CHAPTER 2

LITERATURE REVIEW

In this review, the metabolic process known as fermentation will be defined. The biochemical processes that occur during, as well as the end-products of fermentation that are important in food preservation will be looked at. The organisms involved in fermentation and how they alter food constituents will also be discussed. Examples of some popular traditional African fermented foods, their nutritional properties as well as how food products such as cereals and legumes or dairy products can be combined to improve the nutrient profiles of these foods will be looked at. The effects of fermentation on anti-nutritional factors and the health benefits of fermented foods will be reviewed. The manufacture of yoghurt, *mahewu* and *kishk* will be reviewed. An attempt will be made to show the work that has been done to change the production of some fermented foods from traditional processes to large scale manufacturing where the microbial and nutritional quality of the product and the biochemical processes occurring during fermentation can be controlled.

2.1 The role of the lactic acid fermentation in food preservation

2.1.1 Historical background

The origins of fermented foods in the diets of humans date back many thousands of years and usually predate the existence of written records of their production and consumption (Campbell-Platt, 1987).

Ancient Egyptians kept cows for their milk which was consumed as such or processed into other products. There are many drawings that illustrate the milking of cows and milk processing. It is believed that Egyptian fermented milks were some of the oldest known dairy products in the world (Abou-Donia, 1984).

In Europe, Asia and Africa, from time immemorial, sour milk was known as being more stable and advantageous than fresh milk. Fermentation preserves the high quality nutrients present in a form that has a longer shelf-life (Oberman & Libudzisz, 1998).

2.1.2 Fermentation

Metabolism can be defined as the sum total of all chemical transformations that occur in cells (Stanier, Adelberg & Ingraham 1980). Fermentation is a metabolic process in which carbohydrates and related compounds are oxidised with the release of energy in the absence of any external electron acceptors (Jay, 1978). According to Volk & Wheeler (1984), in fermentation electrons are not passed through an electron transport chain and oxidative phosphorylation does not occur. For example many micro-organisms convert sugars to pyruvic acid and NADH is formed. NADH must pass its acquired electrons on to some acceptor if the organism is to continue to metabolise. This is accomplished by using pyruvic acid or some other product formed from pyruvic acid as a final electron acceptor.

Lactic acid bacteria are perhaps the most widespread of desirable micro-organisms in food fermentations. They are found in fermented cereal products, milks, cheeses and fermented meats (Campbell-Platt, 1987). Lactic acid bacteria convert the available carbohydrate to organic acids and lower the pH of the food. These acids as well as other flavour compounds which include diacetyl, acetaldehyde and acetoin contribute to the desired taste and flavour of the food (Pederson, 1971). The low pH that is created also makes the food unfavourable for the proliferation of spoilage and pathogenic bacteria (Djien, 1982).

2.1.3 Biochemistry of the lactic acid fermentation

Lactic acid fermentations can be divided into two broad categories distinguishable by the products formed from glucose (Stanier et al., 1980). These are referred to as homofermentation and heterofermentation.

Homofermenters convert glucose to glucose -1,6- diphosphate using the Embden Meyerhof (EM) pathway (Dirar & Collins, 1972). The enzyme aldolase cleaves fructose–1,6-diphosphate between C_3 and C_4 to give the phosphate esters dihydroxyacetone phoshate and D-glyceraldehyde-3-phosphate. The reaction favours the production of the glyceraldehyde isomer at equilibrium. The end product in this fermentation pathway is lactic acid (de Vries & Stouthamer, 1968; Doelle, 1975) (Figure 1a).

The overall lactic acid fermentation pathway can be expressed as follows:

$$1\text{glucose} + 2NAD^+ + 2ADP + 2Pi \longrightarrow 2\text{Pyruvate} + 2NADH + 2H^+ + 2ATP$$

The homolactic acid fermentation pathway is important in the dairy industry. It is the pathway responsible for souring milk and is used in the production of yoghurt, cottage cheese and cream cheeses (Atlas, 1995).

In heterolactic fermentation (Figure 1b), the pentose phosphate pathway is used instead of the EM pathway of glycolysis. This type of fermentation produces ethanol and carbon dioxide in addition to lactic acid in the molar ratio 1: 1: 1.

The ethanol and the CO_2 come from the glycolytic portion of the pathway. There are two possible ways by which ethanol is formed (Caldwell, 1995). Acetaldehyde formed by the cleavage of pyruvate by pyruvate decarboxylase is reduced in the presence of alcohol dehydrogenase to form ethanol. Ethanol can also be formed by a combination of acetyl coA reduction to acetaldehyde followed by reduction of acetaldehyde by ethanol dehydrogenase.

Lactate is formed by direct pyruvate reduction with lactate dehydrogenase. Formic acid and acetyl coA are produced by the action of pyruvate-ferredoxin on pyruvate. Acetyl coA is converted to free acetic acid. Vinegars are characterised by a significant acetic acid content. While vinegar is not a food of major importance, in

most traditional African diets, it plays an important role as a highly effective food preseravtive. Formate is converted to CO_2 and hydrogen by the joint operation of formic dehydrogenase and hydrogenase (Volk & Wheeler, 1984).

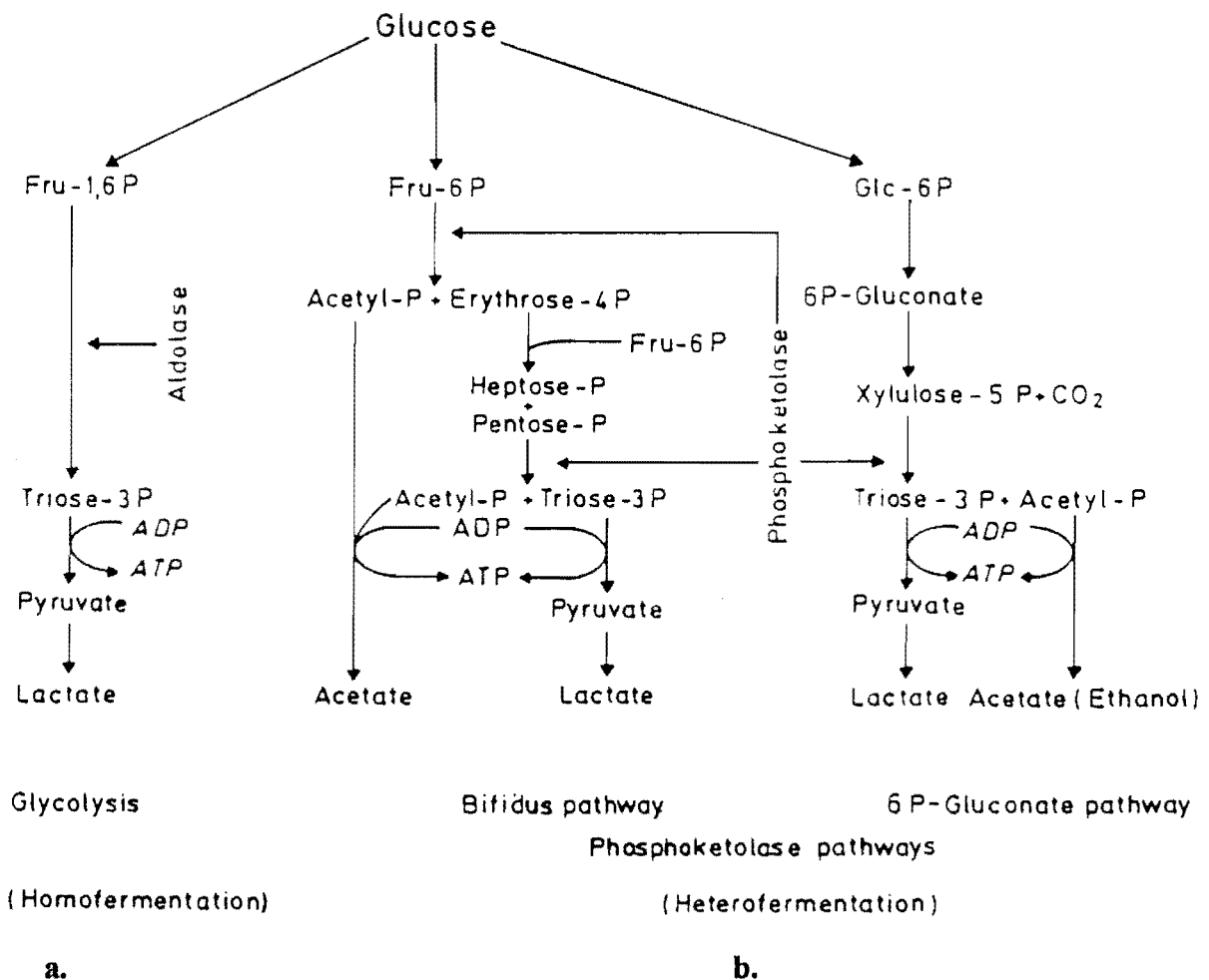The overall reaction for the heterofermentation reaction can be expressed as follows:

Glucose + ADP + Pi $\longrightarrow$ Lactic acid + Ethanol + CO_2 + ATP

Figure 1. The pathway for glucose dissimilation by homofermentative and heterofermentative bacteria (Kandler, 1983)

Mixed acid fermentation is a third type of fermentation that is carried out by members of the family *Enterobacteriaceae* that includes *Escherichia coli* and members of the genera *Salmonella* and *Shigella* (Prescott, Harley & Klein, 1993). These bacteria ferment glucose by the EM pathway to form pyruvate which is converted to succinate, ethanol, lactate, CO_2 and H_2 (Figure 2). According to Atlas (1995), succinate is formed by the carboxylation of phosphoenol pyruvate by phosphoenol pyruvate carboxylase to produce oxaloacetate. The oxaloacetate is converted to

9

succinate by the combined actions of malate dehydrogenase, fumarase and fumarase reductase. The remaining products of mixed acid fermentation arise from pyruvate metabolism. Lactate is formed by the direct reduction of pyruvate with lactate dehydrogenase. Formate and acetyl coA are produced by the action of pyruvate ferredoxin oxidoreductase. Acetyl coA is converted to free acetic acid with the intermediate formation of acetyl phosphate by the actions of phosphotransacetylase and acetyl kinase. This allows the coA bond energy of acetyl coA to generate ATP. A portion of acetyl coA is converted to ethanol by the actions of aldehyde and alcohol dehydrogenases. Formate is converted to CO_2 and H_2 by the joint operation of formic dehydrogenase and hydrogenase
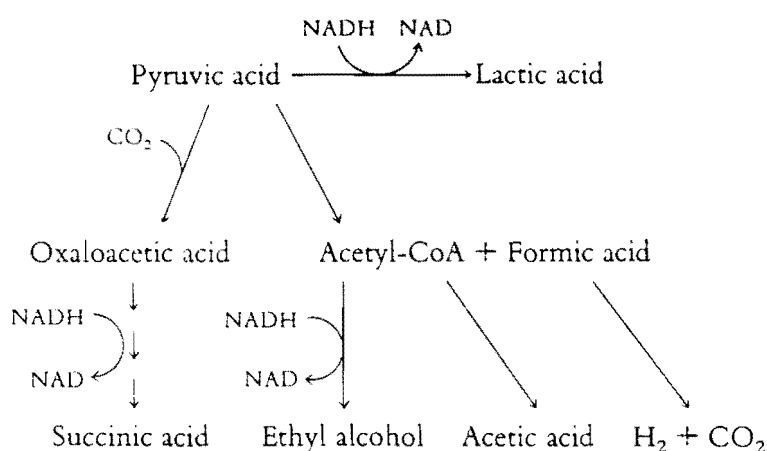

Figure 2. Mixed acid fermentation by some enteric bacteria (Prescott, Harley & Klein, 1993)

2.1.4 Micro-organisms involved in the lactic acid fermentation

The lactic acid bacteria are rod–shaped or spherical micro-organisms. Their name derives from the fact that ATP is synthesised through fermentations of carbohydrates, which yield lactic acid as a major and sometimes the sole end-product (Stanier *et al.*, 1980).

Lactic acid bacteria are unable to synthesise ATP by respiratory means, a reflection of their inability to produce cytochromes and other haem-containing enzymes (Prescott *et al.*, 1993). Lactic acid bacteria are unable to mediate the decomposition of hydrogen peroxide according to the following reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Another distinctive physiological feature of lactic acid bacteria is their tolerance of acid. Although the spherical lactic acid bacteria can initiate growth in neutral or alkaline pH, most of the rod-shaped forms cannot grow in media with an initial pH greater than 6. Growth of all lactic acid bacteria continues until the pH has fallen to a value of 5 or less (Jay, 1992).

The taxonomy of lactic acid bacteria is in a state of flux but the principle genera are *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactosphaera*, *Oenococcus*, *Weissella*, *Tetragenococcus* and *Vagococcus* (Berkeley *et al.* 1994; Adams & Nicolaides, 1997; Jay, 1998)

Lactobacilli are Gram positive, catalase negative rods that often occur in long chains. They can also be almost coccoid. They grow poorly in air but better under reduced oxygen tension. Although those in foods are typically microaerophilic, many true anaerobes also exist. They are chemoorganotrophic and require complex media. Their metabolism is fermentative and at least one of the carbon end-products is lactate. Lactobacilli are common in dairy products and are rarely pathogenic. Their optimum growth temperature is 30 to 40°C.

All members of the genera *Pediococcus* and *Streptococcus* are homofermentative, Gram positive, catalase negative cocci. Pediococci divide in two planes at right angles to produce tetrads of cells but sometimes only pairs of cells are seen. Single cells are rare and chains are not formed. They are facultative anaerobes although some strains

are inhibited on incubation in air. They are chemoorganotrophic and require nutritionally rich media and a fermentable carbohydrate (mainly mono- and disaccharides). Glucose is fermented with the production of acid but no gas. The major product is DL or L(+) lactate. The optimum growth temperature is 25-40°C.

Streptococci divide in one plane to produce chains (Stanier *et al.*, 1980). The cells are spherical or ovoid, occuring in chains or pairs. Some species are encapsulated. They are facultatively anaerobic chemoorganotrophs which require nutritionally rich media for growth and sometimes 5% carbon dioxide. Growth is generally restricted to a temperature of 25-45°C (optimum 37°C).

Leuconostoc are Gram positive, catalase negative cocci that are heterofermentative. The cells are spherical or somewhat longer than broad when in chains or pairs. Sometimes short rods with rounded ends occur in long chains. They grow rather slowly, producing small colonies that may be slimy on media containing sucrose. They are facultative anaerobes and chemoorganotrophic with obligate requirements for a fermentable carbohydrate as well as a nutritionally rich medium. Glucose is fermented with the production of D(-)- lactate, ethanol and gas. The optimum growth temperature is 20-30°C.

Carnobacteria are Gram positive, catalase negative, straight, slender rods. They occur singly or in pairs and sometimes in short chains. They may or may not be motile. They are non-sporing chemoorganotrophs that are heterofermentative. They produce mainly L (+)-lactate from glucose and gas is produced by some species. They grow at 0°C but not at 45°C (optimum 30°C). They differ from Lactobacilli in being unable to grow on acetate medium.

Enterococci were once a sub-group of the genus *Streptococcus*. The cells are spherical or ovoid and occur in pairs or short chains. They are sometimes mobile by means of scanty flagella. They lack obvious capsules, are facultative anaerobic and are chemoorganotrophs with fermentative metabolism. A wide range of

carbohydrates, including lactose, are fermented with the production of mainly L(+)-lactic acid. Gas is not produced. They are Gram positive and most can grow at 10°C and 45°C with an optimum temperature of 37°C.

Lactococci are Gram positive, catalase negative, non-motile spherical or ovoid cells that occur singly, in pairs or in chains. Endospores are not formed. They are non-motile and without capsules. They are facultative anaerobes which can grow at 10°C but not at 45°C (optimum temperature 30°C). They are chemoorganotrophs with fermentative metabolism. A number of carbohydrates are fermented with the production of L(+) -lactic acid but no gas. Their nutritional requirements are complex.

Vagococci are Gram positive, non-sporing, spheres, ovals or short rods which occur singly, in pairs or in short chains. Some motile by peritrichous flagella. They are catalase negative and chemoorganotrophic with a fermentative metabolism. They produce acid but no gas from a number of carbohydrates. Glucose fermentation yields mainly L(+)-lactate. They can grow at 10°C but not at 45°C (optimum temperature 25-35°C).

Lactosphaera, Weissella and Oenococci are heterofermenters. Weissella produce gas from carbohydrates. DL-lactate is the main product from glucose fermentation (Jay, 1998). Weissella were formerly classified as *Leuconostoc confusus* and *Leuconostoc paramesenteroides* (Schleifer & Ludwig, 1995). Oenococci were formerly classified as *Leuconostoc oenos* (Dicks, Dellaglio & Collins, 1995). The reclassification of lactic acid bacteria has been made possible by the development of modern taxonomic tools based on immunoassays, PCR techniques and DNA hybridisation methods (Ehrman, Ludwig & Scheifer, 1994).

Lactic acid bacteria differ with respect to the isomers of lactic acid that they produce. This is determined by the specificity of the lactic dehydrogenases which mediate pyruvate reduction. Some species contain only D-lactic dehydrogenase and hence

form the L-isomer. Some species contain two lactic dehydrogenases of differing stereospecificity and form racemic lactic acid (Stanier *et al.*, 1980).

The ability to convert carbohydrates to lactic acid, acetic acid, alcohol and carbon dioxide with only minor changes in the other food components has made this group of micro-organisms extremely important in the preservation of food (Pederson, 1971; Snoep & de Mattos, 1997). There is little calorific change in the conversion of carbohydrates to lactic acid and very little loss of total nutritive value. The lactic acid produced during fermentation is effective in inhibiting the growth of other bacteria that may decompose the food or make it poisonous (Adams & Hall, 1988; Jeppesen & Huss, 1993; Leisner, Greer, Dilts & Stiles, 1995).

2.1.5 Factors controlling fermentation

A number of intrinsic and extrinsic factors influence the intensity and particular type of fermentation (Tomkins, Alnwick & Haggerty, 1988). Intrinsic parameters are an inherent part of the food or beverage and include pH, water activity, oxidation–reduction potential (Eh) and nutrient content (Jay, 1978).

The pH of a solution describes the hydrogen ion concentration [H$^+$] (Conn, Stumpf, Bruening & Doi, 1987). Bacterial growth rates are greatly influenced by pH values and the effects are mainly based on the nature of proteins (White, Handler & Smith, 1967). Charge interactions within the amino acids of a polypeptide chain strongly influence the secondary and tertiary structure and folding of a protein (Ludescher, 1996). This change in shape of the active site of enzymes affects their function. Enzymes are normally inactive at very high and very low pH values (Atlas, 1995).

Most micro–organisms grow best at pH values around 7 (6.6–7.5). Lactic acid bacteria will grow at low pH (< pH 4) and through the production of lactic acid the pH is lowered further (Tomkins *et al.*, 1988). Many bacteria, particularly the spoilage bacteria and pathogenic bacteria, do not grow at such a low pH. *Listeria monocytogenes* is regarded as showing a poor ability to survive and grow at acid pH

and it is considered that it usually dies at a pH lower than 5.6 (George, Lund & Brocklehurst, 1988; Sorrells, Enigl & Hatfield, 1989). This has important consequences with regard to the shelf–life and safety of fermented foods and beverages.

All bacteria require water for growth and reproduction. Water is an essential solvent and is needed for all biochemical reactions in living systems (Conn, Stumpf, Bruening & Doi, 1987). The availability of water has a marked influence on bacterial growth rates (Jay, 1992). The water requirements of micro-organisms are defined in terms of water activity (a $_w$) in the environment. This parameter is defined by the ratio of the water vapour pressure of the food substrate to the vapour pressure of pure water at the same temperature i.e. $a_w = p/p_o$, where p = the vapour pressure of the solution and p_o = the vapour pressure of the solvent (usually water) (Atlas, 1995). The concept is related to relative humidity, R.H., in the following way: Relative humidity = 100 x a_w. The a_w of most fresh foods is 0.99. Most spoilage bacteria do not grow below a_w of 0.91. With respect to food-poisoning bacteria, *Staphylococcus aureus* has been found to grow at water activities as between 0.83 and 0.86 (Farber, Coates & Daley, 1992) while *Clostridium botulinum* does not grow at water activities below 0.95 (Gaze, 1992; Jay, 1992).

Micro–organisms display varying degrees of sensitivity to the oxidation-reduction potential (O/R, Eh) of their growth medium. The Eh of a substrate is referred to as the ease with which the substrate loses or gains electrons. A substance that readily takes up electrons is a good oxidising agent while one that readily gives up electrons is a good reducing agent (Jay, 1992). The more highly oxidised a substance is, the more positive will be its Eh and the more highly reduced a substance, the more negative will be its electrical potential. Aerobic micro–organisms such as those belonging to the genus *Bacillus* require positive Eh values (oxidised) for growth while anaerobic bacteria such as those belonging to the genus *Clostridium* require negative Eh values (reduced). Some aerobic micro-organisms grow better under slightly reduced

conditions and are often referred to as microaerophilics (Jay, 1992). Examples of microaerophilic bacteria are lactobacilli and streptococci.

To grow, micro-organisms must draw from the environment all the nutrients that they require for the synthesis of their cell materials and for the generation of energy. Water accounts for 80 to 90 % of the total weight of cells and is therefore always the major essential nutrient in quantitative terms (Stanier *et al.*, 1980). Micro-organisms also require carbon as a source of energy. Micro-organisms that are of importance in fermented foods get their carbon from organic nutrients. The nitrogen and sulphur requirements are often met by organic nutrients containing amino acids, proteins, or products of complex protein degradation such as peptones. Growth factors are a group of nutrients that the micro-organisms cannot synthesise. They include amino acids as constituents of proteins, purines and pyrimidines required as constituents of nucleic acids and vitamins (Frazier, 1967). Vitamins are a diverse collection of organic compounds which form parts of the prosthetic groups or active centres of certain enzymes.

Extrinsic parameters of foods are those properties of the storage environment that affect both the foods and their environment (Jay, 1992). Temperature is an important factor. Low temperatures reduce membrane fluidity and hence restrict transport of essential nutrients. Such temperatures also slow down enzyme reactions (Bronck, Madigan, Martinko & Parker, 1994). Below the minimum growth temperature, metabolic processes are too low to meet the requirements of the cell. Within the growth range for a particular micro-organism, there is an optimal growth temperature at which enzyme reaction rates are at their peak, the highest rate of reproduction occurs and growth rate is maximal (Atlas, 1995). The micro-organisms reproduce with the shortest doubling time (McKane & Kandel, 1996). Mesophilic lactic acid bacteria tend to grow best between 10 and 40°C with an optimum around 30°C and they include *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* as well as *Leuconostoc mesenteroides* subsp. *cremoris* (Oberman & Libudzisz, 1998). Thermophilic lactic acid bacteria such as those that are used to produce yoghurt are

represented by the two species *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. Some thermophilic species such as *Lactobacillus leichmanii (delbruekii)*, which is homofermentative, will grow at temperatures of between 48-50°C (Van der Walt, 1956). Raising the temperature above the maximum usually kills the microbial cells by denaturating protein and irreversibly damaging molecules essential to the cell's survival (Bronck *et al.*, 1994).

These various factors, particularly in traditional fermented cereal products where a spontaneous fermentation is often relied upon, result in a sequence of different micro-organisms responsible for the fermentation. Many lactic fermentations are initiated by spherical bacteria such as *Leuconostoc mesenteroides* and *Pediococcus acidilactici*. The rod-shaped bacteria take over and lower the pH to around pH 3.6 (Tomkins *et al.*, 1988). Then yeasts may grow in the final product and spoil it. Alternatively, in alcoholic beverages, the initial fermentation would be alcoholic due to yeasts, followed by a bacterial lactic acid fermentation which would ultimately spoil the product. The complexity of such systems poses great challenges when attempting to elucidate the micro-organisms of importance in a particular product.

2.2 The effect of fermentation on food safety

Diarrhoeal diseases are among the major causes of death in most developing countries and also a major factor in malnutrition in young children (UNICEF, 1988). In Africa cereal gruels and porridges are generally used as weaning food for infants. It can be assumed that a large part of the diarrhoeal diseases are food-borne and it has been confirmed that cereal-based weaning foods and water are important sources of pathogens (Mathur & Reddy, 1983).

The bacteria that are most common aetiological agents include enterotoxinogenic *Escherichia coli*, *Campylobacter*, *Shigella* and *Vibrio cholerae* (Fernandes, Shahani & Amer, 1987). Lactic acid bacteria in fermented gruels significantly suppress the growth of some food-borne pathogens (Svanberg, Sjogren, Lorri, Svennerholm & Kaijser, 1992). The lactic and acetic acids produced when the gruels ferment lower

the pH to less than pH 4.5. This strongly inhibits the pathogenic bacteria which do not grow at such low pH (Jay, 1992). The inhibition of the Gram positive *Staphylococcus aureus* is probably due to the formation of bacteriocins during the lactic acid fermentation as well as the effect of low pH (Svanberg *et al.*, 1992). The use of an inoculum, obtained from a previous gruel, has been found to be more effective than spontaneous fermentation in inhibiting the growth of enteropathogenic bacteria and this appears to be the result of the more rapid decrease in pH (Kingamkono *et al.*, 1995). On the other hand, the emergence of acid resistance in some enteropathogens such as *E. coli* 0157:H7 whereby food products such as yoghurt have been vehicles of infection means that the potential to enhance food safety should be assessed in the light of the total manufacturing process (Adams & Nicolaides, 1997).

A further advantage of fermentation, in addition to inhibiting the growth of pathogenic bacteria, is that it also slows down the rate of microbial spoilage of food (World Health Organisation, 1996). For the maximum benefits, fermented foods must be consumed without heating since such a treatment might eliminate viable bacteria in the product that may be beneficial (Hargrove & Alford, 1980). The importance of hygiene before and during fermentation cannot, therefore, be underestimated.

2.2.1 Fermentation end-products and food preservation

Organic acids

Lactic acid fermentation is characterised by the accumulation of organic acids, primarily lactic and acetic acids, and the accompanying reduction in pH (Berry, Liewen, Mandigo & Hutkins, 1990). Acid production is an efficient tool for inhibiting pathogenic and spoilage bacteria since organic acids produced during fermentation have broad antibacterial activities (Kociubinski, Perez, Anon & Antoni, 1996). Lactic acid, generated *in situ*, is traditionally used for improving food safety and shelf-life (Adams & Hall, 1988). Levels and proportions of organic acids produced depend on the species of the micro-organism involved, the chemical composition of

the culture environment and the physical conditions encountered during fermentation (Fields, Ahmed, & Smith, 1981; Lindgren & Dobrogosz, 1990; Sanni, 1993).

The preservative action of acids may be partly due to the depression of internal (cytoplasmic) pH (Russell, 1992). Undissociated acid molecules are lipophilic and pass readily through the plasma membrane by diffusion. In the cytoplasm (approximately pH 7) acid molecules dissociate into charged anions and protons (Salmond, Roll & Booth, 1984). These cannot pass across the lipid bilayer and accumulate in the cytoplasm thus reducing the pH. The acidified cytoplasm in turn inhibits metabolism, in particular the enzymes of metabolism (Krebs, Wiggins & Stubbs, 1983; Stratford & Anslow, 1998).

A principal target for many antimicrobial compounds is the plasma membrane and an alternative mechanism which may explain how acids act as preservatives is that they eliminate the proton motive gradient (Eklund, 1985). The selective permiability of the plasma membrane to protons allows the cells to create a pH gradient and an electrical potential which together form the proton motive force (Eklund, 1980). In the chemiosmotic theory, the energy contained in the proton motive force is then used to drive the uptake of essential nutrients such as amino acids (Bracey, Holyoak & Coote, 1998). The accumulation of charged acid particles in the cytoplasm disrupts the proton motive force and prevents uptake of amino acids (Freese, Sheu & Galliers, 1973).

Bacteriocins

Lactic acid bacteria are well-known for their production of antimicrobial proteins or peptides collectively known as bacteriocins (Gross & Morell, 1971; Kociubinski *et al.*, 1996). These protein complexes (protein aggregates, lipocarbohydrate proteins, glycoproteins) are active against Gram positive bacteria and normally known to display a narrow range of inhibitory activity that affects closely related species within *Lactobacillaceae* (Klaenhammer, 1988; Klaenhammer, 1993). Bacteriocins have been

isolated from fermented milk and dairy products (Litopoulou-Tzanetaki, 1987) and *mahewu* (Visser, Holzapfel, Bezuidenhout & Kotze, 1986)

The ability of many bacteriocins to inhibit some food-borne pathogens makes them attractive as potential food preservation agents. The best characterised bacteriocin produced by lactic acid bacteria is nisin. Nisin is produced by *Lactococcus lactis* subsp. *lactis* and has been available commercially in concentrated form since 1959 (Coventry, Gordon, Wilcock, Harmark, Davidson, Hickey, Hillier & Wan, 1997). Most micro-organisms require an intact plasma membrane (Bracey *et al.*, 1998). Nisin is strongly attracted to phospholipids in bacterial and liposomal membranes. Cationic nisin molecules initially interact by electrostatic attractions with anionic membrane phospholipids. They reorient themselves in the membrane such that they form non-selective pores (Muriana, 1996). The net result is that nisin makes the cytoplasmic membrane permeable which causes the release of accumulated amino acids from the cells as well as membrane vesicles of sensitive bacteria by leakage (Barrena-Gonzalez, Huot & Petitdemange, 1996; Muriana 1996).

Nisin exhibits broad spectrum inhibitory activity against Gram positive bacteria, including spore-forming bacteria (Klaenhammer, 1988). It inactivates thermophilic spoilage microorganisms in canned goods (Stevens, Sheldon, Klapes & Klaenhammer, 1991). Nisin and pediocin, a bacteriocin produced by *Pediococcus* species, have been shown to be effective in controlling *Listeria monocytogenes* in white pickled cheese, skim milk, yoghurt and other foods (Schaack & Marth, 1988; Abdulla, Davidson & Christen, 1993; Green, Dick, Bruggeman, van Damme & Chikindas, 1997; Ming, Webber, Ayres & Sandine, 1997).

2.3 The effect of fermentation on the nutritive value of foods

Food fermentations are very complex processes since they normally involve the interaction between plant or animal tissue and a group of microorganisms. This means that any changes that occur during fermentation will depend on the available nutrients and nutrient precursors in the raw materials, the metabolic activities of the

microorganisms responsible for the fermentation and any possible interactions of these elements (McFeeters, 1988).

2.3.1 Effect of fermentation on proteins

Changes in the nutritive value of proteins as a result of fermentation are particularly important in cereals and legumes. The proteins in cereals such as sorghum and millets are seriously deficient in the amino acid lysine and are, generally, poorly digestible in their cooked form (Klopfenstein & Hoseney, 1995). These sources of protein are therefore often of lower nutritional quality compared to animal products yet they tend to be the major dietary sources of protein for people with marginal or sub-marginal protein intake (McFeeters, 1988).

Fermentation processes that consistently improve the quality of protein or availability of protein from cereals and legumes could have a positive impact on the diets of many people. Conversely, any fermentation that results in the unnecessary loss of protein content and quality could particularly have a negative effect (Van Veen & Steinkraus, 1970).

Changes produced by fermentation are limited both in time and extent to which microorganisms are allowed to grow. Fermentation is believed to increase total protein content (Kazanas & Fields, 1981; Umoh & Fields, 1981). This is due to the decrease in starch and sugars as a result of hydrolysis by bacterial enzymes with the formation of volatile products. With homofermentative bacteria some of the products formed are lactic acid with small amounts of acetic acid and carbon dioxide. With heterofermentative bacteria other volatile products of fermentation are acetic acid, ethanol, lactic acid and carbon dioxide (Frazier 1967). This leads to changes in proportions of nutrient components. It is believed that the increase in protein content at the expense of starch is beneficial to consumers who need a higher protein intake (Steinkraus, 1994).

The total amino acid composition of yoghurt and other fermented milk products does not differ substantially from that of the milk from which they originate (Fernandes, Chandan & Shahani, 1992). During fermentation some lactic acid bacteria utilise milk proteins as a nitrogen source to ensure their growth. *Lactobacillus helveticus*, in particular, is recognised as possessing efficient protease and peptidase activities with respect to milk proteins (Fernandez, Bhowmik & Steele, 1994). The protein efficiency ratio of milk proteins is already very high and it was found that the bioavailability of yoghurt proteins, as measured using rats, was not improved significantly above that of milk.

Fermentation was found to increase the free amino acids as well as the quantity of the essential amino acids lysine, tryptophan and methionine (Umoh & Fields, 1981; Chavan & Kadam, 1989; Steinkraus, 1994). Contrary to the above- mentioned, results in fermentation studies done with foods of higher nutritional value such as milk, lysine decreased by as much as 40% when skim milk was fermented with *Lactobacillus acidophilus* (Rao, Pulusani & Rao, 1982). On the other hand fermentation studies with *tempeh*, a fermented soyabean product made with *Rhizopus oryzae or Rhizopus arrhizus*, showed little change in either methionine or lysine (Kao & Robinson, 1978).

2.3.2 Effect of fermentation on vitamins

According to McFeeters (1988) fermentations may result in changes in vitamin content by several mechanisms which include:

1. Synthesis of vitamins by the fermenting micro-organisms.
2. Loss of vitamins by metabolism of fermenting micro-organisms.
3. Loss of vitamins by metabolism of the fermenting food.
4. Loss of vitamins by chemical reactions not directly related to fermentation.
5. Increase/decrease in stability of vitamins due to pH changes.
6. Soaking or cooking losses associated with preparation of product prior to or after fermentation

During the manufacture of yoghurt, heat treatment of the milk causes losses in the amount of vitamin B_{12} (Rasic & Panic, 1961). Some lactic acid bacteria require B vitamins for growth while several lactic acid bacteria are capable of synthesising them. *Lactobacillus delbrueckii* subsp. *bulgaricus* require folic acid (Deeth & Tamime, 1981; Gilliland, 1990). Vitamins which increase during the manufacture of yoghurt are niacin and folic acid because they are actively synthesised *by Streptococcus salivarius* subsp. *thermophilus* during the manufacture of yoghurt. *Lactobacillus acidophilus* was found to increase folic acid levels in skim milk (Deeth & Tamime, 1981; Friend, Fiedler & Shahani, 1983).

Fermentation of cowpea flour using, *Rhizopus microsporus* var *oligosporus,* was found to improve the content of folacin, niacin and riboflavin (Prinyawiwatkul, Eitenmiller, Beuchat, McWatters & Phillips, 1996). During the fermentation of sorghum using *Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus fermenti* as test micro-organisms, an increase in niacin, thiamin and riboflavin was observed (Kazanas & Fields, 1981). Improvements in vitamin content, particularly the B vitamins, with fermentation are important and are due to microbial biosynthesis of the vitamins. This is important in foods such as polished rice which is deficient in Vitamin B_1 (Steinkraus, 1988).

2.3.3 The effect of fermentation on anti-nutrients

Raw ingredients such as cereals, legumes and tubers that are used to prepare fermented foods contain significant amounts of antinutritional and toxic components such as phytates, oxalates, tannins, cyanogenic glycosides, saponins, lectins and inhibitors of enzymes such as α-amylase, trypsin and chymotrypsin (Reddy & Pierson, 1994).

Polyphenols

Phenolic compounds have been divided into three categories: phenolic acids, flavonoids and tannins (Serna-Saldivar & Rooney, 1991). Phenolic acids are derivatives of benzoic acid or cinnamic acid. They may occur as free acids, soluble

esters or insoluble esters in cereals and are concentrated in the outer layers of the grain (Harn, Rooney & Earp, 1984). Flavonoids consist of two units: a C_6-C_3 fragment from cinnamic acid and a C_6 fragment from malonyl-coenzyme A (Serna-Saldivar & Rooney, 1991). Tannins are oligomers of flavan-3-ols and flavan-3,4-diols that are joined by a carbon-carbon bond between the C_4 of one unit and C_6 or C_8 of another (Reddy & Pierson, 1994). Tannins are able to precipitate alkaloids, gelatin and other proteins. Tannins are characteristic of the chemical defense of plants and act as barriers to predators such as insects and birds which may feed on such plants (Haslam, 1989).

Many millets test positive for traces of tannins but only finger millet contains condensed (non-hydrolysable) tannins chemically known as proanthocyanidins (reviewed by Serna-Saldivar & Rooney, 1991). White varieties of finger millet have been found to contain tannin levels that are lower than those of brown ones (Ramachandra, Virupaksha & Shadaksharaswamy, 1977).

A relationship between tannin levels and *in vitro* digestibility in some finger millet varieties has been established. Tannins in cereals can bind dietary proteins with the formation of indigestible protein-tannin complexes (Reddy & Pierson, 1994). Proteins interact with tannins by means of hydrogen bonding, hydrophobic interaction, electrostatic attraction and covalent bonding (Butler, Riedl, Lebryk and Blytt, 1984). In addition, dietary tannin could interfere with the digestion by inhibiting digestive enzymes. Phenols inhibit many enzymes *in vitro* including digestive enzymes such as trypsin and α-amylase (Chibber, Mertz & Axtell, 1980; Haslam, 1989).

Fermentation on its own does not lead to a decrease in the amount of tannins. It has been observed, however, that a combination of soaking, sprouting and fermentation reduces the level of assayable tannins probably due to polymerisation (Dhankher & Chauhan, 1987a; Khetarpaul & Chauhan, 1990a).

Enzyme inhibitors

Enzyme inhibitors that specifically inhibit trypsin and chymotrypsin have been identified in sorghum, Japanese millet and pearl millet (Klopfenstein & Hoseney, 1991; Serna-Saldivar & Rooney, 1991). Enzyme inhibitors are more prevalent in legume seeds which tend to contain various protease inhibitors. The peanut trypsin-chymotrypsin inhibitor complex was isolated from peanuts. The complex shows weak antichymotryptic activity while trypsin is rapidly inactivated. Peanut trypsin-chymotrypsin inhibitor possesses two reactive sites for trypsin and one reactive site for chymotrypsin (Ikenaka & Norioka, 1983).

One of the factors limiting the consumption and utilisation of cowpeas is the presence of protease inhibitors (Prinyawiwatkul *et al.*, 1996). As is the case with peanut inhibitors, the protease inhibitors in cowpeas are associated with trypsin. This prevents the complete utilisation of the proteins in cowpeas. Fermentation has been found to be ineffective in reducing trypsin inhibitor activity on its own although soaking and boiling are effective (Reddy & Pierson, 1994).

Phytates

Phytic acid (myoinositol 1,2,3,4,5,6 hexakis dihydrogen phosphate) occurs primarily as a salt of monovalent and divalent cations in discrete regions of cereal grains, legumes, some tubers and roots (Ologhobo & Fetuga, 1984; Reddy & Pierson, 1994).

The presence of high concentrations of phytic acid in cereals and legumes is of nutritional concern because of its ability to reduce the bioavailability of minerals, particularly divalent cations including zinc, calcium, iron and magnesium (McFeeters, 1988). Phytates also interact with enzymes such as trypsin, pepsin, α- amylase and β-galactosidase resulting in a decrease of their activity.

Fermentation of pearl millet has been shown to reduce phytic acid concentrations. When a traditional Indian food *rabadi*, which is made from pearl millet flour and buttermilk, phytic acid contents decreased (Dhanker & Chauhan, 1987a).

Cyanogenic glycosides

Cyanogenic glycosides are widely distributed in beans and tubers such as cassava. Cassava is an important staple food for about 500 million people in developing countries and bitter varieties are potentially toxic because of their cyanide content. In. West Africa cassava is processed into foods such as *gari* and *fufu*. Cassava is one of the few and by far the most important of human food crops in which the content of cyanide creates nutritional problems (Cooke & Coursey, 1981). Acid hydrolysis of cyanogenic glycosides yields hydrocyanic acid (HCN) which is a potent respiratory inhibitor. HCN inhibits cytochrome oxidase, a terminal respiratory catalyst. The glycoside found in cassava is linamarin (Hosel, 1981). The human body is capable of cyanide detoxification but the mechanisms involved increase the requirement for sulphur-containing amino acids. Thiocyanate is one of the detoxification products and it inhibits iodine absorption. This promotes goitre, which is one of the common ailments in developing countries (Narsted & Muller, 1983; Balagopalan, Padmaja, Nanda & Moorthy, 1988).

A combination of grating, fermenting and roasting is required for the effective removal of cyanogenic glucosides from cassava (Nout & Matorjemi, 1997).

In sorghum, although cyanogenic glycosides occur in most varieties their quantities depend on variety and environment The main cyanogenic glycoside, dhurrin, occurs mainly in the leaves of developing sorghum plants (Serna-Saldivar & Rooney, 1991). In the Sudan, the high incidence of goitre has been found to be associated with a goitrogen called thioamide which occurs in pearl millet. Fermentation of grain has not been found to be effective in reducing the levels or activity of goitrogens (Klopfenstein & Hoseney, 1991).

2.4 Apparent health benefits of fermented foods

2.4.1 Improved lactose utilisation

Lactose intolerance describes a situation in which an individual lacks adequate ability to digest lactose. This inability is for the most part due to an insufficient amount of the enzyme β-galactosidase in the small intestine. The usual symptoms associated with this problem include cramps, flatulence and diarrhoea following the consumption of milk products (Fuller, 1989).

Lactose-intolerant individuals can consume certain fermented dairy products without harmful effects. Where beneficial effects have been found, they have been attributed to the reduced level of lactose in the fermented product and to the production of β-galactosidase by the fermenting micro-organisms following ingestion of the products (Blanc, 1984). The bacteria used to make yoghurt contain the enzyme β-galactosidase which can improve lactose utilisation by lactose-intolerant individuals. Being intracellular, β-galactosidase of the yoghurt starter culture bacteria seems to be able to survive passage through the stomach to reach the small intestine (McFeeters, 1988).

2.4.2 Hypocholesterolemic activity

Risks of heart attacks in hypercholesterolemic individuals can be significantly reduced by lowering their plasma cholesterol (Fuller, 1989). It has also been postulated that cholesterol is lowered due to a factor produced or enhanced by the action of the starter culture bacteria during fermentation (Jay, 1992). The factor may lead to decreased synthesis of cholesterol and removal of cholesterol or its precursors from the gastrointestinal tract. The factor may also inhibit cholesterol synthesis in the body (Danielson, Peo, Shahani, Lewis, Whalen & Amer, 1989; Gilliland, 1990; Akalin, Gonc & Duzel, 1997). In another study done with rats, the hypocholesterolemic effects of *Lactobacillus gasseri* were established and they were attributed to the ability of the culture to suppress the reabsorption of bile acids into

the enterohepatic circulation and to enhance the excretion of acidic steroids in faeces of hypercholesterolemic rats (Usman & Hosono, 2000).

2.4.3 Antagonistic actions towards enteric pathogens

Most lactic acid bacteria will exert antagonistic action towards pathogens *in vitro*. *Lactobacillus acidophilus* and *Bifidobacterium bifidum* have received the most attention in their inhibitory activity toward the commonly known food-borne pathogens (Fuller, 1989). Both micro-organisms have been shown to be both preventative and therapeutic in controlling intestinal infections through administration of milk containing one or both micro-organisms. The exact mechanism whereby dietary cultures of lactobacilli may inhibit intestinal pathogens is not clear. It is likely that the acids produced by the micro-organisms may be involved in such antagonistic action (Fuller, 1986). Other studies, however, seem to suggest that neither *Lactobacillus* nor *Bifidobacterium* have an effect on intestinal infections (Fuller, 1989).

2.4.4 Anti-cancer effects

Intestinal microflora may be involved in such intestinal diseases as colon carcinogenesis (Ling, Korpela, Mykkanen, Salminen & Hanninen, 1994; Fonden, Mogensten, Tanaka & Salminen, 2000). Anaerobes such as *Peptostreptococcus* and *Clostridium* as well as *E. coli* produce high amounts of β-glucuronidase and nitroreductase, enzymes which increase the rate of conversion of indirectly acting carcinogens into proximal carcinogens (Cole, Fuller, Mallet & Rowland, 1985). β-glucuronidase influences the enterohepatic circulation of carcinogenic conjugates and nitroreductase enhances the formation of reactive N-nitroso and N-hydroxy intermediates thereby converting aromatic nitro-compounds into potentially harmful amines (Ling *et al.*, 1994).

There is interest in understanding how the colonic flora can be modified through diet. Diet influences the activities of these enzymes in the intestinal tract (Goldin & Gorbach, 1984). Among the food products studied as potential modifiers of the

colonic microflora, dairy products containing viable organisms have received the most attention (Marteau, Pochart, Flourie, Pellier, Santos, Desjieux & Ramboud, 1990; Jay, 1992; Sreekumar & Hosono, 2000). Results by Ling *et al.* (1994) confirm a reduction in faecal β-glucuronidase and nitroreductase activities after ingestion of *Lactobacillus*. They suggested that the partial replacement of the flora in the gastrointestinal tract by *Lactobacillus* could reduce the levels of these enzymes.

2.5 Fortification of fermented foods

Although cereals contribute significantly to the nutritional requirements of a large population in sub-Saharan Africa, their nutritive value is low (Plahar, Leung & Coon, 1983). *Mahewu,* a fermented maize-based beverage, has a low protein content of 7-9% on dry basis (Schweigart & Fellingham, 1963). One litre of mahewu containing 10% solids produces about 4000 kJ, derived from the carbohydrate in the maize meal (Schweigart, Van Bergen, Weichers & de Wit, 1960). If consumed by people on a fully adequate diet, the nutritional value of *mahewu* would be of little importance and there would be no need for fortification. Where malnutrition is prevalent, fortification of mahewu would help to alleviate the problem (Schweigart & Fellingham, 1963). Methods of fortifying *mahewu* that have been suggested include the use of milk, whey, sour milk products, skim milk powder, whey protein, soya flour, food yeast, blood or fish flour (Bates, Wu & Murphy, 1974; Green, Lawhon, Cater & Mattil, 1976; Hesseltine, 1983; Serna-Saldivar, Canett, Vargas, Gonzalez, Bedolla & Medina, 1988). One litre of fortified *mahewu* would then provide about 30% of the daily assimilable protein requirement of an adult human male and between 20% and 30% of the requirement for thiamine and nicotinamide (Schweigart & de Wit, 1960).

Cereal proteins are deficient in some essential amino acids, particularly lysine (Virupaksha & Sastry, 1968; Nche, Nout & Rombouts, 1994). To overcome the problems of protein energy malnutrition, fortification of the commonly used cereal products with inexpensive protein is needed (Del Valle & Perez-Villasenor, 1974). The complementary effect of amino acids from one protein on the nutritive value of another is recognised (Wang & Hesseltine, 1981). An economical and practical way

of improving protein quality and quantity is the combined use of complementary proteins. Vegetable protein sources such as legumes and oil seeds (Bressani, Murillo & Elias, 1974; McPherson & Ou, 1976) are high in lysine, an essential limiting amino acid in most cereals (Shekib, Zoual, Youssef & Mohamed, 1986; Akpapunam & Sefa-Dedeh, 1995). Cereal grains, on the other hand, have adequate amounts of sulphur-containing amino acids while legume proteins are deficient in these amino acids (Wang & Hesseltine, 1981).

Milk can also be used to complement the proteins in cereal grains. An example of a product in which this has been successfully done is *kishk*, a popular fermented food in parts of the Middle East, which is made by mixing boiled, dried and ground wheat grains with fermented milk (Hafez & Hamada, 1984). Dried *kishk* can have up to 23% protein and can also have as much lysine as 310 mg/g nitrogen compared to the Food and Agriculture Organisation provisional pattern of 270 mg/g nitrogen (El-Gendy, 1983). Combining the two proteins in the proper proportions results in a mixture that is nutritionally superior to each one alone (El-Sadek, Zawahry, Mahmoud & El-Motteleb, 1984). The high protein content of kishk and the complementary effect which the milk proteins exert on the lysine deficient wheat may make this product comparable to milk in protein nutritional quality (Hamad & Fields, 1982; Hafez & Hamada, 1984).

Fortification of maize meal with soya flour (Plahar *et al.*, 1983) was found to improve protein quality of fermented maize dough foods in Ghana. The fortified flour was found to be as acceptable as the traditional maize dough.

In Nigeria, inclusion of cashew nut meal, locust bean meal and sesame oil meal in maize-based flour was found to improve the total amino acid profile of fermented gruels that are commonly used as weaning foods (Ekpenyong *et al.*, 1977).

In Ghana the supplementation of maize with cowpea during the preparation of the fermented maize dumpling *kenkey* was found to improve the protein content of the

product. The product also compared well with the traditional product in terms of its sensory characteristics (Nche *et al.*, 1994). Similar studies with a weaning food developed form maize and cowpea again confirmed that fortification improved the protein content of the product (Akpapunam & Sefa-Dedeh, 1995).

Other projects carried out include the fortification of sorghum with green gram which was found to increase the proteins, free amino acids, soluble proteins and *in vitro* digestibility of the protein (Chavan & Kadam, 1989).

In the following section, three examples of fermented products will be discussed as follows: *Mahewu* as an example of a fermented food produced from cereals, yoghurt as an example of a fermented product from milk and, lastly, *kishk* a fermented cereal-milk composite. The discussion will focus on the biochemical and microbiological processes that are involved in the traditional and industrial processing of these products.

2.6 *Mahewu* - An example of a cereal fermented beverage

2.6.1 Background
In southern Africa, *mahewu* is a traditional fermented beverage that is prepared using maize. It contains little or no alcohol, has a pH of about 3.5 and is popular among the black people of southern Africa (Holzapfel, 1989).

2.6.2 Traditional processing of *mahewu*
Traditionally, *mahewu* is prepared by boiling a thin maize porridge containing 8 to 10% solids. Cooking of the maize leads to gelatinisation of the starch. Gelatinisation is characterised by swelling of the starch granules, leaching of the starch components (especially the amylose), increase in the viscosity of the porridge and increased susceptibility to enzymatic digestion (Kent & Evers, 1994; Lii, Tsai & Tseng, 1996). The porridge is allowed to cool to room temperature (25-30°C). In South Africa, a small quantity of wheat flour or bran (2-5% of the maize meal) is added and mixed

throughly into the porridge. In Zimbabwe a handful of malt made from finger millet or sorghum is added instead of the wheat flour or bran. The wheat flour, bran or malt acts as a source of inoculum and as a source of enzymes (Van der Merwe, Schweigart & Cachia, 1964), particularly α-amylase which produces a small amount of maltose which is utilised in the fermentation. The enzyme partially hydrolyses the gelatinised starch making the gruel softer, or even liquefying it. This offers the possibility of making a porridge of acceptable viscosity but with a higher energy density.

The inoculated mixture is left to ferment in a warm place. In Zimbabwe, fermentation is generally carried out in clay pots where the residue in the pot from previous fermentations provides the inoculum for fermentation. The lactic acid bacteria consist of mesophilic strains that are capable of rapid growth in the porridge. Sugars derived form the enzymatic hydrolysis of starch are fermented to lactic acid and other metabolic products such as carbon dioxide, acetic acid or ethanol (Holzapfel, 1989).

The main micro-organisms in native *mahewu* are *Leuconostoc mesenteroides* and *Lactobacillus brevis*. The traditional spontaneous souring process is not suitable for large scale production of *mahewu* because it is too slow and proceeds too irregularly. The development of other types of undesirable bacteria can produce secondary fermentations, the products of which (e.g. acetic acid or butyric acid) influence the taste negatively. *Mahewu* should have a pH of around 3.3, a titratable acidity of 0.4 to 0.5% (Schweigart & De Wit, 1960) and contain very little alcohol [a maximum of 0.25% (w/w)] (South African Bureau of Standards, 1990).

2.6.3 Industrial production of *mahewu*

In South Africa the making of *mahewu* has been industrialised to meet the demand by the increasing urban market of black people. A flow diagram of a modern processing plant is shown in Figure 5. The porridge is prepared by first mixing 8% of maize meal with water at ambient temperature and then pumping the mixture into stainless steel pots where it is heated at 85 to 90°C for 20 to 30 min. Heating not only gelatinises the starch making it more susceptible to enzymatic hydrolysis, it also kills any micro-

organisms in the maize meal that may compete with the starter culture and spoil the product (Holzapfel, 1989).

The porridge is cooled to 50°C before it is transferred to the bioreactors where 1 to 2% sugar and 0.1 to 0.2% wheat flour are added followed by 7 to 12% of the thermophilic *Lactobacillus* starter culture (an adapted pure culture of *Lactobacillus delbrueckii*) (Schweigart, 1970). The sugar provides a readily hydrolysable source of energy for the starter culture. Starch has to be broken down into a mixture of maltose and dextrins by bacterial amylases and then into glucose which means the energy to bacteria is supplied more slowly. During the next 17 to 29 h, the temperature gradually falls to about 30°C and the pH reaches 3.4 to 3.8. When the desired pH has been attained, about 2% sugar (based on the total volume) is added as a sweetener. A sharper decline in pH and culture activity is experienced in winter when the final temperature ranges from 3.5 to 3.9 compared to 3.1 to 3.6 in summer (Holzapfel, 1989).

Initial starter culture preparation occurs in the pre-fermenter where porridge at 50°C is inoculated with 10% of an active culture. Within 18 to 24 h at 30°C, the pH gradually drops and reaches a range of 3 to 3.4.
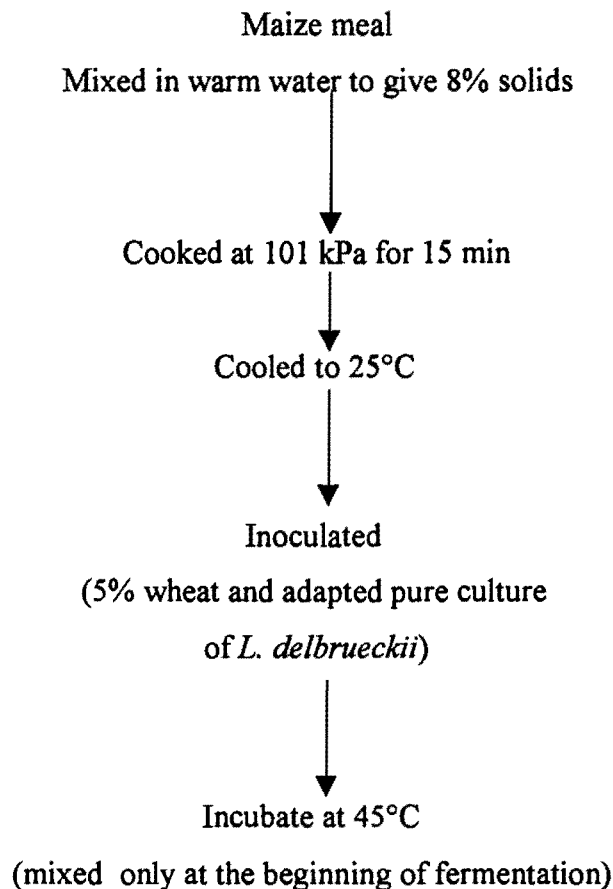
Maize meal

Mixed in warm water to give 8% solids

Cooked at 101 kPa for 15 min

Cooled to 25°C

Inoculated

(5% wheat and adapted pure culture

of *L. delbrueckii*)

Incubate at 45°C

(mixed only at the beginning of fermentation)

Figure 3. Summary for the improved *mahewu* fermentation process (Schweigart & Fellingham, 1963)

Lactobacillus delbrueckii is the micro-organism of choice because its optimum temperature is high and at this temperature the development of undesirable micro-organisms is suppressed. This starter culture is also known to produce large quantities of lactic acid with few by-products, thus giving a relatively pure lactic acid flavour. It remains active until a low pH has been attained and it produces lactic acid by the fermentation of glucose, maltose, sucrose, fructose, galactose and dextrins (Holzapfel, 1989). Fermentation is improved by the addition of bran and high quality proteins also improve the activity of lactic-acid bacteria. To maintain the high rate of acid production, the addition of buffering salts such as $CaHPO_4$ may be necessary. The

degree of sourness required depends on individual taste but is usually on average 0.4-0.5% titratable acidity, calculated as lactic acid, at which the average pH is 3.5 (Schweigart & Fellingham, 1963).

2.6.4 The microbiology of industrial *mahewu* production

During the early 1950s when the first attempts were made to produce *mahewu* on a large scale, the problems that were encountered included the development of undesirable micro-organisms including yeasts, *E. coli* and even clostridia due to the mesophilic conditions (30°C) under which fermentation occurred, irregular unpredictable fermentation due to contamination leading to the production of volatile fatty acids such as acetic and butyric acids and off-flavours (Holzapfel, 1989).

Pure cultures of *Lactobacillus acidophilus*, *L. bulgaricus*, *L. delbrueckii* and *Streptococcus lactis* have been adapted to the maize meal substrate at 51°C *(Streptococcus lactis* at lower temperatures). Factors that have been found to have a stimulatory effect on the starter cultures are buffer salts in the form of KH_2PO_4 and proteins such as yeast extract, soya and whey powder (Schweigart & Fellingham, 1963).

The most important micro-organisms that can cause the spoilage of *mahewu* are yeasts. The major spoilage yeasts are those belonging to the *Pichia* spp. (Holzapfel, 1989). Yeasts will ferment carbohydrates to form ethanol. *Acetobacter liquefaciens* was found to be another major spoilage micro-organism. This micro-organism converts lactic acid into acetic acid leading to off-odours. It also causes discolouration of the product (Holzapfel, 1989).

2.6.5 Biochemical changes during the processing of *mahewu*

The main product of homofermentative *Lactobacillus* cultures is lactic acid which has very little flavour and aroma but has a distinct, refreshing sour taste. In traditionally processed *mahewu* where heterofermentation occurs, in addition to lactic acid, acetic acid and butyric acids are formed which contribute to the flavour and aroma of the product (Schweigart, 1970).

Maize meal has very little buffering capacity and the pH quickly drops to less than 3.5. The addition of buffering salts such as $CaHPO_4$ and protein rich supplements means that the buffering capacity of *mahewu* will be improved and the micro-organisms can produce more acid (Schweigart & Fellingham, 1963).

Sugar is added at a rate of between 1 and 2% in the modern procedure for making *mahewu*. The sugar is readily fermented and provides energy for the starter culture. In the traditionally processed product, fermentation sugars are derived from the wheat flour enzymes hydrolysing the starch in the maize porridge (Schweigart *et al.*, 1960).

2.6.6 The role of starch in fermentation of gruels
In order to appreciate the relationship between viscosity and starch in fermented gruels it is important to look briefly at the structure of starch granules.

Cereals store energy in the form of starch. In addition to its nutritional value, starch is important because of its effect on the physical properties of many foods (Hoseney, 1994). α-D-glucose is the building block of starch and the polymerisation of glucose in starch results in two types of polymers. According to Thomas & Artwell, (1999) amylose is considered to be essentially a linear polymer composed almost entirely of α-1,4-linked D-glucose. Amylopectin is a branched polymer that is much larger than amylose. It is composed of α-1,4-linked glucose segments connected by α-1,6-linkages. Amylopectin has two distinct populations of chain lengths. The smaller chains are thought to be in such close proximity that they interact strongly resulting in crystalline regions that are extensive and arranged regularly with respect to each other throughout the starch granule. Compared with the crystalline areas, amorphous regions are generally degraded more easily by acid and enzymes. While the location of amylose within the granule remains unknown, amylose might be an important component of the amorphous areas (Kent & Evers, 1994).

The increase in viscosity that occurs when a suspension of starch in water is heated is a result of the starch taking up water and swelling substantially. The heat disrupts the hydrogen bonds that hold the polymer chains. It is believed that initial swelling occurs in the amorphous regions of the granule where hydrogen bonds are less numerous and polymers are more susceptible to dissolution (Thomas & Artwell, 1999). With continued heating and swelling, the granule becomes distorted and soluble starch is released into the solution. The soluble starch and the continued uptake of water by the remnants of starch granules are responsible for the increase in viscosity (Hoseney, 1994).

Cooked starch suspensions undergo firming and syneresis with cooling. The process is known as retrogradation. Retrogradation refers to changes that occur in gelatinised starch from an initially amorphous state to a more ordered or crystalline state (Gudmundsson, 1994).

According to Klucinec & Thompson (1999), during retrogradation amylose may form double helical associations of 40-70 glucose units whereas amylopectin forms shorter double helices than amylose due to the restrictions imposed by the branching structure of the molecules and the chain lengths of the branches. Double helices may associate and organise into crystallites and gelation results.

The starch gel is made up of a small amount of solid material which holds a large amount of water and can be visualised as starch chains with layers of water molecules attached by hydrogen bonding (Hoseney, 1994). As the paste is cooled the starch chains become less energetic and the hydrogen bonds become stronger giving a firmer gel.

The viscosity of foods containing starch depends not only on the temperature but also on the presence of such constituents as food acids (Whistler & Daniel, 1985). Acidic conditions have a significant impact on the viscosity and texture of starch gels. Native (i.e. unmodified) starches are typically unstable under acid conditions and tend to

undergo viscosity breakdown at low pH (Lineback & Inglett, 1982). Acid is believed to penetrate the amorphous parts and to hydrolyse the glucosidic bonds. The acid cannot penetrate the crystalline areas and they remain intact (Thomas & Artwell, 1999). The effect of low pH is therefore a reduction in molecular weight of the starch molecules and hence the viscosity of the gels (Hoseney, 1994).

Although a reduction in the viscosity would be desirable in the production of gruels for feeding infants, research on the viscosity reducing effects of fermentation have yielded inconsistent results. Westby & Gallat (1991) found that fermentation before cooking had little effect on the viscosity of sorghum porridges. Mbugua, Ledford & Steinkraus (1983) also concluded that the acidification resulting from the fermentation of *uji* (a thin fermented gruel made from sorghum, maize or millets and popular in East Africa) was not sufficient to reduce the viscosity of the gruel.

On the other hand, work by Lorri & Svanberg (1993) showed a reduction in the viscosity of gruels. They found that the viscosity of sorghum porridge was reduced considerably when the pH was lowered to pH 3.6-3.8 during fermentation with a culture of *Lactobacillus plantarum*. As a result the amount of flour required to produce a porridge of semi-liquid consistency was increased to 14-17% compared to 10-14% in the unfermented porridge.

To sum up, it appears as if the effect of reduced pH on viscosity depends on the method of fermentation and the type of micro-organism responsible.

2.7 Yoghurt - An example of a fermented dairy food

2.7.1 Background
Yoghurt is a traditional food in the Balkans and the Middle East but its popularity has spread throughout the world (Tamime & Deeth, 1980). Yoghurt can be manufactured using milk from cows, sheep, goats, buffalo and camels.

2.7.2 The traditional procedure for making yoghurt

The procedure for making yoghurt has evolved from the traditional fermentation which was carried out under ambient conditions (Figure 3). It involved the successive inoculations of the starter culture. The bacteria whose growth was encouraged under these conditions were *Leuconostoc cremoris, Lactobacillus casei and Lactobacillus plantarum* (Odunfa & Oyewole, 1998). These were natural contaminating micro-organisms present in the milk or residing in the cracks and crevises of the milk containers (Thunell & Sandine, 1985). One of the set-backs of this procedure was that the low incubation temperatures (ambient) resulted in slow acidification of milk. This led to undesirable side-effects such as whey syneresis that adversely affected the quality of the yoghurt. Another set-back was that the procedure provided no control over the level of lactic acid produced during the manufacture (Tamime & Deeth, 1980).

Boil milk to cause partial concentration

(about two thirds of original volume)

↓

Cool to incubation temperature

(i.e. blood or ambient temperature)

↓

► Inoculate with starter culture

↓

Incubate in bulk until coagulum is formed

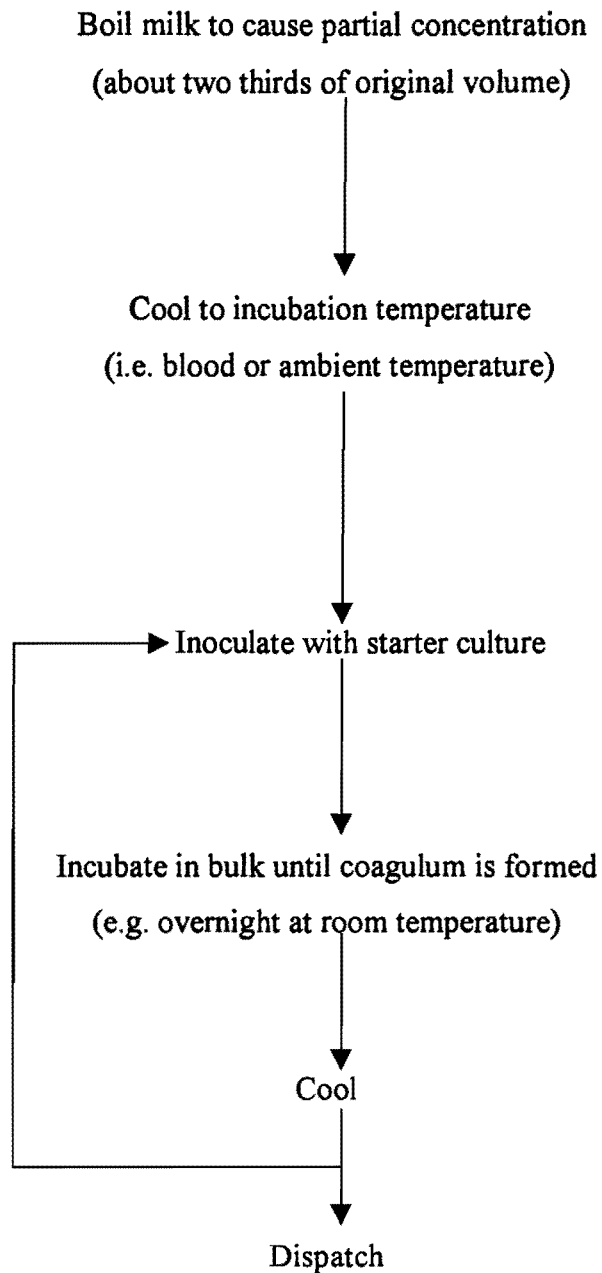(e.g. overnight at room temperature)

↓

Cool

↓

Dispatch

Figure 4. The traditional procedure for making yoghurt (Tamime & Robinson, 1985)

2.7.3 The modern process for making yoghurt

Yoghurt is a coagulated milk product obtained by lactic acid fermentation of milk, with or without additives (whole milk powder, skim milk powder or whey powder) through the action of *Lactobacillus delbruckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (Sharma & Prasad, 1986). In the modern large and small-scale manufacture of yoghurt, the process involves a number of distinct steps (Figure 4).

Increasing the total solids content of milk increases the firmness and viscosity of yoghurt. It is often considered that unless the solids content of milk is increased to 16-18%, the yoghurt gel will be weak and prone to syneresis (Tamime & Deeth, 1980). The solids content of milk can be increased by addition of skim-milk powder, whey protein concentrate or sodium caseinate (Tamime & Robinson, 1985).

Yoghurts can have fat contents ranging from 0.5 to 10%. Fat gives the perception of creaminess and improves the mouth feel of the yoghurt products. Homogenisation of milk for yoghurt manufacture prevents fat separation during storage, improves consistency, increases whiteness and reduces whey separation (Lampert, 1964).

Heat treatment of milk is considered to be one of the most important parameters affecting the texture of acid-milk gels such as yoghurt. Optimum conditions for heating milk are a temperature of 80-85°C with a holding time of 30 min. The objectives of heating are:

1. To eliminate any micro-organisms that might compete with the starter culture to be added later and/or lead to spoilage of the retail product.

Standardisation of fat to 0.5% to 5%

Fortification of milk solids to 14% to 16%

Addition of sugar and/or stabilisers

Homogenisation

(at 55°C-65°C; 15-20 MPa)

Heat treatment of milk

(80-85°C/ 30 min

or 90-95°C/5 min)

Cool to incubation temperature

(43-45°C)

Inoculate with starter culture

(2-3%)

Incubation

41-42°C/ 2-3 h to pH 4.8-4.4
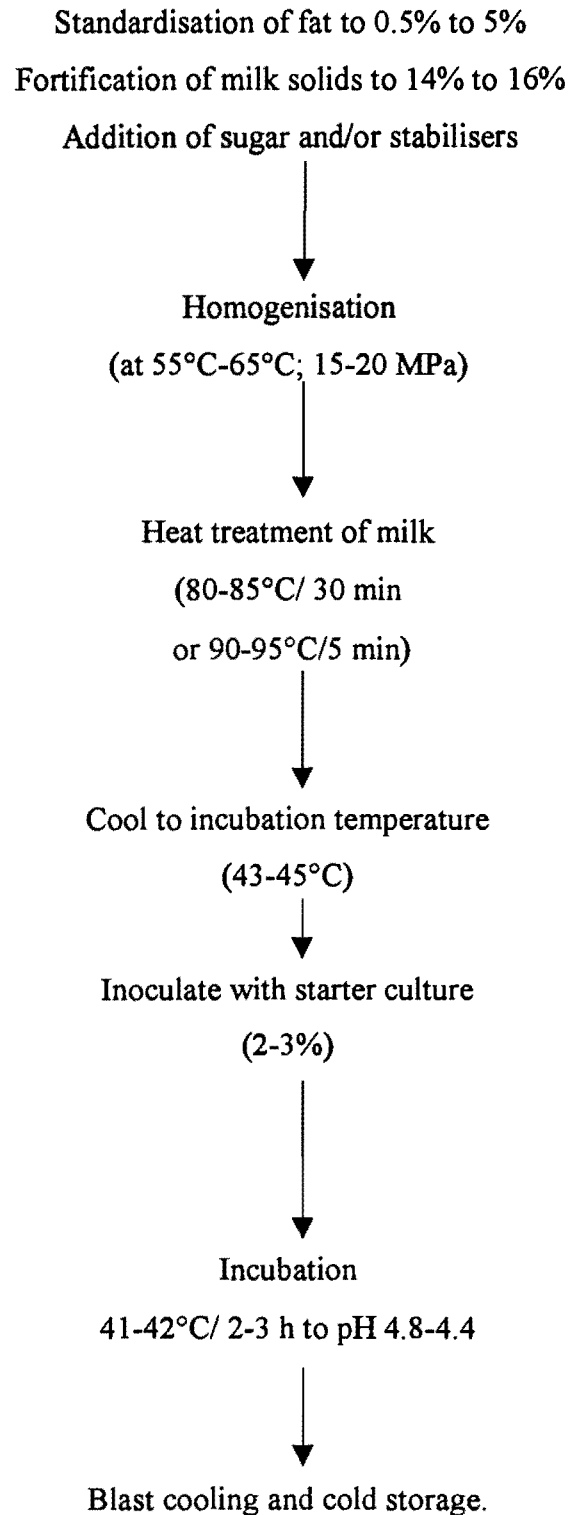
Blast cooling and cold storage.

Figure 5. Flow diagram for the manufacture of set-style plain yoghurt (adapted from Deeth & Tamime, 1981)

2. To induce chemical changes in milk such as expulsion of oxygen and release of free amino acids that encourage the rapid development of starter micro-organisms.

3. To modify the milk proteins in such a way that the physico-chemical properties of the yoghurt are altered. Milk is cooled to 42-43°C prior to inoculation (Lampert, 1964; Tamime & Deeth, 1980).

During the process of fermentation, live bacteria are involved and both the cells and enzymes remain active in the final product (Rosenthal, 1991). The characteristics of the coagulum formed are determined largely by the behaviour of the proteins present in the milk. The incubation temperatures of yoghurt are usually in the range of 40-45°C. The final pH of most yoghurts varies from 4.0 - 4.6. After the pH of the yoghurt has decreased to the desired level, the gel is cooled to less than 10°C (Van den Berg, 1988).

The two cultures commonly used in the manufacture of yoghurt are *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (Singh & Sharma, 1982; Malaton & Sandine, 1986). They are both essential for proper development of flavour and aroma. Lactic streptococci are nutritionally fastidious and require an exogenous supply of pre-formed leucine, valine, methionine, arginine, histidine, glutamic acid and in some cases phenylalanine, proline and cystine (Akpemado & Bracquart, 1983; Novak, Cocaign-Bosquet, Lindley & Loubiere, 1997). Free amino acid concentrations in milk are too low to support the starter growth required in the manufacture of fermented milk products (Law & Sharpe, 1978). The amino acids essential for *Streptococcus salivarius* subsp. *thermophilus*, are liberated by the *Lactobacillus* (Rajagopal & Sandine; Abu-Tarboush, 1996). Formic acid produced by *Streptococcus thermophilus* during fermentation is believed to stimulate acid production by *Lactobacillus delbrueckii* subsp. *bulgaricus* (Bottazzi, Battistotti & Vescovo, 1971).

2.7.4 Biochemical changes that occur during yoghurt fermentation

In milk, lactose is the only carbohydrate available for the production of energy by lactic acid bacteria. The initial step by *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* is the transport of lactose through the cell membranes before utilisation by the intracellular enzyme systems. Lactose is hydrolysed inside the bacterial cell by the enzyme β-galactosidase to glucose and galactose. Glucose is metabolised through the Embden Meyerhof Pathway to pyruvic acid which in turn is converted to lactic acid by the action of the enzyme lactose dehydrogenase. The metabolism of galactose is not clear. It accumulates in yoghurt and is not catabolised to any great extent (Pederson, 1971).

The two main roles of the starter culture during the manufacture of yoghurt are the production of lactic acid and development of flavour in the product. The major flavour components are the carbonyl compounds, acetone, acetoin and diacetyl. The presence of acetaldehyde is also important for good yoghurt flavour. Other compounds associated with flavour enhancement in yoghurt are volatile fatty acids and amino acids (Marth, 1974).

The lactic acid helps to destabilise the casein micelle and this leads to coagulation of the milk protein and formation of the yoghurt gel. The lactic acid also gives the sharp taste to yoghurt and contributes towards the typical aromatic flavour (Herrington, 1948).

2.7.5 The role of protein in the formation of the coagulum

The proteins of milk can be divided into two broad categories namely caseins and serum proteins. Caseins are defined as phosphoproteins which precipitate at pH 4.7 while serum proteins are soluble under these conditions (Walstra & Jenness, 1984). Cows' milk contains about 30-35 g protein per litre of which 80% is present as casein micelles (McMahon & Brown, 1984).

The caseins form aggregates, known as micelles, of up to 680 nm in diameter with a very wide size distribution (Griffin & Anderson, 1983). In cows' milk, casein micelles occur in colloidal dispersion. They are highly hydrated, sponge-like and can bind about 3.7 g water/g protein. Very little of the water is bound to the protein. The remainder is occluded within the micelle and moves with the micelle (Fox, 1989).

The caseins consist of four principal proteins which are αs_1-, αs_2-, β- and κ- caseins (Dalgleish, 1982; Swaisgood, 1989). Caseins have entirely different polypeptide chains and this accounts for the different properties which they exhibit and the different roles that they have during the manufacture of fermented dairy products (Swaisgood, 1989). All caseins are phosphorylated to varying extents (Snoeren, Van Narkwijk, Van Montfort, 1980).

During the fermentation of milk, the colloidal calcium phosphate in the casein micelles progressively solubilises and aggregation of the casein occurs as the isoelectric point (pH 4.6) is approached (Mulvihill & Fox, 1990; Tamime & Robinson, 1999). According to Mulvihill & Fox (1989), the κ- and β-caseins from the micelles gradually diffuse into the aqueous phase of the milk. A size-determining frame-work of αs_2-caseins remains intact. As the pH decreases, the released caseins become positively charged and are readsorbed on the surface of the negatively charged αs_2-caseins leading to the formation of particles that are different from the original micelles in milk. As the pH decreases further the charge on the αs_2-caseins decreases and the particles aggregate into chains and clusters to form the final network. Denatured β-lactoglobulin associates with κ-casein and filamentous appendages are formed on the surface of the micelles. This complex is probably formed by disulphide bonding (Davis, Shankar, Brooker & Hobbs, 1978) and it is believed to protect the micelles from excessive fusion during fermentation (Dannenburg & Kessler, 1988). At a molecular level, an acid-set gel is held together by hydrophobic forces between casein molecules along with some specific interactions provided by hydrogen bonds and ionic interactions (Johnston, Austin & Murphy, 1993).

2.8 *Kishk* - An example of a cereal-dairy composite food

2.8.1 Background

Kishk is a very popular fermented wheat-milk composite common in Egypt and other Middle East countries such Syria, Lebanon and Jordan (Odunfa, 1985). In Turkey it is known as *tarhana* and in Iraq as *kushuk*. It is made by mixing fermented buttermilk with boiled, dried and ground wheat grains (Hafez & Hamada, 1984). It consists of small, round or irregular pieces, yellowish brown in colour, which have a rough surface and hard texture. When moistened, *kishk* becomes white and breaks up as it absorbs moisture. It is used to make a refreshing drink when reconstituted with water. Dried *kishk* is shelf-stable and can be stored for up to three years under ambient conditions in open clay jars (Abou-Donia, 1984). The low moisture content (less than 10%) and low pH of 4.2 of the final product are a safeguard against the growth of pathogenic micro-organisms (Hamad & Fields, 1982).

2.8.2 The traditional process of making *kishk*

The first stage is the preparation of the sour buttermilk, known as *laban zeer*, by processing milk directly into butter. In winter, the buttermilk is used to make cheese. In summer, when temperatures are high, milk becomes contaminated and bacterial loads are high. This leads to the milk coagulating before the butter and cheese have been produced (Tamime & Robinson, 1978). The coagulated milk is stored in earthernware pots. The walls of the pots are porous and thus the moisture evaporates from the buttermilk. The sour buttermilk thickens and salt is added to taste (Abou-Donia, 1984). The pH of the buttermilk drops to 3.5-3.8 while the corresponding titratable acidity ranges from 1.3 to 1.6%. Acetoin is not formed during fermentation (Figure 6)(El-Gendy, 1983).

Milk component

Concentrated, sour buttermilk

Wheat grains

boiled in water until soft

Sour buttermilk diluted with water

Grain dried in the sun

Grain crushed and seed coat removed

Grain moistened with salted water

Mixed to form thin homogenous paste

Fermented for 24 h in clay vessels

Mixture cut into pieces

Drying for 2-3 d
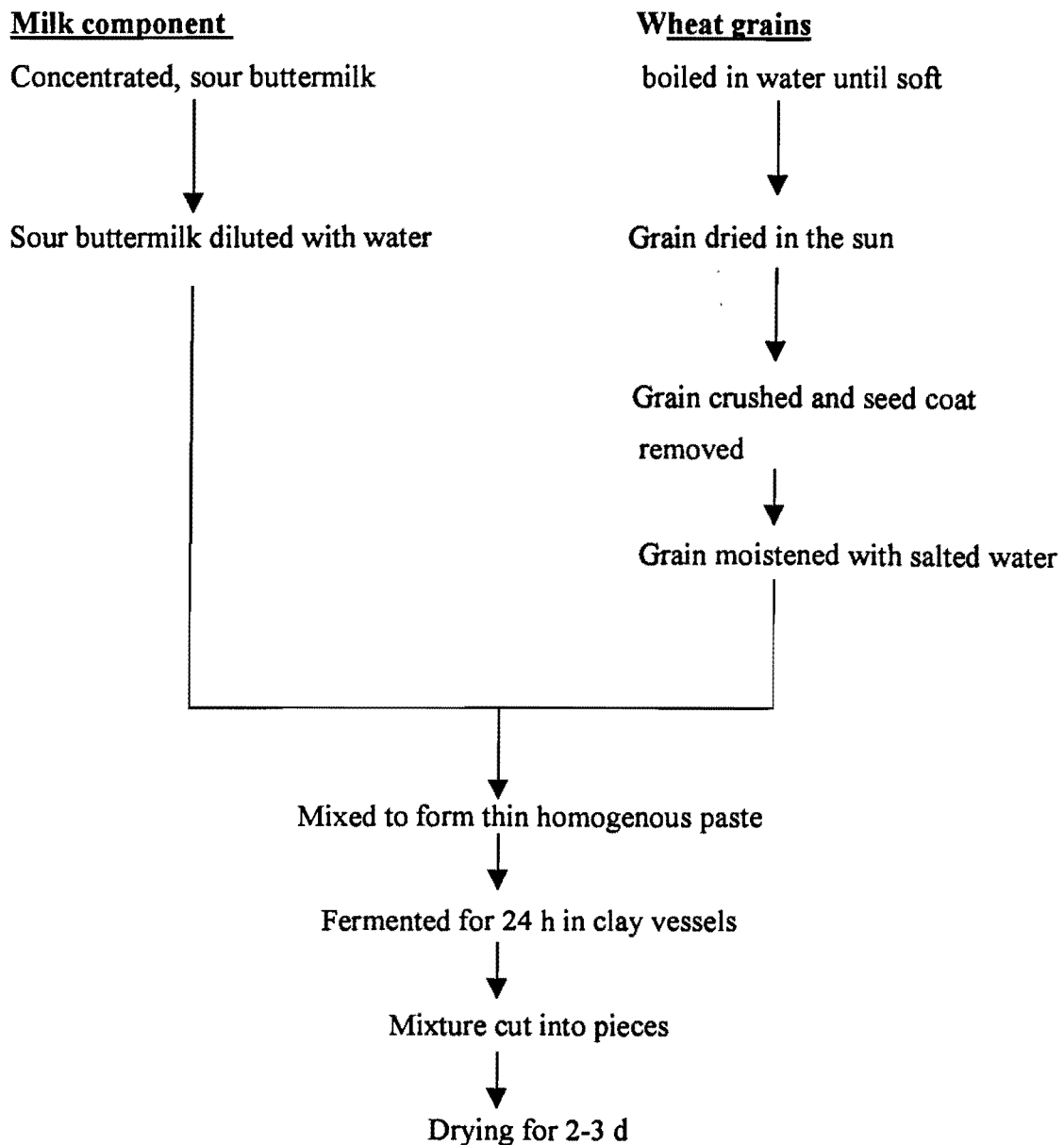
Figure 6. Traditional process for making *kishk* (El-Gendy, 1983)

The second stage involves the preparation of the wheat grains. Fresh wheat grains are boiled until they are soft. Cooking gelatinises the starch. The cooked grains are spread on mats, dried in the sun and ground. After removal of the seed coat, the wheat grains are placed in a large earthenware container and moistened with slightly salted boiling water (El-Gendy, 1983).

The fermented buttermilk is diluted with raw milk or water and added to the wheat grain until a thin homogenous paste of creamy consistency is obtained. The earthenware container is covered with heavy wool cloth and left for 24 h. The mixture of wheat and milk ferments and its volume increases. At the end of fermentation the pH will have dropped from 4.7 to 4.2 and the titratable acidity will be 1.9%. The fermented mixture is thoroughly mixed, divided into small round or irregularly shaped pieces and dried in the sun on straw mats for 2-3 d (El-Gendy, 1983).

2.8.3 The microbiology of traditionally manufactured *kishk*

When the mixture of fermented buttermilk and wheat grains is left for 24 h, it contains a dense population of *Bacillus subtilis* and *Bacillus megaterium*. Although cooking the wheat grains reduces the microbial flora, the population of *Bacillus* species develops during drying. *B. subtilis* produces diastase which increases the reducing sugar content of the mix. The fermented buttermilk is a source of homo- and heterofermentative bacteria. Examples of these bacteria include *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus brevis*. The reducing sugars provide the energy source for the development of lactic acid bacteria. As acidity develops, the *Bacillus* spp. as well as other bacteria are completely inhibited by the high acidity (Abou-Donia, 1984).

Yeast counts increase during fermentation. They produce alcohol and carbon dioxide. The gas is responsible for the increase in the volume of *kishk* during fermentation. Yeasts also synthesise B-vitamins.

2.8.4 The manufacture of a modern '*kishk*-like' product

A *kishk*-type product has been developed using a starter culture containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* at 45°C. Use of a thermophilic starter culture leads to more rapid acid production which suppresses the growth of spoilage and potentially pathogenic bacteria. The procedure used is summarised in Figure 7. The milk is heated for 30 min at 85°C to kill micro-organisms that might compete with the starter culture during fermentation and then

cooled to a temperature at which inoculation can be safely done without destroying the culture. Cooking of the wheat under pressure ensures complete gelatinisation of starch. The protein content of this product compared with that of the traditional product is lower (17% compared to 24%). The carbohydrate content is higher for the *kishk*-like product at 71% compared to 60% in the traditional product (Robinson & Tamime, 1985). The main disadvantage of the *kishk*-like product is that it has lower levels of lysine and threonine . This may be attributed to the process of roller drying that the product goes through to remove moisture.
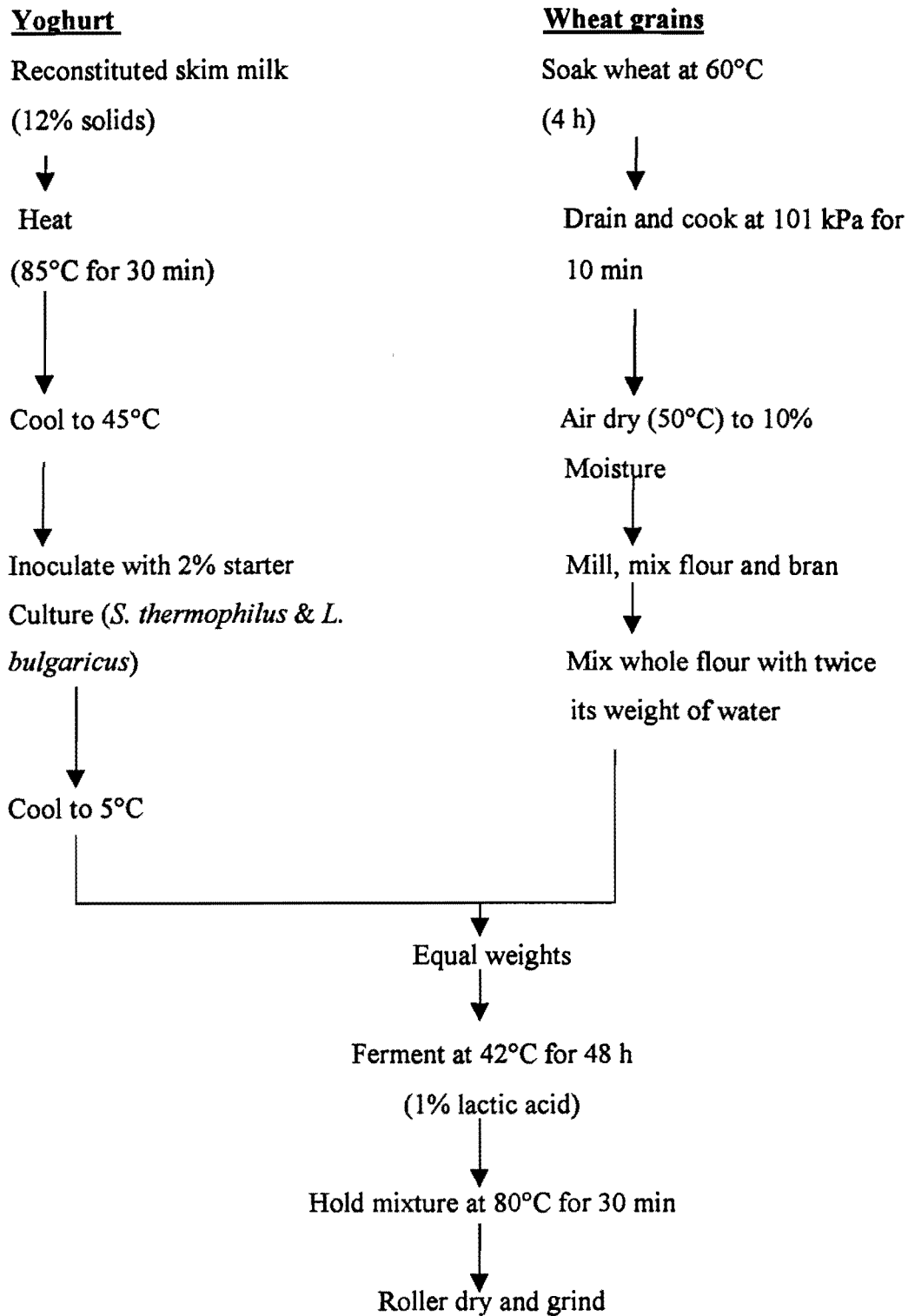
<u>Yoghurt</u>   <u>Wheat grains</u>

Reconstituted skim milk   Soak wheat at 60°C

(12% solids)   (4 h)

   ↓   ↓

 Heat   Drain and cook at 101 kPa for

(85°C for 30 min)   10 min

Cool to 45°C   Air dry (50°C) to 10%

    ↓   Moisture

    ↓   ↓

Inoculate with 2% starter   Mill, mix flour and bran

Culture (*S. thermophilus & L.*   ↓

bulgaricus)   Mix whole flour with twice

    ↓   its weight of water

Cool to 5°C

                    Equal weights
                       ↓

                  Ferment at 42°C for 48 h

                    (1% lactic acid)
                       ↓

               Hold mixture at 80°C for 30 min
                       ↓

                   Roller dry and grind

Figure 7. Flow diagram of a method for the manufacture of a *kishk*-like product (Robinson & Tamime, 1985)

2.9 Concluding remarks

Cereals are known to be low in proteins and deficient in essential amino acids especially lysine. Fortification with high protein sources can be used to overcome this problem. Fortification has been successfully used to improve the protein and amino acid content of fermented foods made with sorghum and maize. Some of the high protein sources that have been used include cowpea, groundnuts, soyabeans, locust bean meal and cashew nut meal. While the use of milk, skim-milk powder, whey protein and sour milk products has been suggested for fortifying *mahewu*, little is known about fortification of finger millet with dairy products and how fortification influences the nutrient content.

The lactic acid produced during fermentation lowers the pH of the fermented gruels to 4.5 or lower. This inhibits the growth of pathogenic bacteria that have been implicated in diarrhoeal diseases in infants and children. Fermentation also slows down the rate of microbial spoilage. It has been observed that the use of starter cultures to ferment composites does not lead to a pH decrease to 4.5 and lower especially when one of the ingredients is milk. The buffering capacity of milk proteins slows down the decrease in pH. There is a need to determine what cultures can be used to ferment both the lactose and starch in cereal composites to sufficiently reduce the pH and produce a safe product. It is important to establish the optimal conditions at which pH reduction occurs and temperature is one of the most important factors. Low incubation temperatures increase the risk of contamination with spoilage and pathogenic bacteria. Time of incubation is another factor and prolonged incubation can lead to over-acidification.

At the same time, the effect of reducing pH on some of the physico-chemical characteristics of the fermented products (e.g. viscosity, consistency and firmness which might affect acceptability of the product) need also to be determined. Firmness influences the amount of solids that can be incorporated into the gruels. This affects the nutritional value of the gruels. Fermentation that leads to an increase in viscosity

of gruels limits their nutrient content while fermentation that leads to an increase in the solids content has the potential to improve the nutrient content of the gruels.