

**A COMPARATIVE ASSESSMENT OF LOCAL,
COMMERCIAL AND HOMEMADE AMAHEWU
WITH RESPECT TO NUTRITIONAL VALUE,
HYGIENE AND OTHER HEALTH BENEFITS
TO THE COMMUNITY.**

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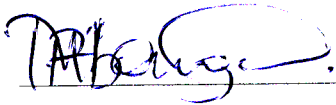
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AUTHOR'S DECLARATION

The experimental work presented in this thesis represents the original work by the author, and has not been submitted in any form to any other University. Where use was made of work of others, it was duly acknowledged in the text.

The research described in this study was carried out under the supervision of Dr N. Gqaleni in the Department of Physiology, Nelson R. Mandela School of Medicine, Durban, during the period of March 2000 to December 2001.



Miss HP Mbongwa

I dedicate this work to my little boy, Langelihle, whose presence continuously gives me strength to persevere.



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POSTERS AND PRESENTATIONS

1. Gqaleni, N., **Mbongwa, H.P.**, and Mokoena M.P. 2001. Role of natural lactic acid bacteria in protection against oesophageal cancer through alteration of fumonisin B₁. 2nd Oesophageal Cancer Research Consortium Conference, Cape Town.
2. **Mbongwa, H.P.**, Gqaleni, N and Dalasile, T. 2001. Mycotoxin analysis of commercially-produced and homemade amahewu. SAAFoST 16th Biennial International Congress, Durban.
3. **Mbongwa, H.P.**, Mokoena, M.P., Carries, S. and Gqaleni, N. 2002. African traditional non-alcoholic fermentations in health promotion and science and technology education. Indigenous Knowledge Systems Conference, Pretoria.

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LIST OF ABBREVIATIONS

ACN	acetonitrile
AFB ₁	aflatoxin B ₁
AME _N	nitrogen corrected apparent metabolisable energy
AOAC	Association of Official Analytical Chemists
ATA	alimentary toxic aleukia
BA	blood agar
CH ₃ COOH	acetic acid
CHO	carbohydrate
CO ₂	carbon dioxide
DCM	dichloromethane
DNA	deoxyribonucleic acid
EEL	endogenous energy loss
ELEM	equine leukoencephalomalacia
EMB	eosin methylene blue
EtOH	ethanol
FAO	Food and Agriculture Organisation
FB ₁	fumonisin B ₁
FDA	Food and Drug Administration
FOS	fructooligosaccharides
g/mol	grams per mol
H ₂ S	hydrogen sulphide
H ₂ SO ₄	sulphuric acid
H ₃ PO ₄	orthophosphoric acid
HCl	hydrochloric acid
HOAc	acetic acid
HPLC	High Performance Liquid Chromatography
KCl	potassium chloride
kJ	kiloJoule
KOH	potassium hydroxide
LAB	lactic acid bacteria
LPS	lipopolysaccharide
MeOH	methanol

mg/kg	milligrams per kilogram
ml	millilitre
mm	millimetre
MR	methyl red
MRS	modified rogosa agar
Na ₂ B ₄ O ₇	sodium tetraborate
Na ₂ SO ₄	sodium sulphate
NADH	nicotinamide adenine dinucleotide
NAG	N-acetylglucosamine
NaHCO ₃	sodium hydrogen carbonate
NAMA	N-acetyl-muraminic acid
NaNO ₂	sodium nitrite
NaOH	sodium hydroxide
nm	nanometer
°C	degrees Celcius
OC	oesophageal cancer
OPA	ortho-phthalaldehyde
PEM	protein-energy malnutrition
ppb	parts per billion
RDA	Recommended Daily Allowance
SAX	strong anion exchange
SDA	Saboraud Dextrose Agar
spp	species
ssp	subspecies
TLC	Thin Layer Chromatography
TME _N	nitrogen corrected true metabolisable energy
TPP	thiamine pyrophosphate
UV	ultraviolet
VP	Voges-Proskauer
VRB	violet red bile
w/v	weight per volume
ZEA	zearalenone

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ABSTRACT

Fermentation is a process by which primary food products are modified biochemically by the action of microorganisms and/or their enzymes. Several societies have, over the years, intentionally carried it out to enhance the taste, aroma, shelf-life, texture, nutritional value and other properties of food. It is used in many parts of the world. However, there are regional differences in use and these depend on the availability of raw materials, consumption habits, and other socio-cultural factors. This study was aimed at (comparatively) assessing, local commercial and homemade amahewu with respect to nutritional value, hygiene and other health benefits to the community. Methods employed were Thin Layer Chromatography (TLC) (mycotoxins), High Performance Liquid Chromatography (HPLC) (mycotoxins, sugars and amino acids), Dumas (proteins), Soxhlet (lipids) and intubation technique (metabolisable energy) to analyse maize meal and amahewu samples from various regions. The regions sampled included maBheleni (South Coast) and kwaNgcolosi (North Coast) villages. Commercial amahewu was analysed with kind permission from Clover SA.

Species from the following genera were isolated and identified from amahewu samples: *Lactobacillus*, *Saccharomyces*, *Leuconostoc*, *Lactococcus*, *Pantoea*, *Enterobacter* and *Klebsiella*. *Saccharomyces* was detected in commercial samples only. Gram-negative strains were identified in most of maBheleni village samples.

No traceable amounts of aflatoxin B₁ (AFB₁), fumonisin B₁ (FB₁) and zearalenone (ZEA) were found in Clover SA samples. AFB₁ was detected in 40% of both maize meal and amahewu samples from maBheleni (range 0.55 – 0.84ng/g and 8.3×10^{-5} – 9.1×10^{-5} ng/g respectively). From the same village, 100% of the maize meal and 80% of the amahewu samples were contaminated with FB₁ (range 4.1 – 47.2ng/g and 1.4 – 6.9ng/g respectively). ZEA was detected in all maize meal samples (range 0.9 – 4.3ng/g). None of the amahewu samples contained detectable levels of ZEA.

All maize meal and amahewu samples from kwaNgcolosi were contaminated with AFB₁ (range 8.3 – 30.1ng/g and 0.04 – 0.102ng/g respectively). FB₁ was detected in 75% of both maize meal and amahewu samples from the same village (range 0.5 – 4.1ng/g and 0.04 – 0.56ng/g respectively). ZEA was also found in all maize meal samples and 75% of amahewu samples (range 3.7 – 16.4ng/g and 0.03 – 0.06ng/g respectively).

MaBheleni, Clover SA and kwaNgcolosi maize meal and amahewu samples contained vitamins B₁, B₂ and B₆ with a range of 0.31±0.21 - 4.48±0.81 B₁; 0.15±0.14 - 1.67±0.33 B₂ and 0.05±0.07 - 0.77±1.45 µg/g B₆. Fat levels ranged from 0.28±0.40 to 4.54±0.05 percentage by weight. The levels of proteins varied from 4.02±0.02 to 8.40±0.04 percentage by weight. Starch concentrations ranged from 31.5±5.28 to 75.9±1.92g/100g. Maize meal samples contained glucose and maltose, while glucose, fructose, sucrose, maltose, M-triose, DP 4 and 5 and DP >15 were detected in amahewu. Apparent and true metabolisable energy for homemade and commercial freeze-dried amahewu was 13.194 and 13.696MJ/kg (AME_N); and 13.605 and 14.106MJ/kg (TME_N), respectively.

This study has shown that lactic acid maize fermentation reduces the levels of AFB₁, FB₁ and ZEA toxins in maize meal, inhibits the growth of most Gram-negative bacteria, and in some instances, fermentation did improve the nutritional value. Metabolisable energy analysis represents an important tool to assess whether or not compounds ingested are converted to sources of energy in the body and utilised. Amahewu fermentation yielded beneficial products (probiotics; reduced mycotoxins levels and reduced starch).

In conclusion, natural lactic acid maize fermentation to produce amahewu will do more good than harm to the consumer, therefore, people need to be advised on how to safely store their maize and also to be encouraged to consume their stored maize in fermented form.

CHAPTER 1

INTRODUCTION

Cereals, in particular maize, form the staple diet in most of the developing countries, especially on the African continent (Steinkraus, 1983). Fermentation is considered as one of the more important ways in which the supply of adequate, safe, wholesome and affordable food to all can be addressed. Most of the fermentation processes in developing countries are carried out indigenously. In the recent years, the value of lactic acid fermentation has been recognised as an alternative technology when cold or hot storage is not feasible (Steinkraus, 1992)

According to Steinkraus (1998), traditional fermentation of foods has 4 benefits, including:

1. Enrichment of the diet through development of a diversity of flavours, aromas and textures in food substrates;
2. Preservation of substantial amounts of food through lactic and acetic acids fermentations;
3. Enrichment of food substrates biologically with protein, essential amino acids, essential fatty acids and vitamins;
4. Detoxification during food fermentation processing.

Rapid displacement of traditional foodstuffs in developing countries with technologies developed in more affluent countries may result in centralised production, distribution problems and less local involvement in food processing. It could also lead to less employment in some areas, less nutritionally adequate substitutions in raw materials, displacement of traditional arts, loss of unique know-how, as well as dependence on importation of equipment and materials. Initially these modern technologies require the use of outside consultants, and otherwise may not meet local needs as fully as traditional fermented products would (Motarjemi and Nout, 1996). On the other hand, a number of problems have been associated with indigenous fermentations. They are uncontrolled and often unhygienic, are normally not integrated into the economic mainstream, difficult to tax, and the impact on nutritive value and safety is questionable (Dicks and Lategan, 1999).

Traditional fermentations are likely to remain an important part of the global food supply. Many may evolve into fermentations involving the use of starter cultures, enzyme additives and controlled environmental conditions, and others may benefit from genetic modification of the cereal or starter bacteria (FAO, 1999).

Further research should be directed towards identifying the benefits and risks associated with specific indigenous fermented cereals; elucidating the contributions of microorganisms, enzymes and other cereal constituents in the fermentation process; and developing starter cultures, unique microbial strains for nutritive improvement and detoxification; and testing of new cereal varieties for their suitability as fermentation substrates (FAO, 1999).

Amahewu is a lactic acid fermented gruel (Campbell-Platt, 1987) enjoyed by Africans in southern Africa. Initially it was one of the indigenously fermented beverages, but now Clover SA also produces it. Even though amahewu is commercially available, it is still very much home made, especially in the rural South Africa.

The World Health Organisation held a workshop in Pretoria, South Africa in 1995 to assess the safety and nutrition of food fermentation. Some of these questions arose when considering the use of fermentation at the household level (Motarjemi and Nout, 1996):

- ◆ What kind of hazards can be controlled through the application of this technology?
- ◆ What risks are associated with fermentation as the result of food handlers' lack of knowledge or unhygienic food preparation conditions?
- ◆ What socio-cultural considerations should be taken into account in order to successfully apply fermentation technology or transfer it to another region?
- ◆ Should fermentation technology be promoted for the prevention of food-borne diarrhoea or as a means of improving the nutritional value of foods?

Even before this workshop, extensive studies have been carried out on fermented foods, however, very few questions have been answered and many more arose. Most studies have concentrated on the biological, chemical, health and nutritional benefits and risks of the fermentation process. The problem is that fermentation is a complex study due to the fact that everything about the process is so diverse, from raw materials, to the procedure itself and to what the final product is required for.

This study was therefore carried out as an attempt to address some of the issues raised by the workshop and to elucidate the role of amahewu in nutrition.

CHAPTER 2

LITERATURE REVIEW

2.1. MAIZE AND ITS USES

2.1.1. Maize

The maize grain is the largest of all cereals (Lasztity, 1984). Its kernel is flattened, wedge-shaped and relatively broad at the apex of its attachment to the cob. The aleurone cells contain much carbohydrates and oil as well as the pigments that make certain varieties appear blue, black or purple (Kent, 1983). Two types of starchy endosperms, horny and floury, are found beneath the aleurone layer. Endosperm contains a higher level of protein. In dent corn varieties, the horny endosperm is found on the sides and back of the kernel and bulges in toward the centre at the sides (Pomeranz, 1987). The floury endosperm fills the crown (upper part) of the kernel, extends downward to surround the germ, and shrinks as dent corn matures, causing an indentation at the top of the kernel. In a typical dent corn, the pericarp comprises approximately 6%, the germ 11% and the endosperm 83% of the kernel (Hoseney, 1986).

2.1.2. Uses of maize and cereals

In general, cereals (including maize) account for as much as 77% of total caloric consumption in African countries, and contribute substantially to dietary protein intake in a number of these countries. A majority of traditional cereal-based foods consumed in Africa are processed by natural fermentation. Fermented cereals are particularly important as weaning foods for infants and as dietary staples for adults (Adams, 1998; FAO, 1999).

Maize grown in subsistence agriculture has been, and is currently being used, as a basic food crop. However, as countries become more developed and shift to a more urban population (with a concurrent increase in demand for wheat flour and animal – derived foods), the primary use for maize shifts to animal feed. As a result, in developed countries over 90% of the maize produced or imported is used for animal feed (Watson and Ramstad, 1991). Major cereals grown in Africa include maize, rice, sorghum and millet.

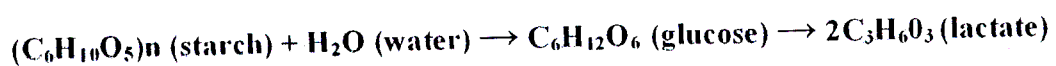
2.2. MICROBIOLOGY OF MAIZE FERMENTATION

2.2.1. Fermentation

Fermentation is the oldest known form of food biotechnology. It was first defined by Pasteur as “life in the absence of oxygen” (Rehm and Reed, 1983). Today, the term is used in the broad sense to refer to any biotransformation and, in the limited sense, a metabolic process in which organic compounds serve both as electron donors and acceptors. Fermentation involves the catabolism of an organic compound (usually a carbohydrate) to compounds whose average oxidation state is the same as that of the initial substrate, but whose total energy content is lower. The result is that both reduced and oxidised products are formed (Heritage *et al.*, 1999).

The most common groups of microorganisms involved in food fermentations are bacteria, yeasts and moulds (FAO, 1995). Plant and animal tissues subjected to the action of microorganisms and/or enzymes to give desirable biochemical changes and significant modification of food quality are, generally, referred to as fermented foods (Campbell-Platt, 1994). For thousands of years lactic acid bacteria (LAB) have played a vital role in many of the fermented foods that are enjoyed today (Goldberg and Williams, 1991).

It is possible to differentiate between homolactic and heterolactic fermentation. Fermentations with a yield of more than 80% of the theoretical value of lactic acid are considered homolactic (Reaction 1).



Reaction 1: Homolactic fermentation of starch into lactic acid.

In heterolactic fermentation, the by-products and lactic acid are produced in approximately equal molar amounts (Dellweg, 1983). Although the type of fermentation occurring depends on the presence of certain genera of LAB; homolactic fermentation, in some cases, can be converted into a heterolactic one by changing the fermentation conditions.

2.2.2. The microbiology of fermentation

A Dutch merchant named Anton van Leeuwenhoek sent a noteworthy letter to the Royal Society of London describing how he used a primitive microscope to observe vast populations of minute creatures. In so doing, he opened a chapter of science that would evolve into the study of microscopic organisms and the discipline of microbiology (Alcarno, 1991).

Microorganisms play integral roles in the cycle of life and death. Some species are used in the production of food and medicine while others, having the ability to grow on and within other organisms, can lead to disease, disability and death as a result of microbial colonisation. The control or destruction of microorganisms residing within the bodies of humans, animals and plants is therefore of paramount importance (FAO, 1995; Prescott *et al.*, 1996).

Bacteria are a large group of unicellular or multi-cellular organisms lacking chlorophyll, with a simple nucleus, multiplying rapidly by simple fission, some species developing a highly resistant resting (spore) phase (Figure 2.1). Some species reproduce sexually, and some are motile. They are spherical, rod-like, spiral or filamentous in shape. They occur in air, water, soil, rotting organic material, animals and plants. Saprophytic forms are more numerous than parasites. A few forms are autotrophic (Walker, 1988). Bacteria generally have strong cell walls that give them shape and may contain components that contribute to their pathogenicity. This structure functions as a means of protection against toxic substances and is the site of action of a number of antibiotics (Prescott *et al.*, 1996).

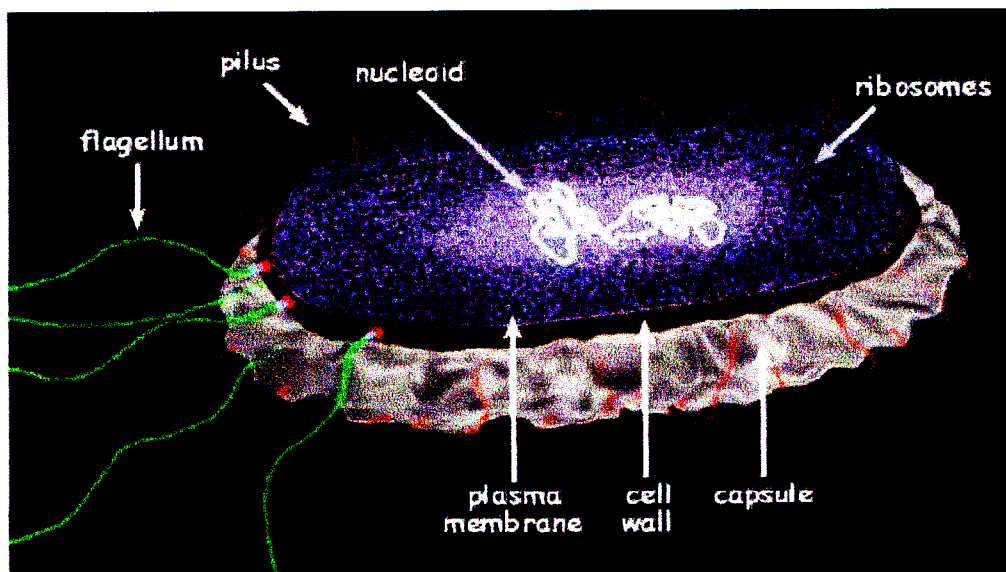


Figure 2.1: Structure of a bacterial cell (www.cellsalive.com)

Several bacterial families are present in foods, the majority of which are concerned with food spoilage. As a result, the important role of bacteria in the fermentation of foods is often overlooked. The most important bacteria in food fermentations are *Lactobacillaceae*, which have the ability to produce lactic acid from carbohydrates (FAO, 1995).

2.2.3. Bacterial cell wall

Bacteria may be divided into two groups based on their response to the Gram staining procedure and these are Gram-positive bacteria, which stain purple and Gram-negative bacteria, which stain red. The staining characteristic is related to differences in the structure of their cell wall. The Gram-positive cell wall consists of a single 20 to 80nm thick homogeneous peptidoglycan layer lying outside the plasma membrane, while the Gram-negative cell wall is more complex and has a 1 to 3nm peptidoglycan layer surrounded by a 7 to 8nm thick outer membrane (Prescott *et al.*, 1996).

a) Gram negative bacterial cell wall

The cell wall of Gram-negative bacteria (Figure 2.2) is much thinner, being comprised of only 20% peptidoglycan. Gram-negative bacteria also have two unique regions that surround the outer plasma membrane: the periplasmic space and the lipopolysaccharide (LPS) layer. The periplasmic space separates the outer plasma membrane from the peptidoglycan layer. It contains proteins that destroy potentially dangerous foreign matter present in this space (Atlas, 1989). The LPS layer is located adjacent to the exterior peptidoglycan layer. It is a phospholipid bilayer construction similar to that of the cell membrane and is attached to the peptidoglycan by lipoproteins. The lipid portion of the LPS contains a toxic substance, called Lipid A, which is responsible for most of the pathogenic affects associated with pathogenic Gram-negative bacteria (Russell and Chopra, 1990). Polysaccharides which extend out from the bilayer also contribute to the toxicity of the LPS. The LPS, lipoproteins, and the associated polysaccharides together form what is known as the outer membrane. The cell wall is porous and thus will let anything pass that can fit through its gaps (Atlas, 1989).

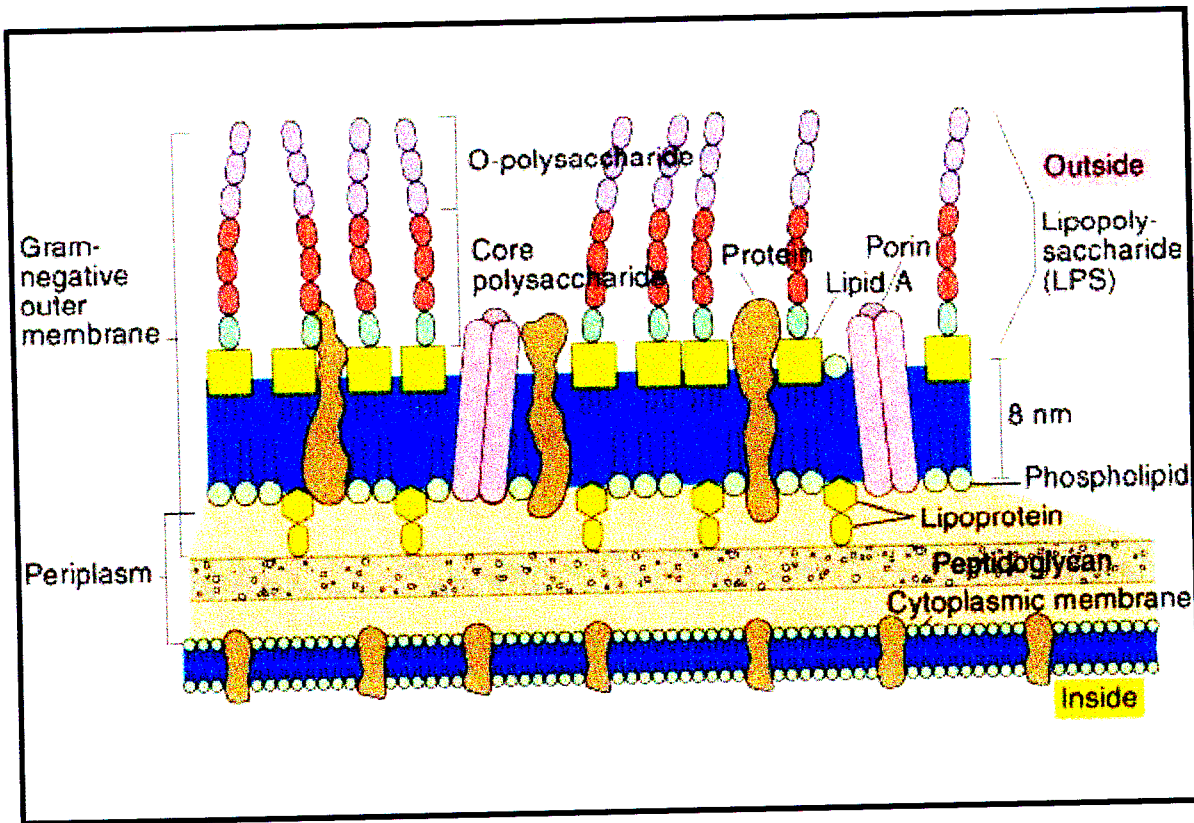


Figure 2.2: Gram-negative bacterial cell wall (www.arches.uga.edu)

b) Gram-positive bacterial cell wall

The cell wall of Gram-positive bacteria (Figure 2.3) consists of many polymer layers of peptidoglycan connected by amino acid bridges. The peptidoglycan polymer is composed of an alternating sequence of N-acetylglucosamine (NAG) and N-acetyl-muramic acid (NAMA). Each peptidoglycan layer is connected, or cross-linked, to the other by a bridge comprising amino acids and amino acid derivatives. The particular amino acids, however, vary among different species. The cross-linked peptidoglycan molecules form a network which covers the cell like a grid. Also, 90% of the Gram-positive cell wall is comprised of peptidoglycan (Atlas, 1989).

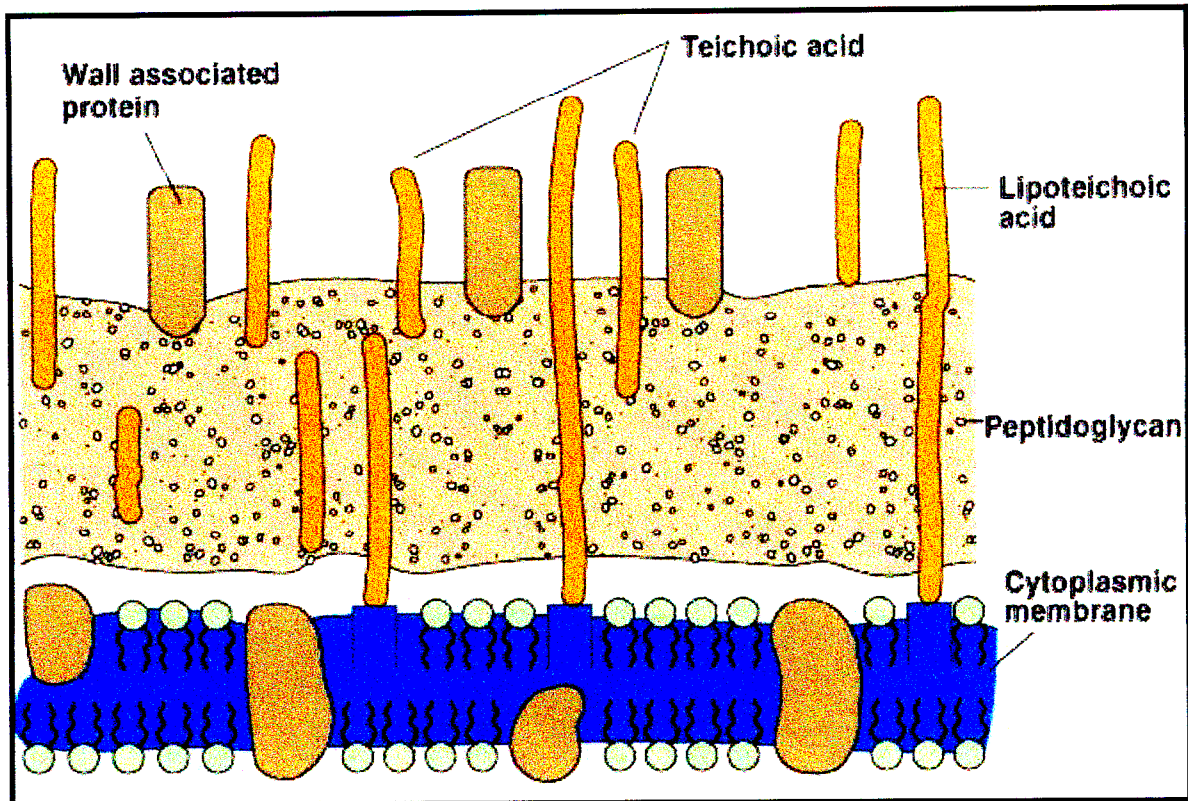


Figure 2.3: Gram-positive bacterial cell wall (www.arches.uga.edu)

c) Pathogenic action

Some pathogenic bacteria destroy host cells, but most cause disease by producing toxins. Pathogenic bacteria of both the Gram-positive and Gram-negative classes produce soluble substances that alter the normal metabolism of host cells with subsequent deleterious effects on the host. Bacterial toxins serve as primary virulence factors and have the capacity to damage the extracellular matrix or the plasma membrane of eukaryotic cells by enzymatic hydrolysis or pore formation. This damage may result in the direct lysis of cells and facilitation of bacterial spread through tissues (Russell and Chopra, 1990; Schmitt *et al.*, 1999). There are two kinds of bacterial toxins, namely endotoxins and exotoxins. Endotoxins are lipopolysaccharides in the outer membrane of all Gram-negative bacteria where they form a shield against the entry of antibiotics. As a result Gram-negative bacteria are not particularly susceptible to antibiotics. Exotoxins are proteins secreted from the growing (primarily Gram-positive) bacterium into the surroundings and are carried through the host's body by the bloodstream (Arms and Camp, 1995).

2.2.4. Enterobacteriaceae

a) *Klebsiella*

These are Gram-negative straight rods, 0.3-1.0µm diameter, 0.6-6.0µm length, arranged singly, in pairs or short chains. The cells are capsulated by a large polysaccharide layer that distinguishes its members from other bacteria in the family. They are non-motile, facultative anaerobes, whose colonies are usually dome-shaped and glistening with varying degree of stickiness. They are oxidase negative, use citrate and glucose as sole carbon source, Voges-Proskauer (VP) test is usually positive, produces lactic, acetic and formic acids in smaller amounts and ethanol in larger amounts. They ferment inositol and hydrolyse urea. Lack of ornithine decarboxylase production or hydrogen sulphide (H₂S) production are their distinctive characters. Some fix nitrogen. They occur in intestinal contents, soil, water and grains, among other environments. They grow on many media since they have no particular growth requirements. A carbohydrate-rich medium gives a better development of the capsule than a carbohydrate-poor medium (Krieg and Holt, 1984).

Species belonging to the genus *Klebsiella* test negative for methyl red (MR) and positive for Voges-Proskauer (VP), meaning that acetoin and 2,3-butanediol are formed from pyruvic acid and that these neutral end products predominate over the acidic end products as a result of sugar fermentation. Many *Klebsiella* species produce bacteriocin (klebecin) (Krieg and Holt, 1984). All *Klebsiella* species are resistant to ampicillin and this resistance may reside in chromosomal genes or be mediated by genes present on the chromosome and on a plasmid. *Klebsiella* are opportunistic pathogens that can give rise to bacteremia, pneumonia, urinary tract and several other types of human infections. In recent years there has been an increase in *Klebsiella* infections, particularly in hospitals, due to strains with multiple antibiotic resistance (Krieg and Holt, 1984).

b) *Enterobacter/Pantoea*

They are straight, (facultative anaerobes), Gram-negative rods of 0.6-1.0µm in diameter and 1.2-3.2µm in length. They are motile by peritrichous flagella, ferment glucose to produce acid and gas, are VP test positive, MR test negative and β-galactosidase (ONPG test) negative. Citrate and malonate are usually utilised as sole sources of carbon and energy. H₂S is not produced from thiosulfate. The optimum growth of this genus is 30°C. *Enterobacter* is negative for DNase, lipase and Tween 80 esterase (these characteristics separates *Enterobacter* from *Klebsiella* and *Serratia* genera in the family *Enterobacteriaceae*) (Krieg and Holt, 1984).

2.2.5. Lactic acid bacteria

Lactic acid occurs widely in nature, being found in humans, animals, plants and microorganisms. The most important producers of lactic acid belong to the family of *Lactobacillaceae* (Dellweg, 1983). The lactic acid bacteria are a group of Gram-positive bacteria, non-respiring, non-spore forming, diplococci, tetrads, streptococci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, sorghum beer, amahewu, all fermented milks, cassava and most pickled vegetables. Historically, bacteria from the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* are the main species involved in food fermentations (Dellweg, 1983; Axelsson, 1998). Lactic acid bacteria are fastidious microorganisms. For normal growth they need, besides a carbon source, nitrogen, partly in the form of amino acids, several vitamins, growth substances, and minerals (Dellweg, 1983). Despite their complexity, the basis of lactic acid fermentation centres on the ability of lactic acid bacteria to produce acid, which then inhibits the growth of other undesirable organisms. All lactic acid producers are microaerophilic (Axelsson, 1998).

a) *Leuconostoc*

Cells may be spherical but are often lenticular, particularly when growing on agar, cells usually occur in pairs and chains, Gram-positive, non-motile, non-spore formers and facultative anaerobes. Colonies are small, usually less than 1mm in diameter, smooth, round and greyish white. Optimum growth temperature is between 20 and 30°C. Growth does, however, occur between 5 and 30°C. These bacteria are chemoorganotrophs, requiring a rich medium often having complex growth factors and amino acid requirements. All species require nicotinic acid, thiamine, biotin and either pantothenic acid or pantothenic acid derivative. Growth is dependent on the presence of a fermentable carbohydrate and glucose is fermented by a combination of the hexose-monophosphate and phosphoketolase pathways. Fructose 1,6-diphosphate aldolase is absent and an active glucose-6-phosphate dehydrogenase is present. Carbon dioxide and D-ribulose-5-P are formed from glucose. Xylulose 5-P phosphoketolase is present and the resulting end products are ethanol and D-(-)-lactic acid. Some strains have an oxidative mechanism and acetic acid is formed in place of ethanol. Polysaccharides and alcohols (except mannitol) are usually not fermented (Sneath *et al.*, 1986). Malate can be utilised and converted to L-(+)-lactate. Catalase negative cytochromes are absent. Arginine is not hydrolysed, non-proteolytic and indole is not formed. Nitrates are not reduced, is non-haemolytic and non-pathogenic to plants and animals (including humans). Growth conditions may affect cell morphology, and not all strains will be influenced in the same way. When cultured in

milk, most strains form coccoid cells in chains which vary in length with the strain. When cultured in broth, cells are elongated and can be mistaken for rods appearing morphologically closer to the lactobacilli than to the streptococci and when cultured on agar, spherical cells are seldom formed. The cell wall of dextran-forming strains contains dextran-sucrase and the cell wall structure is affected by growth in sucrose broth to which strains differ in their response. Although capsular material is apparent in some strains, a true bacterial capsule is not formed. Growth is never rapid. Nutritional requirements vary from strain to strain (Sneath *et al.*, 1986).

Leuconostocs are dependent on the presence of a fermentable carbohydrate, and fermentation ability varies in different species. Glucose is used by all species but fructose is preferred by almost all except *L. mesenteroides* subspecies *cremoris*. Glucose is phosphorylated and all species have an active glucose-6-phosphate dehydrogenase enzyme. Gluconate is decarboxylated and pentose converted to D-(-)-lactate and ethanol by the phosphoketolase pathway. Acetate as well as ethanol may be formed by some strains. Sensitivity to antibiotics and drugs is not known since no species are known to be pathogenic. Type of lactic acid produced separates the D-(-)-forming leuconostocs from L-(+)-forming streptococci (Sneath *et al.*, 1986).

b) *Lactobacillus*

Cells are Gram-positive, non-spore formers, vary from long and slender, sometimes bent rods to short, often coryneform coccobacilli in chains formation. The length of the rods and the degree of curvature is dependent on the age of the culture, the composition of the medium and the oxygen tension. Motility is uncommon, but when present, is by peritrichous flagella (Sneath *et al.*, 1986).

Some species of the gas-producing lactobacilli (*L. fermentum* and *L. brevis*) always exhibit a mixture of long and short rods. At least half of the end-product carbon is lactate which is usually not fermented. These bacteria are microaerophilic, surface growth on solid medium is generally enhanced by anaerobiosis or reduced oxygen pressure and 5–10% carbon dioxide and some are anaerobes on isolation. Nitrate reduction is highly unusual, present only when terminal pH is poised above 6.0. Gelatin is not liquefied and casein is not digested, but small amounts of soluble nitrogen are produced by most strains. Indole and H₂S are not produced. These bacteria are catalase and cytochrome negative (porphyrins absent), however, a few strains decompose peroxide by a pseudocatalase. Complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters and fermentable carbohydrates are generally characteristic for each species, often for particular strains only.

Growth temperature ranges from 2 to 53°C with an optimum temperature of generally 30 - 40°C, are found in dairy products, grain products, meat and fish products, water, sewage, beer, wine, fruit juices, pickled vegetables, sauekraut, silage, sour dough and mash. They are a part of the normal flora in the mouth, intestinal tract and vagina of homothermic animals including man. Pathogenicity is rare (Sneath *et al.*, 1986).

Colonies on agar media are usually small (2-5mm), with entire margins, convex, smooth, glistening and opaque without pigment. Most strains exhibit slight proteolytic activity due to cell wall-bound or cell wall-released proteases and peptidases. Lactobacilli do not develop characteristic odours when grown in common media. However, they contribute to the flavour of fermented food by producing various volatile compounds such as diacetyl and its derivatives and even H₂S and amines in cheese. They are extremely fastidious organisms adapted to complex organic substrates. They require not only carbohydrates as energy and carbon source, but also nucleotides, amino acids and vitamins. While pantothenic acid and nicotinic acid are required by all species with the exception of a few strains, thiamine is only necessary for the growth of the heterofermentative lactobacilli. The requirements for folic acid, riboflavin, pyridoxal phosphate and *p*-aminobenzoic acid are scattered among the various species, with riboflavin being the most frequently required compound. Biotin and B₁₂ are required by only a few strains. The various requirements for essential nutrients are normally met when the media contain fermentable carbohydrate, peptone, meat and yeast extract. Lactobacilli grow best in slightly acidic media with an initial pH of 6.4 – 4.5. Growth ceases when pH 4.0 – 3.6 is reached, depending on the species and strain. Although most strains are fairly aerotolerant, optimal growth is achieved under microaerophilic or anaerobic conditions. Most lactobacilli grow best at mesophilic temperatures with an upper limit of around 40°C (Sneath *et al.*, 1986).

The main fermentation pathways for hexoses are the Embden-Meyerhof pathway converting 1 mol of hexose to 2 mol of lactic acid (homolactic fermentation) and the 6-phosphogluconate pathway, resulting in 1 mol CO₂, 1 mol ethanol (or acetic acid) and 1 mol lactic acid (heterolactic fermentation). Pyruvate, intermediately formed in both pathways, may partly undergo several alternative conversions, yielding either the well-known aromatic compound diacetyl and its derivatives, or acetic acid (ethanol). With hexose limitation, the latter pathway may become dominant and the homolactic fermentation may be changed to heterolactic fermentation with acetic acid, ethanol and formic acid as the main products.

The pH of the medium exerts an influence on the amount of growth of an organism. Some microorganisms, especially under certain environmental conditions, produce acidic or alkaline waste products. These waste products are often produced in such large amounts that further growth ceases because the medium has become either too acidic or too alkaline (Sneath *et al.*, 1986).

c) Lactic *Streptococci/Lactococci*

The genus *Streptococcus* consists of Gram-positive spherical bacteria that grow in chains and ferment sugars to lactic acid. It is this last property, converting sugars into lactic acid during growth, which is useful when streptococci are used to convert milk into curds for cheese making or into yoghurt. The species *Streptococcus lactis* is the most common bacterium used for this purpose (Sneath *et al.*, 1986).

d) Yeasts

Yeasts are unicellular, oval or spherical fungi which increase in number asexually by a process termed budding. A bud forms on the outer surface of a parent cell. The nucleus divides with one nucleus entering the forming bud, and cell wall material is laid down between the parent cell and the bud. Usually the bud breaks away to become a new daughter cell but sometimes, as in the case of the yeast *Candida*, the buds remain attached forming fragile branching filaments called pseudohyphae. Because of their unicellular and microscopic nature, yeast colonies appear similar to bacterial colonies on solid media. Yeasts are facultative anaerobes and can therefore obtain energy by both aerobic respiration and anaerobic fermentation (Potter and Hotchkiss, 1995).

Most yeasts are non-pathogenic and some are of great value in industrial fermentations. For example, *Saccharomyces* species are used for both baking and brewing. Like bacteria, yeasts can have beneficial and non-beneficial effects in foods. The most beneficial yeasts in terms of desirable food fermentation are from the *Saccharomyces* family. Yeasts play an important role in the food industry as they produce enzymes that favour desirable chemical reactions such as leavening of bread and the production of alcohol and invert sugar (FAO, 1995).

2.2.6. African non-alcoholic fermented gruels and beverages

Lactic acid or non-alcoholic fermentation is a type that is carried out by lactic acid bacteria in which simple and complex sugars are converted either entirely or almost entirely to lactic acid (homolactic fermentation) or to a mixture of lactic acid and other products (heterolactic fermentation). There is one important disadvantage to this source of energy, however.

About fifteen times as much glucose is necessary to produce the same amount of ATP in lactic acid fermentation than is necessary for the respiration process (Alexander and Griffiths, 1993). In non-alcoholic fermentation, bacteria will be the first to colonise and grow, followed by yeasts and then moulds. There are definite reasons for this type of sequence. The smaller microorganisms are the ones that multiply and take up nutrients from the surrounding area most rapidly. Bacteria are the smallest microorganisms, followed by yeasts and moulds. The smaller bacteria, such as *Leuconostoc* and *Streptococcus*, grow and ferment more rapidly than their close relations and are therefore often the first species to colonise a substrate. Some microflora may participate in parallel while others may participate in a sequential manner with a changing dominant flora during the course of the fermentation. The specific microflora involved may vary somewhat from village to village and from family to family within the same village as well as the fermentation products (FAO, 1995).

a) Amahewu

This is a fermented maize meal commonly consumed as a staple by the African population of southern Africa. It is traditionally prepared by adding one part of maize meal to 9 parts of boiling water. The suspension is cooked for about 10 - 15 minutes, allowed to cool and then transferred to a fermentation container. At this stage, wheat flour [about 5% (w/w) of the maize meal used] is added to serve as a source of simple sugars. Fermentation occurs in a warm sunny place within 24 hours. *Streptococcus lactis* is the main fermenting organism in traditionally prepared amahewu (FAO, 1999). Amahewu are known to offer some advantages over ogi (in page 16) in that the initial wild fermentation by fungi, etc. is eliminated by boiling both the maize meal and water for steeping. Furthermore, it is pre-cooked and requires only mixing prior to consumption. Amahewu consists of coarse maize particles while ogi contains very fine pasty maize particles (Odunfa and Oyewole, 1998).

Currently, amahewu are produced industrially and literature describing this is available (Wood, 1998). The industrial production of amahewu therefore spurs the need for the development of safer starter cultures. Schweigart and Fellingham (1963) evaluated the use of various lactic acid bacteria as starters in amahewu fermentation and determined that *Lactobacillus delbrueckii* and *L. bulgaricus* produced the most acceptable amahewu at a temperature of 50°C, which was determined to disallow the growth of unwanted microorganisms. van Noort and Spence (1976) of Jabula Foods Limited, South Africa, produced a more acceptable amahewu product at room temperature using a combination of starters including an acid-producing bacterium, a yeast and a non-acid producing bacterium.

The identity of the various organisms used was not however disclosed by these workers (Odunfa and Oyewole, 1998). This non-disclosure therefore represents one disadvantage of industrialisation although advantages include that the process will be able to conform to national standards of food hygiene and safety and it also allows the bulk production of amahewu.

b) Ogi

Ogi is a porridge prepared from fermented maize, sorghum or millet in West Africa. It is a staple of that region, and serves as a weaning food for infants. The traditional preparation of ogi involves soaking of corn kernels in water for 1 to 3 days followed by wet milling and sieving to remove bran, hulls and germ (Odunfa, 1985; Akinrele *et al.*, 1970). The pomace is retained on the sieve and later discarded as animal feed while the filtrate is fermented (for 2-3 days) to yield ogi, which is sour, white starchy sediment. Ogi is often marketed as a wet cake wrapped in leaves or transparent polythene bags. It is diluted to a solid content of 8 to 10% (w/v) and boiled into a soft porridge, or cooked and turned into a stiff gel called "agidi" or "eko" prior to consumption (Odunfa *et al.*, 1994).

Microbiological and nutritional studies by Akinrele *et al.*, (1970) showed that the lactic acid bacterium *Lactobacillus plantarum*, the aerobic bacteria *Corynebacterium* and *Aerobacter*, the yeasts *Candida mycoderma*, *Saccharomyces cerevisiae* and *Rhodotorula* and moulds such as *Cephalosporium*, *Fusarium*, *Aspergillus* and *Penicillium* are the major organisms responsible for the fermentation and nutritional improvement of ogi. Odunfa (1985) determined that *L. plantarum* was the predominant organism in the fermentation responsible for lactic acid production. *Corynebacterium* hydrolysed cornstarch to organic acids while *S. cerevisiae* and *C. mycoderma* contributed to flavour development. Olasupo *et al.* (1995) determined bacteriocin-producing *Lactobacillus* isolates to be active against common food-borne pathogens including *Salmonella*. This bacteriocin also improved the shelf-life of "jellied" ogi, extending it by 10 days (Olasupo *et al.*, 1997).

c) Banku

Banku is a popular staple consumed in Ghana. It is prepared from maize and/or from a mixture of maize and cassava (Owusu-Ansah *et al.*, 1980). The preparation of banku involves steeping the raw material (maize or a mixture of maize and cassava) in water for 24 hours followed by wet milling and fermentation for 3 days. The dough is then mixed with water at a ratio of 4 parts dough to 2 parts water or 4 parts dough to 1 part cassava and 2 parts water.

Continuous stirring and kneading of the fermented dough is required to attain an appropriate consistency during subsequent cooking. Microbiological studies of the fermentation process revealed that the predominant microorganisms involved were lactic acid bacteria and moulds (Beuchat, 1983).

d) Kenkey

This is fermented maize dough which is popularly consumed in Ghana. During the production of kenkey, the dough is divided into two parts: one part, the “aflata” is cooked into a thick porridge, while the other uncooked part is later mixed with the “aflata”. The resulting mixture is moulded into balls and wrapped in dried maize husk or plantain leaves, after which it is steamed (Olsen *et al.*, 1995). It is interesting to note that kenkey varieties vary widely throughout Ghana. In northern Ghana, sorghum is sometimes used instead of maize for preparation of the dough (FAO, 1999). Microbiological studies of kenkey production by Jespersen *et al.*, (1994) highlighted the significance of yeasts and moulds in the production of the fermented maize dough. A mixed flora consisting of *Candida*, *Saccharomyces*, *Penicillium*, *Aspergillus* and *Fusarium* species were found to be the dominant organisms during the preparation of this food product. Halm *et al.* (1993) concluded that a homogenous group of obligatively heterofermentative *Lactobacilli* related to *L. fermentum* and *L. reuteri* play a dominating role during kenkey production.

e) Mawe

Mawe is sour dough prepared from partially dehulled maize meal which has undergone natural fermentation for a one to three-day period. Studies on mawe production were conducted by Houhonigan (1994), an estimated 14-16% of total maize production in Cotonou, Benin is used for mawe production. Mawe is produced using both a traditional (home) process and a commercial process. Traditional mawe production involves cleaning maize by winnowing, washing in water and crushing in a plate disc mill. The crushed maize is screened by sieving whereby grits and hulls are separated by gravity and the fine endosperm fraction collected in a bowl (FAO, 1999).

The grits are not washed but home dehulled, following which they are mixed with the fine endosperm fraction, moistened over a 2 to 4 hour period and milled to dough. The kneaded dough is then covered with a polyethylene sheet and allowed to ferment naturally to sour dough in a fermentation bowl, or wrapped in paper or polyethylene.

The main difference between the traditional and the commercial process of mawe production is that hulls and germs are removed during the commercial processing of mawe. Commercial mawe is whiter in appearance than home-produced mawe and has better swelling and thickening characteristics, but is of lower nutritional value. The crude protein, crude fat, crude fibre and ash contents of home-made mawe were higher than those of commercial mawe since more hulls and germs were retained during home production (Hounhouigan *et al.*, 1993). Dominant microorganisms in mawe preparation include lactic acid bacteria (mainly *Lactobacillus fermentum* and its biotype *L. cellobiosus*, *L. brevis*) and yeasts, *Candida krusei* and *Saccharomyces cerevisiae*.

f) Injera

Injera is the most popular baked product in Ethiopia. It is fermented sorghum bread with a very sour taste (FAO, 1999) and is the undisputed national bread of Ethiopia. The baked product is referred to by different names depending on the locality of production in Ethiopia. The sorghum grains are dehulled manually or mechanically and milled to flour which is subsequently used in the preparation of injera.

On the basis of production procedures three types of injera are distinguishable:

- (i) Thin injera which results from mixing a portion of fermented sorghum paste with three parts of water and boiling to yield a product known as "absit" which is, in turn, mixed with a portion of the original fermented flour.
- (ii) Thick injera, which is reddish in colour with a sweet taste, is a "tef" paste that has undergone only minimal fermentation for 12-24 hours and
- (iii) Komtata-type injera, which is produced from over-fermented paste, and has a sour taste. The paste is baked or grilled to give a bread-like product.

Yeasts are the major microorganisms involved in the fermentation of the sweet type of injera (Beuchat, 1983). There is little variation in the nutrient composition of injera prepared from different cereals, which indicates the potential for the use of cereals other than sorghum in the production of injera.

g) Kisra

This is a thin pancake-like leavened bread prepared from whole sorghum flour. It is a dietary staple in Sudan. This fermented sorghum bread has a very sour taste (FAO, 1999). It is prepared by mixing sorghum flour with water to produce a thick paste which is allowed to ferment for 12-24 hours,

following which the paste is thinned to a desirable consistency with water just prior to baking. El-Tinay *et al.*, (1979), reported that there was a slight increase in protein and fibre and an appreciable decrease in carbohydrate (starch and sugars) during the fermentation of kisra. An amino acid analysis of kisra prepared from three different cultivars of sorghum indicated slight differences in the levels of the various amino acids.

h) Kishk

Kishk is a fermented product prepared from parboiled wheat and milk. It is consumed in Egypt and in most Arabian countries (FAO, 1999). During the preparation of kishk, wheat grains are boiled until soft, dried, milled and sieved in order to remove the bran. Milk is separately soured in earthenware containers, concentrated and mixed with the moistened wheat flour thus prepared, resulting in the preparation of a paste called a hamma. The hamma is allowed to ferment for about 24 hours, following which it is kneaded and two volumes of soured salted milk are added prior to dilution with water. Alternatively, milk is added to the hamma and fermentation is allowed to proceed for a further 24 hours. The mass is thoroughly mixed, formed into balls and dried. Kishk is a highly nutritious food, having a protein content of about 23.5%. It is of a high digestibility, and high biological value. Microorganisms responsible for fermentation include *Lactobacillus plantarum*, *L. brevis*, *L. casei*, *Bacillus subtilis* and yeasts (Beuchat, 1983; Odunfa, 1985). Kishk is usually over-heated to improve its keeping quality.

i) Bogobe

Bogobe is a porridge prepared in Botswana from fermented and non-fermented sorghum. Fermented bogobe is a soft porridge, known as *ting* while the non-fermented bogobe is a thick porridge called *monokwane* (Boising and Nancy, 1982). Information relevant to microorganisms involved in the fermentation of bogobe, and the nutritional changes which occur during fermentation is still scanty.

j) Kunu-zaki

This is a millet-based non-alcoholic fermented beverage widely consumed in the Northern parts of Nigeria. This beverage is however becoming more widely consumed in southern Nigeria, owing to its refreshing qualities. Adeyemi and Umar (1994) described the traditional process for the manufacture of kunu-zaki. This process involves the steeping of millet grains, wet milling with spices (ginger, cloves, pepper), wet sieving and partial gelatinisation of the slurry, followed by the addition of sugar, and bottling. The fermentation which occurs briefly during steeping of the grains in water over 8 - 48 hour period is known to involve mainly lactic acid bacteria and yeasts.

Sopade and Kassum (1992) highlighted the significance of rheological characteristics in processing, quality control, sensory evaluation and structural analysis of kunu-zaki. Increasing temperatures reduced viscosity but did not alter the rheological characteristics of the product. The time of shear (up to 1 hour) did not appreciably alter the viscosity. Storage studies conducted by Adeyemi and Umar (1994) revealed that the product had a shelf-life of about 24 hours at ambient temperature, which was extended to 8 days by pasteurisation at 60°C for 1 hour and storage under refrigeration conditions.

2.3. THE IMPORTANCE AND BENEFITS OF CEREAL FERMENTATION

Fermented foods contribute to about one-third of the diet worldwide (FAO, 1995). Cereals are particularly important substrates for fermented foods in all parts of the world and are staples in the Indian subcontinent, Asia, and Africa. Aside from alcoholic fermentations and the production of yoghurt and leavened bread, food fermentations continue to be important primarily in developing countries where the lack of resources limits the use of techniques such as vitamin enrichment of foods, and the use of energy and capital intensive processes for food preservation. Fermentation causes changes in food quality indices including texture, flavour, appearance, nutrition and safety (Campbell-Platt, 1994).

The benefits of fermentation may include improvement in palatability and acceptability by developing improved flavours and textures; preservation through formation of acidulants, alcohol, and antibacterial compounds; enrichment of nutritive content by microbial synthesis of essential nutrients and improving digestibility of protein and carbohydrates; removal of antinutrients, natural toxicants and mycotoxins; and decreased cooking times (Odunfa and Oyewole, 1998). Another benefit of fermentation is that frequently the product does not require cooking or the heating time required for preparation is greatly reduced (Steinkraus, 1994). Some advantages of traditional fermentations include the fact that they are labour-intensive, integrated into village life, familiar, utilise locally-produced raw materials, inexpensive, have barter potential and the subtle variations resulting, add interest and tradition to local consumers (FAO, 1995).

Starch and fibre tend to decrease during fermentation of cereals (El-Tinay *et al.*, 1979). Although it would not be expected that fermentation would alter the mineral content of the product, the hydrolysis of chelating agents such as phytic acid during fermentation, improves the bioavailability of minerals. Reddy and Pierson (1994) reviewed the effect of fermentation on antinutritional and toxic components in plant foods.

Fermentation of corn meal and soybean-corn meal blends lowers flatus producing carbohydrates, trypsin inhibitor and phytates (Chompreeda and Fields, 1981; Chompreeda and Fields, 1984). The technology of producing many indigenous fermented foods from cereals varies from household to household (Chaven and Kadam, 1989). Prospects for applying advanced technologies to indigenous fermented foods (Wood, 1994) and for the production of value-added additive products, such as colours, flavours, enzymes, antimicrobials, and health products (Cook, 1994) during food fermentations have been reviewed. Special mention should be made of the microbiological risk factors associated with fermented foods. The safety of fermented foods has been recently reviewed (Nout, 1994). Cases of food-borne infection, and intoxications due to microbial metabolites such as mycotoxins, ethyl carbamate, and biogenic amines have been reported in fermented foods. Major risk factors include the use of contaminated raw materials, lack of pasteurisation, and use of poorly controlled fermentation conditions. On the other hand, non-toxicogenic microorganisms can serve to antagonise pathogenic microorganisms and even degrade toxic substances such as mycotoxins in fermented foods (Nakazato *et al.*, 1990).

The optimum health and nutrition of individuals is dependent upon a regular supply of food and a balanced diet. When diets are sub-optimal, the individual's capacity for work and achievements are greatly reduced. The most vulnerable groups are women, children and weaning infants. Availability of food, dietary restrictions and taboos, misconceptions, limited time available for feeding or eating compound to create a group of individuals who are nutritionally disadvantaged. Approximately a third of women consume less than their daily requirements of energy source and at least 40% of women of worldwide suffer from iron-deficiency anaemia (FAO, 1995). Fermentation can enhance the nutritional value of a food product through increased vitamin levels and improved digestibility. Sorghum beer in Southern Africa contains relatively high levels of riboflavin and nicotinic acid, which are important for people consuming a high maize diet. Pellagra (a vitamin deficiency disease associated with high maize diets) is unusual in communities in which sorghum beer is consumed (Odunfa and Oyewole, 1998). Even children benefit from consuming the dregs which contain relatively little alcohol but are rich in vitamins.

Changes in the vitamin content of cereals with fermentation vary according to the fermentation process, and the raw material used in the fermentation. B-group vitamins generally show an increase on fermentation (Chaven and Kadam, 1989). During the fermentation of maize in the preparation of sorghum beer, thiamine levels are virtually unchanged, but riboflavin and niacin contents almost double (Steinkraus, 1994).

Protein energy malnutrition is the most serious nutritional problem affecting pre-school children in developing countries. The low energy and nutrient density of the diet and the presence of anti-nutritional factors contribute to this condition, as do the low frequency of feeding and transmission of diarrhoea-causing pathogens via the food (Svanberg, 1992; FAO, 1995).

Starch porridges are fed to weaning infants in developing countries. The consistency of these gruels, combined with the small capacity of the infants stomach, means that it is physically impossible for the child to take in adequate energy to meet his/her physical and growth demands. By acidifying the porridge through lactic acid fermentation coupled with enzymic digestion, starch is hydrolysed into shorter chains of glucose and dextrose, which reduces the viscosity of the porridge and increase its energy density. Thus, the child has improved chances of taking it in to meet his/her energy requirements (Wood, 1998). The content and quality of cereal proteins may be improved by fermentation (Wang and Fields, 1978; Cahvan *et al.*, 1988). Natural fermentation of cereals increases their relative nutritive value and available lysine (Hamad and Fields, 1979). Bacterial fermentations involving proteolytic activity are expected to increase the biological availability of essential amino acids more so than yeast fermentations which mainly degrade carbohydrates (Chaven and Kadam, 1989).

A prebiotic is described by Fuller (1997) as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host's health. According to Fuller (1997), for a food ingredient to be classified as a prebiotic, it must:

- ◆ Be a selective substrate for one or a limited number of potentially beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated;
- ◆ Neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract;
- ◆ Consequently, be able to alter the colonic microflora towards a healthier composition, for example by increasing the number of saccharolytic species and reducing putrefactive microorganisms such as asaccharolytic *Clostridia*.

The largest group of prebiotics on the market at the moment are oligosaccharides. These complex sugars are required by certain intestinal bacteria for growth and multiplication purposes. By including oligosaccharides in the diet, the growth of specific bacterial populations can be altered (Cummings *et al.*, 2001). Fructo-oligosaccharides (FOS) have been known as prebiotics for some time, but have been joined by galacto-oligosaccharides and other digestion resistant carbohydrates.

FOS are compounds made up of fructose sugar molecules linked together in long chains. FOS are naturally occurring carbohydrates that can be found in foods such as Jerusalem artichoke tubers, onions, leeks, bananas, garlic, tomatoes, some grains and honey (Roberfroid, 2000). FOS cannot be digested or absorbed by humans yet support the growth of *Acidophilus*, *Bifidus* and *Faecium*. Another type of prebiotic is based on arabinogalactan, a naturally occurring carbohydrate in most plant life, including lettuce, tomatoes, spinach and radishes (Roberfroid, 2000).

A probiotic is a cultured product or live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance (Fuller, 1997). Probiotics are an important part of the complex world of foods that are good for health. Probiotics are foods that contain live bacteria. It is the bacteria and metabolites which they produce that give these probiotics their health-promoting properties. The best known example of a probiotic is live-culture yoghurt (Fuller, 1997). Probiotics not only help restore intestinal microflora balance and inhibit the growth of pathogenic bacteria, they also promote good digestion, boost immune function and increase resistance to infection (Collins and Gibson, 1999).

Characteristics of probiotic preparations according to Fuller (1997) include:

- ◆ Preparations should exert effect on the consumer,
- ◆ Microorganisms should be easy to culture, have high viability and good conservation,
- ◆ Capable of surviving passage through the intestinal tract,
- ◆ Capable of establishing a (temporary) niche in the gastrointestinal tract by competing with the resident flora,
- ◆ Not pathogenic/no toxin production, and
- ◆ Available in a multi-strain preparation.

Yoghurt, and the lactic acid-producing bacteria that it contains, have received much attention as potential cancer-preventing agents in the diet. It is usually considered that the mechanism of action is by increasing the numbers of lactic acid bacteria (LAB) in the colon, which modifies the ability of the microflora to produce carcinogens (Collins and Gibson, 1999). Prebiotics such as non-digestible oligosaccharides appear to have a similar effect on the microflora by selectively stimulating the growth of LAB in the colon (Kaplan and Hutkins, 2000). Evidence of cancer-preventing properties of probiotics and prebiotics is derived from studies on faecal enzyme activities in animals and humans, inhibition of genotoxicity of known carcinogens *in vitro* and *in vivo*, as well as suppression of carcinogen-induced preneoplastic lesions and tumours in laboratory animals (Dunne *et al.*, 2001;

Wallowski *et al.*, 2001). Some of these studies indicate that intervention studies provide some, albeit limited, evidence for protective effects of products containing probiotics in humans (Marteau *et al.*, 2001). In essence, prebiotics are a food source for probiotics, which help maintain a healthy intestinal balance by producing compounds, such as lactic acid, hydrogen peroxide and acetic acid that increase the acidity of the intestine and inhibit the reproduction of pathogenic bacteria. Probiotics also produce bacteriocins, substances that act as natural antibiotics and kill undesirable microorganisms (Roberfroid, 2000).

2.4. FUNGI AND AFLATOXIN B₁, FUMONISIN B₁ AND ZEARALENONE MYCOTOXINS

2.4.1. Fungi

Fungi are eukaryotic organisms and include the yeasts, moulds, and fleshy fungi. Yeasts are microscopic, unicellular fungi; moulds are multinucleated, filamentous fungi (such as mildews, rusts, and common household moulds); the fleshy fungi include mushrooms and puffballs. All fungi are chemoheterotrophs, requiring organic compounds for both an energy and carbon source, and obtain nutrients by absorbing them from their environment. Most live off of decaying organic material and are termed saprophytes. Some are parasitic, getting their nutrients from living plants or animals. In general, fungi are beneficial to humans. They are involved in the decay of dead plants and animals (resulting in the recycling of nutrients in nature), the manufacturing of various industrial and food products, the production of many common antibiotics, and may be eaten themselves for food. Some fungi, however, damage wood and fabrics, spoil foods, and cause a variety of plant and animal diseases, including human infections (Frisvad and Samson, 1992).

2.4.2. Mycotoxins

The fungal growth cycle is divided into two phases, trophophase and idiophase (Bu'Lock, 1967). Trophophase is a phase where normal cell growth occurs (primary metabolism) while idiophase is a dormancy stage where spores are produced (secondary metabolism). Secondary metabolism is a phase found in microorganisms (mainly fungi) and plants but not in animals.

In this stage of secondary metabolism, secondary metabolites, among them mycotoxins, are formed. Mycotoxins as fungal metabolites are detrimental to the health of animals, humans and microbes. When ingested, inhaled or absorbed through the skin, they cause lower performance, sickness or death in man and animals, including birds (Pitt, 1996).

2.4.3. Toxicity

At least 300 different mycotoxins have been identified with only about 20 being of a major concern in crops used for human consumption and animal feeds. Growth of fungi in food and feeds is a major concern. Whether the relation between the food products and the fungi is beneficial or detrimental, both situations demand a substantial knowledge of the characteristics of fungal growth in order to understand and control the interactions. Mycotoxins may remain in food and feed long after the fungus that produced them has died, therefore toxins can be present at potentially dangerous levels in products that are not visibly mouldy or do not show positive results on a mould count of the material (Smith and Solomons, 1994).

Diseases in animals and human beings resulting from the consumption of mycotoxins are called mycotoxicoses. The effects in domestic animals include allergic reactions, reproductive failure, unthriftiness, loss of appetite, feed refusal, suppression of the immune system, decreased feed efficiency, and mortality (Smith and Solomons, 1994). Human suffering from mycotoxicoses includes ergot poisoning associated with ingestion of rye flour contaminated with ergot (holy fire, St. Anthony's fire); cardiac beriberi associated with *Penicillium* moulds in rice (yellow rice toxins); and alimentary toxic aleukia (ATA,) associated with *Fusarium* moulds on over-wintered wheat, millet, and barley. Several mycotoxins have been linked to increased incidence of cancer in human beings. These include aflatoxins, sterigmatocystin, zearalenone, patulin, ochratoxin, and fumonisins (Smith and Solomons, 1994).

2.4.4. Conditions of mycotoxin production

Filamentous moulds can propagate in agricultural plants whenever environmental conditions are favourable. If mould growth has occurred, it is likely that one or more of the associated mycotoxins have been produced (Smith and Solomons, 1994). Food can be contaminated by fungi during production, processing, transportation and storage (Frisvad and Samson, 1992). Factors that determine plant fungal infection are listed in Table 2.1 below.

Table 2.1: Factors which determine plant fungal infection (Hesseltine, 1974).

Factors	In the field	At harvest	In storage
Physical factors			
Rapidity of drying	-	+	+
Re-wetting	-	+	+
Relative humidity	+	+	+
Temperature	+	+	+
Mechanical damage	+	+	+
Blending of grain	-	-	+
Hot spots	-	+	+
Time	+	+	+
Chemical factors			
Carbon dioxide	-	-	+
Oxygen	-	-	+
Nature of substrate	+	-	+
Mineral nutrition	+	-	+
Chemical treatment	-	-	+
Biological factors			
Plant stress	+	-	+
Fungus infection	+	-	+
Spore load	+	+	+
Microbiological ecosystem	+	-	+

+ = Determinants and - = Non-determinants

2.4.5. Aflatoxin B₁

Aspergillus flavus, *A. nomius* and *A. parasiticus* produce a class of mycotoxins known as aflatoxins which present a hazard in feed and food due to their potential carcinogenic properties. The most abundant form is aflatoxin B₁ (AFB₁), whose basic skeleton is a condensed bisfuranocoumarin-lactone/cyclopentene ring system (Figure 2.4). Aflatoxin B₁ is soluble in organic solvents such as chloroform and methanol, but is insoluble in water. It is very stable at high temperatures with little or no destruction under ordinary cooking conditions or during pasteurisation (Smith and Moss, 1985).

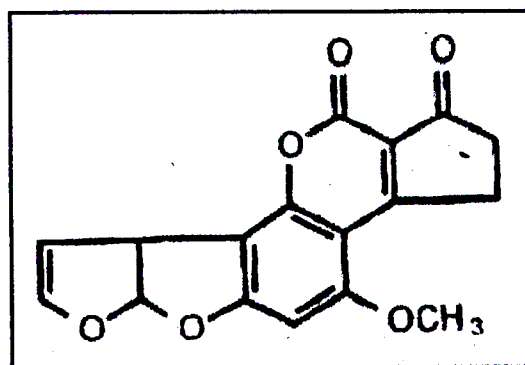


Figure 2.4: Chemical structure of aflatoxin B₁ (Prescott *et al.*, 1996).

Aspergillus can proliferate as a result of drought stress during the latter part of the growing season and will usually produce aflatoxin in the field under stress-humid growing cycles. Hot and humid/moist conditions are favourable for aflatoxin production while commodities are in storage. The best quality control for avoiding this hazard is to test incoming ingredients for aflatoxin and to maintain proper storage conditions – especially temperature and relative humidity (Smith and Moss, 1985).

2.4.6. Fumonisin B₁

Fumonisin, a new group of mycotoxins from *Fusarium verticilloides*, were structurally characterised by Gelderblom *et al.*, (1988). Fumonisin B₁ (FB₁) is the major fumonisin present in culture and naturally contaminated samples. It is a highly polar compound and is soluble in polar solvents such as water, methanol and acetonitrile, but is insoluble in organic solvents (Figure 2.5). Fumonisin B₁ is hydrolysed by strong acids, alkalis and heat to produce tricarbolyllic acid (TCA) and the corresponding aminopentol (Gelderblom *et al.*, 1988).

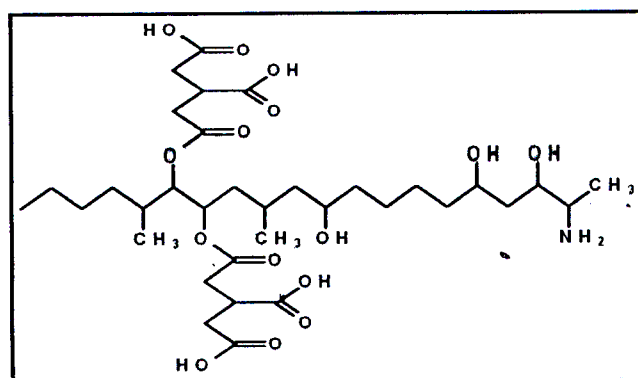


Figure 2.5: Chemical structure of fumonisin B₁ (Gelderblom *et al.*, 1988).

FB₁ causes equine leukoencephalomalacia (ELEM) in horses (Marasas *et al.*, 1988; Kellerman *et al.*, 1990), porcine pulmonary oedema in pigs (Ross *et al.*, 1990), is hepatocarcinogenic in rats (Gelderblom *et al.*, 1991) and is associated with human oesophageal cancer (Rheeder *et al.*, 1992). Fumonisin carcinogenicity and toxicity are due to inhibition of sphinganine (sphingosine) N-acyl transferase (ceramide synthase) (Figure 2.6). This disruption of sphingolipid metabolism is an early event in the onset and progression of diseases associated with fumonisins (Merrill *et al.*, 1996; Riley *et al.*, 1994).

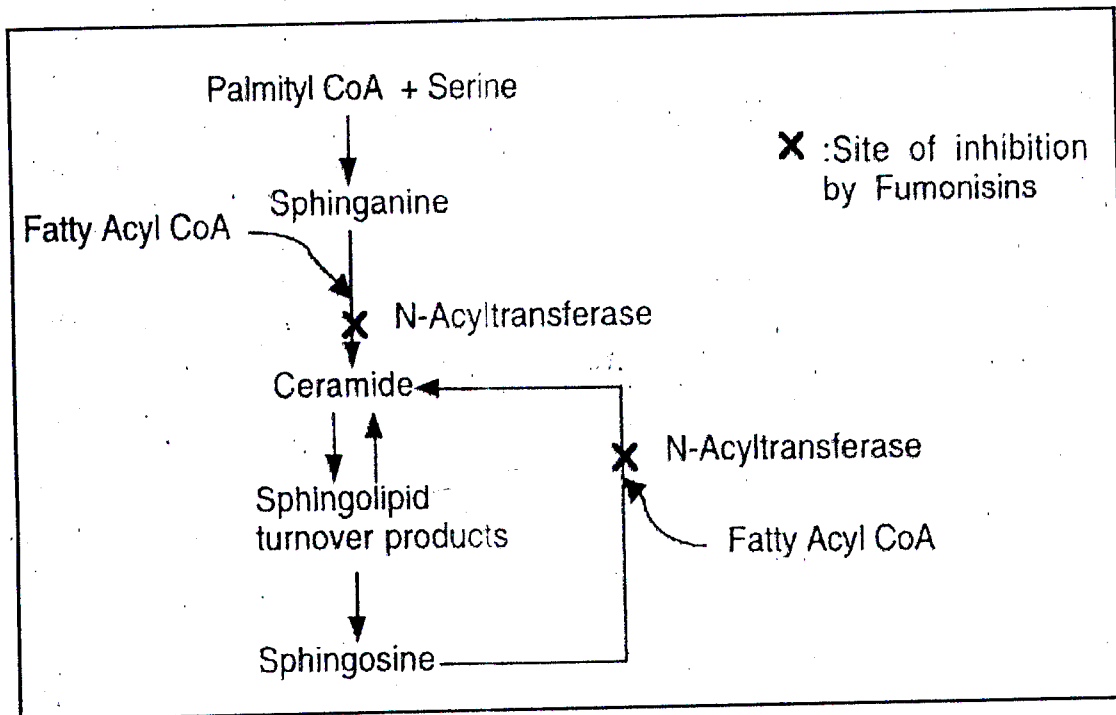


Figure 2.6: The disruption of sphingolipid pathway by fumonisins (www.ansci.cornell.edu)

The major toxic effects of FB₁ are based solely on its disruptive effects on sphingolipid biosynthesis, interference with cellular signal transduction pathways and accumulation of sphingoid bases leading to cell membrane damage (Yoo *et al.*, 1992; 1996). The chemical structure of the backbone of the fumonisin compound is similar to that of the cellular sphingolipids. The specific site of FB₁ action appears to be the enzymes sphinganine and sphingosine-N-acyltransferases as outlined in Figure 2.6. Similarities between fumonisins and sphingoid bases allow them to be recognised as substrates for the enzyme N-transferase (Wang *et al.*, 1991). Inhibition of this metabolic pathway results in depletion of complex sphingolipids, increased intracellular concentrations of free sphinganine and sphingosine (Wang *et al.*, 1991).

Accumulation of sphinganine, a compound that is toxic to many cells, may account for the evidence of varied effects such as toxicity and induction of apoptosis in the liver, kidney of rats, mice and rabbits (Howard *et al.*, 1996; Bucci *et al.*, 1998).

The crop most affected is maize. Fumonisin is usually produced before harvest, but again if storage conditions are favourable, continued toxin production can occur. The effect of fumonisin ingestion on livestock varies with species, amounts ingested, and length of time ingested. Toxic effects on livestock vary from simple excitability to pulmonary oedema. As with all mycotoxins, if consumed in high enough doses death can occur. These toxins are suspected of being able to produce adverse effects in some animals (horses) at levels as low as 1 mg/kg. For most species of livestock the fumonisin levels in feedstuffs should be kept below 2.0 – 5.0 mg/kg (Lacey, 1985).

2.4.7. Zearalenone

Zearalenone (ZEA) is a mycotoxin with oestrogenic activity similar to that of β -oestradiol as it has the characteristic aromatic ring. It is a white crystalline material with molecular weight of 318 g/mol (Figure 2.7). It is soluble in organic solvents such as chloroform, alcohol, acetonitrile, dichloromethane, ethyl acetate and strong alkalis, but is insoluble in water. The most important fungal producers of ZEA are *Fusarium graminearum* and *F. semitectum* (Abbas *et al.*, 1991).

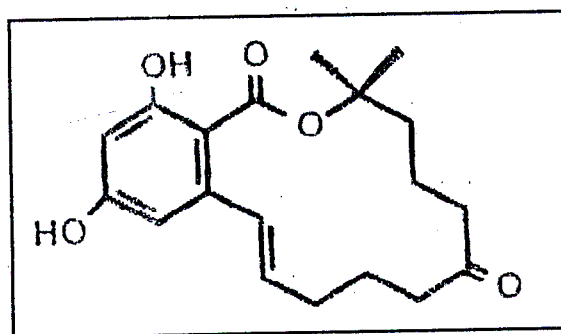


Figure 2.7: Chemical structure of zearalenone (Abbas *et al.*, 1991).

The fungal genus *Fusarium* is one of the most economically important plant pathogens. It includes many species that are toxigenic (Marasas *et al.*, 1984), causing several diseases such as root and crown rots and diseases of the inflorescences such as cob rot of maize and head blight of wheat and other winter cereals (Abbas *et al.*, 1991).

Moulds in the *Fusarium* genus collectively are capable of producing 70 different mycotoxins. Some strains of *Fusarium* may produce as many as 17 mycotoxins simultaneously. Thus *Fusarium* mycotoxins are the most frequently identified group of mycotoxins in grains and feeds. There are at least 7 different species of *Fusarium* moulds that can produce ZEA in a wide variety of crop plants. Although the moulds that produce this toxin most commonly occur in the temperate areas of the world, some ZEA producing moulds have adapted to both sub-tropical and tropical zones. ZEA is often detected in corn, wheat, barley and various silages. Usually it is produced while crops are still in the field. Conducive environmental conditions are similar to that of fumonisin. Like other *Fusarium*-associated toxins, if post harvest storage conditions are favourable, the mould will continue to produce ZEA (Gelderblom *et al.*, 1988).

2.5. CHROMATOGRAPHY AS A TOOL FOR ANALYSING FERMENTED FOODS

2.5.1. Thin Layer Chromatography (TLC)

Over the years TLC has been widely used as a procedure of choice for identifying mycotoxins. It involves spotting of the sample on silica, cellulose, alumina or polyamide coated plate and development in suitable mobile phase solvent/s. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The constituents of a sample can be identified by simultaneously running standards with the unknown (Patterson and Robertson, 1979).

The bottom edge of the plate is placed in a solvent reservoir, and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The developed spots are then visualised under ultra violet (UV) or by spraying the plate with the appropriate reagents to form coloured complexes. A two-dimensional development is preferred to the one-dimensional method as the former gives a better separation of different mycotoxins in a mixture and their separations from other co-extracted substances. After running a sample in one solvent, the TLC plate is removed, dried, rotated 90°, and run in another solvent system. Any of the spots from the first run that contain mixtures can now be separated. In effect, the development of the TLC plate in one direction is a “cleanup” step while the development in the second direction is the actual separation-detection step. The choice of solvents depends very much upon the polarities and the solubilities of the compounds to be separated from the mixture (Snyder *et al.*, 1983).

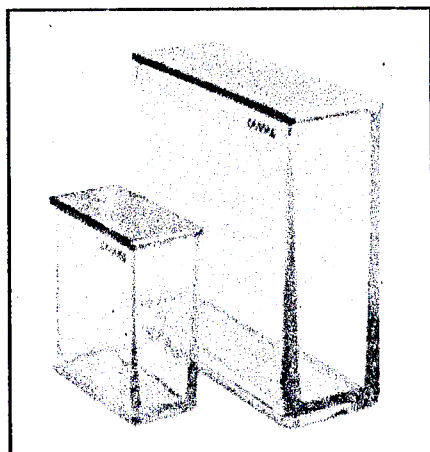


Figure 2.8: Photograph of TLC tanks (www.chromatography.co.uk)

2.5.2. High Performance Liquid Chromatography (HPLC)

The goal of HPLC is to separate components of a sample within a reasonable period of time into separate bands or peaks as they migrate through the column. This is achieved by using high pressure to drive the analyte in solution through a packed chromatographic column, causing separation of the analyte under test which can therefore be identified and quantified using appropriate methods. HPLC plays an increasing role in the analysis of biotechnology products and in the study of biotechnological processes. It provides an essential tool in the analysis of thermally unstable or high molecular weight compounds in fermentation broths (Morovján *et al.*, 1997). The major components of the HPLC system include: the mobile phase reservoir, the pump, autosampler, a separating column, detector/s and a recorder (Figure 2.8) (Willard *et al.*, 1988).

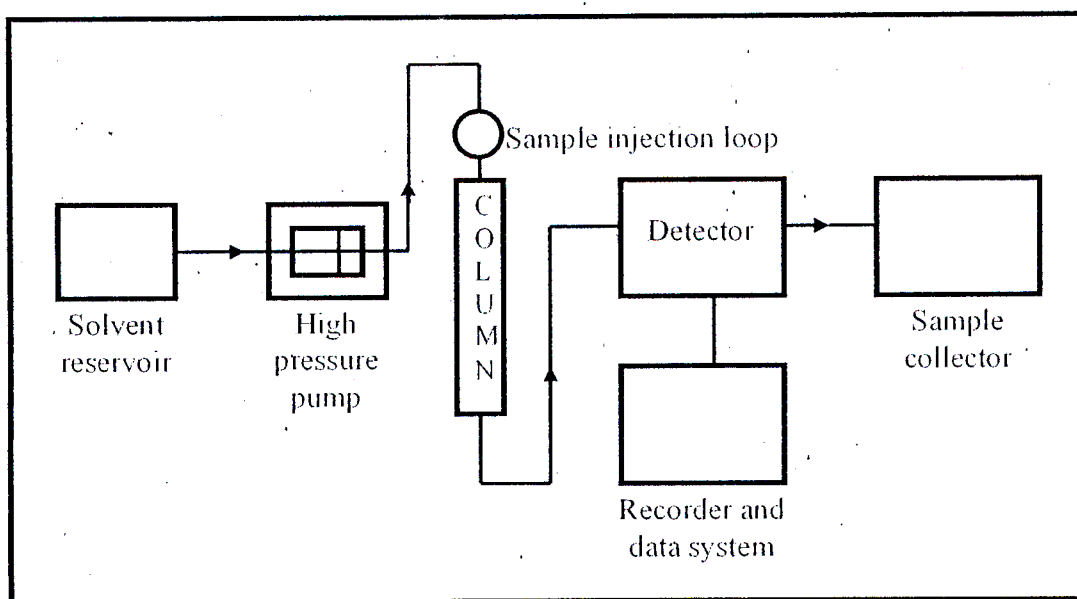


Figure 2.9: A schematic diagram of an HPLC system (Wilson and Walker, 1994).

In summary: cereals and cereal-based foods are still the staple diet in developing countries, especially in Africa. A majority of traditional cereal-based foods consumed in these countries are processed by natural fermentation and previous studies have shown that fermentation has a role to play in:

- ◆ Improving the nutritional value of cereals,
- ◆ Detoxification of xenobiotic compounds,
- ◆ Inhibition of pathogen/s growth, and
- ◆ Health promotion by supplying prebiotic/s and/or probiotic/s.

The objectives of this study, therefore, were to comparatively assess local commercial and homemade amahewu and its starter maize meal with respect to nutritional value, hygiene and other health benefits to the community.

CHAPTER 3

MICROBIOLOGICAL ASSESSMENT OF COMMERCIAL AND HOMEMADE AMAHEWU

3.1. INTRODUCTION

Microorganisms play an integral role in the cycle of life and death. Some species are used in the production of food and medicine while others, having the ability to grow on and within other organisms, can lead to disease, disability and death as a result of microbial colonisation (Prescott *et al.*, 1996).

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria, non-respiring, non-spore forming, diplococci, tetrads, streptococci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations. They are responsible for the fermentation of sour dough bread, sorghum beer, amahewu, all fermented milks, cassava and most pickled vegetables. Historically, bacteria from the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Streptococcus* are the main species involved (Dellweg, 1983; Axelsson, 1998). LAB are fastidious microorganisms which require, besides a carbon source, nitrogen, partly in the form of amino acids, several vitamins, growth substances, and minerals for normal growth (Dellweg, 1983). Despite their complexity, the whole basis of lactic acid fermentation centres on the ability of LAB to produce acid, which then inhibits the growth of other non-desirable organisms. All lactic acid producers are microaerophilic (Axelsson, 1998).

In addition to LAB, most yeasts are non-pathogenic and some are of great value in industrial fermentations. Because of their unicellular and microscopic nature, yeast colonies appear similar to bacterial colonies on solid media. They are facultative anaerobes and can therefore obtain energy by both aerobic respiration and anaerobic fermentation (FAO, 1995).

This study is aimed at assessing local commercial and homemade amahewu with respect to fermenting organisms.

- ◆ Violet Red Bile (VRB) – is a lactose-containing selective medium for the detection and enumeration of coli-aerogenes in water, food and dairy products. Organisms that rapidly attack lactose produce purple colonies surrounded by purple haloes. Non-lactose or late-lactose fermenters produce pale colonies with greenish zones (Cappuccino, 1983).
- ◆ Sabouraud Dextrose Agar (SDA) – is an acid pH medium for the isolation of dermatophytes, other fungi and yeasts (Cappuccino, 1983).

Eleven samples (nine rural and 2 commercial) of amahewu were diluted 1:3 with sterile water. The diluted samples were inoculated onto BA, SDA, EMB, Mac 3, MRS and VRB and incubated anaerobically at 37°C for 24 and 48 hour periods. Well-isolated colonies were sub-cultured into the similar medium and again incubated anaerobically at 37°C for 24 and 48 hour periods.

3.2.1. Gram staining procedure

This procedure was developed by Gram in 1844.

a) Smear preparation

A thin film of the isolated colony was made on a clean glass slide with two drops of water, using a sterile loop. The slide was air-dried, then heat fixed by passing it several times through a flame.

b) Staining procedure

The slide was flooded with crystal violet (60 seconds) and washed with running tap water, flooded with Gram's iodine (30 seconds) and washed with running tap water, was carefully decolourised with acetone-alcohol (3-4 seconds) then washed with running tap water. Lastly, the slide was flooded with safranin (45 seconds) and washed with running tap water, air-dried and was ready to be viewed under microscope.

c) Interpretation of the smear

A drop of immersion oil was added to the dry and stained smear and viewed under the microscope using the oil lens. Organisms that retained the violet-iodine complexes after washing in acetone-alcohol, stained purple and are termed Gram-positive. Those that lose this complex stain red from the safranin counterstain are termed Gram-negative. After organisms were grouped as Gram positive or negative, they were then ready for further identification and confirmation using API kits [API 20 E (Gram negative rods), API 20 STREP (Gram positive streptococci) and API 50 CH using CHL medium (for Gram positive rods and related organisms)].

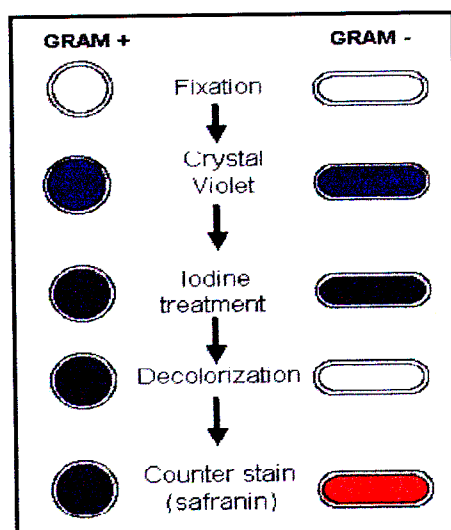


Figure 3.1: Appearance of the Gram-positive coccus and Gram-negative bacillus at different stages of the Gram staining procedure (Birch and Hashimoto, 1996).

3.2.2. API 20 E method

a) Principle

API 20 E is an identification system for *Enterobacteriaceae* and other non-fastidious Gram-negative rods which use 21 standardised and miniaturised biochemical tests and a database. The API 20 E strip consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The microorganisms to be identified with this method were those that grew on VRB, EMB and MacConkey No.3 agar plates (selective media for Gram negatives).

b) Preparation of the strip

An incubation box (tray and lid) was prepared and about 5ml of distilled water was distributed into the honey-combed wells of the tray to create a humid atmosphere. Distilled not tap water was used to avoid the release of gases by chemicals that could be initially in the water. The strip was then removed from its packaging and placed in the incubation box.

c) Inoculation of the strip

Well-isolated colonies were removed from VRB, EMB and Mac No.3 plates using sterile swabs and were carefully emulsified to achieve homogenous bacterial suspensions in Bijoux with 3ml sterile water. Using the same micropipette, both tube and cupule of the tests CIT, VP and GEL were filled

with the bacterial suspension. For the rest of the tests, only the tube was filled and not the cupules. Anaerobiosis in the tests ADH, LDC, ODC, H₂S and URE was created by overlaying with mineral oil. The incubation tray was closed and incubated at 37°C for 24 hours.

d) Reading of the strip

After 24 hours of incubation, each strip was read referring to the reading table and all the spontaneous reactions were recorded on the result sheet. All the tests that required addition of reagents were then read after the addition of the respective reagents.

TDA test: one drop of TDA reagent was added. A reddish brown colour indicated a positive reaction to be added on the result sheet.

IND test: one drop of IND reagent was added. A pink colour developing in the whole cupule indicated a positive reaction and to be added on the result sheet.

VP test: one drop of each of VP1 and VP2 reagents was added and waited at least 10 minutes. A pink or red colour indicated a positive reaction and to be recorded on the result sheet. If a slightly pink colour appears after 10 minutes, the reaction should be considered negative.

3.2.3. API 20 STREP method

a) Principle

API 20 STREP is a standardised method combining 20 biochemical tests that offer widespread capabilities. It enables group or species identification of most streptococci encountered in medical bacteriology. The API 20 STREP strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. The enzymatic tests are inoculated with a dense suspension of organisms, made from a pure culture, which is used to rehydrate the enzymatic substrates:

The metabolic end products produced during the incubation period are either revealed through spontaneous coloured reactions or by addition of reagents. The fermentation tests are inoculated with an enriched medium which reconstitutes the sugar substrates. Fermentation of carbohydrates is detected by a shift in the pH indicator.

b) Selection of the colonies

Once the microorganism to be identified was isolated on BA and verified as a Gram-positive coccus (Gram stain), it was ready for further identification. An incubation box (tray and lid) was prepared and about 5ml of distilled water was distributed into the honey-combed wells of the tray to create a

humid atmosphere. Distilled not tap water was used to avoid the release of gases by chemicals that could be initially in the water. The strip was then removed from its packaging and placed in the incubation box. Using a sterile swab, all the culture from the previously sub-cultured plate was harvested and a dense suspension was made into 3ml of sterile water.

c) Inoculation of the strip

In the first half of the strip (tests VP to ADH), the suspension was distributed with a sterile pipette avoiding the formation of bubbles by tilting the strip slightly forward and placing the tip of the pipette against the side of the cupule.

For the tests VP to LAP: approximately 100µl into each cupule was distributed.

For the ADH test: only the tube was filled.

In the second half of the strip (tests RIB to GLYG):

An ampule of API GP Medium was opened and the remainder of the bacterial suspension was transferred into it and was mixed well. The new suspension was then distributed into the tubes of the remaining tests. To the cupules of the underlined tests (ADH to GLYG), mineral oil was added to form the convex meniscus. The lid was placed to the tray and was incubated in aerobic conditions for 4 hours to obtain a first reading and for 24 hours to obtain a second reading.

d) Reading of the strip

After 4 hours of incubation:

The following reagents were added:

VP test: 1 drop of each of VP1 and VP2 was added

HIP test: 2 drops of NIN was added

PYRA, αGAL, βGUR, βGAL, PAL and LAP tests: 1 drop of each of ZYM A and ZYM B was added. After ten minutes, the reactions were read referring to the reading table. The trays were then re-incubated for the rest of the incubation period. The reading table is outlined in Appendix 1.

3.2.4. API 50 CH method using CHL medium

a) Principle

The API 50 CH strip allows the study of the carbohydrate metabolism of microorganisms. It consists of 50 microtubes each containing an anaerobic zone (the tube portion), for the study of fermentation and an aerobic zone (the cupule portion), for the study of oxidation or assimilation. The first tube contains no substrate and is used as a negative control.

The remaining tubes contain a defined amount of dehydrated substrate, belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids).

These substrates may be metabolised by various biochemical pathways:

Assimilation is indicated by growth of an organism in the cupule, when the substrate is the only source of carbon present, oxidation is shown by a colour change in the cupule portion and is due to the aerobic production of acid detected by a pH indicator included in the chosen medium and fermentation is shown by a colour change in the tube portion and is due to the anaerobic production of acid detected by a pH indicator included in the chosen medium.

b) Inoculation of the strip

Microorganisms were cultured on blood agar and were sub-cultured to blood agar, as a purification step. After the purification step, well-isolated colonies were cultured on MRS agar plates. Colonies from MRS agar plates were Gram stained. Gram-positive bacilli were harvested with sterile swabs into the API 50 CHL medium. Each strip is made up of 5 smaller strips each containing 10 numbered tubes. About 10 ml of distilled water was distributed into the honeycombed wells of the tray to create a humid atmosphere. The joined strips were separated and placed into the incubation tray and the remaining strip was placed next to the others in the tray to complete the strip. The tubes were filled with the inoculated API 50 CHL medium, and all the tests were over-layered with mineral oil. The strips were then incubated aerobically at 37°C over 24 and 48 hour periods.

c) Reading the strips

The strips are read:

The reading table is outlined in Appendix 2.

- ◆ At definite times of incubation (24 and 48 hours), depending on the microorganism and on the type of reaction studied (fermentation, oxidation or assimilation).
- ◆ In a semi-quantitative way: 0 is given to negative reactions and 5 to positive reactions of maximum intensity. Values of 1, 2, 3 or 4 are given to the intermediate reactions (3, 4 and 5 being considered as positive).
- ◆ A positive test corresponded to acidification revealed by the bromocresol purple indicator contained in the medium changing to yellow. For the Esculin test (tube no. 25), a change from purple to black is a positive.

3.3. RESULTS AND DISCUSSION

3.3.1. Solid media results

Table 3.1 gives the summary of physical appearances of different colonies on different media. The differences are according to the general morphology of colonies' texture, colony colour, size of the colonies, media appearance around the colonies, and the margins. They are outlined in Figure 3.2.

Table 3.1: Analysis of colonies growing on different solid media inoculated with amahewu (24 and 48 hours after incubation at 37°C).

MEDIUM	PHYSICAL CHARACTERISTICS ON SOLID MEDIUM.
BA	Cream, big, shiny, circular, convex, δ -haemolysis (<i>Klebsiella</i> spp) Cream, tiny, circular, umbonate, entire, δ -haemolysis (<i>Enterobacter</i> spp)
SDA	Big, cream, shiny, circular, convex, entire (<i>Saccharomyces</i> spp) Big, cream, irregular, umbonate, lobate (<i>Lactobacillus</i> spp) Small, cream, shiny, circular, umbonate, entire (<i>Lactococcus</i> spp)
VRB	Big, pink, shiny, circular, raised, entire (<i>Enterobacter</i> spp)
EMB	Big, pink, shiny, circular, convex, entire (<i>Enterobacter</i> spp) Very small, pink, shiny, circular, convex, entire (<i>Pantoea</i> spp)
MacConkey No.2	Big, shiny, pink, circular, convex, entire (<i>Lactobacillus</i> spp) Very small, pink, circular, shiny, convex, entire (<i>Leuconostoc</i> spp)
MacConkey No.3	Shiny, big, pinkish, circular, convex, entire (<i>Klebsiella</i> spp)
MRS	Small, cream, shiny, circular, umbonate, entire (<i>Lactobacillus</i> spp) Small, cream, shiny, circular, convex, entire (<i>Leuconostoc/Lactococcus</i> spp)

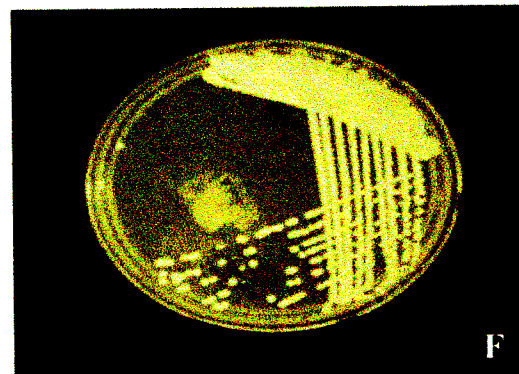
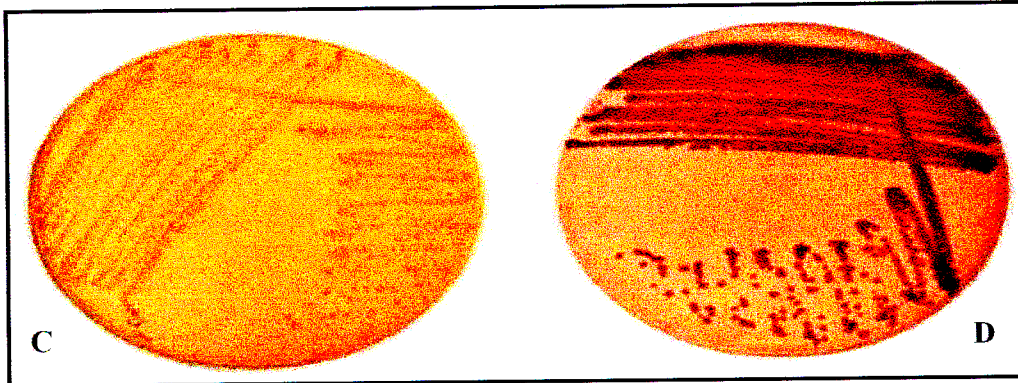
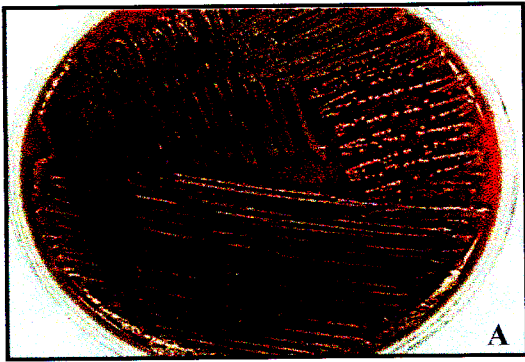


Figure 3.2: Colonies growing on different solid media.

3.3.2. Gram staining results

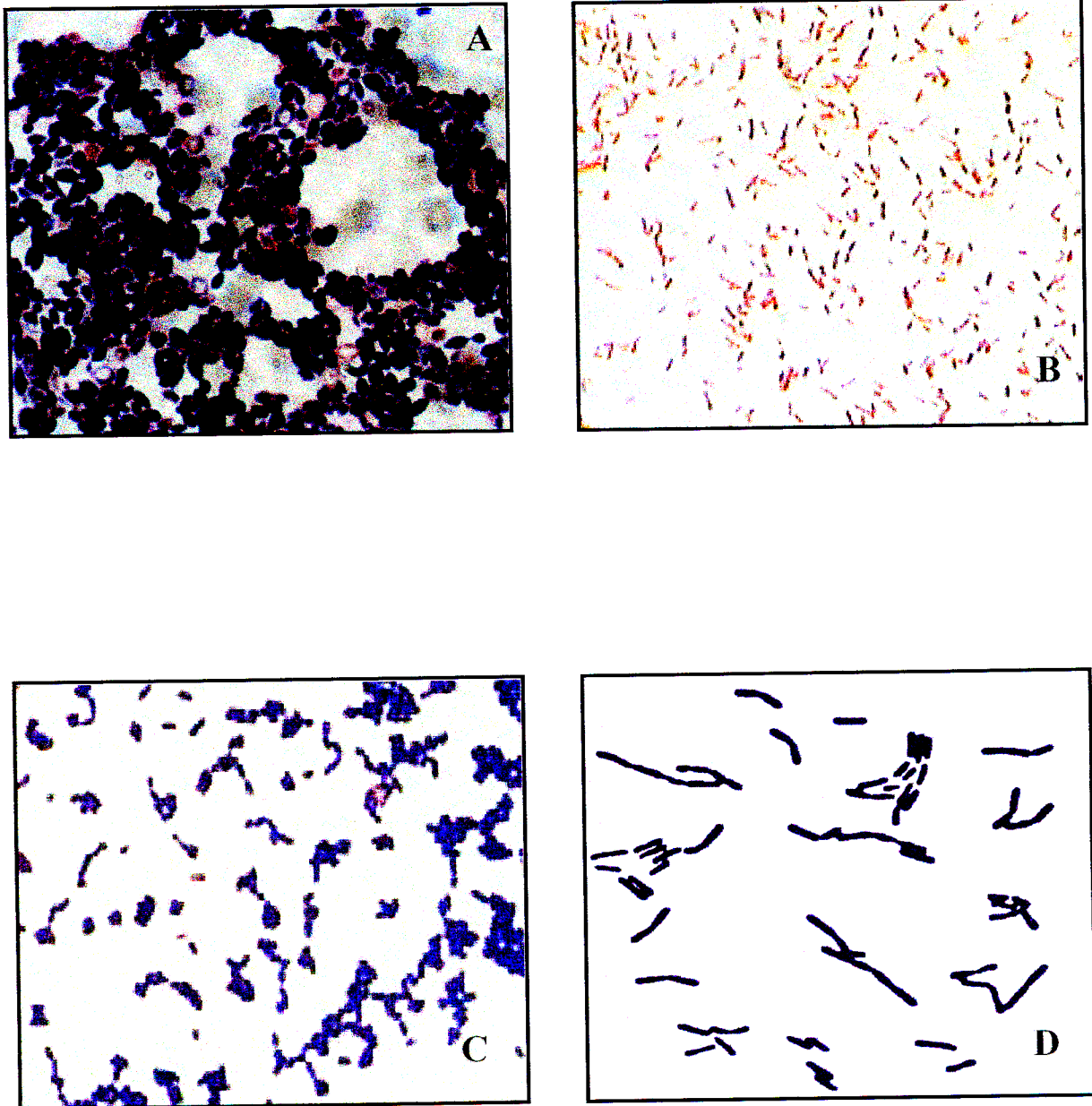


Figure 3.3: Gram reactions of different organisms.

A – Gram reaction of *Saccharomyces cerevisiae*

B – Typical Gram-negative bacilli (Birch and Hashimoto, 1996)

C – Typical Gram-positive cocci (Birch and Hashimoto, 1996)

D - Phase contrast showing Gram positive cells of *Lactobacilli* (Sneath *et al.*, 1986)

Extensive studies have been done on cereal fermentations in African countries. The majority of traditional cereal-based foods consumed in Africa are processed by natural fermentation. Fermented cereals are particularly important as weaning foods for infants and as dietary staples for adults (FAO, 1999). Coliforms were not observed from amahewu samples, none of the colonies that grew in VRB and Mac 3 media appeared purple or intense violet-red, respectively. These results were expected from commercial samples because their preparation is based on a scientific process where the production is controlled therefore there is no growth of unwanted microorganisms, referring to pathogenic strains that may lead to epidemics and even death (Dicks and Lategan, 1999). However, it was interesting not to observe any in homemade amahewu. The reason for this finding to be interesting is that the indigenous acidification process relies on the natural microflora therefore there is no control over the fermentation process and no pasteurisation is carried out on the final product or the ingredients prior to the fermentation process (Dicks and Lategan, 1999).

Table 3.2: Gram positive and negative organisms isolated and confirmed by API 20 E, API 20 STREP and API 50 CHL kits from commercial and homemade amahewu (categories of bacteria found in amahewu samples from maBheleni and kwaNgcolosi rural villages and Clover SA).

Gram positive	Gram negative
<i>Leuconostoc</i> spp † ♦	<i>Pantoea</i> spp 4 † ♦
<i>Lactococcus lactis</i> † ♦	<i>Enterobacter cloacae</i> †
<i>Lactococcus lactis</i> ssp <i>lactis</i> ♦	<i>Klebsiella oxytoca</i> †
<i>Leuc. mesenteroides</i> ssp <i>cremoris</i> ♦	<i>Klebsiella terrigena</i> † ♦
<i>Lactobacillus cellobiosus</i> ♦	<i>Klebsiella planticola</i> †
<i>Lactobacillus delbrueckii</i> † †	
<i>Lactobacillus</i> spp † ♦	
<i>Lactobacillus fermentum</i> †	
<i>Lactobacillus brevis</i> 1 †	
<i>Lactobacillus pentosus</i> ♦	
<i>Saccharomyces cerevisiae</i> †	

† These organisms were found in Clover SA amahewu samples.

‡ These organisms were found in maBheleni (South Coast) samples.

♦ These organisms were found in kwaNgcolosi (North Coast) samples.

3.3.3. API kits results

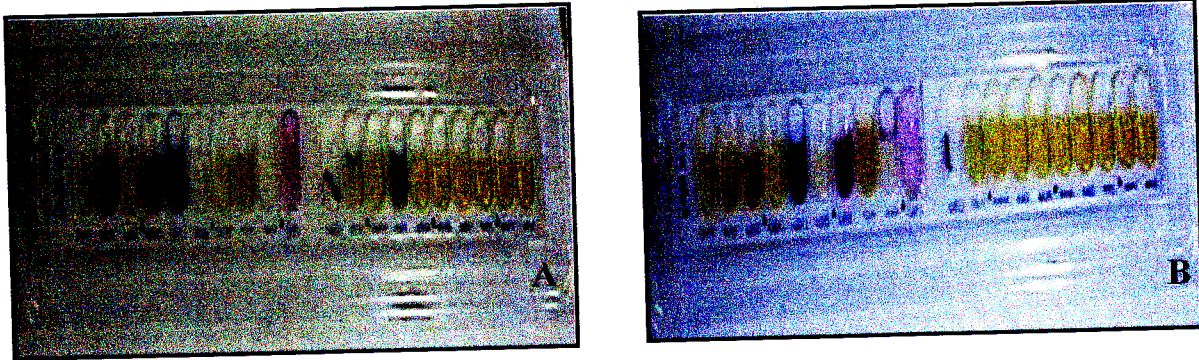


Figure 3.4: *Enterobacter cloacae* (A) and *Klebsiella oxytoca* (B) after 24 hours incubation at 37°C in API 20 E

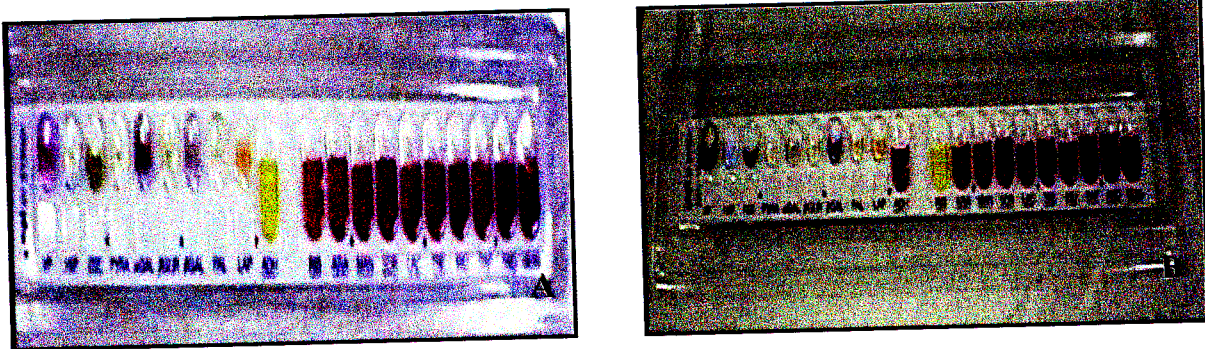


Figure 3.5: *Lactococcus lactis ssp lactis* (A) after 4 hours of incubation and *Leuconostoc sp* (B) after 24 hours of incubation at 37°C in API 20 STREP

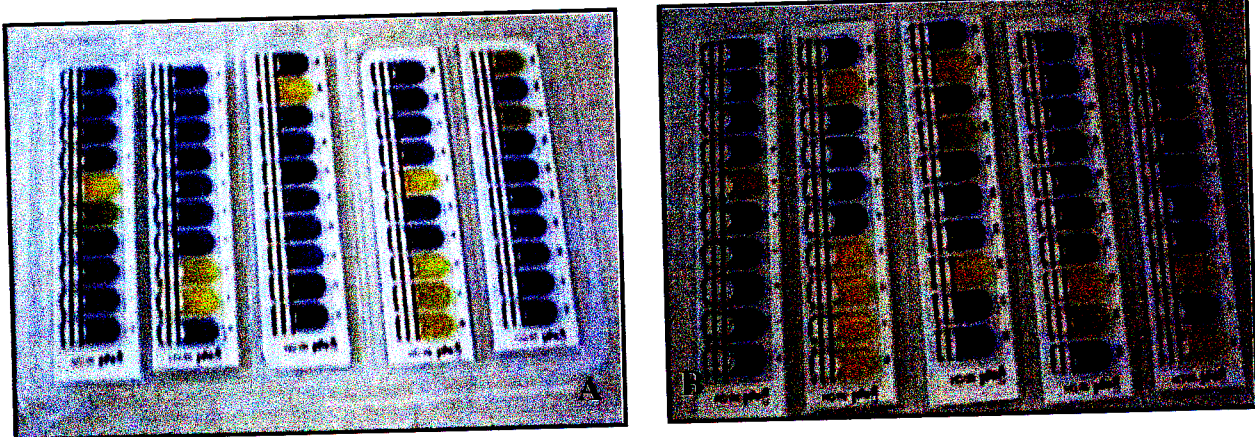


Figure 3.6: *Lactobacillus cellobiosus* (A) and *L. delbrueckii* (B) after 48 hours of anaerobic incubation at 37°C in API 50 CHL

Bacteria from the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus* and *Leuconoctoc* are the main species involved in lactic acid fermentations. Since amahewu fermentation is categorised as lactic acid, the strains responsible for fermentation should be from these genera. All the strains from the genera mentioned above are Gram positive, however, in the results of this study strains from the *Klebsiella*, *Enterobacter* and *Pantoea* genera were also isolated and confirmed. Although tests for the presence of coliform bacilli in general, and *Escherichia coli* in particular, are very useful, it may be desirable to count all the *Enterobacteriaceae* present because some strains of *Citrobacter* and *Klebsiella* species are more heat resistant than *Escherichia* species and their presence is a better indication of inadequate heat processing (Collins *et al.*, 1989) and as is the case with indigenous fermentations.

There is a wide-held belief that lactic acid fermenters exert an antibacterial action against Gram-negative bacteria in a sense that they have long been known to produce various antagonistic substances inhibitory toward many other bacteria. Nisin, an inhibitor produced by *Lactococcus lactis* subspecies *lactis* is bactericidal to Gram-negative bacteria in general (Goldberg and William, 1991). Wood (1998) reviewed tempeh production and mentioned that one bacterial species, *Klebsiella pneumoniae*, was reported to grow in all tempeh fermentations conducted under normal conditions. Steinkraus (1995) first reported its presence in the fermentation, and also demonstrated its involvement in vitamin B₁₂ production.

Klebsiella can grow on all types of media because it has no growth requirements, but a carbohydrate-rich medium provides a better environment for the development of the capsule (means of survival). Amahewu will therefore be an ideal medium for it to grow in and develop its capsule which will protect it better against pH change, bacteriocins, lactic acid, etc, produced by lactic acid bacteria during the fermentation process.

Ogi is a porridge prepared from fermented maize in West Africa. Microbiological studies by Akinrele *et al.*, (1970) showed that the lactic acid bacterium *Lactobacillus plantarum* and *Saccharomyces cerevisiae* yeast are some of the major organisms responsible for the fermentation of ogi. Odunfa (1985) took it further by determining that *Lactobacillus plantarum* was responsible for lactic acid production, *Corynebacterium* hydrolyses starch to organic acids while *Saccharomyces cerevisiae* contributes to flavour development.

Amahewu is a fermented gruel made from maize meal porridge commonly consumed as a staple among Africans of South Africa. Normally, fermentation occurs within 24 hours in a warm place. *Lactococcus lactis* is the main fermenting organism in indigenously prepared amahewu (Odunfa and Oyewole, 1998). Most of amahewu samples prepared indigenously showed to have been fermented by organisms from *Lactococcus* and *Lactobacillus* genera.

In conclusion, this study has proved commercial amahewu to be more hygienic in comparison to the indigenously prepared amahewu because no unwanted microbial strains (pathogens) were found in it. The presence of Gram negative organisms could be due to a number of reasons, one being the fact that there is no pasteurisation step in the preparation method. Further research, however, needs to be conducted in order to determine ways and means that can be used to improve the indigenous methodology of amahewu preparation to yield a safe product and would in turn be easily accessible to the financially challenged communities. Also, further investigation is required to determine why some of the Gram-negative bacterial species are not inhibited during the fermentation process and their safety thereof to the consumer.

CHAPTER 4

REDUCTION OF AFLATOXIN B₁, FUMONISIN B₁ AND ZEARELENONE IN AMAHEWU FERMENTATION

4.1. INTRODUCTION

Non-alcoholic maize fermentation is amongst the oldest known forms of food biotechnology. It is carried out to enhance taste, aroma, shelf-life, texture, nutritional value and other attractive properties of food (Nout, 1994). Traditional fermentations of food have many benefits, including detoxification of natural substances like cyanogenic glycosides during the food fermentation processing, and may include removal of natural toxicants and mycotoxins, amongst other things (Campbell-Platt, 1994; Steinkraus, 1995). Research on health-promoting effects of certain fermented foods and/or the organisms of fermentation, has been overwhelmed by findings both for and against. One of the areas of concern for researchers is anticancer activity (Jay, 1996). Aflatoxin B₁ (AFB₁, a well-known carcinogen), fumonisin B₁ (FB₁, implicated in oesophageal cancer) and zearalenone (ZEA, hyper-oestrogen) presence in food commodities has to be discouraged.

This study is aimed at investigating the potential amahewu offers to the community by lowering the concentrations of AFB₁, FB₁ and ZEA.

4.2. MATERIALS AND METHODS

All chemicals used in this study were of Analar grade supplied by BDH Chemicals (Poole, England) unless otherwise specified. AFB₁, FB₁ and ZEA standards were purchased from Sigma (St Louis, USA).

4.2.1. Solvents and reagents

The following solvents were used in this study: acetonitrile (ACN), potassium chloride aqueous solution (4%w/v), iso-octane, 2-mercapthoethanol, distilled water, acetic acid, sodium hydrogen carbonate (NaHCO₃), dichloromethane (DCM), sodium sulphate anhydrous (Na₂SO₄), acetone, methanol (MeOH), sodium hydroxide (NaOH), ethyl acetate, isopropanol, toluene, formic acid, butanol, cyclohexane, carbon tetrachloride, ethanol, ether, ortho-phthalaldehyde, chloroform,

concentrated sulphuric acid (H_2SO_4), p-anisaldehyde, acetic acid (HOAc), dianisidine, hydrochloric acid (HCl), 10% (w/v) sodium nitrite (NaNO_2), orthophosphoric acid.

Apparatus: Dialysis tubing (Sigma, St Louis, USA), Aluminium-backed fluorescent TLC plates (Merck, Darmstadt, Germany), solid phase extraction (SPE) Bond-Elut strong anion exchange (SAX) cartridges with 500mg sorbent (Analytichem, Harbour City, USA), rotary evaporator, freeze drier, pH meter, Whatman No. 4 filter papers, 250ml separating funnels, 500ml round bottom flasks, 250ml conical flasks, shake incubator, nitrogen gas, TLC tank, ultraviolet cabinet.

4.2.2. Extraction and cleanup

a) Aflatoxin B₁ (AFB₁) and zearalenone (ZEA) extraction and cleanup

The method used for this procedure was that reported by Dutton (1993) summarised in Figure 4.1. Eleven samples of maize meal and eleven samples of amahewu were analysed (nine rural and two commercial)

Step one

Twenty (20) grams (maize meal) or 10g freeze-dried amahewu (from the same maize meal sample) was shake incubated at 150 RPM with 100ml of ACN/4% KCl (9:1) for 1 hour and then filtered through a Whatman No.4 filter paper system attached to a vacuum pump. The filtered aliquot was transferred to a separating funnel and defatted twice with 80ml of iso-octane each time.

To the bottom layer (ACN/4% KCl), 50ml of 1M NaHCO_3 was added and mixed gently. The mixture obtained above was extracted three times with 25ml DCM each time with the lower layer collected into a round bottom flask through a bed of Na_2SO_4 on fluted Whatman No.4 filter paper. The total volume of DCM in the round bottom flask was dried in a rotary evaporator at 60°C.

Step two

Further cleaning of the fraction was carried out using a dialysis tube ($\pm 25\text{cm}$ long, previously soaked in distilled water for at least an hour) by tying a firm knot at one end to form a closed sac. The dry DCM residue from step one was reconstituted in 2ml ACN and was transferred into a dialysis sac using a Pasteur pipette and the top of the sac was tied. The sac was then placed in a boiling tube (15 x 25cm) containing 40ml of aqueous acetone (30% v/v). The top knot lip of the tube was looped over with a piece of aluminium foil and was secured with a rubber band. The tube was shake incubated overnight at 150 RPM. The dialysate was transferred into a 250ml separating funnel and

the boiling tube was washed with a little acetone and the rinsings added to the funnel. The dialysate was extracted three times with 25ml aliquots of DCM, passing each extract through a bed of anhydrous Na₂SO₄ into a clean round bottom flask. The DCM extracts were dried at about 60°C, avoiding excessive use of heat. The residue was reconstituted with 5ml of DCM and transferred to a vial with a Pasteur pipette. The contents of the vial were then dried under a stream of nitrogen gas at 60°C and kept in the fridge until required.

b) Fumonisin B₁ (FB₁) extraction and cleanup

The method used for this procedure is that reported by Dutton (1993) outlined in Figure 4.1. Twenty (20) grams (maize meal) or 10g (freeze-dried amahewu) was shake incubated with 100ml of MeOH/H₂O (3:1) for 1 hour and then filtered through a filter paper system attached to a vacuum pump. The maize meal and amahewu samples were obtained from maBheleni (Port Shepstone) and kwaNgcolosi (Kranskop) villages (rural) and commercial samples were from Clover SA. The pH of the filtrate was adjusted to pH 6.0–6.5 with 1M NaOH. The solid phase SAX cartridges were conditioned by washing with 5ml MeOH and then 5ml MeOH/H₂O (3:1) at a flow rate of 2ml per minute. The flow rate was maintained at 2ml/min and 20ml of the filtrate was passed through the cartridges. The cartridges were washed with 5ml MeOH/H₂O (3:1) and then 3ml MeOH. The flow rate was then adjusted to 1ml/minute. FB₁ was eluted with 10ml of 1% acetate in MeOH. The eluate was then dried under a stream of nitrogen gas at 60°C and was stored at 4 – 8°C until further analysis.

4.2.3. Thin Layer Chromatography

Aluminium-backed precoated silica gel Thin Layer Chromatography (TLC) plates (10 x 10cm) were marked with a pencil line, 1cm from the bottom edge, indicating the origin. The dry FB₁ standard and samples were reconstituted with 200µl MeOH while the AFB₁ and ZEA standards and samples were reconstituted with 200µl DCM.

Twenty microlitres (20µl) of each extract were spotted on the marked origin of the TLC plate and dried in a warm stream of air. The spotted plates were placed in their respective solvent systems (which had been allowed to saturate in the tank prior to use) and allowed to develop until the solvent front reached the top of the plate. The plates were removed, the solvent front was marked, dried and then further developed (at right angles to the first solvent) in the second solvent system..

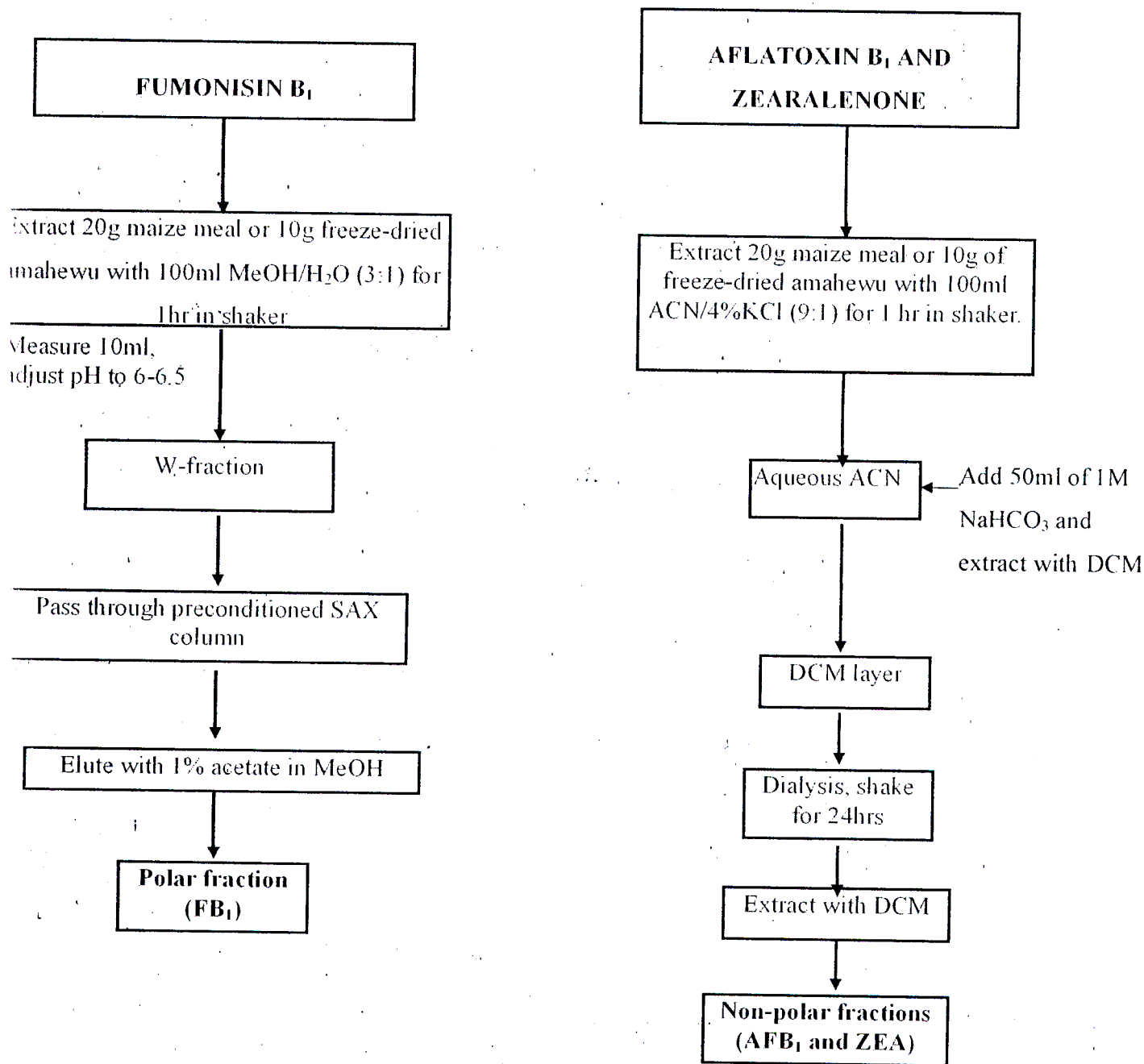


Figure 4.1. Flow diagram of extraction and cleanup of AFB₁, FB₁ and ZEA (Dutton, 1993)

The following solvent systems were used to develop the TLC plates (Appendix 9):

FB₁: 1st and 2nd dimensions were in BWA (12:5:3) solvent system.

AFB₁: 1st dimension, CEI (18:1:1) and 2nd dimension, TEF (6:3:1) solvent systems.

• ZEA: 1st dimension, CEI (19:1) and 2nd dimension, CHL (1:1) solvent systems.

4.2.3.1. Developing sprays

a) Aflatoxin B₁

AFB₁ appeared as blue fluorescing bands when viewed under ultraviolet (UV) light at 254nm (Gallager *et al.*, 1978). No developing spray was required.

b) Fumonisin B₁

The plates were sprayed with *p*-anisaldehyde reagent (prepared by mixing 70ml MeOH, 10ml HOAc, 5ml concentrated H₂SO₄ and 0.5ml *p*-anisaldehyde) and heated in the oven for 3 minutes. The presence of FB₁ indicated by a purple coloured spot (Marasas *et al.*, 1988).

c) Zearalenone

The plates were sprayed with dianisidine spray (prepared by dissolving 0.5g dianisidine in 20ml of 1.5% HCl. The working spray was prepared by mixing equal volumes of 10% sodium nitrite and dianisidine). When developed plates were sprayed with this reagent, ZEA appeared as a visible brick red spot at room temperature (Dutton, 1993).

4.2.4. High Performance Liquid Chromatography (HPLC)

The sensitivity of the HPLC method employed for the determination of the AFB₁, FB₁ and ZEA levels is known to be 0.02 ng/kg, 0.04 ng/kg and 0.1 ng/kg respectively. The detection limits were based on the signal to noise ratio of 4:1 (Dutton, 1993).

a) Solvents

An HPLC-grade water was obtained by filtering water previously purified by a Millipore system through a 0.45µm filter. Methanol and acetonitrile were purchased from Merck and were of HPLC grade. Solvent mixtures were degassed by sonication before use.

b) Instrumentation

The HPLC system consisted of a SpectraSYSTEM P2000 binary pump, a SpectraSYSTEM AS3000 autosampler equipped with a 100µl injection loop and a SpectraSYSTEM FL2000 fluorescence

detector and/or a SpectraFOCUS UV detector (all from ThermoSeparation Products, SMM Instruments, South Africa). The analytical column (for AFB₁ and ZEA) was a Waters NovaPak C₁₈150x3.9mm 4µm cartridge (Waters, MicroSep, South Africa) and a Luna C₁₈ (2) 250x4.6mm 5µm column for FB₁ (Separations, South Africa). Both columns were preceded by a HIRPB-10C guard column (Hichrom Ltd, SMM Instruments).

c) Chromatographic conditions

Aflatoxin B₁ analysis: Mobile phase -- isocratic ACN/H₂O (30:70) at a flow rate of 1.0 ml/min. Detection -- UV at 350 and 360nm. Injection volume was 30µl

Fumonisin B₁ analysis: Mobile phase -- isocratic MeOH/0.1M NaH₂PO₄ buffer (pH adjusted to 3.3 with phosphoric acid) (80:20) at a flow rate of 1.2ml/min. Fluorescence detection (λ_{ex} - 230, λ_{em} - 440nm). Injection volume of a derivatised sample was 50µl.

Zearalenone analysis: Mobile phase -- isocratic ACN/H₂O (50:50 v/v) at a flow rate of 1.0ml/min. Dual detection UV at 274nm and fluorescence (λ_{ex} - 236, λ_{em} - 418nm). Injection volume was 30µl.

d) Derivatisation of samples and FB₁ standard

The derivatising reagent was prepared by dissolving 15mg of OPA (ortho-phthalaldehyde) in 600µl MeOH, to which 20µl of 2-mercaptoethanol was added, followed by 7ml of 0.02M Na₂B₄O₇ solution (pH 10.5). To derivatise a sample/standard, 50µl of the sample/standard solution was mixed for 30 seconds with 100µl of the derivatisation reagent and injected into the chromatographic column within 90 seconds (Sydenham *et al.*, 1992).

4.3. RESULTS AND DISCUSSION

To determine the reduction of AFB₁, FB₁ and ZEA in amahewu, maize meal and amahewu were both qualitatively and quantitatively analysed using TLC and HPLC respectively.

4.3.1. TLC analysis of maize meal and amahewu

TLC plates spotted with extracts from samples showing the presence of AFB₁ (Figures 4.2), FB₁ (Figure 4.3) and ZEA (Figure 4.4) are comparable with that of standards.

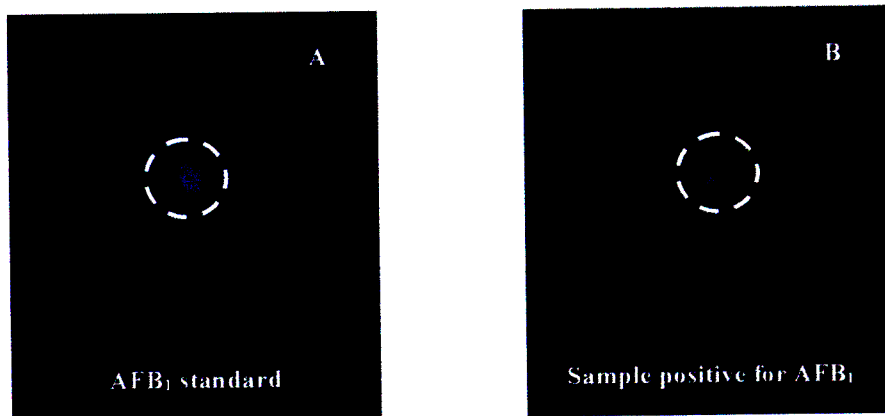


Figure 4.2: A typical TLC plate of AFB₁ standard (A) and a positive sample (B) visualised as a blue spot under UV light (denoted by the dotted circle).

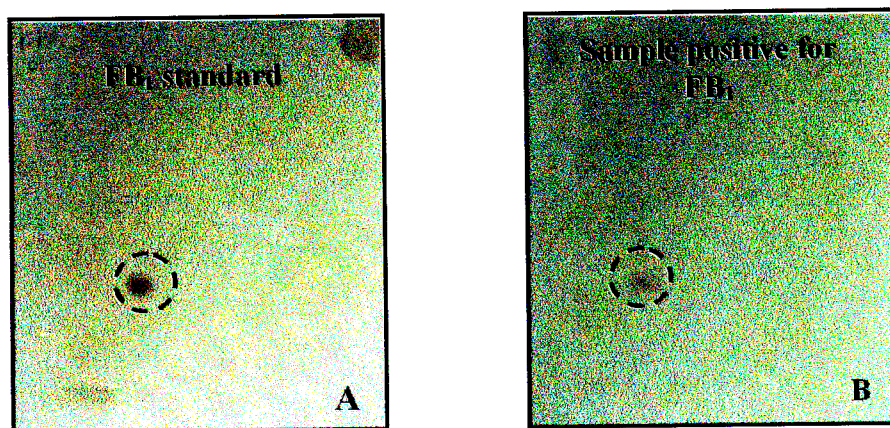


Figure 4.3: A typical TLC plate of FB₁ standard (A) and a positive sample (B) visualised as a purple spot after being sprayed with a developing spray (denoted by the dotted circle).

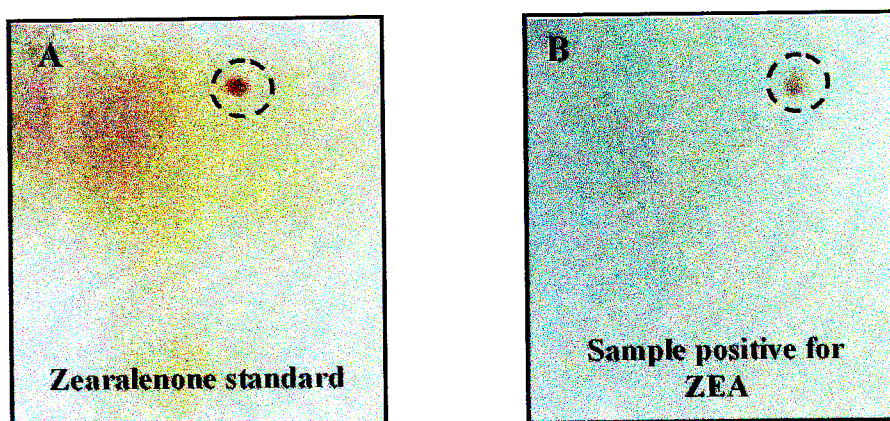


Figure 4.4: A typical TLC plate of ZEA standard (A) and a positive sample (B) visualised as a brick-red spot (denoted by the dotted circle).

4.3.2. Evaluation of results

After the plates were sprayed with developing sprays, they were then carefully inspected under good daylight and marked around any visible spots with a pencil. The plates were also viewed under both long and short wave ultra-violet light and any fluorescing or absorbing spots were also marked with a pencil. The distance from the baseline to the top edge of the plate was measured and this gave the distance migrated by the solvent (DS) in millimetres. The centre of each spot was marked with a pencil and the distance from the base line to it was measured (mm) and this gave the distance migrated by the compound (DC).

Thus, the R_F value for each dimension was calculated as follows:

$$R_F = DC/DS$$

Equation 1: Formula for calculating R_F value

Table 4.1: Distance (in mm) migrated by each toxin analysed in different solvents (Dutton, 1993).

Mycotoxin	Solvent 1	Distance (mm)	Solvent 2	Distance (mm)
Aflatoxin B ₁	CEI	66	TEF	31
Fumonisin B ₁	BWA	50	BWA	50
Zearalenone	CtE	10	ChE	75

CEI – chloroform/ethyl acetate/isopropanol; BWA – butanol/water/acetic acid; CtE – carbon tetrachloride/ether; TEF – toluene/ethyl acetate; ChE – cyclohexane/ether

Nine rural, two Clover SA maize meal, two freeze-dried commercial and nine rural freeze-dried amahewu samples were analysed using TLC. Each sample was analysed in triplicates with a week interval between each analysis. Conclusions were drawn after results of the third analysis were obtained. Table 4.2 show typical results obtained when both amahewu and maize meal samples were analysed for AFB₁, FB₁ and ZEA toxins and are compared to standards. The confirmation of results was done by comparing the distance migrated, in a similar solvent, values (Table 4.1) obtained in this study to those by Dutton, (1993). None of the commercial samples contained detectable amounts of each of the toxins analysed. It should be interesting to note that AFB₁ was only detected in samples from kwaNgcolosi by TLC method, 33% in maize meal and 25% in amahewu, respectively, with similar percentages for ZEA.

FB₁ and ZEA on the other hand could only be detected in some of the rural samples. After the TLC analysis, the same samples were then analysed using the HPLC.

Table 4.2: Analysis of maize meal and amahewu by TLC*

Areas sampled	Aflatoxin B ₁		Fumonisin B ₁		Zearalenone	
	MM	Ama	MM	Ama	MM	Ama
MaBheleni	ND (5)	ND (5)	4 (5)	2 (5)	1 (5)	ND (5)
KwaNgcolosi	2 (4)	3 (4)	3 (4)	1 (4)	2 (4)	3 (4)
Clover	ND (2)	ND (2)	ND (2)	ND (2)	ND (2)	ND (2)

*Number in brackets refers to the total number of samples analysed; ND – None Detected; MM – maize meal; Ama – amahewu

Dutton *et al.*, (1993) screened for mycotoxins in homegrown maize from rural villages in South Africa from 1989 to 1992 using TLC with detection limits of 0.1 µg/kg (AFB₁), 100 µg/kg (FB₁) and 10 µg/kg (ZEA). They also found that although aflatoxin is considered to be ubiquitous, its appearance in homegrown maize was sporadic, despite *Aspergillus flavus* and *A. parasiticus* being routinely found at low levels by fungal screen. AFB₁ is also found in stressed plants, for example, during drought. KwaNgcolosi is located in an arid area of KwaZulu Natal unlike MaBheleni, thus making maize grown in this area prone to stress and subsequent mycotoxin contamination. Also, it is more likely that individuals vary in their methods or storage duration of their maize, considering that up to 50% of the samples from KwaNgcolosi showed detectable levels of the toxin. The number of samples analysed is inadequate to confidently compare them to other findings.

4.3.3. HPLC analysis of maize meal and amahewu

To prove that the absence of the toxins was not caused by losses during the sample analysis, recovery studies were conducted. In these studies, three 20g samples of the commercial maize meal were each spiked with 20 µg of AFB₁, FB₁ and ZEA, meaning that each 20g was spiked with all three toxins. No specific criteria were used to decide on what amount to be used when spiking the maize meal samples. They were extracted and cleaned up according to the procedure outlined in section 3.2.a and 3.2.b. and then analysed by HPLC. Reconstituted extracts from each sample was analysed using HPLC therefore one run per sample for each toxin.

Table 4.3: Recovery of AFB₁, FB₁ and ZEA from commercial maize meal spiked with each toxin and analysed by HPLC.

	AFB ₁ (ng/g)	FB ₁ (ng/g)	ZEA (ng/g)
Original toxin added	20µg/20g	20µg/20g	20µg/20g
Conc. Recovered*	930±5	875±21.8	840±0
Detection limit	0.02 ng/kg	0.04 ng/kg	0.1 ng/kg
% Recovery	93	88	84

*Recoveries are a mean of three replicates.

For calculations to quantify toxins by HPLC, refer to Appendices 10 – 12 for formulae.

$$y = ax$$

Equation 2: Equation used to calculate the toxin amount

Where y - represents the peak area and x - the calculated amount of toxin.

Table 4.4 shows the amounts of AFB₁ toxin recovered from maize meal and amahewu samples from MaBheleni and kwaNgcolosi villages. Of the 5 maBheleni maize meal samples analysed, 2 contained AFB₁ at concentrations 0.55±0.1 and 0.84±0.01 ng/g with a respective 2 amahewu samples, containing amounts of AFB₁ toxin reduced by 99.8 and 99.9% respectively. All 4 samples from kwaNgcolosi analysed contained AFB₁ at concentrations ranging from 11.5±9.03 to 30.1±14.1 ng/g. In general, results of all (9) rural (village) amahewu samples analysed indicated a 98.9 to 99.7% reduction of AFB₁ when compared to the starting maize meal.

Table 4.4: Reduction of AFB₁ concentration in maBheleni and kwaNgcolosi

Samples analysed	MaBheleni			KwaNgcolosi		
	MM [AFB ₁] (ng/g)	Ama [AFB ₁] (ng/g)	Reduction	MM [AFB ₁] (ng/g)	Ama [AFB ₁] (ng/g)	Reduction
1	0.55	8.3x10 ⁻⁵	99.8%	30.1	0.102	99.7%
2	ND	ND	NA	8.3	0.090	98.9%
3	0.84	9.1x10 ⁻⁵	99.9%	11.5	0.040	99.7%
4	ND	ND	NA	14.0	0.040	99.7%
5	ND	ND	NA			

ND – None Detected; NA – Not Applicable; MM – maize meal; Ama – amahewu; [toxin] – concentration of the toxin.

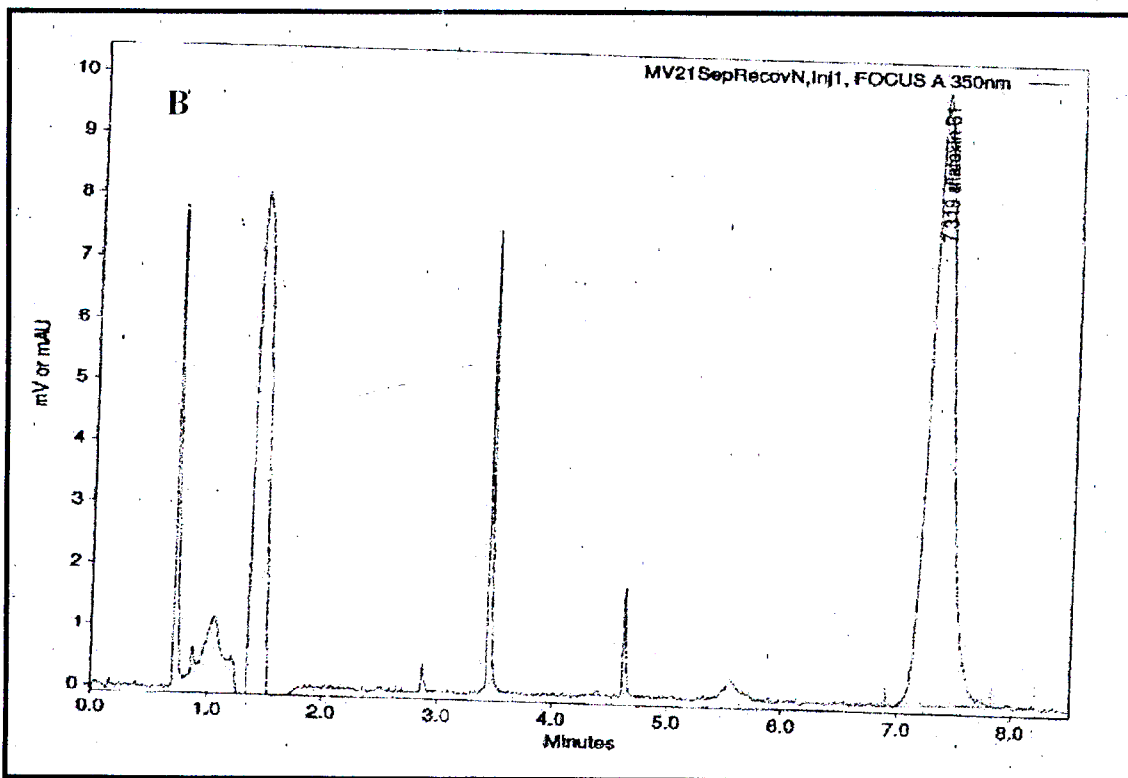
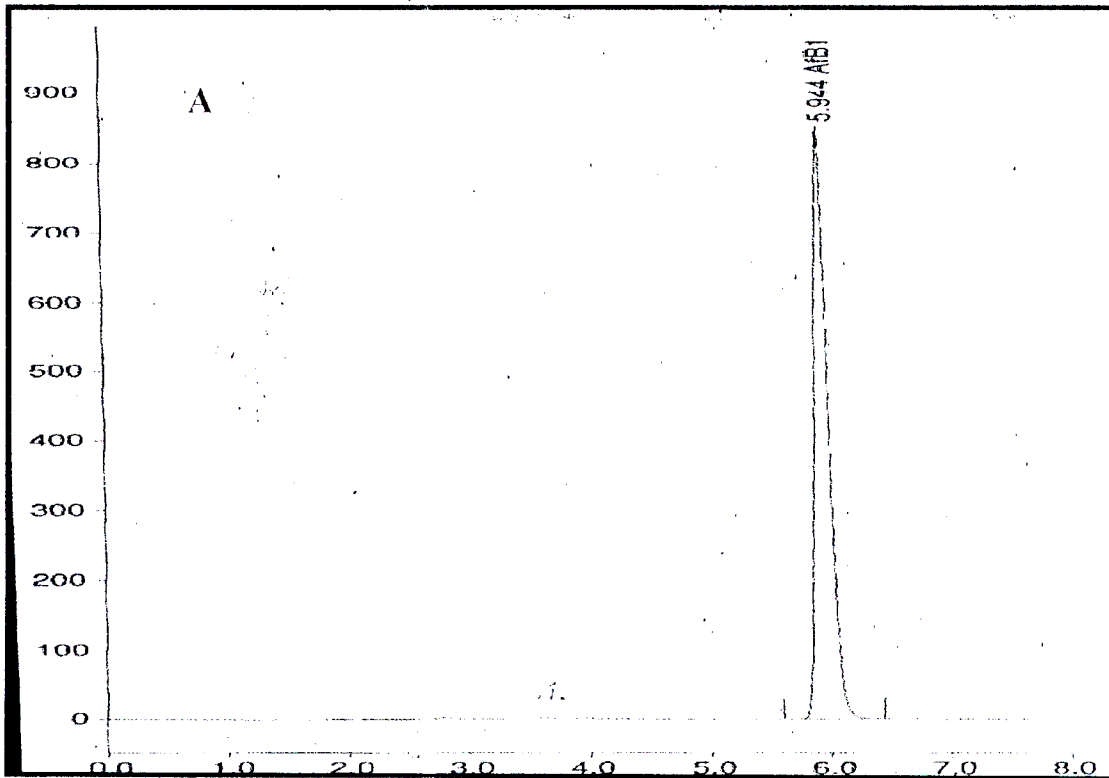


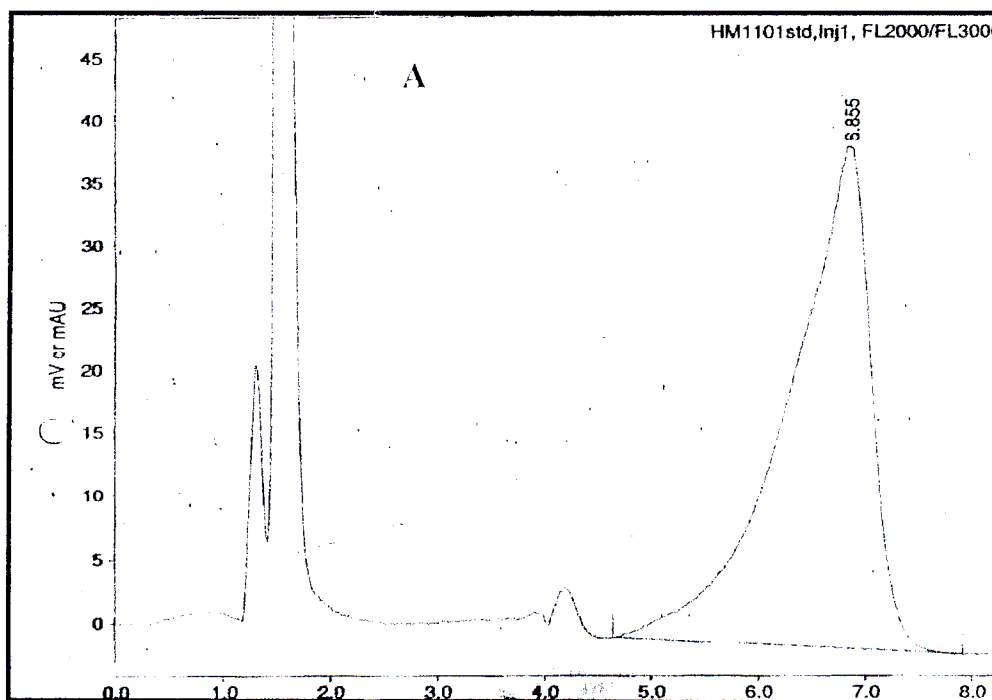
Figure 4.5: AFB₁ chromatograms of the standard (A) and a positive (B) sample after HPLC development.

Table 4.5 shows the results of FB₁ analysed from maize meal and amahewu samples obtained from kwaNgcolosi and maBheleni villages. All the maize meal samples from maBheleni contained FB₁ at concentrations ranging from 4.1 to 47.2 ng/g and amahewu samples showed between 66.5 and 100% reduction in the amounts of the toxin. Of the samples obtained from kwaNgcolosi, 3 of the maize meal samples contained FB₁ at concentrations ranging from 0.5±0.4 to 4.1±3.7 ng/g and 3 of the amahewu samples showed 82.6 to 92% reduction in amounts of FB₁.

Table 4.5: Reduction of FB₁ concentration in maBheleni and kwaNgcolosi maize meal and amahewu samples

Samples analysed	MaBheleni			KwaNgcolosi		
	MM [FB ₁] (ng/g)	Ama [FB ₁] (ng/g)	Reduction	MM [FB ₁] (ng/g)	Ama [FB ₁] (ng/g)	Reduction
1	12.03	1.4	88.4%	0.86	0.15	82.6%
2	24.6	1.4	94.3%	4.1	0.56	86.3%
3	4.1	ND	100%	ND	ND	NA
4	20.6	6.9	66.5%	0.5	0.04	92%
5	47.2	6.3	86.7%			

ND – None Detected, NA – Not Applicable, MM – maize meal, Ama – amahewu, [toxin] – concentration of the toxin



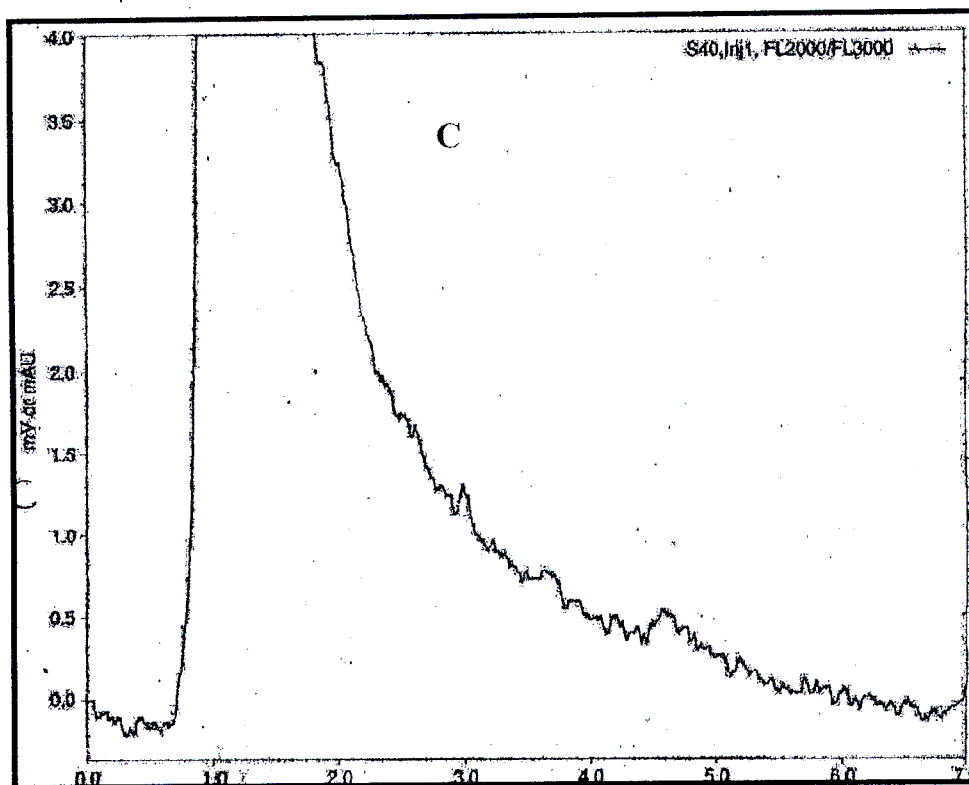
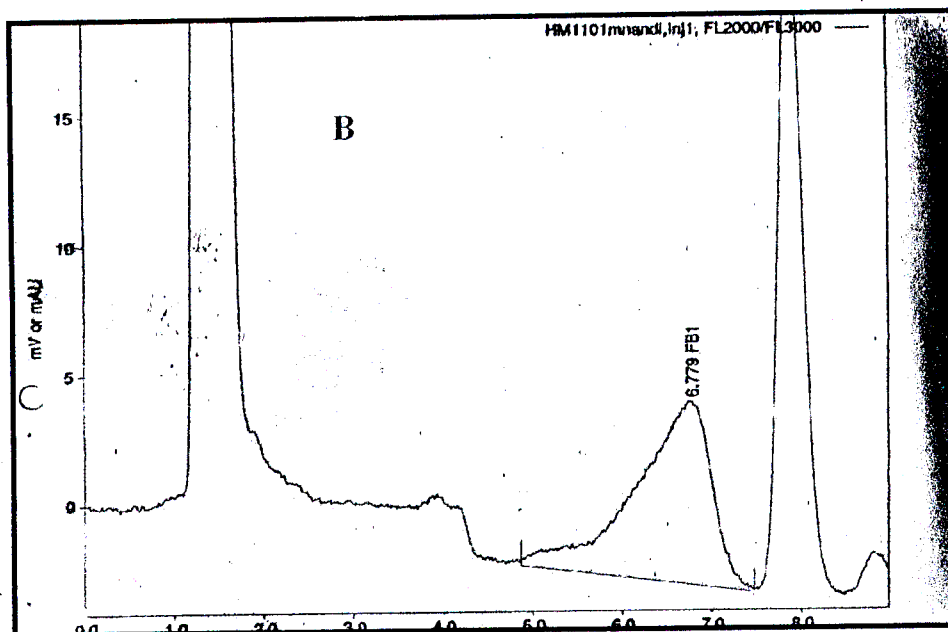


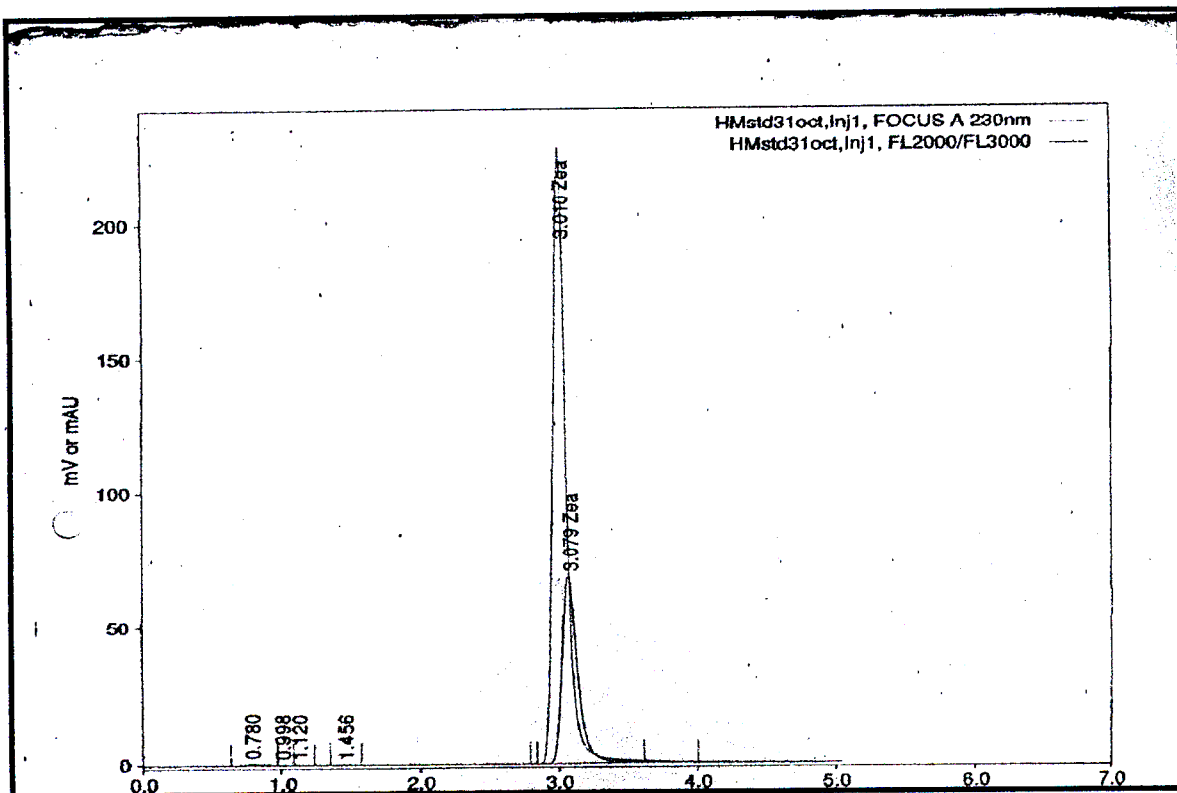
Figure 4.6: FB₁ chromatograms of the standard (A), positive (B) and negative (C) samples after HPLC development.

Table 4.6 shows the reduction of ZEA toxin analysed from maize meal and amahewu samples obtained from maBheleni and kwaNgcolosi villages. All of maBheleni maize meal samples analysed contained ZEA at a concentration range of between 0.92 ± 0.01 and 4.3 ± 0.1 ng/g and none of the amahewu samples contained detectable amounts of ZEA. All of kwaNgcolosi maize meal samples contained ZEA at concentrations ranging from 3.7 ± 6.5 to 16.4 ± 10.7 ng/g and 3 of amahewu samples contained amounts of ZEA with a reduction of between 99.3 to 100%.

Table 4.6: Reduction of ZEA concentration in maBheleni and kwaNgcolosi maize meal and amahewu samples

Samples analysed	MaBheleni			KwaNgcolosi		
	MM [ZEA] (ng/g)	Ama [ZEA] (ng/g)	Reduction	MM [ZEA] (ng/g)	Ama [ZEA] (ng/g)	Reduction
1	4.3	ND	100%	16.4	0.06	99.6%
2	0.9	ND	100%	12.1	0.06	99.5%
3	2.6	ND	100%	4.3	0.03	99.3%
4	2.1	ND	100%	3.7	ND	100%
5	1.3	ND	100%			

ND – None Detected, MM – maize meal, Ama – amahewu; [toxin] – concentration of the toxin



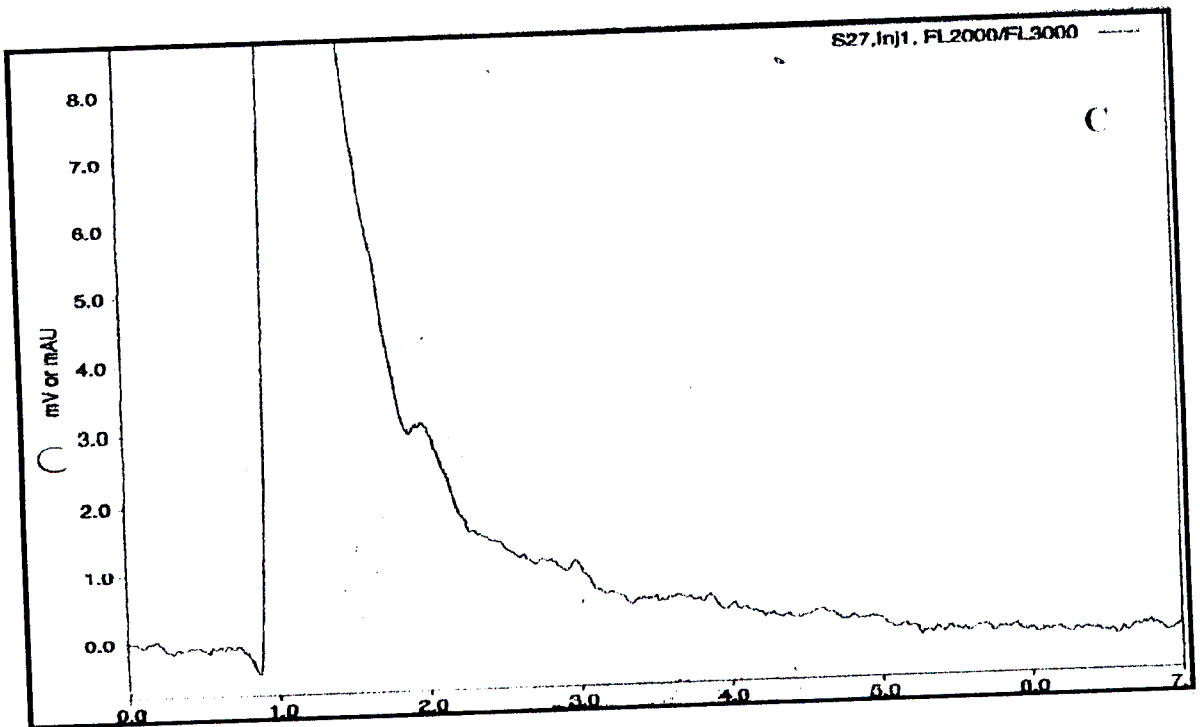
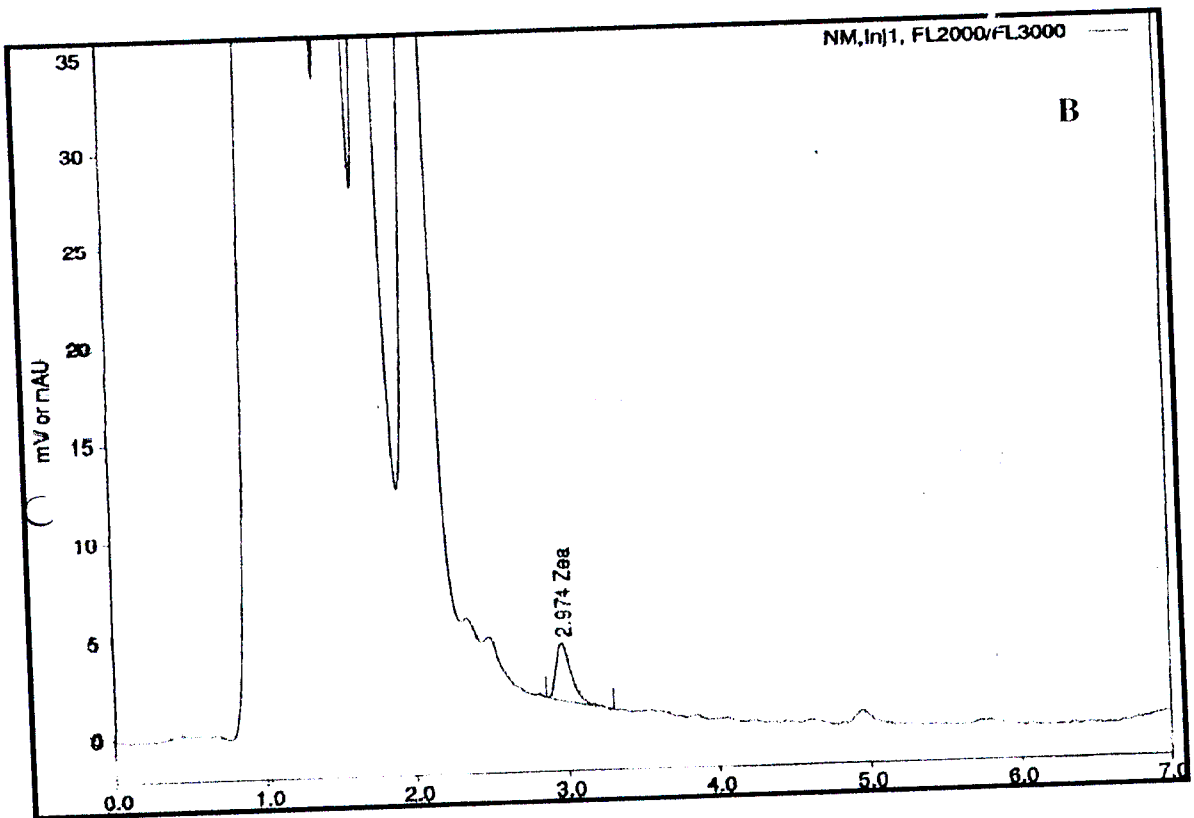


Figure 4.7: ZEA chromatograms of the standard (A), positive (B) and negative (C) samples after HPLC development

All maize meal samples from maBheleni and kwaNgcolosi tested positive for zearalenone. However, none of maBheleni amahewu samples contained detectable amounts of zearalenone and 75% of amahewu samples from kwaNgcolosi tested positive, but at much reduced amounts. These findings agree with a study by Gqaleni *et al.* (1998), which showed that lactate fermentation degrades a number of mycotoxins (although the nature and toxicity of degradation products are not yet known). No detectable amounts of AFB₁, FB₁ or ZEA were found in any of the commercial samples from Clover SA. This was established by following both TLC and HPLC methods for their determination. This therefore means that if any of the mycotoxins under investigation were present in these samples, their concentrations were below the TLC and HPLC detection limits, and thus, safe for human consumption.

Marasas *et al.*, (1993) screened for mycotoxins in commercial maize and maize-based products using HPLC. Of the maize meal samples screened, 88.5% contained FB₁ at a concentration range of 0 – 475ng/g and none was detected from tortillas and corn flakes samples. Tortillas are alkali-treated maize products. These findings presented interesting possibilities for the detoxification of maize contaminated with fumonisins. Also, Viljoen *et al.*, (1993) screened commercial maize meal of the 1990/91 season for mycotoxins using HPLC. No AFB₁ was detected. FB₁ was detected at 214µg/kg concentration and ZEA at only 50µg/kg. The detection limits were 2µg/kg AFB₁, 20µg/kg FB₁ and 4µg/kg ZEA.

Overall, the pattern of mycotoxins reduction was similar from village to village but varied from home to home within the same village and from toxin to toxin analysed. Extensive studies have been done involving non-alcoholic fermentations and their effect on mycotoxin reduction. A study by Gourama and Bullerman (1995), state that non-alcoholic fermentation processes degrade a number of mycotoxins, which, by implication, means that it is possible that the reduction of AFB₁, FB₁ and ZEA was a result of either degradation or conversion to another less toxic compound. Such a result would therefore suggest that fermentation would reduce the chances of toxicity and would prove vital in emphasising the importance of fermentation.

An attempt was made by Mokoena (2003), unpublished, to characterise the metabolic derivatives (by-products) of each mycotoxin following lactic acid maize meal. To achieve this maize meal samples were separately spiked with each of mycotoxin, fermented for four days and screened for specific mycotoxin derivatives (by-products) using GC/MS, HPLC and relevant standards (i.e. partially hydrolysed fumonisin B₁, aflatoxin B_{2a}, α - and β -zearalenol). None of the targeted

derivatives could be detected in the fermented maize meal samples. Aly (2002) conducted a survey of Egyptian corn and corn-based products and by-products showed that the majority of samples had higher limits of aflatoxins. The conducted experiments to determine the fate of distribution of aflatoxin during wet-milling process and investigate aflatoxin destruction during starch conversion to glucose syrup. Eighty nine percent of the aflatoxins were destroyed during starch conversion. Consequently, glucose syrup produced from contaminated starch found to be aflatoxin-free.

The fact that this study has shown 65 – 100% reduction of AFB₁, FB₁ and ZEA in amahewu when compared to that in maize meal has important implications. A study by Chelule *et al.*, (2001) showed no detection of FB₁ in amahewu obtained from homes in kwaNgcolosi and Mphise villages in Kranskop, North Coast of KwaZulu Natal. Also, Nout (1994) reported that fungal and lactic acid fermentations reduce AFB₁, possibly by opening the lactone ring which may result in complete detoxification. HPLC method proved to be more sensitive, as expected, than TLC because it was able to detect toxins from samples in which TLC could not, for example AFB₁ from maBheleni samples. With that said it is also important to remember that TLC is a simple cheap method whereas HPLC is much more expensive quite complicated to operate.

In conclusion, this study has shown that the fermentation of amahewu plays an important role in reducing the concentrations of AFB₁, FB₁ and ZEA mycotoxins. The mechanism/s by which the reduction takes place is still unclear. The reduction of toxin concentrations, however, is beneficial to the community in that it reduces the amounts of toxins that the consumer would otherwise be exposed to. Further research needs to be conducted in order to identify the reduction products and the safety thereof.

CHAPTER 5

NUTRITIVE VALUE ASSESSMENT OF MAIZE MEAL AND AMAHEWU

5.1. INTRODUCTION

Amahewu can be defined as a thin sour porridge consumed as traditional food by a large African population in Africa. The natural/indigenous acidification process used by African tribes is, however, not suited for the commercial production of amahewu, due to the fact that the process takes too long, acidification is irregular or does not occur at all. The quality of the homebrewed product cannot be controlled, due to the presence and growth of unwanted microorganisms and the production of off-flavours. Pathogenic strains may also develop which may lead to epidemics and even death (Dicks and Lategan, 1999).

The manufacturing of commercial amahewu is based on a scientific process where all the above-mentioned problems are eliminated. Specially selected strains of lactic acid bacteria and yeast are used. These organisms are able to metabolise starch and sugars to lactic acid and other aromatic compounds. Just as humans need certain vitamins, proteins, energy compounds, etc. to be able to function, the culture needs specific growth factors and other compounds to grow optimally and produce lactic acid and other flavouring agents. Part of the growth requirements are obtained from maize meal, flour and sugar. The rest is supplied in the form of growth salts and growth factors. Amahewu is mainly an energy food, but the protein levels are low. Proteins, as well as certain vitamins, can be added under certain conditions to increase the nutritional value (Dicks and Lategan, 1999).

This study investigated whether commercial maize meal and amahewu offer similar nutritive values when compared to homegrown maize meal and homemade amahewu.

5.2. MATERIALS AND METHODS

5.2.1. Vitamins B₁, B₂ and B₆ analysis

a) Materials and reagents

The following materials and reagents were used in this study: 250ml Erlenmeyer flasks, demineralised water, Perchloric acid 70% and Orthophosphoric acid 85% (Saarchem Pty Ltd, South Africa), potassium hydroxide (KOH) (Associated Chemical Enterprises), glass filter, nylon filter, magnetic stirring system, pH meter, B₁, B₂ and B₆ vitamin standards (Sigma, St Louis, USA). Nine rural, two Clover SA maize meal, nine rural and two freeze-dried amahewu samples were obtained from maBheleni (South Coast) and kwaNgcolosi (North Coast) villages and Clover SA.

b) Method

Sample extraction and cleanup method was adapted from Chase *et al.*, (1993). Ten (10) grams of each of maize meal and amahewu sample was weighed into 250ml Erlenmeyer flasks. The weighed samples were each mixed with 90ml water. Perchloric acid, 3ml, was added to the mixture whilst continuously stirring and continued stirring for 30 minutes. Carefully, 6M KOH was added dropwise with constant stirring until the pH was $3,2\pm 0,4$. The mixture was then diluted to the 200ml volume with mobile phase (see Appendix 16). The flasks were refrigerated overnight to allow complete precipitation of the proteins and perchlorate. The mixtures were filtered through glass filters and then through nylon filters and were ready for HPLC analysis.

5.2.2. High Performance Liquid Chromatography (HPLC)

a) Solvents

HPLC-grade water was obtained by filtering water previously purified by a Millipore system through a 0.45µm filter. Orthophosphoric acid 85% was purchased from Saarchem Pty Ltd, South Africa) and acetonitrile was purchased from Merck and was of HPLC grade. Solvent mixtures were degassed by sonication before use.

b) Instrumentation

The HPLC system consisted of a SpectraSYSTEM P2000 binary pump, a SpectraSYSTEM AS3000 autosampler equipped with a 100µl injection loop and a SpectraSYSTEM FL2000 fluorescence detector and/or a SpectraFOCUS UV detector (all by ThermoSeparation Products, SMM Instruments, South Africa). The analytical column was a Luna C₁₈ (2) 250x4.6mm 5µm purchased from Separations, South Africa. The column was preceded by HIRPB-10C guard column (Hichrom Ltd, SMM Instruments).

c) Chromatographic conditions

Mobile phase – gradient A: H₂O and H₃PO₄ (0.1% H₃PO₄ of the total volume); B: ACN/H₂O and H₃PO₄ (4:1 and 0.1% H₃PO₄ of the total volume) at a flow rate 1.2 ml/min. Detection – UV at 230 and 290nm. Injection volume was 50 µl.

5.2.3. Fats analysis

Fats were extracted according to the Soxhlet procedure outlined in method 920.39 (JAOAC, 1990), using a Buchi 810 Soxhlet Fat Extractor. The solvent used for extraction was petroleum ether and percentage fat was calculated on the gravimetric analysis.

5.2.4. Amino acids analysis

a) Principle

Amino acids were analysed on a Beckman Amino Acid Analyser System 6300 following a method by Moore and Stein (1984). This is a single column method using three sodium elution buffers [Beckman buffers A (pH 3.3), B (pH 4.0) and C (pH 6.3)], a regenerating reagent, and a Beckman sodium high-performance ion exchange column. The 6300 is a single column ion exchange chromatograph utilising HPLC techniques in an instrument dedicated to amino acid analysis. Two micro flow pumps are used in the analytical flow stream, one buffer pump, and one reagent (ninhydrin for post-column derivatisation) pump. The standard detection system features the classical Moore-Stein ninhydrin reagent, which requires heating the combined column effluent/reagent mixture for a period, prior to survey by the visible photometry at wavelengths of 440 and 520 nm. The injection volume was 20µl.

b) Sample preparation

For the analysis of the total amino acids in any biological material, the sample must first be hydrolysed to break all peptide bonds and liberate the constituent amino acids. Seventy (70) milligrams of the sample was weighed. The standard protein hydrolysis procedure used 6N HCl at 110°C for 24 hours. An internal standard was added to the sample to provide a means of correcting for any inaccuracy that may occur during the sample preparation.

c) Capture and calculation of results

Reproducible chromatography requires accurate delivery of specific flow rates and the capability to proportion changing percentages of three mobile phases over specific time intervals and temperatures (buffer A at ±60, B at ±75 and C at ±90°C). The System Gold Software Package is

designed to control the chromatography while acquiring data from the detector channel. Data from previous runs can be analysed while current runs are in process. Run parameters can be adjusted at any time. The chromatographic and control data is passed back and forth between the System Gold computer system and the Beckman analyser.

5.2.5. Crude protein analysis

Crude proteins were analysed in a LECO FP2000 Nitrogen Analyser using the Dumas Combustion method 990.03 (Cunniff, 1995). The analyser gives results in percentage-per-amount-analysed format.

5.2.6. Sugars analysis

a) Starch analysis

The method used is a modified version of the AOAC method 8.019 (Cunniff, 1995). Samples were extracted by refluxing for 2.5 hours, neutralised and analysed by HPLC as glucose which was then converted to starch using the factor 0.925. A Dionex pump and pulse amperometric detection was used for the analysis.

b) The rest of the sugars analysis

The method used was an in-house method developed by using a Dionex technical note. It is specified for the determination of oligosaccharides up to DP15 (DP = Degree of Polarisation) in solid and liquid samples. At high pH, as used in the mobile phase for chromatography, sugars become negatively charged by abstraction of a proton from the OH groups. They can thus be separated by anion chromatography and detected by triple pulse amperometry.

The applied potentials to the working electrode consisting of:

1. A working potential (usually a small positive voltage).
2. A high positive followed by a high negative potential (to remove oxidation products from the working electrode).

5.3. RESULTS AND DISCUSSION

Three 10g samples of the commercial maize meal were each spiked with 500ng of vitamins B₁, B₂ and B₆ and were extracted and cleaned up according to the procedure outlined in 5.2.1.a. The spiked samples were then analysed by HPLC. The percentage recoveries were 94.5 (B₁), 91 (B₂) and 97.6 (B₆) and are outlined in Table 5.1. Formulae used to calculate the vitamin amounts are listed in Appendices 13 – 15.

$$y = ax$$

Equation 2: The equation used to calculate vitamin amounts

Where, y represents the peak area value and x represents the calculated amount of the vitamin.

Table 5.1: Recovery of vitamins B₁, B₂ and B₆ from commercial maize meal spiked with each vitamin and analysed by HPLC.

	B Vitamins		
	Thiamine (B ₁)	Riboflavin (B ₂)	Pyridoxine (B ₆)
Amounts spiked with (µg/g)	0.05	0.05	0.05
Total amounts recovered (µg/g)	1.527±5.39	1.595±5.76	0.048±3.94
Amounts fortified with (µg/g)*	1.48	1.55	NA
Adjusted amounts (µg/g)**	0.047±5.39	0.045±5.76	0.048±3.94
Lower detection limit (ng/g)	100	25	50
Percentage recovery	94%	90%	96%

*These are the amounts the maize meal used was fortified with by the manufacturer.

**Recovery of three replicates.

Table 5.2 show the results obtained for the analysis of maize meal and amahewu samples for vitamins B₁, B₂ and B₆. MaBheleni maize meal and amahewu samples contained analysed vitamins in the following ranges: B₁ 1.4±0.15 – 0.31±0.21µg/g; B₂ 0.10±0.11 – 0.64±0.40µg/g and B₆ 0.77±0.45µg/g – none detected in amahewu. Vitamins B₁ and B₆ decreased by 78 and 100% respectively, and B₂ increased by 82% when compared to those obtained from maize meal.

KwaNgcolosi maize meal and amahewu samples contained vitamins in the following ranges: B₁ 2.26±0.51 - 4.48±0.81µg/g; B₂ 1.39±0.29 - 1.43±0.72µg/g and B₆ 0.68±0.28 - 0.77±0.22µg/g. Amahewu samples showed a reduction of these vitamins of between 3 and 12%.

Clover SA maize meal and amahewu samples contained vitamins in these amounts: B₁ 3.10±0.05 – 3.95±0.08µg/g; B₂ 1.30±0.05 – 1.67±0.33µg/g and B₆ 0.04±0.05 – 0.12±0.07µg/g. The vitamins amounts contained by amahewu proved to be higher by 17 to 60%.

Table 5.2: Vitamins B₁, B₂ and B₆ analysis from maize meal and amahewu samples

Vitamins (µg/g)	MaBheleni		Clover SA		KwaNgcolosi	
	MM	Ama	MM	Ama	MM	Ama
B ₁	1.4±0.15	0.31±0.21	3.26±0.49	4.09±1.03*	4.48±0.81	4.26±1.97
	ND	ND	3.10±0.05	3.95±0.08*	2.36±0.03	2.26±0.51
	ND	ND			ND	ND
	ND	ND			ND	ND
	ND	ND				
B ₂	0.15±0.14	0.64±0.40	1.38±0	1.67±0.33	1.43±0.72	1.39±0.29
	0.10±0.11	0.56±0.30	1.30±0.05	1.53±0.21	ND	ND
	ND	ND			ND	ND
	ND	ND			ND	ND
	ND	ND				
B ₆	0.77±1.45	ND	0.05±0.07	0.12±0.07	0.77±0.22	0.68±0.28
	ND	ND	0.04±0.05	0.10±0.03	ND	ND
	ND	ND			ND	ND
	ND	ND			ND	ND
	ND	ND				

ND – None Detected, MM – maize meal, Ama – amahewu

*The adjusted amount corrected using Clover SA fortifying amount.

Changes in the vitamin content of cereals with fermentation vary according to the fermentation process, the raw material used in the fermentation and the microorganisms involved. During the fermentation of maize in the preparation of sorghum beer, B₁ levels are virtually unchanged, but B₂ and niacin contents almost double (Steinkraus, 1994). The nutritive value of maize may increase or decrease depending on methods of preparation. Okoruwa and Kling (1996) reported that sprouting or fermentation of maize grains increases the content of some of the B vitamins. In this study, milled grains were used. Milling of cereal grains prior to their consumption is a common practice. During the milling process, a substantial proportion of the nutrients are lost from the refined product (FAO,

1995) and this could offer an explanation as to why there was a loss in some of the B-vitamins amounts when compared to literature. The loss of vitamin B₁ could be due to the findings by Spallholz *et al.*, (1999), that much of it is destroyed by common cooking techniques and by some food processing, like milling.

Table 5.3 shows the results obtained for samples of amahewu and maize meal analysed for fat from maBheleni and kwaNgcolosi villages and Clover SA. The bold arrows represent the reduction of fat amounts. MaBheleni maize meal samples contained fats at concentration ranges between 0.79 ± 0.25 and $4.16\pm 0.55\%$. Amahewu samples on the other hand, contained fats at reduced levels of 0.38 ± 0.65 and $1.47\pm 0.29\%$ by weight. These results show 14 to 89% reduction. KwaNgcolosi maize meal and amahewu samples contained fats at concentrations between 2.08 ± 0.16 and 4.54 ± 0.05 and reduced levels of 1.08 ± 0.31 and $2.68\pm 0.11\%$ by weight. These results show a 12 to 59% reduction. Clover SA maize meal and amahewu samples contained fats at levels between 0.67 ± 0.22 and 1.52 ± 0.40 and reduced levels of 0.28 ± 0.40 and $0.51\pm 0.28\%$ by weight respectively. These results show a 24 to 82% reduction.

Table 5.3: Fat analysis from maize meal and amahewu samples

Sample	Fats analyses (% by sample weight)					
	MaBheleni		Clover SA		KwaNgcolosi	
	MM	Ama	MM	Ama	MM	Ama
1	4.16 ± 0.55	$0.46\pm 0.80 \downarrow$	0.67 ± 0.22	$0.51\pm 0.28 \downarrow$	2.08 ± 0.16	$1.84\pm 0.52 \downarrow$
2	0.79 ± 0.25	$0.38\pm 0.65 \downarrow$	1.52 ± 0.40	$0.28\pm 0.40 \downarrow$	2.63 ± 0.02	$1.08\pm 0.31 \downarrow$
3	1.23 ± 0.06	$0.78\pm 0.47 \downarrow$			4.54 ± 0.05	$2.68\pm 0.11 \downarrow$
4	2.37 ± 2.06	$1.31\pm 0.92 \downarrow$			3.89 ± 0.02	$1.94\pm 0.06 \downarrow$
5	1.61 ± 0	$1.47\pm 0.29 \downarrow$				

MM – maize meal; Ama – amahewu; \downarrow - reduced

Table 5.4 shows the results obtained for samples of amahewu and maize meal analysed for proteins from MaBheleni and kwaNgcolosi villages and Clover SA. The pattern followed by amahewu and maize meal samples analysed was similar for Clover SA, maBheleni and 75% of kwaNgcolosi samples. MaBheleni maize meal and amahewu samples contained proteins at levels between 7.94 ± 0.06 and 8.24 ± 0.05 and reduced levels of 4.33 ± 0.04 and $4.76\pm 0.02\%$ by weight, respectively. These results show a 42 to 47% reduction.

KwaNgcolosi maize meal and amahewu samples contained proteins at a concentrations ranges of between 7.32 ± 0.03 and 8.00 ± 0.03 and 4.02 ± 0.02 and 8.40 ± 0.04 % by weight respectively. These results show a reduction of 11 to 46% and 13% increase in one sample. Clover SA maize meal and amahewu samples contained proteins at concentrations ranges of between 7.50 ± 0.04 and 7.59 ± 0.08 and 4.49 ± 0.01 and 4.96 ± 0.04 % by weight respectively. These results show a 34 to 41% reduction, Cahvan *et al.*, (1988) reported that the content and quality of cereal proteins can be improved by fermentation.

An attempt was made by Carries (2002, unpublished) to prove whether or not different indigenous fermentation processes would yield the same, amongst other things, protein content. Protein content varied for each fermentation process. The most pronounced increase was in the sample containing maize meal, salt, bread flour and yeast. The increase was by 149%. This therefore could be the explanation to the sample from kwaNgcolosi that showed an increase in protein content of amahewu when compared to maize meal.

Table 5.4: Protein analysis of maize meal and amahewu samples

Sample	Proteins analyses (% by sample weight)					
	MaBheleni		Clover SA		KwaNgcolosi	
	MM	Ama	MM	Ama	MM	Ama
1	8.10 ± 0.02	4.33 ± 0.04 ↓	7.59 ± 0.08	4.49 ± 0.01 ↓	7.33 ± 0.01	8.40 ± 0.04 ↑
2	8.24 ± 0.05	4.76 ± 0.02 ↓	7.50 ± 0.04	4.96 ± 0.04 ↓	7.32 ± 0.03	6.56 ± 0.05 ↓
3	8.22 ± 0.02	4.60 ± 0.11 ↓			7.44 ± 0.04	4.02 ± 0.02 ↓
4	7.94 ± 0.06	4.43 ± 0.04 ↓			8.00 ± 0.03	4.57 ± 0.06 ↓
5	8.23 ± 0.07	4.55 ± 0.01 ↓				

MM – maize meal; Ama – amahewu; ↓ - reduced; ↑ - increased

Table 5.5 shows the results obtained for samples of maize meal and amahewu analysed for essential amino acids from Clover SA, maBheleni and kwaNgcolosi. The pattern followed by amahewu and maize meal samples analysed was similar for Clover SA, maBheleni and 75% of kwaNgcolosi samples. There was a reduction in amino acid amounts of amahewu when compared to those of maize meal. For maBheleni village, the reduction was between 37 and 48%; for Clover SA, was between 26 and 41%. For kwaNgcolosi village, reduction was 1.0 to 6.9%, increase was 4.8 to 9.0% and the rest stayed the same for both maize meal and amahewu.

Table 5.5: Essential amino acid analysis of maize meal and amahewu samples

AA (% wt)	MaBheleni		Clover SA		KwaNgcolosi	
	MM	Ama	MM	Ama	MM	Ama
Thr	0.24±0.01	0.14±0.01 ↓	0.20±0.01	0.13±0.01 ↓	0.20±0.00	0.21±0.03 ↑
Val	0.41±0.01	0.24±0.01 ↓	0.35±0.01	0.23±0.02 ↓	0.36±0.02	0.36±0.06 =
Met	0.11±0.01	0.07±0.01 ↓	0.08±0.01	0.08±0.01 =	0.10±0.01	0.11±0.02 ↑
Iso	0.28±0.01	0.17±0.01 ↓	0.28±0.01	0.17±0.01 ↓	0.27±0.01	0.27±0.05 =
Leu	0.98±0.05	0.54±0.03 ↓	1.10±0.03	0.66±0.06 ↓	0.99±0.00	0.98±0.20 ↓
Tyr	0.20±0.01	0.12±0.01 ↓	0.15±0.01	0.10±0.02 ↓	0.15±0.00	0.16±0.02 ↑
Phe	0.36±0.02	0.20±0.01 ↓	0.37±0.01	0.23±0.02 ↓	0.36±0.00	0.35±0.06 ↓
His	0.25±0.01	0.14±0.01 ↓	0.22±0.00	0.15±0.02 ↓	0.22±0.01	0.21±0.02 ↓
Lys	0.21±0.01	0.11±0.01 ↓	0.11±0.01	0.08±0.01 ↓	0.16±0.01	0.17±0.02 ↑

AA – essential amino acids; ↑ - increased; ↓ - reduced; = - the same; MM – maize meal; Ama – amahewu

Natural fermentation of cereals increases their relative nutritive value and available lysine (Hamad and Fields, 1979). In this study there was a very slight increase in the lysine amounts obtained for amahewu from kwaNgcolosi village. Bacterial fermentations involving proteolytic activity are expected to increase the biological availability of essential amino acids more than yeast fermentations which mainly degrade carbohydrates (Chaven and Kadam, 1989). Even though fermentations in maBheleni and kwaNgcolosi samples were carried out by bacteria, their proteolytic activities are not known.

Table 5.6: Detection limits of sugars analysed using HPLC.

Analysis	Unit	Technique used	Detection limit (g/100g)
Glucose	g/100g	HPLC	0.2
Fructose	g/100g	HPLC	0.4
Sucrose	g/100g	HPLC	1.0
Maltose	g/100g	HPLC	0.5
M-triose	g/100g	HPLC	0.4
DP 4	g/100g	HPLC	0.6
DP 5	g/100g	HPLC	0.7
DP >15	g/100g	HPLC	0.5

Table 5.6 shows the detection limits of the sugars obtained in some maize meal and amahewu samples. Table 5.7 show the results obtained for sugar analysis of maBhel:ni, Clover SA and kwaNgcolosi maize meal and amahewu samples. From Clover SA and maBhel:ni maize meal samples, only glucose was detected and fructose and sucrose were detected from amahewu samples. From kwaNgcolosi maize meal samples, on the other hand, glucose and maltose were detected and glucose to maltotriose, DP 4 and 5 and DP>15 were detected from amahewu samples.

Table 5.7: Sugar analysis of maize meal and amahewu samples using HPLC

Analytes (g/100g)	MaBhel:ni		Clover SA		KwaNgcolosi	
	MM	Ama	MM	Ama	MM	Ama
Glucose	0.12±0.11	1.18±0.57	1.06±0.25	8.06±0.62	0.76±0.58	0.84±0.63
Fructose	ND	1.54±0.70	ND	9.25±0.35	ND	2.26±1.43
Sucrose	ND	30.7±2.92	ND	8.47±2.87	ND	8.08±10.7
Maltose	ND	ND	ND	ND	0.26±0.30	9.88±11.7
M-triose	ND	ND	ND	ND	ND	0.93±0.72
DP 4	ND	ND	ND	ND	ND	0.63±0.42
DP 5	ND	ND	ND	ND	ND	0.72±0.4
DP >15	ND	ND	ND	ND	ND	0.85±0.60

ND – none detected; Ama - amahewu; MM – maize meal

Table 5.8 shows the results obtained for samples of amahewu and maize meal analysed for starch from MaBhel:ni and kwaNgcolosi villages and Clover SA. The pattern followed by amahewu and maize meal samples analysed was similar for all samples, in that there was a reduction in starch amounts of amahewu when compared to those of the maize meal. MaBhel:ni maize meal and amahewu samples contained starch at levels of between 66.1±1.60 and 69.4±2.71 levels and reduced levels of between 40.9±0.88 and 47.1±0.25 g/100g, respectively. Clover SA maize meal and amahewu samples contained starch at levels between 75.7±1.45 and 75.9±1.92 and reduced levels of between 53.2±1.29 and 55.0±0.42 g/100g respectively. KwaNgcolosi maize meal and amahewu samples contained starch at levels between 69.6±1.63 and 72.2±2.14 and reduced amounts of between 31.5±5.28 and 53.4±1.30 g/100g respectively.

Table 5.8: Starch analysis of maize meal and amahewu using HPLC.

Sample	Starch (g/100g)					
	MaBheleni		Clover SA		KwaNgcolosi	
	MM	Ama	MM	Ama	MM	Ama
1	69.4±2.71	47.1±0.25	75.7±1.45	55.0±0.42	70.7±1.72	31.5±5.28
2	68.2±1.94	45.3±1.99	75.9±1.92	53.2±1.29	71.5±0.25	53.4±1.30
3	66.1±1.60	40.9±0.88			72.2±2.14	50.1±1.41
4	68.6±0.50	42.2±0.49			69.6±1.63	38.5±1.56
5	67.1±1.48	44.3±0.32				

MM – maize meal; Ama – amahewu

Table 5.8 shows reduced starch after fermentation (hydrolysis of starch to simpler sugars). Table 5.8 clearly shows 32 – 38.5% starch reduction from MaBheleni; 27 – 30% from Clover SA and 25 – 55% from KwaNgcolosi when maize meal is compared to amahewu sample. Table 5.7 shows simpler sugars that resulted after starch was hydrolysed by the fermentation process. Aly (2002) conducted a survey of Egyptian corn and corn-based products and by-products. The conducted experiments to determine the fate of distribution of aflatoxin during wet-milling process and investigate aflatoxin destruction during starch conversion to glucose syrup.

Clover SA ingredients include maize meal, sugar, salt and artificial sweetener (Saccharin). This explains the presence of sucrose in their amahewu samples which otherwise should not be present because starch is made up of only glucose molecules and sucrose is a combination of glucose and fructose. The same explanation can therefore be given for MaBheleni and some of KwaNgcolosi samples. Different homes, even within the same village, use different fermentation methods. All that can be concluded from Table 5.7 is that glucose levels are increased in the fermented product in all the sample groups suggesting that fermentation may also influence the production of increased levels of the monosaccharides and disaccharides observed.

Tables 5.9 and 5.10 represent recommended daily allowances of selected nutrients. Even though some of these nutrients' amounts showed an increase in concentration after fermentation, they are still very much lower than recommended daily allowances. Diets that rely heavily on maize and maize-based foods as an energy source may be deficient in certain nutrients. It is therefore essential to eat foods that complement those deficient nutrients to provide a well-balanced diet.

Table 5.9: Recommended daily allowances of some of the nutrients (by Bender *et al.*, 1998)

Food component	Daily intakes
Total fat	65g
Total carbohydrates	300g
Proteins	50g
Vitamin B ₁ (thiamine)	1.5mg
Vitamin B ₂ (riboflavin)	1.7mg
Vitamin B ₆ (pyridoxine)	2mg

Table 5.10: Recommended daily allowances of essential amino acids (adapted from www.anyvitamins.com.)

Recommended Daily Requirements (mg/kg of body weight)			
Amino acid	Infant (3 – 6 months)	Child (10 – 12 yrs)	Adult
Aromatic amino acids	132	22	16
Histidine	33	Not known	Not known
Isoleucine	80	28	12
Leucine	128	42	16
Lysine	97	44	12
S-containing amino acids	45	22	10
Threonine	63	28	8
Tryptophan	19	4	3
Valine	89	25	14

No distinctive trend was found when the samples were analysed for vitamins. Fats amounts decreased in all amahewu samples. The protein concentrations decreased in all amahewu samples with exception of one sample from kwaNgcolosi. Essential amino acids analysed from maBheleni and Clover SA showed a decrease in concentration after fermentation. However, threonine, methionine, tyrosine and lysine increased after fermentation in samples from kwaNgcolosi. Different microorganisms that were identified and confirmed in amahewu require different growth factors, therefore it is possible that the nutrients in amahewu were then utilised as growth factor/s. Since Clover SA carries out amahewu fermentation under controlled environments, the strains in the fermentation medium are known. The growth factors are incorporated in the medium for the strains to utilise and therefore only ferment the porridge.

CHAPTER 6

APPARENT AND TRUE METABOLISABLE ENERGY ASSESSMENT OF COMMERCIAL AND HOMEMADE AMAHEWU

6.1. INTRODUCTION

Metabolisable energy is the difference between the gross energy of food eaten and the gross energy of faeces and urine voided (Gous and Dennison, 1983). It represents the total amount of energy supplied in the food that the consumer can utilise. Apparent metabolisable energy is the difference between the energy of the feed and the energy of the excreta (Sibbald, 1989). True and apparent metabolisable energies are analysed to determine how much of food taken in ends up in the system of the consumer, especially with new or improved foods and feeds.

$$\text{AME/g of feed} = \frac{(F_i \times \text{GE}_f) - (E \times \text{GE}_e)}{F_i}$$

Formula 2: Apparent metabolisable energy calculation formula

Where F_i is the feed intake (g); E is the excreta output (g); GE_f is the gross energy/g of feed; and GE_e is the gross energy/g of excreta.

Nitrogen-corrected apparent metabolisable energy (AME_N) is the most commonly used estimate of metabolisable energy. It differs from AME because a correction is made for nitrogen retention (NR) which may be either positive or negative. The correction factor is 34.4 kJ/g of retained nitrogen.

$$\text{AME}_N/\text{g of feed} = \frac{[(F_i \times \text{GE}_f) - (E \times \text{GE}_e)] - (\text{NR} \times K)}{F_i}$$

Formula 3: Nitrogen-corrected apparent metabolisable energy calculation formula

Where $\text{NR} = (F_i \times N_f) - (E \times N_e)$; N_f is the nitrogen/g of feed (g); N_e is the nitrogen/g of excreta (g); and K is a constant, 34.4 kJ.

True metabolisable energy is a term used to describe an estimate of ME in which correction is made for metabolic faecal (FE_m) and endogenous urinary (UE_c) energy. The FE_m is that proportion of the faeces other than the feed residues and is present as abraded intestinal mucosa, bile and digestive fluids. The UE_c is that proportion of the urinary energy not of direct feed origin. Combined, the FE_m and UE_c represent a maintenance cost which should not be charged against the feed (Sibbald, 1989).

$$\text{TME/g of feed} = \frac{[(F_i \times GE_f) - (E \times GE_c)] + (FE_m + UE_c)}{F_i}$$

Formula 4: True metabolisable energy calculation formula

Nitrogen-corrected true metabolisable energy (TME_N) bears the same relationship to TME as does AME_N to AME. The correction to nitrogen equilibrium is made in the same manner and its value is subject to the same debate.

$$\text{TME}_N/\text{g of feed} = \frac{[(F_i \times GE_f) - (E \times GE_c) - (NR \times K)] + [(FE_m + UE_c) + NR_o \times K]}{F_i}$$

Formula 5: Nitrogen-corrected true metabolisable energy calculation formula

Where NR and NR_o are estimates of nitrogen retention for fed or fasted birds, respectively.

Figure 6.1 shows the relationship between food energy input and excreta energy with endogenous energy loss correction (a) and food intake, metabolisable energy and true metabolisable energy (b). The linear graph (a) basically shows that excreta energy is directly dependent on food energy input because the EEL factor is constant. The exponential graph (b) on the other hand shows that if the TME is constant, then metabolisable energy is directly related to the amount of food intake. This is true only if small amounts of food are taken in.

Some of the benefits of metabolisable energy analysis are:

- ◆ To ensure that the consumer receives sufficient levels of nutrients for optimum health and performance.
- ◆ The amount of food/feed to be given to the consumer, for maximum performance, will be known.
- ◆ Achieve the target weight, especially in animals.

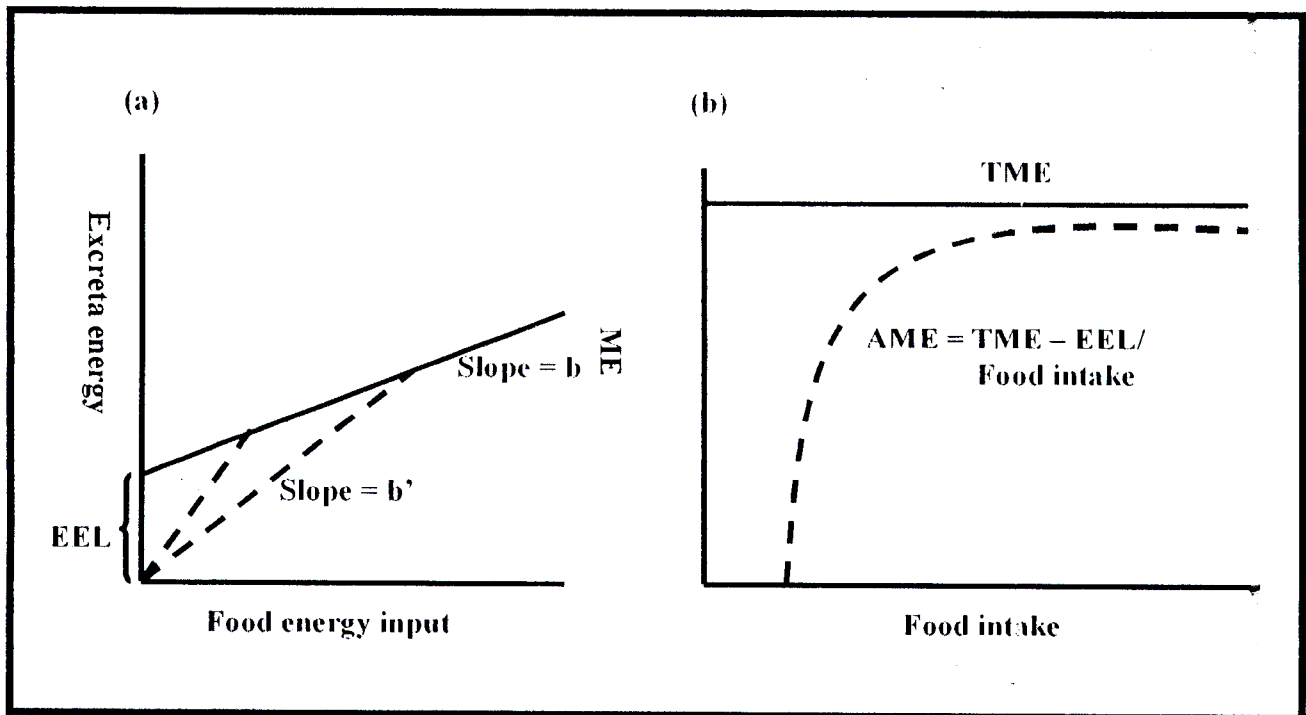


Figure 6.1: (a) Regression of excreta energy on food energy input. (b) Relationship between AME, TME and food intake as derived from (a), assuming that $EEL \neq 0$ (Fisher and McNab, 1988).

6.2. MATERIALS AND METHODS

6.2.1. Materials and reagents

The materials and reagents used in this study were: 18-week old roosters, 100g glucose solution, DDS isothermal CP500 bomb calorimeter, excreta-collecting trays, freeze-dried commercial and homemade amahewu.

6.2.2. Intubation technique

a) Principle

A rapid *in vivo* assay technique involving the feeding of adult roosters with a measured quantity of feed is used, following a 48 hour fasting period. During this fasting period the birds are given glucose in water to ensure that they do not become dehydrated or lacking in energy. After feeding 50g of the test feed, the excreta are collected over the following 48 hours. These are dried, weighed and analysed for energy, to determine the excreta energy output, which is compared with the energy input to determine the digestibility of the energy consumed. A correction is made for endogenous energy losses (EEL), as well as for nitrogen that may either have been retained or lost during the assay period. The resultant AME value does not include a correction for EEL, whereas the TME does. Both values are corrected for nitrogen balance.

b) Method

This is a method developed by McNab and Fisher (1984). Roosters were housed in individual cages. At the start of the assay period food was withheld for 48 hours to ensure that no food remains in the intestinal tract before the test feed was given. After the first 24 hours of fasting, each bird was removed from its cage in turn, and 100g of a glucose solution was fed by intubation. This ensures that the bird remains in positive energy balance, and that it is kept hydrated. Glucose is completely digested, so no feed residue will remain in the intestine following the feeding of this energy source. After 48 hours of fasting each bird was again removed from the cage, in turn, and fed 50g of the test ingredient by intubation.

The feeding process took approximately 45 seconds to complete, and the bird was held securely on the lap of a technician whilst a second technician administered the feed. It was then returned to the cage, with the minimum imposition of stress. The bird remained in the cage for a further 48 hours, during which time the excreta produced were collected in a tray under the cage. After the 48 hour collection period was over the birds were returned to full feeding. Birds were given a week of full feeding to recover from the process. The adult roosters used in the assay were purchased at about 18 weeks of age. They were kept on full feed in holding cages until they were ready to be used for the assay procedure (about 24 weeks of age), and they were culled at 72 weeks of age. The accumulated excreta was analysed further for energy (caloric value) by the DDS isothermal CP500 bomb calorimeter. AME, TME, AME_N and TME_N values of freeze-dried amahewu were determined according to Sibbald (1989).

6.3. RESULTS AND DISCUSSION

Nitrogen-corrected apparent metabolisable energy for homemade and commercial amahewu were 13.194 and 13.696 MJ/kg respectively (Table 6.1). Conversely, nitrogen-corrected true metabolisable energy for the same samples were 13.605 and 14.106 MJ/kg, respectively (Table 6.1). This study contributes information in an area that is not well researched. Several studies have been conducted on metabolisable energy analysis of whole cereals using animals, poultry in particular. Gous and Dennison (1983) screened 13 different types of cereals that are used as poultry feed. They obtained AME_N and TME_N of 13.60 and 13.82 MJ/kg, respectively for maize. Results of this study compare favourably to their study. This study, however, contributes new knowledge on the metabolisable energy analysis of amahewu (processed maize). It is possibly the first of its kind that deals with processed cereals, fermented maize in particular.

Table 6.1: True and apparent metabolisable energy analysis of freeze-dried homemade and commercial amahewu using intubation technique

	Homemade amahewu	Commercial amahewu
AME _N (MJ/kg)	13.19	13.70
TME _N (MJ/kg)	13.61	14.11
Protein (%)	3.5±0.04	4.5±0.13

Table 6.2 shows nitrogen-corrected apparent and true metabolisable energy of some of the cereal grains. According to National Research Council (1994), maize is a high energy cereal when compared to sorghum (AME_N 13.76, TME_N 14.13); wheat (AME_N 12.59, TME_N 13.25); barley (AME_N 11.05, TME_N 12.13); rye (AME_N 10.99, TME_N 12.26) and oat (AME_N 10.67, TME_N 10.98).

Table 6.2: AME_N and TME_N values of some of the cereal grains (adapted from NRC, 1994)

Feed	AME _N (MJ/kg)	TME _N (MJ/kg)
Maize (NRC, 1994)	14.02	14.52
Sorghum	13.76	14.13
Maize (Gous, 1983)	13.60	13.82
Wheat	12.59	13.25
Barley	11.05	12.13
Rye	10.99	12.26
Oat	10.67	10.98

Table 6.3 shows nitrogen-corrected apparent and true metabolisable energy of maize and amahewu (commercial and homemade). This table shows that amahewu (lactic acid bacteria fermented maize) has higher energy than normal maize when fed to animals. Commercial amahewu had slightly higher individual apparent and true metabolisable energy values than those of homemade amahewu. This may be due to individual characteristic values for nitrogen-free extracts, starch and crude fat contents, amongst other things.

The climate in which maize was grown and the soil fertility play important roles in the enrichment of crops whilst they are still in the fields (Saffarzadeh *et al.*, 2000).

Table 6.3: Comparison of AME_N and TME_N of maize and amahewu

Feed	AME _N (MJ/kg)	TME _N (MJ/kg)
Commercial amahewu	21.92	22.58
Homemade amahewu	21.10	21.78
Maize (NRC, 1994)	14.02	14.52
Maize (Gous, 1983)	13.60	13.82

Amahewu has a higher metabolisable energy compared to maize and thus have a potential use for nutrition. Amahewu can also be supplemented in order to enhance its nutritional value.

CHAPTER 7

CONCLUSIONS, RECOMMENDATIONS AND FUTUREWORK

1. A mixture of microorganisms (Gram positive and negative) were identified in amahewu from maBheleni and kwaNgcolosi villages. The Gram positives isolated were from the lactic acid bacteria (LAB) category and Gram negatives identified belonged to the *Enterobacteriaceae* family. Amahewu from Clover SA gave LAB and yeast. Strains from three genera, *Pantoea*, *Klebsiella* and *Enterobacter*, survived the lactic acid fermentation process. Further investigation is required in order to determine how they survived this process.
2. A 66.5 to 100% concentration reduction of AFB₁, FB₁ and ZEA was obtained from maBheleni and kwaNgcolosi amahewu samples when compared to the original maize meal. Since lactic acid fermentation has reduced the various toxin concentrations in amahewu, the rural communities need to be informed of this finding and be encouraged to consume their maize and maize-based foods in fermented forms.
3. Vitamins analysis showed no definite trend; fats were reduced in all amahewu samples; proteins decreased in all samples but one from kwaNgcolosi which showed an increase; most samples showed a decrease in essential amino acids amounts after lactic acid fermentation with an exception of threonine, methionine, tyrosine and lysine which showed an increase in most kwaNgcolosi amahewu samples. Samples from kwaNgcolosi showed an interesting trend in that after lactic acid fermentation, simple and complex sugars most of which are classified as probiotics were present. These sugars are very helpful in promoting the growth of LAB which are very important in regulating the colon microflora, help in the absorption of nutrients in the small intestine and further digestion of carbohydrates in the colon. Further studies need to be conducted to find out how they operate *in vivo*.
4. In the metabolisable energy analysis study, amahewu proved to be more metabolisable when fed to roosters when compared to maize. This is probably because during lactic acid fermentation process, complex sugars are broken down to simpler sugars, which are easier to digest compared to starch. This technique can be used to investigate whether fortification does serve its purpose in terms of the fortificant availability to the consumer.

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APPENDICES

APPENDIX I

TESTS	SUBSTRATES	REACTIONS/ ENZYMES	NEGATIVE RESULTS	POSITIVE RESULTS
VP	Pyruvate	Acetoin production	Colourless	Pink – Red
HIP	Hippurate	Hydrolysis	Colourless 4hrs 24hrs	Dark blue/Violet 4hrs 24hrs
ESC	Esculin	β -glucosidase	Colourless Pale yellow Light grey	Black Black Gray
PYRA	Pyrrolidonyl 2 naphthylamine	Pyrrolidonyl arylamidase	Colourless or very pale Orange	Orange
αGAL	6-Bromo-2-naphthyl α -D-galactopyranoside	α -galactosidase	Colourless	Violet
βGUR	Naphthol AS-BI β -D-glucuronate	β -glucuronidase	Colourless	Blue
βGAL	2-naphthyl- β -D-galactopyranoside	β -galactosidase	Colourless or very pale violet	Violet
PAL	2-naphthyl phosphate	Alkaline phosphatase	Colourless or very pale violet	Violet
LAP	L-leucine-2-naphthylamine	Leucine arylamidase	Colourless	Orange
ADH	Arginine	Arginine dihydrolase	Yellow 4 hrs 24 hrs	Red 4 hrs 24 hrs
<u>RIB</u>	Ribose	Acidification	Red Orange/Red	Orange/Yellow Yellow
<u>ARA</u>	L-Arabinose	Acidification	Red Orange/Red	Orange/Yellow Yellow
<u>MAN</u>	Mannitol	Acidification	Red Orange/Red	Orange/Yellow Yellow
<u>SOR</u>	Sorbitol	Acidification	Red Orange/Red	Orange/Yellow Yellow
<u>LAC</u>	Lactose	Acidification	Red Orange/Red	Orange/Yellow Yellow
<u>TRE</u>	Trehalose	Acidification	Red Orange/Red	Orange/Yellow Yellow
<u>INU</u>	Inulin	Acidification	Red Orange/Red	Orange/Yellow Yellow
<u>RAF</u>	Raffinose	Acidification	Red Orange/Red	Orange/Yellow
<u>AMD</u>	Starch (2)	Acidification	Red Orange/Red	Yellow
<u>GLYG</u>	Glycogen	Acidification	Red or Orange	Orange/Yellow Yellow Bright yellow

The reactions reading table for the API 20 STREP method after 4 and 24 hours of incubation at 37°C.

APPENDIX 2

STRIP 0-9 Tube / substrate	STRIP 10-19 Tube / substrate	STRIP 20-29 Tube / substrate	STRIP 30-39 Tube / substrate	STRIP 40-49 Tube / substrate
0 CONTROL	10 GALactose	20 α -Methyl-D-	30 MELibiose	40 D TURanose
1 GLYcerol	11 GLUcose	Mannoside	31 SUCrose	41 D LYXose
2 ERYthritol	12 FRUctose	21 α -Methyl-D-	32 TREhalose	42 D TAGatose
3 D ARABinose	13 MaNNosE	Glucoside	33 INULin	43 D FUCose
4 L ARABinose	14 SorBosE	22 N-Acetyl-	34 MeLeZitose	44 L FUCose
5 RIBose	15 RHAmmose	Glucosamine	35 RAIfinose	45 D Arabitol
6 D XYLose	16 DULcitol	23 AMYgdalin	36 Starch	46 L Arabitol
7 L XYLose	17 INOsitol	24 ARButin	37 GLYcoGen	47 GlucoNaTe
8 ADOnitol	18 MANnitol	25 ESCulin	38 XylITol	48 2-Keto-
9 β Methyl-D- Xyloside	19 SORbitol	26 SALicin	39 GENtiobiose	Glucenate
		27 CELlobiose		49 5-Keto-
		28 MALtose		Glucenate
		29 LACtose		

Composition of the 50 API 50 CHL microtubes

APPENDIX 3 (Blood Agar Composition)

Formula	gm/litre
"Lab-Lemco" powder	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	15.0
pH 7.3 \pm 0.2	

BA medium was prepared by weighing 40g of powder into a litre of distilled water. The mixture was heated to boiling with frequent stirring until the powder was completely dissolved and then autoclaved for 15 minutes at 121°C. The medium was cooled down to about 50°C and 30ml of red packed cells was added and mixed.

A non-selective general purpose medium which may be enriched with blood or serum. Oxoid blood agar base is a non-selective general purpose medium widely used for the growth of pathogenic and non-pathogenic bacteria:

- ◆ With added blood, the medium is not only enriched, but becomes suitable for the determination of the typical haemolytic reactions which are important diagnostic criteria for *Streptococci*, *Staphylococci* and other organisms.

APPENDIX 4 (Eosin Methylene Blue Agar Composition)

Formula	gm/litre
Peptone	10.0
Lactose	10.0
Dipotassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.065
Agar	15.0
pH 6.8±0.2	

EMB medium was prepared by weighing 36g of powder into a litre of distilled water. The mixture was heated to boiling with frequent stirring until the powder was completely dissolved and then autoclaved for 15 minutes at 121°C. After autoclaving the medium was cooled to 60°C and was shaken in order to oxidise the methylene blue and to suspend the precipitate which is essential part of the medium.

APPENDIX 5 (MacConkey No 3 Agar Composition)

Formula	gm/litre
Peptone	20.0
Lactose	10.0
Bile salts No. 3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0

pH 7.1±0.2

MacConkey No.3 medium was prepared by weighing 51.5g of powder into a liter of distilled water. The mixture was heated to boiling with frequent stirring until the powder was completely dissolved and then autoclaved for 15 minutes at 121°C. It is a selective medium that gives excellent differentiation between coliforms and non-lactose fermenters with inhibition of Gram-positive micrococci. Coliforms produce intense violet-red colonies whilst non-lactose fermenters are colourless.

APPENDIX 6 (Saboraud Dextrose Agar Composition)

Formula	gm/litre
Mycological peptone	10.0
Glucose	40.0
Agar	15.0

pH 5.6±0.2

SDA medium was prepared by weighing 47g of powder into a litre of distilled water. The mixture was heated to boiling with frequent stirring until the powder was completely dissolved and then autoclaved for 15 minutes at 121°C. This is an acid pH medium for the isolation of dermatophytes, other fungi and yeasts.

APPENDIX 7 (Violet Red Bile Agar Composition)

Formula	gm/litre
Yeast extract	3.0
Peptone	7.0
Sodium chloride	5.0
Bile salts No. 3	1.5
Lactose	10.0
Neutral red	0.03
Crystal violet	0.002
Agar	12.0

pH 7.4±0.2

VRB medium was prepared weighing 39.5g of powder into a litre of distilled water. The mixture was heated to boiling with frequent stirring until the powder was completely dissolved making sure that it was not overheated or autoclaved. A lactose-containing selective medium for the detection and enumeration of coli-aerogenes in water, food and dairy products. Organisms which rapidly attack lactose produce purple colonies surrounded by purple haloes. Non-lactose or late-lactose fermenters produce pale colonies with greenish zones. Other related Gram negative bacteria may grow but can be suppressed by incubation at temperatures greater than 42°C or by anaerobic incubation.

APPENDIX 8 (Modified Rogosa Sharpe Agar Composition)

Formula	gm/litre
Peptone	10.0
“Lab-Lemco” powder	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan mono-oleate	1ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate 3H ₂ O	5.0
Triammonium citrate	2.0
Magnesium sulphate 7H ₂ O	0.2
Manganese sulphate 4H ₂ O	0.05
Agar	10.0
pH 6.2±0.2	

MRS medium was prepared by weighing 64g of powder into a litre of distilled water. The mixture was heated to boiling with frequent stirring until the powder was completely dissolved and then autoclaved for 15 minutes at 121°C. This medium is designed to encourage the growth of the lactic acid bacteria which includes species of the following genera: *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*. All these species can produce lactic acid in considerable amounts. They are Gram positive, catalase and oxidase negative and are fastidious in their nutritional requirements. Generally lactic acid bacteria show delayed growth and smaller colony size than other microorganisms. They may be overgrown in non-selective media.

APPENDIX 9

BWA (butanol, water and acetic acid)

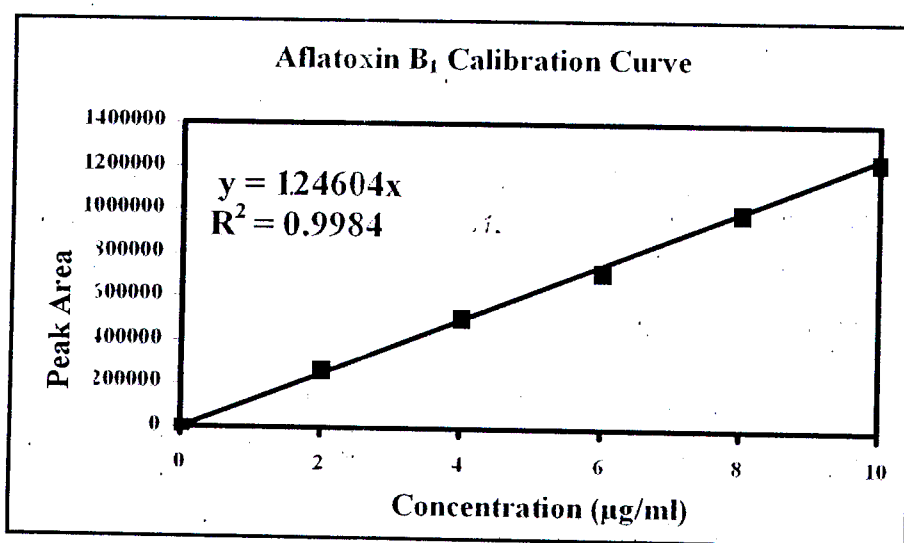
CEI (chloroform, ethyl acetate and isopropanol)

TEF (toluene, ethyl acetate and formic acid)

CtE (carbon tetrachloride and ethyl acetate)

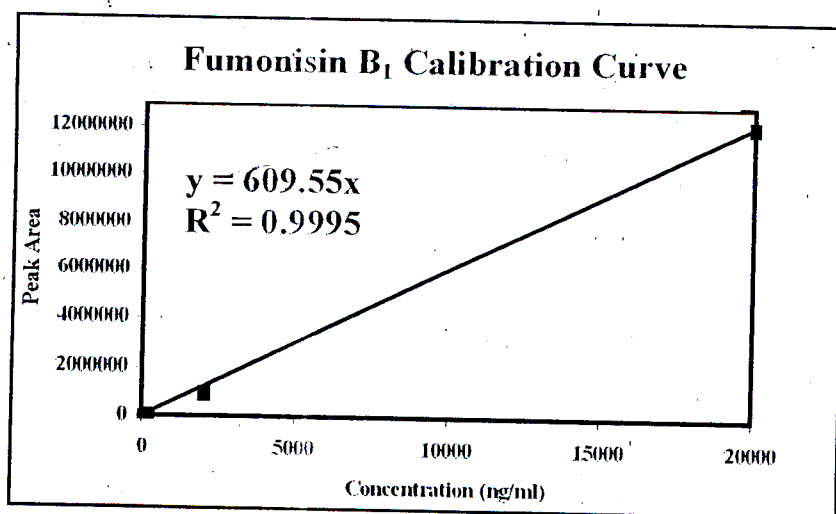
ChE (cyclohexane and ether)

APPENDIX 10



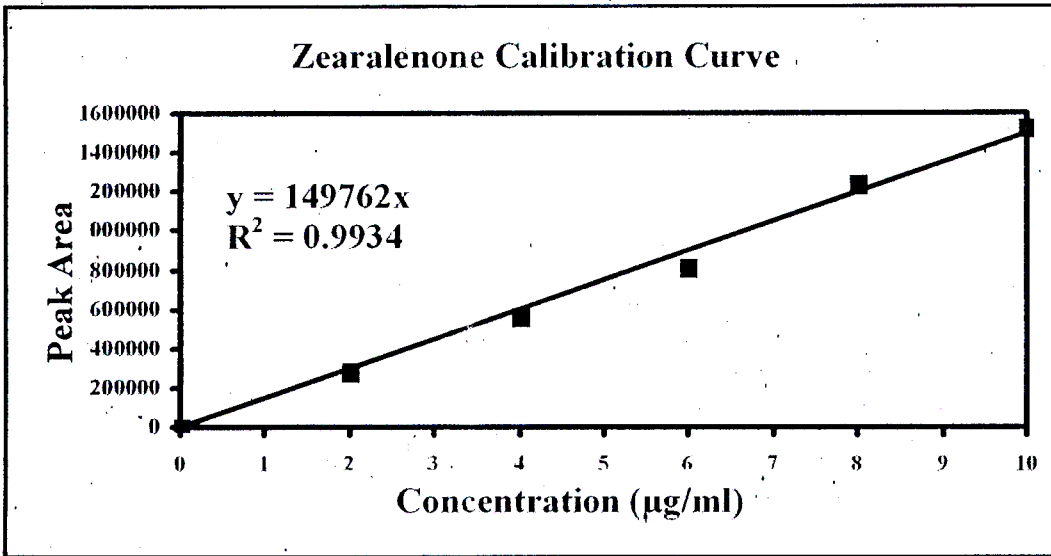
Standard curve for determining aflatoxin B₁ (AFB₁) concentration using HPLC.

APPENDIX 11



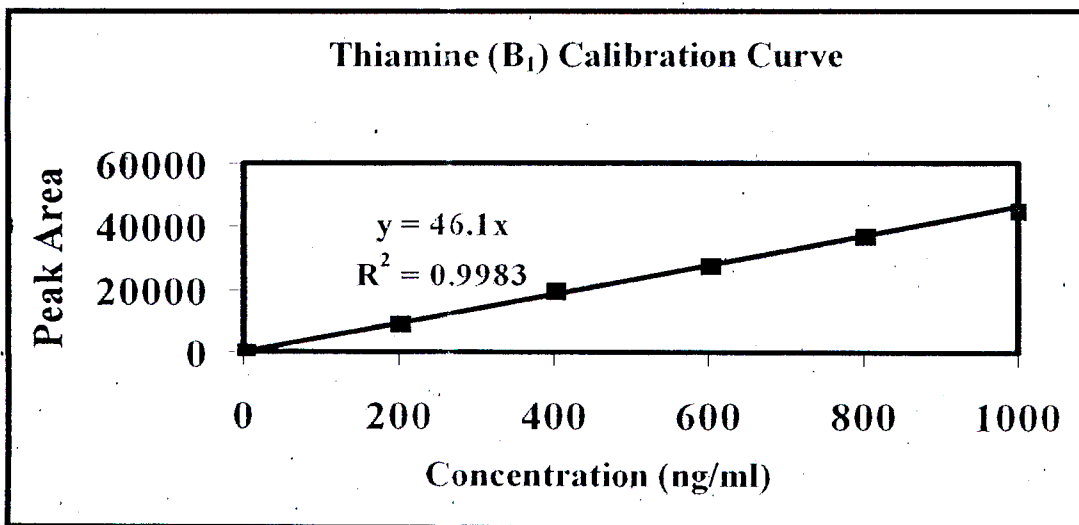
Standard curve for determining fumonisin B₁ (FB₁) concentration using HPLC.

APPENDIX 12



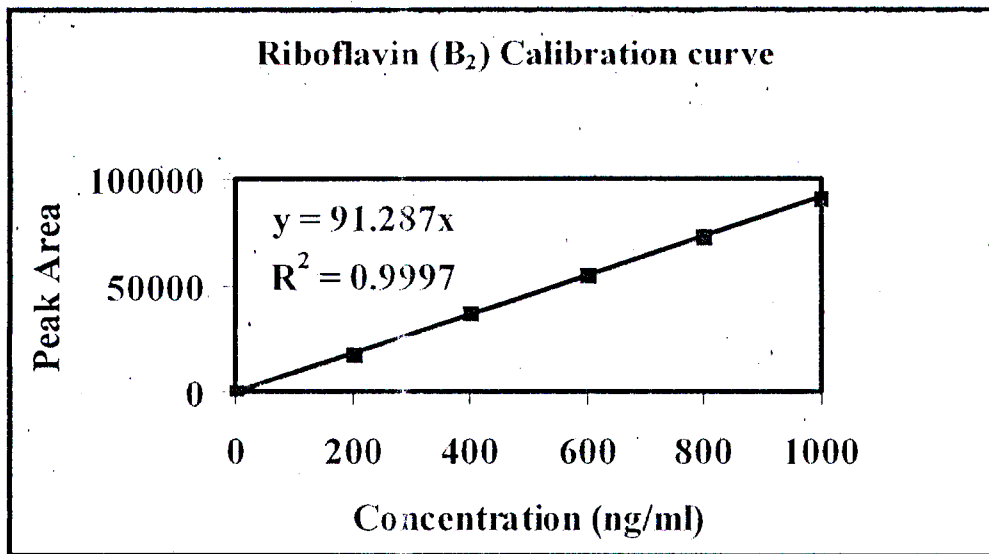
Standard curve for determining zearalenone (ZEA) concentration using HPLC.

APPENDIX 13



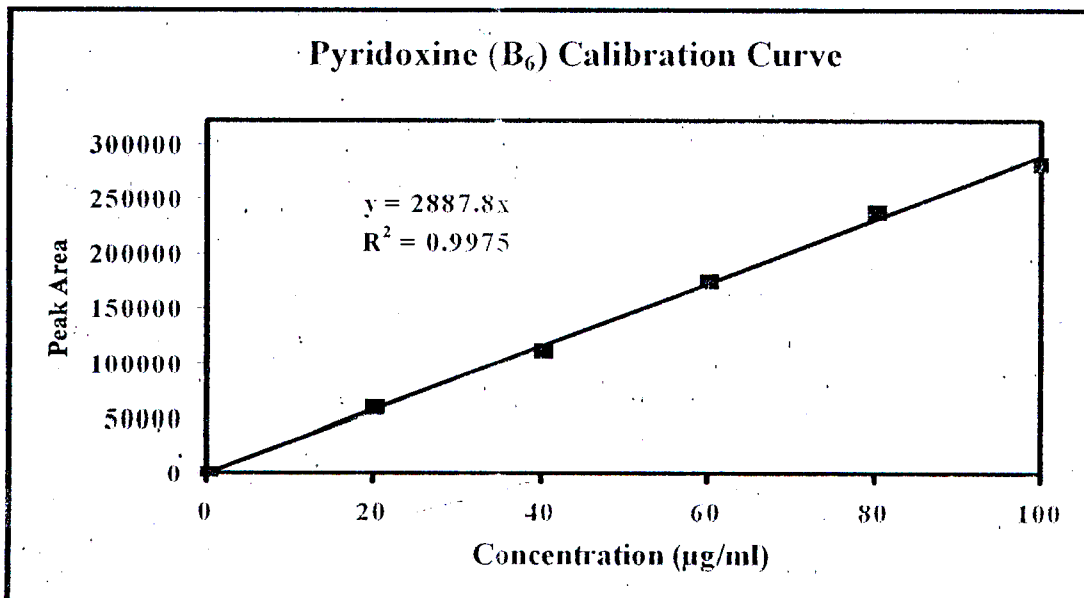
Standard curve for determining thiamine (vitamin B₁) concentration using HPLC.

APPENDIX 14



Standard curve for determining riboflavin (vitamin B₂) concentration using HPLC.

APPENDIX 15



Standard curve for determining pyridoxine (vitamin B₆) concentration using HPLC.

APPENDIX 16

Mobile phase for vitamin samples preparation.

H₂O:ACN:H₃PO₄ (24:1:0.1% of water and acetonitrile total volume).