MICROBIAL INTERACTIONS ASSOCIATED WITH INDIGENOUS FERMENTED MILK

by

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For I know the plans I have for you, declares the Lord. Plans to prosper you and not to harm you, plans to give you hope and a future.

Jer 29:11

Dedicated to my love,

Bernd Theisinger

I Shirleen Coetzer declare that the dissertation hereby submitted by me for the Masters degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I further more cede copyright of the dissertation in favour of the University of the Free State.

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naturally fermented milk

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LIST OF ABBREVIATIONS

%	Percentage
3-4	Three to four
°C	Degree Celsius
&	and
µl	Micro liter
aw	Water Activity
BLAST	Basic Local Alignment Search Tool
Bp	Basepair
BPA	Baird-Parker Agar
C	Control
CFU g^{-1}	Colony forming units per gram
cfu.ml^{-1}	Colony forming units per milliliter
CO_2	Carbon dioxide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic Acid
e.g.	Example
EPS	Exopolysaccharides
et al	et alii (and others)

etc.	Et cetera
ETEC	Enterotoxicenic *E. coli*
FFA	Free fatty acids
g/L	Gram per liter
GRAS	Generally recognized as safe
GS	Gas chromatography
GS-MS	Gas chromatography-mass spectrometry
h/hrs	Hour/Hours
HPLC	High performance liquid chromatography
HS-SPME-GC-MS	Headspace-solid phase micro extraction-gas chromatography-mass spectrometry
HTST	High Temperature Short Time
KOH	Potassium hydroxide
L	Liter
LAB	Lactic Acid Bacteria
LPS	Lipo-polyssacharide
M17	Growth Medium for lactococci
M	Molar
m	Meter

mg/L	milligram per liter
min	Minutes
ml	Milliliter
ml/min	Milliliter per minute
mm	Millimeter
MRS	De Man, Rogosa and Sharpe
MRSV	De Man, Rogosa and Sharpe with Vanocomycin
MS	Mass spectrometry
MSEA	Growth Medium for leuconstocs
NCBI	National Centre for Biotechnology information
nd	Not detected
NFM	Naturally fermented milk
nm	Nanometer
pH	Negative Logarithm of Hydrogen Concentration
PCR	Polymerase chain reaction
RBA	Rose Bengal Agar
rpm	Revolution per minute
SBM	Growth medium for enterococci
SNF	Solids-not-fats

spp.	Species
ST	Heat-stable toxins
subsp.	Sub species
TAE	Tris (2-Amino-2-(hydroxymethyl)-1,3-propandiol)-HCI, Glacial acetic acid and EDTA
TS	Total solids
UFS	University of the Free State
UHT	Ultra high temperature
w/v	Weight per volume
V	Volt
VRB	Violet Red Bile (Agar)
YM	Yeast extract-Malt extract (Agar)

CHAPTER 1

Literature Review

1.1. Introduction

Fermented foods are estimated to constitute about a quarter of the foods consumed worldwide. A wide variety of foods are fermented, including milk, root crops, meat and fish, but the foods of greatest relevance fed to young children are produced by the fermentation of cereals, milk and pulses (Mensah et al., 1991; Simango, 1997). The activities of a group of microorganisms rather than a single microorganism may be the result of some fermented foods (Dirar, 1993).

Research has shown that fermented foods contain essential nutrients needed to maintain optimum health as well as non-nutritional components that contribute to the prevention or delay of the onset of chronic illnesses associated with advancing age (Heller, 2001; Staton et al., 2005). The safety of food fermentation is therefore essential and relies on various contributing factors like food substrates being overgrown by desirable, edible microorganisms and as a result become resistant to the invasion of spoilage or toxic or food poisoning microorganisms (Steinkraus, 1996). Furthermore, fermentations resulting in the production of lactic acid are generally considered as safe (Steinkraus, 1983).

The incorporation of “new bacteria” of intestinal origin into human diet corresponds to the emergence of a new generation of food products, together with the probiotic effects and the reduced level of pathogenic bacteria as seen in fermented foods and beverages. This new generation of food products is especially important when it comes to developing countries where fermented foods have been reported to reduce the severity, duration and morbidity of diarrhoea (Kimmons et al., 1999; Mensah, 1997; Mensah et al., 1991; Mensah et al., 1990; Mitsuoka, 2000; Nout, 1991).

The beneficial role of milk in preventing infection has been recognised for thousands of years. Much of the activity has been attributed to antibodies, but the role of other minor proteins such as lactoferrin, lactoperoxidases and complex sugars in milk as

bioactive agents have only recently been recognised. Fermented milk is one of the most popular fermented foods and has been traditionally consumed for a long time in many countries (Bielecka et al., 2000; Courtin & Rul, 2004; Rademaker et al., 2006). The most ancient lactic fermentation is probably fermented sour milk. Raw milk will rapidly sour because of the lactic acid bacteria present (Steinkraus, 1983). Spontaneous acidification of raw milk by indigenous organisms is how fermented milk emerged (Weigmann, 1905). The composition of cow's milk may also vary considerably depending on the individual animal, stage of lactation, its breed, age and health status. Herd management practice and environmental conditions further influence milk composition. The range of composition of cow's milk is shown in Table 1 (Kebede, 2005; Nagendra, 2000).

Traditional naturally fermented milk (NFM) is still being produced, at the household level in many communities in rural areas of Africa where animals are kept especially for their milk. Although cow's milk is the most common, fermented milk may be made in some areas from camel, sheep or goat's milk. It may be drunk as a refreshing nutritional drink or used as a relish on the staple food (Mutukumira et al., 1995).

The paper reviews the dominant microorganisms present in indigenous fermented milk associated with different regions of Southern African. It also gives a detailed description of the diversity of microbial organisms associated with these products as well as their interactions among them.

1.2. The microbial diversity associated with raw and indigenous fermented milk of Southern Africa countries

In Africa, fermented foods and beverages play a predominant role in the diet. Most often these foods and beverages are produced at household level or at small industrial levels (Iwuoha & Eke,1996;Sanni, 1993; Zulu et al., 1997). Milk is by far the most abundant fermented animal product in Africa, even though the extent to which milk is used in the daily diet varies to a great extent. Fermented milk is mostly used in East Africa and up to 53% of the milk produced in the Kenyan highland has been reported to be consumed as fermented milk. Table 2 shows some examples of African fermented milks (Abdelgadir et al., 2001; Gadaga et al., 2000; Gonfa et al., 2001; Mutukumira, 1996; Odunfa & Oyewole, 1998; Sserunjogi, 1999).

The aim to ensure the safety of milk products, through pasteurisation and/or fermentation have been introduced to small-scale dairies in Africa (Africa Now, 2001; Gran et al., 2002a). Previous studies on the safety of milk in Africa have until now mostly analysed raw milk on the farm or on delivery (Bonsu et al., 2000; Ombui et al., 1992). The nature of fermented products is different from one region to another. The local indigenous microflora, which in turn reflect the climatic conditions of the area. Thus traditional fermented milk in regions with a cold temperature climate contained mesophillic bacteria such as Lactococcus and Leuconostoc spp., whilst thermophillic bacteria, which include mostly Lactobacillus and Streptococcus, prevail in regions with a hot, subtropical or tropical climate (Kurmann, 1994; Tamine & Robinson, 1988; Thomas, 1985).

Various technologies are used in Africa to prepare local varieties of natural fermented milk (Mutukumira et al., 1995), and these technologies also have an effect on the milk in different regions of Africa. Technologies certain to affect product characteristics include heat treatment of the milk, smoking and other treatment of the fermentation container, drainage of whey and the addition of herbs and spices

(Mutukumira et al., 1995; Narvhus, 2003). The fermentation techniques may vary from place to place but a key element influencing the quality of the fermented product is the fermentation vessel. These vessels are usually simple, made from locally available materials such as woven grass, wood fiber, calabash, hollowed wood or animal skin bags (FAO, 1990).

The microorganisms present in milk and naturally fermented milk may originate from the animal itself, from the milking equipment and environment, from personnel or from the previous product batch if back-slopping is used (Mutukumira et al., 1995). As a result, the final product may vary considerably between different regions. In addition, the limited hygiene often practised in the preparation of these products further enhances the presence of a variety of microorganisms. However, most studies of the microflora of African naturally fermented milk have concentrated on the lactic flora whilst recording only the incidence of various groups of indicative organisms such as coliforms and yeasts (Fig. 1).

1.2.1. Botswana

For the preparation of fermented milk in Botswana (madila) raw milk is obtained and transferred to 5L buckets (1L milk/bucket). The open buckets are covered with a piece of linen and kept at ambient temperatures (37ºC) for a day (24h) to allow natural fermentation. After curdling, the curd is transferred to nylon perforated bags and these bags are then hanged in trees. The curd is left in the bag until drainage of all the whey and curd used for consumption (Kebede, 2005).

1.2.2. Namibia

In Namibia most rural women depend on agriculture for household food security and for income generation in order to sustain their family livelihood. Apart from growing vegetables, cereals and raising livestock, fermented milk products are widely used for nutrition and household income generation. Apart from its medical, cosmetic and other usage, sour milk (Omasbikwa) has been developed mainly as a means of providing a variety of foods and of preserving it against spoilage (Van der Berg, 1985).

Processing is based on rural household technology. This involves accumulating milk in a gourd (or other containers) allowing it to ferment naturally for 3-4 days in the presence of Omunkunzi roots (Boscia albitrunca) and agitation (2-3 h) to churn into butter. The sour buttermilk (Omasbikwa) is the main product for the family and for income (Fig. 2). The product has a composition of 3.28% crude protein, 1.6% fat, 89.8% moisture, 0.76% ash, 4.56% lactose, 10.25 total solids (TS), 8.6% solids-not-fats (SNF) with a pH of 3.25 and no whey separation (Bille et al., 2002).

Omasbikwa is an Owambo name for traditional fermented buttermilk produced by local farmers in Namibia. It is consumed as a refreshing drink and as a condiment for other foods like gruel and thick porridge made from maize, pearl millet or sorghum flours (Bille et al., 2002).

1.2.3. Lesotho

Typical indigenous fermented milk from Lesotho is called mafi. These concentrated fermented milks are sour milks obtained by spontaneous acidification of raw milk and are subsequently partly drained. The products are white like whey. Their texture is usually curdy or granular, but some may be semi-fluid when the curd is shaken. Again, the preparations of some of these concentrated fermented milks may involve

addition of certain plant materials or their products into the fermented milk and/or smoking of the fermentation vessels (FAO, 1990; Isono et al., 1994; Kassaye et al., 1991).

1.2.4. Swaziland

Emasi is regarded an important part of people's daily diets as it is a nutritious food product in Swaziland (Beukes et al., 2001; Caplice & Fitzgerald 1999). The product is also of significant value for the people for its therapeutic properties (such as alleviating lactose intolerance and in the treatment and prevention of diarrhoea and constipation), and of social value as well as a source of income (Beukes et al., 2001; Vizoso Pinto et al., 2006).

The fermentation of the emasi milk usually takes 1 -3 days, depending on the ambient temperature (Feresu & Muzondo, 1989, 1990; Gadaga et al., 1999; Gran et al., 2003b; Mutukumira, 1995), and results in a thick lumpy liquid that is consumed as sour milk on its own or together with other food. In order to speed up the fermentation process, it is common practise to back-slop fresh milk with remains of a previous batch of fermented milk (Caplice & Fitzgerald, 1999).

1.3. Microorganisms associated with indigenous fermented milk

When the domains of individual microorganisms overlap, as observed in dairy products, it is likely that interactions will occur. The outcome of natural interactions in nature is evaluated based on the effect they have on population size regardless whether the interactions are detrimental, neutral or beneficial (Steinkraus, 1982). A wide variety of different microorganisms is in present in fermented milk. The different microorganisms are shown in Table 3 (Kurmann, 1994). When a food product is produced, however, the positive or negative aspects caused by interactions between microorganisms become very important. This interaction is important because of the combined physiology, interactions and enzymatic activities

are responsible for major biochemical and nutritional changes that occur in the substrates of fermented milk-based products (Steinkraus, 1982).

1.3.1. Bacterial species associated with interactions in indigenous fermented milk

A variety of bacterial species are known to grow in milk. Some of these bacteria are beneficial while others are harmful (Gombas, 1989; Kebede, 2005). The bacteria present in milk are devided into two categories,namely the lactic acid bacteria which have an important role in the dairy industry (Caplice & Fitzgerald, 1999) and the other bacteria in fermented milk comprise of coliforms (mainly E. coli), Pseudomonas fluorescens, Pseudomonas fragi, Bacillus, Clostridium, Cornebacterium, Arthrobacter, Lactobacillus, Microbacterium, Micrococcus and Streptococcus (Heeschen, 1996).

1.3.1.1. Lactic acid bacteria associated with indigenous fermented milk

Lactic acid bacteria (LAB) are gram-positive, non-sporulating, micro-aerophillic organisms (Axelsson, 1993). Classification and identification of LAB are based on morphology, physiology, carbohydrate fermentation patterns, cell composition and to a degree their ability to metabolize lactose. The type of metabolites produced by lactic acid bacteria can further be utilized to divide LAB into two main groups: the homofermentative and heterofermentative lactic acid bacteria (De Vuyst & Vandamme, 1994; Dillon & Cook, 1994)

Since 50% of lactic acid are formed by converting the carbon source, lactose, by a certain group of microorganisms, the general name 'Lactic Acid Bacteria' has been given to this important group of bacteria.

The presence of lactic acid, defines fermented milks due to the occurrence of LAB and acidity as one of the main properties associated with indigenous fermented milk. This is clearly indicated in the final soured milk products which are mostly consumed by African rural communities (de Vuyst & Vandamme, 1994). The variation in the occurrence of certain LAB in Southern African spontaneously fermented milk was also attributed to different container types (Kebede et al., 2006). LAB not only play a role in health benefits of humans, but their existence in fermented products indicates their importance in successful fermentation processes in industry. Starter cultures for the production of fermented milks consist of LAB (Gran et al., 2003a). Furthermore, the bio-preservation abilities associated with LAB in fermented milk products could assist in producing milk products that are microbiologically safe.

1.3.1.1.1. Aerococcus

Aerococcus is a catalase-negative coccus and has similar biochemical characteristics to enterococci, but does not have a tendency toward chain formation. Aerococcus can also be described as a “putative streptococcus”, largely because of the similarity of its fermentation reactions to those of typical streptococci (Williams et al., 1953).

1.3.1.1.2. Carnobacterium

Carnobacterium species is gram-positive, catalase-negative rods that are phylogenetically closer to enterococci and vagococci than lactobacilli (Jay, 1992). Carnobacterium is an ever-present lactic acid bacterium isolated from cold and temperate environments. They also predominate in a wide range of foods including dairy products, fish and meats (Leisner et al., 2007). Only Carnobacterium divergens and C. maltaromaticum are regularly encountered in the environment and in foods (Leisner et al., 2007).

1.3.1.1.3. Bifidobacterium

Bifidobacterium is gram-positive, non-motile, often branched and anaerobic inhabiting the gastrointestinal tract. Fermented milks using only probiotic strains, mainly belong to Bifidobacterium spp and are often characterised by the lack of desirable sensory features, texture and body (Penna et al., 2006), whereas the physical properties such as firmness and the ability to retain water are the major criteria for quality assessment (Hassan et al., 1996).

It is important that Bifidobacterium survive in fermented dairy products until consumption. The viability of bifidobacteria strains depends on the degree of acidification and on the bacterial strains, fermentation conditions, storage temperature, and preservation methods and is mainly limited by their sensitivity to the acidity (Shah, 1997). The ingestion of specific bifidobacteria could contribute to re-establishment of a bifidobacterial flora in humans after antibiotic therapy. Their establishment will lead to alleviation of constipation, prevention against diarrhoea and other gastrointestinal infections and alleviation of the symptoms of lactose intolerance (O'Sullivan & Kullen, 1998).

1.3.1.1.4. Enterococcus

Enterococcus is a gram-positive, catalyse-negative coccus that is a homofementative lactic acid bacterium (Franz et al., 2003). Although enterococci are commonly found in artisental fermentations, components of some mixed starter cultures play an important and positive role in the production of a variety of traditional food products and may successfully be used as probiotics (Franz et al., 1999, 2003). The most frequently isolated enterococci in dairy products belong to the species Enterococcus faecalis, Enterococcus faecium and Enterococcus durans (Franz et al., 2003).

3.1.1.5. Lactobacillus

The genus Lactobacillus is a gram-positive rod forming microorganism and comprises the largest group of species included in the LAB. Most species of lactobacilli are homofermentative, but some are heterofermentative (Ortu et al., 2007). Nordic ropy milk is the generic name for fermented milks with mesophillic cocci which produce slime (Duboc & Mollet, 2001). While traditionally produced at home, these products are also industrially manufactured. The major LAB used are Lactobacillus delbrueckii spp. bulgaricus and L. helveticus for the thermophilic bacteria (Duboc & Mollet, 2001).

Lactobacillus helveticus contains Ile-ProPro and Val-ProPro which is related to reduce arterial stiffness (Jaunhiainen et al., 2007). Lactobacillus reuteri produces reuterin during stationary phase of growth (Axelsson et al., 1989) and (Hosono et al., 1986) have demonstrated that milk cultured individually with Lactobacillus delbrueckii spp. bulgaricus exhibited antimutagenic activity.

1.3.1.1.6. Lactococcus

Lactococcus species are gram-positive, non-montile, catalase-negative spherical or ovoid cells that occur singly, in pairs or as chains. Lactococci are mesophillic and homofermentative (Weimer et al, 2000). At the turn of the twentieth century identified lactococci as the essential components of the mesophilic microflora in spontaneously fermented cream and milk. This finding led to the introduction of pure starter cultures of lactic acid bacteria to the dairy field for use in the fermentation and ripening of milk (Wiegmann, 1905).

The biochemical and technological functions of lactococci necessary for milk fermentation can be summarized as follows:

1. Formation of lactic acid from lactose. Starter bacteria for this purpose are Lactococcus lactis subsp. lactis and L. lactis subsp. cremoris.

2. Formation of diacetyl from citrate: Is the most characteristic aroma compound provided by Lactococcus lactic biovar "diacetylactis".

The species Lactococcus lactis and its subspecies used on large scale by the dairy industry are generally recognized as safe (GRAS) for human consumption and are therefore deliberately used in the dairy industry as starter cultures for many different products (Ross et al., 2002).

1.3.1.1.7. Leuconostoc

The genus Leuconostoc is gram-positive, facultatively anaerobic, catalase-negative, cocci or coccobacillus. This species is heterofermentative and requires complex growth factors and amino acids (Ross et al., 2002). The major Leuconostoc species used in fermented milk are Leuconostoc mesenteroides spp. cremoris and dextranicum (Duboc & Mollet, 2001).

1.3.1.1.8. Pediococcus

Pediococcus is a homofermentative lactic acid bacterium and these species are not capable to ferment lactose and therefore their application in milk fermentations is restricted (Caldwell et al., 1996; Ross et al., 2002).

1.3.1.1.9. Streptococcus

The genus Streptococcus is gram-positive, non-sporulating, catalase-negative, cocci. Streptococci are also homofermentative lactic acid bacteria (Ross et al., 2002). Hosono et al., (1986) have demonstrated that milk cultured individually with Streptococcus salivarius ssp. thermophilus exhibited antimutagenic and antitumor

activity (Hosoda et al,. 1992). Streptococcus thermophilus metabolize the glucose moiety of lactose and export the galactose moiety into the medium via an antiport sytem for lactose uptake (Hutkins & Ponne, 1991; Poolman, 1993). This species belonging to the Streptococci contributes to the rheological properties of fermented milk (Zacarchenco & Massaguer-Roig, 2006).

1.3.1.1.10. Vagococcus

Vagococcus is defined as a catalase-negative coccus species and its most prominent characteristic is its motility, and therefore they were earlier referred to as motile 'lactic' or group N streptococci. The closest relatives of Vagococcus based on phylogenetic studies are the genera Carnobacterium and Enterococcus (Wallbanks et al., 1990).

1.3.1.2. Metabolism of lactic acid bacteria

Lactic acid bacteria are chemotrophic, they find the energy required for their entire metabolism from the oxidation of chemical compounds. They assimilate sugars by either a homofermentative pathway or a heterofermentative pathway. Based on sugar fermentation patterns, two broad metabolic categories of LAB exist: homofermentative and heterofermentative. The first category, homofermentative LAB, includes some lactobacilli and most species of enterococci, lactococci, pediococci, streptococci, tetragenococci, and vagococci that ferment hexoses by the Embden-Meyerhof (E-M) pathway. The second category, heterofermentative LAB, includes leuconostocs, some lactobacilli, oenococci, and weissella species. The apparent difference on the enzyme level between these two categories is the presence or absence of the key cleavage enzymes of the E-M pathway (fructose 1, 6-diphosphate) and the PK pathway (phosphoketolase).

1.3.1.2.1. Homofermentative metabolic pathway

Homofermentative LAB transforms nearly all of the sugars they use, especially glucose into lactic acid using the glycolytic pathway (Fig. 3).

1.3.1.2.2. Heterofermentative metabolism pathway

Heterofermentative LAB uses the pentose phosphate pathway. This pathway occurs in the cytosol. Its destination is completely different from the homofermentative pathway (Fig. 4) (De Vuyst & Vandamme, 1994; Dillon & Cook, 1994).

1.3.1.3. Importance of lactic acid bacteria as a starter culture

A number of studies have shown that using starter cultures increases the safety of many fermented foods. The major technological importance of starter cultures is to produce large amounts of lactic acid from lactose. A biotechnologically essential starter strain should produce a sufficient intensity of acid during initial stages of the industrial fermentation process and favourable low after-acidification conditions during storage. However, the maximum benefit of using starter cultures depends in such factors as the initial level of contamination of the raw materials, levels of hygiene and sanitation, and starter culture activity (Mortarjemi, 2002).

1.3.1.4. Pathogenic bacteria associated with indigenous fermented milk

Pathogens have the potential to survive under severe environmental conditions and have been isolated from various fermented foods. This indicates that pathogens are capable of growing in the fermented foods or surviving the fermentation process. Pathogens that are found in fermented foods came from the respective raw materials or from the handlers (Nyatoti et al., 1997). The microbial spoilage of milk is generally associated with the growth of bacteria (Bishop & White, 1986; Cousin, 1982).

1.3.1.4.1. Escherichia coli

Escherichia coli are one of the major pathogens isolated from milk. The normal habitat of E. coli is animal faeces, which can contaminate raw milk, especially if the animals have been lying in their own dung. Several studies of naturally soured raw milk have reported high numbers of coliforms (up to 8 log cfu ml^{-1}) and Escherichia coli (up to 7 log cfu ml^{-1}), indicating that spontaneous LAB fermentation does not necessarily eliminate these organisms (Feresu & Nyati, 1990; Gran et al., 2002a; Simango, 1995). An assessment of the infective dose of enterotoxicenic E. coli (ETEC) indicates that a relatively large dose of at least log 5 to log 8 is probably necessary to establish colonization of the small intestine, where these organisms proliferate and produce heat-stable toxins (ST) which induce fluid secretion (Wasteson, 1999). The STs are small, monomeric peptides, which contain multiple cysteine residues. Thus, when large numbers of ETEC are ingested, diarrhoea can be induced (Nataro & Kaper, 1998).

1.3.1.4.2. Listeria monocytogenes

Another major pathogen is Listeria monocytogenes which has also been found to survive in naturally soured raw milk fermentation (Dalu & Feresu, 1996). Listeria monocytogenes has been implicated in several food borne outbreaks associated with consumption of pasteurized milk (Fleming et al., 1985). The pathogen can cause bovine mastitis and is occasionally found in raw milk (Liewen & Plautz, 1988; Louett et al., 1987). Although L. monocytogenes is destroyed by pasteurization, several studies have reported its heat resistance and its ability to survive pasteurization due, in part, to the protective nature of leukocytes in which the pathogen may be present (Doyle et al., 1987; Fleming et al., 1985; Louett et al., 1987).

1.3.1.4.3. Staphylococcus aureus

Staphyloccocus aureus is frequently found in raw milk and just as many times on the human skin. Dissemination of S. aureus from humans to food can occur by direct contact, indirectly by skin fragments, or through respiratory tract droplet nuclei (Jablonski & Bohach, 1997). S. aureus is also commonly found in a mastitis udder (Wellenberg et al., 2002). Milk from mastitis cows could therefore be another reservoir for S. aureus. In food, the minimum amount of S. aureus required to produce intoxication in humans being estimated to be about 5 log CFU g^{-1} (Rørvik & Granum, 1999). To produce sufficient enterotoxin, the pH should be higher than 4.6 and the temperature should be above 15 ºC for more than 3 - 4 h (Rørvik & Granum, 1999). If S. aureus gains access to the milk before fermentation, the pH would have been higher than 4.6 for longer than 6 h, and therefore a definite risk of toxin production during the early part of the fermentation.

1.3.1.4.4. Mycobacterium

Mycobacterium bovis (bovine tuberculosis) and Mycobacterium tuberculosis are also often found in milk and milk products (Bonsu et al., 2000; Schmiedel, 1968; Weinhaupl et al., 2000).

1.3.1.4.5. Campylobacter jejuni

Campylobacter jejuni is also a typical milk-borne pathogen and may cause outbreaks of diseases (Varnam & Sutherlamd, 1994). Symptoms of food-poisoning from Campylobacter include fever, diarrhea and abdominal pain (Hahn, 1994; Nachamkin, 2001).

1.3.1.4.6. Salmonella

Salmonella spp. is small, facultative anaerobic, gram-negative, non-sporing rods. Salmonella grows optimally at 37º C but depending on the substrate or other conditions the growth temperatures range between 5-47º C (D'Aoust et al., 2001). Salmonella spp. is one of the most prevalent pathogens that have resulted in foodborne diseases in humans. Although Standard HTST pasteurization is effective for the destruction of Salmonella in milk, in traditional fermenting processes fermentation usually takes place without pasteurization (Vlaemynck, 1994).

1.3.1.4.7. Yersinia enterocolitica

Facultative anaerobe, gram-negative, psychrotrophic rod, is some of the characteristics to describe Y. enterocolitica. Y. enterocolitica usually contaminates raw milk from cows as well as goats and can cause yersiniosis, a gastro-enteritis of humans (Robins-Browne, 2001).

1.3.1.4.8. Bacillus cereus

Bacillus species are aerobic or facultative anaerobic, gram-positive, catalase-positive, spore-forming rods (Fung, 1987). All the food-poising Bacillus spp. belongs to the mesophilic group with optimum growth temperatures between 30-45º C. Bacillus cereus is a particular difficulty to the dairy industry as it contaminates the udder of the cows and then contaminates the milk during milking. Two types of enterotoxin produced by B. cereus result mainly in either emetic or diarrheal diseases (Gould & Russell, 2003; Granum & Baird-Parker, 2000).

1.3.2. Yeasts associated with indigenous fermented milk

Yeasts are eukaryotic microorganisms and may be defined as unicellular fungi in which asexual reproduction occurs mainly by budding (Deak & Beuchat, 1996). In dairy products yeasts may interact with other microorganisms in three different ways: i) they may inhibit or eliminate microorganisms which are undesired because they cause quality defects or possess potential pathogenic characters: ii) they may inhibit the starter culture, or iii) they may contribute positively to the fermentation or maturation process by supporting the function of the starter culture (Deiana et al., 1984).

Beukes et al., (2001) and Ferezu & Muzondo (1990) conveyed studies on the microorganisms present in African naturally fermented milk and reported on the presence of yeasts, but no indication of the species present. The isolation of yeasts from sethemi, African fermented milk, has been studied by Kebede et al., (2006). Studies confirmed that yeasts can occur at numbers of 1×10^3 ml^{-1} or can be absent in fermented milks (Kebede et al., 2006).

According to Fleet & Balia (2006), there are five prevalent species in fermented dairy products. They are catergorized as follow:

1.3.2.1. Debaryomyces

Debaryomyces hansenii is ahalo-tolerant yeast (Bintsis et al., 2003; Petersen et al., 2002). In recent years, the interest in this species has increased as related to its physiology, biochemistry and genetic aspects with impact in industrial fermentations. In several studies have demonstrated the successful use of D. hansenii to produce flavourful fermented products (Bolumar et al., 2005).

1.3.2.2. Yarrowia

The ability of Yarrowia lipolytica to predominate in real system on the naturally occurring yeast and its compatibility with starter cultures has been evidenced. The released fatty acids can further be transformed into desirable or undesirable volatile or non-volatile compounds with characteristic aroma. Therefore, the selected strain of Y. lipolytica can be used for a co-starter, based on their ability to hydrolyse milk fat (Guerzoni et al., 1998; van den Tempel & Jakobsen, 2000).

1.3.2.3. Candida

Candida kefyr is an example of yeast that is present in fermented milk that has probiotic properties. The fermented milk products kefir and koumis are frequently noted for their health-promoting, probiotic properties. (Beshkova et al., 2002; Frohlich-Wyder, 2003; Oberman & Libudzisz, 1998; Witthuhn et al., 2005).

Organisms such as Candida parapsilosis, Candida tropicalis and Candida albicans are capable of causing human disease in opportunistic circumstances (Hazen, 1995). Candida albicans are well known in this regard, and are responsible for causing a range of mucocutaneous, cutaneous, respiratory, central nervous and systemic infections (Fleet & Balia, 2006).

1.3.2.4. Kluyveromyces

Kluyveromyces lactis uses lactose as a source of carbohydrate to produce fermented milk with a high nutritional value (Vrignaud, 1971).

1.3.2.5. Saccharomyces

It appears that Saccharomyces cerevisiae var. boulardii has been listed as a potential human probiotic (Fleet & Balia, 2006). It produces a screne protease which degrades specific diarrhoea-causing toxins produced by Clostridium diificile, as well as the receptor sites for these toxins on the colonic mucosa (Czeruoke & Rampal, 2002; van der Au Kuhle & Jespersen, 2005). The yeast colonises the intestinal tract, but is eliminated once administration is stopped, or the patient is given fungal antibiotics. The yeast has been reported to be effective in treating antibiotic associated diarrhoea, traveller's diarrhoea, Crohn's disease and other inflammatory bowel disorder (Czerucka & Rampal 2002; Fleet & Balia, 2006).

1.3.3. Moulds associated with indigenous fermented milk

Moulds contamination of dairy products is a disturbing problem in the dairy industry and cases of contamination by different types of moulds are frequently recorded. Moulds can grow well in dairy products when oxygen is present, with the low pH being selective for them. Moulds are commonly found growing in vacuum-packaged cheeses include Penicillium spp. and Clostridium spp. (Hocking & Faedo, 1992). Many mould species are able to utilize most carbon-sources derived from food and some of them can also utilize nitrate, ammonium or organic nitrogen as a nitrogen source. Therefore moulds are able to grow in a wide range of food products (Batish et al., 1997). Some important mould genera associated with dairy products include Aspergillus, Penicillium, Rhizopus, Mucor, Cladosporium, Alternaria, Geotrichum and Fusarium (Roy et al., 1996).

1.3.3.1. Geotricum candidum

Geotrichum candidum usually originate from air, water equipment and staff (Plocková et al., 2001; Roy et al., 1996).

1.3.3.2. Penicillium spp.

Penicillium spp. are commonly isolated from the air and soil. Penicillium roqueforti and Penicillium camemberti are typically used to produce mold-ripend cheeses. Penicillium spp. is also found to be the dominant fungal contaminant in all dairy products (Hoekstra et al., 1998).

1.3.3.3. Aspergillus spp.

Aspergillus spp. are found in the air and soil and are associated with living and decaying plants and animals. A brilliant display of colour is associated with certain species of Aspergillus (Raper & Fennell, 1965). Aspergillus penicillioides and A. versicolor were isolated from cheese factories and warehouses and is found to be a contaminant in a study by Hoekstra et al. (1998).

1.4. Microbial interactions present in fermented dairy products

Milk is an excellent protective medium encouraging the proliferation of many diverse microorganisms (Oberman, 1985). When the domains of individual microorganisms overlap, as observed in dairy products, it is likely that interactions will occur. The outcome of natural interactions in nature is evaluated based on the effect they have on population size (Steinkraus, 1982). In the mixed populations of fermented milk there are different types of microbial interactions. They can be classified on the basis of effects, as direct or indirect interactions. Indirect interactions refer to competition, commensalism, mutualism, ammensialism or neutralism (Linton & Drozd, 1982), and direct interactions to predation and parasitism (Bull & Salter, 1982; Fredrickson, 1977).

It has been well documented that the metabolism of microorganisms can have profound effects on the characteristics of any spontaneously fermented milk product. More complicated is the interaction of complementary metabolisms, where a compound produced by one organism may be metabolised further by another

(Kebede et al., 2006).

Indigenous fermented dairy products are produced predominantly by lactic acid bacteria present in the raw milk containers, acting as starter cultures. The occurrence of yeasts in association with LAB has indicated that there might be interactions between the two microorganisms affecting the product (Narvhus & Axelsson, 2003). Yeasts and LAB growing together might either be stimulation or inhibition of growth of one, or both, of the co-cultured strains (Marshall, 1987; Viljoen, 2001).

1.4.1. Neutral interactions in milk

Three types of mutualism (synergism) occur during fermentation of milk. Firstly between yeasts and lactic acid bacteria (Loretan, 1999; Rossi, 1978). The yeasts provide growth factors like amino acids, vitamins and other compounds for bacterial growth which consequently lead to elevated acid production, while the bacterial end-products are used by the yeasts as an energy source (Loretan, 1999). Stable co-metabolism between LAB and yeasts is common in many foods, enabling the utilization of substances that are otherwise non fermentable (for example starch) and thus increasing the microbial adaptability to complex food ecosystems (Gobetti et al., 1994; Gobetti & Corsetti, 1997; Stolz et al., 1995).

The yeasts and lactic bacteria both have a positive effect on each other. It has been suggested that the proliferation of yeasts in foods is favoured by the acidic environment created by LAB while the growth of bacteria is stimulated by the presence of yeasts, which may provide growth factors, such as, vitamins and soluble nitrogen compounds (Nout, 1991). Growth of yeasts in milk products is attributed to the ability of the yeasts to utilise milk constituents, such as proteins, fat, lactose and citrate (Fleet, 1990). Other reports also attributed this growth in part to symbiosis with other microflora in the mixed culture (Koroleva, 1988). The presence of a lactose-negative but lactate-positive yeasts in co-culture with LAB in milk can initiate a continuum whereby lactate assimilation slightly increase the pH, which then allows

further growth and lactose metabolism by LAB leading to increased lactate production. Evidence of such a synergism was reported (Cheirsilp et al., 2003) from studies based on the co-culture of Lactobacillus kefiranofaciens and S. cerevisiae.
The second mutualistic interaction can be found among bacterial interactions when a Lactobacillus delbrueckii subsp. bulgaricus and Streptoccocus salvarius subsp. thermophilus co-culture are inoculated in milk to produce the characteristical flavour and texture (Kebede, 2005).
The third type of mutualistic interaction exists between filamentous fungi which provide the necessary enzymes for the degradation of complicated substrates like cellulose in co-culture with yeasts by means of commensalisms and mutualism (Viljoen, 2006).

1.4.2. Negative interactions in milk

The negative interactions recorded mainly concern the mutual inhibition of growth. Yeasts are inhibited by LAB-produced compounds such as phenyl-lactic acid, 4-hydroxy-phenyl-lactic and cyclic peptides (Nielsen et al., 1998); conversely, the growth of LAB is inhibited by fatty acids produced by the metabolism of lipolytic yeasts (Broome et al., 1979).

1.4.2.1. Negative effects caused by bacteria

Spoilage bacteria can be sub-divided into three groups of microorganisms, according to their technological characteristics, such as glycolytic, proteolytic and lipolytic activity (Heeschen, 1996; Wouters et al., 2001). The groups of microorganisms are organized as follows:

- Glycoltes degrades carbohydrate (e.g. streptococci and lactobacilli).

- Proteolytes degrade protein (e.g. pseudomonads, enterobacteriaceae, aerobic spore-formers etc.)

- Lipolyte degrade lipids (e.g. pseudomonads, micrococci, cornebacteria etc).

There are also positive correlations between pathogenic microorganisms. The presence of E. coli and S. aureus may in addition suggest cross contamination of the products, indicative that the simultaneous growth of E. coli and S. aureus in milk is synergistic. Studies showed the presence of OP's and high numbers of E. coli and S. aureus in milk products from three small-scale dairies in Zimbabwe (Bonsu et al., 2000; Schmiedel, 1968; Weinhaupl et al., 2000). Raw milk had the lowest number of E. coli and S. aureus cells, but the highest prevalence of opportunistic pathogenic microorganisms, indicating that E. coli and S. aureus are present in milk because of contamination. Fifty-nine percent of the cultured pasteurised milk samples contained neither opportunistic pathogens nor levels of E. coli and S. aureus regarded as harmful (<5 log10 CFU ml^{-1}). Nevertheless, 6 of 27 (22%) cultured pasteurised milk samples were contaminated with both E. coli and S. aureus at counts at > 5 log10 CFU ml^{-1}. Cultured pasteurised milk was prepared from pasteurised milk, and it can therefore be concluded that cultured pasteurised milk was contaminated after pasteurisation. Factors that could have increased the possibility for contamination of the products were limited understanding of hygienic principles, deficient design and layout of equipment and premises, and underdeveloped maintenance and hygiene control systems (Bonsu et al., 2000; Schmiedel, 1968; Weinhaupl et al., 2000).

Milk produced under traditional systems tends to have lower bacterial counts than milk produced under mechanical milking in temperate countries (IDF, 1997). This is characteristic of the most indigenous fermentation processes, and it is obvious that the natural (microflora) of the milk, to a large degree, remains similar. However, in well developed fermentation plants, the production of dairy products is governed by

pasteurization killing all pathogens. Pathogenic bacteria, if present, are only able to multiply and cause food borne diseases by post pasteurization contamination. Therefore the inhibitory effects of microorganisms associated with indigenous fermented products, could assist in developing less contaminated products in fermentation plants. It is important to note that the existence of pathogenic microorganisms is distinct for a specific product.

1.4.2.2. Negative effects caused by yeasts

Yeasts as spoilage organisms role in dairy products is linked with their nutritional requirements, certain enzymic activities and the ability to grow at low temperatures, low pH values, low aw and high salt concentrations (Engel, 1988; Fleet & Mian, 1987; Fleet, 1990; Rohm et al., 1992; Seiler, 1991; Tudor & Board, 1993). Compared with other microbial groups, yeasts are not seen as aggressive pathogens, but they are capable of causing human disease in opportunistic circumstances (Tudor & Board, 1993).

High numbers of yeasts are frequently observed on processing equipment, and in the air of the processing environment (Viljoen & Greyling, 1995; Welthagen & Viljoen, 1998, 1999). Normally, we may attribute the contamination of equipment to poor hygienic practices. Laubscher & Viljoen (1999), however, reported resistance of the dominant dairy associated yeasts to commercial sanitizers and cleaning compounds, that indicates that the yeast are not likely to occur form the raw milk. Yeasts like Debaryomyces hansenii, Candida versatilis, Torulaspora delbrueckii, and other showed strong resistance, even after 60 min of exposure. None of the nine commercial cleaners and sanitizers examined sufficiently inhibited or killed the contaminating yeasts. Therefore it is possible that the yeasts may colonize during cleaning and sanitation cycles (Laubscher & Viljoen, 1999).

Although yeasts are well known for producing fermented foods and beverages, as sources of food ingredients and as spoilage yeasts, their public health significance in foods has largely been overlooked (Fleet & Balia, 2006). Yeasts grow well during the manufacture and ripening of fermented dairy products due to their low tolerance of low pH, low aw and high salt concentrations (Roostita & Fleet, 1996). Such growth can have negative effects such as gas production, yeasty flavours and other off-flavours, discolouration and changes of texture that results from their growth (Jakobsen & Narvhus, 1996).

1.4.3. Positive interactions in milk

The International Dairy Federation (1997) has defined fermented milk as "a milk product fermented by the action of specific microorganisms and resulting in reduction of pH and coagulation. These specific microorganisms shall be viable, active and abundant (at least 10^7 CFU/g) in the product to the date of minimum durability". Many health benefits have been attributed to fermented dairy products by microorganisms (Salminen et al., 1998). In order to exert positive health effects, it is generally assumed that the microorganisms need to be viable. The use of non-viable instead of viable microorganisms would have economic advantages in terms of longer shelf-life and reduced requirements for refrigerated storage (Ouwehand & Salminen, 1998).

Positive microbial interactions in dairy products may contribute differentially to the final product. The association of LAB and yeasts during fermentation may contribute to the production of additional metabolites, which could impart taste and flavour to foods (Akinrele, 1970; Brauman et al., 1996; Halm et al., 1993; Hansen & Hansen, 1996). The commensalistic interaction between Lactobacillus acidophilus and the lactose fermenting yeast, Kluyveromyces fragilis, in acidophilus- yeast milk (Subramanian & Shankar, 1983) relies on the co-existence of both organisms to secure a good product (Subramanian & Shankar, 1983). Furthermore, the combination of low pH produced by the bacterial starter plus the alcohol and CO_2 produced by the yeasts are inhibitory to many undesirable microorganisms (Ferreira & Viljoen, 2003).

1.4.3.1. Flavour to foods

Lactic acid bacteria that produce the lactic acid give the fermented product a sour taste and also result in the formation of a smooth gel. In addition to this, various flavour compounds are formed and these are responsible for the specific taste of different products. Such flavour compounds can be formed from citrate, when the important flavour compounds diacetyl, acetic acid and carbon dioxide are formed. The main attribute of diacetyl during the fermentation of milk is flavouring and enhancing the quality, as observed in nono, a well produced fermented milk in Nigeria (Bankole & Okakbue, 1992).

Other flavours like malty flavour compounds may also be formed from branched chain amino acids by some strains of Lactococcus and Lactobacillus (Ayad et al., 1999; Narvhus et al., 1998).

1.4.3.2. Improved microbial quality

The production of acids and other antimicrobial components in gruel during fermentation may promote or improve the microbiological safety (Kingamkono et al., 1994, 1995; Nout et al., 1989; Svanberg et al., 1992) and stability of the products (Mensah et al., 1991). Yeasts, however, play an essential role in the preparation of certain fermented dairy products (Gobetti & Rossi, 1992; Marshall, 1986; Marth, 1978) and contribute substantially to the final product. These contributions are attributed to various interactions between the yeasts, starter cultures of lactic acid bacteria, and the secondary flora of bacteria and moulds (Welthagen & Viljoen, 1998, 1999).

1.4.3.3. Immune-stimulation

Lactobacilli and their metabolic products have been observed to modify both the immune responses. The host immune system appears to enhance mainly by activating of natural killer cells and T-cells (Kato et al., 1994).

1.4.3.4. Anti-mutagenic activity

It has been suggested that intestinal dysfunctions such as colon cancer are indirectly caused by the altered activity of bacterial enzymes from the indigenous microflora. Several bacterial enzymes have the ability to generate mutagens, carcinogens and tumour promoters from dietary compounds. Modification of the activity of these enzymes is of great interest (Goldin, 1990).

As presented in Table 4, in particular viable probiotic microorganisms appear to have the ability to reduce faecal enzyme activity. For the removal of carcinogens, non-viable microorganisms perform as well or even better, as in the case of aflatoxin binding (El Nezami et al., 1998).

1.4.3.5. Antitumor activity

Antitumor activities of fermented milks and related lactic acid bacteria have been studied, and the majority of the resent published reviews suggested that viable lactic acid bacteria and fermented dairy products possess anticarcinogenic properties (Hosoda et al., 1992).

1.4.3.6. Probiotics

Probiotics are defined as live microbial food supplements which beneficially influence the product by improving its intestinal microbial balance (Ouwehand & Salminen, 1998). Probiotic bacteria are considered 'live microorganisms which when administrated in adequate amounts confer a health benefit on the host' (WHO/FAO, 1996). Benefits include reduction in the incidences of diarrhoea, constipation and bowel cancer, stimulation of the immune system, reduction in serum cholesterol levels, and enhanced nutritional uptake. Fermented milks obtained using only probiotic strains, mainly belong to Bifidobacterium spp, Lb. acidophilus as well as some strains of S. cerevisiae. Isolates from African indigenous fermented foods have been shown to have promising probiotic potential (Penna et al., 2006).

1.4.3.7. Inhibition of spoilage and pathogenic microorganisms

Antimicrobial effects present in fermented products and beverages are attributed to organic acids, antibiotic factors, volatile acids, hydrogen peroxide and to a number of substrates excreted in the products (Bankole & Okagbue, 1992; Borregaard & Arneborg, 1998). These antimicrobial effects are the result of the presence of several kinds of microorganisms involved in the fermentation and putrefaction of products which inevitably lead to beneficial or detrimental interaction among the population (Bull & Slater, 1982). These interactions may lead to the inhibition of the growth of undesired microorganisms by lowering the pH, the secretion of alcohol and CO2 production, or encouraging the growth of the starter cultures by increasing the pH due to the utilization of organic acids (Devoyod, 1990; Kaminarides & Laskos, 1992; Robinson & Tamine, 1990; Schlesser et al., 1992; Seiler, 1991; Welthagen & Viljoen, 1999). The inhibitory properties of fermented foods are usually assessed based on their ability to reduce diarrhoea/ or improve microbial quality and antimicrobial activity in vitro. Mbugua and Njenga (1991) reported that the levels of S. aureus, Salmonella typhimurium, and enteropathogenic E. coli declined during fermentation.

Hence yeasts and LAB have immense potential as tools in tackling the problem of mycotoxins. There are many studies on the fate of mycotoxins during the fermentation of beverages (Daly et al., 1998). Daly et al., (1998) reported that added toxin remained in the spent grains containing yeasts cells indicating possible binding to the cells. However, there are not many reports on levels of different mycotoxins in fermented foods and case control studies on effects of food fermentation on levels of different mycotoxins in fermented food.

1.4.3.8. Bacteriocins

A recent definition of bacteriocins produced by lactic acid bacteria suggests that they should be regarded as extracellularly released primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity. They should include at least some strains of the same species as the producer bacterium against which the producer strain has some mechanism(s) of specific self protection (De Vuyst & Vandamme, 1994; Jack et al., 1995). The possibility of exploiting bacteriocins in food fermentations arises where the inhibitory spectrum includes food spoilage and/or pathogenic microorganisms. The target of bacteriocins is the cytoplasmic membrane is because of the protective barrier provided by the LPS of the outer membrane of gram-negative bacteria, they are generally only active against gram-positive cells (Ray, 1993).

Many bacteriocins are most active at low pH (Garcia-Garcera et al., 1993) and there is evidence that bacteriocinogenic strains can be readily isolated from fresh and fermented milk (Schillinger & Lücke, 1989).

1.4.3.8.1. Classification and nomenclature of bacteriocins

The classification of bacteriocins is based on molecular mass (obtained using retention in dialysis membranes, ultrafiltration, mass spectrometry or molecular sizing) and inhibition spectrum. Based on research of individual properties of bacteriocins, they are classified under the following classes, classes I, II, III and IV (Table 5) (Daly et al., 1998).

Class I

They are small heat-stable proteins containing 19-37 amino acids, and originally contain serine, threonine and cysteine residues which are post-translational modified to obtain a mature bacteriocin. Due to these thioether bridges, a number of intra-molecular rings are formed, conferring a polycyclic structure to lantibiotics andhence they are generally known as Lantibiotics.

It is produced by strains of L lactis subsp. lactis and has a broad inhibitory spectrum against gram-positive bacteria, including pathogens that can prevent outgrowth of Bacillus and Clostridium spores (Deaschel, 1989).

Class II

Class II bacteriocins are called non-lantibiotics and they are bacteriocins which have been considered the largest group of all bacteriocins produced by lactic acid bacteria. In their proteinaceous state they are unmodified, heat stable and can further be dived into three groups, mainly classes II A, B and C (Gonzalez & Kunka, 1987).

Class IIA

Pediocins are produced by Pediococcus spp. and while they are not very efficient against spores they are more effective than nisin in some food systems (Gonzalez & Kunka, 1987).

Class IIB

Class IIB bacteriocins have been classified under both non-lantibiotic and lantibiotic two-peptide bacteriocins. (Abee et al., 1995).

Class IIC

Class IIC bacteriocins contain all other non-lantibiotic bacteriocins, which do not belong to classes IIA or IIB (Abee et al., 1995).

Class III

Bacteriocins belonging to Class III are large (> 15000 Da) heat labile proteins which are inactivated within 10-15 min at 60 – 100 ºC. Examples of Class III bacteriocins include Helveticin J, Acidophilucin A, Lacticin A and B, Caseisin 80 (De Vuyst and Vandamme, 1994).

1.4.3.8.2. Aspects to be considered in the use of bacteriocins in fermented foods

The use of more than one bacteriocin or bacteriocin-producing strain in a specific food system must be carefully controlled so that mutants resistant to one antimicrobial will not be cross-resistant to the others (Rekhif et al., 1994). The implications of resistance arising from general mechanisms such as the alteration of membrane fluidity have to be studied in relation to resistance to other antimicrobial agents. Nisin is the only bacteriocin with GRAS status for use in specific foods and

this was awarded as result of a history of 25 years of safe use in many European countries and was further supported by the accumulated data indicating its nontoxic, non allergenic nature (Federal Register, 1988).

1.4.3.8.3. Application of bacteriocins as biopreservatives

Undoubtedly, the most well-known and studied bacteriocin is nisin, the lantibiotic which has found application as a shelf-life extender in a broad range of dairy and non dairy products worldwide (De Vuyst & Vandamme, 1994).

Nisin has also been investigated and demonstrated to be effective in a range of food products which include processed cheese, cheese spreads and milk products (De Vuyst & Vandamme, 1994).

1.4.3.9. Positive effects associated with yeasts

Yeasts may produce vitamins that enhance the growth of LAB. Furthermore, mutual influence of the microorganisms on each other's metabolism may lead to different profiles of organoleptically important compounds in the fermented milk (Addis et al., 2001; Corsetti et al., 2001). The yeasts as part of the interactions, either contribute to the fermentation by supporting the starter cultures (Jakobsen & Narvhus, 1996), inhibiting undesired microorganisms causing quality defects (Deiana et al., 1984; Gedek, 1991; Siewert, 1986) or adding to the final product by means of desirable biochemical changes like the production of aromatic compounds, proteolytic and lipolytic activities (Besançon et al., 1992; Fernandez Del Poza et al., 1988a,b; Fleet, 1990; Hostin & Palo, 1992; Lubert & Frazier, 1955; Machota et al., 1987; Nunez, 1978; Szumski & Cone, 1962).

Saccharomyces cerevisiae the best known yeast worldwide, have been found to stimulate the growth of other microorganisms, including lactic acid bacteria, by providing essential metabolites such as pyruvate, amino acids and vitamins. On the other hand, S. cerevisiae has been reported to utilise certain bacterial metabolites as

carbon sources (Gadaga et al., 2001b; Leroi & Pidoux, 1993). However, the mechanisms have not been described in detail. S. cerevisiae as well as several other yeast species have been reported to have pectinase activity that could be of importance for the substrate availability for other microorganisms and for subsequent microbial degradation of complex molecules. For S. cerevisiae pectinase activity has been ascribed to an endo-polygalacturonase encoded by a PGU1 gene on chromosome X (Blanco et al., 1998, 1999). The presence of pectinase activity in yeasts could especially be of importance in the fermentation of cocoa and coffee, as well as in other indigenous fermented products where degradation of pectic substances is desired (Agate & Bhat, 1996).

Despite the fact that Saccharomyces isolates have been reported to have probiotic effects (Gedek, 1991; Jakobsen & Narvhus, 1996), the probiotic effects of strains of S. cerevisiae isolated from indigenous fermented products have never been investigated. In clinical trials Saccharomyces isolates have been reported to be effective in the treatment of acute infantile gastroenteritis and diarrhoea following treatment with antibiotics (Rodrigues et al., 1996), and have been shown to inhibit infections with C. albicans (Berg et al., 1993; Ouwenhand & Salmines, 1998), Salmonella typhimurium and Shigella flexneri (Rodrigues et al., 1996) as well as Clostridium difficile (McFarland et al., 1994). For the latter the effect has been shown to be due to a reduction in the binding of C. difficile toxins A and B by the inhibition of toxin-receptor binding, probably due to secretion of a protease that digests both the toxins and the intestinal receptor for these toxins (Castagliuolo et al., 1999). Furthermore, Saccharomyces strains have been found to combat cholera toxins probably by the adhesion of the toxin to receptors on the yeast surface (Brandão et al., 1998). Saccharomyces isolates have also been observed to modulate the host immune response by stimulating sIgA production and the phagocytic system in mice (Rodrigues et al., 2000).

The application of yeasts as therapeutic agents has been described for Saccharomyces boulardii (Klein et al., 1994). Further, the 'killer factor', a well-known phenomenon in industrial yeasts, has been reported to be effective against

enterobacteria (Brugier & Patte, 1975; Polonelli & Morace, 1986). It should be mentioned that yeasts produce metabolites like short-chain fatty acids and other specific compounds, with known toxic effects against undesired microorganisms in the intestinal tract (Gedek, 1991). It appears that S. cerevisiae can survive passage through the intestinal tract, with live cells detectable in the small intestine (Gedek, 1991; Gedek & Hagenhoff, 1988). This further accentuates the possible use of yeasts as probiotics.

Except for the effect of yeasts on pathogenic bacteria there is an interactive relationship between yeasts and filamentous fungi that consists primarily of the antagonistic application of yeasts as biocontrol agents against fungi (Viljoen, 2006). S. cerevisiae together with C. krusei have been observed to have an inhibitory effect on the growth of mycotoxin-producing moulds such as Penicillium citrinum, Aspergillus flavus and Aspergillus parasiticus. The inhibitory effects of the yeasts were shown to be mainly due to substrate competition, but inhibition of spore germination might also occur due to the production of high concentrations of organic acids (Halm & Olsen, 1996).

1.4.3.10. Positive effects associated with lactic acid bacteria

Lactic acid bacteria are the dominant microorganisms in fermented milk. The specific antimicrobial mechanisms of LAB exploited in the biopreservation of foods include the production of organic acids, bacteriocins, diacetyl, carbon dioxide, hydrogen peroxide, reuterin and ethanol (Adams & Nicolaides, 1997; Helander et al., 1997; Holzapfel et al., 1995).

Bacillus cereus is associated with spoilage problems in the dairy industry (Andersson et al., 1995; Larsen & Jorgensen, 1997; Mayr et al., 1999). Psychrotrophic strains of B. cereus can grow in foods at temperatures as low as 4-6 ºC (Borge et al., 2001; Dufrenne et al., 1994; Griffiths & Phillips, 1990; Rowan & Anderson, 1998; van Netten et al., 1990) and this raises a concern about the safety of cooked, refrigerated foods with extended shelf lives. Studies on the inhibition of B.

cereus by LAB have been reported in non-fat milk medium (Wong & Chen, 1988). The rate of pH reduction during the early stages of fermentation was crucial for optimum inhibition of B. cereus (Røssland et al., 2003). Low pH and high acidity were the major factors for inhibition of growth of B. cereus (Byaruhanga et al., 1999).

Different types of lactic acid bacteria have been isolated from fermented milk and all play a substantial role in the outcome of the final product. Lactobacillus acidophilus has inhibitory activity against pathogenic species which is an important criterion for its use as a dietary adjunct. This microorganism has been reported to produce antibiotic-like compounds such as acidolin, acidophilin lactocidin (Gupta et al., 1996). Lactococcus lactis strains most important properties are their ability to produce acid in milk and to convert protein into flavour components (Crow et al., 1993; Desmazeaud & Cogan, 1996) whereas Streptococcus diacetylactis inhibited the food borne pathogens Pseudomonas fluoroescens, Staphylococcus aureus and Clostridium perfringens when co-cultured (Daly et al., 1970). This inhibition was most likely a direct result of acid production by the starter L. lactis subsp. lactis biovar. diacetylactis strain.

It is important that bifidobacteria survive in fermented dairy products until consumption. The viability of bifidobacteria depends on the degree of acidification and on the bacterial strains, fermentation conditions, storage temperature, and preservation methods and is mainly limited by their sensitivity to the acidity (Shah, 1997). The ingestion of specific bifidobacteria could contribute to re-establishment of a bifidobacterial flora in humans after antibiotic therapy. Their establishment will lead to alleviation of constipation, prevention against diarrhoea and other gastrointestinal infections and alleviation of the symptoms of lactose intolerance (O'Sullivan & Kullen, 1998).

1.5. Conclusion

The microbial diversity of fermented milk in Southern Africa lands is of great importance, because fermented foods in Africa play an essential role in feeding of various people on the continent. The milk is produced locally in different regions of Africa, and has an effect on the quality and microbial composition of the milk. Many studies have been done on the fermented milk of different countries, comparing the composition, taste and starter cultures present within specific regions. For commercialized fermented milk, it is important that the composition, the flavour as well as the microorganisms in the milk must be the same at all times within a region.

Fermented foods like fermented milk are of most importance to humans, not only as a food supplement but it also have a nutritional and probiotic affect. In fermented milk it is a heterogenic (mixed) population of microorganisms, and that may be the result of the aroma flavours, non-toxic compounds, and nutritional value.

The difference between fermented and non-fermented milk in protein, lactose and mineral composition is limited, but the importance of fermented milk relies on the effect that the microorganisms in the fermented milk has the ability to eliminate all kinds of pathogens.

The interactions in fermented milk can either be the stimulation between organisms or there could be competition between the organisms. The two major groups of organisms present in fermented milk are lactic acid bacteria (LAB) and yeasts. The lactic acid bacteria have two main functions in fermented milk. It inhibits al undesired microorganisms, and it creates an acceptable environment for the yeasts to help with the fermentation process. Some of these lactic acid bacteria that are used as starter cultures produce antimicrobial compounds such as bacteriocins, hydrogen peroxide, formic acid, acetate and diacetyl. The yeasts on the other hand affect the quality of the final product significantly.

Previous studies on how to use one common starter culture has been done, but the techniques and equipment in the different regions of Africa still varies too much. Enormous research needs to be done on the safety of fermented milk in these regions, and how the share similarities by producing the same beneficial end product in all regions.

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1.7. Tables and Figures

Table 1: Composition of cow□s milk before and after fermentation.

Main constituent	Whole milk, Raw[1] (%)	Fermented milk[2] (%)
Water	85.5 – 89.5	V
Total solids	10.5 – 14.5	~14 – 18
Fat	2.5 – 6.0	0.1 – 10
Proteins	2.9 – 5.0	4 – 6
Lactose	3.6 – 5.5	2 – 3
Lactic acid	0.14 – 0.16	0.6 – 1.3
Minerals	0.6 – 0.9	~0.6 – 0.9

[1]Source: O□ Mahony (1988), [2]Source: Oberman (1985).

v = variable depending on the extent of whey removal.

Table 2: Some examples of African fermented milks.

Local name of the fermented milk	Country	Reference
Iria ri matii	Kenya	Kimonye and Robinson (1991)
Susa	Kenya	Kurmann (1992)
Maas, Inkomasi	South Africa	Keller and Jordan (1990)
Nono	Nigeria	Atanda and Ikenebomeh (1991), Bankole and Okagbue (1992), Olasupo and Azeez (2001)
Suusaac	Somalia	FAO (1990)
Garoor	Somalia	FAO (1990)
Laben rayeb	Egypt	Kurmann *et al*, (1992)
Irgo	Ethiopia	O□Mahony and Peters (1987 a & b); Kurmann *et al* (1992)

Arrera	Ethiopia	FAO (1990)
Ititu	Ethopia	Bekele and Kassaye *et al* (1987); Kassaye *et al*, (1991); Kurmann *et al*, (1992)
Amasi	Zimbabwe	Mutukumira *et al*, (1995)
Rob	Sudan	Abdelgadir *et al*, (1998)
Kadam	Mali	FAO (1990)
Nyaamme	Ghana	FAO (1990)
Ikuvugota	Zaire	FAO (1990)
Lait caille	Mauritania	FAO (1990)
Pindidaam	Cameroon	FAO (1990)

Table 3: Microorganisms used in the production of traditional fermented milks.

Genus	Species
Bacteria	
Lactobacillus	*L. delbrueckii*
	L delbrueckii ssp. *lactis*
	L delbrueckii spp. *bulgaricus*
	L. helveticus
	L. acidophilus
	L. casei
	L. fermentum
	L. brevis
	L. kefir
Lactococcus	*L. lactis* spp. *lactis*
	L. lactis spp. *lactis* var. *Diacetylactis*
	L. lactis spp. *cremoris*
Leuconostoc	*L. mesenteroides*
	L. mesenteroides spp.

	dextranicum
	L. mesenteroides spp. *cremoris*
	L. lactis
Streptococcus	*S. thermophilus*
Pediococcus	*P. pentosaceus*
	P. acidilactici
Acetobacter	*A. aceti*
Yeasts	
Kluyveromyces	*K. marxianus* spp. *marxianus*
	K. marxianus spp. *bulgaricus*
	K. lactis
Candida	*C. kefyr*
Saccharomyces	*S. cerevisieae*
	S. lactis
Yarrowia	*Y. lipolytica*
Debaryomyces	*D. hansenii*
Moulds	
Geotrichum	*G. candidum*

Table 4: Anti-mutagenic activity of viable and non-viable probiotic bacteria.

Effect	Administration	Microorganism	Viable	Non-viable	Refrence
Faecal enzyme activity	Oral	*L. rhamnosus* GG in yoghurt	ß-Glucuronidase Glucocholic acid hydrolase Nitroreductase Urease	ß-Glucoronidase Glycocholic acid hydrolase Nitroreductase Urease	Ling *et al.*, (1994)
Faecal enzyme activity in atrophic	Oral	Yoghurt	Nitroreductase Azoreductase	Nitroreductase Azoreductase	Pedrosa *et al.*, (1995)

gastritis subjects					
Faecal enzyme activity	Oral	*L. gasseri* ADH	ß-Glucuronidase Nitroreductase Azoreductase	ß-Glucuronidase Nitroreductase Azoreductase	Pedrosa *et al.*, (1995)
Chemically induced DNA damage in colon cells	Oral	*L. acidophilus*	87% intact cells	46.5% intact cells	Pool-Zobel *et al.*, (1996)
Aflatoxin binding		*L. rhamnosus* GC *L. rhamnosus* LC-705	51-54%	100º -killed 80-82%	El-Nezami *et al.*, (1997)
Binding of tryptophan pyrolyzates		*Lc. cremoris* *Lc. lactis* ssp. *diacetylactis* *B. bifidum*	94-97%	121ºC,81-96% 80ºC not different from viable	Zhangand Ohta (1990)
Chemically induced DNA damage in colon cells		*L. acidophilus*	57% intact cells	Heat-killed 41% intact cells	Pool-Zobel *et al.*, (1996)

Table 5: Classes of bacteriocins produced by lactic acid bacteria.

Class	Subclass	Description
I		Lantibiotics-small, heat stable, containing unusual amino acids
II		Small (30-100 amino acids), heat stable, non-lantibiotic
	IIa	Pediocin-like bacteriocins, with anti-listerial effects
	IIb	Two peptide bacteriocins
	IIc	Sec-dependent secretion of bacteriocins
III		Large (> 15000 Da)
IV		Complex bacteriocins with glycol-and/or lipidmoieties

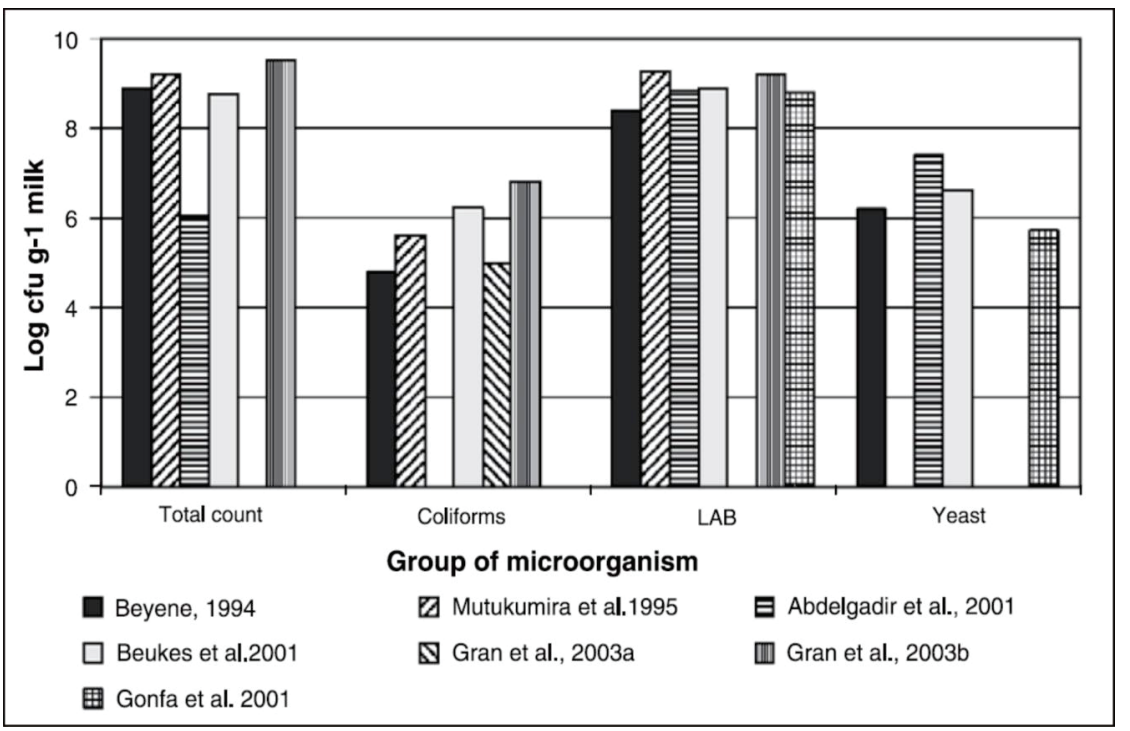

Figure 1: Microbial groups associated with indigenous fermented milks in Africa.

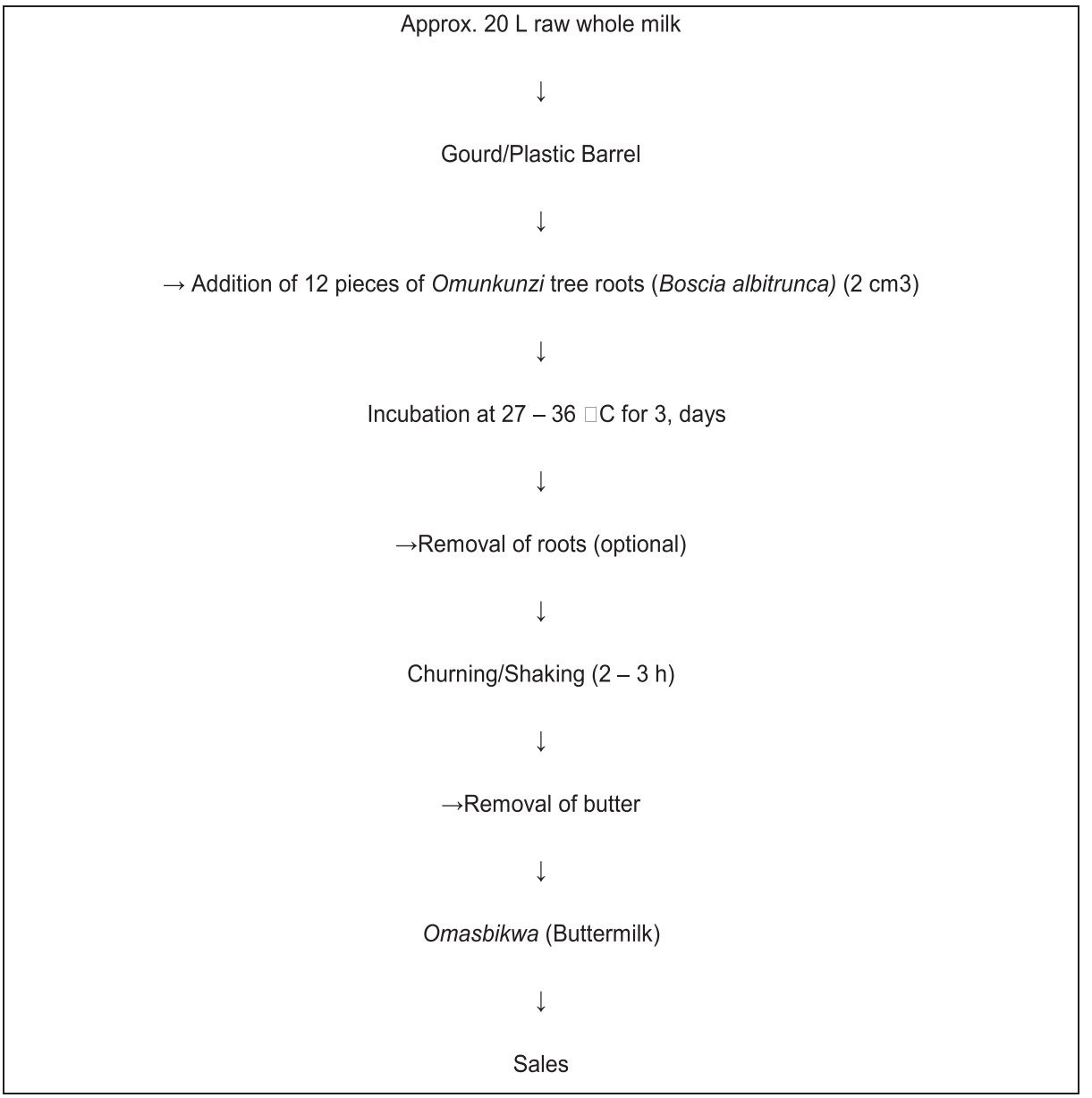

Figure 2: Procedure for the production of traditional *Omasbikwa* in Namibia.

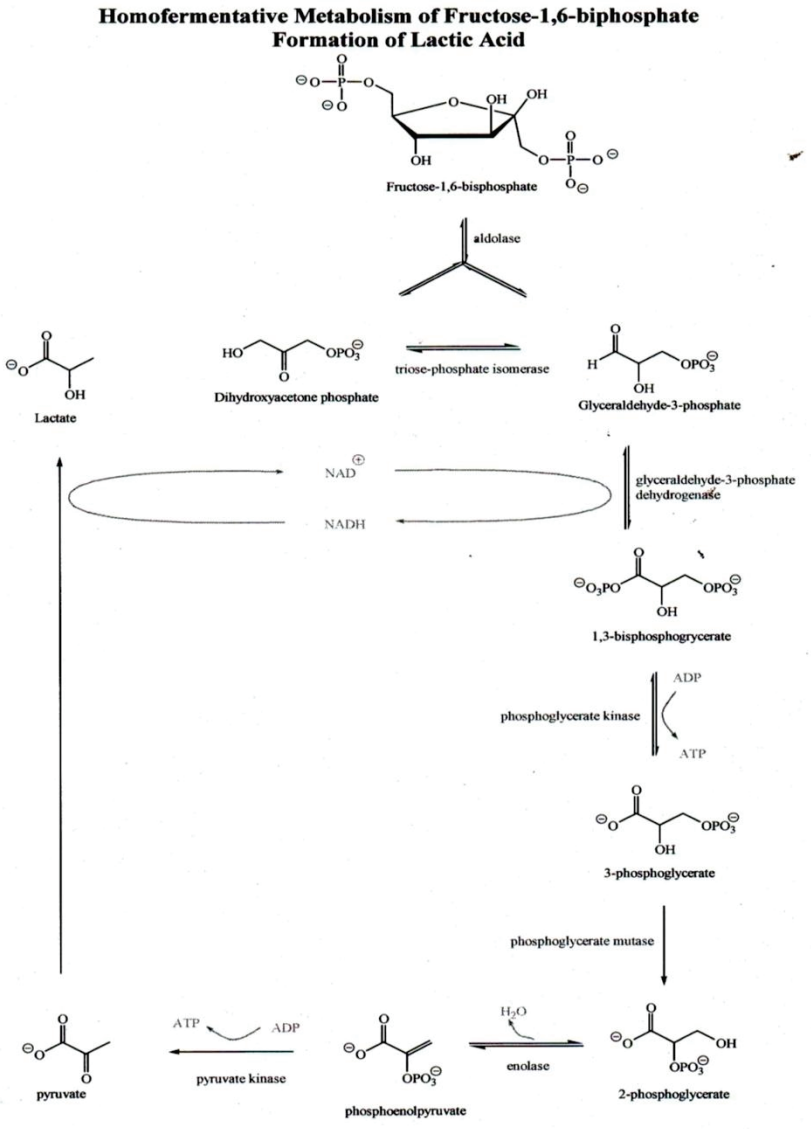

Figure 3: Homofermentative metabolism of lactic acid bacteria.

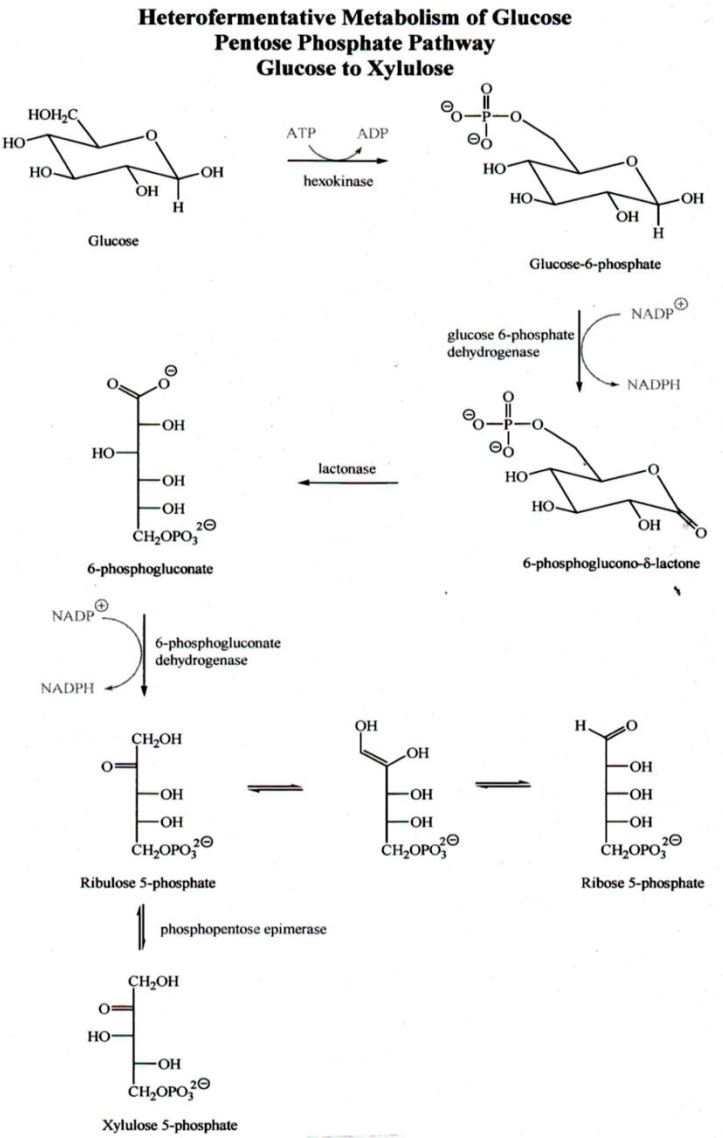

Figure 4: Heterofermentative metabolism of lactic acid bacteria.

CHAPTER 2

Comparison of dominant microorganisms associated with indigenous raw and naturally fermented milk of Southern Africa countries

Abstract

Samples of raw and indigenous fermented milk were collected from Botswana, Namibia, Lesotho and Swaziland. Coliforms and enterococci were found at high numbers in the raw milk, whereas the numbers of yeasts were at low numbers and LAB (lactococci, lactobacilli and leuconostocs) totally dominated especially after fermentation of milk. For the identification of the dominant microbes found in NFM of the different regions two techniques was used. DGGE is a technique which is used to represent the dominant microbial organisms within a particular sample. DGGE were compared against culture sequencing for the best results.

The dominant LAB strain isolated from culture sequencing was Enterococcus durans (32%). Other species isolated were identified as Lactococcus lactis spp. lactis, Leuconostoc mesenteroides, Enterococcus faecalis, Staphylococcus cohnii, Enterococcus lactis and Leuconostoc pseudomesenteroide. The dominant LAB varied between the different regions. In Namibia's fermented milk, Lactococcus lactis was ubiquitous in this environment while in Lesotho and Botswana distinct bands of Streptococcus sp. and Lactococcus lactis were obtained. For Swaziland no prominent bands was formed during the DGGE analysis. These results showed that the final product may vary between the regions depending on the different methods and environment.

2.1. Introduction

In Africa the fermented foods and beverages play a predominant role in the diet (Iwuoha & Eke, 1996; Sanni, 1993; Zulu et al., 1997). Milk is by far the most abundant fermented animal product, even though the extent to which milk is used in the daily diet varies to a great extent. The nature of these fermented products is different from one region to another reflected by the climatic conditions of each area. Traditional fermented milk in regions with a cold temperature climate contained mesophillic bacteria such as Lactococcus and Leuconostoc spp., whilst thermophillic bacteria, which include mostly Lactobacillus and Streptococcus, prevail in regions with a hot, subtropical or tropical climate (Kurmann, 1994; Tamine & Robinson, 1988; Thomas, 1985).

Lactic acid bacteria and yeast populations are the two dominant microbial groups indigenous to raw and naturally fermented milk obtained in Southern Africa. Lactic acid bacteria (LAB) are gram-positive, non-sporulating, micro-aerophillic (Pot et al., 1994). Classification and identification of LAB are based on morphology, physiology, carbohydrate fermentation patterns, cell composition and to a degree their ability to metabolize lactose. The type of metabolites produced by lactic acid bacteria can further be utilized to divide LAB into two main groups: The homofermentative and heterofermentative lactic acid bacteria (De Vuyst & Vandamme, 1994; Dillon & Cook, 1999). The presence of lactic acid, defines fermented milks due to the occurrence of LAB and acidity is one of the main properties associated with indigenous fermented milk. This is clearly indicated in the final soured milk products which are mostly consumed by African rural communities (Ganguly et al., 1999). Furthermore, the bio-preservation abilities associated with LAB in fermented milk products could assist in producing milk products that are microbiologically safe.

Yeasts are eukaryotic microorganisms and may be defined as unicellular fungi in which asexual reproduction occurs mainly by budding (Deak & Beuchat, 1996). In dairy products yeasts may interact with other microorganisms in three different

ways: i) they may inhibit or eliminate microorganisms which are undesired because they cause quality defects or possess potential pathogenic characters; ii) they may inhibit the starter culture, or iii) they may contribute positively to the fermentation or maturation process by supporting the function of the starter culture (Beukes et al., 2000; Ferezu & Muzondo 1990) conveyed studies on the microorganisms present in African naturally fermented milk and reported on the presence of yeasts, but no indication of the species present.

Limited research has been done to identify and characterize the dominant microflora of some of the Southern Africa raw and fermented milk (Beukes et al., 2001: Keller & Jordan, 1990; Loretan et al., 2003). Madila is naturally fermented milk which is produced by the people of Botswana by fermenting the raw milk in bags (Kebede, 2005). In Namibia most fermented milk products are widely used for nutrition and household income generation. Sour milk (Omasbikwa) processing is based on rural household technology (Bille et al., 2002). Typical indigenous fermented milk from Lesotho is called mafi. These concentrated fermented milks are sour milks obtained by spontaneous acidification of raw milk and are subsequently partly drained (FAO, 1990; Isono et al., 1994; Kassaye et al., 1991). Emasi is regarded an important part of people's daily diets as it is a nutritious food product in Swaziland and South Africa (Beukes et al., 2001; Caplice & Fitzgerald 1999). The fermentation of the emasi milk usually takes 1 -3 days, depending on the ambient temperature (Feresu & Muzondo, 1989, 1990; Gadaga et al., 1999; Gran et al., 2003b; Mutukumira, 1995).

To our knowledge, limited research has been done on the studies of the microflora of African raw and naturally fermented milk, especially of Botswana, Namibia, Lesotho and Swaziland. Thus the objective of this study was to isolate and identify the dominant organisms indigenous to these types of milk, as well as to compare the microbial composition of the milk indigenous to these certain regions in Southern Africa.

2.2. Materials and Methods

2.2.1. Sample collection

Twenty samples of raw milk and naturally fermented milk, prepared by the traditional methods, were collected on five occasions in sterilized plastic bottles from different regions in Botswana, Namibia, Lesotho and Swaziland. These samples were frozen (-30ºC) and then transported in portable cooler boxes lined with ice packing to the Department of Microbiology, University of the Free State (UFS), as per International Dairy Federation Guidelines (IDF, 1997). On receipt at the UFS the samples were immediately frozen (-30ºC), until they were used for identification.

2.2.2. Microbial enumeration

The procedures used for the microbial analysis of milk samples were followed as methods outlined in “Laboratory Methods in Food and Dairy Microbiology”. Serial dilutions were prepared by diluting milk samples in 9ml volumes of 0.1% Bacteriological Peptone (Oxoid, Basingstoke, England). Appropriate dilutions were spread plated onto selective media (Table 1) and single colonies were selected for further identification procedures.

2.2.3. Identification of the dominant microbes

2.2.3.1. DNA Extraction

Bacterial genomic DNA was extracted from 4 fermented milk samples obtained from Namibia (Nam), Lesotho (LES), Swaziland (HER) and Botswana (ILA). Norgen's Milk Bacterial DNA Isolation Kit (designed for the rapid preparation of genomic DNA from milk) (Norgen, Biotek Corporation).

DNA was isolated firstly by adding digestion buffer, digestion solution and Proteinase K to the milk sample. The sample was incubated for 55º for 30 minutes. The Binding solution and ethanol were added and centrifuged to bind the bacterial DNA. The DNA was washed twice with Wash Solution. DNA was then eluted by the Elution Buffer, centrifuged again and ended with pure bacterial genomic DNA.

Following DNA isolation, purified DNA was quantified using the Nano Drop ND-3000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE). DNA presence was confirmed by separating DNA preparations on a 0.8% Agarose gel, staining with ethidium bromide and visualization under UV transillumination (ChemiDoc XRS from Bio-Rad Labarotories).

2.2.3.2. DGGE

DGGE is a molecular fingerprinting method that is used to separate PCR generated products. These products can be targeted for Eukaryotes, Bacteria or Archaea. If environmental DNA is used as template for a PCR in which these DNA fragments are produced, a variety of different DNA fragments (of the same size) of differing DNA sequence are produced. These represent many of the dominant microbial organisms within the particular sample. This approach overcomes the limitation of separating PCR products based on their size: by separating DNA fragments based on sequence differences that result from differential denaturing characteristics of the DNA (Muyzer & Smalla, 1998).

The DNA from each of the samples were used to PCR amplify the DNA fragments required for the DGGE, making use of the universal primers 341F-GC/517R to PCR amplify the ~230bp DNA fragment from the bacterial 16S rDNA fragment.

Amplification products were separated on an 8% (w/v) DGGE polyacrylamide gel (acrylamide: bisacrylamide ratio 40:1) with a urea/formamide denaturing gradient ranging from 30% to 60% (100% denaturant corresponds to 7M urea and 40% (w/v) formanide) in a Dcode Universal Detection System (BioRad). Electrophoresis was

performed in 1x TAE buffer, pH 8.0 at a constant voltage of 200V at 60ºC for 3 hours. Gels were stained with SYBR Gold and viewed with a UV transillumination.

2.2.3.3. Sequencing

2.2.3.3.1. DGGE Sequencing

After DGGE analysis was performed followed the sequence analysis and the analysis of DNA sequences homology searches were completed with the BLAST server of the National Center for Biotechnology Information (NCBI) using BLAST algorithm for comparison of a nucleotide query sequence against a nucleotide sequence database (Muyzer & Smalla, 1998).

2.2.3.3.2. Culture Sequencing

From the different milk samples, single colonies from M17 (4), MRS (4), MRSV (6) and SBM (4) agar plates were purified by subculturing on the same media from which they were collected. The products were sequenced by Ingaba Biotech and the results obtained from Finch Tv were completed with the BLAST server of the National Center for Biotechnology Information (NCBI) using BLAST algorithm for comparison of a nucleotide query sequence against a nucleotide sequence database (Muyzer & Smalla, 1998).

2.3. Results and Discussion

Raw and naturally fermented milk play a predominant role in the diet and still widely used in many parts of the world (Iwuoha & Eke, 1996; Sanni, 1993; Zulu et al., 1997). When the domains of individual microorganisms overlap, as observed in dairy products, it is likely that interactions will occur. The outcome of natural interactions in nature is evaluated based in the effect they have on population size (Viljoen, 2001). Table 2 show counts of the majority and indicator microbial populations of the different samples.

Since the occurrence of coliforms and enterococci in fermented milk is often associated with potential health hazards (Gran et al., 2003b), it is strongly suggested for adequate sanitary measures. Coliform populations reached counts as high as 1.01×10^{9} cfu/ml (Fig. 1) obtained on VRB and enterococci populations reached 9.5×10^{8} cfu/ml (Fig. 2) obtained in SBA were only found before fermentation in the raw milk. During the fermentation all coliforms and enterococci were destroyed, and is no counts were found in the final fermented product.

Low numbers of lactococci were found in the raw milk of Lesotho. Reaching 1.6×10^{56} cfu/ml and the highest counts of lactococci were found to be in Botswana's raw milk, reaching counts of 9.6×10^{6} cfu/ml. For the lactobacilli, also low numbers were found in Swaziland's raw milk, reaching counts of 1.2×10^{6} cfu/ml and the highest counts of lactobacilli were found to be in Botswana's raw milk, reaching counts of 7.2×10^{6} cfu/ml. Leuconostocs had the lowest counts of all the lactic acid bacteria tested in the raw milk and fermented milk samples. With Botswana's raw milk reaching as low as 2.2×10^{4} cfu/ml and Lesotho's raw milk with the highest leuconostocs, reaching counts of 9.7×10^{4} cfu/ml. This indicates that lactococci (M17), lactobacilli (MRS) and leuconstocs (MSEA) were only obtained in low numbers in the raw milk samples.

In contrast, these three populations were the dominant organisms obtained from the NFM (Figs. 3-5). Leuconstocs had a definite climb in numbers during the fermentation process, but were still found in significantly lower numbers than the lactococci and lactobacilli. With Namibia's NFM reaching the highest counts (4×10^6 cfu/ml). Lactobaciili reached counts as high as 2×10^8 cfu/ml, with Botswana's fermented milk reaching the highest counts of lactobacilli (8.5×10^8 cfu/ml). Since the highest number of microorganisms in the naturally fermented milk was found on M17, it is possible that the microflora was dominated by strains of Lactococcus, where the population reached counts of as high as 1.04×10^9cfu/ml.

No staphylococci (BPA) were detected in either the raw or fermented milk samples. The LAB dominated the microbial populations was compared with similar studies on fermented milks within the East African region. According to (Isono et al., 1994; Mathara, 1999) they used naturally fermented milk of regions in East Africa and reported a similar range of bacterial counts.

The yeasts, as part of the interactions, contribute to the fermentation by supporting the starter culture (LAB). Literature showed that yeasts create desirable changes like the production of aromatic compounds, proteolytic and lipolytic activities that contributes to the growth of the bacteria (Viljoen, 2001). Although fermented milk products are regarded as predominantly lactic fermentations, the frequent co-occurrence of yeasts and LAB has led to the suggestion that interactions may occur that can influence product characteristics and quality (Marshall, 1987; Viljoen, 2001). The chemical composition of milk will support the growth of yeast species with a diverse range of biochemical and physiological properties. Although raw and pasteurized milks are frequently contaminated with yeasts, the populations reached around 1.13×10^9cfu/ml (Fig. 6). After fermentation, the yeast numbers decreased two to three logarithmic units, suggesting that the faster growing bacteria restricted yeasts growth which corresponded with literature (Deak, 1991: Fleet, 1990).

For the identification of the dominant microbes found in the NFM of the different regions two different techniques were compared. 18 Strains that was found to be the dominant population during the microbial enumeration were further identified to species level by Ingaba Biotech (Table 3). All the homologies were above 99% and were considered to represent the indicating species identity (Stackebrandt & Goebel, 1994). The dominant LAB strains isolated and identified from the fermented milk of the different regions were identified as Enterococcus faecium (32%), Lactococcus lactis spp. lactis (22%), Leuconostoc mesenteroides (22%), Enterococcus faecalis (6%), Staphylococcus cohnii (6%), Enterococcus lactis (6%), Leuconostoc pseudomesenteroide (6%). Namibia had two dominant organisms present in their NFM, Lactococcus lactis spp. lactis and Leuconostoc mesenteroides. The NFM of Swaziland also had two dominant LAB, Leuconostoc pseudomesenteroide and Lactococcus lactis spp. lactis. Three dominant microbes were identified in the NFM of Lesotho and they were identified as Lactococcus lactis spp. lactis, Leuconostoc mesenteroides and Leuconostoc pseudomesenteroide. Botswana's NFM had the greatest variety of dominant microbes. The four dominat microbes were identified as Enterococcus faecium, Enterococcus faecalis, Lactococcus lactis spp. lactis and Enterococcus lactis.

The majority of the isolates belonged to the genera Enterococcus. A reason for the abundance of Enterococcus could be due to that enterococci can grow in a wide range of temperatures as well as restrictive environments, including high salt and pH ranges between pH4.0-9.6. The genus has been isolated on many occasions from similar African fermented foods (Giraffa, 2003). However the Lactococcus lactis spp. lactis has been the main species isolated from fermented milk products such as Amasi (South Africa) and Ergo (Ethiopia) (Mutukumira, 1996). Based on the results Lactoccus lactis spp. lactis was represented at low numbers. The genus Leuconostoc strains were also found in high numbers, this genus contributes to the development of flavour in fermented products (Togo et al., 2002).

Fermented milk is an excellent protective medium encouraging the proliferation of many diverse microorganisms as observed by Oberman (1985). Molecular techniques were used to determine the total diversity of microorganisms present in the NFM. Following genomic DNA extraction, agarose gel electrophoresis was an indicative of sufficient concentrations of DNA required for further downstream applications (Fig. 11). Nanodrop ND-3000 qauntifications indicated very low DNA concentrations (Table 4). However these were sufficient for PCR amplification of the ~ 230 bp DGGE-fragment (Fig. 12).

In comparison between the microbial analysis, culture sequencing and molecular identification (DGGE) it was found that the nature of fermented products was different from one region to another (Kurmann, 1994; Tamine & Robinson, 1988; Thomas, 1985). Because of the relatively simple microbial composition of the samples, the DGGE profiles were also expected to be rather simple (Fig. 13). Each of the bands on the DGGE profile seen on the gel theoretically represented a different species of bacteria as described in section (Mat en Met). The samples from Site 1 (Namibia) showed a prominent band, suggesting that this one species of bacteria (Lactococcus lactis) is ubiquitous in this environment, where there for the microbial analysis of the NFM of Namibia (Fig. 7) showed that lactobacilli were the dominant population, reaching numbers around 5.7 x 10^8cfu/mL, although lactococci strains were also found in high numbers reaching counts of 3.0 x 10^8cfu/mL. Site 2, 3 (Lesotho and Botswana) showed distinct bands (Streptococcus sp., Lactococcuslactis). While Site 4 (Swaziland) showed no prominent bands.Lactococci, lactobacilli and leuconostocs were found to be the dominant bacteria in these region's fermented milk (Figs. 8, 9 and 10) as observed by Kebede (2005), Moore (2008) and Van Jaarsveld (2008).

The microorganisms present in milk and naturally fermented milk may originate from the animal itself, from the milking equipment and environment, from personnel or from the previous product batch if back-slopping is used (Mutukumira et al., 1995). As a result, the final product may vary considerably between different regions.

2.4. Conclusion

Although fermented milk products are regarded as predominantly lactic fermentations, the frequent co-occurrence of yeasts and LAB has led to the suggestion that interactions may occur that can influence product characteristics and quality. The combination of the yeasts and bacteria creates an environment that restricts pathogen growth and the yeasts stimulate the growth of the bacteria, followed by a rapid decrease in viable numbers.

Different interactions exist within dairy products, depending on the environmental stresses and as expected, lactic acid bacteria including Lactococcus and Lactobacillus species totally dominated the NFM of the different Southern Africa countries as observed through microbial analysis and molecular identification.

It is clear that until NFM from different countries or areas are more accurately characterized according to desirable and non-desirable sensory properties, the selection of suitable starter microorganisms will be a difficult, if not impossible task.

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2.6. Tables and Figures

Table 1: General and selective media for microbial counts.

Microbial group	Selective media	Temperature	Time
Aerobic mesophilic bacteria	Plate count agar with 1% skimmed milk (PCAM; Merck, Darmstadt, Germany)	30ºC	72h
Lactococci	M17 agar (M17A; Scharlau)	30ºC	48h
Lactobacilli	de Man, Rogosa and Sharpe agar (MRSA; Merck)	32ºC	72h
Leuconostocspp.	Mayeux, Sandine and Elliker agar (MSEA; Biokar Diagnostics, Beauvais, France)	25ºC	120h
Enterococci	Slanetz and Bartley agar (SBA; Merck)	44ºC	24h
Coliforms	Violet red bile glucose agar (VRBGA)	30ºC	48h
Staphylococci	Baird-Parker agar (BPA; Merck)	37ºC	24h
Yeasts	Rose Bengal Agar (RBA; Merck)	30ºC	48h

Table2: Diagram of sampling of the microbial counts of different microbial groups in the raw and naturally fermented milk of the four countries (Log cfu/ml).

	Sample (Country)							
Microbial groups	**Swaziland Raw**	**Swaziland Fermented**	**Namibia Raw**	**Namibia Fermented**	**Lesotho Raw**	**Lesotho Fermented**	**Botswana Raw**	**Botswana Fermented**
Total aerobic counts	6.643453	9.143015	7.064458	9.376577	6.763428	8.579784	8.176091	9.064458
Enterococci	8.977724	Nd	7.90309	nd	5	Nd	6.176091	Nd
Coliforms	8.544068	Nd	5.69897	nd	8.799341	Nd	9.004321	Nd
Staphylococcus	Nd	Nd	nd	nd	Nd	Nd	nd	Nd
Lactococci	6.748188	8.995635	6.20412	8.477121	6.20412	8.69897	6.982271	9.017033
Lactobacilli	6.079181	8.30103	6.633468	8.755875	6.230449	8.477121	6.857332	8.929419
Leuconostoc	4.431364	6.544068	4.447158	6.60206	4.986772	6.491362	4.342423	6.255273
Yeasts	9.053078	3.69897	8.60206	3.30103	6.39794	4.30103	6.556303	4.041393

nd, not detected.

Table 3: Identification of dominant LAB from indigenous fermented milk of different regions.

Isolate	Accession	Description	Query coverage	E value	Max identity
SM1	JQ411245.1	*Lactococcuslactis* strain SS11A 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	AB673464.1	*Lactococcus* sp. Cd31 gene for 16S rRNA, partial sequence	100%	0.0	99%
SM2	JQ364952.1	*Lactococcuslactis* subsp. *lactis* strain YF11 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ411245.1	*Lactococcuslactis* strain SS11A 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	AB673464.1	*Lactococcus* sp. Cd31 gene for 16S rRNA, partial sequence	100%	0.0	99%
	JQ364952.1	*Lactococcuslactis* subsp.*lactis* strain YF11 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
SM3	AB673464.1	*Lactococcus* sp. Cd31 gene for 16S rRNA, partial sequence	100%	0.0	100%
	JQ364952.1	*Lactococcuslactis*subsp . *lactis* strain YF11 16S	100%	0.0	100%

		ribosomal RNA gene, partial sequence			
SM4	HE646374.1	*Lactococcuslactis* subsp. *lactis*bv. diacetylactis partial 16S rRNA gene, strain BGAL1-1	100%	0.0	100%
	AB673464.1	*Lactococcus* sp. Cd31 gene for 16S rRNA, partial sequence	100%	0.0	98%
	JQ364952.1	*Lactococcuslactis*subsp .*lactis*strain YF11 16S ribosomal RNA gene, partial sequence	100%	0.0	98%
	HE646437.1	*Lactococcuslactis*subsp . *lactis*bv. diacetylactis partial 16S rRNA gene, strain ZG9-11	100%	0.0	98%
SM5	HE646373.1	*Leuconostocpseudome senteroides* partial 16S rRNA gene, strain BGLE1-36	100%	0.0	100%
	JF411965.1	*Leuconostocpseudome senteroides* strain 22678 16S ribosomal RNA gene, partial sequence	100%	0.0	100%
	JF411962.1	*Leuconostocpseudome senteroides* strain 22593 16S ribosomal RNA gene, partial sequence	100%	0.0	100%

SM6	JN853602.1	*Leuconostocmesenteroides* strain YML003 16S ribosomal RNA gene, partial sequence	100%	0.0	100%
	AB690198.1	*Leuconostoc* sp. JCM 8610 gene for 16S rRNA, partial sequence, strain: JCM 8610	100%	0.0	100%
	JN990379.1	*Leuconostocmesenteroides* strain CR1_1417 16S ribosomal RNA gene, partial sequence	100%	0.0	100%
SM7	JQ067695.1	*Enterococcus faecium* strain JZ1-4 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ067691.1	*Enterococcus faecium* strain BX2-2 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ067690.1	*Enterococcus faecium*strain BX2-1 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
SM8	JQ607681.1	Bacterium NLAE-zl-P849 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ607665.1	Bacterium NLAE-zl-P847 16S ribosomal RNA gene, partial sequence	100%	0.0	99%

	JQ446562.1	*Enterococcus lactis* strain Chyp6 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
SM9	JQ607681.1	Bacterium NLAE-zl-P849 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ607665.1	Bacterium NLAE-zl-P847 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ067695.1	*Enterococcus faecium* strain JZ1-4 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
SM11	JN853602.1	*Leuconostocmesenteroides* strain YML003 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	AB690198.1	*Leuconostoc* sp. JCM 8610 gene for 16S rRNA, partial sequence, strain: JCM 8610	100%	0.0	99%
	JN990379.1	*Leuconostocmesenteroides* strain CR1_1417 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
SM12	GU132842.1	*Leuconostocmesenteroides* strain CYR1 16S ribosomal RNA gene, partial sequence	99%	0.0	100%

	JN853602.1	*Leuconostocmesenteroides* strain YML003 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	HE616211.1	*Leuconostocmesenteroides* partial 16S rRNAgene, strain BGVL2a-20	100%	0.0	99%
SM13	JN853602.1	*Leuconostocmesenteroides* strain YML003 16S ribosomal RNA gene, partial sequence	100%	0.0	100%
	AB690198.1	*Leuconostoc* sp. JCM 8610 gene for 16S rRNA, partial sequence, strain: JCM 8610	100%	0.0	100%
	JN990379.1	*Leuconostocmesenteroides* strain CR1_1417 16S ribosomal RNA gene, partial sequence	100%	0.0	100%
SM15	JN128237.1	*Staphylococcus cohnii* strain HNS003 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JF799887.1	*Staphylococcus cohnii* strain CIFRI D-TSB-9-RS 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JF784040.1	*Staphylococcus cohnii* strain CIFRI P-TSB-54 16S ribosomal RNA gene, partial sequence	100%	0.0	99%

SM17	JQ067695.1	*Enterococcus faecium* strain JZ1-4 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ067691.1	*Enterococcus faecium* strain BX2-2 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ067690.1	*Enterococcus faecium* strain BX2-1 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
SM18	CP003351.1	*Enterococcus faecium* Aus0004, complete genome	99%	0.0	99%
	JQ619997.1	*Enterococcus faecium* strain RK 203 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
	AB512765.1	*Enterococcus faecium* gene for 16S rRNA, partial sequence	99%	0.0	99%
SM19	JQ067695.1	*Enterococcus faecium* strain JZ1-4 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ067691.1	*Enterococcus faecium* strain BX2-2 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ067690.1	*Enterococcus faecium* strain BX2-1 16S	100%	0.0	99%

		ribosomal RNA gene, partial sequence			
SM20	FJ538584.1	*Enterococcus faecium* strain CSI35MX 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ607725.1	Bacterium NLAE-zl-P921 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ607703.1	Bacterium NLAE-zl-P867 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
SM21	AB712374.1	*Enterococcus faecalis* gene for 16S rRNA, partial sequence, strain: NP-10011	100%	0.0	99%
	JQ404478.1	*Enterococcus faecalis* strain XJ149-N2F1 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JF772098.1	*Enterococcus faecalis* strain FCC120 16S ribosomal RNA gene, partial sequence	100%	0.0	99%

Table 4: DNA concentrations.

Sample ID	Date	Time	ng/ul
H	29/4/2010	12:20 PM	13.99
IL	29/4/2010	12:21 PM	19.76
LES	29/4/2010	12:22 PM	9.69
NAM	29/4/2010	12:23 PM	12.08

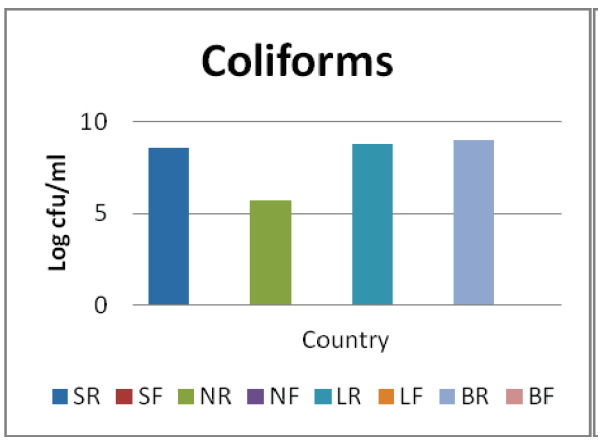

Figure 1: Coliform (VRB) counts of the four regions in Africa for before and after fermentation.

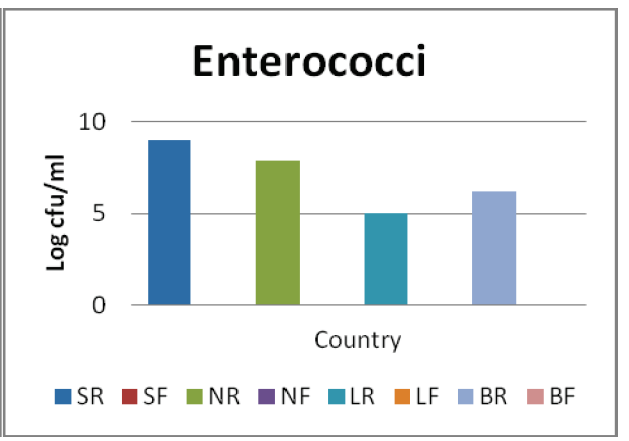

Figure 2: Enterococci (SBA) counts of the four regions in Africa for before and after fermentation.

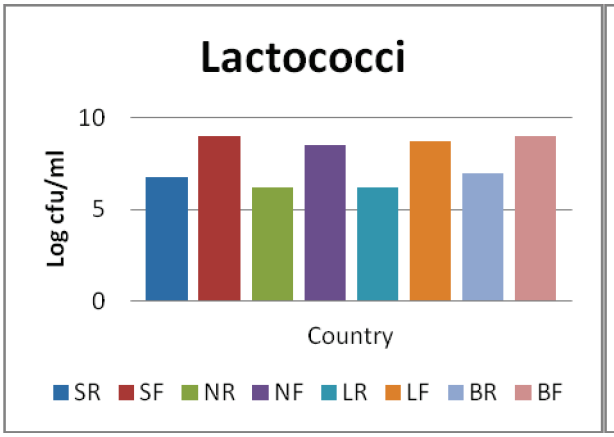

Figure 3: Lactococci (M17) counts of the four regions in Africa for before and after fermentation.

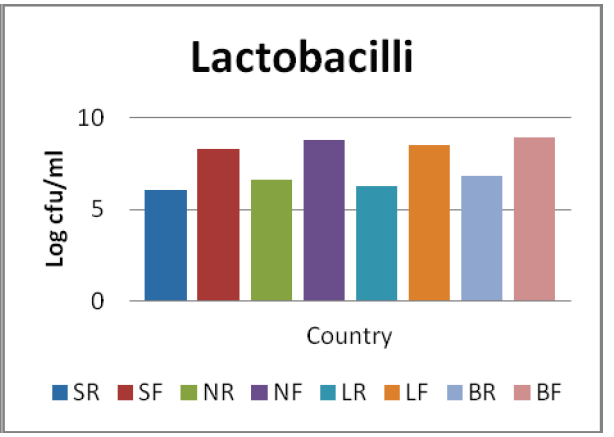

Figure 4: Lactobacilli (MRS) counts of the four regions in Africa for before and after fermentation.

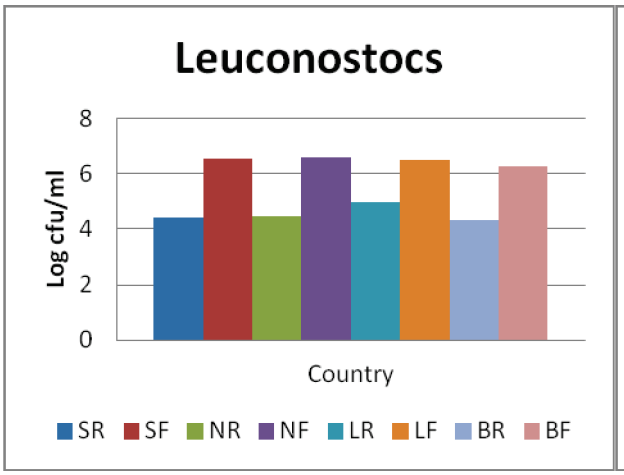

Figure 5: Leuconostocs (MSEA) counts of the four regions in Africa for before and after fermentation.

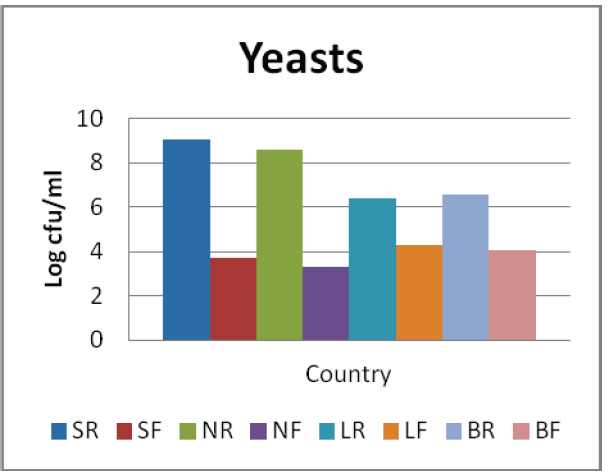

Figure 6: Yeasts (RBA) counts of the four regions in Africa for before and after fermentation.

Figure 1-6:

SR- Swaziland raw milk	SF – Swaziland end of fermented milk
NR – Namibia raw milk	NF – Namibia end of fermented milk
LR – Lesotho raw milk	LF – Lesotho end of fermented milk
BR –Botswana raw milk	BF – Botswana end of fermented milk

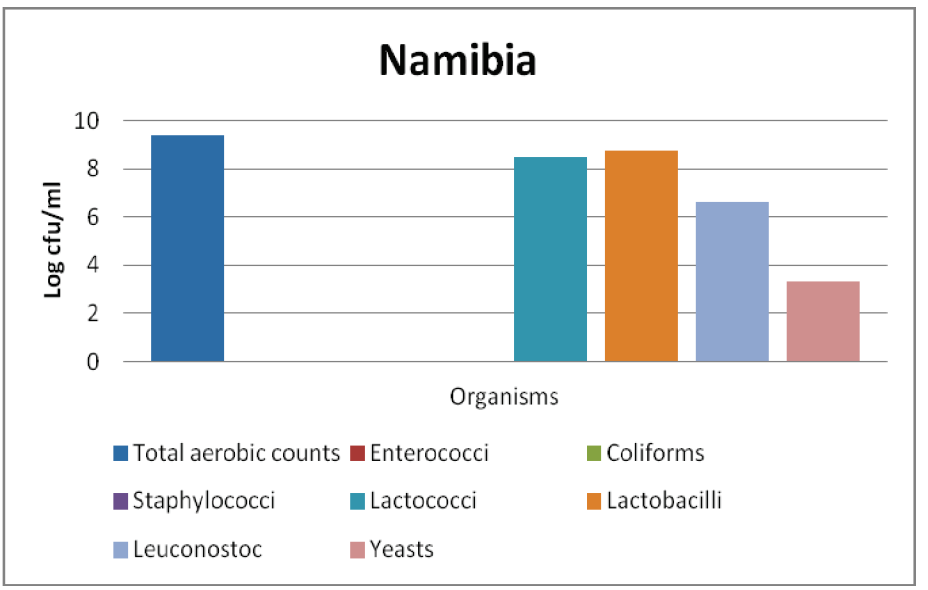

Figure 7: Dominant population observed in NFM of Namibia.

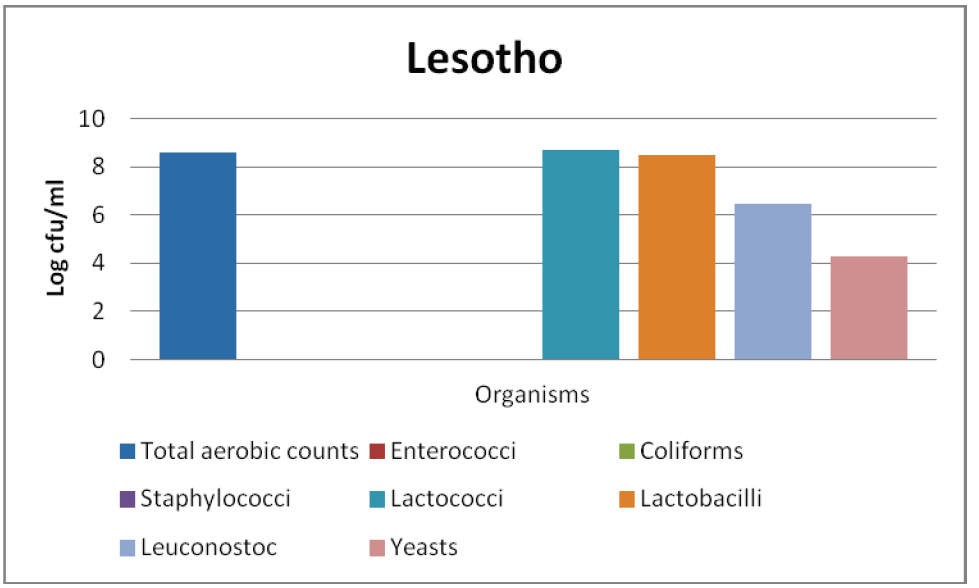

Figure 8: Dominant population observed in NFM of Lesotho.

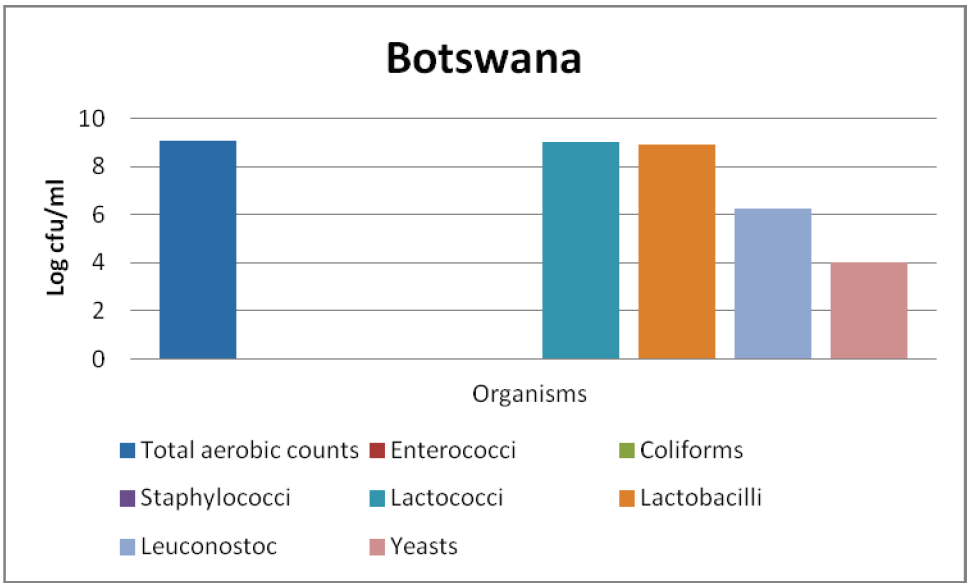

Figure 9: Dominant population observed in NFM of Botswana.

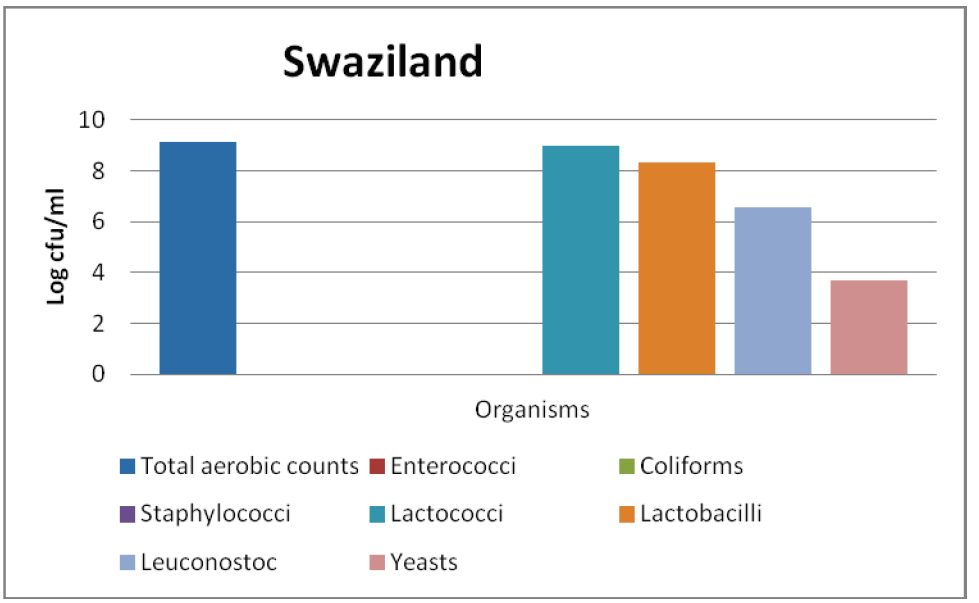

Figure 10: Dominant populations observed in NFM of Swaziland.

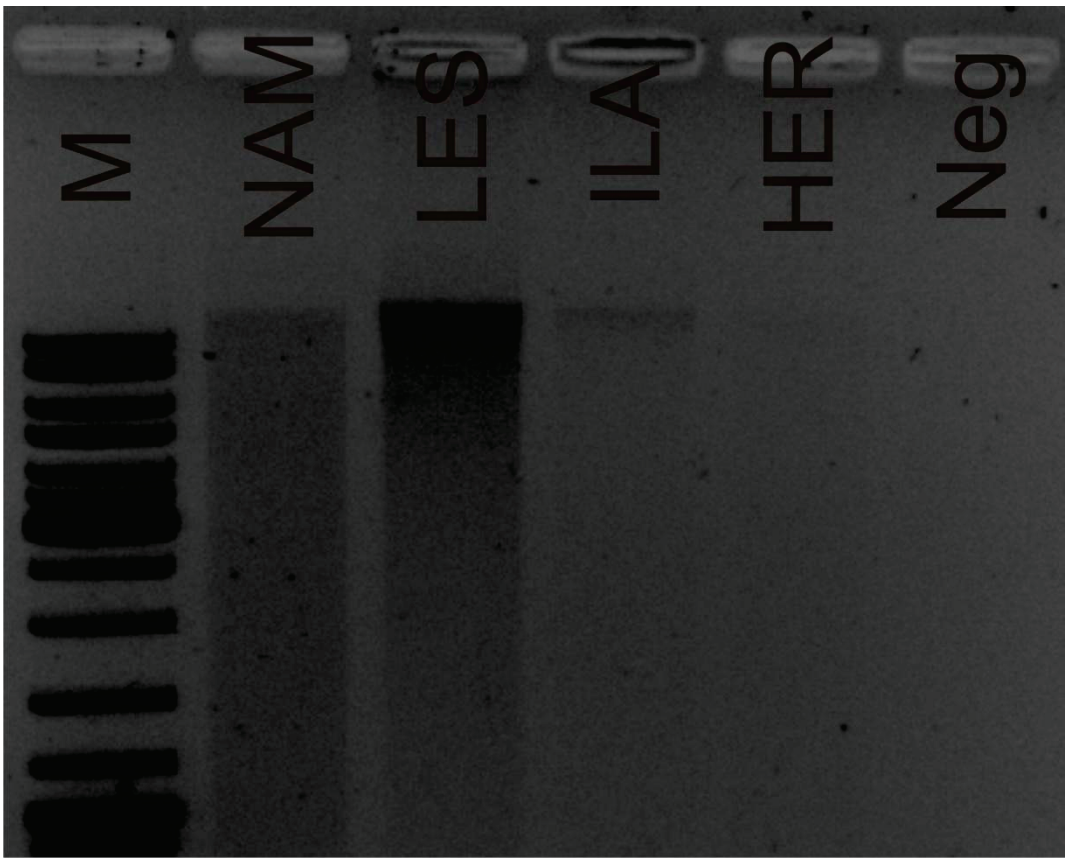

Figure 11: Agarose gel of the NFM of the four regions in Africa.

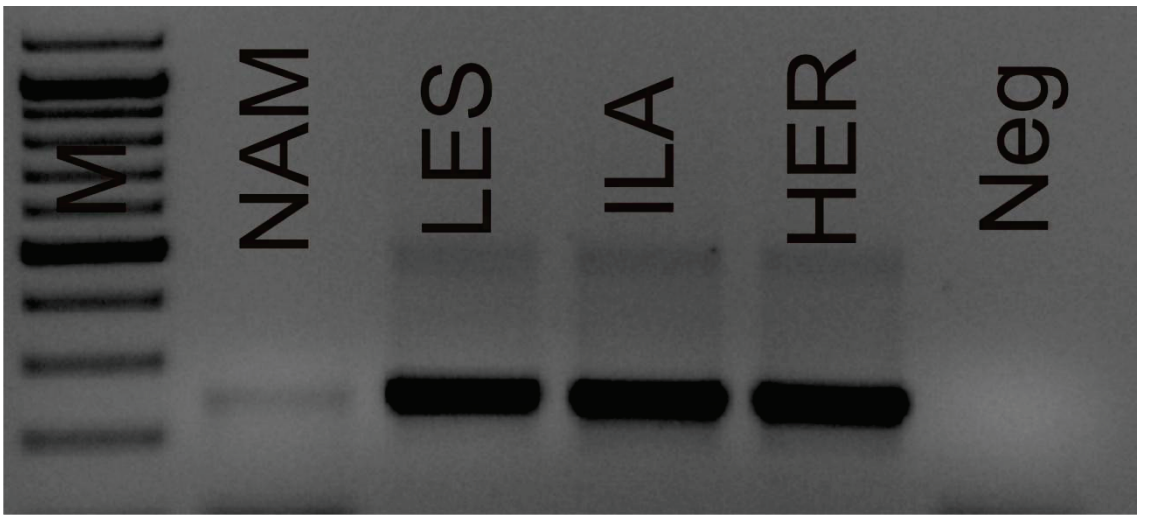

Figure 12: DGGE PCR of the NFM of the four regions in Africa.

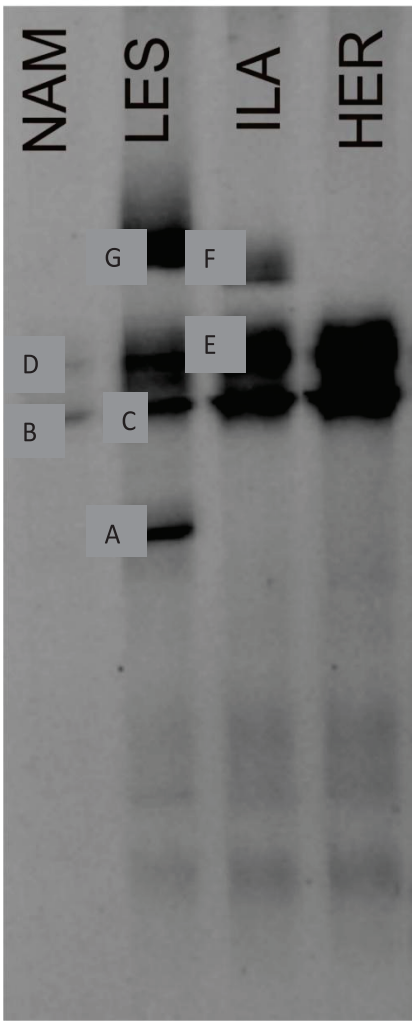

Figure 13: DGGE of the NFM of the four regions in Africa.

Band	***Hit***	**D**	*Lactococcus lactis*
A	*Streptococcus thermophilus*	**E**	*Lactococcus lactis*
B	*Lactococcus lactis*	**F**	*Lactococcus lactis*
C	*Streptococcus* sp.	**G**	*Streptococcus* sp.

CHAPTER 3

Growth and interaction of selected lactic acid bacteria against spoilage yeasts, isolated from indigenous naturally fermented milk

Abstract

The fermentation of milk with lactic acid bacteria leads to certain characteristics of the product. Depending on the microorganisms involved different compounds can be produced. The co-culture between yeasts and LAB is frequently observed in milk. The interaction between LAB with inhibitory activities and specific undesirable yeasts was studied to see if there is stimulation or inhibition between these two groups of microorganisms. During the growth study (Day 0 – Day 6) it was observed that the LAB had an inhibitory affect against the yeasts. The LAB reached maximum counts of log 9.5cfu/ml after 1 day whilst in combination with the yeasts. In contrast, the yeasts had much lower log numbers through the whole growth trail. The combination between *Enterococcus durans* and *Zygosaccharomyces bailli* had the lowest pH, 4.50 on day 1. While the LAB strains alone started with a pH of around 5.56 – 5.73 (Day 0) and ended up with a pH of 4.4 – 4.63 (Day 6) due to the organic acids and other compounds produced by LAB. Lactic acid and citric acid were the most prevalent organic compounds produced during the chemical analysis. In co-culture volatile compounds detected were predominantly ethanol and acetone.

The results showed that the change in chemical composition has an effect on the nutritional value and creates an environment incapable for pathogens to survive.

3.1. Introduction

Lactic acid bacteria (LAB) have received considerable attention during the past few years due to the preserving ability of the fermented end products as well as the microbiological stability and the production of antimicrobial compounds in fermented foods (De Vuyst & Vandamme, 1994). LAB needs sugar such as lactose for growth, as well as amino acids, vitamins and other growth factors to survive in milk. The fermentation of milk with lactic acid bacteria leads to certain organoleptic characteristics of the product due to the conversion of lactose to lactic acid and the coagulation of milk protein (Oberman & Libudzisz, 1998). Flavouring compounds such as diacetyl, acetaldehyde, acetone etc., which are non-existent in the raw milk, but present in fermented milk are the resultant from the metabolic activities of microorganisms, including lactic acid bacteria (Kebede, 2005), and contribute to unique characteristics of the fermented end-product.

Depending on the microorganisms involved, milk fermentations proceed via the glycolisis pathway with the formation of lactic acid and via pentose phosphate pathway with the formation of lactic and acetic acids (Driessen & Puhan, 1988; Urbiene & Leskauskaite, 2006). Free fatty acids (FFA) are liberated as a result of lipolysis and these compounds are involved in forming the sensorial quality of the product (Beshokova *et al.,* 1998; Parodi, 2003; Prandini *et al.,* 2007).

Enterococci are found in a variety of dairy products. *Enterococcus durans* can easily grow in raw and finished food products, especially milk. They are tough bacteria, important in fermented food due to their ability to tolerate low pH and high temperature (Adams & Moss, 2000a; Franz *et al.,* 1999; Sarantinopoulos *et al.,* 2001). The main volatile compounds derived from *E. durans* are the sensory compounds acetaldehyde, ethanol and acetone (Franz *et al.,* 1999; Sarantinopoulos *et al.,* 2001). In the search of exopolysaccharides (EPS)-producing LAB strains for potential industrial application. They found a

Lactobacillus fermentum strain isolated from traditional dairy products in Inner Mongolia of China produced a viscous EPS when grown in milk.

Lactococcus lactis and spp. *lactis biovar.diacetyl lactis* isolated from traditional fermented milk has been used to prepare cultured milk of good quality at laboratory scale (Mutukumira *et al*., 1996; Narvhus *et al.,* 1998). *L. lactis*is encountered in numerous food fermentation processes. Its contribution primarily consists of the formation of lactate from the available carbon source, which results in rapid acidification of the raw material (Mansour *et al.,* 2009). Some strains of *Lactococcus lactis* spp. *lactis* have the ability to metabolise citric acid and are added to milk in order to produce aroma compounds and carbon dioxide (CO_2) in e.g. cultured milk. Citrate negative *L. lactis* spp. *lactis* is usefull for fast development of acid, which contributes to the flavour of the product, controls growth of unwanted microorganisms and creates the right environment for activity of flavour producers (Sserunjogi *et al.,* 1999).

The cell structure of *Streptococcus thermophilus* allows the bacteria to endure elevated temperatures, such as the many industrial dairy fermentation processes that require high temperatures. *Streptococcus thermophilus* also lacks genes which contain surface proteins. This is important because harmful bacteria use these surface proteins to attach to mucosal tissues and hide from the body's defensive actions. Ongoing research and experimentation have improved the *Streptococcus thermophilus* strains even beyond its natural beneficial state. This improved strain is responsible for the consistent taste and texture of many dairy products (European Bioinformatics Institute, 2009).

Some strains of yeasts, also isolated from traditional fermented milk (Gadaga *et al.,* 2000), were able to grow in ultra high temperature UHT-treated milk and produce flavour compounds that could be important to the characteristics of the cultured milk (Gadaga *et al.,* 2000). The origin of yeasts present in dairy products may be varied: fresh milk, ambient environment, utensils, brine, the massaging solutions and also the starter cultures (Baroiller & Schmidt, 1990). Depending on their properties and

concentrations, yeasts may have negative (unpleasant taste or appearance) effects (Eliskases-Lechner & Ginzinger, 1995b; Lenoir *et al.,* 1985). Lactose fermentation and assimilation, lipolysis and proteolysis are the important reactions of yeasts which are responsible for the diverse flavour compounds reported in many dairy products (Roostita & Fleet, 1996). Yeasts can be related to cases of mycotic mastitis in goats and cow, being responsible for economic losses due to the reduction of milk production and augmentation of costs of the production. The species of the genus *Candida* are the yeasts more commonly isolated from milk (Spanamberg *et al.,* 2009). Stored Domaiti cheese also contained diverse yeast species involving isolates of the pathogenic yeast *C. albicans.* This raises the possibility of dairy products being vehicles of transmission of pathogenic yeasts (El-Sharoud,2009).

In co-culture, LAB mainly affects the final pH and metabolite content. Some LAB-yeast co-cultures showed enhanced production of flavour compounds like ethanol, acetaldehyde and malty components, and this thought to be an indicator of interaction between the LAB and yeasts (Gadaga *et al.,* 2001a).

Therefore, the objectives of this study were to study in greater detail the growth and metabolism during the growth of LAB and spoilage yeasts in pure and mixed cultures.

3.2. Materials and Methods

3.2.1. Inhibition of yeasts cultures test

The 15 characterized lactic acid bacteria cultures were individually streaked out on MRS agar (Merck), and aerobically incubated for 24 hrs at 30°C, while the yeasts were inoculated in YM broth (Merck) and incubated for 24 hours at 30°C in a shaking incubator. Melted YM agar was inoculated with viable yeasts from the YM broth (0.5ml of the inoculated YM broth per 50ml YM agar), and poured on top of the bacterial incubated MRS agar plates. After 24 hours of incubation at 30°C, the plates were examined for clear zones of inhibition and the diameter of the zones measured.

3.2.2. Growth and interaction in UHT-milk

Strains of LAB and yeasts were inoculated as log 6units per ml (LAB) and log 4 units per ml (yeasts) in 10ml UHT milk (Full cream, Clover, S.A (Pty) Ltd) in 20ml sterile McCarthy bottles with screw cap. Each strain was inoculated individually and in pairs of yeasts and LAB, as represented in Table 1. All inoculation procedures were done before the microbial co-culture was started; starting with L1-L4, Y1-Y5, combination between L1-L4 and Y1-Y5 and then also a control (C) was performed. This was done in duplicate.

3.2.3. Enumeration of LAB and yeasts

The enumeration of cell numbers was done on day 0, 1, 3 and day 6, at the same time every day as shown in Fig. 1. LAB were enumerated by pour plating appropriate serial dilution of the UHT milk (0.1ml) on sterile M17 and MRS agar (Merck). The plates were incubated at 30°C for one day. Spoilage yeasts were enumerated by spread plating (0.1ml) on RBCA (Oxoid). The plates were incubated at 25°C for three days. This was done in duplicate.

3.2.4. Chemical analysis

Samples L1-L4 as well as combinations between L1-L4 and Y1-Y5 were performed as represented in Table 1. Approximately 2ml of the culture were transferred into 2ml Eppendorf tubes and centrifuged at 14 000 rpm for 5 min at 4°C. Approximately 1.8ml of the supernatant was transferred into a new tube and 109.8µl of 35% Perchloric acid added. The tubes were then placed on ice for 10min. 99µl of 7N KOH was then added to the tubes and left on ice for 1 hr. The samples were then centrifuged at 14 000 rpm for 10 min at 4°C, 250µl of the supernatant removed and carefully transferred avoiding toprecipitate. This was repeated two times. 250µl of the sample were injected into the HPLC.

3.2.4.1. Analysis of organic acids and carbohydrates

HPLC analysis was carried out on a Shimadzu Prominence system with a UV/VIS detector. Analytes were separated on a Bio-Rad HPX 87 H (7.8 x 300mm) ion exchange columns maintained at 65°C. Elution was performed with 5mM sulphuric acid at a flow rate of 0.5 ml per minute. Organic acids were detected at 202 and 276nm. Carbohydrates were detected with a Waters 2414 RI detector connected in series. RID cell temperature was 40°C. Data recording and analysis were carried out with Shimadzu LC solution software.

3.2.4.2. Analysis of volatile compounds

The product of volatile compounds was analysed by headspace-solid phase micro extraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS). Samples were cultured in 20 ml headspace vials and frozen for 24 hours. When ready for analysis the vials were thawed and equilibrated at 60°C for 1 hour in a block heater. A SPME fibre coated with Carboxen/Polydimethylsiloxane, sorbent thickness 75µm was exposed to the headspace for 20min. Thermal desorption was carried out in the

injection port of the gas chromatograph which was fitted with a 0.75 mm inner diameter glass liner at 250°C for 2 min in split less mode.

The GC-MS system consisted of a Trace GC ultra gas chromatograph and a DSQ mass spectrometer (Thermo Electron Scientific). The analytical column was a Varian FactorFour VF-5ms, 30 m in length, an inner diameter of 0.25mm and film thickness of 0.25µm. Helium was the carrier gas at 2 ml/min. The GC oven was initially held at 40°C for 5 minutes after which the temperature was ramped to 200°C at 4°C per minutes and held for 5 minutes. The MS transfer line was held at 250°C.

Mass analysis was carried out by electron impact ionization at 70eV, source temperature 200°C and a mass range of 40-400m/z. Data was captured and processed with Xcalibur 1.4 MS software. Compounds were identified by spectral comparison with authentic samples and the NIST 11 spectral library.

3.2.5. Determining pH

The pH of each of the samples was determined using a digital pH meter (Cyberscan 510, Eutech Instruments, Germany) fitted with an FC 200 electrode (CE, Singapore. The pH meter was calibrated using commercial buffers (Merck) of pH 4 and 7.

3.3. Results and Discussion

Fermented milk products are usually regarded as predominantly lactic acid fermentations. The frequent occurrence of yeasts and LAB simultaneously in fermented products however can influence the quality and characteristics of the product (Narvhus & Gadaga, 2003) and therefore studies on the mechanisms of interaction, such as the production of metabolic products that inhibit each other's growth, are important.

The 15 lactic acid bacteria isolates with inhibitory activity were tested against five prominent yeasts species commonly found in dairy products. The inhibition test based on clear zones showed no inhibition zones, which indicated that the LAB tested were not able to inhibit the growth of the yeasts. It might be that the LAB did not inhibit the yeasts sufficiently to get a clear inhibition zone, and only being adequate when the yeasts are totally inhibited. The yeasts strains not affected by the lactic acid bacteria can therefore proliferate and some of these yeasts can cause contamination and consequently pose a health risk to the consumer. However, in previous studies in our laboratory, distinct zones were obtained between LAB and some yeasts (Van Jaarsveld, 2010). No exact answer was formulated at the time for the reason for the reason of inhibition.

Since no inhibition was observed on the rapid screening test, it was decided to grow the different combinations in co-culture in a liquid medium. As stated in literature lactic acid bacteria inhibition mechanisms include the production of organic acids, carbon dioxide, hydrogen peroxide, diacetyl, reuterin and bacteriocins (Caplice & Fizgerald, 1999; De Vuyst & Vandamme, 1994). The four lactic acid bacteria isolates which showed the most enhanced inhibitory activity were grown in co-culture with five selected types of yeasts frequently observed in dairy products to see if any interaction; inhibition or stimulation take place. As a control, all LAB and yeasts were also grown alone in UHT-milk. The results are presented graphically showing all combinations between LAB and yeasts. Growth of each microorganism for specified time zones is shown in Figs 2 and 3.

The LAB in pure cultures had the best growth after the first day of incubation. At Day 0, the lactic acid bacteria started with a maximum of between log 5.4 cfu/ml (*Enterococcus durans* L2) and log 5.5 cfu/ml (*Lactobacillus curvatus* L3). At Day 1 the LAB in pure cultures reached there maximum growth. Reaching between log 7.2 cfu/ml (*Enterococcus durans* L2) and log 8.3 cfu/ml (L1). After Day 1 the LAB pure cultures started to decrease in microbial numbers, reaching as low as log 4.2 cfu/ml (*Lactococcus lactis* subsp. *lacits* L1, *Lactobacillus curvatus* L3 and *Lactobacillus fermentum* L4).

For the combination between the LAB and yeasts, the LAB microbial numbers started with log 5.3 cfu/ml (*Enterococcus durans* and *Debaryomyces hansenii* L2Y3) and log 5.5 cfu/ml on Day 0. The maximum growth rate was during the first 24 hours, at Day 1 the LAB in combination with the yeasts reached as high as log 9.6 cfu/ml (*Enterococcus durans* and *Zygosaccharomyces bailii* L2Y5). After day 1 the growth of the LAB in combination with the yeasts started to decrease and ended up with a microbial count of between log 6.2 and 6.5 cfu/ml. (*Enterococcus durans* and *Zygosaccharomyces bailii* L2Y5) was the best combination in terms of the growth rate of the LAB. *Enterococcus durans* (L2) had the best microbial count at Day 1 and also was still found in high number after Day 3 (log 8.5 cfu/ml).

Yeasts had lower log numbers than the LAB on each occasion during the entire growth trail. All five yeasts started off with a log 5 cfu/ml on Day 0 and the microbial numbers increased to between log 7 cfu/ml (*Candida sake* Y2) and log 7.5 cfu/ml (*Kluyveromyces marxianus* Y1, *Yarrowia lipolytica* Y4 and *Zygosaccharomyces hansenii* Y5) on Day 1. After Day 1 the microbial growth started to decrease and the reduction of growth from day 3 to day 6 was limited.

During the growth trails of the yeasts in co-culture with the LAB it was observed that the yeasts struggle to survive in the presence of LAB. On Day 0, the yeasts in combination with the LAB started with a microbial count of between log 4.3 cfu/ml

and log 5.2 cfu/ml. After 24 hours *Yarrowia lipolytica* (Y4) in combination with *Lactobacillus fermentum* (L4) had the highest microbial count, reaching log 5.4 cfu/ml. While *Zygosaccharomyces bailli* (Y5) struggle in combination with *Lactobacillus curvatus* (L3) only reaching a microbial count of log 4 cfu/ml on Day 1. After Day 1 the microbial counts started to decreased and reached as low as log 2.7 cfu/ml on Day 6. *Yarrowia lipolytica* (Y4) in combination with *Lactobacillus fermentum* (L4) had the highest microbial growth during the growth trails.

Co-cultures with LAB seemed to discourage the yeast's growth conditions after 1 day incubation. This is probably due to acid and other compounds produced by LAB (Gadaga *et al.,* 2001b). This indicated that the yeasts thrived best alone and was inhibited by the different LAB.

The pH of the control (pure UHT milk) had a pH of 6.49 at Day 0 and ended up at around a pH 5.45 on Day 6. The LAB strains alone started with a pH of around 5.56-5.73 (Day 0) and ended up with a pH of 4.4 – 4.63 (Day 6). With *Lactobacillus fermentum* (L4) reaching the lowest ph of 4.4 on Day 6, this corresponds with literature that indicates that lactobacilli have the ability to produce a pH at values as low as 4 (Ortu *et al.,* 2007). The yeasts on its own had a slight reduction in pH, reducing the pH of the milk between 5.2 and 5.3 on Day 6. The highest reduction in pH of yeast grown in single culture was after 6 days for *Candia sake* (Y4), (Fig. 4).

The reduction in pH during the fermentation went just as fast for the LAB alone as it did with co-cultures of LAB and yeasts. *Enterococcus durans* (L2) in combination with *Zygosaccharomyces bailli* (Y5) had the lowest pH, 4.50 on Day 1. The combination between *Lactobacillus curvatus* (L3) and *Yarrowia lipolytica* (Y4) had the highest pH, 4.64 on Day 1, but all the co-cultures ended up with pH values around 4.2 – 4.7 at Day 6 (Figs 5 and 6). Lowest pH values were detected for combinations showing the most enhanced growth.

The presence of different organic acids for sample L1-L4 and the combination between L1-L4 and Y1-Y4 were tested over a period of six days of incubation. The presence of organic acid in milk samples is very important, since it is known that organic acids are important for the flavour compounds in fermented milk. Organic acids also play an important role in producing a safe fermented product (Rubin *et al.,* 1982). The organic acids detected included small amounts of citric acid as well as high amounts if lactic acid. All results are shown in Table 2.

According to the HPLC results, for L1-L4 there were low concentrations of citric acid, while the lactic acid was more prominent especially after 3 days of incubation. *Lactobacillus fermentum* (L4) had the lowest presence of citric acid whereas *Lactococcus lacits* spp. *lactis* (L1) had the highest amount of citric acid (2.16 g/l) after 3 days of incubation. This was stilla small amount of organic acids in comparison of the lactic acid presence in these single inoculations of LAB. Lactic acid production was as high as 47.5 g/l (*Lactobacillus curvatus* L3) after 3 days of incubation (Figs 7-10).

The combination between the LAB and yeasts definitely had a positive effect on the production of lactic acid. The production of citric acid was still found at low values. Citric acid plays an important role in aroma development during day 1 and 3 of fermentation but rapidly disappears after this because of the action of lactic acid bacteria that use it as a substrate for secondary reactions. Leuconostocs and *Lactococcus lactis* spp. *lactis* can convert citric acid to oxaloacetate and acetat (Hugenholtz, 1993). Lactic acid present in milk can play a significant role in improving shelf-life and safety of the product (Davidson, 2001). A high value of lactic acid was obtained from the combination between LAB and yeasts reaching as high as 64. 33 g/l (*Lactobacillus curvatus* and *Yarrowia lipolytica* L3Y4) even after 1 day of incubation. The best combinations between the LAB and yeasts are shown in Figs. 11-20.

Results obtained from the lactose analysis were not expected and are present in Table 3. Lactose is present in UHT milk, but extremely high concentrations of lactose were present even on Day 0. For the LAB in single cultures in the UHT milk, *Enterococcus durans* (L2) converted more lactose on Day 0 reaching 32.73 g/l lactic acid while *Lactococcus lactis* spp. *lactis* (L1) converted the most lactose, reaching 36.04g/l lactic acid on Day 3 (Figs. 21-24).

In co-culture with the yeasts it was observed that after Day 3 hydrolysis of lactose started. Lactose is a fermentable sugar that can be used as an energy source. The lactose content decreased during the fermentation to a final value as low as 9.82 g/l on Day 6, which confirmed that the lactose was metabolized by the LAB to lactic acid (Gilliand & Rich, 1990) as observed in Figs 25-34.

For the production of volatile compounds (ethanol and acetone) during the growth trail, the pure cultures of LAB were lower than in co-culture with the yeasts. The highest production of ethanol by single cultures was *Lactobacillus fermentum* (L4) 3.66mg/l on day 0. While the highest production of acetone by pure cultures was by *Enterococcus durans* (L2) reaching 4.24 mg/l on day 0 (Figs. 35-38). Acetone presence here as product can be contributed to either the continuous production and reduction of diacetyl or via direct decarboxylation of α-acetolactate (Mathews *et al.,* 2000b).

For the co-cultures high amounts of ethanol and acetone was produced at day O, but after the production of the volatile compounds it slowly decreased endingup with around 1.05 to 0.17 mg/l at day 6 (Table 3). The combination between *Lactococcus lactis* spp. *lactis* and *Kluyveromyces marxianus* (L1Y1) had the highest production of ethanol on day 0, reaching 10.89mg/l. The concentration of ethanol produced by this bacterium is most likely due to its ability to reduce acetaldehyde to ethanol with alcohol dehydrogenase and the yeast speices capable to ferment lactose (Nicholson, 2003). The combination between *Lactococcus lactis* spp. *lactis* and

Yarrowia lipolytica (L1Y4) had the highest production of acetone on Day 0 reaching 11.56mg/l (Figs. 39-48).

3.4. Conclusion

During this study, metabolic interactions between lactic acid bacteria and yeasts in UHT milk were observed. In co-culture the LAB had much higher growth rates, indicating that the yeasts supported the growth of the LAB. *Enterococcus durans* had the best growth in co-culture with the yeasts. During the growth studies between these organisms in UHT milk it possible that fermentation will occur. As typical of a fermentation process the pH of the samples progressively decreased until Day 6. The lactose was hydrolyzed, which is common to be found during the fermentation process. Ethanol and acetone was also found in low numbers. Lactic acid appeared to be the dominant organic acid. The combination of the LAB-yeasts definitely had a positive effect on the production of lactic acid. This is clearly a result that the fermentation of milk is by the combination of mixed microorganisms and not only by a single microorganism. This change in the chemical composition has the ability to enhance the nutritional value, organoleptic characteristics as well as creating an environment incapable for pathogens to survive.

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3.6. Tables and Figures

Table 1: Combination of samples used in trails.

Yeasts / LAB		Y1	Y2	Y3	Y4	Y5
		Y1 (5)	Y2 (6)	Y3 (7)	Y4 (8)	Y5 (9)
L1	L1 (1)	L1/Y1 (10)	L1/Y2 (11)	L1/Y3 (12)	L1/Y4 (13)	L1/Y5 (14)
L2	L2 (2)	L2/Y1 (15)	L2/Y2 (16)	L2/Y3 (17)	L2/Y4 (18)	L2/Y5 (19)
L3	L3 (3)	L3/Y1 (20)	L3/Y2 (21)	L3/Y3 (22)	L3/Y4 (23)	L3/Y5 (24)
L4	L4 (4)	L4/Y1 (25)	L4/Y2 (26)	L4/Y3 (27)	L4/Y4 (28)	L4/Y5 (29)

L1 – *Lactococcus lactis* subsp. *lactis*	L2 – *Enterococcus durans*
L3 – *Lactobacillus curvatus*	L4 – *Lactobacillus fermentum*
Y1 – *Kluyveromyces marxianus*	Y2 – *Candida sake*
Y3 – *Debaryomcyces hansenii*	Y4 – *Yarrowialipolytica*
Y5 – *Zygosaccharomyces bailii*	

Table 2: Organic acid production during growth study of LAB and yeasts in combination in UHT milk.

Sample Name	Concentration (g/L) Citric acid	Detector response	Sample Name	Concentration (g/L) Lactic acid	Detector response
A01	0.19	439817	A01	0.17	12199
A02	0.31	700210	A02	0.85	60635
A03	0.04	88593	A03	0	0
A04	0.11	243331	A04	0.2	14158
	0.07	151251	A10	0	0

A10					
A11	0.11	250782	A11	0.24	16949
A12	0.25	559164	A12	0.58	41448
A13	0.29	659648	A13	0.18	13226
A14	0.21	472240	A14	0.52	36971
A15	0.04	89197	A15	0	0
A16	0.16	373027	A16	0.21	15323
A17	0.32	728884	A17	0.81	58366
A18	0.03	67606	A18	0	0
A19	0.18	402385	A19	0.41	29699
A20	0.14	315000	A20	0.28	20008
A21	0.19	423350	A21	0.75	53477
A22	0.22	502237	A22	0.98	70206
A23	0.23	517025	A23	0.32	23262
A24	0.05	115848	A24	0	0
A25	0.12	269865	A25	0	0
A26	0.34	774500	A26	1.14	82000
A27	0.06	130209	A27	0	0
A28	0.04	101645	A28	0	0
A29	0.13	291708	A29	0.2	14318
B001	0.5	1130250	B001	9.43	675402
B002	0.03	78028	B002	1.4	100540
B003	0.06	128265	B003	2.08	148791
B004	0.05	116351	B004	1.9	136364
B010	2.07	4705205	B010	36.76	2633033
B011	1.71	3880851	B011	52.18	3738086
B012	1.81	4103017	B012	36.73	2631502
B013	1.26	2868847	B013	49.99	3580920
B014	1.95	4429789	B014	53.49	3831959
B015	2.05	4662317	B015	54.8	3925815
B016	1.5	3408683	B016	51.69	3703150
B017	2.04	4636776	B017	49.36	3535952
B018	2.38	5406952	B018	61.23	4386407
B019	2.89	6567805	B019	52.55	3764368
B020	1.2	2719409	B020	45.18	3236834
B021	1.28	2906945	B021	43.6	3123410
B022	0.91	2058569	B022	32.33	2316191
B023	1.28	2919194	B023	64.33	4608448
B024	1.57	3572071	B024	48.86	3500399
B025	1.6	3641697	B025	54.46	3901027
B026	0.22	496845	B026	9.9	709352
B027	1.18	2690644	B027	35.45	2539393

B028	1.53	3471025	B028	66.85	4788819
B029	1.24	2811944	B029	46.38	3322669
C001	2.16	4914942	C001	42.63	3054158
C002	1.91	4337717	C002	43.99	3151055
C003	1.62	3691326	C003	47.5	3402627
C004	0.11	256717	C004	1.75	125283
C010	1.58	3595888	C010	44.59	3193942
C011	2.36	5353536	C011	48.67	3486871
C012	1.66	3773170	C012	31.88	2283810
C013	2.38	5403536	C013	49.41	3539870
C014	1.38	3130136	C014	43.7	3130761
C015	1.66	3772889	C015	39.42	2823740
C016	2.06	4689212	C016	46.68	3343915
C017	1.12	2550102	C017	25.82	1849624
C018	2.34	5322753	C018	52.62	3769711
C019	2.03	4603985	C019	46.89	3358899
C020	1.2	2728395	C020	28.94	2072964
C021	1.46	3321969	C021	35.69	2556391
C022	1.18	2677709	C022	38.53	2759856
C023	1.18	2671932	C023	40.02	2867021
C024	1.47	3333117	C024	38.28	2741994
C025	1.34	3037099	C025	47.8	3423850
C026	1.79	4072055	C026	46.98	3365424
C027	1.77	4017155	C027	39	2793866
C028	1.66	3765451	C028	47.49	3402200
C029	1.16	2634559	C029	42.96	3077638
D01	0.04	81716	D01	0.71	50897
D02	0.08	185827	D02	2.38	170272
D03	0.07	149804	D03	3.07	219850
D04	0.1	222471	D04	5.02	359822
D10	1.55	3526085	D10	39.19	2807058
D11	1.47	3342060	D11	40.25	2883152
D12	0.88	2008500	D12	28.34	2030227
D13	1.27	2889580	D13	37.59	2693040
D14	0.77	1738971	D14	24.93	1785907
D15	1.71	3880487	D15	48.87	3500912
D16	1.09	2476925	D16	38.23	2738655
D17	1.49	3379098	D17	45.06	3227688
D18	1.75	3974075	D18	49.98	3580169
D19	1.61	3649477	D19	50.22	3597886
D20	0.7	1585066	D20	37.96	2719539

D21	1.23	2791424	D21	49.74	3563271
D22	1.23	2796557	D22	54.36	3894011
D23	1.26	2864769	D23	58.32	4177484
D24	0.95	2147402	D24	42.92	3074888
D25	0.81	1846365	D25	34.98	2505854
D26	0.83	1895101	D26	38.61	2765658
D27	0.73	1660640	D27	34.83	2495238
D28	0.68	1537327	D28	30.13	2158417
D29	0.86	1951280	D29	36.96	2647378

A	Day 0
B	Day 1
C	Day 3
D	Day 6

Table 3: Hydrolyzing of carbohydrates during growth study of LAB and yeasts in combination in UHT milk.

Sample Name	Concentration (g/L) Lactose	Sample Name	Concentration (g/L) Lactose
A01	20.29	C001	36.04
A02	32.73	C002	35.29
A03	4.96	C003	8.0
A04	7.25	C004	15.28
A10	7.362	C010	33.16
A11	13.623	C011	42.55

A12	27. 98	C012	26.99
A13	29.99	C013	34.81
A14	20.21	C014	32.75
A15	6.97	C015	36.04
A16	16.31	C016	33.35
A17	24.26	C017	28.14
A18	3.14	C018	43.91
A19	16.79	C019	43.92
A20	12.59	C020	26.05
A21	19.77	C021	33.77
A22	16.02	C022	30.30
A23	18.51	C023	34.47
A24	5.87	C024	33.33
A25	11.05	C025	33.31
A26	30.02	C026	33.29
A27	6.70	C027	23.13
A28	2.47	C028	28.05
A29	11.60	C029	28.54
B001	32.73	D01	1.81
B002	33.23	D02	6.68
B003	7.55	D03	1.37
B004	10.59	D04	2.72

B010	36.63	D10	32.80
B011	40.87	D11	25.78
B012	32.56	D12	16.95
B013	33.16	D13	23.63
B014	45.22	D14	15.45
B015	49.22	D15	33.53
B016	30.22	D16	22.08
B017	27.23	D17	9.82
B018	13.75	D18	29.98
B019	45.19	D19	32.23
B020	34.31	D20	17.36
B021	29.49	D21	31.29
B022	21.52	D22	33.23
B023	30.41	D23	24.56
B024	24.54	D24	24.54
B025	28.68	D25	18.01
B026	4.36	D26	19.24
B027	23.33	D27	14.79
B028	33.45	D28	15.53
B029	26.24	D29	16.03

A	Day 0

B	Day 1
C	Day 3
D	Day 6

Table 4: Volatile production during growth study of LAB and yeasts in combination in UHT milk.

Sample name	Concentration (mg/L)	Sample name	Concentration (mg/L)
	Ethanol		Acetone
A1	2.36	A1	2.27
A2	3.28	A2	4.24
A3	3.29	A3	3.19
A4	3.66	A4	2.44
A10	10.89	A10	8.31
A11	6.81	A11	9.12
A12	3.55	A12	8.71
A13	1.52	A13	11.56
A14	9.43	A14	10.69
A15	10	A15	16.55
A16	7.25	A16	9.12
A17	3.05	A17	6.62
A18	2.79	A18	12.66
A19	9.12	A19	9.47
A20	8.59	A20	8.48

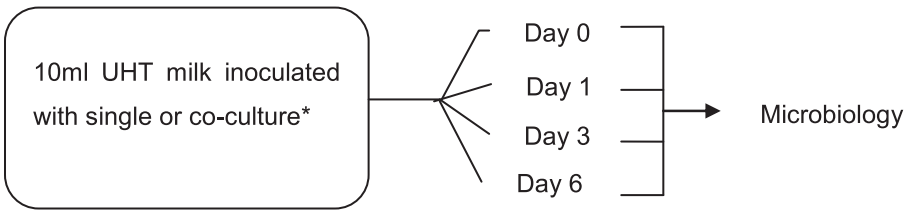
A21	7.26	A21	6.97
A22	3.94	A22	8.02
A23	1.34	A23	7.03
A24	9.41	A24	10.45
A25	7.92	A25	9.12
A26	6.2	A26	7.09
A27	6.78	A27	4.53
A28	7.15	A28	3.48
A29	6.09	A29	3.31
B1	2.38	B1	1.86
B2	3.03	B2	2.79
B3	2.96	B3	1.92
B4	2.74	B4	0.58
B10	6.37	B10	3.37
B11	6.82	B11	3.89
B12	5.4	B12	5.46
B13	2.75	B13	4.59
B14	5.05	B14	5.52
B15	6.04	B15	5.81
B16	6.19	B16	3.83
B17	2.98	B17	2.79

B18	2.72	B18	4.65
B19	6.16	B19	4.3
B20	5.17	B20	3.72
B21	2.55	B21	3.02
B22	2.93	B22	3.66
B23	2.34	B23	3.48
B24	3.66	B24	3.31
B25	4.83	B25	3.89
B26	4.61	B26	5.11
B27	4.32	B27	3.37
B28	4.69	B28	3.89
B29	3.27	B29	3.25
C1	0.68	C1	0.41
C2	2.91	C2	2.56
C3	1.77	C3	1.34
C4	1.1	C4	0.52
C10	2.18	C10	1.16
C11	3.53	C11	2.44
C12	2.58	C12	4.01
C13	1.59	C13	2.5
C14	2.64	C14	3.95

C15	2.8	C15	2.32
C16	3.49	C16	2.85
C17	2.22	C17	1.22
C18	1.35	C18	2.15
C19	3.75	C19	3.08
C20	3.19	C20	1.86
C21	0.38	C21	0.35
C22	1.6	C22	2.73
C23	1.3	C23	1.45
C24	2.1	C24	2.32
C25	2.28	C25	2.44
C26	3.06	C26	2.96
C27	2.25	C27	2.44
C28	2.47	C28	3.02
C29	1.45	C29	3.02
D1	0.38	D1	0.41
D2	0.76	D2	0.76
D3	0.24	D3	0.52
D4	0.54	D4	0.46
D10	0.88	D10	1.05
D11	0.78	D11	0.76

D12	0.7	D12	1.1
D13	0.58	D13	0.81
D14	0.58	D14	1.05
D15	0.78	D15	1.28
D16	0.8	D16	0.81
D17	0.48	D17	0.41
D18	0.31	D18	0.81
D19	0.91	D19	0.76
D20	0.67	D20	0.64
D21	0.17	D21	0.17
D22	0.62	D22	0.58
D23	0.35	D23	0.64
D24	0.77	D24	0.64
D25	0.72	D25	0.64
D26	0.88	D26	1.1
D27	0.38	D27	0.58
D28	0.56	D28	0.7
D29	0.49	D29	2.27

A	Day 0
B	Day 1
C	Day 3
D	Day 6



*LAB inoculated to log 6 units per ml, and yeasts to log4 units per ml.

Figure 1: Illustration of the growth and interaction study in the trail.

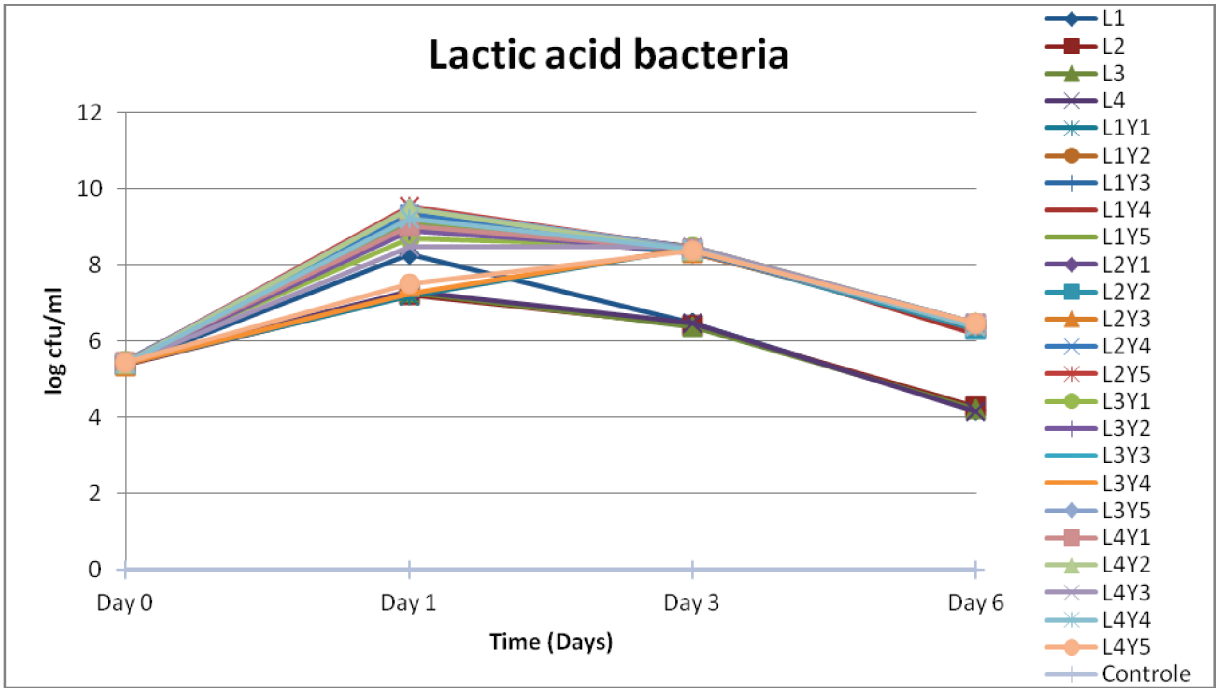


Figure 2: Growth of lactic acid bacteria alone and in combination with yeasts.

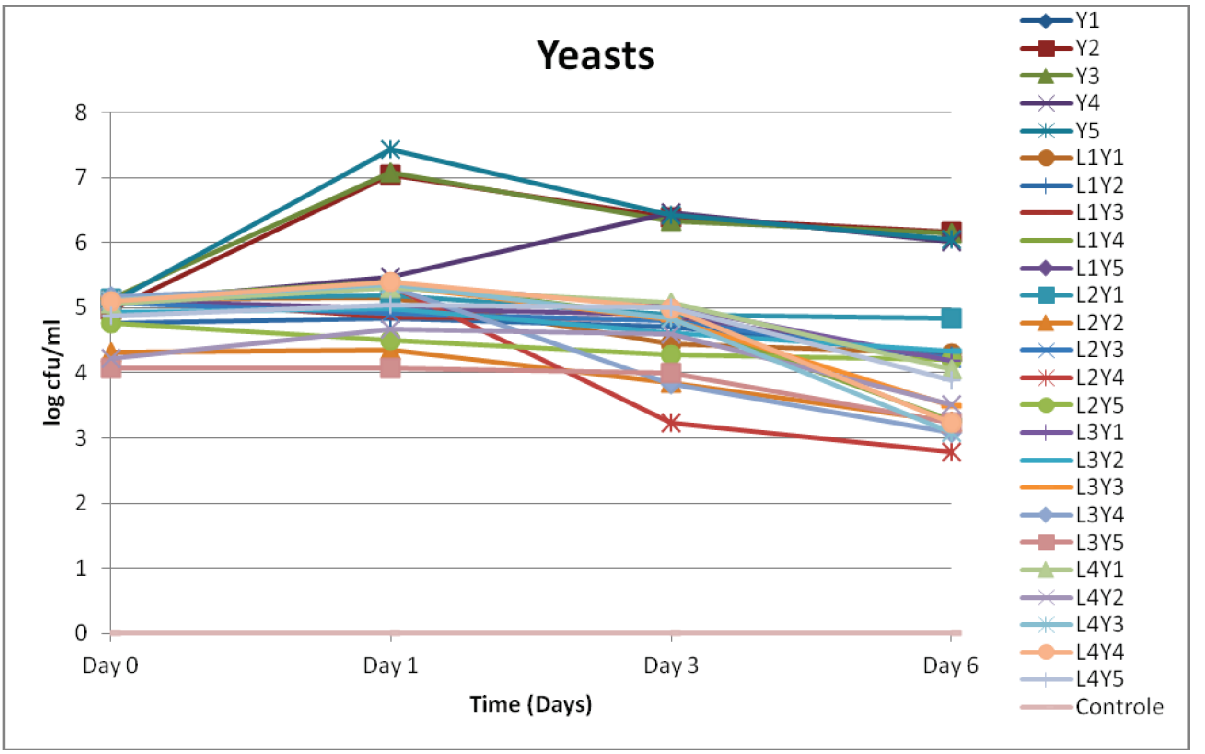


Figure 3: Growth of yeasts alone and in combination with lactic acid bacteria.

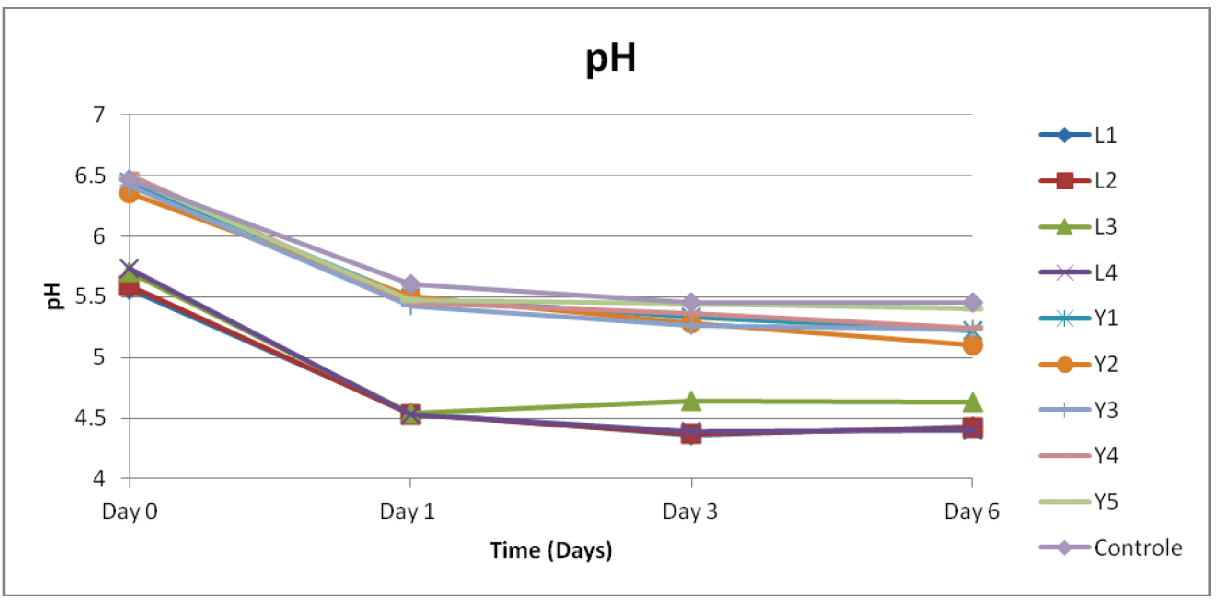


Figure 4: Changes in pH values during growth studies of LAB and yeast combination in UHT milk.

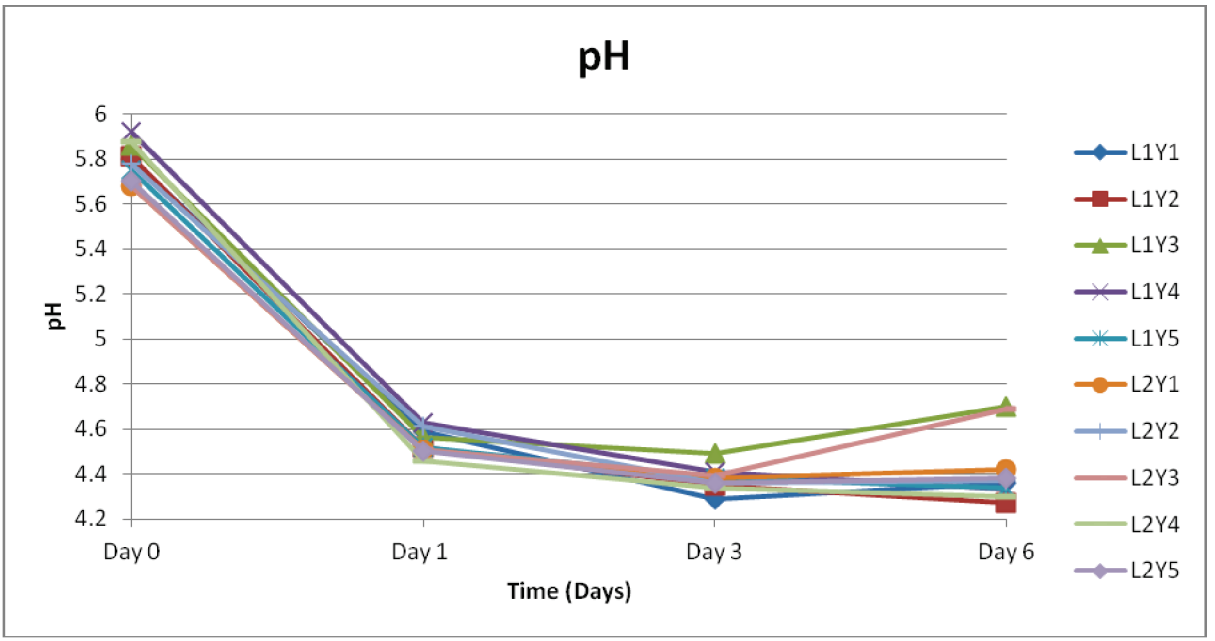


Figure 5: Changes in pH values during growth studies of LAB and yeast combination in UHT milk.

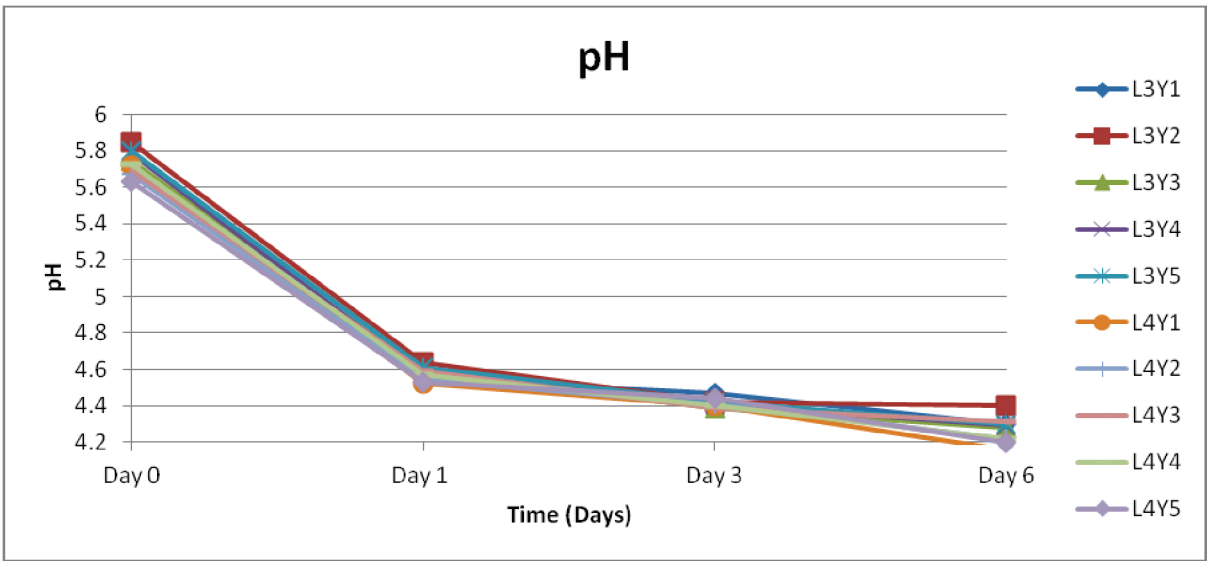


Figure 6: Changes in pH values during growth studies of LAB and yeast combination in UHT milk.

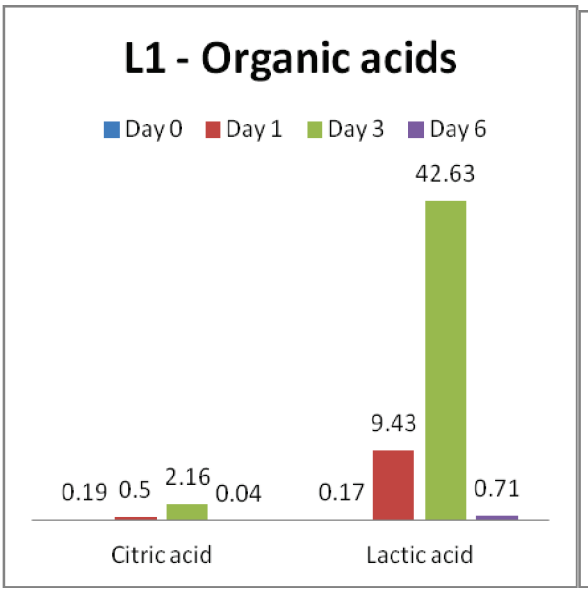


Figure 7: Production of organic acids during growth study of L1.

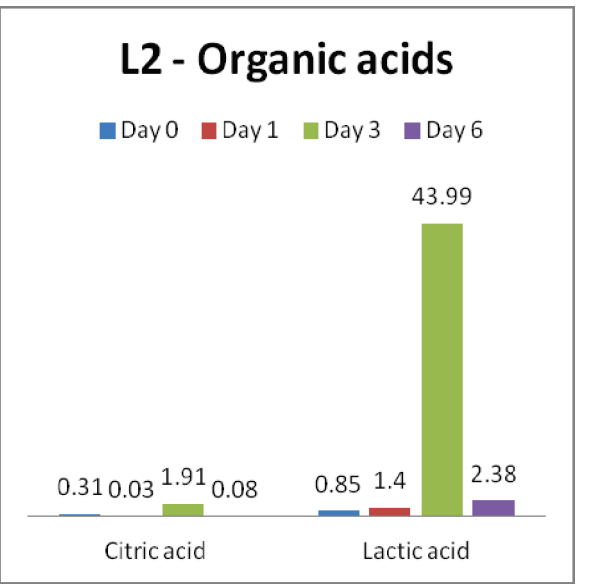


Figure 8: Production of organic acids during growth study of L2.

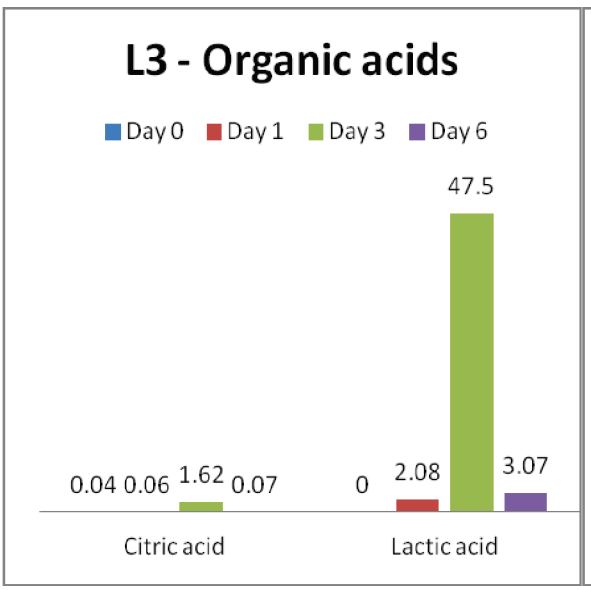


Figure 9: Production of organic acids during growth study of L3.

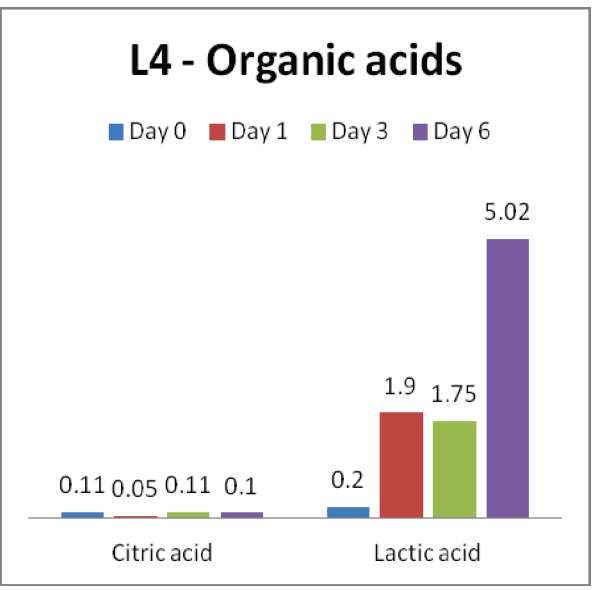


Figure 10: Production of organic acids during growth study of L4.

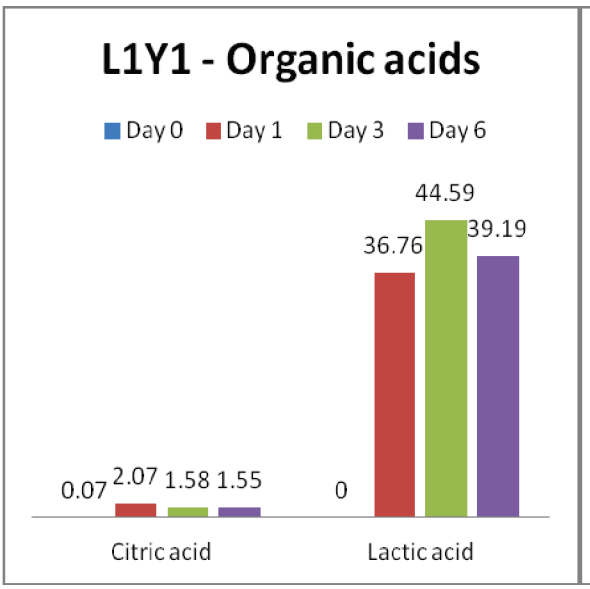


Figure 11: Production of organic acids during growth study of L1Y1.

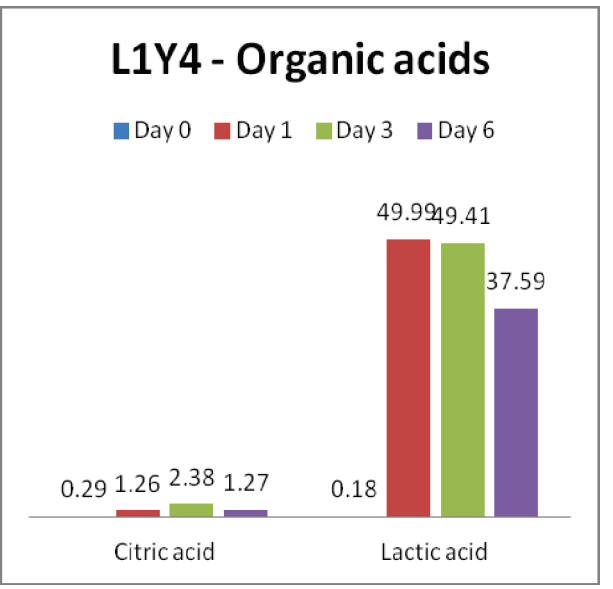


Figure 12: Production of organic acids during growth study of L1Y4.

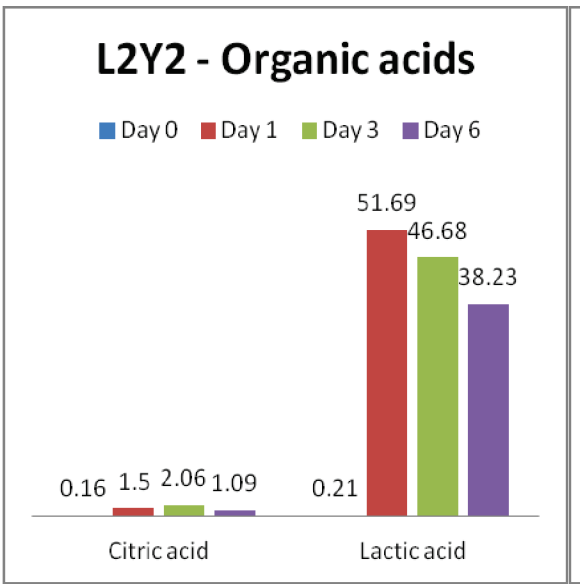


Figure 13: Production of organic acids during growth study of L2Y2.

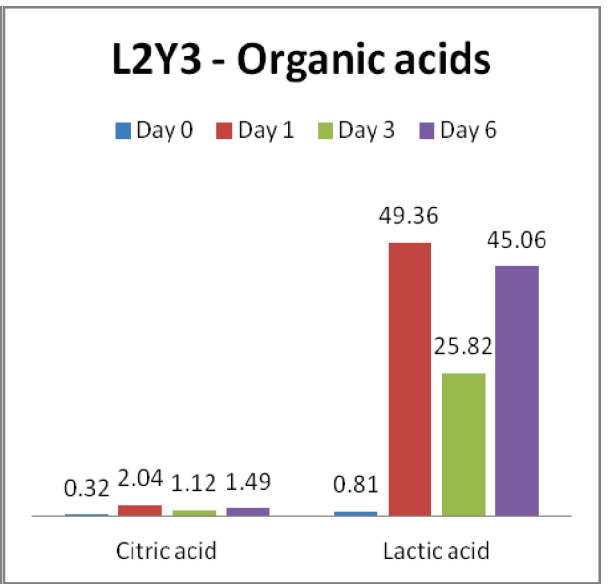


Figure 14: Production of organic acids during growth study of L2Y3.

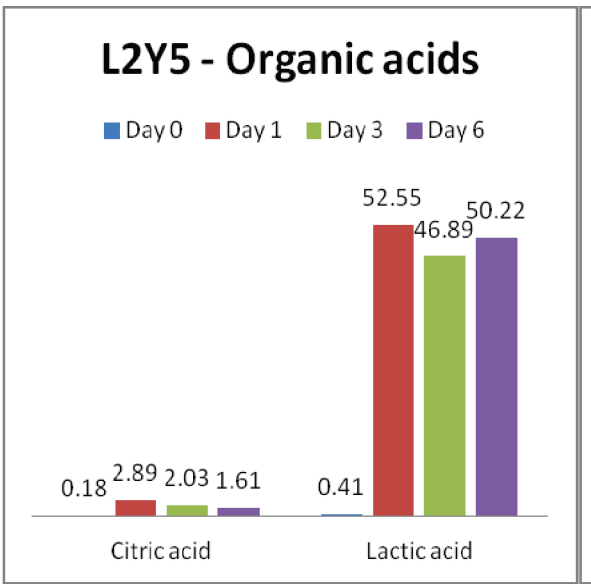


Figure 15: Production of organic acids during growth study of L2Y5.

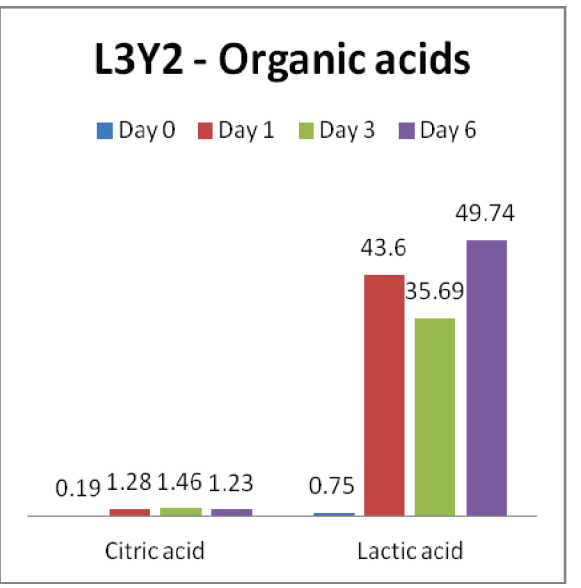


Figure 16: Production of organic acids during growth study of L3Y2.

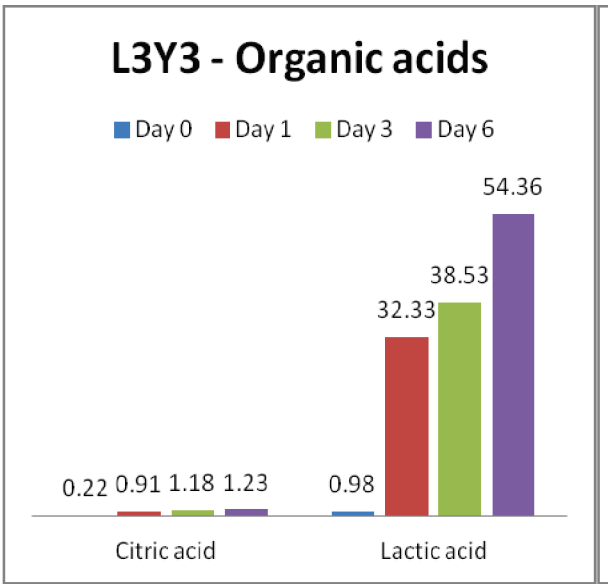


Figure 17: Production of organic acids during growth study of L3Y3

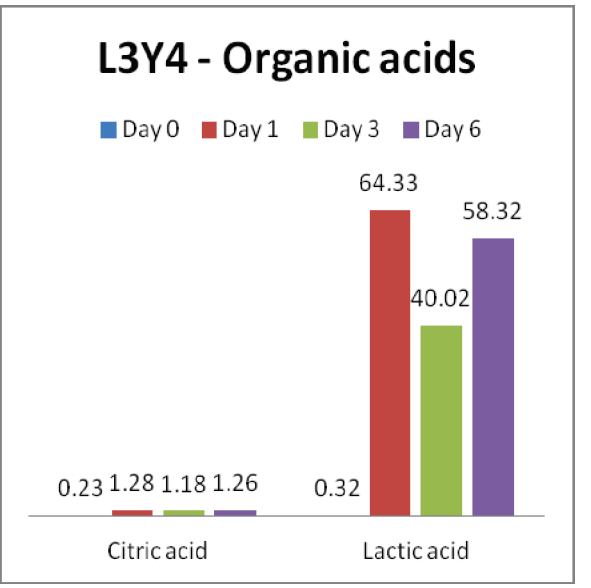


Figure 18: Production of organic acids during growth study of L3Y4.

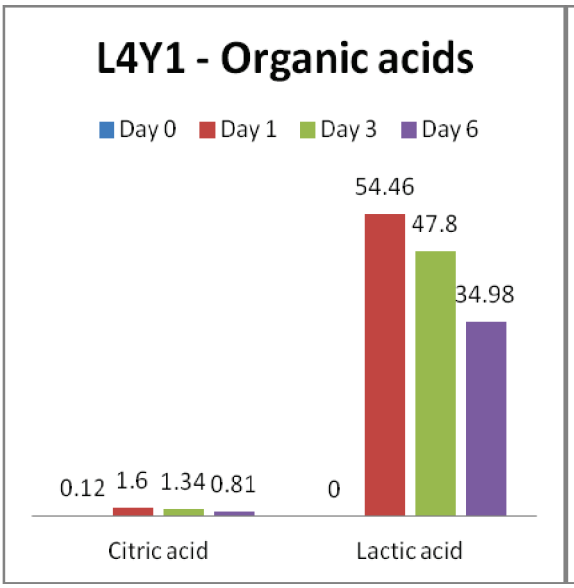


Figure 19: Production of organic acids during growth study of L4Y1.

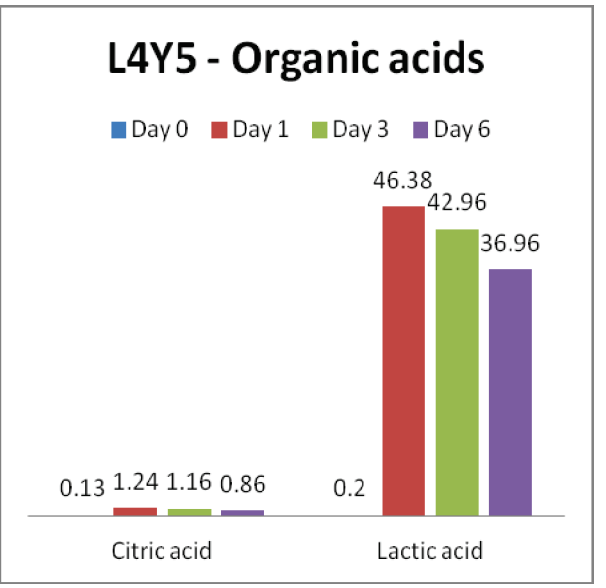


Figure 20: Production of organic acids during growth study of L4Y5.

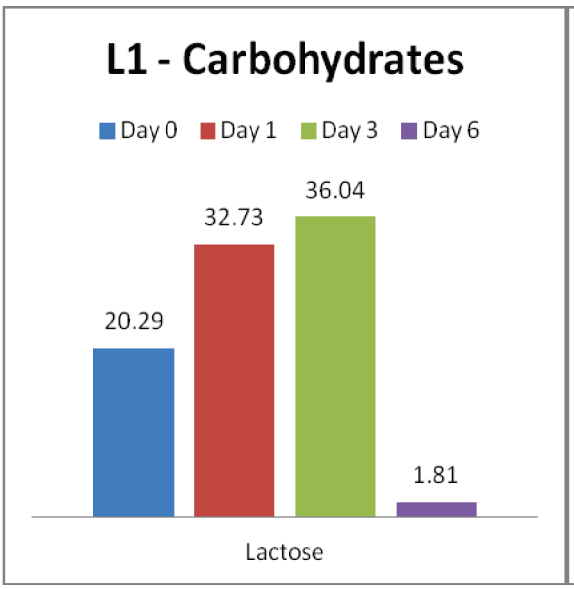


Figure 21: Hydrolyzing of carbohydrates during growth study of L1.

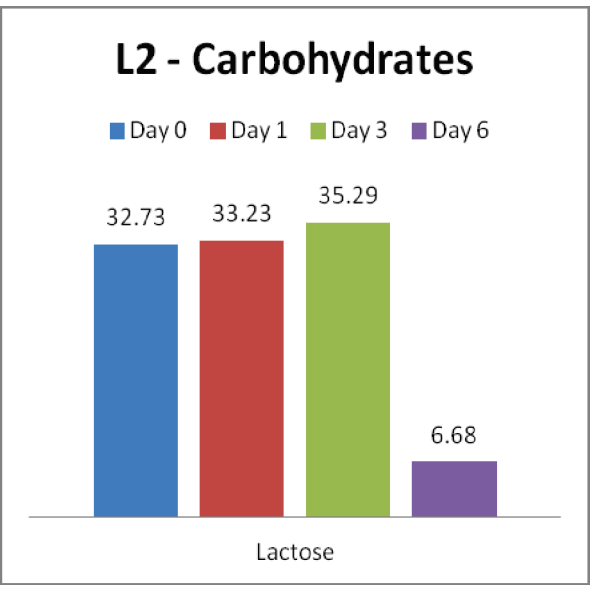


Figure 22: Hydrolyzing of carbohydrates during growth study of L2.

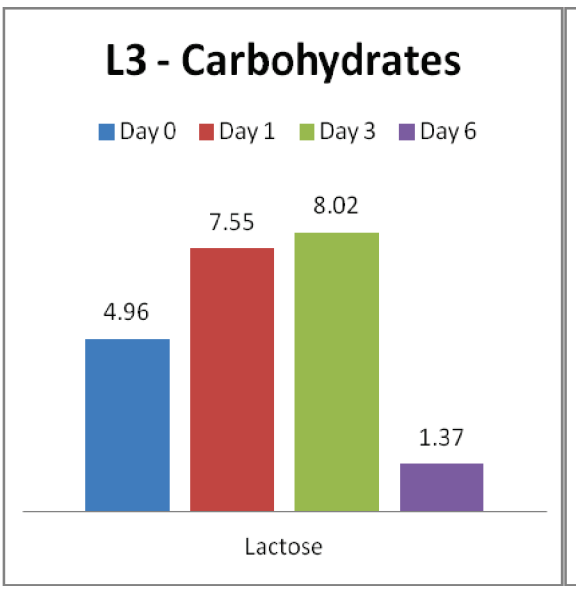


Figure 23: Hydrolyzing of carbohydrates during growth study of L3.

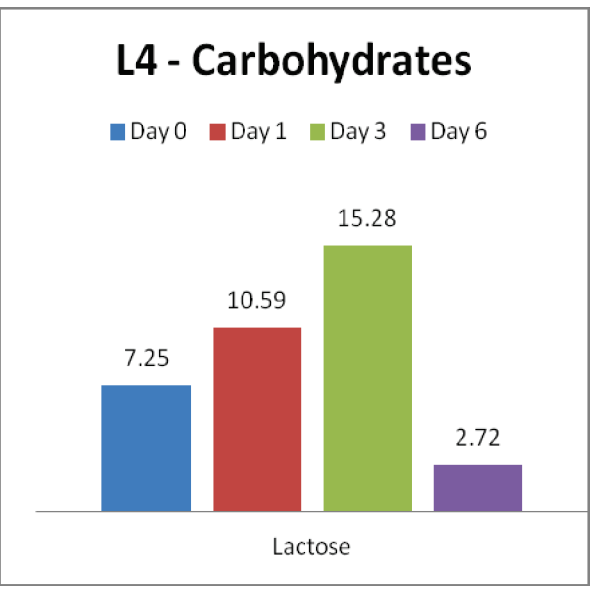


Figure 24: Hydrolyzing of carbohydrates during growth study of L4.

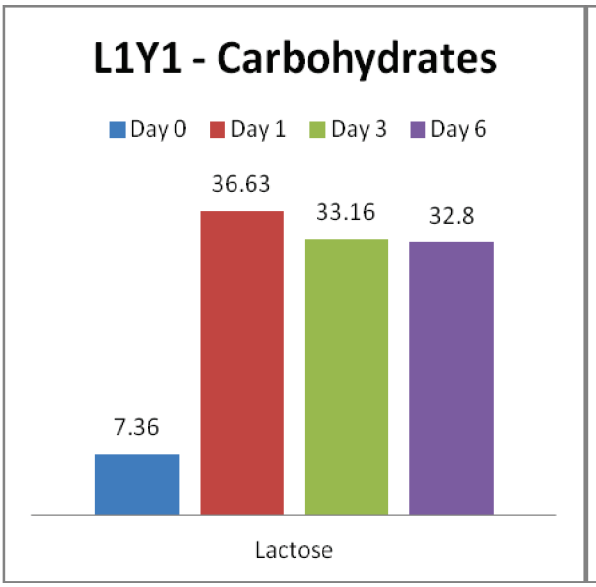


Figure 25: Hydrolyzing of carbohydrates during growth study of L1Y1.

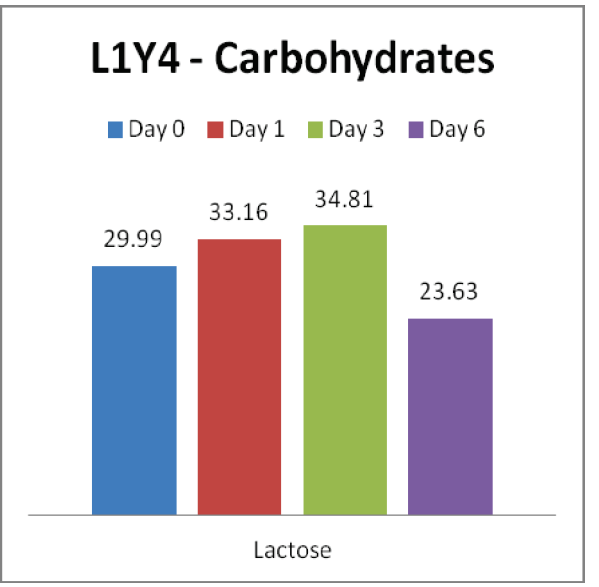


Figure 26: Hydrolyzing of carbohydrates during growth study of L1Y4.

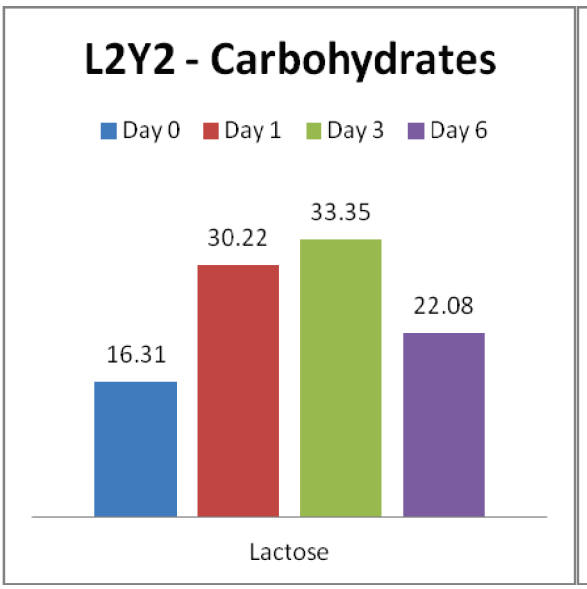


Figure 27: Hydrolyzing of carbohydrates during growth study of L2Y2.

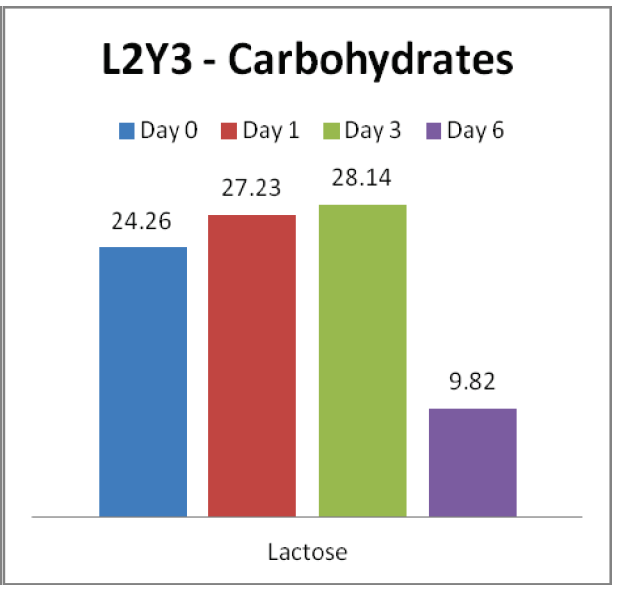


Figure 28: Hydrolyzing of carbohydrates during growth study of L2Y3.

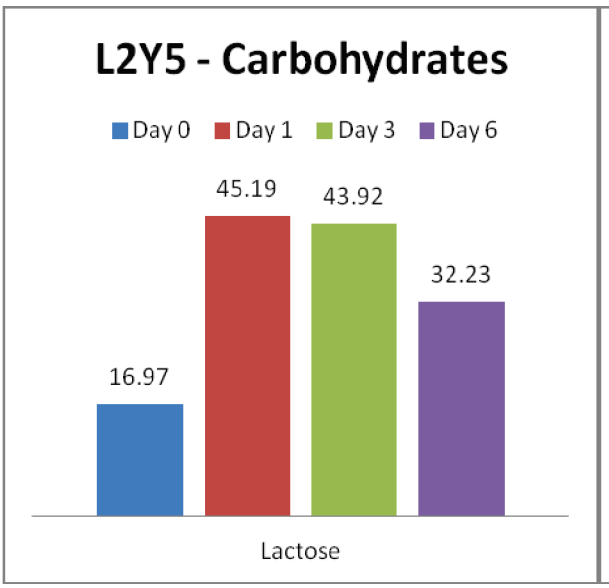


Figure 29: Hydrolyzing of carbohydrates during growth study of L2Y5.

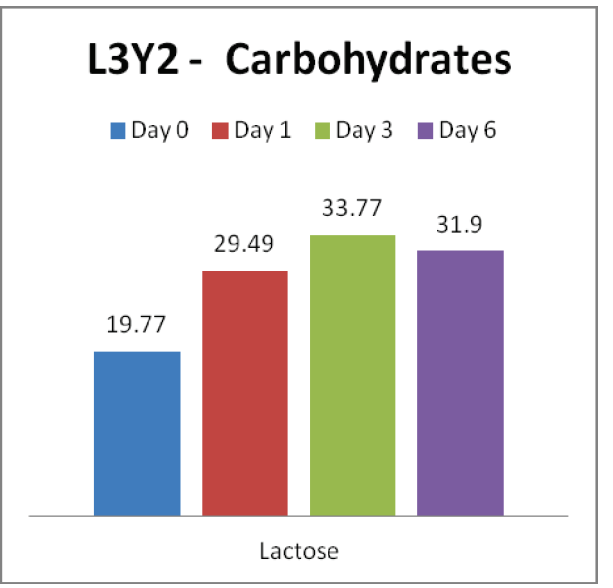


Figure 30: Hydrolyzing of carbohydrates during growth study of L2Y3.

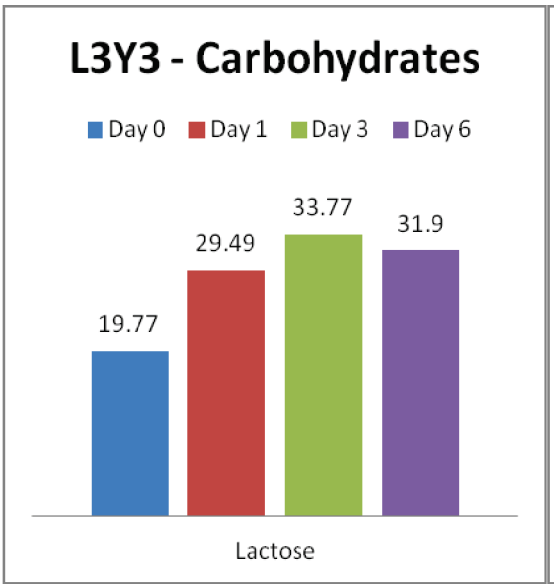


Figure 31: Hydrolyzing of carbohydrates during growth study of L3Y3.

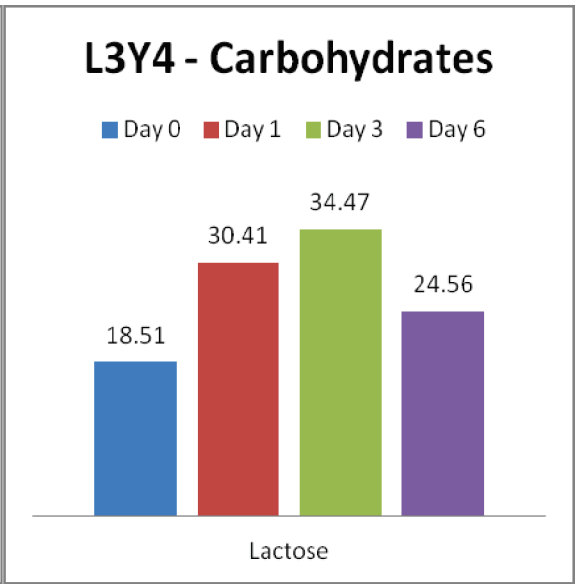


Figure 32: Hydrolyzing of carbohydrates during growth study of L3Y4.

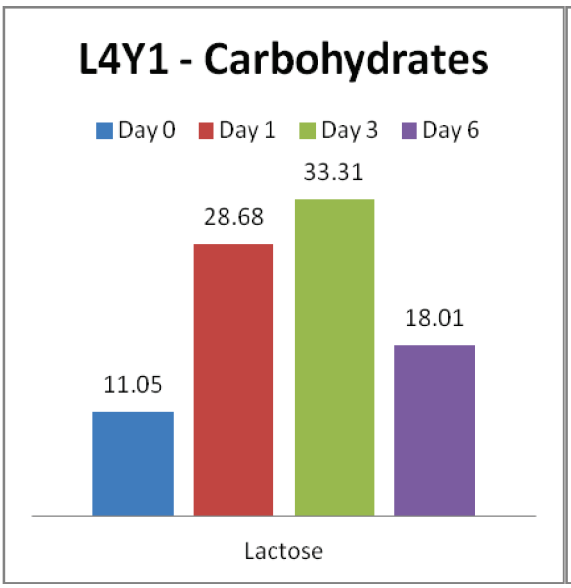


Figure 33: Hydrolyzing of carbohydrates during growth study of L4Y1.

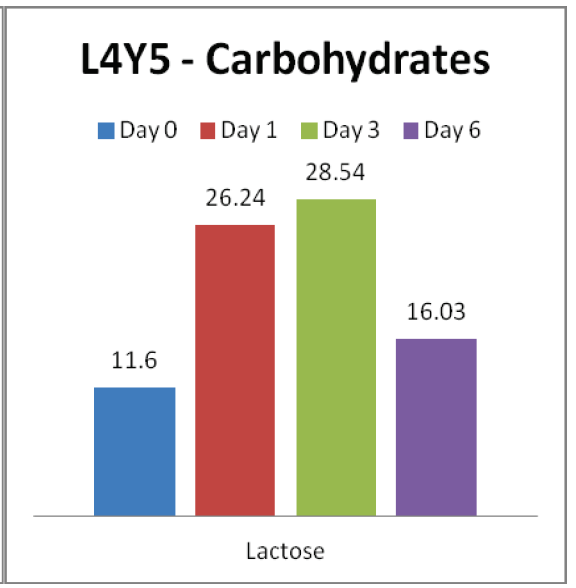


Figure 34: Hydrolyzing of carbohydrates during growth study of L4Y5.

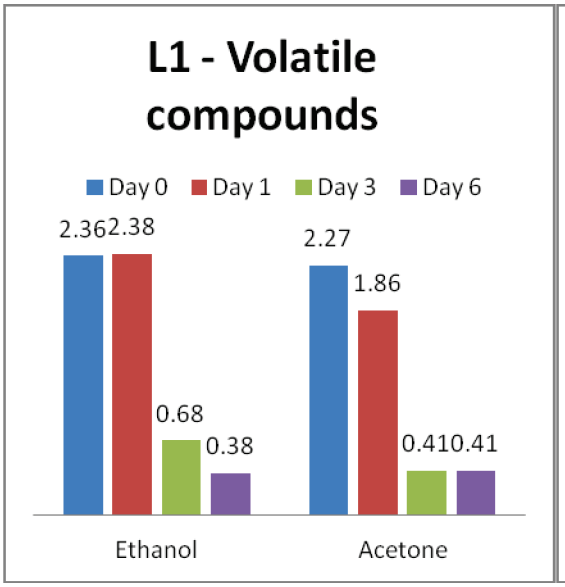


Figure 35: Production of volatile compounds during growth study of L1.

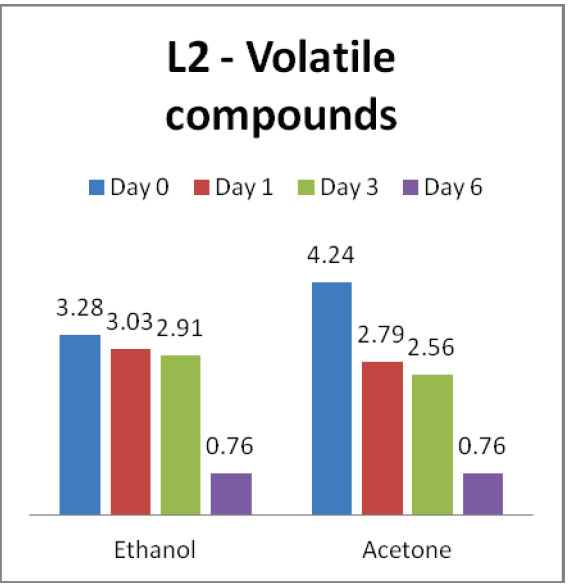


Figure 36: Production of volatile compounds during growth study of L2.

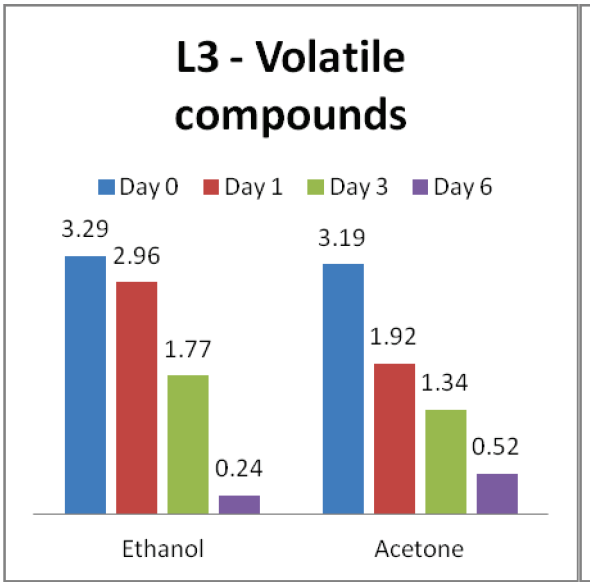


Figure 37: Production of volatile compounds during growth study of L3.

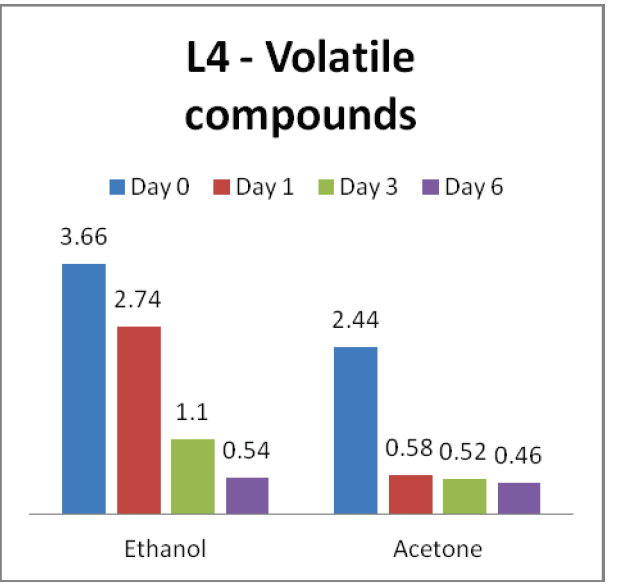


Figure 38: Production of volatile compounds during growth study of L4.

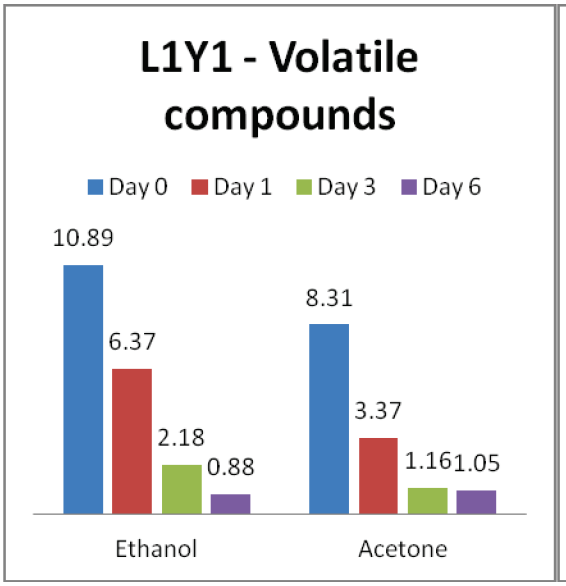


Figure 39: Production of volatile compounds during growth study of L1Y1.

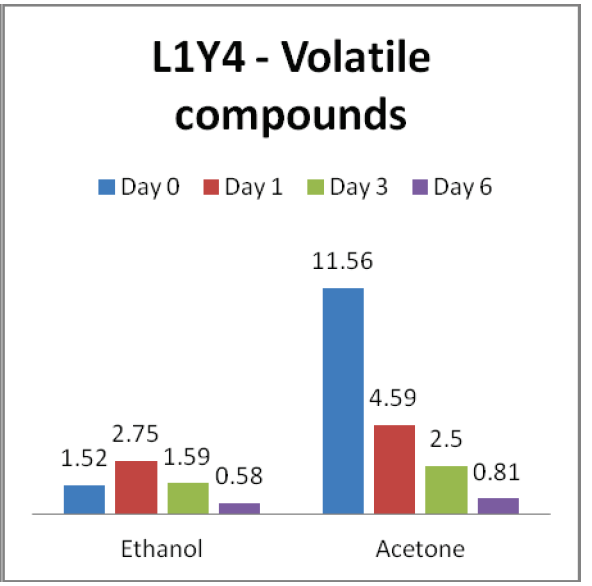


Figure 40: Production of volatile compounds during growth study of L1Y4.

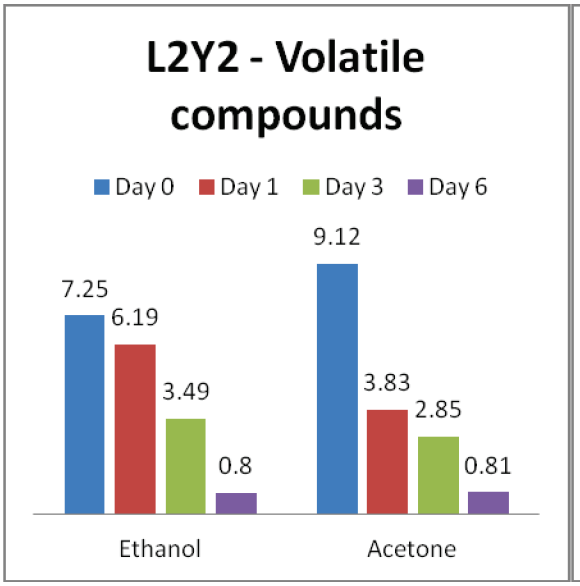


Figure 41: Production of volatile compounds during growth study of L2Y2.

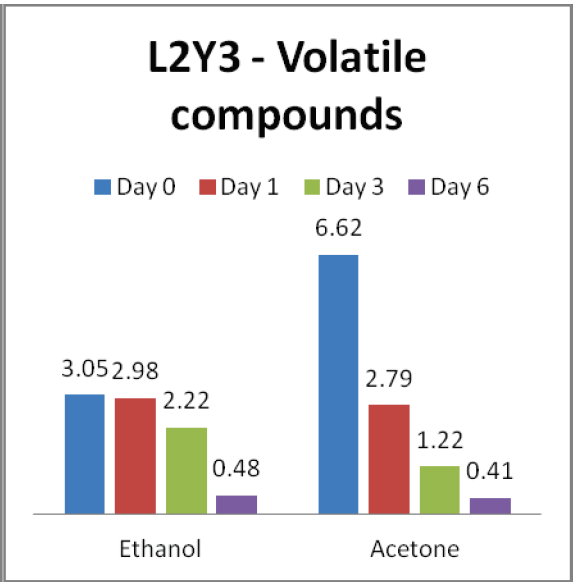


Figure 42: Production of volatile compounds during growth study of L2Y3.

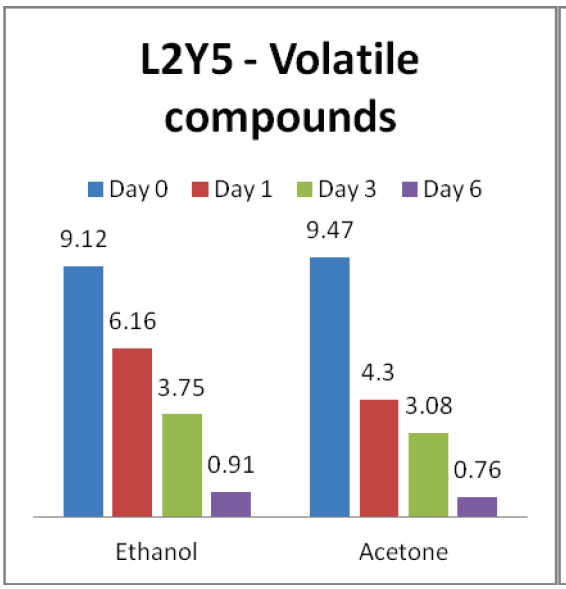


Figure 43: Production of volatile compounds during growth study of L2Y5.

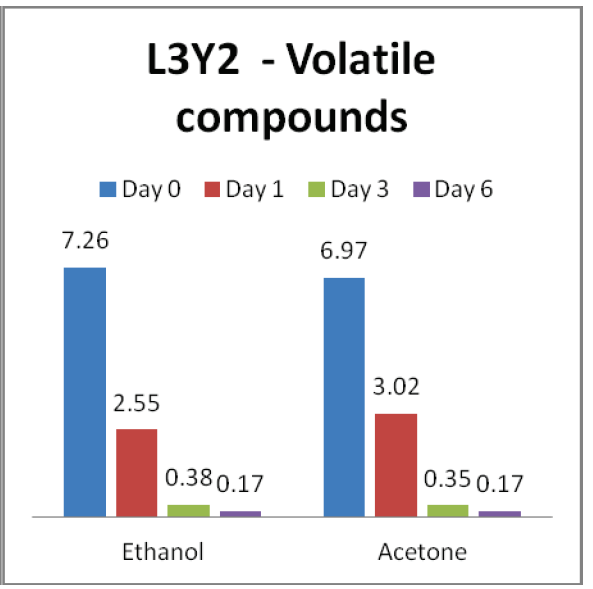


Figure 44: Production of volatile compounds during growth study of L3Y2.

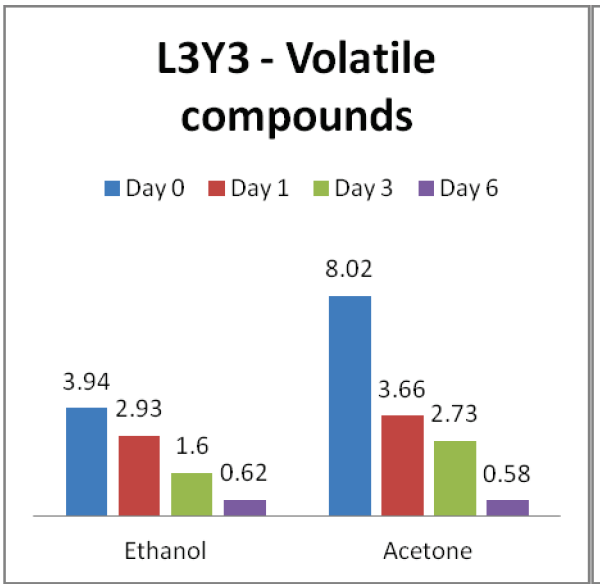


Figure 45: Production of volatile compounds during growth study of L3Y3.

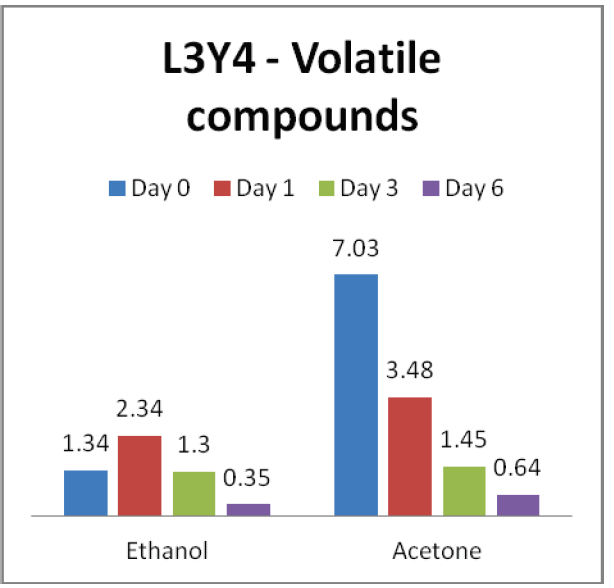


Figure 46: Production of volatile compounds during growth study of L3Y4.

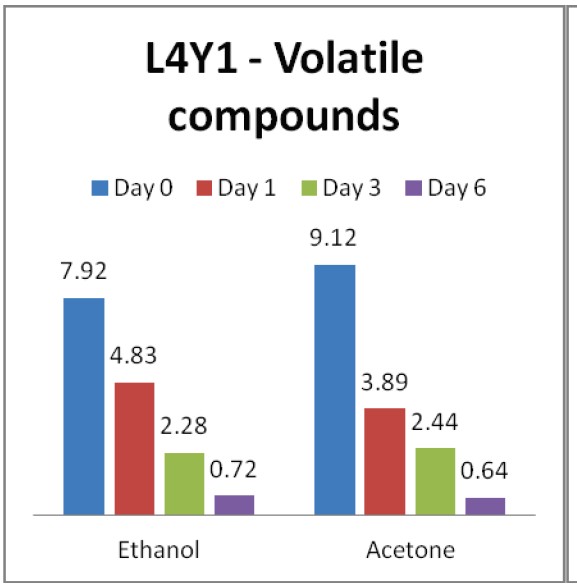


Figure 47: Production of volatile compounds during growth study of L4Y1.

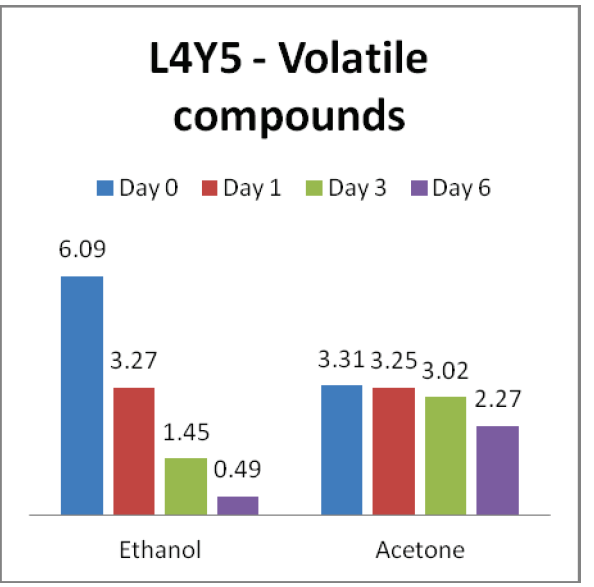


Figure 48: Production of volatile compounds during growth study of L4Y5.

CHAPTER 4

Changes in microbial loads during the fermentation process of indigenous fermented milk of Lesotho

Abstract

Twenty five milk samples and fermented product (raw milk, day 1 – 4 after fermentation) were collected from a small scale farm in Lesotho. The fermentation process was evaluated by the enumeration of microorganisms, pH measurements as well as the inhibitory activity of the LAB against pathogens found in milk. It was observed that potential pathogens (Enterococci, coliforms and Escherichia coli), yeasts and LAB were found in the raw milk. As the fermentation process proceeded the potential pathogens were inhibited by the combination of the yeasts and LAB. In the final fermented milk product there were 79% LAB (lactococci, lactobacilli and leuconostocs) and 21% of yeasts. The pH of the milk decreased gradually with fermentation time. It started with a pH of 6.92 at day 1 and ended up with a pH of 4.34 at day 4. Visually different LAB isolates (32) were tested for antimicrobial activity against typical dairy associated pathogenic organisms. Fifteen showed inhibitory activity, and six of these showed inhibition against all four (Salmonella enterica, Shigella sonnei, Staphylococcus aureus and Escherichia coli) presumptive pathogens. Inhibition diameters between 6 and 40mm were detected. The majority of these LAB isolates belonged to Lactobacillus plantarum (83%) and a small fraction comprised of Enterococcus mundtii/faecium (17%).

In this study it was observed that the production of acids and other antimicrobial components during fermentation may promote or improve the microbiological safety and the stability of the final products.

4.1. Introduction

In developing countries fermented food products are extremely important. The consumption of fermented foods in these regions has many advantages including enhanced nutritional value, digestibility, therapeutic benefits and safety against pathogens (Mutukumira, 1995). Traditional fermentation of fresh milk has been practised in Africa as a means of preserving milk for centuries among the Egyptians.

According to the IDF (1988), 'fermented milk is a milk product prepared from milk, skinned or not, with specific cultures; the microflora is kept alive until sale and my not contain any pathogenic microorganisms'. During fermentation of milk, the main metabolic product of LAB fermentation is lactic acid. The other microbial groups such as yeasts could contribute to the overall characteristics of the fermented milk (Mutukumira et al., 1996). In Kenya, Sudan, Ethiopia, Egypt, Lesotho and many parts of West Africa all making use of similar methods using lactic acid fermentation of milk as a way of preservation. These fermentations are carried out in storage gourdes after allowing the milk to coagulate in the hot weather (Morcos et al., 1973; Nyanga et al., 1982; Tauxe et al., 1988).

The Basotho people of Lesotho ferment milk from as early as 1861. Traditional Mafi is produced from cow's milk which is allowed to ferment spontaneously on an earthenware (clay) pot or gourd (“calabash”) for 2 to 3 days at ambient temperature. The microflora responsible for the fermentation is derived from the air, raw milk and surfaces of the containers. After coagulation, the whey is drained through a plugged hole at the bottom of the container. Although normally consumed with thick corn-meal porridge, Amasi is also consumed between meals with ground sorghum, similar to muesli.

In most countries where fermentation is commonly practiced characteristically lack safe water and adequate sewage disposal facilities, which allows bacterial contamination of the food as found in the rural areas of Lesotho (Esrey & Feachem,

1989). This bacterial contamination in the milk may cause food poisoning and can be responsible for illnesses like septic sore throat, tuberculosis, brucellosis, typhoid fever and diphtheria (Baird-Parker, 1994; Bryan, 1983). For the pathogens to grow in fermented milk the microorganisms must overcome such hurdles as low pH, low aw, low redox potential and in some cases, heat treatment and natural antimicrobial compounds(Byaruhanga et al., 1999). Table 1 lists some recorded cases where pathogens have been detected in fermented foods (Gadaga et al., 2004). Pathogens such as Escherchia coli O157:H7, Listeria monocytogenes, Salmonella enteritidis/enterica and Staphylococcus aureus have been reported to survive the growth in fermented milks (Feresu & Nyathi, 1990).

Fermentation of dairy products is dominated by LAB genera such as: Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Streptococcus and Pediococcus. LAB genera such as Enterococcus, Lactococcus and Lactobacillus have competitive effects in fermented milk that inhibit pathogens like Escherichia coli, Bacillus cereus, and Listeria monocytogenes. This is mainly attributed to their ability to produce lactic acid as well as other metabolites (Mathara et al., 2004).

The objective of the study was to evaluate the microbial quality of the indigenous fermented milk of Lesotho, to follow the changes in microbial loads during fermentation and to assess whether antimicrobial activities derived from LAB isolates from the indigenous fermented milk of Lesotho exist against a variety of food-borne pathogens.

4.2. Materials and Methods

4.2.1. Milk sample collection

Five samples of raw milk (day 1) and five samples during the fermentation process (day 2-4) were collected from the Musi family farm in Lesotho. The above was repeated on five occasions resulting in 25 samples at a specific point. These samples were collected in sterilized plastic bottles, frozen (- 30°C) and then transported in portable cooler boxes lined with ice packing to the Department of Biotechnology, University of the Free State (UFS), as per International Dairy Federation Guidelines (IDF, 1997). On receipt at the UFS the samples were immediately frozen (-30ºC), until they were used for analysis.

4.2.2. Microbial enumeration

The procedures used for the microbial analysis of milk samples were followed as methods outlined in “Laboratory Methods in Food and Dairy Microbiology”. Serial dilutions were prepared by diluting milk samples in 9ml volumes of 0.1% Bacteriological Peptone (Oxoid, Basingstoke, England). Appropriate dilutions were spread plated onto selective media (Table 2) and single colonies were selected for further identification procedures.

4.2.3. Determining pH

The pH of the raw milk (Day1) and during fermentation (Day 2-4) was determined during the sample collection using a digital pH meter (Cyperscan 510, Eutech Instrument, Germany) fitted with an FC 200 electrode (CE, Singapore). The pH meter was calibrated using commercial buffers (Merck) of pH 4 and 7. All pH determining were done in duplicates.

4.2.4. Presumptive pathogenic indicator strains

Indicator strains Salmonella enterica (strain C), Staphylococcus aureus (ATCC 25923), Shigella sonnei (strain H) and Escherichia coli (ATCC 10418) were obtained from the Department Food Science, UFS, Bloemfontein as single colonies on Nutrient Agar plates (Merck). The single colonies obtained from working cultures were used for inhibition strains.

4.2.5. Detection of inhibition

All 32 characterized LAB isolates obtained from Lesotho's fermented milk were screened for inhibition against all 4 presumptive pathogens. All the characterized LAB were individually streaked out on MRS agar plates whereas the four pathogens were streaked out on Nutrient agar plates (Merck). After 24 hrs at 37°C an inoculation loop full of each pathogen was streaked over the LAB and incubated for 24 hrs at 37°C to screen for inhibition.

4.2.6. Antimicrobial assay

The 15 LAB isolates that screened positive for inhibition activity were streaked out individually across the surface of MRS plates (Merck), and aerobically incubated for 24 hrs at 37°C. A lawn with 5ml Nutrient agar (Merck) previously inoculated with 100 µl of the respective indicator strain which was grown in Nutrient broth (Merck) for 24 hrs at 37°C. These plates were incubated for 48 hrs at 37°C under aerobic conditions and examined for inhibition zones of inhibition.

4.2.7. Culture Sequencing

From the antimicrobial assay six LAB showed inhibition against all four presumptive pathogens (L7, 8, 15, 16, 21 and 25). These different isolates were purified by sub culturing them on MRS agar from which they were collected. The products were sequenced by Ingaba Biotech and the results obtained from Finch Tv were completed with the BLAST server of the National Centre for Biotechnology Information (NCBI) using BLAST algorithm for comparison of a nucleotide query sequence against a nucleotide sequence database (Muyzer & Smalla, 1998).

4.3. Results and Discussion

The microbial composition of fermented milk products depends on the composition of the raw milk, the air and the surfaces of the container (Cooke et al., 1987). Results obtained from the raw milk (Day 1) showed that coliforms (18%), enterococci (4%) and Escherichia coli (14%) were present at the initial stages. These organisms are often associated with poor hygiene and their occurrence in the final fermented product may pose a potential health risk (Gran et al., 2003b). Other organisms found in the raw milk were LAB (lactococci (22%), lactobacilli (14%) and leuconostocs (20%) as well as yeasts (8%) (Fig.1). These organisms originated either from the raw milk, the air or walls of the container.

During the fermentation (Day 2, 3) the counts of the coliforms, enterococci and Escherichia coli decreased untill there was none present after fermentation (Day 4). Fermented milk is the result of the activity of a group of microorganisms rather than a single microorganism (Boddy & Wimpenny, 1992). This indicated that the LAB and yeasts interactions or individually were responsible for the elimination of these undesired organisms (Viljoen, 2001). Lactococci, lactobacilli and the leuconstocs continued to increase during the fermentation process (Day 2, 3) and clearly ended up as the predominant group of microorganisms after fermentation (Day 4) representing 79% of the total populations. The number of LAB rapidly increased from 56% at day 1 to a total viable number of 79% at the end of fermentation. The yeasts also played an important part in contributing to fermentation by supporting the LAB. The chemical composition of milk support the growth of yeasts, but the faster growing bacteria restricts the growth of the yeasts (Deak 1991). After fermentation the yeasts were the only other microorganisms present representing 21% of the populations in the final fermented milk product (Figs. 2, 3 and 4). Yeast numbers increased from 8% at day 1 to 21% at the end of fermentation.

The pH of the raw milk (Day 1) started with an initial value of 6.92. The pH of the milk decreased gradually during fermentation (Day 2) to a pH value of 4.82. After 3 days

of fermentation (Day 4) the pH reached the lowest value 4.34 (Fig. 5). The pH of fermented milk of Lesotho corresponded to pH values of fermented milk of other countries (El Hadi & Tsenkova, 2007). Acidification develops in milk at different times, depending on the growth rate of the LAB species, strains and the nutritional characteristics of milk as media (Mc Kay et al., 1976). The high concentration of lactic acid together with other organic acids produced by LAB, all contributed to a decrease of the pH value during fermentation.

The screening results for antimicrobial activity of all 32 isolated LAB from indigenous fermented milk of Lesotho against the four presumptive and/or food-borne pathogen is represented in Table 3. Of the 32 LAB isolates, 15 screened positive for inhibition and were further studied using the antimicrobial assay. Of the selected 15 LAB isolates, six showed inhibition against all four presumptive food-borne pathogens showing clear inhibition zones ranging between 6 and 40mm. Results are indicatedin Table 4. Since only a limited number of LAB showed positive activity against the pathogens, it might be speculated that the inhibition is not only pH driven, but some unknown compounds also play a role.

The inhibition zone obtained with Salmonella enterica reached a diameter range between 10 and 30mm (Figs. 6, 7), while the zone range for Shigella sonnei and Staphylococcus aureus ranged between 6 and 35mm (Figs. 8, 9). Most notable was the inhibition zones when applied to Escherichia coli, as enhanced inhibition zones between 16 and 40 mm (Figs. 10, 11) were detected. Large positive inhibition zones were observed for both the Gram-positive and Gram-negative bacteria. The inhibition of pathogens by LAB has been appreciated by man for more than 1000 years and has been used widely in the preservation of fermented dairy products (Savadogo et al., 2004).

From the antimicrobial assay six LAB isolates showed inhibition against all four presumptive pathogens (L7, 8, 15, 16, 21 and 25). These six strains were identified to species using 16S rDNA (Table 5). All the homologies displayed were above

99%, which proofed to be species identity (Stackebrandt & Goebel, 1994). The majority of the isolates belonged to Lactobacillus plantarum (83%) and with only a small fraction belonging to Enterococcus mundtii/faecium (17%). Lactobacillus plantarum is occasionally found in raw and fermented milk. The reason for the abundance of Enterococcus mundtii/faecium from indigenous fermented milk of Lesotho is due to its versatility growing in a wide range of temperatures as well as restricted environments (Domig et al., 2003; Giraffe, 2003).

4.4. Conclusion

It was observed that potential pathogenic organisms can be present in the raw milk, but during the fermentation process the combination between the LAB and yeasts present created an environment incapable for the pathogens to grow.

During fermentation the yeasts stimulated the growth of the LAB and the LAB were the dominant organisms while the yeasts growth was restricted, but more comprehensive studies are needed to determine the role of the co-occurrence of yeasts and LAB during these interactions in dairy products and to assess its value in the outcome in the final product.

Clear inhibition zones of some pathogens were visible based on their inhibition ability induced by selected lactic acid bacteria. These species comprised of antimicrobial activity are all capable to inhibit the pathogenic strains. This clearly reflects that LAB plays an important role in inhibiting potential pathogens, and therefore an essential role in keeping the quality of the product. Further studies on the characterization of these inhibitory properties should be investigated to utilize theseLAB into dairy products as starter cultures.

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4.6. Tables and Figures

Table 1: The citied occurrence of pathogens in African fermented foods.

Pathogen	Food Product
Bacillus cereus; *Staphylococcus aureus*	Banku kenkey and Fanti kenkey
E. coli	Mahewu
Enteropathogenic E. coli	Sour milk
B. cereus, E. coli	Fermented sorghum meal
S. aureus and *Klebsiella* spp.	Wara
E. coli, Salmonella spp. and *Klebsiella* spp.	Nono
B subtilis, E. coli, S. aureus, Klebsiella spp. and *Enterococcus faecalis*	Ogi and kuni-zaki
Enterotoxigenic E. coli, S. aureus, Klebsiella pneumonia, Enterobacter cloacae, Proteus mirabilis, Hafnia alvei, Citrobacter spp., *Serretia marcescens* and *Aeromonas hydrophilia*	Cultured pasteurized milk and naturally sour milk

Table 2: General and selective media for microbial counts.

Microbial group	Selective media	Temperature	Time
Aerobic mesophilic bacteria	Plate count agar with 1% skimmed milk (PCAM; Merck, Darmstadt, Germany)	30ºC	72h
Lactococci	M17 agar (M17A; Scharlau)	30ºC	48h
Lactobacilli	de Man, Rogosa and Sharpe agar (MRSA; Merck)	32ºC	72h
Leuconostoc spp.	Mayeux, Sandine and Elliker agar (MSEA; Biokar Diagnostics, Beauvais, France)	25ºC	120h
Enterococci	Slanetz and Bartley	44ºC	24h

	agar (SBA; Merck)		
Coliforms	Violet red bile glucose agar (VRBGA)	30ºC	48h
Staphylococci	Baird-Parker agar (BPA; Merck)	37ºC	24h
Yeasts	Rose Bengal Agar (RBA; Merck)	30ºC	48h

Table 3: Screening for antimicrobial activity of LAB against pathogens.

Isolates	Pathogens			
	S. sonnei **Strain H**	***E. coli*** **ATCC 10418**	***Salmonella***	***S. aureus*** **ATCC 25923**
L1	-	-	-	-
L2	++	+	++	+
L3	-	-	++	-
L4	-	-	-	+
L5	-	-	-	-
L6	+++	+++	+++	+++
L7	+	+	+	+
L8	++	++	++	++
L9	-	-	-	-
L10	-	-	-	-
L11	+	++	++	+
L12	-	+	+	-
L13	+	++	+	+
L14	+	+	++	++
L15	+	-	-	-
L16	++	-	-	++
L17	-	-	-	-
L18	-	-	-	-
L19	-	-	-	-
L20	-	+	+	-
L21	++	++	-	-
L22	-	-	-	-
L23	-	-	-	-

L24	-	-	-	-
L25	-	-	-	+++
L26	-	-	-	-
L27	-	-	-	-
L28	-	-	-	-
L29	-	-	-	-
L30	-	-	-	-
L31	-	-	-	-
L32	-	-	-	-

-: No inhibition

+: Poor inhibition

++: Medium inhibition

+++: Strong inhibition

Table 4: Diameter of zones of inhibition (mm) produced by lactic acid bacteria on pathogenic indicator strains.

Isolates	**Pathogens**			
	S. sonnei **Strain H**	***E. coli*** **ATCC 10418**	***Salmonella***	***S. aureus*** **ATCC 25923**
L2	20	-	15	10
L3	-	22	11	-
L4	18	16	24	19
L6	-	-	-	-
L7	21	20	25	25
L8	30	40	20	-
L11	-	-	-	-
L12	-	26	16	17
L13	-	-	-	-
L14	-	-	-	-
L15	14	24	30	9
L16	35	39	30	18

L20	-	-	-	-
L21	11	20	10	6
L25	11	18	16	16

Table 5: Identification of LAB from indigenous fermented milk of Lesotho.

Isolate	Accession	Description	Query coverage	E value	Max identity
L7	AB601179.1	*Lactobacillus plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni729	99%	0.0	99%
	AB601168.1	*Lactobacillus plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni344	99%	0.0	99%
	JQ801725.1	*Lactobacillus plantarum* strain L544 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
L8	AB601179.1	*Lactobacillus plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni729	100%	0.0	99%
	AB601168.1	*Lactobacillus plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni344	100%	0.0	99%
	JQ811213.1	*Lactobacillus pentosus* strain Reyan 20 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
L15	JN853603.1	*Lactobacillus plantarum* strain YML007 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
	JN247633.1	*Lactobacillus plantarum* strain M74 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
	JN247632.1	*Lactobacillus plantarum* strain M68 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
L16	AB601179.1	*Lactobacillus plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni729	99%	0.0	99%
	AB601168.1	*Lactobacillus plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni344	99%	0.0	99%
	JQ811213.1	*Lactobacillus pentosus* strain Reyan 20 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
L21	JF690892.1	*Enterococcus mundtii* strain	100%	0.0	99%

		M.D.E.YAN1-4 16S ribosomal RNA gene, partial sequence			
	JF690896.1	*Enterococcus mundtii* strain M.D.E.YAN3-4 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
	JQ429446.1	*Enterococcus faecium* strain BS-13 16S ribosomal RNA gene, partial sequence	100%	0.0	99%
L25	HQ441200.1	*Lactobacillus plantarum* strain LP-01 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
	GQ359860.1	*Lactobacillus* sp. 0-C-2 16S ribosomal RNA gene, partial sequence	99%	0.0	99%
	JN853603.1	*Lactobacillus plantarum* strain YML007 16S ribosomal RNA gene, partial sequence	98%	0.0	99%

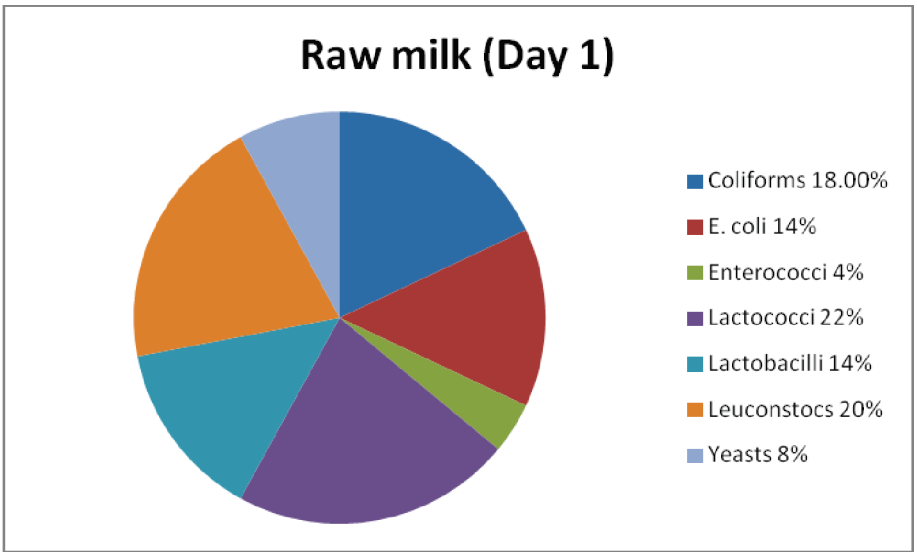

Figure 1: Microbial population in raw milk (Day 1).

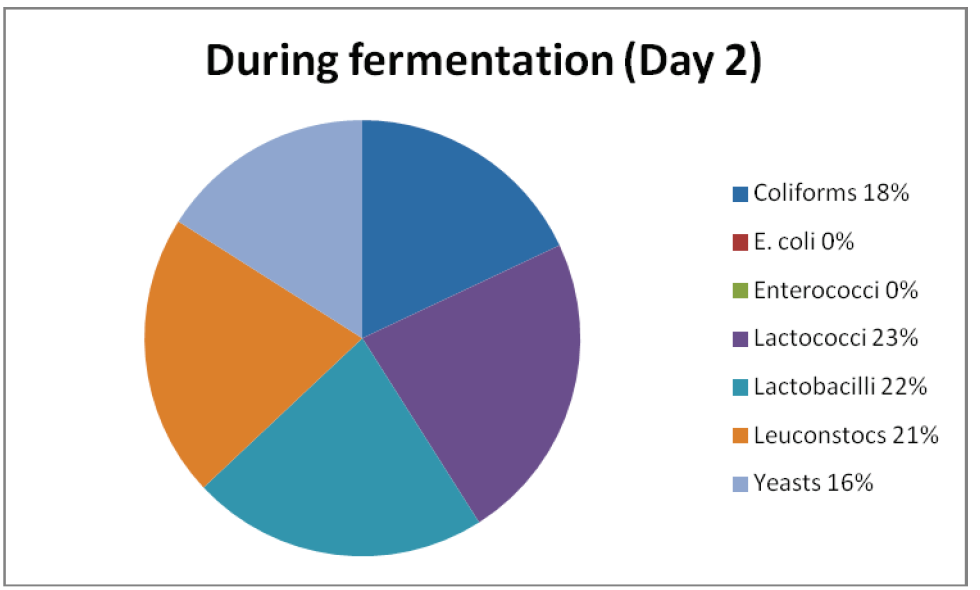

Figure 2: Microbial population during fermentation (Day 2).

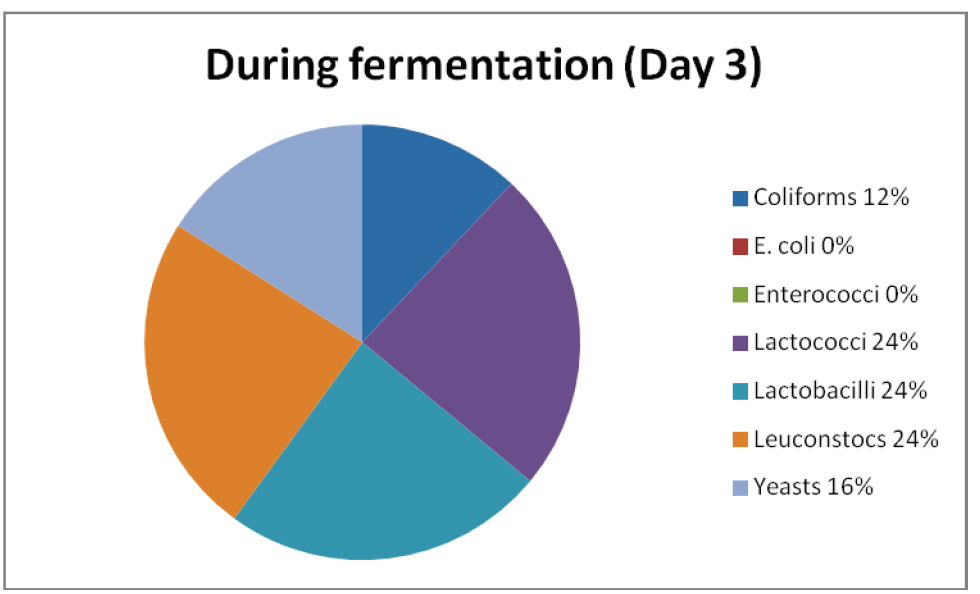

Figure 3: Microbial population during fermentation (Day 3).

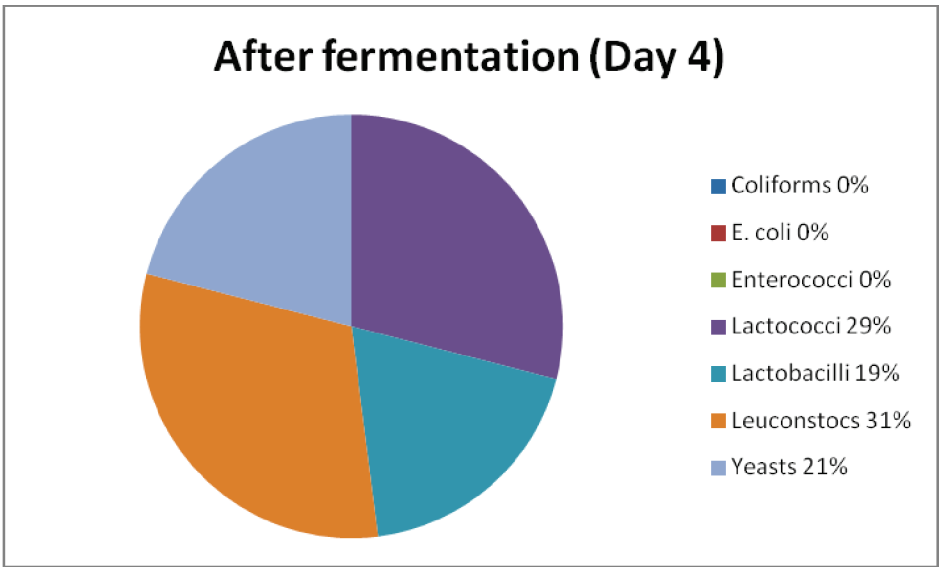

Figure 4: Microbial population after fermentation (Day 4).

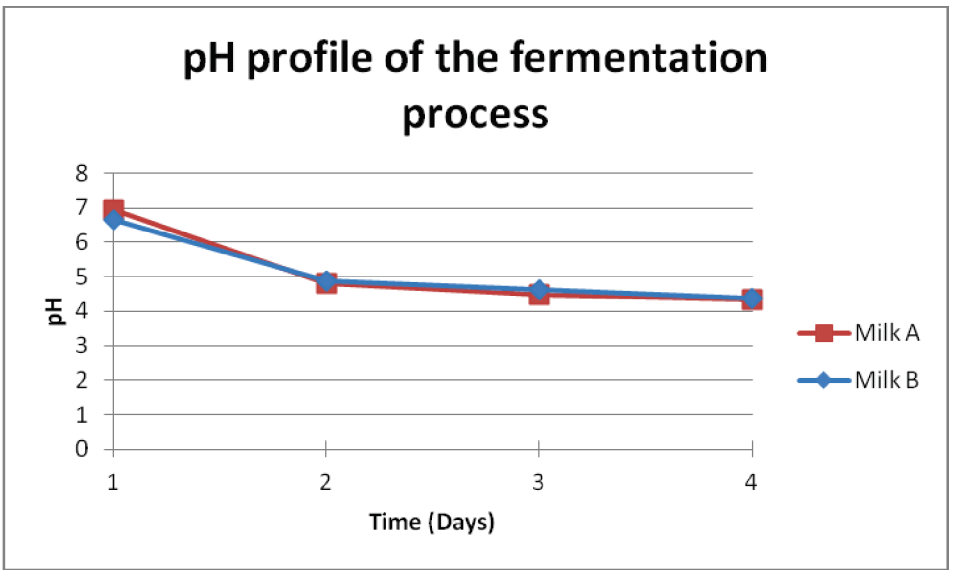

Figure 5: Profile of pH during the fermentation process of raw milk from Lesotho.

Milk A = Samples obtained from Lesotho

Milk B = Duplicate of pH determining of Milk A

Figure 6: Inhibition of *Salmonella enterica* by LAB L15.

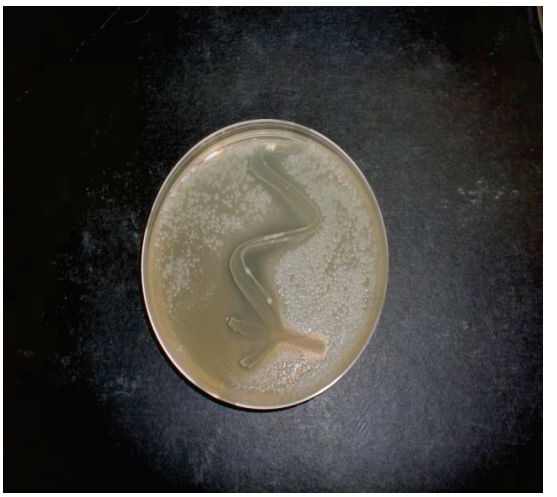

Figure 7: Inhibition of *Salmonella enterica* by LAB L16.

Figure 8: Inhibition of *Shigella sonnei* by LAB L25.

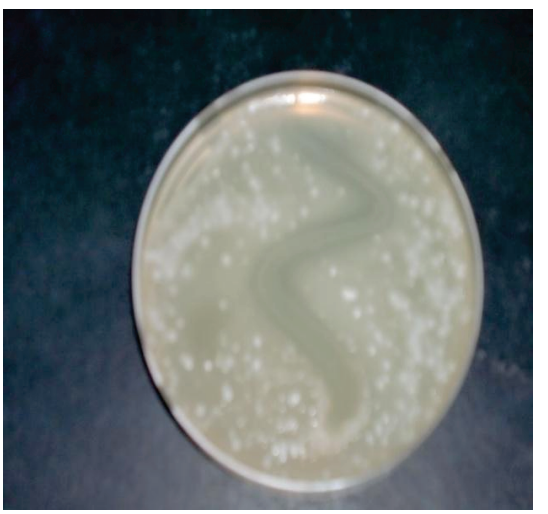

Figure 9: Inhibition of *Staphylocccus aureus* by LAB L25.

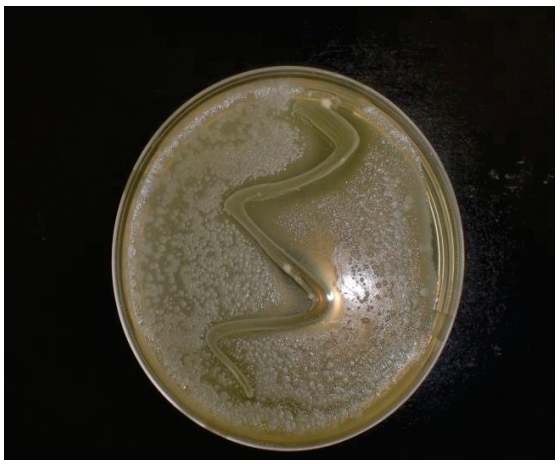

Figure 10: Inhibition of *Escherichia coli* by LAB L12.

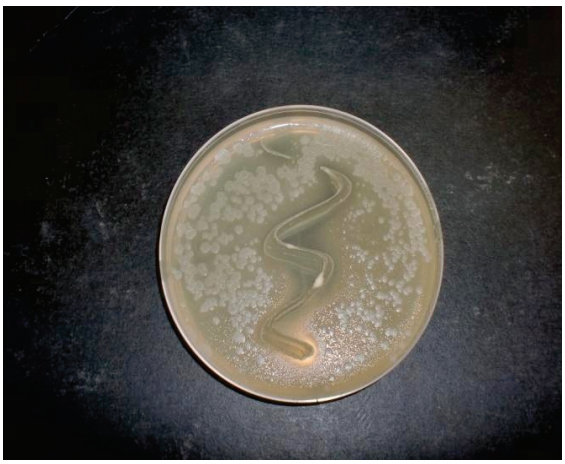

Figure 11: Inhibition of *Escherichia coli* by LAB L15.

CHAPTER 5

General discussion and conclusion

Research has shown that fermented foods contain essential nutrients needed to maintain optimum health as well as non nutritional compounds that contribute to the prevention of spoilage and enhance the probiotic effect (Steinkraus, 1996). Fermented food products are extremely important in developing countries, not only do they result as spontaneous foods, but further serve as products enriched in vitamins, high in energy and capital-intense processes for food preservation (Mutukumira, 1995). Fermented foods are the result of the activity of a group of microorganisms rather than a single microorganism (Dirar, 1993).

Traditional naturally fermented milk is still being produced at household levels in many communities in Africa were animals are kept for their milk. It has been observed that the final product may vary considerably between different regions. This may be due to the different regions with different climate conditions, different fermentation techniques, and different types of containers and if back-slopping is used (Mutukumira et al., 1995). In this study there was focus on the indigenous naturally fermented milk of four different regions in Africa, mainly; Botswana (Madila), Namibia (Omasbikwa), Lesotho (mafi) and Swaziland (amasi).

In naturally fermented milk yeasts and LAB are frequently encountered together. These microorganisms are responsible for the direct and indirect interactions as observed by Viljoen (2001). As literature shows it is possible that undesirable organisms, LAB and yeasts may be present in the raw milk samples. After fermentation the composition of the organisms present also shows that the fermentation process is not a close unit (Kebede et al., 2006). The results obtained (Chapter 2) conveyed that the predominant organisms found in the raw milk were coliforms and enterococci, but after fermentation high numbers of LAB were observed. This proofed that fermented products are of higher microbial quality compared to the raw product and fermentation is responsible for the change in microbial loads. It was also observed that the yeasts are present in the raw as well as the fermented milk. Yeasts play an important role in providing certain vitamins

and other compounds for the starter culture (LAB) but are later out competed by the faster growing lactic acid bacteria and that is why they are only found at low numbers in the final product. This corresponds with results obtained from previous studies (Marshall, 1987; Viljoen, 2001). Culture sequencing and DGGE techniques were used to determine the dominant lactic acid bacteria present in the different regions of Africa. Results indicated that the dominant lactic acid bacteria primarily comprised of Enterococcus and Lactococcus species. Various other lactic acid bacteria associated with these fermented milks were also present, but appeared at lower numbers. The respective lactic bacterial groups included species of Leuconostoc and Streptococcus.

The interaction between lactic acid bacteria and yeasts frequently observed in dairy products was studied in Chapter 3. Depending on their properties and concentrations, yeasts may have a negative (unpleasant taste or appearance) effect or positive when supporting the LAB, add to taste etc. on the dairy products (Lenoir et al., 1985). In this chapter we focused on the interaction between LAB with inhibitory activities and specific yeasts to see if there is stimulation or inhibition between these organisms. During the growth trail it was clearly observed that the yeasts stimulated the growth of the bacteria but it seemed that the LAB discouraged the growth of the yeasts. This was probably due to acid and other compounds, or just normal outcompeting growth by LAB (Gadaga et al., 2001b). Results showed that during the growth between the LAB and yeasts the pH value decreased as seen during fermentation. Lactic acid concentrations increased considerably to very high levels during fermentation and thus also contributed to a rapid decrease in pH values. During the chemical analysis it was found that lactic acid and citric acid were produced by the LAB at low amounts, but in combination with the yeasts these compounds were found at much higher concentrations, indicating that the yeasts contributed to the fermentation process. Lactose present was utilized by the organisms present after day three and probably converted to lactic acid. Volatile compounds such as ethanol and acetone were also produced.

In many Africa countries as found in Lesotho, fermentation is commonly practiced where there is a lack of safe water and adequate sewage disposal facilities. This can allow bacterial contamination. For the pathogens to survive in fermented milk the microorganisms must overcome hurdles such as low pH and antimicrobial compounds (Byaruhanga et al., 1999). The fermentation process from indigenous fermented milk of Lesotho was studied as well as the production of antimicrobial compounds against pathogens was done in Chapter 4. Before fermentation potential pathogens, yeasts and LAB were found. As the fermentation process proceeded the potential pathogens were inhibited by the combination of the yeasts and LAB. In the final fermented milk product there were 79% LAB (lactococci, lactobacilli and leuconostocs) and 21% yeasts. During the fermentation the pH also decreased indicating that lactic acid is being produced as the fermentation process evolved.

During the past decade, microorganisms such as Salmonella sp., Escherichia coli, Shigella sp. and Staphylococcus aureus were reported as the most common foodborne pathogens present and are able to survive in milk and fermented milk products (Cangella et al., 1998). A number of researchers have reported that the survival and growth of pathogenic bacteria may be inhibited by metabolites excreted by lactic acid bacteria (Holzapfel, 2002). Of the 15 selected LAB isolates from Lesotho, six isolates showed inhibitory activities against all four presumptive pathogens. The majority of these LAB isolates belonged to Lactobacillus plantarum (83%) and a small fraction to Enterococcus mundtii/faecium (17%). These results obtained were in agreement with work done by other workers (Bhattacharya & Das, 2010; Savadogo et al., 2004; Tadese et al., 2005).

The microbial populations exist due to the initial composition of raw milk utilized before fermentation. The main sugars, lactose serves as a vital component for the growth of lactic acid bacteria (Orla-Jensen, 1919). These microorganisms have been recognized for both their health benefits in individuals consuming the final fermented milk products, as well as allowing longer keeping quality in well

fermented dairy products (Liu et al., 2002). Therefore the isolation of novel lactic acid bacteria species from indigenous fermented products could assist dairy industries in improving upon the starter cultures utilized as well as prolonging the keeping quality of the products.

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CHAPTER 6

Summary

Fermented foods contain essential nutrients needed to maintain optimum health as well as other components that help in the delaying and prevention of diseases such as chronic illnesses. Indigenous naturally fermented milk of different regions in Africa play an important role in their daily diet and it helps rural woman to generate a household income. The literature review focused on the microbial composition of fermented milk in different regions in Africa (Botswana, Lesotho, Namibia and Swaziland). Furthermore, the positive and negative interactions associated with fermented milk were discussed in detail.

Fermented milk is the result of the activity of a group of microorganisms. Each of these organisms plays an important role in the production of the final fermented product. Before fermentation, undesired microorganisms were present in the samples, but after a four day fermentation the yeasts and lactic acid bacteria (LAB) interactions created an environment impossible for pathogens to survive. The yeasts provide essential growth compounds for the starter culture (LAB) but remained at low numbers at the end of fermentation because of being outcompeted by faster growing LAB.

The dominant LAB of the different regions was determined by DGGE and culture sequencing. Enterococci and Lactoccoci were found to be the dominant LAB followed by Leuconostocs and Streptococci. Enterococcus durans species comprised 32% of the isolated LAB. The microbial composition has beneficial effects not only for the consumer but may assist in the improvement of the final product.

The microbial interaction between dominant LAB and yeasts frequently observed in dairy products was also monitored during a study in Lesotho. During the growth studies the LAB in combination with the yeasts had a much better growth rate, but in contrast the yeasts grew better on its own compared to those in combination with LAB. For the change in chemical composition it was also found that if in co-culture

more organic acids were produced resulting in a rapid decrease in pH levels.

During fermentation a small number of LAB proofed to have antagonistic effects against a series of pathogenic bacteria and were further evaluated by evaluating the fermentation process of milk of Lesotho and the LAB's inhibition effect. Salmonella anterica, Shigella sonnei, Staphylococcus aureus and Escherichia coli were completely inhibited by Lactobacillus plantarum and Enterococcus mundtii/feacium. Further studies need to be done in the isolation of novel lactic acid bacteria to provide insight on the preservation and safety of fermented milk products.

(Keywords: Africa indigenous milk, fermented, microbial interactions, lactic acid bacteria, pathogenic inhibition)

Opsomming

Gefermenteerde voedsel besit belangrike nutriente wat nodig is vir 'n optimal gesondheid, dit besit ook ander komponente wat help in vertraaging en voorkoming van kronies siektes. Inheemse natuurlike gefermenteerde melk van verskillende gebiede in Afrika speel 'n belangrike rol in die daaglikse diet en help landelike vrouens om 'n huislike inkomste by te dra. Die literatuur oorsig fokus op die mikrobiese komposisie van gefermenteerde melk in verskillende gebiede in Afrika (Botswana, Lesotho, Namibia en Swaziland). Die positiewe en negatiewe interaksies geassosieer met gefermenteerde melk word ook volledig bespreek.

Gefermenteerde melk is die resultaat van die aktiwiteite van 'n groep mikroörganismise. Elk van die organismis speel 'n belangrike rol in die vervaardiging van die finale gefermenteerde projek. Voor fermentasie kom onplesierige mikroörganismis voor in die produk, maar na fermentasie vorm die giste en melksuurbakterieë 'n omgewing wat dit onmoontlik maak vir patogene om te oorleef. Die giste voorsien vir die begin kultuur (melksuurbakterieë), maar aan die einde van fermentasie kom die giste slegs voor in lae getalle as gevolg van die melksuurbakterieë wat vinniger groei.

Die dominante melksuurbakterieë van die verskillende gebiede was bepaal deur DGGE en kultuur basispaaropeenvolgingbepalings. Enterococci en Lactococci was die dominante melksuurbakterieë gevolg deur Leuconostocs en Streptococci. Enterococcus durans spesies bestaan uit 32% van die geïsoleerde melksuurbakterieë. Die mikrobiese samestelling het 'n voordelige effek nie net vir die verbruiker nie maar help ook in die bevordering van die finale produk.

Die mikrobiese interaksies tussen die dominante melksuurbakterieë en die giste wat gereeld gevind word in suiwel produkte is ook gemonitor. Tydens die groei studies van die melksuurbakterieë in kombinasie met die giste het die melksuurbakterieë 'n

baie beter groei tempo, maar in kontras the giste groei beter op hulle eie as in kombinasie met melksuurbakterieë. Vir die verandering in die chemiese komposisie is daar gevind dat wanner in mede-kultuur daar meer organiese sure geproduseer word en dat die pH ook verlaag wat duidelik aandui dat die giste dra by tot die vermenigvuldige optimale groei van die melksuurbakterieë.

Tydens fermentasie is dit bewys dat 'n klein hoeveelheid melksuurbakterieë antagonistiese effek kan uit oefen teen a reeks patogeniese bakterieë en is verder geevalueer deur die fermentasie proses van die melk van Lesotho en die melksuurbakterieë inhibisie effek te monitor. Salmonella anterica, Shigella sonnei, Staphylococcus aureus en Escherichia coli was heeltemal geinhibeer deur Lactobacillus plantarum en Enterococcus mundtii/feacium. Verder studies moet nog gedoen word op die isolasie van nuwe melksuurbakterieë om insig te kry op die preservasie en veiligheid van gefermenteerde melk produkte.

(Sleutel woorde: Afrika inheemse melk, fermentasie, mikrobiese interaksie, melksuurbakterieë, patogeniese inhibisie)