	**Group 4**
	C015, CO14, C030	**C037, C021, C011, C001, C012.**	**C028, C003**	**C006, C010**
Gram stain	+	+	+	+
Catalase test	-	-	-	-
Carbon dioxide from glucose	+	+	-	-
Arginine hydrolyzed	-	-	-	V
Type of lactic acid	D [-]	D [-]	L [+]	L [+]
Growth at 10^0c	+	+	+	+
Growth at 15^0c	-	+	+	+
Growth at 45^0c	+	+	-	-
Litmus test				
Acid	-	-	+	+
Coagulation	-	-	+	+

Key

Coding C – Cocci, [D] – Dextrorotary [L] – Levorotary

Positive reaction (+), negative reaction (-).

Table 4.4.2. Biochemical characteristics and identification profile of isolated cocci on API® 50 CHL test strips

Substrate	Homogenous LAB Clusters			
	Group 1	Group 2	Group 3	Group 4
Glycerol	-	-	-	+
Erythritol	-	-	-	-
D Arabinose	-	-	-	-
L Arabinose	+	+/-	-	-
Ribose	-	+	-	+
D Xylose	-	+	+	-
L Xylose	-	-	-	-
Adonitol	-	-	-	-
B Methly-D Xyloside	-	-	-	-
Galactose	+	+	+	+
Glucose	+	+	+	+
Fructose	+	+	+	+
Mannose	+	+	+	+
Sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
α-Methyl-D-Mannoside	-	-	-	-
α-Methyl-D-Glucoside	+	+	+	-
N-Acetyl-Glucosamine	+	+	+	+
Amygdalin	+	+/-	+	+
Arbutin	+	+/-	+	+
Esculin	+	+	+	+
Salicin	-/+	+/-	+	+
Cellobiose	+	+/-	+	+
Maltose	+	+	+	+
Lactose	-	+	-	+
Melibiose	-	+	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Inulin	-	-	-	-
Melezitose	-	-	-	-
Raffinose	-	+/-	+	-
Starch	-	-	+	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
Gentiobiose	+	+	+	+
D Turanose	+	+	+	-
D Lyxose	-	-	-	-
D Tagatose	-	-	-	-
D Fucose	-	-	-	-
L Fucose	-	-	-	-
D Arabitol	-	-	-	-
L Arabitol	-	-	-	-
Gluconate	+	-	+	+
2-Keto-Gluconate	-	-	+	-
5-Keto-Gluconate	-	-/+	-	-
Identity	*Leuconostoc citreum*	*Leuconostoc mes.* ssp. *mesenteroides*	*Lactococcus raffinolactis*	*Lactococcus lactis* ssp. *lactis*

4.4.2 Identification and characterization of the rods isolated in *kirario.*

The biochemical characteristics and identification profiles of the isolated rods are shown in Table 4.4.3 to 4.4.4. Generally the coded species in groups six to eight were all Gram-positive, catalase-negative, and capable of growth at 10^0c, 15^0c but not at 45^0c as shown in Table 4.4.3. All the species were able cause acidification and coagulation of litmus milk. The isolates in group 6 and group 7 were able to hydrolyse arginine to produce ammonia while those in group 8 were able to grow at 45^0c unlike the rest of the isolates. The species in group 6, 7 and 8 produced carbondioxide from glucose hence were tentatively classified as heterofermenters.

Further identification was carried out based on the biochemical tests described above to give the identification profile shown in Table 4.4.4. From API 50 CH strips, all the lactobacilli strains fermented D-glucose, D-fructose, ribose, gluconate, N-acetylglucosamine, esculin, maltose and sucrose. The species were identified as *Lactobacillus plantarum*, *Lactobacillus brevis, Lactobacillus collinoides* and *Lactobacillus coprophilus.* The largest cluster (Group 5) consisted of 14 *Lactobacillus plantarum* strains which were characterized by their rod-shaped cells, production of DL-lactate, fermentation of ribose and other pentose sugars, and inability to utilize arginine (Table 4.4.3 & 4.4.4). *L. plantarum* was categorized as a streptobacteria, which is homofermentative, and thus did not produce Co_2 from glucose fermentation. This strain was unable to produce arginine from ammonia hydrolysis and being a mesophile it was able to grow at 15^0c and not 45^0c. One strain of L. *plantarum* was misidentified as L.

rhamnosus but was differentiated by the later's ability to grow at 45^{0}c, produce L-lactate and may be identified biochemically by its negative raffinose, melibiose, fructose fermentation and ability to ferment rhamnose after 48 hours of incubation. L. *rhamnosus* is heterofermentative as opposed to L. *plantarum*, which is homofermentative.

Lactobacillus collinoides and *Lactobacillus brevis* were identified as heterofermentative lactobacilli hence tentatively were categorized as betabacterium. These species produced CO_2 from glucose fermentation and ammonia from arginine hydrolysis. The two strains belong to the L. brevis-buchneri-group which is able to ferment ribose, melibiose but unable to ferment cellobiose and melezitose. *Lactobacillus coprophilus* was able to grow at 45^{0}c and hydrolyze arginine to produce ammonia. *L. coprophilus* is a new variety of the subspecies Betabacterium.

Table 4.4.3 Biochemical characteristics of the rods isolated from *Kirario*.

Characteristics	Homogenous LAB clusters			
	Group 5	Group 6	Group 7	Group 8
	R040,R044,R027,R006,R031, R038,R002,R041,R025,R042, R049,R027,R046,R032	R008, R024	R007	R003
Gram stain	+	+	+	+
Catalase test	-	-	-	-
Carbon dioxide from glucose	-	+	+	+
Arginine hydrolyzed	-	+	+	+
Type of lactic acid	DL- Lactate	DL-Lactate	DL-Lactate	DL-Lactate
Growth at 10^0c	+	+	+	+
Growth at 15^0c	+	+	+	+
Growth at 45^0c	-	-	-	+
Litmus test				
Acid	+	+	+	+
Coagulation	+	+	+	+
KEY **Positive reaction (+), negative reaction (-). Coding, R-Rods**				

Table 4.4.4 Biochemical characteristics and identification profile of isolated rods on API® 50 CHL test strips

Substrate	Homogenous LAB Clusters			
	Group 5	**Group 6**	**Group 7**	**Group 8**
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D Arabinose	-	-	-	-
L Arabinose	+	+	+	-
Ribose	+	+	+	+
D Xylose	-	+	+	+
L Xylose	-	-	-	-
Adonitol	-	-	-	-
B Methly-D Xyloside	-	-	-	-
Galactose	+	+	+	+
Glucose	+	+	+	+
Fructose	+	+	+	+
Mannose	+	+	-	+
Sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	+	-	-	-
Sorbitol	+	-	-	-
α-Methyl-D-Mannoside	+	-	-	-
α-Methyl-D-Glucoside	-	-	-	-
N-Acetyl-Glucosamine	+	+	+	+
Amygdalin	+	+	-	+
Arbutin	+	+	-	+
Esculin	+	+	+	+
Salicin	+	+	-	+
Cellobiose	+	-	-	+
Maltose	+	+	+	+
Lactose	+	-	-	-
Melibiose	+	+	+	-
Sucrose	+	+	+	+
Trehalose	+	+	-	-
Inulin	-	-	-	-
Melezitose	+	-	+	-
Raffinose	+	+	-	-
Starch	-	-	-	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
Gentiobiose	+	-	-	+
D Turanose	+	+	-	-
D Lyxose	-	-	-	-
D Tagatose	-	-	-	-
D Fucose	-	-	-	-
L Fucose	-	-	-	-
D Arabitol	-	-	-	-
L Arabitol	-	-	-	-
Gluconate	+	+	+	+
2-Keto-Gluconate	-	+	-	-
5-Keto-Gluconate	-	-	-	-
Identity	*Lactobacillus plantarum 1*	*Lactobacillus brevis 1*	*Lactobacillus collinoides*	*Lactobacillus coprophilus*

CHAPTER 5: DISCUSSION

5.1 Traditional Fermentation of *Kirario.*

The fermentation of *Kirario* is a spontaneous process, which has its own unique features. The raw materials used in the preparation are unique since green maize is used as opposed to other documented lactic fermented beverages where dry cereals are used. This unique feature is likely to influence the type of lactic acid bacteria involved in the fermentation of *Kirario*. Several researchers [Akinrele, 1970; Nout, 1980; Holzafel, 2002] have indicated that the microorganisms involved in the natural fermentation of cereals are essentially the microflora of the raw materials and equipment.

Women involved in preparation of *Kirario* re-use the fermentation vessels [clay pots, plastic buckets and jars] several times to ensure satisfactory fermentation. This implies that the microorganisms retained in the fermentation vessels from the previous batch of fermentation help to steer the process in the right direction [Mathara, 1999; Holzapel, 2002; FAO, 1990]. However, the vessels are rinsed with clean water before re-use. Deliberate back slopping was however not observed in the *Kirario* process but aspects of it are demonstrated in this practice of re-use of vessels previously used for fermentation. Thus, the sources of fermenting organisms in *Kirario* are likely to be the raw materials, fermentation vessels, blending water, grinding personnel, as well as milling and mixing utensils. The resulting starchy material from wet-grinding has characteristic sour taste and aroma which has become an essential and desired element in the product that is subsequently cooked before consumption (Campbell-Platt, 1987). Novellie (1981) and

Campbell-Platt (1987) have reviewed several African fermented products like porridges and dumplings, which are prepared by soaking and wet-milling processes. Examples of local cereal-based products that have been scientifically studied are *ogi* and *agidi* of Nigeria, *koko, akasa*, and *kenkey* of Ghana, *uji* of East Africa, and *mahewu* of Southern Africa. Many of these products are also made commercially for local markets (Odunfa, 1985).

5.2 Microbial Growth during *Kirario* Fermentation.

A high microbial load was observed in *Kirario* with very high initial counts of between 6-8 $\log_{10}$ CFU/ml. This initial count could be attributed to its unique raw material (green maize) and unintentional back slopping. Studies have shown that maturing green maize kernel contains carbohydrates other than starch i.e. simple sugars. The total sugars found in the green maize range between 1 and 3 percent, with sucrose, the major component (Ingle et al 1965). Higher levels of monosaccharides, disaccharides and trisaccharides are present in the mature green maize (Khan and Bressan., 1987). These relatively high levels of reducing sugars and sucrose are possibly the reason why there were high counts of LAB during the fermentation of *Kirario* and the low final pH achieved. The carbon sugars are readily fermentable substrates by the lactic acid bacteria during catabolic processes in fermentations of cereals. The initial counts increased to high levels of 9.3-9.5 log CFU/ml during fermentation. The high microbial load of 9.3-9.5 log CFU/ml achieved in *Kirario* is similar to microbial counts reported in the Zimbabwean *mangisi* [Zvauya et al, 1997]. Akinrele (1970) also reported that in a fully fermented slurry of *ogi*

(a Nigerian lactic fermented cereal-based porridge), microbial load of more than 10^9 CFU/g, very similar to the results showed for *Kirario,* were achieved.

The results obtained from the laboratory-produced *Kirario* showed similar microbial counts and profile, indicating the ability to replicate *Kirario* production under laboratory conditions. This was vital for further research work on *Kirario*. The low pH of 3.0-4.5 in *Kirario* is also in agreement with other cereal based fermented beverages with pH values in the range of 3.0-4.8 like Tanzanian *togwa* [Lorri, 1993], Ethiopian *tella* [Sahle and Gashe, 1991], Sudanese *merissa* [Dirar, 1993], Zimbabwean m*angisi* [Zvauya et al, 1997] and Egyptian *bouza* [Morcos, 1977]. The low pH in *Kirario* can be linked to the high lactic acid bacteria (LAB) counts observed in the product with an average count of 9.50 $\log_{10}$ CFU/ml. The count of lactococci was high initially with an average count of 7.96 log CFU/ml and increased slowly as the fermentation progressed. Lund et al., 2000 found that the lactococci group of LAB decreases during fermentation as a result of the decrease in pH. They reported that the lactococci group, especially the *Leuconostoc, is* less resistant to low pH than the *Lactobacillus.*

5.3 Biochemical and microbiological changes during *Kirario* fermentation.

The biochemical and microbial development in *Kirario* was characterized by steady increases in the total viable counts and the LAB within the 48 hours. The lactococci yeasts and moulds were fairly constant throughout the fermentation time with a sharp decline in the yeast and mould counts towards the end of fermentation. The pH decreased rapidly from 6.4 to 3.0 while the titratable acidity increased from 1.04 to 3.15. The titratable acidity describes the degree of free [H+] present in a sample and hence its acid content. Lactic acid fermented cereal gruels inhibit the proliferation of pathogenic bacteria provided that the pH is below 4.0 [Lorri, 1993; Nout et al., 1989]. Thus the low pH achieved during the spontaneous fermentation of *Kirario* can be expected to contribute to its safety. This explained the decline of coliforms, which were undetectable due to the dramatic decline of pH through rapid acidification.

The results obtained for total viable and LAB counts in *Kirario*, and the predominance of the LAB are similar to those reported by authors who have studied the microbial profiles of traditional fermented cereal based gruels similar to *Kirario*. Kabede et al [2002] reported an average aerobic mesophilic count [AMC], LAB and yeasts counts of 9.9, 10.1 and 8.1 log CFU g^{-1} respectively in the Ethiopian fermented beverage, *borde*. Studies carried out on the microbial groups involved in the spontaneous fermentation of a Sudanese cereal-based product *medida*, showed that *Lactobacillus spp.*, and *acetobacter spp.*, were the predominant microbes in the product [Abdel-Gadir & Mohamed, 1983].

The decrease observed in yeasts and moulds to levels of <2 log CFU/ml indicate that they were destroyed by the more dominant LAB as the fermentation progressed.

The biochemical and microbiological changes that occur during production of m*asvusvu* and *mangisi*, both traditional Zimbabwean cereal-based beverages are similar to those of *kirario*. Zvauya et al (1997) showed that during fermentation to produce *mangisi*, total aerobic mesophilic and lactic acid bacteria increased with fermentation time. However, the total titratable acidity increased from 0.51 to 4.10 percent lactic acid, and pH decreased from 6.10 to 3.98. These results are slightly different to those obtained in *kirario* whereby the titratable acidity increased from 1.04 to 3.15 percent lactic acid, and pH decreased from 6.4 to 3.0. This is a vital characteristic in *Kirario* fermentation in terms of microbial and lactic acid development. The high levels of fermentable simple sugars and lack of nutrient inhibitors in the green maize were attributed to the high initial count of LAB and rapid acid development

5.4 Characterization of Lactic Acid Bacteria in *Kirario*.

From 30 isolates of cocci and rods presumptively classified as LAB, eight species were identified which accounted for various percentages of the total. The bacterial populations in the *Kirario* samples consisted of *Lactobacillus*, *Leuconostoc* and *Lactococcus* species. The predominance of lactic acid bacteria in indigenous fermented porridges/gruels in Africa has been reported by many authors (Mbugua, 1985; Akinrele, 1970; Odunfa,

1985; Rombounts and Nout 1995; Zvauya, 1997; Nokuthula et al, 1999; Hamad et al, 1999; Kabede et al, 2002; Abdel-Gadir and Mohamed, 1983; Lorri, 1993; Sahle and Gashe, 1991; Dirar, 1993 and Morcos, 1977). The main reported genera are *lactobacilli, leuconostocs* and *lactococci.*

The majority of the isolates characterized in this study belonged to the genera *Lactobacillus* (60% of the isolates examined) and *Leuconostoc* (26% of the isolates examined). Previous studies have showed that naturally fermented cereal-based African foods are dominated by *Lb. plantarum*, *Lactobacillus fermentum*, *Lactobacillus reuteri, Lc. mesenteroides, P. pentosaceus,* and *Lc. lactis* strains [Rombouts & Nout, 1995]. From the API 50 CH profiling results, all the LAB species isolated from *Kirario* were able to ferment sucrose, maltose, glucose, and fructose, which are the main soluble sugars of maize (Boyer and Shanon, 1987), reflecting adaptation to their habitat. The results from *Kirario* fermentation illustrated the predominance of *Lactobacillus plantarum. Lactobacillus plantarum* is often the dominant *Lactobacillus* sp. in traditional lactic acid fermented foods based on plant material (Rombouts, 1995). The results obtained from the biochemical and microbial profile characteristics of LAB in the fermentation of *Kirario* also show the predominance of *Lb. plantarum* (47% of the total isolates) and its tolerance to low pH (pH 3.0-3.8). Mbugua [1985] showed that the *Lactobacillus plantarum* dominated the traditional fermentation process of *uji*, accounting for over 70% of the isolates examined.

There were 28 isolates that did not belong to any major clusters (not identified), although 22 of these isolates were very similar to the *Lactobacillus* and *Lactococcus* species as shown the results. Two strains remained unidentified by the API 50 CH. The significance of this finding can be illustrated by the fact that screening of 200 strains from traditional fermented products always resulted in only a few strains (< 2%) which could not be identified by the database used for the study (Van den Berg et al., 1993). The isolates not identified by the database are significant for further research due to the likelihood of identifying new and important strains of LAB with unique traits.

From the previous studies it is apparent that two of the LAB species (*Lactobacillus coprophilus* and *Lactobacillus collinoides*) identified in *Kirario* has not been isolated from other lactic fermented cereals in Africa (Hammes et al., 1995 & 1992). In comparison to other African cereal-based fermented foods, *Kirario* thus appears to be a food ecosystem in which other types of LAB species have been developed. The occurrence of this species could be attributed to the unique ecosystem associated with fermentation of *Kirario*. This indicates the possibility of new species and subspecies of LAB in *Kirario*. Some of the unique selective factors for lactic acid bacteria in *Kirario* include the low pH (3.0) and carbohydrates (green maize), which supports the predominance of LAB.

Low pH values have been reported to favour the growth of *lactobacillus* strains much more than the *lactococcus* strains [Mathara, 1999; Wood and Holzapfel, 1995 and Nakazawa and Hosono, 1992]. *Kirario* had very low pH values during the fermentation period as shown in Figure 4.2.2. In general, the *lactococcus* and *leuconostocs* genera

require a slightly more alkaline pH as compared with their more acidophilic relatives namely *Lactobacillus* and *Pediococus*. *Leuconostocs* are generally inhibited below a pH of about 4.5 and therefore are usually active in the initial stages of fermentation [Garvie, 1967 and 1984].

Results obtained from studies on microbial profile of *mahewu*, (a lactic fermented cereal based product from South Africa) have shown the predominance of *Lb. plantarum* and *Lc. mesenteroides* strains during the fermentation process [Nokuthula et al. 1999]. This is very similar to *Kirario*, which is dominated by *Lb. plantarum* and *Lc. mesenteroides* ssp. *mesenteroides*. Moss et al. (1984) found that the fermentation of *ting* of Southern Africa, a sour porridge made from maize or sorghum meal was meditated first by Gram negative rods, which rapidly gave way to LAB, both rods and cocci. The strains of *Lb. fermentum* and *Lb. plantarum* were found to give the best aroma as well as acid producers.

During *Kirario* fermentation the *Lactobacillus* species were the most frequently isolated strains especially towards the end of fermentation period. In studies of the microflora found in the spontaneously fermented cereal flour in Sudan, seven strains of lactobacillus were isolated which represented the dominant flora [Hamad et al., 1999]. Studies have shown that the fermented cereal-based foods can be inoculated with *Lb. plantarum* directly from the plant, since *Lb. plantarum* is present in plant materials though in low numbers (less than 10 CFU/g of plant material), (Mundt and Hammer, 1968). There is a great possibility that the lactic acid bacteria present in the raw materials and the readily

available simple sugars in the mature green maize led to the high initial load of LAB in *Kirario.*

Odunfa [1985] determined that *Lb. plantarum* was the predominant organism in the fermentation of ogi responsible for lactic acid production. Other fermented gruels which have LAB as the predominant organism include *bogobe* of Botswana, *koko* and *kenkey* of Ghana, *mawe* of S. Africa, *kisra* of Sudan and *bushera* of East Africa to name but a few.

5.4.1 Identification and characterization of Lactic acid cocci isolated in *Kirario.*

The species in group 1, 2, 3 & 4 (Fig. 4.3), which consisted of only four strains, were *Lactococci* and *Leuconostoc* clusters. The natural habitat of lactococci is milk, but *L. lactis* subsp. l*actis* has been isolated previously from plants, vegetables, and cereals [Salama et al., 1995].

The *Lactococci* and *Leuconostoc* species were identified as *Leuconostoc mesenteroides* ssp. *mesenteroides/detranicum 1, Leuconostoc citreum, Lactococcus raffinolactis* and *Lactococcus lactis ssp.lactis 1&2.* These species have been reported to produce diacetyl, a flavour compound that is important in aroma production of the fermented gruel [Jay, 1986; Jay, 1992 and Garvie, 1978]. The genus *leuconostoc* also produces other products of heterofermetative fermentation like D [-] lactic acid, ethanol, acetic acid and CO_2 via the Hexose monophosphate pathway [Jay, 1992]. *Lc.mesenteroides* ssp. *mesenteroides* has been used in the manufacture of detrans, which have been used as blood plasma extenders [Garvie, 1974]. This is of great importance in studies of *Kirario* due to the biotechnological applications of these species.

Lc. lactis ssp. *Lactis* and *Lc. raffinolactis* isolated from *Kirario* are homofermentative cocci and produced ammonia from arginine and L [+] lactic acid from fermentation of glucose. These species are able to grow at 15^{0}C but not at 45^{0}C, and are unable to grow at pH 9.6 and in 6.5% NaCl. These species also are able to acidify, coagulate and reduce litmus milk.

5.4.2 Identification and characterization of the *Lactobacilli* isolated in *Kirario*.

The rods isolated from *Kirario* were identified as *Lactobacillus plantarum*, *Lactobacillus brevis, Lactobacillus collinoides* and *Lactobacillus coprophilus.* Lactobacilli are commonly associated with plant herbage. They are also commonly associated with the gastrointestinal tract of humans and animals (Kleerebezem et al, 2003). *Lactobacillus plantarum* was the predominant lactic acid bacteria in *Kirario* (47% of the isolates identified). All the species identified as *Lb. plantarum* were typical streptobacteria which were able to ferment lactose, maltose and mannitol. Usually the atypical streptobacteria are able to ferment both lactose and maltose, but are unable to ferment mannitol. Hitchner (1982) however, found no strains of atypical streptobacteria capable of fermenting both lactose and mannitol (Hichner et al 1982).

Lactobacillus coprophilus isolated in *Kirario* has been renamed as *Lb. confusus.* Originally it was so named because of confusion with *leuconostoc* species, and was isolated from ensilages of grass, dirty water, sugarcane, raw milk, banana leaves, garlic mix, sewage, carrot juice and juice producing fruits (Hammes et al 1995). However, the

genus *Weissella* has been separated recently from the genus *Lactobacillus* through reclassification using DNA molecular techniques (Collins et al., 1993). *W. confusa* previously known as *Lactobacillus confusus* is now the current name for *Lactobacillus coprophillus* (Stiles et al., 1997). *Lb. coprophilus* is present in the normal microflora of human intestines and has been isolated from foods such as Greek salami (Schleifer et al., 1992) and Malaysian chili bo (Leisner et al., 1999). *Lb. collinoides* and *Lb. coprophilus* strains have not been isolated in lactic acid fermented cereals (Carr and Davies, 1972). These points out the possibility of unique microbial profile of *Kirario* when other modern techniques are used for further identification studies. This is of interest due to their biotechnological potential and with regard to possibility of isolation of new bacterial species and strains in *Kirario* (Leisner et al., 1999).

Lactobacillus plantarum frequently occurs spontaneously in high numbers in most lactic acid fermented foods and especially when the food is based on plant material. Examples of plant foods from where it has been isolated include: sourdoughs, Nigerian *ogi* [made from maize or sorghum], East African *uji* [made from maize, millet or sorghum], Ethiopian *Kocho* [made from starch from *Ensete ventrcosum*], Ethiopian sourdough made from *tef* [*Egragrostis tef*], and cassava (Lönner et al, 1995, Johansson, 1995, Gashe, 1995 and Nigatu, 1998). Thus, it is obvious that individuals consuming lactic acid fermented products of plant origin also consume large numbers of *Lb. plantarum*.

CHAPTER 6: CONCLUSIONS

The traditional art of *Kirario* preparation as documented with regard to the study region, Meru involves spontaneous fermentation of green maize and millet gruel at ambient temperature for 24-48 hours. Traditional fermenting vessels like earthen ware pots or clay pots [nyung'o] are rarely used currently and have been replaced by the more popular plastic jars and buckets. Fermenting vessels that are frequently used for fermentation are most preferred for use during the preparation of *Kirario* based on the belief by the local women that they accelerate the fermentation of the gruel. The fermented porridge is cooked by boiling to a thick consistency and served into calabashes and plates; the consumer uses a spoon [usually wooden] to drink. It was noted that the consumption of *Kirario* is mostly for thirst quenching purposes, a special drink during festivities/rituals and is a special drink for the invalids, lactating mothers, infants and circumcised youth who are undergoing recovery.

The main objective of this work was to study the identity and characteristics of the microbial strains in *Kirario* by use an API 50 CHL test. The fermentation process of *Kirario* involved an interaction of mixed microflora of lactic acid bacteria species. Biochemical characterization of 30 Gram positive, catalase negative, non-motile isolates resulted in the identification of lactic acid bacteria species that accounted for various percentages of the total. In this study, members of several genera of lactic acid bacteria were recovered from *Kirario* fermented samples. The dominant groups found in the fermented porridge samples were *Lactobacillus plantarum 1* and *Leuconostoc*

mesenteroides ssp. *mesenteroides*. Two unique LAB species were isolated in *kirario* which opens new fields of investigations on the microbial diversity of the fermented product. Relatively high acid contents were observed in both the traditional and laboratory-produced *kirario*, corresponding to pH 3.0 to 3.5, which indicated relatively high activity of the lactic acid bacteria in *kirario*. However, further studies using more recent methods of identification will be required to characterize the LAB to the subspecies level and isolate any new species in the product. There is still a considerable lack of information with regard to lactic fermented porridges (soured porridges and dumplings), in spite of the significance of these foods for millions of people in Africa and Asia.

CHAPTER 7: RECOMMENDATIONS

The identification of microorganisms in this study was based mainly on fermentation of carbohydrates, morphology, and gram staining. Although these methods are still widely used, the taxonomy has changed considerably in the recent years with increasing knowledge of the genomic structure and phylogenetic relationships between *Lactobacillus* spp. (Klein et al., 1998, Stiles et al., 1997). The identification of some *Lactobacillus* species by biochemical methods alone is not reliable (Charteris et al., 1997), hence more recent and reliable methods need to be used in further identification of LAB in K*irario* in future research. This includes genotypic methods for strain typing like PCR methods (e.g., randomly amplified polymorphic DNA [RAPD] analysis) or variations of restriction enzyme analysis (e.g., pulsed-field gel electrophoresis [PFGE] and ribotyping).

Research on the technological properties and modification need to be conducted on the fermented *Kirario* and organisms selected for further tests (production of bacteriocins and volatile compounds. The potential biotechnological applications of lactic acid bacteria found in *Kirario* were not examined. This indicates the need for further research on this fermented product. The information obtained from this study may also serve as a basis for further investigation on process optimization and eventually to establish small-scale *Kirario* production. Consumer acceptability of *Kirario* when alternative process techniques and replacement of earthenware pots are incorporated should also be investigated.

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9. APPENDIX 1

INTERVIEW SHEDULE FOR SURVEY OF KIRARIO PREPARATION

Raw Materials

- What are the major raw materials used in the preparation of *kirario*?
- Are the raw materials locally available or are they bought from other areas?
- Are there substitutes used for the raw materials?
- What are the hygienic practices used in handling and use of these raw materials?

Containers

- What are the local names of the containers/ gourds used to ferment and store the porridge?
- Describe the process of preparing these gourds for use. How are the containers stored?
- How often are new containers made?
- What methods are there for the cleaning of the containers?

Wet milling

- What equipment is used for wet milling?
- What material is it made of?
- What steps are carried out in the wet milling of the raw materials?
- What group of people are usually involved in this activity- wet milling/
- Is the equipment normally washed after milling?
- What are the implications of the equipment used in the wet milling on the fermentation of the product?

Fermentation

- Is the porridge left to ferment spontaneously of is some previously fermented porridge added as starter?
- Are there any additives to the porridge during preparation process?
- How long does the fermentation process take? [IMPORTANT].
- Measure the range of fermentation temperature.
- Describe the appearance of he fermented product [color: smell; taste; appearance; consistency e.tc.]
- How long is the product stored?