ANALYSIS OF MICROBIAL POPULATIONS ASSOCIATED WITH A SORGHUM-BASED FERMENTED PRODUCT USED AS AN INFANT WEANING CEREAL

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ABSTRACT

The incidences of diarrhoeal episodes in infants and children have mostly been associated with the consumption of contaminated weaning foods. This is especially true in developing countries where factors such as the lack of sanitation systems and electricity have been found to contribute to an increase in the incidence of microbiologically contaminated weaning foods. The process of fermentation has been found to reduce the amount of microbiological contamination in such foods as a result of the production of antimicrobial compounds such as organic acids, peroxides, carbon dioxide and bacteriocins. In this study, microbiological surveys were conducted on sorghum powder samples and their corresponding fermented and cooked fermented porridge samples collected from an informal settlement of the Gauteng Province of South Africa. The process of fermentation was found to result in significant decreases ($P>0.05$) in Gram-negative counts and spore counts, while aerobic plate counts decreased slightly. Lactic acid bacteria counts, however, increased significantly ($P>0.05$). The cooking process was found to result in further significant decreases ($P>0.05$) in all counts. Sorghum powder samples and fermented porridge samples were found to be contaminated with potential foodborne pathogens, including *Bacillus cereus, Clostridium perfringens* and *Escherichia coli*, however, none of the pathogens tested for were detected in any of the cooked fermented porridge samples. SDS-PAGE and phenotypic analysis of 180 lactic acid bacteria isolated from sorghum powder samples and their corresponding fermented and cooked fermented porridge samples showed that a majority of the isolates were lactobacilli and leuconostocs, however, some isolates were identified as pediococci and lactococci. These results demonstrated the heterogeneity of the lactic acid bacteria isolates that were associated

with fermentation processes in this study. Of the lactic acid bacteria identified, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* strains were found to have the highest distribution frequencies, being distributed in 87% and 73% of the households, respectively. Analysis of *Lactobacillus plantarum* (58) and *Leuconostoc mesenteroides* (46) strains isolated from sorghum powder samples and corresponding fermented and cooked fermented porridge samples by AFLP fingerprinting showed that they originated from a common source, which was sorghum powder. There was, however, evidence of strains that may have been introduced at household level. Antimicrobial activity of selected lactic acid bacteria was found to be mainly due to a decrease in pH in fermented and cooked fermented porridge samples. None of the lactic acid bacteria tested seemed to produce bacteriocins.

DECLARATION

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any university. Where use has been made of the work of others it is duly acknowledge in the text.

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18th Day of March 2000

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PREFACE

The experimental work described in this dissertation was carried out in the School of Molecular and Cellular Sciences, University of Natal, Pietermaritzburg and in the Department of Microbiology, University of the Witwatersrand, Johannesburg, From January 1996 to September 1998, under the supervision of Professors John W. Hastings and Alexander von Holy. Some sections of the work conducted for this thesis have been presented as papers or posters elsewhere.

CHAPTER 2:

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

This study will give an insight on the microbial populations usually found in infant weaning foods and the effects of lactic acid fermentation on microbiologically contaminated infant weaning foods. A discussion on the current taxonomic status of the lactic acid bacteria responsible for the fermentation, the techniques used in their identification and the antimicrobial substances produced by these lactic acid bacteria will also be covered.

1.2 WEANING FOODS

1.2.1 The microbiology and epidemiology associated with weaning foods

Weaning infants off breast milk has been recognised to put them at risk of illnesses such as diarrhoea because of the introduction of solid foods (Harrison *et al.,* 1993). The bacterial quality of weaning foods and water given to infants has been a matter of concern as poor hygienic quality has been found to result in diarrhoeal diseases (Mensah *et al.,*1990; Simango and Rukure, 1992). Such foodborne diseases have been reported to cause severe and long term damage to health including acute watery and bloody diarrhoea, meningitis and chronic diseases affecting renal, cardiovascular, respiratory and immune systems. These diseases have been found to affect the growth of infants and children after weaning is initiated, due to a lower food intake aggravated by loss of nutrients from diarrhoea, vomiting and malabsorption (Motarjemi *et al.,* 1993).

In developing countries, the weaning process in human infants has been associated with an increase in diarrhoeal episodes resulting from consumption of microbiologically contaminated weaning foods. It has been estimated that 1500 million infants and children under the age of 5 years suffer from diarrhoeal infections each year and in 1990, more than 3 million died as a result (Motarjemi and Nout, 1996). These diarrhoeal infections have been associated with enteric pathogens such as *Aeromonas* spp., *Campylobacter* spp., *Escherichia (E.) coli, Shigella,* spp., and *Salmonella* spp. and toxin producing bacteria such as *Bacillus (B.) cereus* and *Clostridium (C.) perfringens* (Simango and Rukure, 1992; Motarjemi *et al.*, 1993).

Several practices have been identified that usually result in the proliferation of disease causing microorganisms in foods. These include eating raw foods of animal origin, insufficient

cooking or reheating of foods to reduce or eliminate pathogens, preparation of foods several hours prior to their consumption and storage at temperatures that favour growth of pathogenic bacteria and/or formation of toxins (Klontz *et al.*, 1995; Motarjemi and Nout, 1996). The consumption of instant weaning foods reconstituted with water has been found to encourage the use of warm water instead of boiled water, resulting in increased chances of contamination. The preparation of safe foods has been found to be affected by several factors such as the type of water supply and sanitary conditions (Immong *et al.*, 1989; Mensah *et al.*, 1990; Simango and Rukure, 1992). In underdeveloped countries, the lack of facilities, such as electricity and in-house sanitation systems, has been reported to support the rapid proliferation of foodborne pathogens, especially weaning foods like porridge, which are usually prepared once on a daily basis for use during the whole day (Nout, 1991; Lorri and Svanberg, 1995). The need for nutritional weaning foods that could be used throughout the day has necessitated the processing of such foods in a way that would impart antimicrobial activity to them to prevent post-preparation contamination.

1.2.2 Fermented weaning foods

Fermentation is increasingly used as an alternative method of preserving foods, where cold or hot storage is not possible (Motarjemi and Nout, 1996). Fruits, vegetables, cereals, root crops, legumes, oil seeds and dairy products have all been used in the production of fermented foods (Rombouts and Nout, 1995). The fermentation process has been found to impart desirable effects to food products, including increasing the shelf life and enhancing the taste, aroma, texture and nutritional value of fermented food products. Certain of these fermented products are used as infant weaning foods. In addition to the nutritional benefits attributed to such weaning foods by the fermentation process, they also offer good keeping quality as a result of the organic acids produced by lactic acid bacteria.

Lactic acid fermented food products constitute the bulk of foods given to infants and children and they generally form part of the daily main dishes of the average individual in Africa. They are consumed in more than 70% of the African continent (Oyewole, 1995). Traditional weaning foods in eastern and southern Africa are based on local staple foods which are usually cereals such as maize, sorghum, rice or millet (Lorri and Svanberg, 1995). Some of the

fermented weaning foods widely used in Africa include uji (Kenya), ogi (Nigeria), kenkey (Ghana), magai (Tanzania) and mawè (Bènin), which are all made from fermented maize or sorghum (Mensah *et al.*, 1991; Lorri and Svanberg, 1994; Kingamkono *et al.*, 1995). South African fermented weaning food products include mageu, which is produced as a non-alcoholic drink (Lorri and Svanberg, 1994), and 'ting' or 'incwancwa', which is made from sorghum or maize. In Africa, fermented foods are produced at the household level and as such the fermentation process is spontaneous and is initiated by a mixed microflora in the raw food, however, fermentation vessels of previous ferments usually serve as sources of inoculum for the initiation of the process. In South Africa, a prevalent method of obtaining a fermented product is soaking the maize/sorghum in water for 2-3 days resulting in a succession of naturally occurring microorganisms dominated by lactic acid bacteria. The process is usually accelerated by the addition of a starter culture from a previous batch of fermented gruel. The fermented gruel is cooked as a soft porridge and is first given to infants between the ages of 1-6 months (Mensah *et al.*, 1990; Bentley *et al.*, 1991; Guptill *et al.*, 1993).

1.2.3 Effects of fermentation on foodborne pathogens associated with weaning foods

Fermented sorghum and maize cereals used as weaning foods have been found to inhibit growth of food pathogens including enteric *E. coli, Campylobacter (C.) jejuni, B. cereus, Salmonella (S.) typhimurium, Shigella (S.) flexneri and Staphylococcus (S.) aureus*, within 24 hours of incubation, as a result of the production of lactic acid and other antimicrobial substances by lactic acid bacteria (Mensah *et al.*, 1991; Kingamkono *et al.*, 1995; Olsen *et al.*, 1995; Rombouts and Nout, 1995). Studies on uji, which is a fermented maize-based weaning food product, showed complete inhibition of coliform bacteria after a 48 hour fermentation period (Mensah *et al.*, 1991). The same study reported a decrease in the incidence of *E. coli* from 7 log cfu/g to < 2 log cfu/g and the inhibition of 17 strains of *S. flexneri* strains after 48 hours of fermentation. Similar results were obtained by Simango and Rukure (1992) where growth of pathogenic bacteria in fermented sorghum cereals was completely inhibited within a 48 hour period, whereas lactic acid bacteria reached levels of 10^7 to 10^9 cfu/g during fermentation (Nout, 1991).

1.3 POTENTIAL FOODBORNE PATHOGENS ASSOCIATED WITH WEANING FOODS AND THEIR DETECTION

A critical step in preventing outbreaks of foodborne diseases is quantifying levels of pathogenic foodborne bacteria in food products and identifying possible sources of contamination. In most cases, the detection and identification of foodborne pathogens is achieved using phenotypic methods. The limitations to currently used methods of pathogen detection include time requirements and costs of the tests. These limitations necessitate the development of techniques that are sensitive, specific, rapid, less labour intensive and cost effective. Although several genotypic methods exist which are highly sensitive (Pillai and Ricke, 1995; Venkitanarayanan *et al.*, 1996; Wagner *et al.*, 1996), the use of biochemical tests has been found to be easy and cost effective in the tentative identification of microorganisms and because of that, the detection and identification of many food pathogens still relies heavily on phenotypic methods. In most cases, the isolation of foodborne pathogens requires culturing the bacteria in enrichment media prior to growing on selective media because the pathogens may be present in low concentrations (ICMSF, 1996). Several foodborne pathogens and toxin producing bacteria have previously been associated with weaning foods and these are discussed below.

1.3.1 *Aeromonas* spp.

Aeromonas spp. are commonly isolated from water, water associated animals, and contaminated food. They have been associated with foods of animal origin including raw meat and milk. Although they are established fish pathogens, some species have been identified as potential human pathogens and have been associated with traveller's and infantile diarrhoea (Popoff, 1984). *Aeromonas* are characterised by Gram-negative rods which are cytotoxin C3 positive. Illnesses due to *Aeromonas* infection range from mild diarrhoea to fatal cholera-like diseases. *Aeromonas* are thought to produce enterotoxins that elicit the same reaction as the *Vibrio (V.) cholerae* toxin (ICMSF, 1996). The enterotoxins, in combination with other virulence factors associated with colonization of the intestine, are thought to be the most probable cause of diarrhoea. Although they have been found to grow rapidly at refrigeration temperatures, their growth is completely inhibited at pH 4.5 under the same conditions. The first step in the detection of *Aeromonas* is enrichment in a liquid medium, such as alkaline

peptone water, and isolation on selective agar such as starch ampicillin agar, since most Gram-negative bacteria cannot hydrolyse starch and are inhibited by ampicillin (Palumbo *et al.*, 1985). Other selective media include brilliant green phenol red agar (BGA), on which colonies appear colourless. Identification of *Aeromonas* spp. includes the use of the API 20E system, catalase test and resistance to antibiotics such as novobiocin (Popoff, 1984; ICMSF, 1996).

1.3.2 *Bacillus cereus*

B. cereus is a common contaminant of milk, dried milk products, dairy products and surfaces of milking equipment (Becker *et al.*, 1994) and has been isolated from soil, dung, straw hay and other fodder as well as contaminated rice, spices, (Damgaard *et al.*, 1996), meat, cereals and dried products (Tan *et al.*, 1997). Toxins produced by *B. cereus* have been found to be responsible for two distinct food borne illnesses, one characterised by diarrhoeal symptoms and the other by emetic symptoms (Jackson, 1993; in't Veld *et al.*, 1993). The diarrhoeal toxin is sensitive to heat and therefore can be inactivated by cooking, and is also sensitive to proteolytic enzymes and acids. The emetic toxin, however, is resistant to proteolytic enzymes and extreme pH (Luby *et al.*, 1993; Tan *et al.*, 1997). The production of toxins by *B. cereus* is reportedly inhibited at low temperatures ranging from 6^0C to 21^0C, with amounts of toxin increasing with an increase in temperature and at pH above 5 (Becker *et al.*, 1994). *B. cereus* toxins are inactive at pH <4 and are only produced at high bacterial densities (>10^4 cfu/ml) (Baker and Griffiths, 1994, Tan *et al.*, 1997). Antibiotic resistance of *B. cereus* includes ampicillin, colistin and polymyxin while certain strains have shown resistance to tetracycline (ICMSF, 1996). The ability of *B. cereus* to form spores has been found to allows it to survive extreme conditions such as dryness, high temperatures and low pH. Detection of *B. cereus* includes enrichment in a liquid medium such as Robertson's meat broth, and isolation on selective agar such as blood agar and polymyxin-pyruvate-egg yolk mannitol-bromothymol blue agar (PEMBA). Typical *B. cereus* colonies are indicated by haemolysis on blood agar and blue colouration as well as production of lecithinase on PEMBA (Holbrook and Anderson, 1980; Kramer and Gilbert, 1989). Positive identification can be further achieved using a series of biochemical tests that distinguish them from other bacilli (Slepecky and Hemphill, 1992).

1.3.3 *Campylobacter* spp.

Campylobacters have been isolated from rivers, estuaries and coastal waters, unchlorinated water, raw milk and raw poultry. *C. jejuni* strains have reportedly been responsible for most human enteritis illnesses, resulting in diarrhoea accompanied by severe dehydration in infants and the elderly (ICMSF, 1996). Illnesses have been found to result from the ingestion of as few as 500-800 cells in milk. Campylobacters have been found to produce enterotoxins and cytotoxins which have been implicated in cases of bloody diarrhoea. Growth of campylobacters is inhibited at low temperature and low pH and they are unable to grow in lactic acid fermented food products. They can survive in chilled foods but fail to grow at pH $\leq$ 4.7. Detection of campylobacters includes enrichment in a liquid medium such as Preston's broth, containing rifampicin, cycloheximide and polymyxin B, and isolation, usually on Preston or Skirrows agars and incubated at 42^{0}C under micro-aerophilic conditions. Identification includes the use of the oxidase test, catalase test, haemolysis, triple iron sugar test and sensitivity to nalidixic acid (Bolton, 1983; Beuchat, 1987; Stern and Kazmi, 1989; ICMSF, 1996).

1.3.4 *Clostridium perfringens*

Clostridia have been isolated from dust, soil, flies and vegetation. They are transmitted by stored cooked meat, poultry and dehydrated food products. Pathogenicity involves the production of exotoxins and enterotoxins which may be produced in foods resulting in illness, however, illnesses have been reported to result from lysis of sporulating cells in the intestine. Food poisoning occurs following the consumption of a large number of clostridial cells and symptoms include diarrhoea and abdominal pains. The enterotoxins are reportedly heat labile and sensitive to protease and pronase degradation but are not sensitive to degradation by trypsin, chymotrypsin and papein. Clostridial spores have been found to be resistant to high temperatures up to 100^{0}C, however, resistance to heat is decreased in acidic conditions (ICMSF, 1996). Although clostridia have a very short generation time of less than 10 min at optimum temperatures, the generation time decreases in acidic conditions and their growth is inhibited at pH < 5. They are easily detected in foods after enrichment in a liquid medium such as Robertson's broth followed by plating on selective agar such as tryptose sulphite cycloserine (TCS) agar, on which the colonies will have a black colouration. Clostridia strains

are identified by their characteristic morphology, Nagler's reaction and growth on selective media (Labbe, 1989).

1.3.5 *Escherichia coli*

There are four main types of pathogenic *E. coli* that have been associated with foodborne diarrhoeal diseases, namely, enteropathogenic (EPEC) *E. coli*, enterotoxigenic (ETEC) *E. coli*, enteroinvasive (EIEC) *E. coli*, and the highly pathogenic enterohaemorrhagic (EHEC) *E. coli*, exemplified by *E. coli* 0157:H7, which is a newly evolved *E. coli* serotype that has become pathogenic through the acquisition of virulence factors (Smith and Francisco, 1995). This serotype is reportedly the fourth most common enteric pathogen recovered from human stool samples and results in fatal haemorrhagic colitis in infants and immunocompromised individuals (Johnson *et al.*, 1995; Huang and Chang, 1996). This seroptype is often characterised by the inability of its strains to ferment sorbitol, however, there are some exceptions. Enterotoxigenic and enterohaemorrhagic *E. coli* are reportedly major causes of infantile diarrhoea in developing countries (ICMSF, 1996). The diarrhoeal outbreaks resulting from *E. coli* contamination have been associated with the use of contaminated water (Weagant *et al.,* 1995) and consumption of contaminated food products such as raw milk and raw or undercooked beef (Glass *et al.*, 1992). Diarrhoeal infections are caused by toxins referred to as *Shigella*-like toxins (SLT) or vero toxins (Smith and Francisco, 1995). Although toxin production has been reported to occur at bacterial levels of 10^4-10^5, the infectious dose of *E. coli* 0157:H7 has been found to be extremely low (Bennett *et al.*, 1996). Sensitivity to pH depends on the type of acid since they have been found to be more sensitive to lactic acid than hydrochloric acid. Pathogenic *E. coli* have been found to be unable to grow on fermented food products. The detection of all four serotypes in foods has been found to be hindered by the fact that some serotypes do not survive the enrichment process and some do not have typical characteristics of *E. coli* such as inhibition by bile salts, inability to ferment lactose and inability to grow at elevated temperatures (Doyle and Schoeni, 1984; Doyle and Padhye, 1989). *E. coli* 0157:H7 has, however, been distinguished from other *E. coli* strains by its inability to ferment sorbitol in 24 hours, inability to produce β-glucoronidase and poor growth at temperatures above 42^0C (Johnson *et al.*, 1995; Vernozy-Rozand, 1997). Detection and isolation of pathogenic *E. coli* include plating samples on selective media such as MacConkey

agar and tellurite-ceffixine-sorbitol MacConkey agar (TCS MAC) (Johnson *et al.*, 1995). Identification involves the use of biochemical tests, phage typing, stereotyping, ELISA and DNA probing methods (Weagant *et al.*, 1995).

1.3.6 *Listeria monocytogenes*

Listeria (*L.*) *monocytogenes* has been isolated from soil, silage, sewage, food processing environments, raw meats and poultry (ICMSF, 1996). These microorganisms have been found to infect a wide variety of animals including cattle, sheep, birds, rodents, fish and humans. Outbreaks of human listeriosis have being linked to the consumption of coleslaw, celery, tomatoes, lettuce, unpasteurised milk, milk products and undercooked chicken. Pathogenic *L. monocytogenes* strains are hemolytic and are thought to have an infective dose of over 100 viable cells (Lovett *et al.*, 1987). Growth is reportedly very slow at 0^0C while it is inhibited at pH <5 and during fermentation of food products, however, re-contamination after fermentation has been found to be possible. *L. monocytogenes* has been found to produce endotoxins resulting in listeriosis and although the endotoxins are heat resistant, the resistance decreases in acidic conditions. Detection of *L. monocytogenes* includes enrichment in a liquid medium supplemented with nalidixic acid and acryflavin. They are isolated by plating on selective agars such as *Listeria* agar and incubated for periods of up to 5. Positive identification of *Listeria monocytogenes* is achieved after analysis of colonies using a series of biochemical tests (Lovett *et al.*, 1987).

1.3.7 *Salmonella* spp.

Salmonella reside in the intestinal tracts of infected animals and have been isolated from contaminated foods of animal origin and those subject to sewage pollution. Contamination is believed to be spread to humans during animal slaughter and processing and symptoms of infection include diarrhoea. This pathogen is primarily a contaminant of foods of animal origin such as meat, poultry, eggs and milk, however, it has also been found to contaminate foods of non-animal origin such as cereals and spices. *Salmonella* strains are invasive microorganisms that invade the small bowel and penetrate the intestinal mucosa. Infection by *Salmonella typhimurium* strains results in salmonellosis, which is a gastrointestinal infection characterised by fever, diarrhoea and intestinal cramps. Although the rates of mortality as a

result of infection are low, the disease has been found to be severe in infants, children and the elderly. The infective dose for a healthy person is reportedly 10^7 cells, but may vary, depending on its ability to survive during transit through the stomach. Growth of *Salmonella* has been found to be inhibited at temperatures below 5^0C and at pH below 4. Detection includes pre-enrichment in buffered peptone water followed by selective enrichment in tetrathionate, selenite, and Gram-negative broths. Isolation is usually on selective media such as BGA and Xylose Lysine Desoxycholate agar (XLD) and identification is confirmed by biochemical tests (API) and stereotyping (ICMSF, 1978; Arvanitidou *et al.*, 1998).

1.3.8 *Shigella* spp.

Shigella strains are primarily transmitted through contaminated food and from person to person. They have the ability to cause bacillary dysentery or acute enteritis in man and have been found to produce endotoxins which are responsible for diarrhoeal infections. *Shigella* strains are also invasive pathogens which penetrate the epithelial tissue and cause ulcerative lesions in the mucosa. The infective dose has been found to be very small (10 - 100 cells) and as such food containing low numbers of *Shigella* may cause disease. Their growth is inhibited at temperatures below 5^0C and at pH below 4, however, *Shigella sonnei* has been reported to survive in solutions at pH 3 and at 15% NaCl concentrations. Detection and isolation of *Shigella* strains from food samples has been found to be difficult because the strains are often masked by indigenous microflora including coliform bacteria. The first step towards detection of *Shigella* is usually enrichment in liquid media such as selenite broth and Gram-negative broth. Although these bacteria can be isolated on XLD agar and BGA agar, positive identification is achieved by further biochemical tests and stereotyping (Ewing, 1986).

1.3.9 *Staphylococcus aureus*

S. aureus is an ubiquitous organism occurring in mucus membranes and skin of most warm blooded animals. It is an opportunistic pathogen that causes illness due to changes in host physiology. This organism has previously been implicated in food poisoning incidences involving the consumption of contaminated food such as dairy products and unpasteurised milk. *S. aureus* strains reportedly produce up to seven different enterotoxins which result in diarrhoea. They compete poorly with other bacteria in foods, however, the toxins produced are

very heat stable, often surviving cooking and sterilisation processes of low acid canned foods. Growth of *S. aureus* as well as toxin production is reportedly inhibited at temperatures below 4 and at pH values below 4.3. Detection of *S. aureus* includes plating the food sample on selective agar such as Baird-Parker agar, which contains tellurite, glycine and lithium chloride, supplements which are selective for *S. aureus*. This medium also contains pyruvate which assists in the recovery of damaged cells and egg yolk which is used as the main diagnostic characteristic. The identity of colonies growing on Baird-Parker plates is further confirmed by the blood plasma coagulase test and the thermostable nuclease test (ICMSF, 1978).

1.3.10 *Vibrio cholerae*

Vibrio (V.) cholerae has been frequently isolated from rivers and inshore marine waters. Contamination of fresh and cooked foods has been found to be a major factor in the spread of cholera during an epidemic as the disease is commonly associated with the ingestion of sewage contaminated drinking water and/or contaminated foods. Pathogenicity is obtained by adherence of the bacteria to the small intestines where they produce enterotoxins. Symptoms include severe diarrhoea and dehydration, which are fatal it not treated, especially in malnutritioned individuals. Vibrios are sensitive to high temperature, acid and dry conditions and their survival in acid foods, dried foods, salted fish and fruit juices has been found to be minimal. Detection of *V. cholerae* includes enrichment in peptone water, followed by isolation on TCBS agar which is a highly selective high pH medium containing bile salts. Positive identification is achieved on the basis of growth on TCBS agar, morphology, oxidase reaction, non-gaseous fermentation of glucose and a series of other biochemical tests. Different serotypes have been distinguished by using polyvalent O-antisera (Madden and McCardell, 1989).

1.4 CONTROL OF FOODBORNE PATHOGENS IN WEANING FOODS

Many different approaches have been used in the control of food contamination by foodborne pathogenic bacteria, including the use of chemical additives, increased osmotic pressure, irradiation and low and high temperature processing and storage (Sofos, 1993; WHO, 1993; Pillai and Ricke, 1995; Smith and Francisco, 1995). There has been a general tendency, however, to decrease the use of chemical preservatives in food product and this has increased

research attention on the use of naturally occurring compounds produced by selected bacteria to inhibit the growth of pathogenic bacteria in foods (De Vuyst and Vandamme, 1994). The process of fermentation has been recognised as an alternative method of preserving food products. Fermentation processes effected by lactic acid bacteria have been found to extend the shelf life of food products as a result of the production of organic acids and bacteriocins. Fermentation has been found not only to reduce growth of foodborne pathogens but also to inhibit toxin production (Simango and Rukure, 1992; Becker *et al.*, 1994; Lorri and Svanberg, 1995). For this reason, a detailed study of the lactic acid bacteria involved in food fermentations is given below.

1.5 LACTIC ACID BACTERIA

1.5.1 Lactic acid bacteria associated with fermented foods

Lactic acid bacteria are a heterogenous group of bacteria that are generally regarded as safe (GRAS) for use in food and food products (Gancel *et al.*, 1997). Their use in food products dates back to ancient times, mainly because of their contribution to the flavour, aroma and increased shelf life of the fermented products (Nes *et al.*, 1996). They have been isolated from traditional fermented foods, plant material, dried marine algae, sea foods, fungi, spoiled foods and beverages (Aguirre and Collins, 1993; Damelin *et al.*, 1995). Lactic acid bacteria have been used commercially as starter cultures in the manufacture of food products including dairy products (Salama *et al.*, 1995), fermented vegetables (Leisner *et al.*, 1996), fermented doughs (Vogel *et al.*, 1994), alcoholic beverages (Patarata *et al.*, 1994), probiotics in animal feeds (Castellanos *et al.*, 1996) and as starter cultures in meat products (Vogel *et al.*, 1993). They have also been used as food additives (*Lactobacillus delbrueckii and Lactobacillus helveticus*), and in the production of dextran (*Leuconostoc mesenteroides*) and silage (*Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus rhamnosus*) (Aguirre and Collins, 1993).

1.5.2 Beneficial effects of lactic acid bacteria

Lactic acid bacteria used in fermented foods have been found to impart nutritional and therapeutic effects on these food products, such as improving their nutritional quality. In cereals specifically, they have been found to contribute to lysine enrichment and in the overall stimulation of the metabolism by producing vitamins (folic acid) and enzymes (lactase). Their

ingestion is thought to contribute in stabilising the intestinal microflora and in excluding pathogenic bacteria, including enteropathogenic *E. coli, Salmonella, Shigella*, and *S. aureus* strains, due to their adhesion to intestinal walls. Lactic acid bacteria reportedly have a protective role against intestinal and urinary infections through the production of antibacterial substances and have been found to decrease the risk of colon cancer by detoxifying carcinogens and toxic compounds. They have also been found to contribute to the breakdown of antinutritional factors such as trypsin inhibitors and phytic acids (De Vuyst and Vandamme, 1994).

Certain lactic acid bacteria such as *Lactobacillus acidophilus*, are considered to possess health promoting attributes such as anti-carcinogenic and hypocholesterolemic properties apart from their antagonistic activities against intestinal and foodborne pathogens. The anti-carcinogenic activity is thought to be a result of the production of compounds inhibiting the proliferation of tumour cells, the suppression of microorganisms that convert pro-carcinogens to carcinogens and the degradation of already formed carcinogens. The hypocholesterolemic activity is attributed to the inhibition of 3-hydroxy-3-methyl glutaryl CoA reductase which is a rate-limiting enzyme in endogenous cholesterol biosynthesis in the body (De Vuyst and Vandamme, 1994). The ability of this microorganism to survive in the gastrointestinal tract after ingestion allows it to have beneficial effects that accrue over a long period compared to those microorganisms that cannot colonise the gut (Mital and Garg, 1995). Lactic acid bacteria have also been found to contribute to the reduction of naturally occurring toxins in plants such as cyanides, synthesis of certain amino acids, reduction of non-digestible oligonucleotides and improvement of the availability of certain B-group vitamins by degrading phytate, a plant constituent often forming complexes with essential ions in diet (WHO, 1996).

1.5.3 Potential undesirable effects of lactic acid bacteria

Although lactic acid bacteria have been generally regarded as safe (GRAS) in food products and are of major interest in the food industry (Gancel *et al.*, 1997), some strains have been found to produce food poisoning compounds such as histamines and tyramines (Leisner *et al.*, 1994). They have also been found to be significant spoilage agents of vacuum packed meat and meat products, however such spoilage organisms can be controlled by bacteriocigenic

non-spoiling lactic acid bacteria (Leisner *et al.*, 1996). Lactic acid bacteria have also been implicated in clinical cases, as in the case of *Lactobacillus rhamnosus* (Aguirre and Collins, 1993) which is a functional microorganism in cheese manufacturing. Although it is very unlikely that it was a causative agent, it has been isolated from clinical cases associated with endocarditis. Most of the reported cases of infection resulted from underlying diseases or immunosuppression and infection in previously healthy humans has been found to be extremely rare. For this reason, it has been placed in group II (small risk) of lactic acid bacteria (Verrips and van den Berg, 1996). *Leuconostoc* strains have also been isolated from immunocompromised patients and a major part of the disease contraction was found to occur while patients were hospitalised. Their isolation from body fluids or tissue in the presence of fever and other systematic signs and symptoms is indicative of a causative pathogenic role and in many cases, portal entries have been reported to be provided by surgical procedures such as the insertion of venous catheters and endotraechal tubes (Aguirre and Collins, 1993). Pediococci have also been isolated from patients who were severely immunocompromised, and for many cases the role of pediococci and portal entries of infections remained uncertain (Weiss, 1992). Lactococci have also been isolated from clinical cases as exemplified by the isolation of a *Lactococcus lactis* strain from the blood of an otherwise healthy patient with a history of systolic murmur. It was thought that the resultant endocarditis was caused by an infected tooth gum as a result of ingesting sour cream. Lactococci have also been isolated from stools of children suffering from diarrhoea, urinary tract infections and wounds (Aguirre and Collins, 1993).

1.5.4 Antimicrobial activity of lactic acid bacteria

One of the major beneficial characteristics of lactic acid bacteria is the production of antimicrobial compounds which are being used in the inhibition of spoilage and pathogenic organisms in food products. Lactic acid bacteria have been found to inhibit other bacteria through the production of bacteriocins, aldehydes, peroxides, carbon dioxide, diacetyl, ammonia, ethanol, organic acids and bacteriolytic enzymes (Daeschel, 1989; De Vuyst and Vandamme, 1994; Olsen *et al.*,1995; Dessie *et al.*, 1996).

1.5.4.1 Organic acids, H_2O_2 and other antimicrobial compounds

There has been numerous reports on the inhibitory effects of lactic acid bacteria on food pathogens as a result of lactic acid production resulting in the lowering of pH (Mensah *et al.*, 1991; Nout *et al.*, 1991; Simango and Rukure, 1992; Rombouts and Nout, 1995). Wong and Chen (1988) showed that the growth of *B. cereus* and the germination of *B. cereus* spores were inhibited when it was co-cultured with certain lactic acid bacteria, due to the production of formic, lactic and acetic acid. The inhibitory activity of hydrogen peroxide, however, is thought to be due to membrane permeability as a result of peroxidation of membrane lipids and the destruction of basic molecular structures of nucleic acids and proteins due to its oxidation effects (Piard and Desmazeaud, 1991). Carbon dioxide has been found to create an anaerobic environment which is inhibitory to growth of many bacteria by replacing existing molecular oxygen, decreasing extra- and intracellular pH and destroying the cell wall (Eklund, 1984). Diacetyls reportedly interfere with arginine utilisation by reacting with arginine-binding proteins of Gram-negative bacteria, resulting in cell death (Jay, 1982).

1.5.4.2 Bacteriocins

Bacteriocins are antimicrobial peptides or proteins that have bactericidal or bacteriostatic activities against closely related species (Klaenhammer, 1993; Hastings *et al.*, 1991; Farías *et al.*, 1996). Some bacteriocins produced by lactic acid bacteria have been found to have antimicrobial activities against food pathogens and food spoilage organisms (Tichaczek *et al.*, 1994). Bacteriocins have been described from all genera of lactic acid bacteria (Table 1.1) (Daeschel, 1989; De Vuyst and Vandamme, 1994). There are 4 major classes of bacteriocins, namely, lantibiotics (nisin, lactonin), small heat-stable peptides (pediocin, sakacin), large heat-labile proteins (helveticin) and complex proteins that require carbohydrates or lipid moieties for bacteriocin activity (leuconocin S) (Klaenhammer, 1993). The existence of the fourth class was supported by the observation that some bacteriocin activities obtained in cell-free supernatants were abolished not only by protease treatment, but also by glycolytic and lipolytic enzymes. Such bacteriocins have, however, not been adequately characterised at the biochemical level and the recognition of this class is therefore premature (Nes *et al.*, 1996).

Bacteriocins offer an alternative food preservative method in that they are produced by lactic

acid bacteria which have been ingested throughout history. They have been reported to be active against foodborne pathogens including *B. cereus, C. botulinum, L. monocytogenes, S. aureus*, *Salmonella* and enterococci strains (Table 1.1). Many bacteriocins are heat stable, making them applicable in combination with heat treatments. They are reportedly food stable, biodegradable, digestible, and are active at low concentrations (De Vuyst and Vandamme, 1994). Bacteriocins are either plasmid (Hastings and Stiles, 1991; Tichaczek *et al.*, 1993) or chromosome (Barefoot and Klaenhammer, 1983) encoded. Some lactic acid bacteria strains have been found to produce more than one bacteriocin, as in the case of *Lactobacillus plantarum* C11 (3), *Carnobacterium piscicola* LV17B (2), *Lactobacillus lactis* subsp. *cremoris* (3) and *Leuconostoc mesenteroides* (3) (Tichaczek *et al.*, 1994, Nes *et al.*, 1996; Papathanasopoulos *et al.*, 1997). The mode of action is thought to be at the cytoplasmic membrane level, as exemplified by nisin which is a lantibiotic that permeabilizes the membrane, thereby dissipitating the membrane potential (Borrena-Gonzalez, 1996; Goff *et al.*, 1996).

1.5.5 Screening for inhibitory lactic acid bacteria

The techniques used in the detection of inhibitory lactic acid bacteria are based on the diffusion of antimicrobial compounds through solid culture media to inhibit growth of sensitive microorganisms. Screening for antimicrobial compounds is usually performed on solid media and involves the detection of inhibition zones in the growth of sensitive strains growing in an agar overlay. Two methods frequently used (Vescovo *et al.*, 1996) are, the simultaneous/direct antagonism method, where both the producer and sensitive strains are grown simultaneously, and the deferred antagonism method, where the test strain is grown first to allow it to produce its antimicrobial compound and is then over layered with the indicator strain and re-incubated to develop zones of inhibition. Growth inhibition is measured as a clear zone around the lactic acid bacteria spot (De Vuyst and Vandamme, 1994). The proteinaceous nature of the inhibition is usually tested by spotting proteases next to the lactic acid bacteria spot resulting in the formation of half zones.

1.5.6 The current taxonomic status of lactic acid bacteria

Lactic acid bacteria are Gram-positive, catalase-negative, non-sporing and non-motile rod- and

Table 1.1 Bacteriocins produced by representative strains of lactic acid bacteria and their spectrum of activity

Producer organism	Bacteriocin	spectrum of activity	reference
Carnobacterium piscicola	Carnobacteriocin	LAB, enterococci, *Listeria*	Ahn and Stiles, 1990
Enterococcus faecum	Enterococcin	*Listeria monocytogenes*	Arihara *et al.*, 1993; Farías *et al.*, 1996
Lactobacillus acidophilus TK9204	Acidocin A	LAB[1], *Listeria monocytogenes*	Kanatani *et al.*, 1995
Lactobacillus bavaricus	Bavaricin A	LAB, Gram-positive bacteria, *Listeria monocytogenes*	Larsen *et al.*, 1993
Lactobacillus curvatus	Curvacin A	*Listeria monocytogenes, Enterococcus faecalis*	Tichaczek *et al.*, 1993; Vogel *et al.*, 1993; Leisner *et al.*, 1996
Lactobacillus plantarum	Plantaricin S and T	LAB, Gram-negative bacteria	Larsen *et al.*, 1993
Lactobacillus sake LTH673	Sakacin P	LAB, gram-negative	Tichaczek *et al.*, 1993
Leuconostoc gelidum UAL187	Leucocin A	LAB, *Listeria monocytogenes, Enterococcus faecalis*	Hastings *et al.*, 1991,
Lactobacillus reuteri	Reuterin	LAB, Gram positive bacteria	Ståhl and Molin, 1994)
Lactococcus lactis	Nisin	LAB, Gram-positive, Gram-negative	Leisner *et al.*, 1996
Pediococcus acidilactici	Pediocin	LAB, Gram- negative and Gram-positive bacteria	Goff *et al.*, 1996
Streptococcus thermophilus	Thermophilin 13	Gram-positive bacteria	Marciset and Mollet, 1993

[1]Lactic acid bacteria

coccus-shaped organisms which may be homo- or hetero-fermentative, producing lactic acid, CO_2, ethanol and acetic acid from glucose (Hammes, *et al.*, 1992; Stiles and Holzapfel, 1997). They are grouped into the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (De Vuyst and Vandamme, 1994; Vandamme *et al.*, 1996). Those lactic acid bacteria that are important in the food industry include members of the genera *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus* and *Weissella*. The discovery of new species and reclassification of previously mis-classified ones has resulted in significant changes in their taxonomic classification and some of these changes are discussed below.

1.5.6.1 Genus *Carnobacterium*

The genus *Carnobacterium (C.)* was proposed by Collins *et al.*, (1987). Although they were originally isolated with lactobacilli, members of this group have since been distinguished from the lactobacilli by their inability to grow on acetate agar, aciduric nature and their ability to grow at pH 9.5. The carnobacteria include isolates from fish and poultry, some of which are fish pathogens, and their removal from the genus *Lactobacillus* rendered the lactobacilli a homogenous group of non-pathogenic bacteria that are beneficial to humans and animals (Collins *et al.*, 1987; Hammes *et al.*, 1992).

1.5.6.2 Genus *Lactobacillus*

The genus *Lactobacillus (Lb.)* is heterogenous and comprises more than 50 validly recognised species. Members of this group are widely distributed in nature and have been isolated from fermented cabbage leaves (Kagermeier-Callaway and Lauer, 1995), sewage and silage (Collins *et al.*, 1989), poultry (Collins *et al.*, 1987) as well as oral and intestinal cavities of warm blooded animals (Hiu *et al.*, 1984). Lactobacilli are strictly fermentative and have complex nutritional requirements (Schleifer and Ludwig, 1995). They are Gram-positive, catalase-negative, non-sporing and usually non-motile bacteria (Takahashi *et al.*, 1992). Three groups have been recognised, based on fermentation characteristics, namely, the *Lb. delbrueckii* group consisting of homofermentative lactobacilli, the *Lb. casei-Pediococcus* group consisting of facultative heterofermentative lactobacilli and the *Leuconostoc* group consisting of obligately

heterofermentative lactobacilli (Schleifer, 1987). They are aciduric or acidophilic, producing pH ≤ 4.0. They are used as starter cultures for food products including cheese, fermented plant foods, fermented meats, wine, beer and sourdoughs (Stiles and Holzapfel, 1997). The classification of the genus has gone through dramatic changes, including the establishment of new genera, namely, *Carnobacterium* (Collins *et al.*, 1987), *Oenococcus* (Dicks *et al.*, 1995) and *Weissella* (Collins *et al.*, 1993), while other species have been found to be synonymous, as in the case of *Lb. sake* and *Lb. bavaricus* (Kagermeier-Callaway and Lauer, 1995) and *Lb. fermentum* and *Lb. cellobiosus* (Stiles and Holzapfel, 1997).

DNA-DNA homology studies prompted the reclassification of *Lb. acidophilus* strains having same biochemical characteristics but different DNA-DNA homologies into six homology groups at species level instead of three (Fujisawa *et al.*, 1992). *Lb. casei* strains have been found to be phenotypically and genotypically heterogenous. On the basis of phenotypic criteria, five subspecies were recognised. A new species *Lb. paracasei* was proposed (Collins *et al.*, 1989) because the majority of strains designated *Lb. casei* subsp. *casei* belonged in the same homology group. Dellaglio and his group (1991), however, suggested the rejection of the name *Lb. paracasei*, and *Lb. casei* subsp. *casei* ATCC393 and *Lb. rhamnosus* ATCC15820 were reclassified as *Lb. zeae nom. rev.* strains (Dicks *et al.*, 1996). Strains belonging to *Lb. pentosus* species which were genotypically different from *Lb. plantarum* (Van Reenen and Dicks, 1996), were treated as synonymous to *Lb. plantarum* because of the omission of the name *Lb. pentosus* in the Approved Lists of Bacterial Names. A re-examination of the genetic relationship of the two species prompted a revival of the name. Certain strains, however, could not be classified as either of the two, and a new species, *Lb. paraplantarum,* was proposed (Curk *et al.*, 1996). The taxonomy of lactobacilli from fish and poultry has also changed following the reclassification of *Lb. piscicola* from fish and *Lb. divergens* from poultry in a new genus *Carnobacterium* (Collins *et al.*, 1987; Mauguin and Novel, 1994).

1.5.6.3 Genus *Lactococcus*

This genus comprises coccoid, non-motile, usually non-hemolytic homofermentative bacteria that produce L (+) lactic acid from glucose. They can be distinguished from streptococci and enterococci by their ability to grow at 10^{0}C but not 45^{0}C (Schleifer, 1987). Although certain

Lactococci are commonly isolated from plant material, they appear to originate from dairy products (Stiles and Holzapfel, 1997). The genus *Lactococcus (L.)* was originally included under the genus *Streptococcus* (*S.*), which originally comprised a wide range of organisms including the highly pathogenic *S. pneumoniae, S. pyogenes and S. agalactiae,* the intestinal streptococci including *S. faecalis* and *S. faecum* and the economically important strains of *L. lactis* used as starter cultures in the dairy industry. A revision of the genus resulted in the separation of the streptococci into 3 genetically distinct groups, namely, *S. sensu stricto*, *Enterococcus* and *Lactococcus* (Stiles and Holzapfel, 1997). The *L. lactis* strains were transferred to the genus *Lactococcus*. The taxonomy of this genus has also undergone major taxonomic changes, largely because most lactococci were mis-classified as lactobacilli as a result of their ovoid shape due to elongation in the plane of chain formation, thus appearing as coccobacilli (Stiles and Holzapfel, 1997). As a result, *Lb. xylosus* was reclassified as *L. lactis* subsp. *lactis* and *Lb. hordniae* as *L. lactis* subsp. *hordniae* (Schleifer *et al.*, 1985). *L. lactis* subsp. *diacetylactis* strains producing diacetyl from citrate were reclassified as, *L. lactis* subsp. *lactis* (Stiles and Holzapfel, 1997) because of findings that citrate utilisation was plasmid-mediated and thus an unstable characteristic.

1.5.6.4 Genus *Leuconostoc*

The genus *Leuconostoc* (*Lc.)* consists of a diverse group of Gram-positive, catalase-negative cocci or coccoid rods which share many characteristics with lactobacilli (Garvie, 1984). They are usually isolated from milk, dairy products, plant material and meat products (Farrow *et al.*, 1989; Hastings and Stiles, 1991), except for *Lc. oenos* which is only isolated from wines and related habitats (Patarata *et al.*, 1994). Leuconostocs have a weak acidifying activity and are generally considered as flavouring agents in foods and also play a role in the spoilage of food. They differ from the lactobacilli in their inability to produce ammonia from arginine and in the production of D(-)-lactate from glucose (Schillinger *et al.*, 1989). The genus initially comprised four species (Garvie, 1984) namely, *Lc. mesenteroides, Lc. paramesenteroides, Lc. lactis and Lc. oenos*, based on morphological and phenotypic characteristics, immunological relationships of dehydrogenases, cellular fatty acid content and DNA homologies (Shaw and Harding, 1989). Six more species have, however, since been defined, namely, *Lc. carnosum, Lc. gelidum, Lc. pseudomesenteroides, Lc. citreum/amelibiosum, Lc. argentinum* and *Lc. fallax*

(Dicks *et al.*, 1993; Villani *et al.*, 1997). *Lc. oenos* was later reclassified as *Oenococcus oeni* (Dicks *et al.*, 1995) and *Lc. paramesenteroides* and related species were reclassified in a new genus *Weissella* (Villani *et al.*, 1997).

1.5.6.6 Genus *Pediococcus*

Pediococci were thought to be the only lactic acid bacteria that divided in two planes to produce tetrads or pairs (Garvie, 1986), which aided in their identification, however, 2 more genera have since been found to form tetrads. Pediococci are homofermentative, produce DL-lactate from glucose and can tolerate high salt concentrations. Eight pediococci species have been recognised in Bergeys manual of systematic bacteriology (Sneath *et al.*, 1986). The genus can be separated from all other Gram-positive cocci by not producing gas from glucose (Weiss, 1992). Pediococci can be divided into two sections, plant pediococci and animal pediococci. Of interest to the food microbiologist are the plant pediococci such as *Pediococcus(P.) pentosaceus* and *P. acidilactici* which occur in plant material. Industrially they have been used in the production of cheese, fermentation of plant material and in the preservation of meat products (Weiss, 1992).

1.5.7 Identification of lactic acid bacteria

Most of the changes in the taxonomy of the lactic acid bacteria have been a result of the emergence of new taxonomic techniques. The heterogenous nature of lactic acid bacteria dictates that a polyphasic approach be adopted when classifying and characterising them. Although different methods have been employed, the classical approach to the taxonomy of lactic acid bacteria was originally based on phenotypic methods which included morphological, physiological and biochemical methods, whole cell protein analysis and cell wall composition analysis (Stiles and Holzapfel, 1997). The advent of new techniques used to manipulate DNA such as DNA hybridisation and PCR prompted a shift from phenotypic studies to genotypic studies. The choice of which method to use has been found to depend on the application and the organism under investigation (Vos *et al.*, 1995). The criteria for evaluating different identification methods include factors such as reproducibility, discriminatory power, ease of interpretation of results and ease of performance (Farber, 1996).

1.5.7.1 Phenotypic methods

Phenotypic methods are those that detect characteristics that are expressed by the microorganism including physiological and biochemical characteristics (Farber, 1996). Biochemical methods include carbohydrate fermentations, cell wall analysis, gas chromatography and fatty acid content analysis (Farrow *et al.*, 1983; Collins *et al.*,1986; Rizzo *et al.*, 1987; Takizawa *et al.*, 1994). These methods have, however, been found to be time consuming and ambiguous, owing to the high number of lactic acid bacteria species (Pot *et al.*, 1993; Villani *et al.*, 1997). The combined use of these methods has been found to successfully distinguish lactic acid bacteria to species level, however, the methods were not sufficient in distinguishing them to subspecies and strain level (Dykes *et al.*, 1994; Villani *et al.*, 1997). Furthermore, the use of these methods in the identification of certain lactic acid bacteria species has been found to be unsatisfactory due to inter-laboratory variation of techniques, strain to strain variations and inconsistent biochemical and physiological properties with known species (Villani *et al.*, 1997). The use of electrophoretic patterns of total soluble protein together with computer-assisted analysis of the resultant protein profiles is well established in bacterial taxonomy and has been found to give results that correlate well with DNA-DNA hybridisation studies (Dykes and von Holy, 1993; Pot *et al.*, 1993; Vogel *et al.*, 1994; Klein *et al.*, 1996). This technique allows fast screening of a large number of strains for comparative purposes and has been successfully applied to the taxonomic studies of lactic acid bacteria from meat and dairy products as well as other environments. It is hindered by the fact that it can only yield discriminative information at the species level, requiring some degree of pre-identification. This problem has been overcome by the creation of a database of digitised and normalised protein patterns for most known species of lactic acid bacteria (Pot *et al.*, 1993).

1.5.7.2 Genotypic methods

The introduction of molecular subtyping techniques resulted in the ability to distinguish between strains belonging to the same species, strain being defined as an isolate or group of isolates that can be distinguished from other isolates of the same genus and species using phenotypic and genotypic characteristics. Such techniques are directed at the nucleic acids, RNA and DNA molecules and they are the most dominant and most reliable of all taxonomic

techniques since they reflect natural relationships as encoded in the DNA. They include DNA base ratio methods, DNA-DNA hybridisations studies, rRNA homology studies as well as DNA-based typing methods such as plasmid profiling, ribotyping, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP), which are DNA fingerprinting techniques that detect DNA sequence polymorphisms using principles such as restriction endonuclease analysis and PCR amplification (Huys *et al.*, 1996; Vandamme *et al.*, 1996; Aarts *et al.*, 1998).

The DNA-DNA hybridisation method was once considered to be one of the most reliable techniques in bacterial taxonomy (Dellaglio *et al.*, 1975) and has contributed largely to the improved taxonomic knowledge on the generic and supra generic relationships of lactic acid bacteria (Collins *et al.*, 1993). Different regions of the rRNA have been used such as the 16S rRNA and 23S rRNA (Vogel *et al.*, 1993). Hybridisation using the 23S rRNA has been found to be reliable for identification at species level but no detectable diversity in this rRNA region has been found that would be specific for strains of a single species (Vogel *et al.*, 1993). One of the disadvantages of ribosomal RNA sequence analysis is its requirement for a large database of rRNA sequences (Pot *et al.*, 1993).

The use of directly visualised restriction enzyme digests of chromosomal DNA from bacteria has been found to offer a rapid and reproducible method for the characterisation and classification of isolates applicable to ecological, epidemiological and population genetics studies. Isolates can be grouped together on the basis of similar or identical numerical profiles after a single electrophoretic run (Forbes *et al.*, 1991). One of the problems associated with such methods is the choice of restriction enzymes used. The criterion for selection of a restriction enzyme for use in DNA fingerprinting is that it should cut the DNA into fragments which are suitable for analysis both in size and in frequency when separated by electrophoretic methods. Enzyme selection may be based on the mol % G+C content of enzyme restriction site (Janssen *et al.*, 1996), however, this is a poor predictor of restriction frequency (Forbes *et al.*, 1991). High frequency of restriction is observed with restriction enzymes that have a four-base recognition site as compared with those having a six-base recognition site, irrespective of the genome (Forbes *et al.*, 1991). Several techniques using this principle have

been reported to successfully differentiate between closely related strains and these include DNA Amplification Fingerprinting (DAF), Pulse Field Gel Electrophoresis (PFGE), RAPD, RFLP and Ribotyping (Farber, 1996; Huys *et al.*, 1996; Arias *et al.,* 1997). A brief discussion on some of the genotypic techniques is given below.

DAF is a technique that is based on the detection of polymorphisms between genomic DNA of closely related species. It is similar to RAPD in the use of a single primer to amplify arbitrary regions of the template DNA but differs in that it has different optimal conditions for amplification due to different lengths and concentrations of primers, template concentrations, general amplification conditions and separation methods of amplified products. The use of this technique requires no prior knowledge of the sequence of the genome. The primers used are usually between 5 and 8 nucleotides long and the amplification procedure is carried out at high annealing temperatures. The limitations of the technique include factors such as preferential amplification of certain sequences (Breen *et al.*, 1995), however, it is considered to be one of the best fingerprinting tools because it uses the simplest and most relaxed amplification conditions, the shortest primers and offers the highest resolution. The technique is mostly used in fingerprinting plant genomes, however, it has been widely applied in typing bacteria and has previously been used for subtyping *Streptococcus uberis* strains isolated from clinical cases (Jayarao *et al.*, 1992).

PFGE was development in an attempt to eliminate some of the problems which occur during restriction enzyme analysis whereby hundreds of different bands can appear on a gel. This technique is based on the use of infrequent cutters, i.e., enzymes recognizing 6- or 8-base sequences resulting in a small number of fragments (5-20) of very large sizes ranging from 10-800 Kb. It works on the principle that large molecules cannot normally be separated by conventional gel electrophoresis but would require a modified version of the electrophoretic procedure. The modification involves changing the orientation of the electric field across the gel (Farber, 1996). The technique involves digestion of genomic DNA with enzyme, separation of fragments on agarose gels by continually changing the orientation of the electric field across the gels, transfer of fragments to membrane and hybridization with labelled probe. The disadvantage of this method are that it is tedious, time consuming and is not cost effective

since it requires the use of a large number of restriction enzymes. The technique has previously been used to characterise *Lc. mesenteroides* strains and was found to be suitable for species discrimination but could not differentiate between the *Lc. mesenteroides* subspecies (Villani *et al.*, 1997). It has also been used to differentiate between individual strains of *Lc. oenos* isolated from New Zealand wines (Kelly *et al.*, 1993).

RAPD is a PCR-based technique that is also referred to as AP-PCR (Power, 1996). It is relatively fast, technically simple and has a high frequency of identification of polymorphisms (Cocconcelli *et al.*, 1995). This technique does not require the use of restriction enzymes. The primers used in this technique possess a base sequence that is arbitrarily defined, hence the procedure does not require any prior knowledge of base sequence for the genome to be amplified. Short primers (10 bases long) are usually used for the random amplification of sequences (Newbury and Ford-Lloyd, 1993; Prabhu and Gresshoff, 1994). On average a 10 base primer will hybridize once every million bases to any strand of DNA. Primers are arbitrary but chosen carefully to have a G-C content of more than 50% and no internal complementarity. The low stringency conditions under which the amplification is carried out result in poor reproducibility of results between laboratories, due to weak complex formations between primer and template (Morell *et al.*, 1995), as such Southern blotting is required to confirm the presence of the species-specific product (Day Jr. *et al.*, 1997). The fact that only one primer is used suggests that RAPD analyses possible differences from a small percentage of total genome (Farber, 1996). This technique is also sensitive to many factors such as selection of primers, magnesium concentration in the PCR buffers and the thermocycler used (Lin *et al.*, 1996). It has previously been used to discriminate between *Lb. Plantarum* and *Lb. pentosus* isolates, which have been found to have very similar phenotypic characteristics and group in the same protein profile cluster (Van Reenen and Dicks, 1996)

The RFLP technique is mostly used in genetic mapping and is based on the differences in the lengths of DNA fragments as a result of digestion with restriction endonucleases. The procedure makes use of the hybridization technique, where cloned DNA sequences are labelled (radioactively or non-radioactively) and used as probes to identify differences in the sizes of specific genomic DNA fragments, after digestion by restriction enzymes (Newbury and Ford-

Lloyd, 1993). The method involves the extraction and purification of genomic DNA, digestion with specific restriction endonucleases and separation of the resulting fragments by gel electrophoresis. The fragments are transferred to a membrane and specific fragments are revealed using labelled DNA probes that hybridize to them. The disadvantages of the technique include the requirement for large amounts of DNA, defined nucleic acids probes and labourious hybridization procedures (Lin *et al.*, 1996). RFLP has been used to characterise *Lb. plantarum* isolates which varied widely in their phenotypic characteristics and had different plasmid profiles (Johansson *et al.*, 1995).

Ribotyping refers to the use of nucleic acid probes to recognise ribosomal genes. This technique takes advantage of the heterogeneity found in the spacer regions that exist between genes coding for rRNA in microorganisms (Farber, 1996). Ribosomal RNA makes up about 82% of total RNA in any typical bacterial cell. Genes coding for the RNA are highly conserved and rRNA occurs in high copy number. The more copies of rRNA present, the more the discriminatory power for that particular bacterium. The procedure involves isolation and restriction of DNA, separation of fragments in agarose gels and the transfer of highly conserved regions of rRNA (16S, 23S and 5S rRNA) to a membrane to be used as probes. A fingerprinting pattern is then created, consisting of 1-15 bands, that can be easily compared among isolates (Farber, 1996). The technique allows for easy reading and is based on stable genetic characteristics. It is, however, tedious, time consuming and expensive, since it requires screening for a restriction enzyme that gives the highest resolution and a probe giving the brightest signal (Arias *et al.*, 1997). This technique has been used in the characterisation of *Lb. sake* strains which were found to contaminate vacuum-packaged sliced cooked meat products. The technique was used to discriminate between strains from equipment surfaces and spoiled products in an attempt to trace the origin of the contamination (Björkroth and Korkeala, 1996).

Ideal fingerprinting methods, however, should not require prior investments in terms of the amount of DNA to be analysed, sequence analysis, primer synthesis or characterisations of DNA probes. This requirement is met only by a few of these techniques (Vos *et al.*, 1995), an example of which being a recently developed DNA fingerprinting technique termed AFLP. This technique has been found to generates fingerprints from DNA of any origin and from any

complexity, without prior sequence knowledge. The electrophoretic patterns depend on the sequence of the PCR primers and the nature of the template DNA (Vos *et al.*, 1995). The technique overcomes many of the problems experienced using other techniques such as, not requiring large amounts of DNA and defined nucleic acid probes, labourious hybridisation procedures (Lin *et al.*, 1996), sensitivity to variations in the template DNA concentrations and analysis of portions of the genome (Janssen *et al.*, 1996). The technique is an ideal system for detecting genetic variation because variation is assessed at a large number of independent loci, the markers are neutral and not subject to natural selection and the variation is revealed in any part of the genome (Majer *et al.*, 1996). It is one of the most sensitive discriminatory tools for assessing intraspecific variability (Huys *et al.*, 1996; Arias *et al.*, 1997) and is highly reproducible (Janssen *et al.*,1996) with reproducibility values of 100%. It has been used in identification, epidemiology and taxonomic studies (Vos *et al.*, 1995; Janssen *et al.*, 1996). The AFLP technique was originally designed for typing plant crops (Smith and Smith, 1992) but has recently been modified for typing of fungi (Majer *et al.*, 1996) and bacteria (Vos *et al.*, 1995). It has since been successfully used in determining genetic diversity and genetic relatedness withing the genomic species in the genera *Aeromonas* (Huys *et al.*, 1996) and *Acinetobacter* (Janssen *et al.*, 1997), and within genospecies III of *Pseudomonas syringae* (Clerc *et al.*, 1998).The technique successfully determined that the ongoing anthrax epidemic in Canada and the northern united States was due to a single strain introduction of a *Bacillus anthracis* strain that has been stable for over 30 years (Keim *et al.*, 1997). AFLP fingerprinting has also been used in the identification of different *Salmonella* serotypes (Arias *et al.*, 1997) and in intraspecific differentiation of *Vibrio vulnificus* biotypes (Aarts *et al.*, 1998) to determine its applicability in epidemiological and ecological studies. The AFLP technique has also been used in determining pathogenicity groupings within *Xanthomonas translucens* strains (Bragard *et al.*, 1997).

The AFLP technique involves three steps (Figure 1.1), the first of which being the digestion of total cellular DNA with two restriction enzymes having different restriction frequencies and ligation of restriction half site-specific adaptors to restriction fragments. The second step is the selective amplification of those fragments with two PCR primers that have corresponding adaptor- and restriction-site sequences as their target sites, and the last step is the

electrophoretic separation of the PCR product.

Restriction fragments are generated by a rare cutter enzyme which has a 6-base recognition site and a frequent cutter enzyme having a 4-base recognition site. Only those fragments having a rare cutter sequence on one end a frequent cutter sequence on the other end are predominantly amplified. The rationale for using two restriction enzymes is to limit the number of fragments and to generate fragments of small sizes that will amplify well and are in the optimal size range for separation on denaturing gels. Therefore, using two different enzymes allows for greater flexibility in tuning the number of fragments to be amplified (Vos *et al.*, 1995).

AFLP adaptors consist of a core sequence and an enzyme-specific sequence, whereas AFLP primers consist of a core sequence, an enzyme-specific sequence and selective extension sequence (Figure 1.1). The sequence of the adaptors and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments. Selective nucleotides are included at the 3' ends of the PCR primers in order to prime DNA synthesis from a subset of the restriction sites, such that only the restriction fragments in which the nucleotide flanking the restriction site match the selective nucleotide will be amplified. Only a subset of fragments will be amplified because primers contain at their 3' end one or more selective bases complementary to the nucleotide flanking the restriction sites. Since the reaction conditions are such that only perfectly matched primers will initiate DNA synthesis, approximately one out of four restriction fragments will be amplified for each selective base used (Janssen *et al.*, 1996). The number of polymorphic bands is determined by the complexity of the genome and the number of choices of selective nucleotides at the 3' end of the restriction enzyme primers. One selective nucleotide is usually used for bacterial genomes (Figure 1.1) (Lin *et al.*, 1996).

Amplified fragments are usually visualised using radioactive labelling or silver staining. Silver staining has been found to detect nucleic acids in the nanogram range. It is a preferable method of visualising bands because it is less hazardous than radioactive labelling (Bassam *et al.*, 1991), however, bands in the lower molecular mass range are usually not efficiently visualised.

This problem in avoided by radioactive labelling since the radioactive label is equally incorporated via standardised PCR conditions (Janssen *et al.*, 1996).

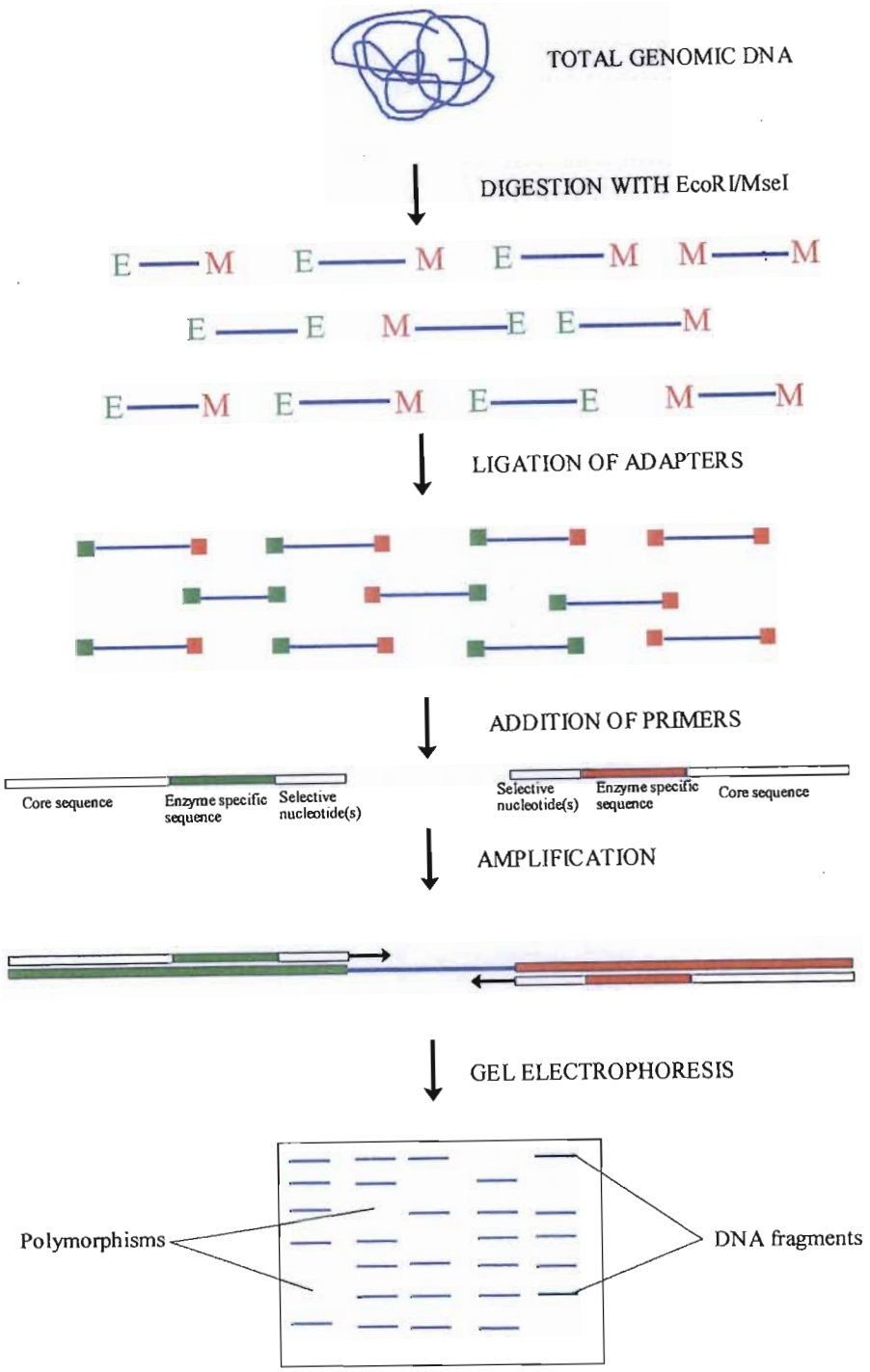

Figure 1.1 A modified description of the AFLP technique as adapted from Janssen (1996). E -EcoRI restriction enzyme, M - MseI restriction enzyme, ■- EcoRI adaptor, ■- MseI adaptor

1.6 MOTIVATION

In recent years, a number of bacteria have emerged as foodborne pathogens and have resulted in foodborne disease outbreaks. These foodborne diseases have had a detrimental effect on infants and children and have often resulted in high mortality rates. This is especially true in third world and developing countries where a large number of the population lives in rural and informal settlements where there is lack of essential basic facilities such as electricity, proper water supplies and sanitation systems, resulting in the prevalence of unhygienic conditions (Smith and Fratamico, 1995).

Numerous studies have shown that weaning foods prepared under unhygienic conditions are frequently heavily contaminated with foodborne pathogenic bacteria, making them a major risk factor in the transmission of diseases, especially diarrhoeal diseases. In one study, milk-based cereals prepared for infants were found to be most frequently contaminated with foodborne pathogens than food prepared for adults and the contamination increased dramatically after storage of the food (Motarjemi *et al.*, 1993). These factors necessitate the development of safe and hygienic methods of preparing and storing food. The process of fermentation is a widely accepted preservation method which has been found to imparts desirable sensory and nutritional properties to fermented products and does not require refrigeration of the food products. This factor makes it an ideal method of food preservation for communities living in informal settlements.

The objectives of this study, therefore, were to analyse the bacterial content of sorghum powder and corresponding fermented and cooked fermented porridge samples mostly used in South African communities as a weaning cereal, especially those living in rural and informal settlements. The microbial populations present in sorghum during the different stages of its preparation and storage as a fermented weaning cereal were quantified, and the samples were screened for the presence of foodborne pathogens usually associated with weaning foods. An investigation on the effects of the fermentation and cooking processes on foodborne pathogens was conducted and lactic acid bacteria associated with sorghum powder and corresponding fermented and cooked fermented porridge samples were identified. This study also investigated the genotypic relationships of selected lactic acid bacteria isolated from sorghum

powder and corresponding fermented and cooked fermented porridge samples using AFLP fingerprinting. This was done in an attempt to differentiated lactic acid bacteria strains within the same species, so as to trace their origin and to study their distribution patterns in samples from the same household and between samples from different households. The antimicrobial activity of selected lactic acid bacteria was also investigated.

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CHAPTER 2

BACTERIAL POPULATIONS ASSOCIATED WITH A SORGHUM-BASED FERMENTED WEANING CEREAL

ABSTRACT

Microbiological surveys were conducted on 45 sorghum samples comprising dry powders and corresponding fermented and cooked fermented porridge samples collected from households in an informal settlement of the Gauteng Province of South Africa. Mean aerobic plate counts of sorghum powder decreased by 0.48 and 2.91 log cfu/g, while mean bacterial spore counts decreased by 1.58 and to < 2.00 log cfu/g in fermented and cooked fermented porridge, respectively. Mean Gram-negative counts of sorghum powder decreased to <2 log cfu/g, whereas mean lactic acid bacteria counts increased by 4.98 log cfu/g but decreased slightly by 0.24 log cfu/g in fermented and cooked fermented porridge, respectively. The mean pH value of sorghum powder samples decreased by 2.4 and 2.1 log cfu/g in fermented and cooked fermented porridge, respectively. *Bacillus (B.) cereus* was detected in all 15 sorghum powder samples, while *Escherichia (E.) coli* was detected in 53%, *Clostridium perfringens* in 27%, *Listeria monocytogenes* in 13% and *Aeromonas* spp., *Salmonella* spp., *Staphylococcus aureus* and *Shigella* spp. each in 7% of sorghum powder samples. Of the fermented porridge samples, 40% contained *B. cereus* and 7% contained *E. coli*. None of the pathogens tested for were detected in the cooked fermented porridge samples. *B. cereus (53%)*, *B. subtilis (21%)*, *B. thuringiensis (13%)*, *B. licheniformis (10%)* and *B. coagulans (3%)* were identified from one hundred and twenty isolates randomly selected from spore count plates of the highest dilution showing growth.

2.1 INTRODUCTION

In developing countries, the beginning of the weaning process in human infants has been associated with an increase in diarrhoeal episodes as a result of consumption of contaminated weaning foods. High incidences of diarrhoeal diseases have been reported in children between the ages of 6 to 18 months (Rowland and McCollum, 1977; Black *et al.*, 1982; Motarjemi *et al.*, 1993). It is estimated that worldwide, 1500 million children under the age of five years suffer from diarrhoeal diseases each year and in 1990, more than 3 million children died as a result (Motarjemi and Nout, 1996).

The causative agents of diarrhoeal diseases in infants and children reportedly include toxin-producing strains of *B. cereus* and *Clostridium (C.) perfringens*, and infectious pathogens such as *E. coli Campylobacter*, *Salmonella* and *Shigella* species (Jackson, 1993; Luby *et al.*, 1993; Kingamkono *et al.*, 1995). Cereal-based products used as weaning foods are reportedly a major source of these microorganisms (Black *et al.*, 1982). Of 261 dried milk products and infant food samples from 17 European and Asian countries, 54% were contaminated with *B. cereus* (Becker *et al.*, 1994). Unfermented maize porridge samples prepared for infants and children in a Ghanaian village were reported to be contaminated with pathogenic bacteria including *Aeromonas* spp., *B. cereus*, *Salmonella* spp., *Staphylococcus (S.) aureus and Vibrio (V.) cholerae* (Motarjemi *et al.*, 1993), while weaning foods and water samples obtained from rural Bangladesh villages were reportedly contaminated with faecal coliforms (Black *et al.*, 1981; Henry *et al.*, 1990). In the Asian country of Myanmar, 505 out of 775 weaning food samples that were tested for 4 enteric pathogens contained *E. coli* only, whereas 28 contained *V. cholerae* non 01 only and 6 contained *Salmonella* spp., *E. coli* and *V. cholerae* non 01 (Motarjemi *et al.*, 1993).

Traditional weaning foods in eastern and southern Africa are based on local staple foods. These are usually cereals such as maize, sorghum, rice or millet (Lorri and Svanberg, 1995). Some of the fermented weaning foods widely used in Africa include uji (Kenya), ogi (Nigeria), kenkey (Ghana), magai (Tanzania) and mawè (Bènin), which are all made from fermented maize or other cereals including sorghum (Rombouts and Nout, 1995). South African fermented weaning foods include mageu, which is made from maize and is produced as a non-

alcoholic drink (Lorri and Svanberg, 1994), and ting or incwancwa, which is made from sorghum or maize.

The process of fermentation by lactic acid bacteria has been shown to lower the pH to below 4 in food products, including sorghum-based fermenting cereal gruels, resulting in growth reduction of pathogenic bacteria (such as *B. cereus, Campylobacter* spp., enterotoxigenic *E. coli, Salmonella* spp., *Shigella* spp. and *S. aureus* (Nout *et al.*, 1989; Mensah *et al.*, 1991; Nout, 1991; Simango and Rukure, 1992; Kingamkono *et al.*, 1995). In rural and informal settlements of South Africa where electricity and sanitary facilities are not available, fermented sorghum and maize are frequently used as weaning foods mainly because they are inexpensive, can be stored for long periods at ambient temperatures and do not require re-heating before consumption. They are prepared from commercial grain available in supermarkets. At the household level, sorghum powder is usually stored in plastic or enamel containers until used. Its preparation is similar to that of 'akasa' or 'koko', a traditional Ghanaian maize-based weaning food (Mensah *et al.*, 1991), where the sorghum is soaked in hot (60 - 90°C) pre-boiled water in a plastic container and incubated in a warm place for 2 to 3 days. Microorganisms which occur naturally in the cereal ferment the sorghum, resulting in a gruel which is mixed with boiling water, cooked for a period of up to 30 min and then consumed as a sour porridge. This type of fermented product is most common as a weaning food in underdeveloped areas such as informal settlements and rural areas of South Africa.

Although there has been extensive research on fermented weaning foods from several African countries, no report exists on traditional weaning foods widely used in rural and informal settlements of South Africa. The aim of this study was to assess bacterial populations found in sorghum before, during and after its preparation as a fermented weaning cereal at the household level.

2.2 MATERIALS AND METHODS

2.2.1 Sample preparation

Samples were collected between March and April from an informal settlement in the Gauteng Province, situated on the East Rand and consisting of shacks built from corrugated iron (Figure

2.1). Electricity and sanitary facilities were not common in this area. Samples were collected from 15 households, each of which was provided with commercial packets of pure grain sorghum ("Supertieng", Nola, Randfontein, South Africa) purchased from a local supermarket (Figure 2.2) a week before samples were collected. The sorghum powder was transferred to dedicated storage containers available in the households. A fermented gruel was prepared by mixing sorghum powder with pre-boiled hot (60 - 90°C) water (ratio 3:1) in plastic containers to make a slurry. The containers were covered with plastic lids or saucers and incubated at ambient temperature (20 - 30°C) for 24 - 48 h Figure 2.2). The cooking process involved mixing the resultant fermented slurry with boiling water followed by cooking for 15 - 30 min. The cooked porridge was then transferred to smaller plastic or enamel containers, often using the same utensils (spoons) used before and during the cooking process and covered with plates or lids from pots for storage. The cooked porridge was stored at ambient temperature.

2.2.2 Sampling procedure

Cooked, fermented porridge samples, together with corresponding sorghum powder and fermented porridge samples, were collected 3 - 4 h after cooking. All samples (*ca.* 200g) were aseptically collected, using sterile wooden spatulas, in sterile Whirl Pak bags (Nasco, USA), transported at ambient temperatures and tested within 24 h of collection.

2.2.3 Sample processing and analysis

2.2.3.1 Bacterial and spore counts

For each sample, 20g was homogenized for 3 min in 180ml diluent (0.1% peptone, 0.85% NaCl) (10^{-1} dilution) in a Colworth 400 Stomacher (Seward Medical, London, UK). Tenfold serial dilutions in the same diluent were plated in duplicate onto different media and incubated aerobically for 48 h (Table 2.1). Plates showing between 30 and 300 colony forming units (cfu), or the highest number if below 30, were counted. Bacterial spore counts were obtained by heating 10ml of the 10^{-1} dilution at 80°C for 10 min in a water bath, followed by cooling on ice and duplicate plating (Table 2.1) (Becker *et al.*, 1994). Six packets of sorghum powder were analysed for aerobic plate counts (APC) directly after purchase and before delivery to the households as controls, as described above (Table 2.1).

Corresponding bacterial counts were averaged and compared between sample categories by analysis of variance (ANOVA) at the 95% confidence level. The pH of all samples was measured immediately after preparation of the 10^{-1} dilution.

2.2.3.2 Identification of colonies isolated from spore count plates

The 120 colonies isolated from plates of the highest dilution showing growth from sorghum powder (60) and fermented porridge (60) samples were purified on Plate Count Agar (Oxoid, Basingstoke, UK). Gram-positive, catalase positive isolates were identified according to Norris *et al.*, (1981).

2.2.3.3 Analysis for foodborne pathogens

Aeromonas spp.

To enrich for *Aeromonas* spp., samples (25g) were mixed with 225ml *Vibrio* enrichment broth (Biolab, Midrand, South Africa) and incubated at 37°C for 48 h, then spread (1ml) onto blood agar plates (Oxoid) (*Kirov et al.*, 1990). All Gram-negative colonies were identified using biochemical tests as described by Popoff (1984).

Bacillus cereus

Robertson's heated cooked meat (HCM) (Merck, Darmstadt, Germany) (100ml) was mixed with 50ml of the 10^{-1} dilution and incubated at 37°C for 48 h (Kramer and Gilbert, 1989). The broth (1ml) was spread plated on blood agar plates and Polymyxin-Pyruvate-Egg Yolk Mannitol- Bromothymol Blue Agar (PEMBA) (Oxoid) plates and incubated aerobically at 37°C for 24 h (ICMSF, 1996). Typical *B. cereus* colonies indicated by a blue colouration on PEMBA plates and haemolysis on blood agar plates were streaked on Willis and Hobbs agar (Oxoid) and incubated for 24 h anaerobically (H_2/CO_2 Gas Generating Kit, Oxoid), to test for production of lecithinase.

Campylobacter spp.

Preston broth (225ml) (Oxoid) was mixed with 25g of sample and incubated for 24 h at 42°C (ICMSF, 1996). The broth (1ml) was spread on blood-free *Campylobacter* agar (Oxoid) (Beuchat, 1987) and Colombia blood agar (Oxoid) (Stern and Kazmi, 1989) plates and

Table 2.1 Culture media, incubation temperatures and plating techniques used for the microbiological analyses of 15 sorghum powder samples and corresponding fermented and cooked fermented porridge samples

Count type	Incubation temperature (°C)	Plating technique	Growth medium
Aerobic plate count (APC)	35	Pour-plate	Plate count agar (Oxoid)
Gram-negative count (GNC)	30	Pour-plate	Violet red bile glucose agar (Oxoid)
Spore count (SC)	37	Spread-plate	Plate count agar (Oxoid)
Lactic acid bacteria count (LABC)	25	Spread-plate	MRS agar (Oxoid) supplemented with 0.1% L-cysteine monohydrochloride (Sigma) (Dykes *et al.*, 1991) and 40µg/ml cycloheximide (Merck) (Nout *et al.*, 1987).

Figure 2.1 **Typical scenes from an informal settlements. A - one of the households from which sorghum powder samples and their corresponding fermented and cooked fermented porridge samples were collected. B , C and D - Plastic containers of industrial origin, containing water which was collected from communal taps and stored inside the households next to other kitchen utensils and food products. These stores of water were used during the preparation of the fermented and cooked fermented porridge samples**

A

B

Figure 2.2 **A - An example of the sorghum powder samples used in the study. B - Fermenting sorghum which was prepared by mixing sorghum powder with pre-boiled water and stored in a warm place on top of a coal stove for a period of 2 to 3 days and incubated under micro-aerophilic conditions at 42°C, for 48 h.**

Since no positive colonies were obtained, confirmation tests for *Campylobacter* (Bolton, 1983; Beuchat, 1987) were not conducted.

Clostridium perfringens

Aliquots of 1ml of the 10^{-1} dilution were spread onto Reinforced clostridial agar (Oxoid) plates and incubated aerobically and anaerobically (H_2/CO_2 Gas Generating Kit, Oxoid) for 48 h at 37°C to test for growth on the anaerobic plates and scanty or no growth on the aerobic plates Catalase-negative colonies on anaerobic plates were streaked on egg yolk agar (Oxoid) plates and examined for α-toxin activity (Nagler's reaction), resulting in the formation of insoluble degradation products of egg yolk (lecithin). Lecithinase producing colonies were streaked on egg yolk agar plates containing *C. perfringens* Type A antitoxin (Oxoid). Colonies showing inhibition of lecithinase production by the antitoxin were identified as *C. perfringens* (Labbe, 1989; ICMSF, 1996).

Escherichia coli

Duplicate tubes (10ml) of MacConkey broth (Oxoid) with Durham tubes were each mixed with 1ml of the 10^{-1} dilution and incubated at 37°C for 24 h. Tubes showing gas and acid production were inoculated (1ml) into brilliant green bile broth (Oxoid) and tryptone water (Oxoid) and incubated at 44°C overnight. Production of gas in the brilliant green bile broth and indole in tryptone water (red colouration) when reacted with Kovacs reagent (Merck), indicated the presence of *E. coli*. An aliquot of 1ml of the 10^{-1} dilution was spread onto duplicate MacConkey sorbitol agar (Oxoid) plates and incubated at 37°C overnight to select for sorbitol-negative *E. coli* O157:H7 colonies (Vernozy-Rozand, 1997).

Listeria monocytogenes

Dairy enrichment broth (225ml) (Oxoid) supplemented with 15mg/l acriflavin HCl (Sigma, Saint Louis, USA), 40mg/l nalidixic acid (Sigma) and 50mg/l cycloheximide (Sigma) was inoculated with 25g of sample (Lovett, 1987) followed by incubation for 5 consecutive weeks at 30°C. At the end of each of the five weeks, the broth (1ml) was spread on Mcbride *Listeria* agar (Oxoid) plates, incubated at 37°C for 24 h and analysed for the presence of black colonies. No positive colonies were obtained and therefore none of the identification tests

(Lovett, 1987) were conducted.

Salmonella spp.

Pre-enrichment was carried out by homogenising 25g sample in 225ml buffered peptone water (Oxoid) for 2 min followed by incubation at 37°C for 24 h (Arvanitidou *et al.*, 1998). This was followed by selective enrichment whereby the buffered peptone water (10ml) was inoculated into 100ml Tetrathionate medium (Oxoid) and incubated at 43°C for 24 - 48 h. The buffered peptone water (10ml) was further inoculated into 100ml selenite medium (Difco, Detroit, USA) and Gram -negative medium (Merck) and then incubated at 37°C for 24 - 48 h (ICMSF, 1978; (Arvanitidou *et al.*, 1998; ISO 6579, 1993). The 3 broths were streaked onto BGA (Oxoid) and XLD agar (Difco) plates and incubated for 24 h at 37°C. Lactose-negative colonies were further characterised using biochemical and serological tests (ICMSF, 1978; (Arvanitidou *et al.*, 1998).

Shigella spp.

Gram-negative Medium (100ml) was mixed with 25ml of the 10^{-1} dilution and incubated for 24 h at 37°C. The broth was streaked onto XLD agar plates and incubated at 37°C for 24 h (Wachsmuth and Morris, 1989). Typical colonies were subjected to further biochemical tests as described by Ewing (1986).

Staphylococcus aureus

Triplicate bottles each containing 20ml *Staphylococcus* enrichment broth (Difco) were mixed with 1ml of the 10^{-1} dilution and incubated for 48 h at 37°C after which they were examined for acid production. The broth was streaked onto Baird-Parker agar (Oxoid) plates and jet-black colonies forming a fibrin clot in plasma mannite tubes (Difco) and clear zones on DNA agar (Oxoid) were regarded as *S. aureus* (ICMSF, 1978).

Vibrio spp.

Pre-enrichment was carried out by homogenising 25g sample in 225ml buffered peptone water (Madden and McCardell, 1989). An aliquot of 50ml of the buffered peptone water was inoculated into two bottles containing 50ml of *Vibrio* enrichment broth (Oxoid) followed by

incubation for 24 h, one at 37°C and the other at 42°C . Loopfuls from the surface of the broths were streaked onto duplicate Thiosulphate Citrate Bile Sucrose (TCBS) agar (Difco) plates, incubated at 37°C and examined for the presence of flat yellow round colonies with a diameter of 2-3mm and/or smooth green colonies with a diameter of 3-5mm (Madden and McCardell, 1989).

2.3 RESULTS

2.3.1 Bacterial counts of sorghum samples

Bacterial counts of the six commercial sorghum powder controls exhibited significant differences ($P<0.05$) between Aerobic plate counts (APC) from different packets, ranging from 3.20 - 5.02 log cfu/g, with a mean of 4.64 log cfu/g. The mean APC were significantly lower ($P<0.05$) by 2.78 log cfu/g when compared to the APC of corresponding sorghum powder samples after distribution and storage at the household level (Table 2.2).

For sorghum powder samples from the 15 households, APC were the highest, followed by spore counts (SC) and counts of lactic acid bacteria (LABC), while Gram-negative counts (GNC) were the lowest (Table 2.2).

Compared to sorghum powder samples, the mean APC of fermented porridge samples decreased slightly by 0.48 log cfu/g, while the mean SC were significantly lower ($P<0.05$) by 1.58 log cfu/g. LABC exhibited significant increases ($P<0.05$) by 4.98 log cfu/g while Gram-negative bacteria decreased to below the lower detection limit (<2.00 log cfu/g) in fermented porridge samples (Table 2.2). The APC of cooked fermented porridge samples were significantly lower ($P<0.05$) by 2.91 log cfu/g when compared to the sorghum powder samples, whereas an increase of 0.23 log cfu/g in LABC was not significant ($P>0.05$). Compared to the fermented porridge samples, APC and LABC of cooked fermented porridge samples showed significant decreases ($P<0.05$) by 2.43 log cfu/g and 4.75 log cfu/g, respectively. GNC of cooked fermented porridge samples remained below the lower detection limit (>2.00 log cfu/g) while SC decreased significantly ($P<0.05$) to below the lower detection limit when compared to corresponding counts of sorghum powder and uncooked fermented porridge samples (Table 2.2).

Table 2.2 Bacterial counts (log cfu/g) of sorghum powder and corresponding fermented and cooked fermented porridge samples.

Analysis	Sorghum powder (15)	Fermented porridge (15)	Cooked fermented porridge (15)
APC[a]	7.42 ± 1.45[b]	6.94 ± 1.28	4.51 ± 0.89
	(5.30 - 9.25)[c]	(4.99 - 9.81)	(2.00 - 5.89)
SC	5.42 ± 1.38	3.84 ± 1.01	<2.00
	(3.34 - 7.75)	(2.30 - 5.69)	
GNC	2.50 ± 0.74	<2.00	<2.00
	(<2.00 - 4.00)	-	-
LABC	3.07 ± 1.23	8.06 ± 1.08	3.31 ± 1.13
	(2.00 - 5.80)	(5.32 - 9.69)	(2.04 - 5.90)
pH	6.3 ± 0.3	3.9 ± 0.2	4.2 ± 0.2
	(5.9 - 6.8)	(3.6 - 4.4)	(3.8 - 4.5)

[a] for abbreviations, see Table 2.1

[b]mean ± standard deviation

[c]range

2.3.2 pH

The mean pH of dry sorghum powder samples was 6.3, but dropped significantly ($P<0.05$) to 3.9 and 4.2 in fermented and cooked fermented porridge samples, respectively. The differences observed between the pH of fermented and cooked fermented porridge samples were not significant ($P>0.05$) (Table 2.2).

2.3.3 Incidence of foodborne pathogens

All sorghum powder samples contained *B. cereus*. *E. coli* was detected in 53%, *C. perfringens* in 27% and *L. monocytogenes* in 13%. *S. aureus*, *Aeromonas*, *Salmonella* and *Shigella*, species were each detected in 7% of sorghum powder samples, whereas *E. coli* O157:H7, *Campylobacter* spp. and *V. cholerae/parahaemolyticus* were not detected in any of the sorghum powder samples (Table 2.3). *B. cereus* was also detected in 40% of fermented porridge samples and *E. coli* in 7%. None of the pathogens tested for were detected in any of the cooked, fermented porridge samples.

2.3.4 Identification of isolates from spore count plates

All 120 isolates were catalase-positive, rod-shaped and spore-forming. Of these, 53% were *B. cereus*, which were isolated from all sorghum powder samples and 60% of the fermented porridge samples. The remaining isolates were identified as *B. subtilis* (21%), *B. thuringiensis* (13%), *B. licheniformis* (10%) and *B. coagulans* (3%). Of these, *B. subtilis* was isolated from 53% of the sorghum powder samples and 46% of the fermented porridge samples while *B. thuringiensis* was isolated from 60% of the sorghum powder samples and 27% of the fermented porridge samples. *B. licheniformis* was isolated from 40% of the sorghum powder samples and 20% of the fermented porridge samples while *B. coagulans* was isolated from 20% of the sorghum powder samples and 7% of the fermented porridge samples.

2.4 DISCUSSION

The wide range of APC (3.20 - 5.02 log cfu/g) from commercial sorghum powder packets suggested varying levels of contamination of sorghum powder. Since the mean APC of the sorghum powder samples from households were significantly higher ($P<0.05$) by a log value of 2.78 cfu/g (Table 2.2) compared to commercial sorghum powder controls, this suggested

Table 2.3 Incidence of foodborne pathogens in 15 sorghum powder samples and corresponding fermented and cooked fermented porridge samples

Pathogen[a,b]	Sorghum powder (15)	Fermented porridge (15)	Cooked fermented porridge (15)
Aeromonas sp.	1 (7%)	ND[c]	ND
Bacillus cereus	15 (100%)	6 (40%)	ND
Clostridium perfringens	4 (27%)	ND	ND
Listeria monocytogenes	2 (13%)	ND	ND
Salmonella sp.	1 (7%)	ND	ND
Staphylococcus aureus	1 (7%)	ND	ND
Shigella sp.	1 (7%)	ND	ND
Escherichia coli	8 (53%)	1 (7%)	ND

[a]*Campylobacter*, sorbitol negative Enterobacteria and *V. cholerae/parahaemolyticus* were not detected in samples tested.

[b]All pathogens were detected using the enrichment approach, except for *E. coli* 0157:H7 and *C. perfringens*, in which cases the detection limit was 10 cfu.

[c]not detected.

additional contamination at the household level. A general lack of hygienic practices and cleanliness observed during visits to the households indicated a strong likelihood of cross-contamination between cooking utensils, food handlers and the weaning food samples. Spore counts suggested high contamination levels of sorghum powder and uncooked fermented porridge samples. The identification of randomly selected colonies from spore count plates of the highest dilution resulted in more than 50% of those colonies being identified as *B. cereus*. Further confirmation of contamination by *B. cereus* was obtained following their detection in all dry sorghum samples and in 40% of uncooked fermented porridge samples (Table 2.3). These bacteria are reportedly common contaminants of agricultural commodities such as cereal crops, including sorghum crops, vegetation and soil (Kramer and Gilbert, 1989). The other *Bacillus* species identified from spore count plates of the highest dilution were insect pathogens that have previously been isolated from soil, plant material, and food ingredients such as spices, sugar, cereals milk and milk products (Norris *et al.*, 1981; Damgaard *et al.*, 1996; Damgaard *et al.*, 1997). Of these, *B. subtilis* and *B. licheniformis* have been associated with food poisoning incidences resulting in diarrhoea (Norris *et al.*, 1981; Tan *et al.*, 1997). Gram-negative bacteria were detected at low levels (2.50 log cfu/g) in sorghum powder, in agreement with previously reported contamination levels of sorghum powder before fermentation (Mensah *et al.*, 1990; Nout, 1991). Lactic acid bacteria counts and pH of sorghum powder samples in the present study were also similar to results obtained by Nout (1991) who reported LABC of 2.80 log cfu/g at the start of fermentation of sorghum-based infant foods.

Although APC of fermented porridge samples decreased by 0.48 log cfu/g compared to sorghum powder samples, the bacterial populations are likely not to have been the same due to changed conditions brought about by the fermentation process. These include the lowering of pH due to the production of organic acids which would support the proliferation of acid-tolerant bacteria. The reduced levels of Gram-negative bacteria and bacterial spores found in fermented porridge samples indicated the effectiveness of fermentation in reducing these populations. This reduction has been attributed to the production of organic acids by lactic acid bacteria (Kingamkono *et al.*, 1995) and possibly other inhibitory substances such as bacteriocins, peroxides, aldehydes, carbon dioxide and diacetyl (De Vuyst and Vandamme,

1994). Previous studies on the survival of spores at low pH have reported a decrease in the counts of *C. perfringens* (Smith, 1963) and *B. cereus* (Wong and Chen, 1988) in media with pH values below 5. Lactic acid bacteria predominated in fermented porridge samples, reinforcing results obtained by Rombouts and Nout (1995) who reported LABC of 9.50 - 9.70 log cfu/g in fermented sorghum. The mean pH of fermented porridge samples in the present study was 3.9, which was similar to pH 3.7 for fermented sorghum-based infant food samples analysed by Nout (1991).

Interviews held in each household indicated cooking times for fermented porridge samples to have been 15 min or longer, which reportedly kills vegetative bacterial cells (WHO, 1993). Residual APC and LABC in cooked fermented porridge samples suggested that re-contamination occurred during the transfer and storage period of the weaning food samples. Although this may also suggest a potential re-contamination risk by foodborne pathogens, the low pH of cooked fermented porridge samples (range 3.8 - 4.5), however, would inhibit their growth. The low levels of Gram-negative bacteria (<2.00 log cfu/g) found in cooked fermented porridge samples were similar to those found in samples analysed by Mensah *et al.*, (1991). Although the fermentation process was found to result in a significant decrease ($P<0.05$) in SC of fermented porridge samples, the combination of fermentation (low pH) and cooking (high temperature) reduced the spore count further to below the lower detection limit.

The high incidence of *B. cereus* confirmed previous reports on its prevalence in sorghum powder (Norris *et al.*, 1981; Travers *et al.*, 1987). These *B. cereus* isolates originated from all sorghum powder samples and 40% of fermented porridge samples (Table 2.3). This was in agreement with spore count results for sorghum powder and fermented porridge samples (Table 2.2).

Although it is possible that the *E. coli* detected in dry sorghum and fermented porridge samples could have resulted from contamination during manufacture and packaging, contamination could also have occurred at the household level. For example, the absence of in-house sanitation systems necessitated the use of surrounding bushes as toilets, and the tap water used in the preparation of the samples was collected from communal taps and stored in

plastic containers of industrial origin for periods of 3 days and longer inside the shack. In most households, other types of food such as meat, eggs, fruits and vegetables were stored next to water supplies, creating further possible sources of cross contamination through handling of food. Other sources of contamination could have been dust, pets and parasites. The reduction of the incidence of *E. coli* to only 7% in fermented porridge samples suggested that fermentation was effective in reducing the incidence of Gram-negative bacteria, including enteric pathogens. Previous studies have reported growth reduction of foodborne pathogens, including *Campylobacter jejuni*, *Salmonella typhimurium*, enterotoxigenic *E. coli*, *Shigella sonnei*, *S. aureus*, *L. monocytogenes* and *B. cereus*, in fermented sorghum or maize cereal samples similar to the weaning foods in this study (Nout *et al.*, 1989; Simango and Rukure, 1992; Kingamkono *et al.*, 1995).

2.5 CONCLUSION

This study highlighted sorghum powder as a source of *E. coli*, spore forming bacteria such as *B. cereus* and *C. perfringens*, and lactic acid bacteria. The process of fermentation was found to be capable of significantly reducing the incidence of Gram-negative bacteria and non-sporing bacterial pathogens, however, a combination of fermentation (low pH) and cooking (high temperature) was found to reduce these populations even further. The spontaneous fermentation process employed by households to prepare weaning foods was found to reduce bacterial counts and enhance the safety of the weaning food samples.

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CHAPTER 3

IDENTIFICATION OF LACTIC ACID BACTERIA FROM SORGHUM-BASED FERMENTED WEANING FOODS BY SDS-PAGE ANALYSIS OF SOLUBLE PROTEINS AND DETECTION OF STRAIN VARIATIONS BY AFLP FINGERPRINTING

ABSTRACT

Lactic acid bacteria represent one of the most diverse groups of bacteria known and their identification has been extensively studied using different techniques. In this study, 180 members of this group which were isolated from sorghum powder (44) and corresponding fermented (93) and cooked fermented porridge samples (43) were identified by biochemical and physiological methods as well as by the analysis of electrophoretic profiles of total soluble proteins. Of the 180 strains isolated, 58 were identified as *Lactobacillus plantarum*, 47 as *Leuconostoc mesenteroides*, 25 as *Lactobacillus sake/curvatus*, 17 as *Pediococcus pentosaceus*, 13 as *Pediococcus acidilactici* and 7 as *Lactococcus lactis* strains. *Lactobacillus plantarum* and *Leuconostoc mesenteroides* strains were found to dominate during the fermentation process and were found to have the highest frequency of distribution between the households, being distributed in 13 and 11 of the 15 households, respectively. These findings prompted the analysis of 58 *Lactobacillus plantarum* and 46 *Leuconostoc mesenteroides* strains by AFLP fingerprinting, in order to trace their source of origin and to determine patterns of distribution in the sorghum powder samples and their corresponding fermented and cooked fermented samples. The primary source of *Lactobacillus plantarum* and *Leuconostoc mesenteroides* strains was established as the sorghum powder samples by AFLP analysis. The spontaneous fermentation processes from which the lactic acid bacteria isolates were obtained were found to result in heterogenous populations of lactic acid bacteria. Seventy two lactic acid bacteria isolates were tested for antimicrobial activity against selected foodborne pathogens and toxin producing bacteria including *Bacillus cereus, Bacillus subtilis, Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus* strains. Antimicrobial activity was also tested against selected lactic acid bacteria strains isolated from sorghum powder and corresponding fermented and cooked fermented porridge samples. Broad spectrum antimicrobial activity, as a result of lactic acid production, was demonstrated by all selected lactic acid bacteria isolates. None of the isolates tested seemed to produce bacteriocins.

3.1 INTRODUCTION

Lactic acid bacteria are a heterogenous group of bacteria that are generally regarded as safe (GRAS) for use in food and food products (Gancel *et al.*, 1997). Those important in the food industry include members of the genera *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus* and *Weissella* (De Vuyst and Vandamme, 1994; Vandamme *et al.*, 1996). Their use in food products dates back to ancient times, mainly because of their contribution to flavour, aroma and increased shelf life of fermented products (Nes *et al.*, 1996). Various members of this group are being used commercially as starter cultures in the manufacture of food products including, dairy products (Salama *et al.*, 1995), fermented vegetables (Leisner *et al.*, 1996), fermented doughs (Vogel, *et al.*, 1994), alcoholic beverages (Patarata *et al.*, 1994; Pattison *et al.*, 1998) probiotics in animal feeds (Castellanos *et al.*, 1996) and as starter cultures in meat products (Vogel *et al.*, 1993). They have also been used in lactic acid fermentation of sorghum- or maize-based cereals used as infant weaning foods (Lorri and Svanberg, 1994; Olsen and Jakobsen, 1995; Rombouts and Nout, 1995; Motarjemi and Nout, 1996). These cereals have been reported to be dominated by lactic acid bacteria belonging to the *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* species (Nout, 1991; Kingamkono *et al.*, 1995; Oyewole, 1995). Lactic acid bacteria have also been associated with the production of antimicrobial compounds, including the production of lactic acid, hydrogen peroxide, diacetyl, carbon dioxide and bacteriocins (De Vuyst and Vandamme, 1994). Bacteriocin production has been described from all genera of lactic acid bacteria (Klaenhammer, 1993; Daeschel, 1989; De Vuyst and Vandamme, 1994) and producer strains are being used industrially in the inhibition of spoilage and pathogenic microorganisms (Leisner *et al.*, 1996)

Various techniques have been employed in identifying lactic acid bacteria including phenotypic and genotypic methods, however, the classical approach was based on phenotypic methods which included morphological, physiological and biochemical methods, whole cell protein analysis and cell wall composition analysis. (Stiles and Holzapfel, 1997). The combined use of these methods is reportedly successful in distinguishing bacteria to species level, however, they are not sufficient in distinguishing bacteria to subspecies and strain level (Dicks *et al.*, 1990; Dykes *et al.*, 1994). Use of electrophoretic patterns of total soluble

proteins and computer-assisted analysis of the resultant protein profiles is well established in bacterial taxonomy (Pot *et al.*, 1993; Dykes and von Holy, 1993; Klein *et al.*, 1996). This technique has been successfully applied to the taxonomic studies of lactic acid bacteria from meat, dairy products and other environments, but is hampered by the fact that it can only yield discriminative information at species level, requiring some degree of pre-identification. This problem has been overcome by the creation of a database of digitised and normalised protein patterns for most known species of lactic acid bacteria (Pot *et al.*, 1993).

Genotypic methods include DNA base ratio methods, DNA-DNA hybridisation studies, rRNA homology studies and DNA-based typing methods such as plasmid profiling, ribotyping, RFLP and RAPD (Vandamme *et al.*, 1996; Aarts *et al.*, 1998). Although they have been successfully used in different applications, they are hampered by factors such as, non-reproducibility of results between experiments (RAPD) and the requirement for large amounts of time and labour (stereotyping and biotyping) (Aarts *et al.*, 1998). Recently, a DNA fingerprinting technique termed Amplified Fragment Length Polymorphism (AFLP), was developed, which detects genetic variation in organisms. The variation is assessed at a large number of independent loci and is revealed in any part of the genome and from any complexity, without prior sequence knowledge (Vos, *et al.*, 1995). This technique has since been successfully used in taxonomic studies of *Acinetobacter* (Janssen *et al.*, 1996) and *Aeromonas* strains (Huys *et al.*, 1996), and in epidemiological studies of *Bacillus anthracis* (Keim *et al.*, 1997), *Salmonella* (Aarts *et al.*, 1998) and *Xanthomonas translucens* (Bragard *et al.*, 1997) strains as well as *Vibrio vulnificus* biotypes (Arias *et al.*, 1997). This technique has also been applied in distinguishing molecular evolutionary origins and geographic correlations of *Pseudomonas syringae* strains (Clerc *et al.,* 1998).

The aims of this study were to identify the lactic acid bacteria that occur naturally in sorghum powder and corresponding fermented and cooked fermented porridge samples by the combined use of physiological and biochemical methods and analysis of total soluble protein patterns. The study also aimed to determine which lactic acid bacteria dominated during the fermentation process, and to detect strain variation within a species by AFLP analysis, in order to determine the source of the strains responsible for the fermentation. The antimicrobial

activity of lactic acid bacteria against food pathogens and toxin-producing bacteria which were detected in sorghum powder samples and corresponding fermented and cooked fermented porridge samples was also investigated.

3.2 MATERIALS AND METHODS

3.2.1 Culture selection and maintenance

The 180 lactic acid bacteria strains used in this study were obtained from a previous microbiological survey of 45 sorghum samples collected from an informal settlement of the Gauteng Province of South Africa (Kunene, *et al.*, 1999). These lactic acid bacteria were isolated from sorghum powder samples (44) and corresponding fermented (93) and cooked fermented (43) porridge samples. Presumptive lactic acid bacteria colonies were randomly selected from MRS agar (Oxoid, Hampshire, England) plates supplemented with 0.1% L-cysteine monohydrochloride (Sigma, Saint Louis, Mo., USA) (11) and 40µg/ml cycloheximide (Merck, Darmstadt, Germany) The isolates were purified on MRS agar at 25°C for 48 h. Working cultures were maintained on MRS agar plates and subcultured every 3 weeks. Stock cultures were maintained in MRS broth (Oxoid) supplemented with 30% glycerol and stored at -70°C. Fourteen reference strains were included in the study (Table 3.1).

3.2.2Analysis of total soluble protein profiles

3.2.2.1 Extraction of total soluble protein

Protein extracts were obtained from 180 strains by a previously described method (Dykes and von Holy, 1993). Cells were disrupted on ice in Eppendorf tubes using a Virsonic 60 (Virtis Company Inc., NY, USA), at 20 watts for 5 min. Undisrupted cells and debris were removed by centrifugation at 6000 rpm for 1 hour. The protein extracts were concentrated under vacuum down to 100µl and stored at -20°C until use.

3.2.2.2 Electrophoresis

Protein extracts were fractionated, using the principle of Laemmli, on 12% polyacrylamide gels using a Hoefer SE 600 electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, USA). Aliquots of 20µl of the protein extracts were mixed in a 1:1 ratio with loading buffer, boiled at 100°C for 5 min and after electrophoresis at 20mA, the gels were

stained with Commassie blue. Mid-range molecular weight markers (Boehringer Mannheim, Darmstadt, Germany) were used as standards (Dykes and von Holy, 1993).

3.2.2.3 Data analysis

Images were captured on an image analyser (UVP Image store 5000, Ultra Violet Products Ltd., Cambridge, UK). Electrophoretic patterns were analysed using the software package GelManager, version 1.5 (Biosystematica, Devon, UK). Levels of similarity between the patterns were calculated using the Pearson product moment correlation coefficient. The Unweighted Pair Group Method of Arithmetic Average Clustering (UPGMA) was used for cluster analysis and the clusters were delineated at an arbitrary level of r = 0.60.

3.2.3 Physiological and biochemical tests

Seventy two isolates randomly selected from clusters obtained from electrophoretic profiles of total soluble protein were subjected to biochemical and physiological tests. Of the 72 isolates, 24 were from cluster I, 13 from the cluster II, 3 from cluster III, 5 from each of clusters IV and V, 10 from cluster VI and 12 from cluster VII. Each isolate was grown on MRS agar for 48 hours and Gram-stained. Cell morphological characteristics were examined microscopically using a Kontron image analyser (Vidas, Darmstadt, Germany). The fermentation of carbohydrates and identification of isolates by biochemical traits was conducted as described by Schillinger and Lücke (1987). In summary, sterile sugars were aseptically added to the sterile basal medium containing 0.04% chlorophenol red to reach a final concentration of 0.5% (w/v). Tubes were incubated at 25 °C for 2 and 5 days. Isolates were tested for the fermentation of acetate, adonitol, arabinose, cellobiose, erythritol, fructose, galactose, glycerol, inositol, lactose, maltose, mannitol, mannose, melibiose, rhamnose, ribose, saccharose, sorbitol, sucrose, trehalose and xylose. The terminal pH of the broth cultures was measured after a 48 hour incubation period in MRS broth at 25 °C (Gancel *et al.*, 1997) and growth at pH 3.9 was determined on MRS broth (Schillinger and Lücke, 1987). Tolerance to NaCl was examined by testing for growth in MRS broth containing 7% and 10% NaCl. The ability to grow at different temperatures was determined by growth in MRS broth after incubation at 4 °C for 7 days and at 15 °C and 45 °C for 3 days. Slime production was tested on MRS agar containing sucrose instead of glucose (Schillinger and Lücke, 1987). Cell walls

Table 3.1 Reference strains used in this study

Strain	Culture collection number
Lactobacillus alimentarius	[1]DSM 20249^T
Lactobacillus brevis	DSM 20054^T
Lactobacillus casei	DSM 20011^T
Lactobacillus casei	DSM 20258
Lactobacillus confusus	DSM 20196^T
Lactobacillus coryniformis	DSM 20001^T
Lactobacillus curvatus	DSM 20019^T
Lactobacillus plantarum	DSM 20174^T
Lactococcus lactis subsp. *cremoris*	DSM 20069
Lactococcus lactis subsp. *lactis*	DSM 20481
Leuconostoc mesenteroides	DSM 20343^T
Leuconostoc paramesenteroides	DSM 20288^T
Pediococcus acidilactici	DSM 20284
Pediococcus pentosaceus	DSM 20336

[1]Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany)

were examined for the presence of meso-diaminopimelic acid (mDAP) as described by Keddie and Cure (1977) using paper chromatography (Rhuland *et al.*, 1955). The configuration of lactic acid enantiomers was determined enzymatically using D- and L-lactate dehydrogenase (Boehringer Mannheim) (Dykes *et al.*, 1994). The production of gas from glucose, H_2S and the hydrolysis of arginine was determined as described by Schillinger and Lücke (1987).

3.2.4 Amplified Fragment Length Polymorphism (AFLP)

3.2.4.1 DNA extraction

Genomic DNA was extracted from 104 strains, selected from the *Lactobacillus (Lb.) plantarum* (58) and *Leuconostoc (Lc.) mesenteroides* (46) clusters that were obtained from the electrophoretic profiles of total soluble proteins, according to a previously described cetyltrimethyl ammonium bromide (CTAB) method (Bickley *et al.*, 1996). The DNA was resuspended in a final volume of 35µl TE buffer. RNA was digested by incubating the DNA solution with 1µl DNAse-free RNAse (Boehringer Mannheim) at 37°C for 1 hour. The purity and concentration of the DNA was determined on 1% agarose gels with Lambda DNA (Boehringer Mannheim) used as a concentration standard. Pure DNA was stored at -20°C until used.

3.2.4.2 AFLP analysis

The AFLP procedure was carried out using a Perkin Elmer AFLP Ligation and Pre-selective Amplification kit, according to manufacturer's instructions (Perkin Elmer, Foster City, California, USA). DNA was cleaved with restriction enzymes EcoRI and Tru9I (MseI isoschizomer) and ligated by using T4 DNA ligase (Boehringer Mannheim). An enzyme master mix was prepared immediately before use, containing 0.5µl EcoRI (10U/µl), 0.1µl Tru9I (10U/µl) and 1µl T4 DNA ligase (1U/µl), per reaction. The restriction-ligation reaction was carried out at 37°C overnight in Eppendorf tubes containing 1µl T4 DNA ligase buffer, 0.5µl nuclease-free NaCl (0.5M) (BDH, Dorset, England), 0.5µl nuclease-free BSA (10mg/ml) (New England Biolabs, Massachusetts, USA) and 1.6µl of the enzyme master mix per reaction. Pre-selective amplification was carried out using EcoRI and MseI primers with single 3' A and 3' C selective bases, respectively. A Gene Amp PCR system 2400 (Perkin Elmer) was used for PCR. The sequences of primers and adaptors are not supplied by the manufacturer.

3.2.4.3 Electrophoresis

Glass plates were treated according to the Promega protocol (1996) with the exception that the short plate was treated with Bind Silane (Promega, Madison, Wisconsin, USA) instead of SigmaCote. Aliquots of 4µl of the PCR reactions were mixed with equal volumes of loading buffer (95% formamide, 20mM EDTA pH 8, 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 95°C for 5 min and snap-cooled on ice before loading. PCR products were separated on 4% denaturing polyacrylamide gels at 55W, on a S2 Sequencing gel electrophoresis apparatus (Gibco BRL, Life Technologies, NY, USA), with 1X TBE as the electrophoresis buffer. The lower compartment buffer was supplemented with 0.5M sodium acetate to correct "frowning" or "smiling" gels (Aarts *et al.*, 1998). A reference marker was included every 4 tracks as a standard for normalisation of patterns in different gels. The gels were stained using a modified silver staining method (Bassam *et al.*, 1991). They were fixed in 12% acetic acid, stained with 1g/l silver nitrate and then developed in a solution containing 0.5ml of sodium thiosulphate (10mg/ml) and 2.5ml of formaldehyde (37%) per litre.

3.2.4.4 Data analysis

The gels were air-dried and scanned with a Hewlett Packard ScanJet IIcx. The electrophoretic patterns were analysed using the software package GelManager, version 1.5. Levels of similarity between AFLP fingerprints were calculated using the Dice coefficient. The UPGMA algorithm was used for cluster analysis and the clusters were delineated at an arbitrary level of r = 0.70.

3.2.5 Antimicrobial studies

Antimicrobial activities of 72 selected lactic acid bacteria were tested against *Bacillus (B.) cereus* and *Bacillus (B.) subtilis* isolates originating from the sorghum samples, *Escherichia (E.) coli*, *Listeria (L.) monocytogenes*, *Staphylococcus (S.) aureus* and *Carnobacterium* (C.) *mobile* 4848 isolates from the departmental collection and selected lactic acid bacteria isolates from the *Lb. plantarum*, *Lb. sake/curvatus*, *Lc. mesenteroides*, *Pediococcus (P.) pentosaceus*, *P. acidilactici* and *Lactococcus (L.) lactis* clusters. Antimicrobial activity was tested on MRS agar at 30°C by the spot-on-lawn-assay. Lactic acid bacteria cultures were grown overnight and spotted on MRS agar plates until growth was visible and then overlaid with soft agar (7g/l)

seeded with 1% of indicator organism. Growth inhibition was measured as a clear zone around the lactic acid bacteria spot. The proteinaceous nature of the activity was tested by spotting Proteinase K (Boehringer Mannheim) next to the lactic acid bacteria spot (Hastings and Stiles, 1991).

3.3 RESULTS

3.3.1 Total soluble protein profiles and biochemical and physiological characteristics

The results are tabulated in Table 3.2 and 3.3. All isolates were positive for utilization of fructose and negative for utilization of adonitol, erythritol, inositol and rhamnose. Computerised numerical analysis of protein electrophoretic patterns grouped the isolates into 7 major clusters (Figure 3.1, Table 3.3).

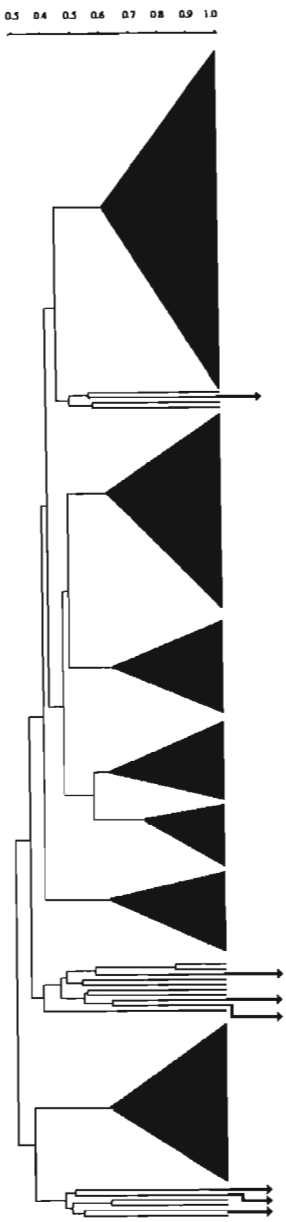
Cluster I was the largest cluster and consisted of isolates identified phenotypically as *Lb. plantarum* strains (Table 3.2). These strains were rod-shaped and contained mDAP in their cell walls. The reference strain *Lb. plantarum* DSM 20174^T was also grouped in this cluster. A total of 58 isolates were recovered, of which 20 originated from sorghum powder samples, 27 from fermented porridge samples and 11 from cooked fermented porridge samples (Figure 3.1). Isolates from sorghum powder and fermented porridge samples had the highest frequency of distribution, having being recovered from 11 and 13 of 15 households respectively. The 11 isolates originating from cooked fermented porridge samples were distributed in 4 households.

Cluster II was the second largest cluster and consisted of 28 isolates identified as *Lc. mesenteroides* strains. Isolates in this cluster produced the D- isomer of lactic acid and dextran from sucrose but did not produce ammonia from arginine. A total of 23 isolates originated from fermented porridge samples and were distributed in 6 households, whereas 5 isolates originating from cooked fermented porridge samples were distributed in 5 different households. None of the isolates originated from sorghum powder samples.

Cluster III was the smallest cluster and consisted of isolates identified as *L. lactis* strains. These isolates were coccoidal in morphology and produced the L- isomer of lactic acid. The terminal pH of the media after a 24 hour growth period was >4.0. These isolates were

Table 3.3 Distribution of lactic acid bacteria in sorghum powder and corresponding fermented and cooked fermented porridge samples as obtained by analysis of total soluble protein profiles and biochemical and physiological tests

	Cluster						
	I	II	III	IV	V	VI	VII
Source	*Lb. plantarum*	*Lc. mesenteroides*	*L. lactis*	*P. pentosaceus*	*P. acidilactici*	*Lc. mesenteroides*	*Lb. sake/curvatus*
Sorghum powder (SP)	20	0	3	2	8	3	7
Household distribution	11/15	-	3/15	1/15	3/15	3/15	215
Fermented porridge (FP)	27	23	2	11	4	13	11
Household distribution	13/15	6/15	1/15	3/15	2/15	11/15	4/15
Cooked fermented porridge (CP)	11	5	2	4	1	3	7
Household distribution	4/15	5/15	1/15	3/15	1/15	1/15	3/15



Cluster no.	Reference strains	No. of isolates	No. of isolates in food type		
			SP	*FP*	*CP*
I	*Lactobacillus plantarum* group *Lactobacillus plantarum* 20174	58	20	27	11
	Lactobacillus alimentarius 20249				
II	*Leuconostoc mesenteroides* group	28	0	23	5
III	*Lactococcus lactis* group *Lactococcus lactis* subsp. *lactis* 20481 *Lactococcus lactis* subsp. *cremoris* 20069	7	3	2	2
IV	*Pediococcus pentosaceus* group *Pediococcus pentosaceus* 20366	17	2	11	4
V	*Pediococcus acidilacti* group *Pediococcus acidilacti* 20284	13	8	4	1
VI	*Leuconostoc mesenteroides* group *Leuconostoc mesenteroides* 20343	19	3	13	3
	Lactobacilllus confusus 20196				
	Lactobacillus casei 20011 *Lactobacillus casei* 20258				
VII	*Lactobacillus sake/curvatus* group	25	7	11	7
	Lactobacillus brevis 20054 *Lactobacillus coryniformis* 20001 *Lactobacillus curvatus* 20019				

Figure 3. 1 **Simplified dendrogram derived from whole cell protein profiles of 180 lactic acid bacteria isolates obtained from sorghum powder (SP) and corresponding fermented (FP) and cooked fermented porridge (CP) samples. The electrophoretic profiles were analysed using GelManager software, version 1.5. Similarity patterns were calculated using the Pearson product moment similarity coefficient and clusters were delineated at r = 0.6**

recovered with the reference strains *L. lactis* subsp. *lactis* DSM 20481 and *L. lactis* subsp. *cremoris* DSM 20069. Seven isolates were recovered in this cluster of which 3 were from sorghum powder samples and 2 each from fermented and cooked fermented porridge samples. The 3 isolates originating from sorghum powder were distributed in 3 different households, whereas the 2 isolates originating from each of the fermented and cooked fermented porridge samples were recovered from the same household.

Cluster IV was identified as a *P. pentosaceus* cluster and was composed of 17 isolates. Microscopic examination revealed a coccoid morphology and the cells appeared as pairs or tetrads. They utilised arginine, cellobiose, galactose, melibiose and sorbitol and produced the DL- isomer of lactic acid. The terminal pH of the broth after a 48 hour incubation period was <4.0. Two isolates originated from sorghum powder, 11 from fermented porridge samples and 4 from cooked fermented porridge samples. These isolate were grouped with the reference strain *P. pentosaceus* DSM 20366. Isolates from fermented porridge samples were distributed in only 4 households.

Cluster V was identified as a *P. acidilactici* cluster with a total of 14 isolates, including the reference strain *P. acidilactici* DSM 20284. These isolates exhibited a coccoidal morphology, forming tetrads or pairs. The arginine test was negative and they could not grow in 10% NaCl. They differed from the *P. pentosaceus* cluster in the utilisation of sorbitol, xylose, melibiose, galactose, cellobiose, arabinose and in the hydrolysis of arginine (Table 3.2). Eight of these isolates were from sorghum powder samples and were distributed in 3 households, where as 4 isolates originated from fermented porridge samples and were distributed in 2 households. One isolate originated from a cooked fermented porridge sample.

Cluster VI contained 19 isolates also identified as *Lc. mesenteroides* isolates. These isolates produced the D- isomer of lactic acid, dextran from sucrose but no ammonia from arginine. They differed from the first *Leuconostoc* cluster (II) in the utilisation of arabinose, lactose, ribose and trehalose (Table 3.2). These isolates were grouped with the reference strain *Lc. mesenteroides* DSM 20343^T. Of the 19 isolates recovered in this cluster, 13 originated from fermented porridge samples and were distributed in 11 households, whereas 3 isolates

originated from sorghum powder were distributed in 3 different households and the other 3 isolates originating from cooked fermented porridge samples were from the same household.

Cluster VII consisted of 25 isolates, identified as *Lb. sake/curvatus* isolates. The isolates were rod-shaped, produced gas from glucose and the DL- isomer of lactic acid. They did not contain mDAP in their cell walls. Eleven of these isolates originated from fermented porridge samples and were distributed in 4 households, while 7 isolates originating from sorghum powder samples were distributed in 2 households and 7 isolates originating from cooked fermented porridge samples were distributed in 3 households.

Thirteen isolates could not be sufficiently identified phenotypically and did not group with any of the reference strains. No isolates were grouped with *Lb. alimentarius* DSM 20249^T, *Lb. brevis* DSM 20054^T, *Lb. casei* DSM 20258, *Lb. casei* DSM 20011^T, *Lb. coryniformis* DSM 20001^T, *Lb. curvatus* DSM 20019^T, *Lb. confusus* DSM 20196^T *(Weissella confusa)* and *Lc.(Weissella) paramesenteroides* DSM 20288^T. The two *Lb. casei* reference strains were, however, grouped together at a similarity level of 0.60.

3.3.2 AFLP analysis

Analysis of DNA electrophoretic patterns of 58 *Lb. plantarum* strains separated them into 5 major clusters (Figure 3.2, Table 3.4). Cluster A was composed of 6 strains, 3 of which were isolated from sorghum powder samples originating from 3 households (4, 1 and 12) and 3 from fermented porridge samples originating from 2 households (1 and 6). Cluster B was the largest cluster, with 38 strains. Strains from sorghum powder samples were distributed in 9 households (2, 3, 5, 6, 7, 8, 9, 13 and 14) while those from fermented porridge samples were distributed in 11 households (1, 2, 3, 5, 6, 7, 8, 9, 11, 12 and 15) and those from cooked samples were distributed in 6 households (2, 3, 4, 5, 7 and 8). Cluster C contained 4 strains, of which 3 originated from the same household (13) but different food categories. The remaining strain originated from a cooked porridge sample from household 9. Cluster D was composed of 6 strains, of which 2 originated from sorghum powder (households 3 and 10), 3 from fermented porridge samples (households 10 and 11) and 1 from a cooked fermented sample (household 5). Cluster E

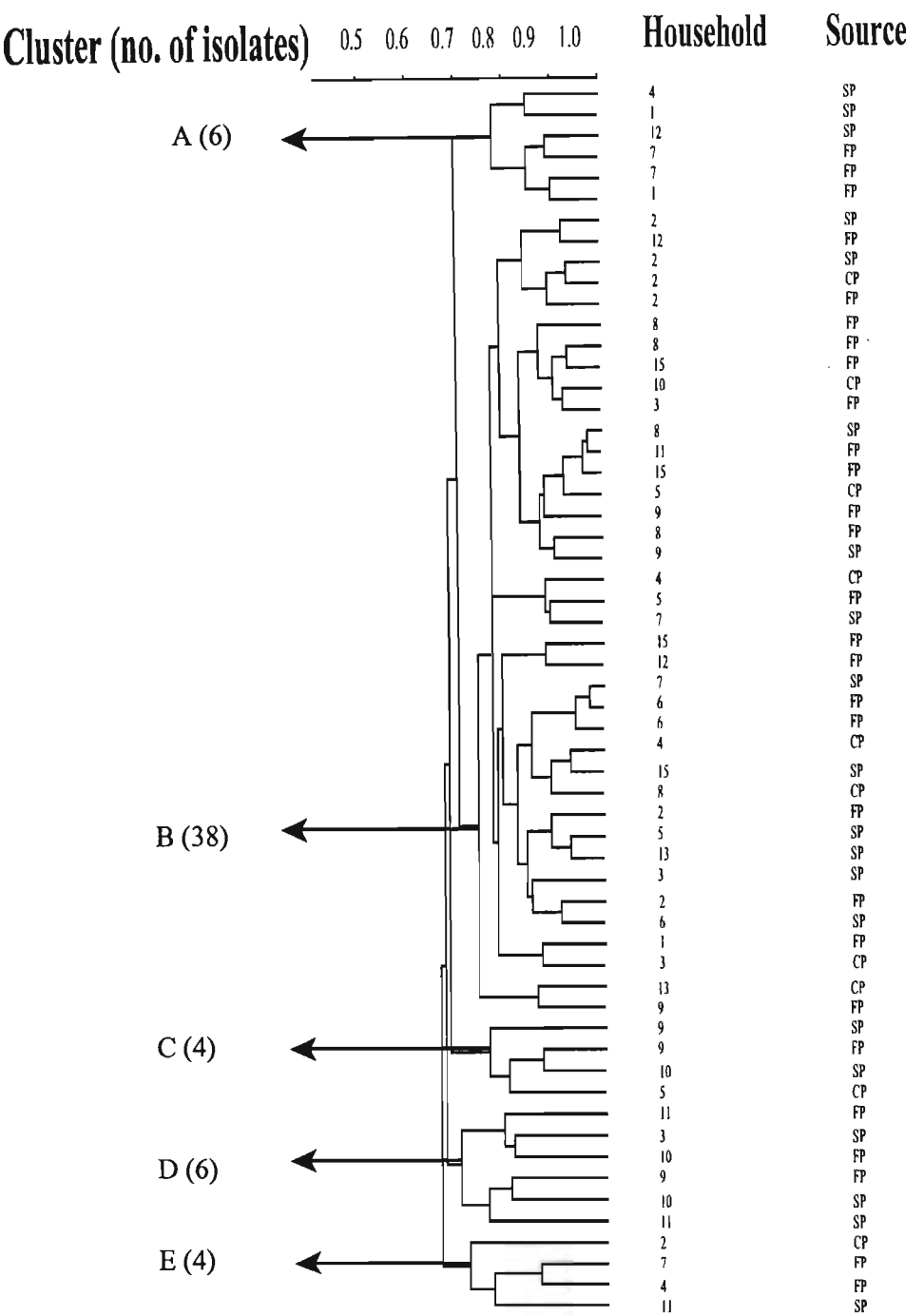


Figure 3.2 **Dendrogram of 58 *Lactobacillus plantarum* strains derived from unweighted pair group average linkage cluster analysis of AFLP patterns. The AFLP patterns were analysed using GelManager software version 1.5. Similarity between the AFLP fingerprints was calculated using the Dice coefficient and clusters were delineated at an arbitrary level of r = 0.70. The origin of isolates is indicated by SP (sorghum powder), FP (fermented porridge) and CP (cooked fermented porridge)**

Table 3.4 Distribution of *Lactobacillus plantarum* strains in sorghum powder and corresponding fermented and cooked fermented porridge samples as obtained from AFLP analysis

	Clusters				
Source	**A**	**B**	**C**	**D**	**E**
Sorghum powder (SP)	3	12	1	2	1
Household distribution	3/15	9/15	1/15	2/15	1/15
Fermented porridge (FP)	3	19	2	3	2
Household distribution	2/15	11/15	1/15	3/15	2/15
Cooked fermented porridge (CP)	0	7	1	1	1
Household distribution	-	6/15	1/15	1/15	1/15

contained 4 strains, of which 2 originated from sorghum powder (household 10 and 11) and 1 each from a fermented (household 7) and cooked fermented porridge (household 1) sample.

The *Lc. mesenteroides* isolates were grouped into 2 major groups (Figure 3.3, Table 3.5), similar to the 2 clusters obtained from the soluble protein profiles dendrogram (Figure 3.1). Cluster F was the largest cluster with 15 strains. Only 1 strain originated from sorghum powder (household 11) while 14 originated from fermented porridge samples (households 4, 7, 9, 11, 12, 14 and 15). The second cluster (G) was composed of 9 strains, 5 from fermented porridge samples (households 7, 8 and 12) and 4 from cooked fermented porridge samples (households 8 and 10). The third cluster (H) was composed of 4 strains, 3 of which originated from fermented porridge samples (households 12 and 13) while 1 strain originated from a cooked fermented porridge sample (household 10). The fourth cluster (I) was composed of 3 strains, each from different households (1,11 and 10) and from different food categories. The fifth cluster (J) consisted of 5 strains, 4 from fermented porridge samples (households 3, 5 and 6) and 1 from a cooked fermented porridge sample (household 5). The sixth cluster (K) was composed of 7 strains, of which 6 originated from fermented porridge samples (households 4, 5, 7, 9, 10 and 11) and 1 from a cooked fermented porridge sample (7). The last cluster (L) consisted of only 2 strains both originating from fermented porridge samples from households 2 and 3.

3.3.3 Antimicrobial studies

Selected lactic acid bacteria from each group were found to be inhibitory against all indicator strains used (Table 3.6). Antimicrobial activity was indicated by a clear zone around lactic acid bacteria colonies (Figure 3.4). Antimicrobial activity against other groups of lactic acid bacteria was observed only when the lactococci were used as indicator strains. The inhibition zones were not affected by proteases.

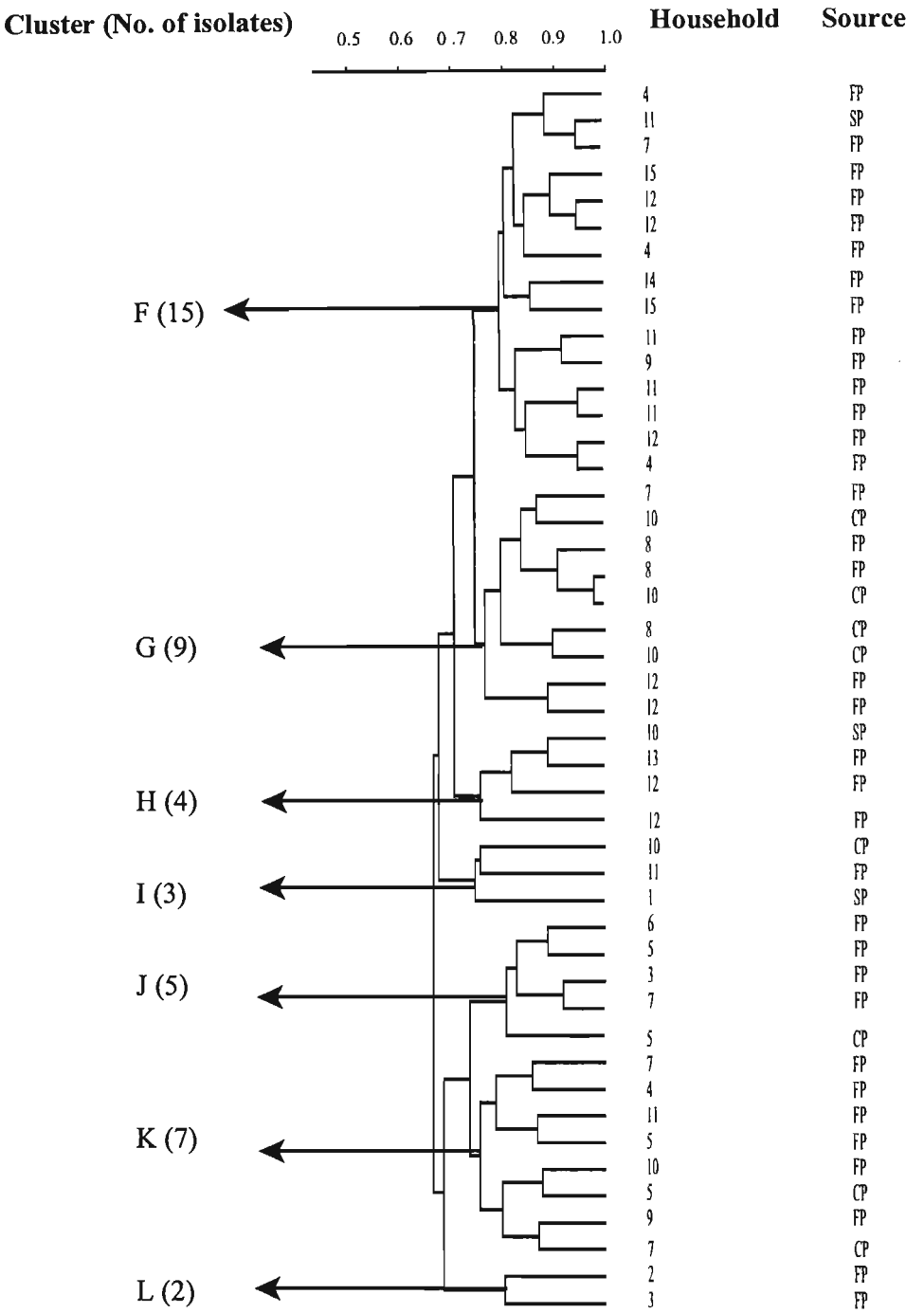


Figure 3.3 **Dendrogram of 45 *Leuconostoc mesenteroides* strains derived from unweighted pair group average linkage cluster analysis of AFLP patterns. The AFLP patterns were analysed using GelManager software version 1.5. Similarity between the AFLP fingerprints was calculated using the Dice coefficient and clusters were delineated at an arbitrary level of r = 0.70. The origin of isolates is indicated by SP (sorghum powder), FP (fermented porridge) and CP (cooked fermented porridge).**

Table 3.5 **Distribution of *Leuconostoc mesenteroides* strains in sorghum powder and corresponding fermented and cooked fermented porridge samples as obtained from AFLP analysis**

	Cluster						
Source	**F**	**G**	**H**	**I**	**J**	**K**	**L**
Sorghum powder (SP)	1	0	1	1	0	0	0
Household distribution	1/15	-	1/15	1/15	-	-	-
Fermented porridge (FP)	14	5	3	1	4	6	2
Household distribution	7/15	3/15	2/15	1/15	4/15	6/15	2/15
Cooked fermented porridge (CP)	0	4	0	1	1	1	0
Household distribution	-	2/15	-	1/15	1/15	1/15	-

Table 3.6 Antimicrobial activity of 77 lactic acid bacteria isolates selected from each cluster resulting from the analysis of the electrophoretic profiles of total soluble protein

	Number of isolates active against the indicator strains (number of isolates tested)						
Indicator strains	***Lb. plantarum*** **(25)**	***Lb. sake/curvatus*** **(13)**	***Lc. mesenteroides*** **(13)**	***Lc. mesenteroides*** **(10)**	***P. acidilactici*** **(6)**	***P. pentosaceus*** **(7)**	***Lactococcus*** **(3)**
B. cereus	23/25	12/13	13/13	10/10	6/6	7/7	3/3
B. subtilis	25/25	13/25	13/13	10/10	6/6	/7	3/3
E. coli	25/25	12/13	13/13	10/10	5/6	7/7	3/3
L. monocytogenes	23/25	12/13	11/13	8/10	6/6	5/7	3/3
S. aureus	23/25	8/13	9/13	9/10	0/6	1/7	0/3
C. mobile 4848	21/25	8/13	9/13	8/10	6/6	6/7	2/3
Lb. *plantarum* cluster	0/25	0/13	2/13	1/10	1/6	2/7	3/3
Lb. sake/curvatus	7/25	0/13	0/13	0/10	3/6	0/7	3/3
L. mesenteroides	4/25	0/13	2/13	0/10	0/6	0/7	3/3
P. pentosaceus cluster	3/25	2/13	1/13	3/10	2/6	0/7	2/3
P. acidilactici cluster	7/25	0/13	0/13	0/10	0/6	0/7	3/3
L. lactis	23/25	0/13	0/13	0/10	0/6	0/7	0/3

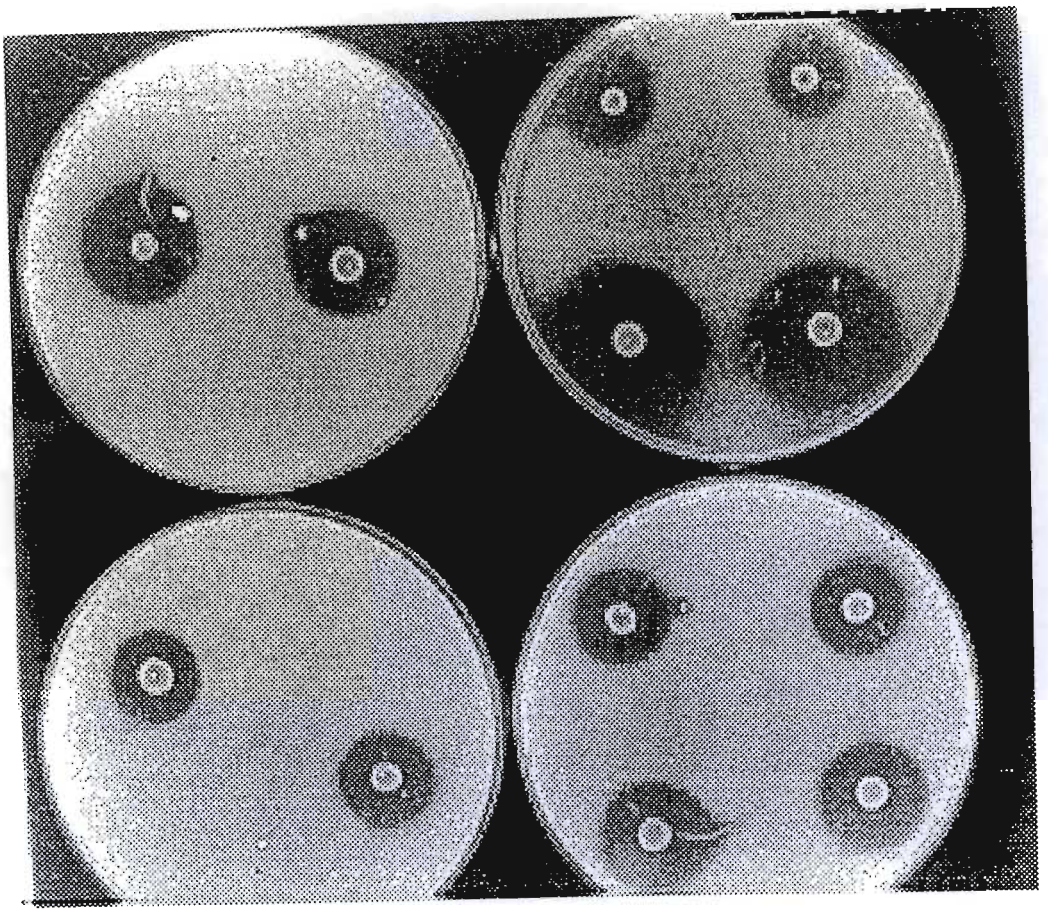


Figure 3.4 **Antimicrobial activity of selected lactic acid bacteria isolates from sorghum powder and corresponding fermented and cooked fermented porridge samples. The antimicrobial activity was indicated by a zone of inhibition around the lactic acid bacteria colony.**

3.4 DISCUSSION

The bacterial populations found in sorghum powder samples and corresponding fermented and cooked fermented porridge samples in this study included isolates from the *Lactobacillus, Lactococcus*, *Leuconostoc* and *Pediococcus* species of lactic acid bacteria. Previous studies on the composition of lactic acid bacteria populations found that naturally fermented cereal-based African food products were dominated by *Lb. plantarum, Lb. fermentum, Lb. reuteri, Lc. mesenteroides, P. pentosaceus* and *L. lactis* strains (Oyewole, 1995; Rombouts and Nout, 1995).

In this study, the majority of the isolates (83/182) were identified as belonging to the genus *Lactobacillus,* The clusters with the most isolates were the *Lb. plantarum* and *Lc. Mesenteroides* clusters. Members of these species have been previously isolated from a variety of food products and from the environment, and have been reported to be two of the most dominant species during the fermentation of sorghum-based infant weaning foods (Nout, 1991; Olsen *et al.*, 1995; Leisner *et al.*, 1996). The wide household distribution of *Lb. plantarum* and *Lc. mesenteroides* strains originating from sorghum powder and fermented porridge samples indicated their occurrence in sorghum powder before fermentation, suggesting that sorghum may be a natural habitat for these species. The detection of isolates in cooked fermented porridge samples suggested re-contamination by sorghum powder strains, however, re-contamination of these samples by strains present in the household before the study was conducted was also possible.

The third largest cluster was the *Lb. curvatus/sake* cluster. Members of this group belong to the group of atypical streptobacteria which are phenotypically diverse and are usually separated by the fermentation of melibiose and arginine hydrolysis. *Lb. curvatus* is negative for both tests (Klein *et al.*, 1996). The cluster obtained in this study seems to contain a mixture of the two species. Previous studies have reported on the phenotypic similarities of *Lb. sake and Lb. curvatus* (Kagermeier-Callaway and Lauer, 1995) and on the inability of biochemical tests and total soluble protein profiles to consistently differentiate between strains of these two species (Hastings and Holzapfel, 1987; Dykes and von Holy, 1993). Strains from the *Lb. sake/curvatus* cluster were distributed in 5 households and were most prevalent in fermented

porridge samples. Members of this group have been reported to have important associations with foods. They form part of the microflora of vacuum-packaged meat and meat products (Björkroth and Korkeala, 1996) and vacuum packaged smoked and salted fish (Gancel *et al.*, 1997). They also cause spoilage of vacuum-packaged chill stored meat products (Von Holy *et al.*, 1991). Although the habitats of *Lb. Sake/curvatus* are diverse and include plant material (Klein *et al.*, 1996), it is also possible that they may have been a result of contamination at the household level, introduces through mishandling of food products.

Other lactic acid bacteria species that were found in this study were *L. lactis*, *P. acidilactici* and *P. pentosaceus* species. The lactococci did not seem to form part of the microorganisms involved in the fermentation of sorghum since only 7 isolates were recovered. This may have been due to their inability to compete efficiently with other lactic acid bacteria for nutrients, compounded by their inability to grow at pH<4 (Table 3.3). The natural habitat of the lactococci is milk, but *L. lactis* subsp. *lactis* has been isolated from green plants and vegetables, including cereals (De Vuyst and Vandamme, 1994; Salama *et al.*, 1995). Pediococci have often been found in low numbers together with leuconostocs and lactobacilli on plant material and in various foods, they have been are widely used as starter cultures in the fermentation of sausages and have been extensively used in the control of food pathogens in vegetables (Vescovo *et al.*, 1996). They have previously been isolated from fermented cereal gruels and were found to inhibit growth of food pathogens including *B. cereus* and *E. coli* (Kingamkono *et al.*, 1994). The low households distribution levels of lactococci and pediococci suggested that they may have been introduced at the household level.

The presence of isolates that did not group in any of the major clusters was an indication of the high diversity of lactic acid bacteria strains naturally occurring in sorghum powder. It is possible that they could belong to any of the other species frequently found in fermented cereal products, such as *Lb. fermentum* and *Lb. reuteri* (Rombouts and Nout, 1995).

The results suggest that the fermentation processes in this study were effected by a mixed culture of lactic acid bacteria dominated by strains belonging to the *Lactobacillus, Leuconostoc and Pediococcus* species. Although fermentation was largely effected by lactic

acid bacteria originating from the sorghum powder, introduction of undesirable lactic acid bacteria at household level was also possible. No household was found to yield a homogenous population of lactic acid bacteria. It should be mentioned, however, that the fermentation processes from which these lactic acid bacteria were isolated were not lengthy enough for natural selection to result in a stable microbiological composition. Homogenous populations are usually established after several cycles of the fermentation process have occurred (Nout (1991) and in our case, the samples were collected after the first cycle lasting 36 - 72 hours. Acquisition of homogenous populations of lactic acid bacteria can, however, be achieved by the use of suitable and appropriate starter cultures.

Strain variation in the *Lb plantarum* and *Lc. mesenteroides* clusters was demonstrated by grouping of the strains into several clusters after AFLP analysis. The recovery of 38 *Lb. plantarum* strains in one cluster (B), suggested the preferred dominance of this particular strain of *Lb. plantarum* in sorghum powder samples. The distribution of these strains in 9 and 11 households for sorghum powder and fermented porridge samples, respectively, suggested a common source of origin for all the strains in this cluster. Findings that contaminating strains from cooked fermented porridge samples originated from the same households as strains from sorghum powder and fermented porridge also suggested a common source for these strains. This was further supported by observations made during sample collection where utensils used during the cooking process were also used during transfer of cooked fermented porridge samples into storage containers. In clusters C, D and E (Figure 3.2, Table 3.4), however, there was no relationship between contaminating strains from cooked porridge and those strains originating from sorghum powder, suggesting that the contamination may also have been at the household level.

The separation of the *Lc. mesenteroides* strains into 2 major clusters (Figure 3.2) confirmed earlier results obtained from the analysis of the total soluble protein profiles (Figure 3.1) of the presence of two *Leuconostoc* groups. The division of these two *Leuconostoc* clusters into smaller clusters demonstrated the ability of the AFLP technique to discriminate further between strains belonging to the same species. These clusters may represent different groups within the *Lc. mesenteroides* species. The largest cluster in this group (F) contained 15 strains,

14 of which originated from fermented porridge samples and were distributed in 7 households, suggesting a common source for the strains. Strains in cluster (K) were widely distributed between households, further suggesting a common source of origin. Although these strains could be traced to the sorghum powder (clusters J and K), contamination at the household level cannot be ruled out.

The lactic acid bacteria selected for antimicrobial studies exhibited a broad spectrum of inhibition against all indicator strains selected (Table 3.6). Antimicrobial activity against the lactococci was probably due to their inability to grow at pH <4. The inhibition did not seem to be due to bacteriocins as it could not be inactivated with proteolytic enzymes in the agar spot assay. The inability of selected lactic acid bacteria populations found in this study to produce bacteriocins could have contributed to the heterogenous nature of the bacterial populations involved in these fermentation processes, since bacteriocin-producing strains would have inhibited the growth of closely related strains. Several authors have reported on the production of bacteriocins by lactic acid bacteria from strains isolated from sorghum-based products, including *Lb. curvatus* (Vogel *et al.*, 1993) and *Lb. plantarum* (Larsen *et al.*, 1993; Olsen *et al.*, 1995).

3.5 CONCLUSION

Several lactic acid bacteria species were found to occur naturally in sorghum powder, however, spontaneous fermentation of sorghum powder resulted in dominance by *Lb. plantarum* and *Lc mesenteroides* species, with pediococci and *Lb. sake/curvatus* strains also contributing to fermentation process. AFLP analysis was found to distinguish between strains that were originally in the sorghum powder and those that were introduced at the household level. It was further successfully applied in determining inter-household relationships between strains. In the event that this type of product is commercialised, appropriate starter cultures will need to be developed in order to eliminate the possibility of interference by undesirable lactic acid bacteria during fermentation. It is suggested that at the household level, a starter culture from a previous batch be used to begin the fermentation process in order to achieve a homogenous culture. Although only broad spectrum antimicrobial activity was exhibited by the lactic acid bacteria in this study, the activity was found to be sufficient to inhibit growth

of selected foodborne pathogens that may contaminate infant weaning foods.

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CHAPTER 4

SUMMARISING DISCUSSION AND CONCLUSION

Previous studies have shown that one of the major causes of diarrhoeal diseases in infants and children is the consumption of microbiologically contaminated weaning foods. In most cases, microbiological contamination was found to result from the preparation of foods under unhygienic conditions, usually in environments that lack basic facilities such as electricity, proper water supplies and sanitation systems. Communities that live in such environments have to resort to other methods of food preservation that would not require refrigeration or storage of the food at high temperatures. The process of fermentation is one such method and in addition to that, it has also been found to impart desirable sensory and nutritional properties to fermented products.

The objectives of this study, therefore, were to analyse the bacterial populations in sorghum powder, which is a raw material for weaning foods, and corresponding fermented and cooked fermented porridge samples mostly used in South African informal and rural settlements. The effects of the fermentation and cooking processes on these bacterial populations were analysed and lactic acid bacteria responsible for the fermentation process were isolated and identified. We also investigated the genotypic relationships of selected lactic acid bacteria using AFLP fingerprinting, in order to discriminate between strains from the same species, so as to trace their origin and to study their distribution patterns in samples from the same household, and between samples from different households. The antimicrobial activity of selected lactic acid bacteria was also investigated.

Microbiological surveys indicated high levels of bacterial contamination of sorghum powder, which were found to decrease in fermented and cooked fermented porridge samples. Spore counts and Gram-negative counts of sorghum powder showed significant decreases when compared to fermented and cooked fermented porridge samples, while lactic acid bacteria counts were found to increase significantly in fermented porridge but decreased in cooked fermented porridge. This suggested the ability of the fermentation process to reduce the level of contamination. Further decreases in bacterial counts were observed in cooked fermented porridge suggested the ability of the cooking process to reduce even further the level of contaminating bacteria and bacterial spores. These results clearly demonstrated the advantages of the combination of the fermentation and cooking processes in enhancing the safety of the

fermented weaning cereal.

The detection of pathogens from sorghum powder, such as, *E. coli, L. monocytogenes S. aureus*, *Aeromonas, Salmonella,* and *Shigella* spp., and toxin-producing bacteria such as *B. cereus* and *C. perfringens,* indicated the susceptibility of sorghum powder to contamination by pathogenic bacteria. The high frequency of detection of *B. cereus* suggested that it may have been part of the normal flora of sorghum powder, especially because it has previously been isolated from cereal crops, vegetation and soil. *B. cereus* also forms spores which have the ability to survive extreme conditions such as dryness and high temperatures. The detection of *E. coli*, however, suggested that the contamination may have been introduced at the household level, especially after considering the conditions that prevailed in the informal settlement where these food samples were collected. These included factors such as the non-availability of sanitation systems and the fact that water was collected and stored in plastic containers inside the households for long periods of time. Contaminating *E. coli*, therefore, may have been introduced as a result of unhygienic practices such as handling and preparing food products, including the sorghum powder, with unwashed hands. The decrease in the incidence of all foodborne pathogens tested for in fermented and cooked fermented porridge further indicated the ability of the combination of the fermentation and cooking processes in reducing the levels of contaminating foodborne pathogens, thus enhancing the safety of the food products.

Computer analysis of electrophoretic protein profiles grouped the lactic acid bacteria isolates into 7 major clusters. Identification of these clusters revealed that lactic acid bacteria species present in sorghum powder and corresponding fermented and cooked fermented porridge included *Lb. plantarum, Lb. sake/curvatus, L. lactis, Lc. mesenteroides and Pediococcus* species, with the majority of the isolates being members of the genus *Lactobacillus*. This indicated the diversity of lactic acid bacteria found in sorghum. Dominant species were, however, found to be *Lb. plantarum* and *Lc. mesenteroides.* Various studies have reported on the domination of these two species in spontaneous fermentation processes of traditional fermented food products, specifically sorghum-based cereals used as weaning foods. Their wide distribution patterns, in sorghum powder obtained from different households, suggested

that they may form part of the normal flora of sorghum powder. Their detection in cooked fermented porridge suggested re-contamination by sorghum powder strains, however, re-contamination by household strains was also possible.

It is unlikely that members of the other lactic acid bacteria species (lactococci, *Lb. sake/curvatus* and pediococci) isolated from sorghum samples originated from sorghum powder. This is mainly because they were isolated in low numbers and originated from a few of the households. Although they have been isolated from plant crops, they have been largely associated with other food products such as meat (*Lb. sake/curvatus* and *Pediococci)* and milk (*Lactococci)* products. It is also possible that they may have been a result of contamination at the household level, introduced through mishandling of food products. These results indicated that the spontaneous fermentation processes from which the isolated lactic acid bacteria were obtained were effected by mixed cultures of lactic acid bacteria dominated by strains belonging to the *Lactobacillus, Leuconostoc and Pediococcus* species.

Analysis of *Lb. plantarum* and *Lc. mesenteroides* strains by AFLP fingerprinting was found to distinguish between strains that were originally in the sorghum powder and those that were introduced at the household level. The results suggested that although most of the strains originated from sorghum powder, there were some strains that were introduced at the household level. In addition to that, strains from the same household that were isolated from sorghum powder were also detected in fermented porridge. Further research should focus on the use of starter cultures in the production of this type of traditional fermented product, in order to compare the effects of spontaneous fermentations against controlled fermentation.

Although only broad spectrum antimicrobial activity was exhibited by the lactic acid bacteria in this study, the activity was found to be sufficient to inhibit growth of selected foodborne pathogens that may contaminate infant weaning foods. The use of lactic acid bacteria that can produce bacteriocins, however, would be an added advantage to this process and may result in the complete inhibition of the growth of foodborne pathogens and toxin producing bacteria.