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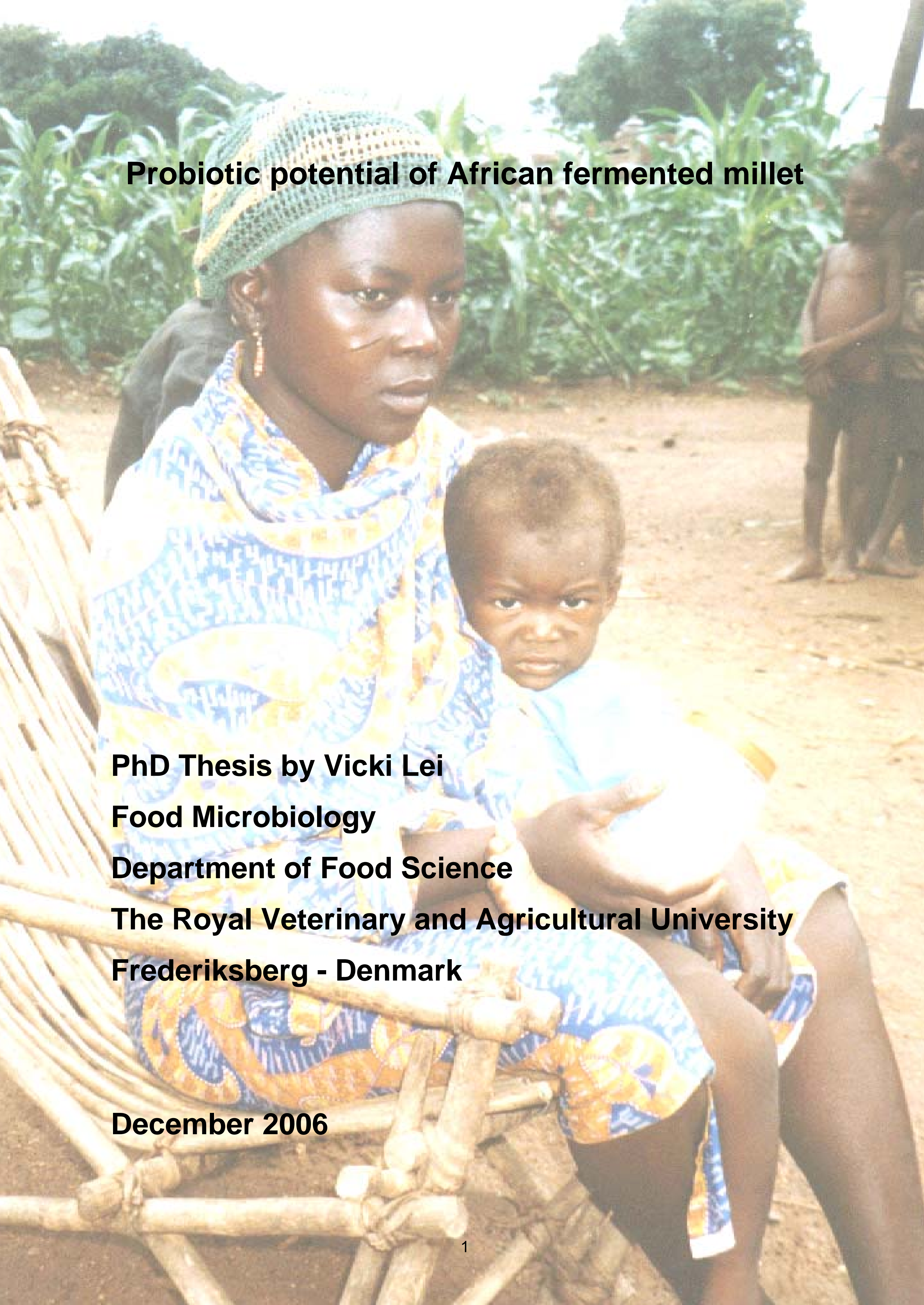
Publication date:
2006

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Lei, V. (2006). *Probiotic potential of African fermented millet*. Department of Food Science, University of Copenhagen.

Download date: 07. Apr. 2020



Probiotic potential of African fermented millet

PhD Thesis by Vicki Lei

Food Microbiology

Department of Food Science

The Royal Veterinary and Agricultural University

Frederiksberg - Denmark

December 2006

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Front page cover: Mother and child from Kalba, Northern Region, Ghana enrolled in the intervention study. Mother holding ‘koko sour water’ container. Photo by Vicki Lei, August 2001.

Preface and acknowledgements

The present Thesis was carried out by Vicki Lei (LC2004) at Food Microbiology, Dept. of Food Science, The Royal Veterinary and Agricultural University (KVL) as well as at the UDS/DANIDA Microbiology Laboratory, Faculty of Applied Sciences, University for Development Studies, Tamale, Ghana. The Thesis is presented to fulfil the requirements for a Ph.D. degree at KVL. The project was funded by the Danish Council for Development Research (Rådet for Ulandsforskning - RUF) (project no. 90963). The funding is acknowledged and highly appreciated. Supervisor of the project was Prof. Mogens Jakobsen, Dept. of Food Science, KVL and co-supervisor Prof. Kim F. Michaelsen, Dept. of Human Nutrition, KVL. The Thesis comprises of a literature survey and four publications.

I am deeply devoted to my daily mentor and supervisor, Prof. Mogens Jakobsen, for his continuous support and belief in me, as well as his excellent scientific guidance. In addition, great appreciation and thanks go to Prof. Kim F. Michaelsen (KVL) and Ass. Prof. Henrik Friis (KU) for their help and knowledge concerning the intervention study. ‘High five’ to all my colleagues and friends at Food Microbiology. You are inspiring and great fun to be around. Special thanks go to Henrik Siegumfeldt for his magic touch, when it comes to computers and to my office mates over the years; Kamilla Munk Petersen and Irene Ouoba, with whom I have shared conversations on many levels. I would also like to thank ‘my students’ for their work on the project; Gitte Maegaard Knudsen, Vicky Kastbjerg and Anne Hvid Karsten.

A whole lot of thanks and thoughts go to West Africa and especially Northern Ghana, which I now consider my second home. Warm thanks to the people of Nyankpala and all the health staff involved in the intervention study, especially Mr. George Tambro who is a great inspiration. I thank Richard Glover for leaving me avail of the facilities of the UDS/DANIDA Microbiology laboratory in Nyankpala and Moses Mengu for helping creating initial contacts to the health clinics. A special warm thought goes out to all the hard working women of Africa.

Heartfelt thanks to Danish as well as Irish family and friends for believing in me and supporting me when needed. Special thanks to Noah’s mormor and morfar. Finally, from the bottom of my heart I want to thank my everyday superheroes, Derek and Noah, for being you, and for always putting a smile on my face. You are my world!

Table of Contents

Preface and acknowledgements ..... **I**
Summary ..... **IV**
Sammendrag ..... **VIII**
1. Introduction **1**
2. African fermented cereal foods **6**
2.1 Background 6
2.3 Fermentation 7
2.3 African non-alcoholic fermented cereal foods 9
2.3.1 Millet 'koko' and koko sour water (KSW) 9
2.3.2 Fermented cereal based weaning products 13
2.4 Nutritional aspects of fermented cereals 14
2.5 Microorganisms involved in fermentation of cereals 16
2.5.1 The need for identification of lactic acid bacteria in relation to food safety and control of fermentation 21
2.5.2 Lactic acid bacteria predominant in millet 'koko' fermentation 26
2.6 Safety of fermented cereals 41
2.6.1 The role of lactic acid bacteria in food safety 41
2.6.2 Mycotoxins in fermented cereals 45
3. Probiotic potential of lactic acid bacteria **46**
3.1 Definitions of probiotics, prebiotics and synbiotics 46
3.2 Probiotic effects of lactic acid bacteria 48
3.2.1 Diarrhoea 49
3.2.3 Stimulation of the immune system 51
3.3 In vitro and in vivo evaluation of probiotic efficacy of lactic acid bacteria 55
3.4 Lactic acid bacteria marketed as probiotics 59
3.5 Safety of lactic acid bacteria as probiotics 62
3.5.1 Resistance to antibiotics 67
4. Probiotic properties of indigenously fermented cereals on childhood diarrhoea **71**
4.1 Probiotics and childhood diarrhoea 71
4.2 Traditional fermented cereal foods and diarrhoea 76
5. Discussion **80**

6. Conclusions and perspectives 87
References 89
Appendices 119

I Lei, V. and Jakobsen, M. (2004). Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *Journal of Applied Microbiology,* 96, 384-397.

II Lei, V., Friis, H. and Michaelsen, K.F. (2006). Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: An intervention study in Northern Ghana. *International Journal of Food Microbiology,* 110, 246-253.

III Sawadogo-Lingani, H., Lei, V., Diawara, B., Nielsen, D.S., Møller, P.L., Traoré, A.S. and Jakobsen, M. The biodiversity of predominant lactic acid bacteria in dolo and pito wort, for production of sorghum beer. *Journal of Applied Microbiology* (Accepted for publication).

IV Ouoba, L.I.I., Lei, V, Jensen, L. and Jakobsen, M. Resistance of lactic acid bacteria from African and European origins to antibiotics: determination and transferability of the resistance genes to other bacteria. *Journal of Applied Microbiology* (In preparation).

Summary

Approximately 2 million children die yearly from diarrhoea-related diseases in the African region. From this perspective alone there is a pressing need for low-cost, easily accessible and acceptable ways of preventing and treating diarrhoea.

According to the literature, defined probiotic cultures have been shown to reduce and prevent diarrhoea in children. However, the use of defined probiotic cultures in an African setting does not meet the requirements described above. Fermented products contain a high number of LAB with a probiotic potential and the use of traditional African fermented products for treatment and prevention of diarrhoea is a possibility.

Studies with defined probiotic cultures and diarrhoea have been carried out in mainly industrialised countries however; morbidity and mortality from diarrhoea are first and foremost a grave matter of concern in developing countries. The potential of being able to use a locally produced product as a probiotic treatment is considered to be immense. With the low cost and the widespread availability in some populations with high prevalence of

The hypothesis of this Thesis is, that traditional African fermented foods possess a probiotic potential, which would alleviate and prevent diarrhoea in African children, hence the aim was to investigate whether Ghanaian spontaneously fermented millet possessed a probiotic potential, which could alleviate and prevent diarrhoea in Ghanaian children. This was done by first studying the occurrence of lactic acid bacteria (LAB) in spontaneously fermented millet from the Northern Region of Ghana and isolating and identifying the predominant lactic acid bacteria. Second to estimate the ability of predominant lactic acid bacteria isolates to survive the passage of the gastro-intestinal tract by *in vitro* studies and then screen the isolates for antimicrobial activity. Finally, a spontaneously fermented millet drink was investigated in a human intervention study for ability to alleviate and prevent diarrhoea in children in Northern Ghana.

The spontaneously fermented millet porridge ‘koko’ as produced in Northern Ghana was the product investigated in the present Thesis. In addition, an un-cooked intermediate part of the ‘koko’, called ‘koko sour water’ (KSW) was investigated and used as the therapeutic agent in the intervention study. KSW contains a level of 10^8 colony forming units per ml of lactic acid bacteria.

The predominant microflora of ‘koko’ and KSW were identified using Intergenic Transcribed Spacers (ITS)-PCR Restriction Fragment Length Polymorphism (RFLP) and sequencing of a part of the 16S rRNA gene. In total 215 predominant isolates were selected from different production sites and different stages of the ‘koko’ production. Of these, *Lactobacillus fermentum* was found to be the dominant lactic acid bacteria. Other lactic acid bacteria found in significant numbers were *Weissella confusa* followed by *Lactobacillus salivarius* and *Pediococcus* spp.

The biodiversity at strain level of the predominant lactic acid bacteria was investigated using Restriction Enzyme Analysis with Pulsed-Field Gel Electrophoresis (REA-PFGE). The isolates showed a pronounced taxonomic biodiversity at strain level of all species throughout the different production stages, indicating that no microbial succession of single strains was taking place.

A pronounced variation at species level in the distribution of predominant lactic acid bacteria between the millet ‘koko’ production sites was observed; however a consistency in predominant lactic acid bacteria from production to production was seen within the individual production sites.

Ability to survive the passage through the digestive system is desirable for probiotic microorganisms. This ability was studied *in vitro* for all isolates and it was found that 70% of all isolates were capable of surviving four hours in physiological levels of acid and bile, indicating a the potential of the isolates to reach the gastro-intestinal tract in a viable form.

The antimicrobial activity of the 215 LAB isolated from ‘koko’ and KSW was tested towards two different test organisms; *Listeria innocua* was used as a model organism

for the human pathogen *Listeria monocytogenes*, and *Lactobacillus sakei* was used due to its sensitivity towards bacteriocins. The majority of the LAB isolates (approx. 90%) showed a weak inhibition of *L. innocua*, indicating production of antimicrobial substances with a little effect against to *L. innocua* and a competition for nutrients as the mechanisms. In contrast, only a few of the isolates (approx. 2%) showed weak inhibition of *L. sakei*, indicating that the isolates overall did not produce bacteriocins sensitive to *L. sakei*. Furthermore, pH-neutralised supernatants of the isolates did not show any inhibition of either of the two test-organisms, indicating that the weak inhibition found could be due to competition for nutrients.

Eight selected isolates from fermented millet were tested for resistance towards 24 antibiotics. All were found resistant to vancomycin, colistin, spectinomycin, ciproflox-acin, apramycin, trimethoprim, nalidixan, neomycin and sulphamethoxazole and sensitive towards gentamycin, penicillin, chloramphenicol, florfenicol and cephalothin. For the antibiotics to which the lactic acid bacteria were found resistant, it was investigated whether the isolates were in possession of well known resistance deter-minants for these antibiotics. None of the isolates showed positive PCR amplicons for the investigated resistance genes, indicating that the isolates are not likely to transfer antibiotic resistance genes to other bacteria.

The use of KSW as a therapeutic agent was assessed in an intervention study with children with diarrhoea. Children below five years of age coming to Northern Ghana health clinics for treatment of diarrhoea were randomised into two groups (inter-vention group and control group). Children of both groups received treatment for diarrhoea given at the local health clinic. In addition, the intervention group received up to 300 ml KSW daily for five days after enrolment. The clinical outcome of diarrhoea and reported well-being were registered every day for the five-day inter-vention and again 14 days after diagnosis. Among 184 children (mean age 17.4, standard deviation 11.3 months) included, no effects of the intervention were found with respect to stool frequency, stool consistency and duration of diarrhoea. The fact that no effect of KSW on diarrhoea was observed short term could be, because many children had a mild form of diarrhoea, and many were treated with antibiotics.

A possible long term effect was observed for KSW. This protective effect of KSW was seen as an overall better well-being, as well as tendencies to less diarrhoea and other illnesses of the children having consumed KSW two weeks prior to these findings. The effect found is speculated to be either a protective effect against new incidences of acute diarrhoea in the children, a prevention of antibiotic-associated diarrhoea or a combination of both.

Further investigations on the probiotic potential of African fermented cereals are needed to establish a possible beneficial effect. Fermented cereal foods have many advantages over non-fermented foods and the potential in being able to use a locally produced product as a probiotic treatment is immense. These fermented products are widely accepted among Africans and are also cheap and easily accessible to the local population. Because of the high number of LAB it is likely that traditionally fermented foods have an important role in preventing diarrhoea.

Sammendrag

I Afrika dør op mod 2 millioner børn årligt som følge af diarré-relaterede sygdomme. Ud fra denne betragtning alene, er der brug for billige, let tilgængelige og acceptable midler til forebyggelse og behandling af diarré.

Tidligere studier har vist at definerede probiotiske kulturer kan anvendes til at behandle og forebygge diarré hos børn. Anvendelsen af definerede probiotiske kulturer i Afrika imødekommer dog ikke ovenstående behov. Fermenterede produkter indeholder et højt antal mælkesyrebakterier med probiotisk potentiale og anvendelsen af traditionelle fermenterede produkter fra Afrika til behandling og forebyggelse af diarré er en mulighed.

Hypotesen for denne Afhandling er, at traditionelle afrikanske fermenterede fødevarer er i besiddelse af probiotiske egenskaber. Det var således formålet at undersøge om ghanesisk spontant fermenteret hirse besad probiotiske egenskaber, som kunne lindre og forebygge diarré hos ghanesiske børn. Dette blev udført ved først at studere forekomsten af mælkesyrebakterier i spontant fermenteret hirse fra Ghanas nordlige region og isolere og identificere de dominerende mælkesyrebakterier. Dernæst blev mælkesyrebakteriernes evne til at overleve passagen gennem mave-tarmkanalen undersøgt ved *in vitro* undersøgelser. Isolaterne blev ligeledes undersøgt for deres antimicrobielle egenskaber. Til sidst blev en spontant fermenteret hirse drik undersøgt i et humant interventions studium for dens evne til at lindre og forebygge diarré hos børn fra Nord-Ghana.

Den spontant fermenterede hirsegrød 'koko', bliver produceret i Ghanas nordlige region og et delprodukt af 'koko', kaldet 'koko sour water' (KSW), blev ydermere anvendt som terapeutisk middel i interventionsstudiet. KSW indeholder et niveau af 10^8 kolonidannende enheder per ml af mælkesyrebakterier.

Den dominerende mikroflora af 'koko' og KSW blev identificeret vha. ITS-PCR RFLP samt sekventering af en del af 16S rRNA genet. Ialt blev 215 dominerende mælkesyrebakterier isoleret fra forskellige produktionssteder samt fra de forskellige produktionstrin af 'koko'. Den dominerende mælkesyrebakterie blandt disse isolater var

Lactobacillus fermentum. Ligeledes blev *Weissella confusa*, efterfulgt af *Lactobacillus salivarius* og *Pediococcus* spp. også isoleret i et højt antal.

Biodiversiteten på stammeniveau af de dominerende mælkesyrebakterier blev undersøgt ved anvendelse af REA-PFGE. Isolaterne udviste en udtalt taksonomisk biodiversitet af alle species gennem de forskellige produktionstrin, hvilket indikerer at ingen mikrobiel succession af enkelte stammer finder sted.

Der var en udtalt variation i fordelingen af de dominerende mælkesyrebakterier på artsniveau mellem 'koko' produktionsstederne, hvorimod der blev observeret en ensartethed i de dominerende mælkesyrebakterier fra produktion til produktion ved de individuelle produktionssteder.

Det er ønskeligt for en probiotisk mikroorganisme at kunne overleve passagen gennem fordøjelsessystemet og denne evne blev undersøgt *in vitro* for samtlige isolater. 70% af alle isolater var i stand til at overleve fire timer i fysiologiske koncentrationer af syre og galde, hvilket indikerer isolaternes potentiale til levende at nå mave-tarmkanalen.

De 215 isolater blev undersøgt for deres antimikrobielle egenskaber mod to forskellige indikator bakterier; *Listeria innocua* blev anvendt som model for den human-patogene bakterie *Listeria monocytogenes*, og *Lactobacillus sakei* blev anvendt grundet dens bakteriocin-sensitivitet. Størstedelen (ca. 90%) udviste svag inhibering af *L. innocua*, hvilket indikerer produktion af antimikrobielle forbindelser med lille effekt mod *L. innocua* samt mulig konkurrence om næringsstoffer. Modsat viste kun meget få (ca. 2%) af isolaterne en hæmning af *L. sakei*, hvilket indikerer at isolaterne generelt ikke producerede bakteriociner, som *L. sakei* var følsom overfor. Yderligere hæmmede pH-neutraliserede supernatanter ingen af de to indikator bakterier, hvilket forstærker teorien om af hæmningen skyldes konkurrence om næringsstoffer.

Otte udvalge isolater fra fermenteret hirse blev testet for deres resistens overfor 24 antibiotika. Alle otte isolater blev fundet resistente mod vancomycin, colistin, spectinomycin, ciprolfoxacin, apramycin, trimethoprim, nalidixan, neomycin og sulpha-

methoxazole, samt følsomme for gentamycin, penicillin, chloramphenicol, florfenicol og cephalothin. Isolaterne blev undersøgt for kendte resistensfaktorer overfor de antibiotika som de var resistente mod. Ingen af isolaterne viste positive PCR fragmenter for de undersøgte resistens gener, hvilket indikerer at isolaterne ikke er i stand til at overføre deres antibiotika resistensgener til andre bakterier.

Anvendelsen af KSW som terapeutisk middel blev undersøgt i et interventions-studium med børn med diarré. Børn under fem år der kom til de lokale lægehuse for behandling mod diarré, blev randomiseret i to grupper (interventionsgruppen og kontrolgruppen). Samtlige børn modtog behandling mod diarré som normalt hos de individuelle lægehuse. Interventionsgruppen modtog ligeledes op til 300 ml KSW dagligt i fem dage efter indskrivning. Det kliniske udfald af diarré samt rapporteret vel-befindende blev registreret hver dag gennem de fem dages intervention og igen 14 dage efter indskrivning. Af de 184 børn (gennemsnit alder = 17.4 mdr., standard-afvigelse = 11.3 mdr.) indskrevet blev der ikke fundet effekt af interventionen mhp. afførings-hyppigheden, afføringens konsistens, samt diarréens varighed. At der ikke blev fundet nogen effekt på kort sigt kan skyldes, at hovedparten af børnene havde en mild diarré, og at mange af børnene blev behandlet med antibiotika.

Der blev fundet en mulig effekt af KSW på længere sigt. Den beskyttende effekt af KSW blev set som et generelt bedre velbefindende hos de børn der havde modtaget KSW to uger før disse observationer. Denne effekt kan skyldes en beskyttende effekt overfor nye tilfælde af akut diarré hos børnene, en forebyggelse af antibiotika-associeret diarré, eller en kombination af begge.

Yderligere undersøgelser af det probiotiske potentiale af afrikanske fermenterede kornprodukter er nødvendige for at fastslå en mulig gavnlig effekt. Fermenterede kornprodukter har mange fordele frem for ikke-fermenterede produkter og der synes at være et stort potentiale i at kunne anvende probiotiske produkter fremstillet lokalt. Disse fermenterede produkter er generelt accepterede af afrikanere og er desuden billige og let tilgængelige for den lokale befolkning. Det er sandsynligt, at traditionelt fermenterede produkter har en vigtig rolle i at forbygge diarré, grundet det høje indhold af mælkesyrebakterier.

1. Introduction

Food fermentation is one of the oldest known uses of biotechnology. All over the world, fermented foods continue to constitute an important part of our diet and fermented foods and beverages are estimated to provide some 20-40% of our food supply world-wide (Campbell-Platt, 1994). Particularly in developing countries, where refrigeration is not always an option, the fermentation process is widely used and of crucial importance, since fermentation prolongs the shelf-life of foods in addition to improving the nutritional value and reducing the risk for food borne illness. Fermented foods can even have beneficial health effects, when the fermenting microorganisms possess probiotic activity.

The word *probiotic* is derived from Greek and means “for life”. Even though probiotic products *per se* have always existed, it was Metchnikoff at the beginning of the 20th century that first acknowledged the health benefits related to the regular consumption of fermented milks (Metchnikoff, 1907). In 1965 Lilley and Stillwell defined probiotics “*as substances secreted by one microorganism to stimulate growth of another – as an antonym for antibiotic”*. Since then many authors (e.g. Parker, 1974; Fuller, 1989; Naidu *et al*., 1999, Salminen *et al*., 1999) have modified and developed the definition of probiotics. One of the more detailed current definitions of probiotics is; “*a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract”*, in addition to probiotic-active substances defined as; “*a cellular complex of LAB that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independent of LAB’s viability*” (Naidu *et al*., 1999). Salminen *et al.* (1999) put this in short to; “*probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host”*.

Mainly specific strains of lactobacilli, bifidobacteria, enterococci and yeast are today used commercially as probiotics, however lactobacilli still remain the most commonly used microorganisms in this respect (e.g. Naidu *et al*., 1999; Holzapfel and Schillinger, 2002; Saxelin *et al*., 2005). One of the most well documented effects of probiotics, is the reduction of the diarrhoea period (e.g. Isolauri *et al*., 1991; Guarino

et al., 1997; Shornikova *et al*., 1997a,b,c; Guandalini *et al*., 2000; Simakachorn *et al*., 2000; Rosenfeldt *et al*., 2002a,b). Of the estimated 10.6 million yearly deaths in children below the age of five, 18% are estimated to be directly attributable to diarrhoea (Bryce *et al*., 2005). This amounts to approx. 2 million children yearly. Among the deaths, 42% occur in the African region alone (Bryce *et al*., 2005). From this perspective alone there is a pressing need for low-cost, easy accessible and acceptable ways of treating and preventing diarrhoea.

Ghana (Figure 1.1) is a country estimated as of 2004 to have approx. 21.7 million people on 240,000 km^2 (UNICEF, 2006). The major food crops produced by the country are, in descending order cassava, yam, plantain, cocoyam, maize, millet, guinea corn and rice. Due to differences in climate, southern Ghana is the major root crop producing zone and the northern part the major grain producing zone. The major cash crops in Ghana are cocoa, coconuts, groundnuts, limes and lemons (Atta-Quayson, 1999).

Ghana is divided into ten regions. The Northern Region is 70,384 km^2 with a population of close to 1.7 million in 1999. Tamale is the regional capital of the Northern Region. Average annual rainfall in the Northern Region is 1000-1250 mm, with most rainfall in August and September (approx. 200 mm per month) (Atta-Quayson, 1999). The rainy season from June to September is also the period for peak of malaria and diarrhoea incidences (Lei *et al*., 2006 – Appendix II).

The “infant mortality rate” and “under-five mortality rate” have steadily declined in Ghana in recent years. However there are regional disparities between the north and the south of the country, partly due to poverty and to lack of, or poor access to medical treatment and other health services. In northern Ghana, the “infant mortality rate” is twice as high and the “under-five mortality rate” three times as high, as in the capital region. Malaria, acute respiratory infections, diarrhoea, malnutrition and measles remain the five leading killer diseases of children in Ghana (UNICEF, 2006). Table 1.1 shows baseline statistics for children in Ghana, respectively in 1990 and 2004, and for comparison, children in Denmark in 2004.

Table 1.1 Baseline statistics for children in Ghana, respectively in 1990 and 2004, and for children in Denmark in 2004 (UNICEF, 2006).

	Crude birth rate	Infant mortality rate	Under-five mortality rate (and ranking)
1990, Ghana	40	75	122 (-)
2004, Ghana	31	68	112 (42)
2004, DK	12	4	5 (172)

Crude birth rate = annual number of births per 1,000 population.

Infant mortality rate = probability of dying between birth and exactly one year of age expressed per 1,000 live births.

Under-five mortality rate = probability of dying between birth and exactly five years of age expressed per 1,000 live births.

Under-five mortality raking = list ranking countries in descending order of their estimated 2004 under-five mortality rate, a critical indicator of the well-being of children.

The level of morbidity and mortality from diarrhoea in African children *per se* is high and a cause for concern (UNICEF, 2006). Medical treatment is not always a possibility, which creates a necessity for effective, acceptable, cheap and easily accessible means to alleviate and reduce the incidence of diarrhoea.

Many lactic acid bacteria (LAB) possess effects beneficial to the host and specific strains of LAB have proven to reduce and prevent diarrhoea in children (e.g. Isolauri *et al*., 1994; Guarino *et al*., 1997; Shornikova *et al*., 1997a,b,c; Rosenfeldt *et al*., 2002a,b; Weizman *et al*., 2005). African spontaneously fermented products meet the needs of being easily accessible, accepted by the population and are low in cost. In addition, the fermented products contain large numbers of LAB.

Based upon the above, the hypothesis of this Thesis is, that traditional African fermented foods possess a probiotic potential, which can alleviate and prevent diarrhoea in African children.

To test this hypothesis the objectives of the Thesis are to:

- study the occurrence of lactic acid bacteria in a selected spontaneously fermented millet from the Northern Region of Ghana and to isolate and identify the predominant LAB.
- estimate the ability of predominant lactic acid bacteria isolates to survive the passage of the gastro-intestinal tract by *in vitro* studies.
- screen isolates for antimicrobial activity.
- Investigate the ability of the spontaneously fermented millet drink to alleviate diarrhoea in children in Northern Ghana by a human intervention study.

A literature survey was performed in order to support the experimental work carried out for this Thesis. The survey gives an introduction to African fermented foods, their benefits as well as their microbiology. Furthermore, the survey reviews the need for identification of microorganisms in relation to food safety and control of fermentation, as well as different methods for identification. An introduction to probiotics is also presented, and last the survey discusses the probiotic potential of indigenously fermented foods, with special attention to alleviation and prevention of diarrhoea.

Figure 1.1 Ghana with its ten regions.

2. African fermented cereal foods

2.1 Background

As reviewed by Campbell-Plat (1994) the origin of fermented foods in our diets goes back many thousands of years, and pre-dates the existence of written records of their production and consumption. Fermented foods provided then, as well as now, preservation, flavours and variety to the diet. More importantly, but perhaps unknown in the early days, fermented foods supply important nutrients, in particular proteins and amino acids and improve food safety. Furthermore, the microorganisms responsible for the actual fermentation may also have beneficial effects on human health.

Production of fermented foods may have started as 'natural' processes in which nutrient availability and environmental conditions selected particular microorganisms, which modified and preserved the food. People became familiar with the particular fermented foods produced in their part of the world, and many of these foods became an integral part of the local diet and were to become regarded as essential. Migration of people then helped the technological transfer of fermented foods (Campbell-Plat, 1994).

Fermented foods are produced and consumed in most parts of the world, however, in Africa alone fermented foods are of critical importance to the people from a nutrition and health perspective. The range of raw materials used in lactic fermentation processes in Africa includes mainly cereals, root crops, legumes and milk. Unlike other parts of the world, lactic fermentations of vegetables, fish and meat are not common in Africa (Steinkraus, 1996). The list of African fermented products is vast and will not be presented in detail in this Thesis. Several authors have made thorough reviews of a number of these products (Hesseltine, 1979; Odunfa, 1985; Wood, 1991; Dirar, 1993; Iwuoha and Eke, 1996; Olasupo *et al.*, 1997a; Oyewole, 1997; Steinkraus, 1996, 1997; Odunfa and Oyewole, 1998; Gadaga *et al.*, 1999).

Classification of fermented products is useful when studying African foods, since the many native languages and localities make it difficult to differentiate the products into

specific groups. Classification of the fermented foods can be carried out in different ways depending on the desired focus, specifically;

- by the fermenting microorganisms -as bacteria, yeast or moulds
- by classes -as e.g. beverages, cereal products or dairy products
- by food group -as e.g. cereal, fruits or roots
- by commodity -as e.g. alcoholic beverages or fermented vegetable proteins
- by production method -as e.g. back-slopping, spontaneous fermentation or starter culture
- by geographical location -as e.g. products from a specific country or region in a country.

(Dirar, 1993; Iwuoha and Eke, 1996; Steinkraus, 1997; Gadaga *et al*., 1999).

Food fermentation, and especially lactic acid fermentation, is an important technology in Africa. The technology is indigenous and is adaptable to the culture of the people. The fermentation process meets the requirements of being low-cost, preventing food spoilage and food-borne diseases with respect to consumers living in a climate, which favours the rapid deterioration of food. In addition, fermented foods are of particular importance in ensuring adequate intake of protein and/or calories in the diet (Motarjemi and Nout, 1996; Oyewole, 1997).

As will become evident in the following Sections, the lactic fermentation technology in Africa has developed indigenously and boasts an extensive range of products. Lactic fermented food products constitute the bulk of foods given to children and in general fermented foods form a large part of the main dishes consumed daily by the average individual.

2.3 Fermentation

Spontaneous (also called natural) fermentations are carried out by the micro-organisms occurring on the raw material and in the environment of the production site (Oyewole, 1997). Practically all indigenous fermented foods are still produced in this way. However, experience derived through trial and error, has shown that "inoculation" of raw materials with the residue of a previous batch (so-called back-slopping), accelerated the initial fermentation phase and controlled desirable

changes in the process. Many traditional African fermented foods are today processed this way in addition to other types of “inoculation” methods (Nout *et al.*, 1995; Holzapfel, 1997).

Spontaneously fermented foods in Africa are still mostly home-based, small scale productions. The method involves either soaking of the raw materials, submerged in water contained in a fermenting vat, for example clay pots, for length of time, or an initial size reduction of the raw material by grating or milling in the wet form, before being allowed to ferment (e.g. Müller, 1970; Odunfa, 1985; Oyewole, 1997; Odunfa and Oyewole, 1998; Salovaara, 2004). Although this is an inexpensive technique that can be applied in simple environments, product quality and safety is difficult to predict and standardise. This has lead to the development of starter cultures; where the substrate to be fermented is inoculated with defined pure culture(s) in order to obtain specific desired changes (Holzapfel, 1997, 2002; Nout, 2005). Starter cultures for African fermented cereal products have been developed in order to enhance fermentation (Halm *et al.*, 1996; Hounhouigan *et al.*, 1999; Mugula *et al.*, 2003), improve the ability of reducing pathogens (Olukoya *et al.*, 1994), enhance reduction of anti-nutritional factors (Khetarpaul and Chauhan, 1989; Sharam and Kapoor, 1996; Murali and Kapoor, 2003), improve nutrition (Khetarpaul and Chauhan, 1991b; Sanni *et al.*, 1998 and 1999a,b), and to improve aroma properties (Annan *et al.*, 2003a,b).

However, commercially available starter cultures for small scale processing of traditional African foods have yet to become accessible and economically advantageous. Therefore the spontaneous fermentation method is likely to be among the dominating production methods in Africa for many years to come.

Yeast, moulds and bacteria are capable of fermentation; however this Thesis will focus solely on the lactic fermentation carried out by LAB. With respect to glucose fermentation, LAB are divided into two groups based on the end products of the fermentation: The *homofermentative* that produce lactic acid as the major or sole product of glucose fermentation and the *heterofermentative* that produce equal molar amounts of lactate, carbon dioxide, and ethanol from hexoses. The latter can again be divided into two groups: the obligate- and facultative heterofermentative. The

heterofermentative LAB produce more flavour and aroma components, such as acetylaldehyde and diacetyl, than the homofermentative (Hammes and Vogel, 1995; Axelsson, 2004).

2.3 African non-alcoholic fermented cereal foods

The lactic fermented cereal-based products in Africa include porridge, dumplings, bread and both alcoholic and non-alcoholic beverages. The cereals most commonly fermented are maize, sorghum, millet, tef and occasionally rice and wheat (Oyewole, 1997). Some of the most well known and widely used fermented cereal products are shown in Section 2.5; Table 2.2.

2.3.1 Millet 'koko' and koko sour water (KSW)

Millet is known as "one of the lost crops of Africa". Millet grows well on poorly fertilized and dry soils, particularly in regions with hot climates and short rainfall periods. Millet is unique due to its short growing season and its capability of producing good yields of grain under conditions unfavourable to most other cereals. This is an important consideration for areas where food is limited. However, the average yields of millet are lower than those of maize even in semi-arid areas of Africa (FAO, 1995).

In Ghana the name 'koko' is used for a viscous liquid gruel made from cereal grains. In Southern Ghana 'koko' is traditionally prepared from maize (Andah and Muller, 1973; Halm *et al*., 1996; Lartey *et al*., 1999) and from millet in Northern Ghana and Nigeria (Oyeyiola, 1991; Lei and Jakobsen, 2004 - Appendix I). Preparation of 'koko' is traditionally carried out on a small scale (30-50 litres/day) by local women in the villages. The detailed preparation process of millet 'koko' as produced in Northern Ghana, is described by Lei and Jakobsen (2004 - Appendix I), in brief it includes; overnight steeping of pearl millet (*Pennisetum glaucum*) followed by wet-milling with spices such as ginger, chilli pepper, black pepper, and cloves. Addition of water to the flour makes a thick slurry, which then is sieved and left to ferment and sediment for 2-3 h. The fermented top-layer is then decanted to a pot and boiled for 1-2 h. After boiling, the thicker, un-boiled sediment from the fermentation is added until the desired consistency is achieved. Figure 2.1 shows the process photographically

depicted, whereas Lei and Jakobsen (2004 - Appendix I) present a detailed schematic flow diagram of the production process.

The daily production of ‘koko’ is normally ready around early afternoon, and is consumed as lunch or an afternoon snack. ‘Koko’ is a traditional product enjoyed by both adults and children. It is consumed from plastic bags or from bowls, normally with addition of sugar. ‘Koko’ is acidic in taste with a strong flavour of spices; especially ginger (Lei and Jakobsen, 2004 - Appendix I). ‘Koko’ has a pH of about 4.0 (Lei and Jakobsen, 2004 - Appendix I) and preliminary investigations show that koko can be kept at ambient temperatures up till 72 hours before palatability and odour becomes unacceptable (unpublished results).

During the studies on ‘koko’ in northern Ghana, it was observed that the fermented, but un-boiled part of the ‘koko’ product (the fermented top-layer) occasionally was used in some areas, as a refreshing drink during fastening instead of water, or for alleviation of an up-set stomach for children and adults. This fermented, but uncooked top-layer is in the present Thesis referred to at koko sour water (KSW) (Lei and Jakobsen, 2004 - Appendix I; Lei *et al*., 2006 – Appendix II).

Figure 2.1 Photos from preparation of ‘koko’ produced by the woman Samata in Nyankpala (Nyankpala A production site), Northern Region, Ghana (Figure 2.1 continues next page).

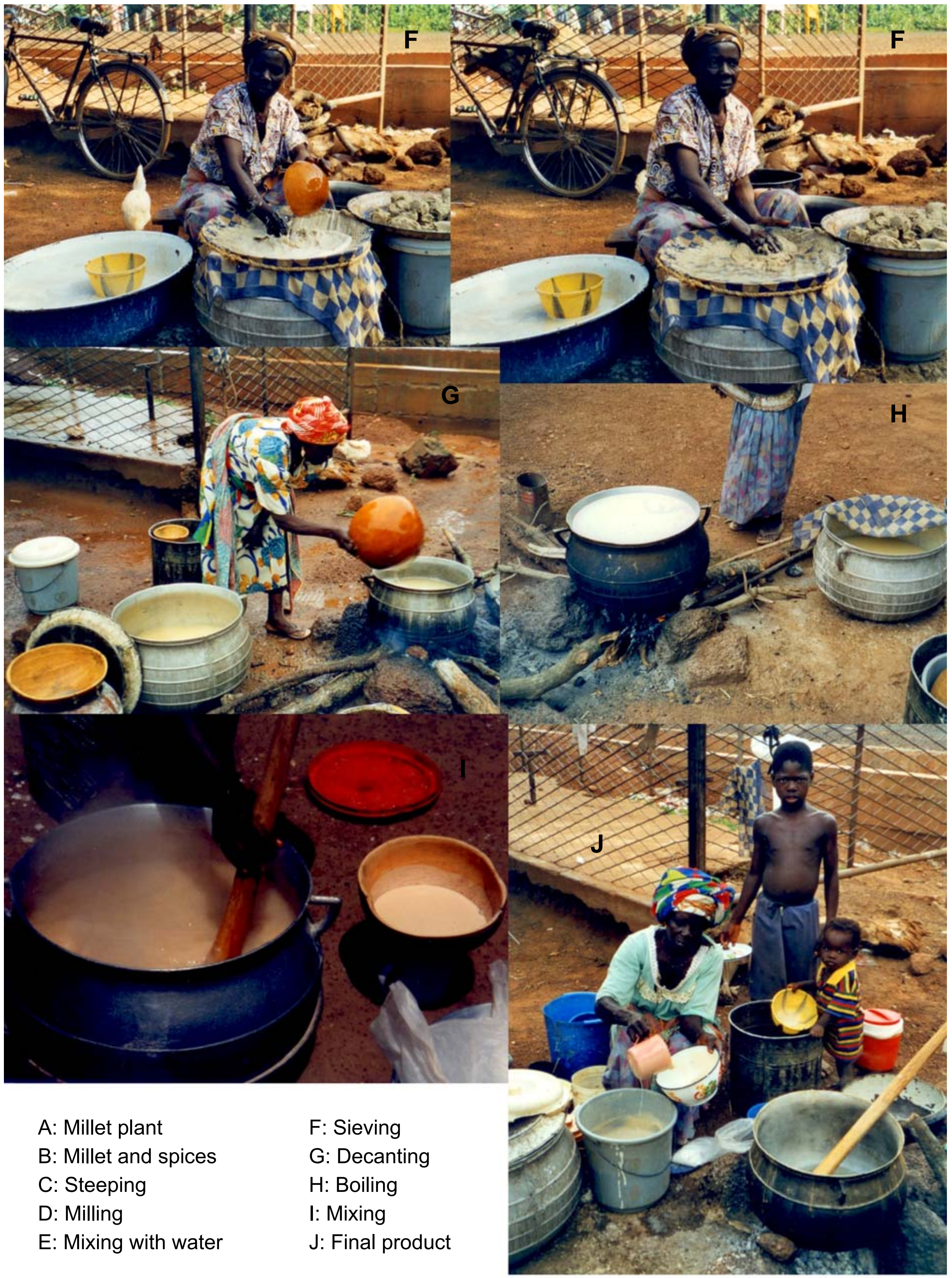

Figure 2.1 *-cont.* Photos from preparation of ‘koko’ produced by the woman Samata in Nyankpala (Nyankpala A production site), Northern Region, Ghana.

2.3.2 Fermented cereal based weaning products

Exclusive breastfeeding is usually adequate from birth and up to a year, but at some point during this period, breast milk becomes increasingly inadequate to support the nutritional demands of the growing infant. In this period, called the weaning period, before the infant is introduced to the family diet, there is a need to introduce soft, easily swallowed food to supplement the infant's feeding early in life. Especially in Africa where socio-economic factors, taboos, and ignorance are pronounced, weaning can be a period of problems and vulnerability for the survival of a child (Michaelsen and Friis, 1998; Onofiok and Nnanyelugo, 1998; UNICEF, 2006).

Using fermented foods as weaning products have the benefits of enhancing the nutritive value and the food safety. Fermentation can also reduce the high bulk of unfermented products by reducing the viscosity of the cereal gruel and hence increase the density of the nutritional value and energy intake (Graham *et al.*, 1986; Khetarpaul and Chauhan, 1990; Armar-klemesu *et al.*, 1991; Ezeji and Ojimelukwe, 1993; Darling *et al.*, 1995, Simango, 1997). This is important since the volume of traditional diets is too large to allow the child to ingest all the food necessary to cover its energy needs. Cereal based diets have lower nutritional value than animal based ones; however, meat is often not an option in Africa and use of fermented food seems to be the best option available (Michaelsen and Friis, 1998; Onofiok and Nnanyelugo, 1998).

Fermented food as weaning products is widespread in Africa. Most often the weaning food is prepared from cereals, typical maize, rice, sorghum or millet and sometimes starchy foods such as cassava, potato and plantain (Svanberg and Lorri, 1997; Onofiok and Nnanyelugo, 1998). Millet is generally recommended as weaning food, because it is considered as one of the least allergenic and most digestible grains (FAO, 1995). Millet is suitable for making non-sticky porridge and gruel because it has a low content of water extractable dietary fibre, contrary to wheat and oat (FAO, 1995). Table 2.1 shows the most traditional weaning foods from West Africa.

Table 2.1 Summary of the most widely used fermented cereal weaning foods in West Africa. Modified after Oyewole (1997), Onofiok and Nnanyelugo (1998) and Tou *et al.* (2006).

Country	Food name	Description
Nigeria	Ogi, pap, akamu, koko	Fermented maize, sorghum or guinea corn
Ghana	Koko, kenkey	Fermented millet or maize porridge
Sierra Leone	Ogi, couscous ogi	Fermented maize or sorghum gruel
Benin	Ogi	Fermented maize, sorghum or millet gruel
Burkina-Faso	Ben-saalga	Fermented millet porridge

In the recent years it has, however, become evident that even fermented cereal weaning foods are lacking somewhat in nutritional terms in order to prevent malnutrition. A study from Ghana by Kwaku *et al.* (1998) showed that liquid weaning diets were introduced months earlier than recommended, and that the energy and protein intakes of the children were low, meeting only 49% and 90% of their respective recommended daily intakes. Efforts have now turned to fortifying and improving the weaning products available and extensive literature exists in this respect (Ashworth and Feachem, 1985; Ashturkar *et al.*, 1992; Jansen, 1992; Ezeji and Ojimelukwe, 1993; Darling *et al.*, 1995; Olukoya *et al.*, 1994; Annan-Prah and Agyeman, 1997; Michaelsen and Friis, 1998; Onofiok and Nnanyelugo, 1998; Lartey *et al.*, 1999; Mugula and Lyimo, 1999; Onilude *et al.*, 1999; Sanni *et al.*, 1999a,b; Mugula and Lyimo, 2000; Egounlety *et al.*, 2002; Moore *et al*, 2003; Thaoge *et al.*, 2003; UNICEF, 2006).

2.4 Nutritional aspects of fermented cereals

The main nutritional diseases in the developing world are kwashiorkor -the result of protein deficiencies; marasmus -caused by a combination of protein and calorie deficiencies; xerophthalmia -childhood blindness due to vitamin A deficiency; beri-beri -due to thiamine deficiency; pellagra -due to niacin deficiency; riboflavin deficiency; rickets -caused by vitamin D deficiency; anaemia -due to vitamin B-12 deficiency and anaemia due to insufficient iron in the diet (Golden, 1993; Halsted, 1993; McLaren *et al.*, 1993).

Fermentation is known to improve the nutritional value of raw materials and by using fermented foods in the diet; the nutritional status of the individual can be improved (Motarjemi and Nout, 1996). African cereal weaning foods have been shown to

improve in nutritional value when fermented, either spontaneously (Ezeji and Ojimelukwe, 1993; Tou *et al.*, 2006) or by using starter cultures (Sanni *et al.*, 1999a,b). In addition, fermentation causes a decrease in the viscosity of starchy food mixed with water, which enables an increase in the concentration of dry matter while maintaining the desirable semi-liquid consistency, -an important feature for weaning foods (Motarjemi and Nout, 1996).

Cereals contain anti-nutritional factors such as phytic acids, tannins, polyphenols and trypsin inhibitors, and they are responsible for the low availability of proteins and minerals (Svanberg and Lorri, 1997). The phytate present in cereals form complexes with protein or polyvalent cations such as iron, zinc, calcium and magnesium, which are not digestible in this form. Seeds have a natural content of phytases, which can make the minerals bio-available, but also sprouting and fermentation can be used to increase the phytase activity (Svanberg and Lorri, 1997).

Fermentation is one of the most economic and effective measures for reducing the content of anti-nutritional factors. Studies have shown that both spontaneous fermentations as well as fermentations with starter cultures significantly reduced the content of phytic acid in millet (Sharma and Kapoor, 1996; Elyas *et al.*, 2002; Murali and Kapoor, 2003). One study found starter culture fermentations were to be more effective than spontaneous fermentations (Murali and Kapoor, 2003). Similarly, as a result of lactic acid fermentation, the protein digestibility can be elevated (Antony and Chandra, 1998; Taylor and Taylor, 2002; Ali *et al*, 2003; Onyango *et al.*, 2004) and the tannin content may be reduced in some cereals, leading to the increased absorption of iron (Khetarpaul and Chauhan, 1989, 1990; Motarjemi and Nout, 1996; Antony and Chandra, 1998; Sanni *et al.*, 1999b; Elyas *et al*, 2002; Onyango *et al.*, 2005).

Unfermented whole millet grains contain about 90% dry matter, 84% carbohydrate and 12% protein and many minerals; however unfermented millet grains are normally not consumed directly (e.g. Hulse *et al.*, 1980; Nkama *et al.*, 1994; Abdalla *et al.*, 1998). Millet is a rich source of dietary fibre and primary nutrients, in addition to minerals, but the bioavailability is low, due to the presence of anti-nutritional factors, such as phytate, phenols, tannins and trypsin inhibitors (Chung and Pomeranz, 1985;

Malleshi and Hadimani, 1993). Millet has a well-balanced protein content, except for its lysine deficiency, with high concentration of threonine and lower (but adequate) leucine than sorghum protein. Tryptophan levels are generally higher in millet than in other cereals (Chung and Pomeranz, 1985). Millet is also a good source of calcium (Malleshi and Hadimani, 1993) and is recommended to people with celiac disease (gluten intolerance), who cannot eat wheat, rye and barley. Further, millet is an important source of vitamin-E (tocopherol and tocotrienols) (FAO, 1995).

Other studies on nutritional changes in fermented millet have found improvement of the *in vitro* protein digestibility (Antony and Chandra, 1998; Ali *et al.*, 2003) and a significant reduction in total polyphenols and phytic acid content (Obizoba and Atii, 1994; Sharma and Kapoor, 1996; Antony and Chandra, 1998; Elyas *et al.*, 2002; Tou *et al.*, 2006). The effects of fermentation on tannins are variable. A reduction was reported by Antony and Chandra (1998) and no reduction in tannin content was reported by Elyas *et al.* (2002). Furthermore, an increase in starch digestibility (Antony and Chandra, 1998), increase in total free amino acids (Antony and Chandra, 1997), increase in minerals (Antony and Chandra, 1998), and a reduction in trypsin inhibitor activity (Antony and Chandra, 1998) have been found when fermenting millet.

As a final remark on the nutrition of fermented foods, it can be noted that cereals contain water-soluble fibre such as beta-glucan and arabinoxylan, oligosaccharides such as galacto- and fructo-oligosaccahrides and resistant starch, all of which have been suggested to fulfil the prebiotic concept (Charalampopoulos *et al.*, 2002). Prebiotics are further reviewed in Chapter 4.

2.5 Microorganisms involved in fermentation of cereals

Even though the methods for cereal fermentation have been known for centuries, the microorganisms responsible for the spontaneous fermentations were not identified before the 1960’s. Since then, new identification methods, especially molecular methods have been developed, and the nomenclature of the microorganisms has been altered several times.

Cereals are most commonly fermented by LAB, and mainly by the four genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* (reviewed by Salovaara, 2004). Since *Weissella* in 1993 was described as a new genus (Collins *et al*., 1993) it has in a few studies been isolated as one of the predominant species in fermenting cereal (Ampe *et al*., 1999b; Nigatu, 2000; Corsetti *et al*., 2001; Lei and Jakobsen, 2004 – Appendix I; Mugula *et al*., 2002). The fermenting microorganisms of the lactic fermentations will be the focus of the present Section.

Characteristics of cereal fermentations are;

- that the fermentation processes, being "by chance inoculation", usually are initiated by mixed microbial population
- that non-lactic acid microorganisms are eliminated with increasing acid production in the medium
- that there is a microbial succession for the LAB which involves inactivation of some and survival of others
- that the LAB, that survive the fermentation processes, usually do this in association with some yeast

(Odunfa and Adeyele, 1985; Oyeyiola, 1991; Hamad *et al*., 1992; Halm *et al*., 1993; Hounhouigan, 1993a,b; Hounhouigan *et al*., 1994; Jespersen *et al*., 1994; Olsen *et al*., 1995; Brauman *et al*., 1996; Hamad *et al*., 1997; Holzapfel, 1997; Oyewole, 1997; Antony and Chandra, 1998; El Nour *et al*., 1999; Sawadogo-Lingani *et al*., App. III).

Spontaneous fermentations typically result from the competitive activities of different microorganisms. Strains best adapted and with the highest growth rate will dominate during particular stages of the process, the so-called microbial succession (Holzapfel, 1997). A similar succession to the above described also takes place when using a starter culture or "back-slopping" in the preparation of African fermented food (Oyewole, 1990; Hamad *et al*., 1992, 1997, Olasupo *et al*., 1997a; Calderon *et al*., 2003).

In a review carried out of research carried out over the past years, many indigenous lactic fermented products have been investigated for their dominating microorganisms. Table 2.2 presents this overview of typical examples of African LAB fermented cereal products and the references to various key published investigations.

Table 2.2 Examples of different African lactic acid bacteria fermented cereal products and their dominating microflora. Microorganisms marked in **bold** are regarded as the predominant at the end of the fermentation. Products marked with a symbol, have been investigated in numerous studies.

Dominating LAB	Product	Product description	Country	Reference
Lactobacillus brevis *Lactobacillus fermentum* *Lactobacillus plantarum* *Lactobacillus paracasei*	'bushera'	Sorghum and millet non-alcoholic beverage	Uganda	Muyanja *et al*. (2002)
Lactobacillus fermentum *Weissella confusa*	'koko'	millet porridge	Northern Ghana	Lei and Jakobsen (2004 - Appendix I)
Lactobacillus fermentum	'dolo'	alcoholic sorghum beverage	Benin	Sawadogo-Lingani *et al*. (Appendix III)
Lactobacillus fermentum	'pito'	alcoholic sorghum beverage	Northern Ghana	Sawadogo-Lingani *et al*. (Appendix III)
Lactobacillus fermentum ***Lactobacillus amylovorus*** *Lactobacillus reuteri*	'kisra'	sorghum dough	Sudan	Hamad *et al*. (1992, 1997)
Lactobacillus fermentum *Lactobacillus brevis* *Lactobacillus salivarius* *Pediococcus* spp.	'mawé'	maize dough	Benin & Togo	Hounhouigan *et al*. (1993a,b, 1994)
Lactobacillus fermentum	'kenkey'*	maize dough	Ghana	Halm *et al*. (1993)
Lactobacillus helveticus *Lactobacillus salivarius* *Pediococcus damnosus* *Lactobacillus casei*	'busaa'	maize and millet beer	Kenya	Odunfa & Oyewole (1998)
Lactobacillus plantarum	'uji'	Maize + millet or sorghum porridge	Eastern Africa	Mbugua (1981)
Lactobacillus plantarum		sorghum based weaning food	Africa	Nout (1991)

Table 2.2 continues

Table 2.2 –cont.

Dominating LAB	Product	Product description	Country	Reference
Lactobacillus plantarum *Lactobacillus fermentum* *Lactobacillus brevis* *Lactobacillus delbrueckii* *Lactobacillus acidophilus* *Lactobacillus casei* *Lactobacillus cellobiosus*	'kenkey'*	maize dough	Nigeria	Olasupo *et al*. (1997a)
Lactobacillus plantarum *Pediococcus pentosaceus*	'kamu'	millet starch-cake	Nigeria	Oyeyiola (1991)
Lactobacillus plantarum *Lactobacillus brevis* *Lactobacillus fermentum*	'injera' dough	tef dough	Ethiopia	Nigatu (2000)
Lactobacillus plantarum *Lactobacillus coryniformis*	'injera' bread	kocho bread	Ethiopia	Nigatu (2000)
Lactobacillus plantarum *Leuconostoc mesenteroides*		sorghum weaning food	South Africa	Kunene *et al*. (2000)
Lactobacillus plantarum *Bacillus* spp. *Streptococcus lactis*	'obiolor'	non-alcoholic sorghum and/or millet beverage	Nigeria	Achi (1990)
Lactobacillus plantarum				Christian (1970)
Lactobacillus plantarum	'ogi'‡	maize starch-cake	Africa	Akinrele (1970)
Lactobacillus plantarum	'ogi'‡	maize or sorghum porridge	Nigeria	Johansson *et al*. (1995)

Table 2.2 continues

Table 2.2 –cont.

Dominating LAB	Product	Product description	Country	Reference
Lactobacillus plantarum *Lactobacillus casei* *Lactobacillus delbrueckii* *Lactobacillus brevis* *Lactobacillus jensenii*	'ogi'‡	Maize porridge	Nigeria	Olasupo *et al*. (1997a)
Lactobacillus plantarum *Lactobacillus brevis* *Lactobacillus cellobiosus* *Lactobacillus fermentum* *Weissella confusa* *Pediococcus pentosaseus*	'togwa'	sorghum, maize, millet or maize+sorghum weaning gruel or refreshment drink	Tanzania	Mugula *et al*. (2002)
Lactobacillus plantarum ***Streptococcus lactis***	'ogi-baba'	sorghum gruel	West Africa	Odunfa and Adeyele (1985)
Lactobacillus saccharolyticum *Gluconobacter oxydans* *Acetobacter xylinum* *Saccharomyces cerevisiae*	'hussuwa'	sorghum dough or drink	Sudan	El Nour *et al*. (1999)
Lactobacillus salivarius *Lactobacillus casei* *Lactobacillus acidophilus* *Lactobacillus jensenii* *Lactobacillus cellobiosus* *Lactobacillus plantarum*	'kanu-zarki'	millet porridge	Nigeria	Olasupo *et al*. (1997a)

2.5.1 The need for identification of lactic acid bacteria in relation to food safety and control of fermentation

The ability to identify microorganisms from fermented food is important in order to obtain knowledge on the biodiversity of the microflora, as well as the safety and quality of the product. Knowledge of the biodiversity is a prerequisite for risk assessment, which in the end should lead to the ability to control the fermentation and hence produce safe foods of a known quality.

Standardised criteria for assessment of microorganisms to be used in food production would be a useful tool in food safety management. The GRAS (Generally Recognised As Safe) terminology is already known from USA, however, the European Food Safety Authority (EFSA) recently proposed a new concept; "*Qualified Presumption of Safety (QPS) of microorganisms in food and feed*". QPS is modified from GRAS to take account of the different regulatory practices in Europe. It aims at harmonising approaches for the safety assessment of microorganisms used in feed and food for all European food safety authorities and to ensure a better use of assessment resources by focussing on those organisms which represent the greatest risks or uncertainties. When used in practice, QPS should permit the identification of what is required to make an adequate safety assessment. The assessment requires determinations of taxonomy, familiarity, end use, presence of acquired antibiotic resistance factors, pathogenic potential and production of undesirable metabolites (EFSA, 2005).

Following the QPS concept, the first task is identification of the microorganism(s) to be assessed (EFSA, 2005). Concerning fermented products the first step would be to isolate the microflora and second to differentiate between the various species present in order for them to be identified. Traditional methods for species typing of LAB comprise of morphological, physiological and biochemical methods (Axelsson, 2004), which are both laborious and time consuming. Simplified methods for LAB identification based on fermentation and assimilation profiles exist, e.g. API 50 CHL (bioMerieux sa, Marcy-l'Etoile, France). These simplified methods have made identification work easier, but still laborious. In addition, there are indications that such fermentation and assimilation tests are not satisfactory for the identification of

LAB species, since the genetic basis of such tests is either unknown or known to be controlled by a single or few genes that do not appear to be of phylogenetic significance. Many of these phenotypic characters are unstable and can be due to a single mutation (Axelsson, 2004). This problem was also experienced by Lei and Jakobsen (2004 - Appendix I) when using API 50 CHL on the *Weissella* group, easily distinguished by ITS-PCR RFLP; the API 50 CHL was unable to define these isolates as a single genus. Also Nigatu (2000) found discrepancies when API 50 CHL results of LAB isolated from fermented 'tef' and 'kocho' was compared with two molecular identification methods.

With the rapid development of molecular biology during the last decades, new techniques for identification and typing of microorganisms have emerged as an attempt to simplify and reduce time in the laboratory. Close relationships (at species and sub-species levels) can be determined with DNA-DNA homology studies. For determining phylogenetic positions of species and genera, ribosomal RNA (rRNA) is more suitable, since the genes contain both well-conserved and less conserved regions (Axelsson, 2004). In the light of the above mentioned problems with the phenotypic tests, molecular methods seem overall to result in a more accurate identification. Examples of different molecular methods used for identification at species and strain level will be given in the following.

Identification of lactic acid bacteria from fermented foods at species level

The invention of the Polymerase Chain Reaction (PCR) by Saiki *et al.* (1988) was a huge break through for modern microbiology and gave rise to the development of new and fast techniques within the area of fingerprinting. The technique results in an amplification of specific DNA sequences by an enormous factor. DNA fingerprinting or species typing by use of PCR can be based on amplification of either a specific part or a non-specific part of the genome (Towner and Cockayne, 1993).

Ribosomal (r)RNAs (the 5S, 16S and 23S molecules) are essential elements in protein synthesis and are present in all living organisms. Because of the conserved functions of these molecules they have changed very little during evolution (Priest and Austin, 1993). The 16S rRNA gene has a size of about 1500 bp and contains

nine variable regions (ITS regions). Intergenic Transcribed Spacer (ITS)-PCR is an example of fingerprinting based on amplification of a specific part of the genome. As shown in Figure 2.2 ITS-PCR relies on amplification of the region spanning the non-coding ITS between the 16S rDNA and 23S rDNA and/or 23S rDNA and 5S rDNA; the former being the most widely used. The ITS regions are much less evolutionary conserved than the rRNA coding genes and therefore appear to be useful in detecting genetic variability among species, which is valuable for taxonomic purposes and for species identification (Barry *et al.*, 1991; Nour, 1998).

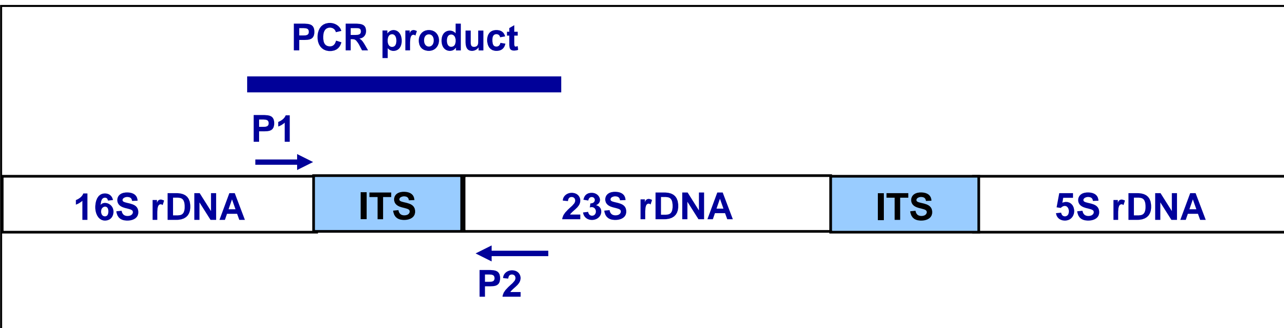

Figure 2.2 Ribosomal DNA repeat unit and location of intergenic transcribed spacers (ITS), location of primers and the resulting ITS-PCR product (Drawn from Nour, 1998).

In order to better differentiate the amplified sequences obtained by ITS-PCR, application of a restriction enzyme to the amplified sequences can be used, followed by examination of the Restriction Fragment Length Polymorphisms (RFLPs) after being separated by electrophoresis (Towner and Cockayne, 1993).

For the initial identification of the LAB in the present Thesis, ITS-PCR RFLP was applied to the 215 isolates from ‘koko’ and KSW (Lei and Jakobsen, 2004 - Appendix I) as well as the 556 isolates from ‘pito’ and ‘dolo’ (Sawadogo-Lingani *et al.*, Appendix III). Using this method, the isolates from ‘koko’ and KSW were divided into four distinct groups. Of the 556 isolates from ‘pito’ and ‘dolo’, 96% were divided into seven groups; with the three largest groups accounting for respectively 33%, 26% and 22% of the isolates. Overall, the method gave a good initial separation of the species; however, other molecular methods had to be applied in order to differentiate the isolates at strain level. The ITS-PCR RFLP method was also successfully used by Barrangou *et al.* (2002) on LAB isolated from sauerkraut (fermented cabbage).

Differentiation of LAB species from fermented foods have effectively been carried out in other studies using different molecular approaches. Corsetti *et al*. (2001) and Paludan-Müller *et al*. (2002) used the ITS-PCR method without RFLP to separate the LAB species found in respectively wheat sourdoughs and a Thai fermented fish product. Muller *et al*. (2000) differentiated and identified LAB from sour dough by applying PCR to different 16S rRNA regions of the isolates. Leisner *et al*. (2001) used SDS-PAGE and compared whole cell protein patterns of LAB isolated from a lactic acid fermented condiment from Malaysia and Kunene *et al*. (2000) used amplified fragment length polymorphism (AFLP) fingerprinting to identify LAB isolated from fermented sorghum.

In addition, nucleic acid sequencing has been shown to be a useful tool for identification of microorganisms at species level. The majority of the sequencing is focusing on the ribosomal genes, especially the 16S rRNA gene and the 16S-23S ITS region (Priest and Austin, 1993; Morelli *et al*., 2004). The basic principle of the "chain terminator technique" was developed in 1977 (Sanger *et al*., 1977) and is still used today for sequencing.

The sequencing method of a specific part of the 16S rRNA gene proved useful for species differentiation of the LAB isolates in the present Thesis (Lei and Jakobsen, 2004 - Appendix I; Sawadogo-Lingani *et al*., Appendix III). Likewise; Hamad *et al*. (1997), ben Omar *et al*. (2000), Escalante *et al*. (2001), Paludan-Mûller *et al*. (2002) and Sanni *et al*. (2002) found the method useful when identifying LAB from traditional fermented products at species level.

In line with the fast development of molecular methods, new demands have been set for identification of microorganisms directly from their natural habitat. By having to isolate and propagate the microorganisms, the microorganisms unable to grow outside of their habitat will be lost. Hence a need for culture-independent techniques arose. Denaturing Gradient Gel Electrophoresis (DGGE) is one such a culture independent technique and has successfully been applied directly on fermented cereal foods (Ampe *et al*., 1999a,b; ben Omar and Ampe, 2000; Lee *et al*., 2005; Meroth *et al*,. 2003) as well as non-cereal products as cassava (ben Omar *et al*., 2000), coffee

(Masoud *et al*., 2004) and cocoa (Nielsen *et al*., 2005) in order to identify the fermenting microorganisms.

Identification of lactic acid bacteria from fermented foods at strain level

Restriction enzyme analysis (REA) involves as an example the digestion of chromosomal DNA with restriction endonucleases. The selection of an appropriate restriction enzyme, or set of enzymes, is important for obtaining revealing band patterns. The fragments obtained from LAB by REA are smaller than 50,000 base pairs in size, and can be separated in an agarose gel by use of electrophoresis as pulsed field gel electrophoresis. When using REA in combination with pulsed field gel electrophoresis the whole genome can be investigated and this gives the technique superior discriminatory power to many other molecular methods (Towner and Cockayne, 1993).

Pulsed field gel electrophoresis (PFGE) is a type of gel electrophoresis which is designed for the purpose of improved resolution of, or to separate and resolve DNA molecules up to 2 Mbp. PFGE involves periodically changing the orientation of the electric field, thereby enabling the separation of high-molecular-weight fragments. PFGE allows the use of rare-cutting restriction endonucleases, which generates a low number of fragments, resulting in a band pattern that is easily interpreted. This type of DNA fingerprint typically consists of 5 to 20 large well-resolved fragments, ranging in size from 10 to 1000 Kbp. It is a highly discriminatory and reproducible method capable of differentiating strains (Towner and Cockayne, 1993).

PFGE-REA was applied to the in total 771 isolates represented in this Thesis (Lei and Jakobsen, 2004 - Appendix I; Sawadogo-Lingani *et al*., Appendix III). Of the 215 isolates from 'koko' and KSW more than 80% were found to have a unique PFGE-REA pattern. This great biodiversity at strain level among the dominating species was confirmed by Sawadogo-Lingani *et al*. (Appendix III) where PFGE was applied to the 556 isolates from the production of sorghum 'pito' and 'dolo'. A large biodiversity among the dominant LAB species in spontaneously fermented cereal products have been confirmed by Hamad *et al*. (1997), Hayford *et al*. (1999) and ben Omar and Ampe (2000).

Another widespread molecular method for strain level identification of LAB isolated from traditional fermented foods is the Randomly Amplified Polymorphic DNA (RAPD) analysis (Hamad *et al.*, 1997; Hayford *et al.*, 1999; Nigatu, 2000; Paludan-Mûller *et al.*, 2002). It is a PCR-based method and differs from the traditional PCR in that a short primer (~10 nucleotides) with no known homology to the template DNA is used against two longer primers with known homology for the conventional PCR method. These primers under low stringency (low annealing temperature at which primer binds to template DNA) bind to specific and non-specific sequences on the template DNA and the PCR reaction then amplifies fragments of the genome where the correct orientation of the primer has annealed. The results of RAPD analysis are a number of DNA amplicons of different sizes occurring and giving a characteristic genomic fingerprint of the organisms (Farber, 1996; Roy *et al.*, 2000).

An alternative method of generating fingerprints directly, i.e. without the use of restriction endonucleases, is repetitive element sequenced-based PCR (rep-PCR) (Towner and Cockayne, 1993). The term rep-PCR refers to the general methodology involving the use of oligonucleotide primers based on short repetitive conserved sequence elements that are dispersed throughout the bacterial genome (Towner and Cockayne, 1993). The method has been proven useful for identification of a wide range of lactobacilli at the species, strain and potentially sub-species level (Gevers *et al.*, 2001).

In the end, still great many investigations identify LAB from fermented foods using the traditional identification methods by studying fermentation and assimilation patterns in combination with morphological and physiological characteristics (e.g. Kunene *et al.*, 1999; Sanchez *et al.*, 2000; Muyanja *et al.*, 2002; Thapa and Tamang, 2004). Seen from a technological viewpoint, these methods should not be under-estimated, but are recommended to be used in connection with molecular methods.

2.5.2 Lactic acid bacteria predominant in millet 'koko' fermentation

Lei and Jakobsen (2004 - Appendix I) investigated for the first time, the microbial succession in millet 'koko' from two production sites in Northern Ghana. Figure 2.3 shows a graphic distribution of LAB during the production. The figure gives an

overview of the percentage distribution of the predominant LAB at the different stages during the production of ‘koko’.

At the Tamale ‘koko’ production site, no LAB were isolated from the millet grains, due to overgrowth of moulds, however, moulds were not isolated in any of the remaining production stages. *Lactobacillus fermentum* was found dominant in the following production stages; steep water after steeping, mixture before and after sieving, in the top- and bottom-layer after fermentation, i.e. all the later production stages (Figure 2.3, Tamale production site). At the two stages where *L. fermentum* was not dominating, i.e. steep water before steeping and in the milled millet, it was found to be the second most dominant LAB species (Figure 2.3, Tamale production site). *Weissella confusa* and *Pediococcus* spp. were found to dominate in the steep water before steeping, however, *Pediococcus* spp. were only hereafter isolated from the bottom-layer (Figure 2.3, Tamale production site). *Weissella confusa* was isolated from all production stages, except in the mixture before sieving and in the top-layer. In addition to being dominant in the steep water before steeping, it was also dominating in the milled millet (Figure 2.3, Tamale production site). *Lactobacillus salivarius* was isolated from all production stages, however never as the dominating LAB (Figure 2.3, Tamale production site). *Lactobacillus fermentum* and *L. salivarius* were the only species isolated from all stages (Figure 2.3, Tamale production site). Finally, no LAB was isolated from the final product, due to extensive heat treatment (Figure 2.3, Tamale production site).

At the Nyankpala A production site *W. confusa* was found to dominate throughout the production of ‘koko’. Only on the millet grains was it found to dominate together with *L. fermentum* and *Pediococcus* spp. *Weissella confusa* was found as the sole LAB in the steep water before and after steeping and in the mixture before and after sieving (Figure 2.3, Nyankpala A production site). *Lactobacillus fermentum* was found in low numbers in the milled millet, in the top- and bottom-layer as well as in the final product. *Pediococcus* spp. was only isolated from the millet grains and in the bottom-layer (Figure 2.3, Nyankpala A production site).

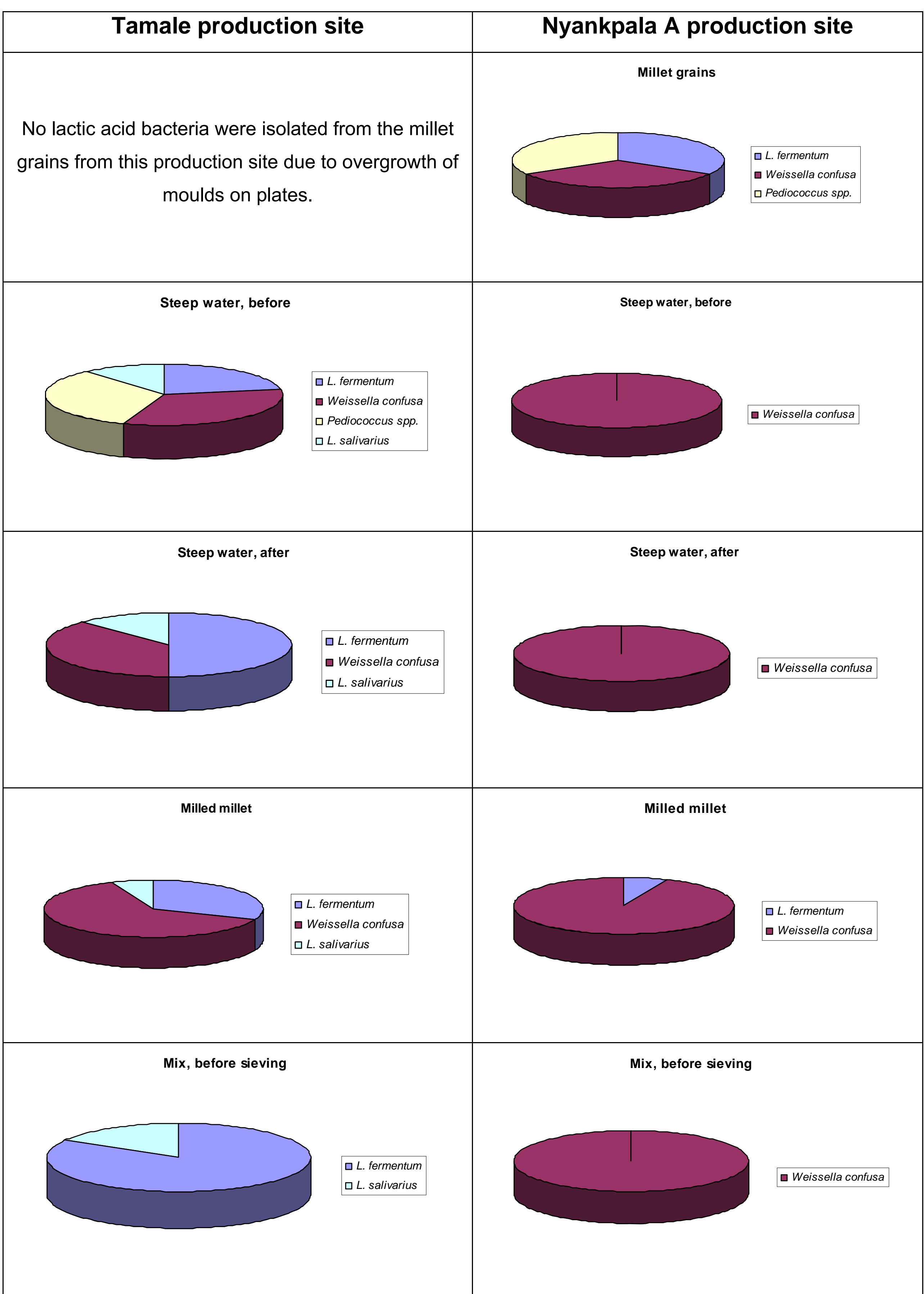
Tamale production site
Nyankpala A production site
No lactic acid bacteria were isolated from the millet grains from this production site due to overgrowth of moulds on plates.
Millet grains
L. fermentum
Weissella confusa
Pediococcus spp.
Steep water, before
L. fermentum
Weissella confusa
Pediococcus spp.
L. salivarius
Steep water, before
Weissella confusa
Steep water, after
L. fermentum
Weissella confusa
L. salivarius
Steep water, after
Weissella confusa
Milled millet
L. fermentum
Weissella confusa
L. salivarius
Milled millet
L. fermentum
Weissella confusa
Mix, before sieving
L. fermentum
L. salivarius
Mix, before sieving
Weissella confusa

Figure 2.3 *-cont.*

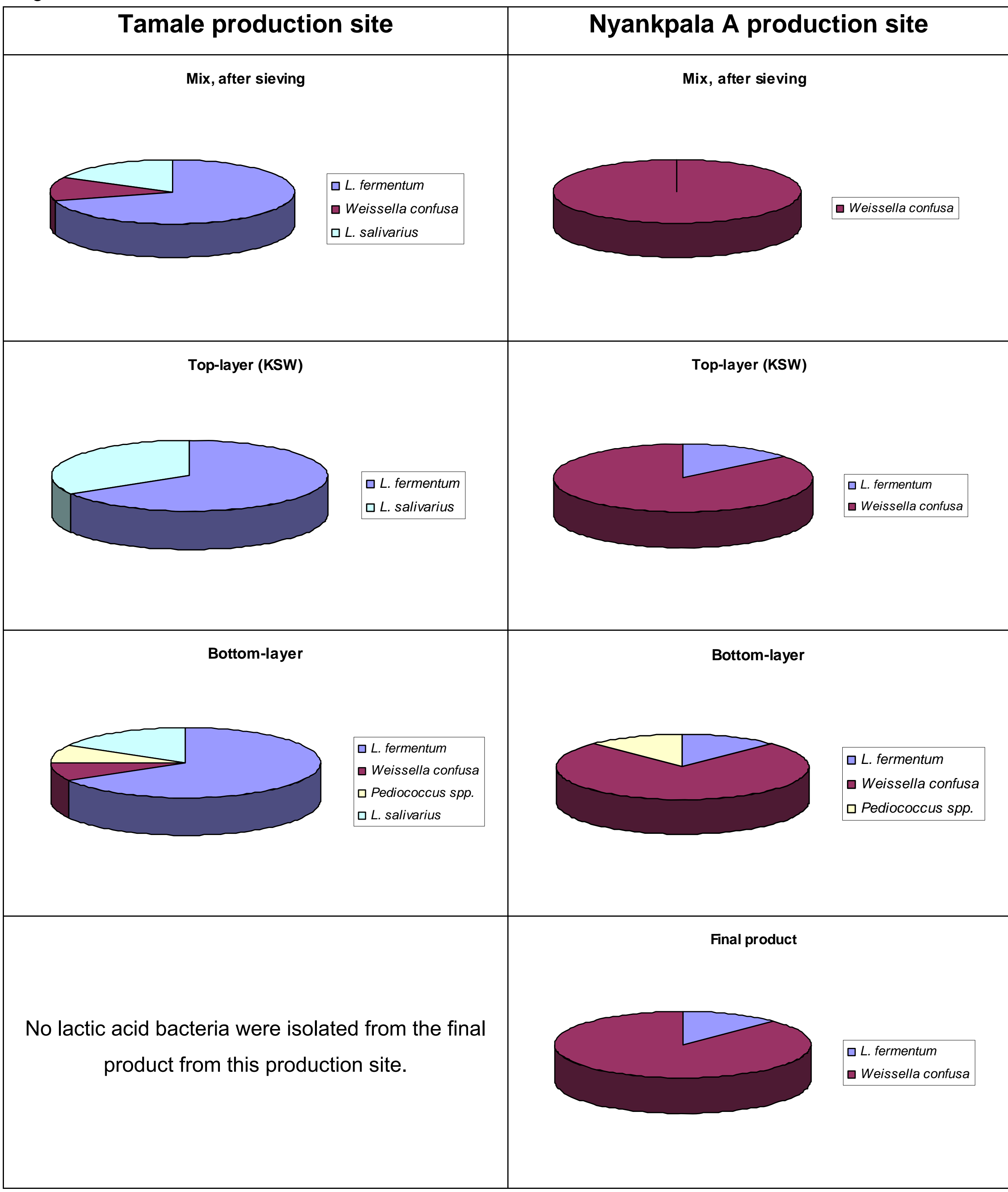

Figure 2.3 Percentage distribution of isolated lactic acid bacteria during the production of millet koko from two production sites, Nyankpala A and Tamale, respectively (Lei and Jakobsen, 2004 - Appendix I).

Lei and Jakobsen (2004 – Appendix I) used the chemometric tool “ANOVA Partial Least Square Regression” (APLSR) to show the distribution of LAB in the various stages from the two production sites (Figure 2.4 and 2.5). From the APLSR analysis of Tamale production site (Figure 2.3) two significant components were found, having a validated explained variance in PC1 and PC2 of 55 and 31%, respectively. From Figure 2.4 (and Figure 2.3) it can be interpreted that the *W. confusa* isolates were highly correlated with the milled millet, as well as *L. fermentum* isolates were correlated with all other stages except water for steeping. Moreover, *Pediococcus* spp. isolates were highly correlated with the stages, water for steeping and bottom-layer as was also indicated in Figure 2.3.

The APLSR plot from Nyankpala A production site (Figure 2.5) depicted *Pediococcus* spp. as highly correlated with the stages of millet grains and bottom-layer, and *W. confusa* as being dominant in all production stages. The APLSR had two significant components with validated explained variance in PC1 and PC2 of 77 and 16%, respectively. When comparing Tamale and Nyankpala A production sites, it was found that Nyankpala A throughout the ‘koko’ production had a very uniform microbiota compared to the Tamale site. In an APLSR plot this was confirmed by isolates of the two production sites being oppositely correlated, indicating that the LAB from the two productions were differently distributed throughout the production stages (plot not shown).

Few authors have previously used Principal Component Analysis (PCA) to interpret band patterns in order to group microorganisms obtained from molecular methods (Couto *et al*., 1995; Wilkström *et al*., 1999; Mugula *et al*., 2002; Lay *et al*., 2005), and it seems that APLSR has not previously been used to visualise the correlated LAB species with their processing stages and production sites. By using this type of chemometric tool to depict the distribution of LAB in relation to the production stages or production sites, the reader gets an “easy-to-interpret” overview of how closely related certain species are to the individual production stages or production sites. Seen from this “user-friendly” perspective, it appears that multivariate data analysis is not being used to its full potential as yet in the interpretation of microbiological results of this nature.

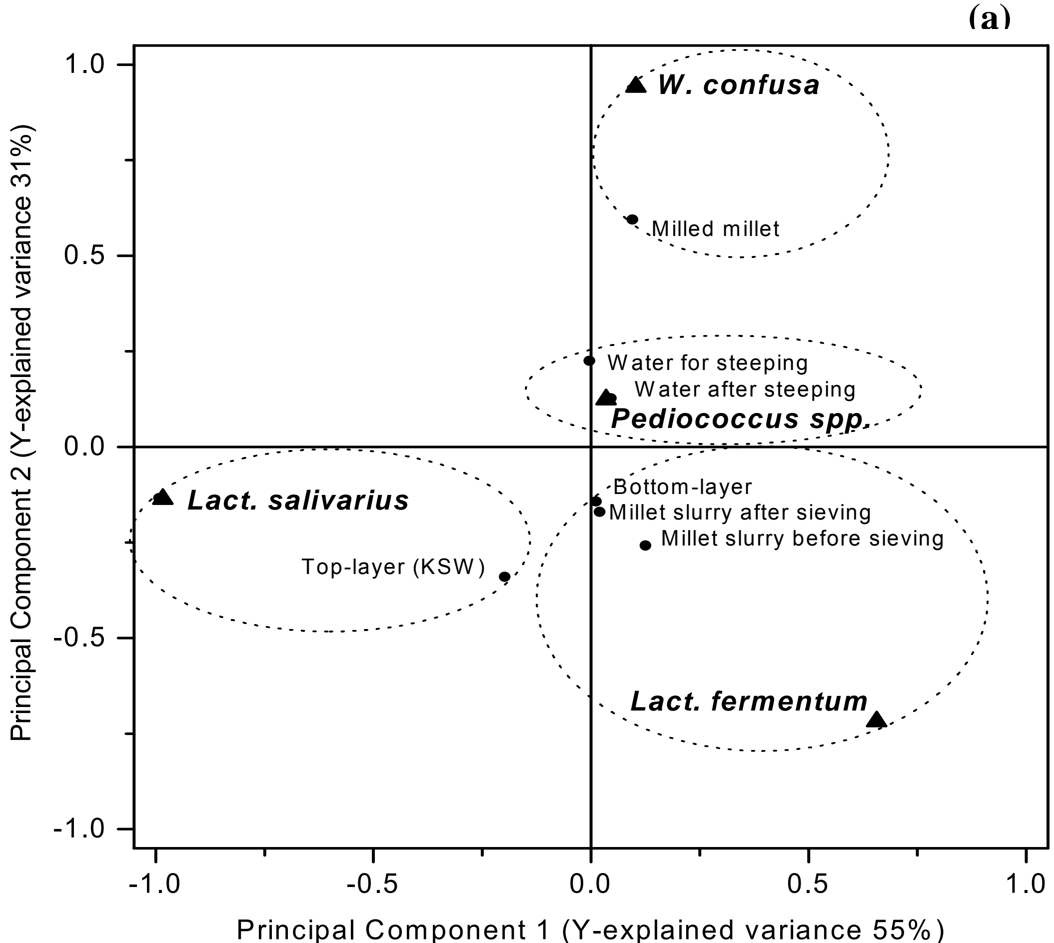

Figure 2.4 ANOVA Partial Least Squares Regression (APLSR) correlation loadings plot. Distribution of lactic acid bacteria from Tamale production site for the various stages of *koko* production. Shown are the loadings of the X- and Y-variables for the first 2 PCs. ▲ = isolates and ● = stage of production. X variables were 0/1 design variables for the production stages and the LAB isolates and the Y matrix was set as the base pair patterns from ITS-PCR RFLP fragments of the LAB isolates (response variables). Dashed ellipses are visual aid to interpretation of correlations.

(b)

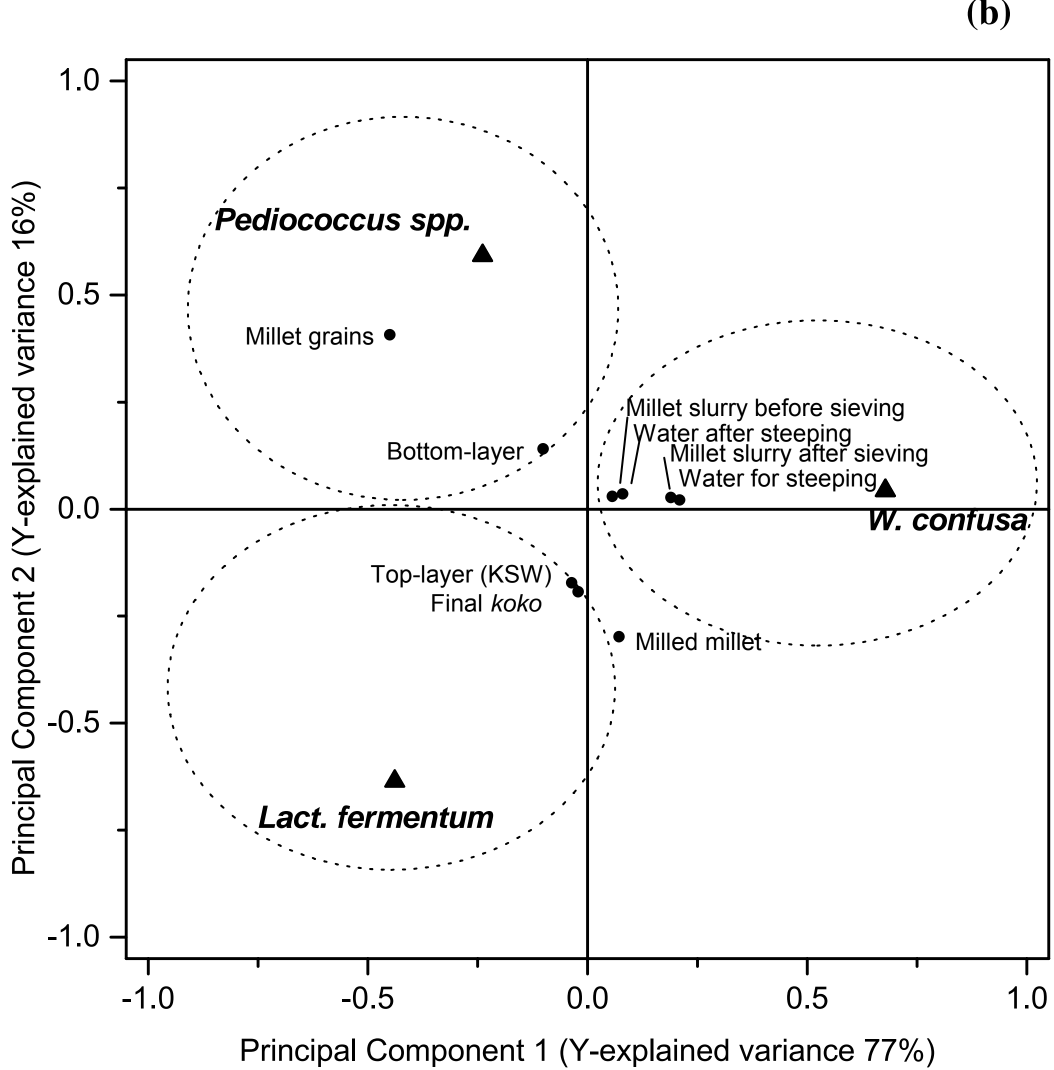

Figure 2.5 ANOVA Partial Least Squares Regression (APLSR) correlation loadings plot. Distribution of lactic acid bacteria from Nyankpala A production site for the various stages of *koko* production. Shown are the loadings of the X- and Y-variables for the first 2 PCs. ▲ = isolates and ● = stage of production. X variables were 0/1 design variables for the production stages and the LAB isolates and the Y matrix was set as the base pair patterns from ITS-PCR RFLP fragments of the LAB isolates (response variables). Dashed ellipses are visual aid to interpretation of correlations.

The microbiology of the fermented, but un-cooked top-layer of 'koko' (KSW) was investigated in an additional three production sites, i.e. Nyankpala B, Savelugu and Pong-Tamale (Lei and Jakobsen, 2004 - Appendix I). This product (without the addition of spices to the production) was used as the intervention product in the study by Lei *et al.* (2006 - Appendix II). Figure 2.6 shows the distribution of LAB in KSW from the five production sites investigated in total (Lei and Jakobsen, 2004 - Appendix I). From four of the five sites, i.e. Tamale, Nyankpala B, Savelugu and Pong-Tamale, *L. fermentum* was found to be the dominant species, whereas KSW from Nyankpala A was dominated by *W. confusa*. *Lactobacillus salivarius* and *L. paraplantarum* were only found from a single production site, i.e. Tamale and Savelugu, respectively.

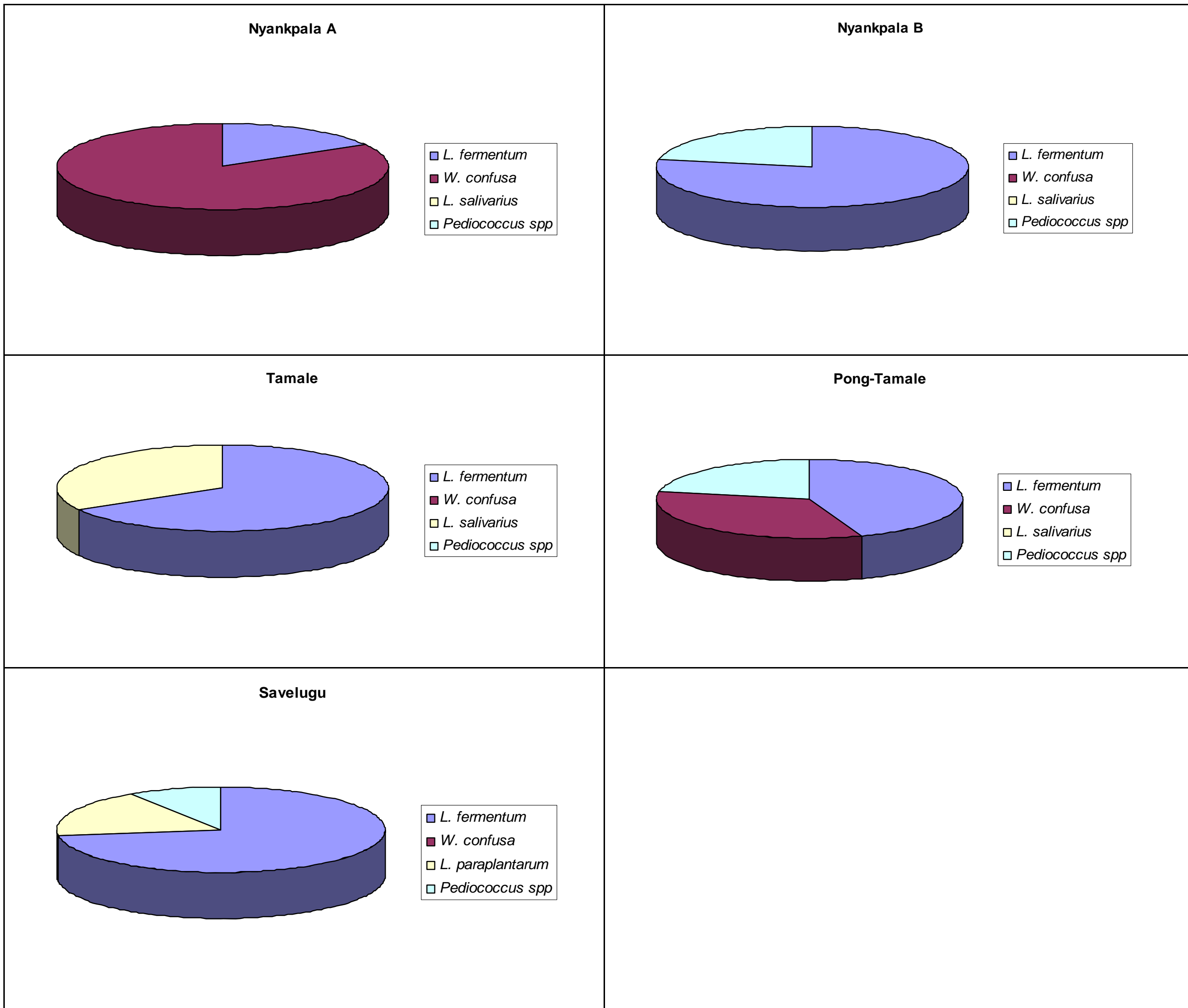

Figure 2.6 Percentage distribution of isolated lactic acid bacteria in koko sour water (KSW) from five production sites; Nyankpala A, Nyankpala B, Tamale, Pong-Tamale and Savelugu, respectively.

Figure 2.7 shows the APLSR correlation loading plot of the distribution of LAB isolates from KSW from the five production sites. It gives an overview of the dominating LAB and their relation to the different production sites. From Figure 2.7 it can been seen that a strong positive correlation existed between *W. confusa* and the sites Nyankpala A and Pong-Tamale. *Lactobacillus fermentum* was seen to be highly correlated with Nyankpala B and Savelugu and *L. salivarius* highly correlated with Tamale. Isolates of *Pediococcus* spp. were found in three of the five production sites and are also seen not to correlate strongly with any single production site (Figure 2.7). In the APLSR plot depicting PC2 and PC3, it was made clear that *Pediococcus* spp. isolates were highly correlated with Nyankpala B productions sites (not shown). This could also be said to be evident to a degree in the first 2 PCs of (Figure 2.7). The APLSR had three significant components with validated explained variance in PC1, PC2 and PC3 of 48, 23 and 19%, respectively.

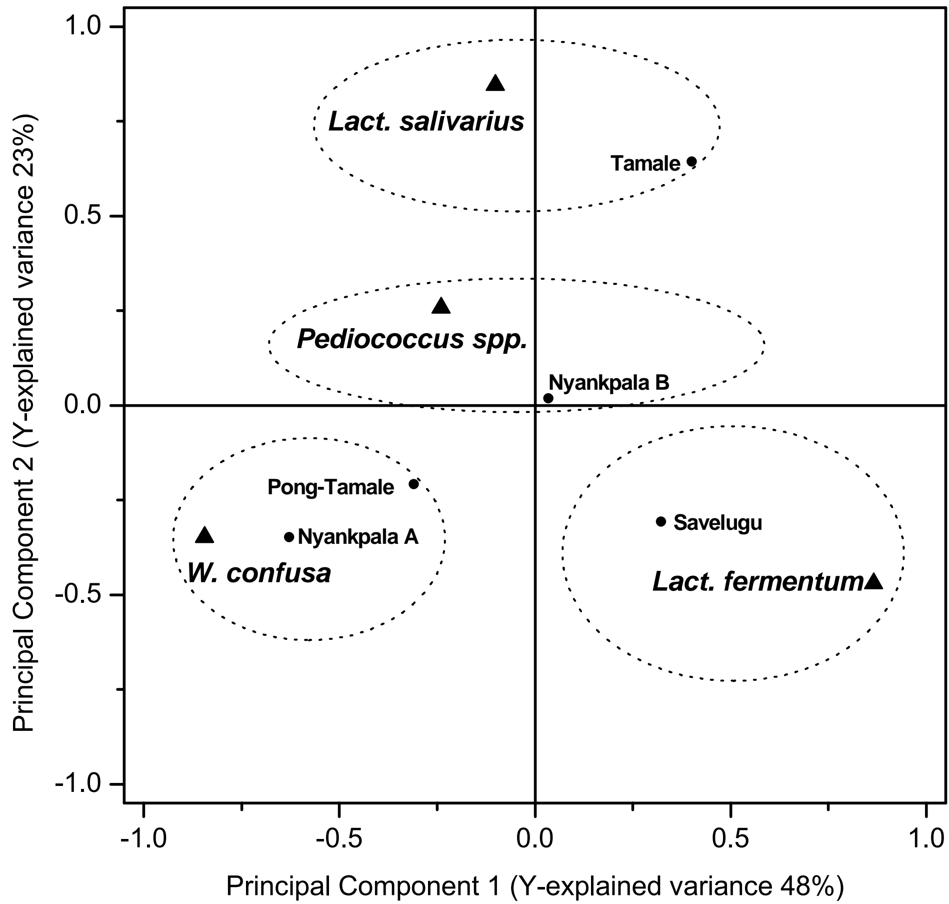

Figure 2.7 ANOVA Partial Least Squares Regression (APLSR) correlation loadings plot. Distribution of lactic acid bacteria isolates from *koko sour water* (KSW) from the five production sites Tamale, Nyankpala A, Savelugu, Pong-Tamale and Nyankpala B. Shown are the loadings of the X- and Y-variables for the first 2 PCs. ▲ = isolates and ● = site of production. X variables were 0/1 design variables for the production sites and the LAB isolates and the Y matrix was set as the base pair patterns from ITS-PCR RFLP fragments of the LAB isolates (response variables). Dashed ellipses are visual aid to interpretation of correlations.

In summary, it was established by Lei and Jakobsen (2004 - Appendix I) that 'koko' and KSW contained two dominating LAB, namely *Lactobacillus fermentum* and *Weissella confusa*. In addition, *Lactobacillus salivarius*, *Lactobacillus paraplantarum* and *Pediococcus* spp. were also identified, but in a less dominating role. Moreover, these LAB occurred in an irregular and inconsistent way and no distinct microbial succession at the species level was observed. Moreover, Lei and Jakobsen (2004 – Appendix I) isolated moulds from the millet grains for 'koko' production, however

moulds were never isolated from the KSW product. Yeasts were isolated in the order of 10^4 cfu per ml in KSW, and the number of coliforms in KSW was always low, i.e. 10^2 cfu per ml or below.

It was evident, that there was a great variation in the distribution of LAB between the production sites (Figure 2.3 – 2.7). This was probably due to the so called "house-flora" variation, where dominating LAB are found in high numbers in the processing water, on the vessels for production and on the hands of the food producer (Oyewole, 1997; Holzapfel, 2002). That cleaning between each production batch reduces the number of predominant species towards a uniform microflora, was shown by Sawadogo-Lingani *et al*. (Appendix III). This study investigated four production sites. At one of the production sites a cleaning procedure had been implemented, and *L. fermentum* was found as the only species throughout the production. The other production sites also had *L. fermentum* as the predominant species, but in cooperation with *L. delbrueckii*, *Pediococcus acidilactici*, *Lactococcus lactis* and *Leuconostoc lactis*.

Spontaneously African fermented millet products similar to 'koko' have been investigated for their dominant fermenting microflora. 'Kamu' from Nigeria is a fermented millet starch-cake (Oyeyiola, 1991) prepared as 'koko' (Lei and Jakobsen, 2004 – Appendix I). When eaten, 'kamu' is diluted with water to make a porridge. Oyeyiola (1991) found *L. plantarum* to be the dominating microorganism responsible for the fermentation; however *Pediococcus pentosaceus* were also isolated in high numbers. Olasupo *et al*. (1997a) investigated a fermented millet product 'kunu-zaki' and found the following microorganisms starting with the most predominant: *L. salivarius*, *L. casei*, *L. acidophilus*, *L. jensenii*, *L. cellobiosus* and *L. plantarum*. In 2002, Mugula *et al*. investigated 'togwa', a fermented millet food from Tanzania and found *L. plantarum*, *L. brevis*, *L. fermentum*, *L. cellobiosus*, *Pediococcus pentosaceus* and *Weissella confusa* to be the microflora dominating the fermentation process.

Other parts of the world also find LAB to be the predominant microflora of spontaneously fermented millet products. In India, Antony and Chandra (1998) investigated the fermentation of finger millet flour during 48 hours and found

Pediococcus spp. to represent more that 80% of the isolates, followed by species of *Leuconostoc* and *Lactobacillus*. Thapa and Tamang (2004) found *Pediococcus pentosaceus* and *L. bifermentans* dominating in 'kodo ko jaanr', a fermented millet beverage from the Himalayas.

In addition to millet, other African cereal products have been investigated for their dominating fermenting microflora. Differences in the microbial composition have been found, but overall the most often isolated fermenting LAB from cereal products are *L. plantarum* and *L. fermentum* (see Table 2.2 for references). Studies of indigenous fermented cereal foods from other parts of the world tend to agree with the above findings of the African products (e.g. Hesseltine, 1983 (review); Fields *et al.*, 1981; Joshi *et al.*, 1989; Infantes and Tourneur, 1991; Lee, 1997; Ampe *et al.*, 1999a,b; Ben Omar and Ampe, 2000; Ben Omar *et al.*, 2000; Escalante *et al.*, 2001).

The predominant LAB of 'koko' processing and KSW, as well as *L. plantarum*, due to its great importance in African fermented foods, is briefly reviewed as follows:

Lactobacillus fermentum is a member of the obligatively heterofermentative group of lactobacilli, and have 52-54 mol% G+C content in their DNA (Kandler and Weiss, 1986; Collins *et al.*, 1991; Schleifer and Ludwig, 1995). *Lactobacillus fermentum* has been isolated from milk products, sour dough, fermenting plant material, manure, sewage, mouth and faeces of man (Kandler and Weiss, 1986). *Lactobacillus fermentum* was isolated as the fermenting and dominant microflora in the African products of 'kisra' (Hamad *et al.*, 1992, 1997), 'mawé' (Hounhouigan *et al.*, 1993a,b), 'kenkey' (Halm *et al.*, 1993), 'koko' (Lei and Jakobsen, 2004 - Appendix I), and 'pito' and 'dolo' (Sawadogo-Lingani *et al.*, Appendix III). In KSW, *L. fermentum* was found to be the dominant species in four out of five production sites (Figure 2.6) as well as predominant in most stages during production of 'koko' (Figure 2.3) (Lei and Jakobsen, 2004 – Appendix I).

Lactobacillus plantarum has 44-46 mol% G+C of DNA and belongs to the facultative heteromentative group of LAB (Schleifer and Ludwig, 1995). The species is a versatile LAB found in a range of environmental niches (de Vries *et al.*, 2006). It

is also said to be the most predominant microorganism in spontaneous fermentations and has been identified as the dominating microorganism in the following African fermented products; 'uji' (Mbugua, 1981), sorghum based weaning food (Nout, 1991; Kunene *et al*., 1999, 2000), 'kenkey' (Olasupo *et al*., 1997a), 'kamu' (Oyeyiola,1991), 'injera' dough and bread (Nigatu, 2000), 'ogi' (Akinrele, 1970; Johansson *et al*., 1995; Olasupo *et al*., 1997a), 'togwa' (Mugula *et al*., 2002) and 'ogi-baba' (Odunfa and Adeyele, 1985). *Lactobacillus plantarum* was not isolated from 'koko' or KSW (Lei and Jakobsen, 2004 – Appendix I); however *L. paraplantarum* was identified in low numbers in KSW, in one out of five productions sites (Figure 2.6).

As mentioned, *L. plantarum* seems to be the most frequently isolated species in connection with spontaneously fermented products. In addition to the above mentioned African cereal products, *L. plantarum* has also been isolated as one of the dominant microorganisms in the following indigenous products; 'tempoyak' - fermented durian fruit from Malaysia (Lesiner *et al*., 2001), 'pozol' -a maize doump-ling from Mexico (Ampe *et al*., 1999b; Escalante *et al*., 2001), cassava fermentation (Brauman *et al*., 1996; ben Omar *et al*., 2000) and 'almagro' –fermented eggplants (Sanchez *et al*., 2000).

Lactobacillus salivarius is obligately homofermentative and has 34-36 mol% G+C of DNA (Schleifer and Ludwig, 1995). *Lactobacillus salivarius* rarely seems to be the dominant organism in fermented products. It has only been found predominant in 'kanu-zaki', a fermented millet porridge from Nigeria (Olasupo *et al*., 1997a), however, it has been found as one of the few LAB species surviving the microbial succession throughout the production stages (Odunfa and Oyewole, 1998; Lei and Jakobsen, 2004 - Appendix I). At one production site (Tamale), *L. salivarius* was the only LAB species together with the dominating *L. fermentum*, which was found in all production stages (Figure 2.3). However, *L. salivarius* was only found in this production (Tamale) out of the five sites investigated for microbiological diversity of KSW (Figure 2.6).

Weissella is a relatively new genus within the LAB. In 1993, *Weissella* were suggested by Collins *et al*. (1993) as a separate genus for a distinct phylogenetic

cluster of obligately heterofermentative LAB, consisting of species previously assigned to *Leuconostoc* and some heterofermentative *Lactobacillus* spp. *Weissella* is the first genus in the LAB group that by definition can include both cocci and rods (Collins *et al*., 1993). Differentiation of the genus *Weissella* from *Leuconostoc* requires the use of a combination of characters for particular species. The species *W. confusa* has 45-47 mol% G+C of DNA (Schleifer and Ludwig, 1995) and can be distinguished from leuconostocs by their ability to hydrolyse arginine and by the formation of DL-lactate (Collins *et al*., 1993).

In the study by Lei and Jakobsen (2004 – Appendix I) one production site in particular (Nyankpala A) had *W. confusa* as the dominant microflora throughout the production of millet 'koko' (Figure 2.3). From the second production site (Tamale) *W. confusa* was dominating in the earlier production stages, but was only isolated in lower numbers in the latter production stages, overtaken by *L. fermentum* (Figure 2.3). Similarly, *W. confusa* has been isolated from a great variety of fermented food products, such as maize 'pozol' from Mexico (Ampe *et al*., 1999b), 'som-fak' (fermented fish) and 'som-fak' production ingredients from Thailand (Paludan-Mûller *et al*., 1999, 2002), wheat sourdough from Italy (Corsetti *et al*., 2001), 'tempoyak' (acid fermented condiment) from Malaysia (Leisner *et al*., 2001), 'bushera' (fermented cereal beverage) from Uganda (Muyanja *et al*., 2002), 'togwa' (fermented cereal gruel) from Tanzania (Mugula *et al*., 2002) and 'kimchi' (fermented vegetable food) from Korea (Lee *et al*., 2005).

In addition to being a natural inhabitant of plant material, *W. confusa* has also been found present in the gastro-intestinal tract (GIT) of humans, along with many other LAB species (Walter *et al*., 2001).

Pediococcus spp. has the key characteristics of tetrad formation and having spherical shapes. *Pediococcus* spp. are homofermentors. Despite their morphological distinctiveness, a relationship between the pediococci and the lactobacilli of the *L. casei* group has been demonstrated (Collins *et al*., 1991). The current taxonomical schemes for the heterogeneous genera of *Lactobacillus* and *Pediococcus* are not in agreement with the phylogenetic relationships revealed by 16S ribosomal

DNA sequences, and hence, further changes are likely in the future (Stiles and Holzapfel, 1997; Axelsson, 2004).

When investigating the different production stages of 'koko' production, *Pediococcus* spp. was isolated from the millet grains and from the water for steeping, but hereafter only from the bottom-layer (Figure 2.3). This indicates the ability of *Pediococcus* spp. to survive the microbial succession throughout the production (Lei and Jakobsen, 2004 – Appendix I). In addition, *Pediococcus* spp. was isolated from three out of five KSW production sites (Figure 2.6) (Lei and Jakobsen, 2004 – Appendix I).

Sequencing of the 16S rRNA revealed that the *Pediococcus* isolated, were respectively *P. acidilactici* and *P. pentosaceus*. These two species have been known to be difficult to distinguish using fermentation patterns (Axelsson, 2004); however the use of molecular biology methods can clearly differentiate the two species (Collins *et al*., 1990; Axelsson, 2004; Lei and Jakobsen, 2004 – Appendix I), as well as the inability of *P. acidilactici* to ferment maltose and grow at 50°C (Simpson and Taguchi, 1995). *P. acidilactici* and *P. pentosaceus* contain respectively 38-44 and 35-39 mol% G+C (Simpson and Taguchi, 1995).

Pediococcus spp. are widely distributed in fermenting plant material (Simpson and Taguchi, 1995), however they have rarely been isolated as the dominating micro-organism in spontaneous cereal fermentations (see Table 2.2). One study from India by Anthony and Chandra (1997) found *Pediococcus* to dominate at the end of millet fermentation; however most often *Pediococcus* spp. have been isolated as less predominant during production; i.e. in production of 'kamu' (Oyeyiola, 1991), natural sour doughs from France (Infantes and Tourneur, 1991), 'mawé' (Hounhouigan *et al*., 1993a,b), Moroccan sour-dough bread (Boraam *et al*., 1993), 'kenkey' (Halm *et al*., 1993; Olsen *et al*., 1995), cassava sour starch (ben Omar *et al*., 2000), sorghum-based weaning food (Kunene *et al*., 2000), 'togwa' (Mugula *et al*., 2002), 'kodo ko jaanr' –fermented millet from Himalaya (Thapa and Tamang, 2004), and sorghum beer (Sawadogo-Lingani *et al*., Appendix III).

As previously mentioned for *W. confusa, P. pentosaseus* has also been found present in the GIT of humans (Walter *et al*., 2001).

2.6 Safety of fermented cereals

2.6.1 The role of lactic acid bacteria in food safety

The fermentation itself is only one part of the production process that should be considered when discussing safety of fermented cereals. Also water quality, cleaning of grains, steeping, grinding, cooking and the use of germinated grains should be taken into consideration. However, the safety issues discussed here will focus mainly on the role of LAB.

The principal anti-microbial factor of lactic acid fermentations is the ability of all LAB to produce organic acids and hence decrease the pH of foods in which they grow. Other factors such as the production of bacteriocins, hydrogen peroxide, carbon dioxide, ethanol and diacetyl as well as competition for nutrients also play a contributory role in assuring the safety of fermented cereal foods, however their overall impact is thought to be secondary compared to the impact of the organic acids (Adams, 2001).

Fermentation inhibits growth, survival and toxin production of a number of pathogenic bacteria. The major inhibitory effect of fermentation is the presence of organic acids, mainly lactic acid. The extent of the inhibition depends on the microorganism concerned, the temperature, the amount of un-dissociated acid and the buffering capacities of the food. Fermented cereals are generally weakly buffered and will therefore easily achieve a low pH (e.g. Nout, 1992; Ali *et al*., 2003; Mugula *et al*., 2002; Lei and Jakobsen, 2004 – Appendix I; Sawadogo-Lingani *et al*., Appendix III). Efficient lactic acid fermentation will normally produce a pH of 4 or less, at which the growth of bacterial pathogens is inhibited. However, the level of food contamination depends on factors that are often difficult to quantify, such as the initial level of contamination from raw material or water, level of hygiene and sanitation, as well as the degree of acidification (Adams and Nicolaides, 1997; Nout and Mortarjemi, 1997). The first step in the production of ‘koko’ is steeping the millet grains overnight (Lei and Jakobsen, 2004 – Appendix I). Already during steeping the environment

becomes acidic and reaches a pH on average of 4.3. This pH increases to 4.7, when water is added to the millet flour to make a slurry, but only to quickly decrease again to below pH 4 for the rest of the fermentation and production (Lei and Jakobsen, 2004 – Appendix I). Hence, the acidic environment created already early in the production of millet 'koko' presents a hostile environment to microorganisms which are sensitive to organic acids and low pH.

That the inhibitory potential of the lactic fermentations, however, is caused by other effects than the acids and the low pH alone has been observed in studies by Mensah *et al*. (1991) and Simango (1995). Bacteriocins are peptide anti-microbials produced by bacteria, which are inhibitory to other, normally very closely related, bacteria (Adams and Nicolaides, 1997). The target of bacteriocins is the cytoplasmic membrane and because of the protective barrier provided by the lipopolysaccharide layer of Gram-negative bacteria, bacteriocins are generally only active against Gram-positive cells (Abee *et al*., 1995).

The antimicrobial activity of the 215 LAB isolated from 'koko' and KSW was tested towards two different test organisms; *Lactobacillus sakei* was used due to its sensitivity towards bacteriocins, and *Listeria innocua* was used as a model organism for the human pathogen *Listeria monocytogenes*. The majority of the LAB isolates (approx. 90%) showed a general weak inhibition of *L. innocua*, indicating production of antimicrobial substances or a competition for nutrients. In contrast, only a few of isolates (approx. 2%) showed weak inhibition of *L. sakei*, indicating that the isolates overall did not produce bacteriocins. Furthermore, the pH-neutralised supernatants of the isolates did not show any inhibition of either of the two test-organisms, indicating the weak inhibition found was most likely due to competition for nutrients (Lei and Jakobsen, 2004 - Appendix I). These findings are in agreement with findings from other studies with fermented cereal (Olasupo *et al*., 1995; Olsen *et al*., 1995; Hayford, 1998; Jacobsen *et al*., 1999). Overall the same trend was seen in the study by Lei and Jakobsen (2004 - Appendix I), in that the isolates showed a general moderate inhibition towards the organisms tested, as well as no inhibitory effect from supernatants.

Carbon dioxide produced during heterolactic fermentation can assist in creating an anaerobic environment and is toxic to some aerobic food microorganisms through its action on cell membranes and ability to reduce intra- and extracellular pH (Eklund, 1984). Microorganisms vary in their sensitivity to carbon dioxide. Moulds and oxidative Gram-negative bacteria are more susceptible than lactobacilli and some yeast. The role of carbon dioxide in lactic acid fermentations has yet to be defined; however, its greatest contribution is likely to be at the start of the fermentation where carbon dioxide would affect the large numbers of aerobes present (Adams and Nicolaides, 1997; Caplice and Fitzgerald, 1999).

Since LAB lack true catalase, they are unable to degrade the hydrogen peroxide produced in the presence of oxygen. It is argued that the hydrogen peroxide can accumulate during fermentation and be inhibitory to some microorganisms. Inhibition by hydrogen peroxide is mediated through the strong oxidising effect. However, because of the presence of other enzymes during fermentation that can break down hydrogen peroxide, it is not clear if hydrogen peroxide contributes to any antibacterial activity (Marshall, 1979; Lindgren and Dobrogosz, 1990; Kullisaar *et al*., 2002).

In addition to the above mentioned antimicrobial factors, also factors such as nutrient depletion and competition contribute to the accumulative microbial inhibitory effect during the fermentation process and in fermented products (Adams and Nicolaides, 1997; Caplice and Fitzgerald, 1999; Lei and Jakobsen, 2004 – Appendix I)).

Several studies have been carried out where pathogenic bacteria have been added to the fermented cereal product in order to observe their ability to survive. Svanberg *et al*. (1992) showed that Gram-negative intestinal pathogenic bacteria, as well as the Gram-positive *Bacillus cereus* and *Staphylococcus aureus* were strongly inhibited in sour gruel made from respectively maize and sorghum. However, *Staphylococcus aureus* still showed a slow growth seven hours after inoculation, in the fermented gruel samples which contained viable LAB, implying that low pH (<4.0) alone is not sufficient to sustain the inhibition of the growth of *Staphylococcus aureus* in this particular experiment (Svanberg *et al*., 1992). These results, showing a significant reduction in numbers, in addition to the ability of pathogens to survive, are supported

by the findings of Mensah *et al*. (1991), Odugbemi *et al*. (1991 and 1993), Simango and Rukure (1991 and 1992), Simango (1995), Annan-Prah and Agyeman (1997), Olasupo *et al*. (1997b), Antony *et al.* (1998), Bakare *et al*. (1998), Muyanja *et al*. (2002), Thaoge *et al*. (2003) and Tetteh *et al*. (2004). The same trend was found when “naturally occurring” pathogens were followed during sorghum fermentation (Kunene *et al*., 1999). In two studies (Mensah *et al*., 1988; Kingamkono *et al*., 1998) the authors were unable to detect any of the spiked pathogens after a few hours after start of fermentation.

Since children during weaning are in a very vulnerable state, special attention has been paid to weaning foods and their level of pathogenic contamination (Nout *et al*., 1989; Odugbemi *et al*., 1993; Motarjemi *et al*., 1993; Afifi *et al*., 1998). Nyatoti *et al*. (1997) and Kimmons *et al*. (1999) compared fermented weaning foods with non-fermented and found less pathogens in the fermented foods. Kunene *et al*. (1999) found a significant reduction in “natural” pathogens during fermentation of sorghum-based weaning product; however, pathogens were still detected in the end product. The same observation was made when pathogenic bacteria were added to the weaning foods (Barkare *et al*., 1998). Lei and Jakobsen (2004 - Appendix I) found the fermented millet product KSW, which was used for the intervention study with children having diarrhoea, to contain $<10^2$ colony forming units (cfu) coliforms per ml.

Yeasts seem to be able to resist and survive the lactic environment during fermentation. Studies of fermented foods show that the yeast species also have a succession pattern with different yeast species initially, followed by a reduction to only few dominant species during fermentation and in the end product (Jespersen *et al*., 1994; Jespersen, 2003). The surviving yeast species seem to reach stationary phase at a far lower level (i.e. 10^4-10^6 cfu per ml) than the LAB, probably due to the inhibitory effect of the fermentation (Elfaki *et al*., 1991; Oyeyiola, 1991; Hamad *et al*., 1992; Halm *et al*., 1993; Hounhouigan *et al*., 1994; Antony and Chandra, 1997; Hamad *et al*., 1997; Muyanja *et al*., 2002; Lei and Jakobsen, 2004 - Appendix I; Sawadogo-Lingani *et al*., Appendix III).

Except for the specific foods fermented by moulds, moulds in general are unwanted in food products due to their ability to produce mycotoxins. Lactic acid fermentation has been reported to have a significant ability to reduce the levels of moulds present (Oyeyiola, 1991; Halm *et al*., 1993; Jespersen *et al*., 1994; Ben Omar and Ampe, 2000). However, if moulds initially have been present, there is a possibility that mycotoxins have been produced. The possible effect of fermentation on mycotoxins is discussed below.

2.6.2 Mycotoxins in fermented cereals

In contrast to fermentation being able to improve the nutritional status and significantly reduce the ability of pathogens to survive in the products, the potential hazard of mycotoxins cannot be easily avoided. Mycotoxins, especially aflatoxins and fumonisins are a major risk factor in stored cereals, and even though moulds are significantly reduced during fermentation, already produced mycotoxins are able to remain in the product. Studies have shown detectable levels of mycotoxins in fermented maize products with undetectable or low mould levels (Jespersen *et al*., 1994; Kpodo *et al*., 1996).

Reports indicate, however, that some mycotoxins may be degraded or inactivated during fermentation of cereals (Adegoke *et al*., 1994; Moss, 2001). The possible mechanism for inactivation or degradation is suggested to be by enzymatic degradation (Teniola *et al*., 2005; Alberts *et al*., 2006) or by binding of the mycotoxin to the fermenting microorganisms (Haskard *et al*., 2000; El-Nezami *et al*., 2001; Peltonen *et al*., 2001; Shetty and Jespersen, 2006). The latter has also been suggested for yeast (Shetty and Jespersen, 2006). In addition, a reduction in mycotoxin levels has been observed when the product is cooked for longer periods (Adegoke *et al*., 1994; Kpodo *et al*., 1996).

In summary; lactic fermented foods have a significant ability to reduce and inhibit unwanted microorganisms; though, survival of pathogens is still possible along with the possibility of unknown levels of toxic compounds left, more or less, unaffected by the fermenting microorganisms. However, the positive effects of fermenting foods far outweigh the negative effects, especially in the African setting. Section 3.5 reviews further on LAB and their safety when used as probiotics.

3. Probiotic potential of lactic acid bacteria

The influence of diet and of some living bacteria on health has been known for more than a hundred years. Ever since Metchnikoff around 1907 in the Caucasus hypothesised the importance of LAB for human health and longevity, it has been assumed that LAB possess beneficial properties (Metchnikoff, 1907). During recent decades, knowledge of these properties has increased greatly. The great interest in the topic is assured from the enormous amount of literature in the subject. To date more than 3000 papers have been published within this theme (Web of Science).

The aim of this Chapter is to give a brief introduction into some of the beneficial effects claimed for probiotics. However, the main emphasis will be on the probiotic effect on diarrhoea, since these effects were the focus of the intervention study (Lei *et al*., 2006 – Appendix II). Furthermore the probiotic effect on stimulation of the immune defence will be described by use of examples, since it is argued that this effect is an underlying reason for probiotic effects *per se*. The mechanisms behind the beneficial effects are not fully known and understood and will not be dealt with in detail here. Chapter 5 will present further detail with respect to probiotics in connection to childhood diarrhoea and the potential of traditional fermented food to alleviate diarrhoea.

3.1 Definitions of probiotics, prebiotics and synbiotics

Several definitions have over the years been proposed for *probiotics*. Naidu *et al*. (1999) attempted a more detailed definition by dividing the definition into probiotic and probiotic-active substances. They defined probiotic as "*a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract*". Further they defined probiotic-active substances as "*a cellular complex of LAB that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independent of LAB's viability*". Thus, both viable and non-viable LAB have proven to possess beneficial effects (Ouwehand and Salminen, 1998; Naidu *et al*., 1999; Pessi *et al*., 1999; Rayes *et al*., 2002a,b; Simakachorn *et al*., 2000; Klingberg *et al*., 2005). Salminen *et al.* (1999) put this in

short to; "*probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host*".

In addition to probiotics, the terms *prebiotics* and *synbiotics* are often used. A definition for prebiotic was developed by Gibson and Roberfroid (1995), however in the light of published research, the authors redefined this in 2004 to: *"A probiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health"* (Gibson *et al.,* 2004). Most commercial prebiotics are carbohydrates, predominantly oligosaccharides and some polysaccharides. The non-digestible character of prebiotics is a feature shared with dietary fibre, but their physiological functions are often different (Macfarlane *et al*., 2006). The prebiotics are selective in their growth stimulation and at the same time they are reported to suppress pathogenic bacteria present in the GIT because they can only use the prebiotic ingredient for growth to a limited extend or not at all (Bengmark, 2000; Holzapfel and Schillinger, 2002; Macfarlane *et al*., 2006). Hence, the prebiotic principle is based on selective stimulation of microorganisms able to hydrolyse the prebiotics to carbohydrate monomers and use these for growth in the GIT (Bielecka *et al*., 2002; Bomba *et al*., 2002; Macfarlane *et al*., 2006).

Other suggested beneficial effects of prebiotics are: improvement of calcium bioavailability and reduction in the risk for cardiovascular diseases, non-insulin-dependant diabetes, obesity, osteoporosis, colon cancer as well as reduction of traveller's diarrhoea, however evidence to support these effects are still preliminary (Roberfroid, 1998; Macfarlane *et al*., 2006).

A synbiotic refers to a food product, which contains both a probiotic and a prebiotic (Roberfroid, 1998). This combination has shown to confer benefits, which are beyond the benefits of the pro- and prebiotic on their own. If the prebiotic carbohydrate is utilised by a probiotic strain, its growth and proliferation in the gut will be selectively promoted (Holzapfel and Schillinger, 2002; Bengmark, 2003). It could be argued that fermented cereal products, such as for example 'koko', are potential synbiotic products, in that they contain LAB (potential probiotics) as well as water-soluble

fibres, oligosaccharides and resistant starch (potential prebiotics). However, the present study will be limited to the probiotic effect of LAB.

3.2 Probiotic effects of lactic acid bacteria

This Section is based upon examples rather than description and explanation of the probiotic mechanisms, which is not fully understood. When considering the health effects of probiotics, it is important to recognise that different strains, species and genera of bacteria may have different effects. Clinical studies on probiotics are usually carried out with one defined strain or a defined blend of strains (see Table 4.1). Although any one study may not give a complete picture of effectiveness, an evaluation of the research done on probiotic cultures suggests that certain strains, consumed at adequate levels, positively influence human health. This aspect will be described in the following examples, in particularly those relevant to the present study; i.e. alleviation of diarrhoea symptoms and stimulation of the immune defence.

A healthy intestinal epithelium, in association with an optimal intestinal flora, provides a vital barrier against invasion or uptake of pathogens, antigens and harmful compounds from the gut lumen. The stable barrier ensures host protection and serves as support for normal intestinal function and immunological resistance. In situations where the stable barrier of an individual is challenged, probiotics can, to some extent, help in maintaining the stability (Rolfe, 2000; Holzapfel and Schillinger, 2002; Guslandi, 2003). Overall, it appears that many probiotic effects are mediated through immune regulation, and especially through control of pro-inflammatory cytokines, thereby suggesting the use of probiotics as innovative tools to alleviate intestinal inflammation, normalise gut mucosal dysfunction, and down-regulate hypersensitivity (Isolauri *et al*., 2001; Holzapfel and Schillinger, 2002).

In addition to the effects to be reviewed as mentioned above, pro- and prebiotics have been suggested to have a positive effect on;

- Lactose intolerance (Gendrel *et al.*, 1990; Vesa *et al.*, 1996; Hove *et al.*, 1999; Ouwehand and Salminen, 1998; Boudraa *et al.*, 2001; Holzapfel and Schillinger, 2002).
- Constipation (Malkki and Virtanen, 2001; Scheppach *et al.*, 2001; Moore *et al.* (2003).
- Gastric *Helicobacter pylori* infections (Kabir *et al.*, 1997; Michetti *et al.*, 1999; Armuzzi *et al.*, 2001; Felley *et al.*, 2001; Lorca *et al.*, 2001; Sakamoto *et al.*, 2001; Sheu *et al.*, 2002, Cremonini *et al.*, 2002a).
- Elevated serum cholesterol and lipids (Agerbaek *et al.*, 1995; Tahri *et al.*, 1995; Richelsen *et al.*, 1996; du Toit *et al.*, 1998; Bertolami *et al.*, 1999; Rossi *et al.*, 1999; Agerholm-Larsen *et al.*, 2000; Delzenne and Kok, 2001; Kiessling *et al.*, 2002; Sindhu and Khetarpaul; 2003).
- Cancer (Reddy, 1998; Wollowski *et al.*, 2001; Ohashi *et al.*, 2002; Perdigon *et al.*, 2002; Rafter, 2003).

Even though these studies indicate a positive effect of probiotics, further investigations and documentation is needed.

3.2.1 Diarrhoea

The ability of probiotics to decrease the incidence or duration of certain diarrhoeal illnesses is probably the most substantiated of the probiotic health effects. Investigations suggest that treatment with probiotics has most effect towards diarrhoea caused by rotavirus, followed by bacterial and parasitic diarrhoea (Shornikova *et al.*, 1997c; Guandalini *et al.*, 2000; Heyman, 2000; Heyman and Ménard, 2002). Studies on diarrhoea, observing a significant reduction in stool frequency and stool consistency after probiotic therapy have been conducted (Isolauri *et al.*, 1991; Raza *et al.*, 1995; Pant *et al.*, 1996; Guarino *et al.*, 1997; Shornikova *et al.*, 1997a,b,c; Pedone *et al.*, 1999; Guandalini *et al.*, 2000; Pedone *et al.*, 2000; Simakachorn *et al.*, 2000; Boudraa *et al.*, 2001; Rosenfeldt *et al.*, 2002a,b).

Lactic acid bacteria are capable of producing antimicrobial substances which inhibit certain microorganisms; among others many of the diarrhoea causing pathogens.

The activity of these antimicrobial substances was described in Chapter 2 of the present Thesis. It is believed that by producing these substances LAB contribute to the maintenance of the existing intestinal microflora and the inhibition of pathogenic microorganism and thereby reduce or prevent diarrhoea (e.g. Rolfe, 2000; Holzapfel and Schillinger, 2002; Guslandi, 2003). It is likely that factors other than antimicrobial substances are involved in the mechanisms behind the reducing or preventing effect of probiotics on diarrhoea; however they are difficult to prove *in vivo* and therefore are still largely unknown. Based on mainly *in vitro* studies it is generally recognised that the anti-infective properties described for lactobacilli are due to their ability to [1)]adhere to surfaces and inhibit the adhesion of pathogens, [2)]inhibit the growth of pathogens, [3)]deplete nutrients and [4)]modulate the host immune response and microenvironment, such that risk of infection is reduced, as reviewed by Ouwehand *et al*. (1999a), Heyman (2000), Rolfe (2000), Isolauri (2001); Dunne and Shanahan (2002); Gionchetti *et al*. (2002); Heyman and Ménard (2002), Holzapfel and Schillinger (2002), Ried and Burton (2002), Penner *et al*. (2005) and Santosa *et al*. (2006).

In the human intervention study by Lei *et al*. (2006 – Appendix II) a fermented millet product (KSW) was tested as a probiotic product. However, initially the antimicrobial activity of 215 isolates of LAB from the product was tested *in vitro* (Lei and Jakobsen, 2004 - Appendix I) against two Gram-positive bacteria (bacteriocin-sensitive *Lacto-bacillus sakei* and *Listeria innocua*). The details were described in Section 2.6, but overall the results indicated, that the antimicrobial activity found, was not caused by bacteriocins and was most likely due to competition for nutrients (Lei and Jakobsen, 2004 - Appendix I). Olsen *et al*. (1995) studying the antimicrobial potential of a fermented cereal specifically pointed out, that the inhibitory potential of the fermented products seemed to depend on the mixed population of the LAB with different types of individually less pronounced antimicrobial factors. They explained that the combined antimicrobial effect was due to the combined effects of acids, compounds sensitive to proteolytic enzymes and other compounds with antimicrobial activity. They further established that the pattern of antimicrobial factors was not species-specific. Diarrhoeal diseases and probiotics are further reviewed in Chapter 4 of the present thesis.

The purpose of antibiotics is to kill harmful bacteria. Unfortunately, antibiotics are often active in the GIT towards a broad spectrum of bacteria, harmful as well as non-harmful, resulting in disruption of the bacterial flora, leading to diarrhoea and other intestinal disturbances. Treatment with probiotics during and after antibiotic therapy has been shown to minimise disruptive effects of antibiotic use (Arvola *et al.*, 1999; Vanderhoof *et al.*, 1999; Thomas *et al.*, 2001; Armmuzzi *et al.*, 2001; Johnston *et al.*, 2006). However, not all studies have shown positive results in the prevention of antibiotic associated diarrhoea, or other symptoms associated with antibiotic therapy (Cremonini *et al.*, 2002b).

Probiotics do not only alleviate disease symptoms, but have also shown positive results in healthy individuals. Prevention of diarrhoea as well as antibiotic-associated diarrhoea has been widely investigated. Even though the effect is less significant than that seen for direct treatment of acute diarrhoea, studies show that probiotics are capable of reducing the risk of diarrhoea (Oksanen *et al.*, 1990; Saavedra *et al.*, 1994; Oberhelman *et al.*, 1999; Vanderhoof *et al.*, 1999; Szajewska *et al.*, 2001). Lei *et al.* (2006 - Appendix II) found a tendency towards diarrhoeal prevention when children ingested a fermented millet product (KSW) containing high numbers of viable LAB.

3.2.3 Stimulation of the immune system

The gut is the major immune organ in humans and it provides the primary defence against microbial pathogens. The gut immune system can be divided into three major compartments: organised gut-associated lymphoid tissue (GALT), the mucosal lamina propria and the epithelium. The immune system is extremely complex, involving both cell-based and antibody-based responses to potential infectious agents. Immunodeficiency can result from certain diseases (e.g. cancer, AIDS and leukaemia) or, to a lesser extent, from more normal conditions such as old age, pregnancy or stress. In addition, autoimmune diseases (e.g. allergies, rheumatoid arthritis and inflammatory bowel diseases) can also occur due to malfunction of the immune system (Vaarala, 2003).

The ability of probiotics to stimulate the immune system is most likely also the underlying reason for many of the probiotic effects described in this Section. The complexity of the stimulation of the immune system by probiotics is reviewed by e.g. Gill (1998), Matsuzaki and Chin (2000), Walker (2000), Isolauri *et al.* (2001), Vanderhoof and Young (2001) and Vaarala (2003).

In the following a number of key immune-related diseases will be reviewed in relation to probiotics:

Atopic disease (allergy based conditions) is a constantly increasing problem in the industrialised world. It is estimated that about 20% of the population in the industrialised world suffer from atopic diseases, which are also the most common chronic diseases of childhood. Genetic factors alone are unlikely to explain the emergence of atopic eczema, allergic rhinitis and asthma. To explain the rise in allergic conditions, scientists have proposed a hypothesis known as the “hygiene hypothesis”. This hypothesis is based on observations that lower allergy incidence is associated with environments that have greater numbers of microbes, such as day care centres, farms, or in homes with siblings or pets. It is believed that the increased sanitary living environments and the consumption of processed foods have limited the number of microbes in the diet, which has lead to an increase in atopic disease (Alm *et al.*, 1999; Kirjavainen *et al.*, 1999b; Isolauri *et al.*, 2001; Laiho *et al.*, 2002a,b; Boyle and Tang, 2006). Figure 3.1 depicts different factors suggested to influence atopic diseases.

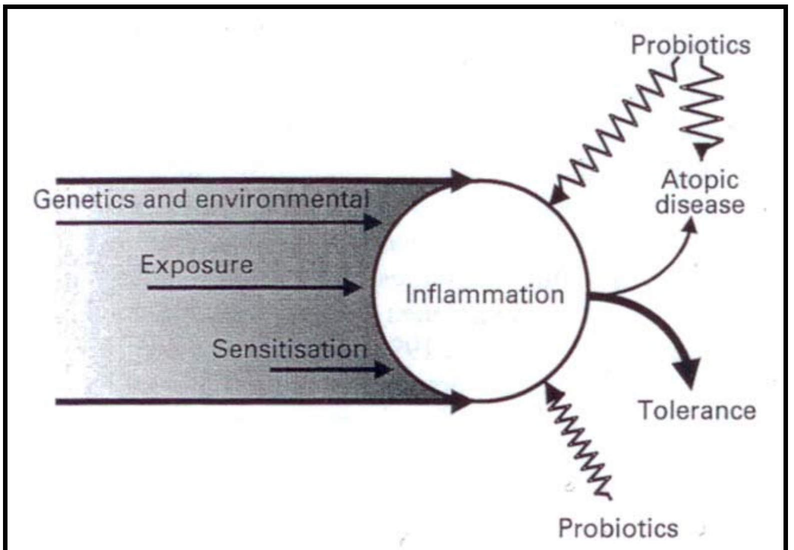

Figure 3.1 Schematic drawing of factors influencing atopic diseases. Genetic factors together with environmental exposures and sensitisation lead to allergic inflammation which may flavour the development of atopic disease. Probiotics may counteract early allergic inflammation as well as that in established atopic disease (Laiho *et al.*, 2002a).

Studies have been conducted supplying evidence that probiotics are capable of alleviating the symptom of atopic disease. A study by Kalliomaki *et al.* (2001 and 2003) supplied pregnant mothers, two-to-four weeks before delivery and newborn babies through six months of age with *Lactobacillus rhamnosus* GG and reported a 50% drop in incidence of recurring atopic eczema in the group receiving the probiotic supplement. A follow-up study of the same children showed same trend at 4 years of age. Other studies support these findings when investigating probiotics in relation to atopic diseases (Isolauri *et al.*, 2000; Pessi *et al.*, 2000; Rosenfeldt *et al.*, 2003; Viljanen *et al.*, 2005a,b). However, one study by Helin *et al.* (2002) showed no effect of probiotic treatment on birch-pollen allergy. The results of the above mentioned studies suggest that exposure to the right types of microbes early in life may decrease the risk of allergy.

In addition to the described capability to reduce atopic diseases, studies have also indicated that ingestion of probiotics can prevent development of allergies (Kalliomaki *et al.*, 2001, 2003; Szajewska *et al.*, 2004).

Irritable Bowel Syndrome (IBS) is a functional bowel disorder that can be characterised by symptoms of abdominal pain, cramps, gas, bloating, diarrhoea or constipation. It is estimated that 8-22% of the adult population suffer from the condition and that women are three times more often affected than men (Madden and Hunter, 2002). Only a small number of controlled studies have been conducted evaluating probiotics and IBS (Nobaek *et al.*, 2000) and prebiotics and IBS (Hunter *et al.*, 1999). The studies report on a relief from IBS symptoms, especially from diarrhoea or abdominal pain or bloating upon intake of probiotic LAB (Nobaek *et al.*, 2000; Madden and Hunter, 2002; Saarela *et al.*, 2002).

Inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease are serious intestinal diseases that can ultimately lead to the surgical removal of the colon. The causes of these diseases are not fully known, but it has been hypothesised that an intolerance to the normal bacteria flora in the gut leads to inflammation and resulting pathology. Furthermore, it has recently been shown that there is a reduced microbial diversity in inflammatory bowel disease (Manichanh *et al.*, 2006; Ott and Schreiber, 2006). It is believed that probiotic treatment is capable of maintaining the state of reduced inflammation that occurs during recovery from the diseases (Rutgeerts, 2002; Shanahan, 2002; Sullivan and Nord, 2002). Controlled clinical trials with probiotic therapy have shown alleviation of symptoms (Gupta *et al.*, 2000; Ulisse *et al.*, 2001; Guarner *et al.*, 2002; Kuisma *et al.*, 2003).

Even though it has been established that probiotics do possess certain beneficial effects with respect to human health, the mechanisms behind the effects have been difficult to establish. Most attempts have been carried out *in vitro* and the difficulties in extrapolating these results to an *in vivo* situation have been realised (Mattila-Sandholm *et al.*, 1999; Heyman, 2000; Holzapfel and Schillinger, 2002). It is believed that the effects of probiotics can involve one or several components of the immune response. However, the specific mechanisms of the observed changes are still poorly understood. Overall, the results of the *in vitro* and *in vivo* findings can be said to conclude that probiotics effect the immune mechanisms by effecting mucosal barrier mechanisms and help the functional maturation of the immune system (Erickson and Hubbard, 2000; Vaarala, 2003). In brief, this can be caused by tightening and

reducing permeability of epithelial cells (Klingberg and Budde, 2005), activating monocytes and macrophages, and hence influencing the cytokine profile and stimulation of IgA and IFN-γ secretion (reviewed by Vaarala, 2003).

3.3 *In vitro* and *in vivo* evaluation of probiotic efficacy of lactic acid bacteria

When investigating a potential probiotic strain there are certain criteria generally agreed upon that have to be met. However, to date, no standard as to specifically which criteria should be met has been published. However, it is expected that the outcome of the EU 5th Framework Programme Integrated Project 'PROSAFE' will be a set of recommendations for the assessment of probiotics used for human consumption (PROSAFE, 2002). The recommendations will be on taxonomy, antibiotic resistance, *in vitro* assessment of virulence and *in vivo* assessment of safety (PROSAFE, 2002). As described in Section 2.5.1, EFSA (2005) has in addition suggested the QPS concept be applied for any microorganism to be used in food or feed, in order to improve food safety. The QPS concept will be further described in Section 3.5.

A set of selection criteria considered to be relevant for any potential probiotic micro-organism has been proposed by Ouwehand *et al.* (1999a) and later up-dated by Tuomola *et al.* (2001) and Saarela *et al.* (2002). These include, apart from proven and documented beneficial effects, that the probiotic also possesses certain physiological, technological, sensory and safety requirements. The safety issues of probiotics will be discussed later in this Chapter, however technological and sensory requirements will not be further dealt with in this Thesis.

In order for a probiotic microorganism to exhibit beneficial effects, the following physiological factors are considered to be important:

Effective dose

The dose given in order to obtain an effect should be considered. Shornikova *et al.* (1997b) found a significant faster recovery from diarrhoea in children given 10^{10} cfu per day in a capsule compared to a capsule containing 10^{7} cfu. In addition Donnet-Huges *et al.* (1999) investigated modulation of non-specific mechanisms of the

immune defence (i.e. respiratory burst activity and phagocytic activity of peripheral blood leukocytes) in healthy volunteers and found a significant difference when subjects were given a product containing 10^7 cfu per ml probiotic compared to the same product containing 10^6 cfu per ml and stored for a period of 28 days before use. Saxelin *et al*. (1995) observed that for doses from 10^6 to 10^8 cfu per day, recovery of *Lactobacillus rhamnosous* GG from stool samples of healthy volunteers was not possible. However, when a daily dose of $>10^{10}$ cfu was given; recovery of viable cells from all subjects was made.

Considering African fermented products, Lei *et al*. (un-published results) investigated whether there was a difference between children having consumed a higher amount of KSW (total intake >600 ml) compared to children having received less KSW (total intake <600 ml) during the intervention study. One hundred ml KSW would correspond to a total of 10^{10} LAB. When comparing the two groups, few significant differences were found. The group receiving more than 600 ml KSW in total had a significantly more solid stool consistency on the fifth day of intervention compared to the group receiving less KSW ($P = 0.03$). However, the stool consistency of neither of the groups was defined as diarrhoea (WHO, 1990), indicating that the children of both groups had recovered. Further, when investigating the group of high and low intake of KSW it was found that significantly more children receiving <600 ml KSW, were associated with a better well-being compared to the children receiving more than 600 ml KSW ($P = 0.04$) (un-published results). This was in contrast to the overall results of the intervention study showing KSW associated with greater reported well-being 14 days after start of the intervention compared to not receiving KSW ($P = 0.02$) Lei *et al*. (2006 – Appendix II). These results indicate that it was not due to inadequate intake of KSW, that no overall effect on diarrhoea was seen.

Ability to survive the passage of the gastro-intestinal tract

An important requirement for a probiotic is tolerance for physiological levels of acid and bile in the GIT. In order for the probiotic to reach the intestine, where the probiotic actions are effectuated, it has to pass the first part of the human digestion system from mouth to stomach. This environment is very hostile and it is a prerequisite for the probiotic to be able tolerate low pH and bile salt in order for it to

reach the GIT in viable form (Fooks and Gibson, 2002). Even though it has been established that also non-viable cells have probiotic activity (Salminen *et al.*, 1999; Klingberg *et al.*, 2005), probiotic activity is considered higher using viable cells (Ouwehand *et al.*, 1999a; Salminen *et al.*, 1999).

Many studies have investigated the *in vitro* tolerance of LAB to physiological concentrations of acid and bile salts (Chateau *et al.*, 1994; Charteris *et al.*, 1998a; Garriga *et al.*, 1998; Jaya *et al.*, 1998; Kimoto *et al.*, 1999; Godward *et al.*, 2000; Zarate *et al.*, 2000; Bernardeau *et al.*, 2001; Haller *et al.*, 2001; Shinoda *et al.*, 2001; Gardiner *et al.*, 2002; Cebeci and Gurakan, 2003). Lei and Jakobsen (2004 - Appendix I) found that 70% of the 215 tested LAB isolates from spontaneously fermented millet were able to survive 4 hours incubation in pH 2.5 and 0.3% (v/v) oxgall bile, however unable to grow during incubation. Furthermore, no difference was found between the species tested. Other studies of LAB isolated from traditional fermented foods agree with these findings (Hayford, 1998; Haller *et al.*, 2001; Sanni *et al.*, 2002). The conclusion of these studies was that most LAB are capable of surviving the acid and bile concentration in the digestive system, however without growing. It should also be taken into consideration that probiotics most often are incorporated in a product and that the food matrix is likely to have a significant protective effect on the probiotic.

Adhesion and colonisation

Another selection requirement for a probiotic is the ability to attach to the intestinal mucosa (Ouwehand *et al.*, 1999b; Saarela *et al.*, 2002). The main reason for this is that adhesion is considered as a prerequisite for colonisation, which again prolongs the time the probiotic resides in the GIT and hence prolongs the time available to exhibit probiotic activity (Kirjanainen *et al.*, 1998). In addition, the ability to adhere is also, to some extent, likely to be connected to the ability to stimulate the immune defence (Ouwehand *et al.*, 1999b; Plant and Conway, 2002). However, permanent colonisation by the probiotics is unwanted and it remains unsolved whether colonisation is critical for probiotics to have their effect at all (Ouwehand *et al.*, 1999b; Fedorak and Madsen, 2004).

Adhesion to intestinal mucosa is difficult to investigate *in vivo* and hence several model systems have been developed. Tissue culture cells are commonly used for this purpose. Each individual cell line has advantages and disadvantages when representing the intestinal tract and this should be taken into consideration when planning a study. The human colon carcinoma cell lines Caco-2 and HT-29 are the most generally used cell lines (Blum *et al*., 1999). The HT-29MTX cell line is a mucus secreting cell line (Bernet *et al*., 1993). Mucus is considered an important site for colonisation, since it covers the enterocytes in the intestine (Fioramonti *et al*., 2003). An alternative to the tumorigenic cell lines is the normal porcine jejunal epithelial cell line IPEC-J2 (Berschneider, 1989).

Both *in vitro* and human intervention studies have investigated different adhesion abilities of different probiotic strains. *In vitro* studies: Coconnier *et al.* (1992), Greene and Klaenhammer (1994), Crociani *et al*. (1995), Hayford (1998), Tuomola and Salminen (1998), Jacobsen *et al*. (1999), Kimoto *et al*.(1999), Kirjavainen *et al*. (1999a), Shinoda *et al*. (2001), Todoriki *et al*. (2001) and Morita *et al*. (2002). Human intervention studies: Golding *et al*. (1992), Johansson *et al*. (1993), Saxelin *et al*. (1995), Alander *et al*. (1997, 1999), Jacobsen *et al*. (1999), Fujiwara *et al*. (2001), Goossens *et al*. (2003), Klingberg and Budde (2006) and Rochet *et al*. (2006). However, the term “colonisation” used here, for the intervention studies refers to an ability to recover the probiotic culture within a period of two to three weeks after cessation of ingestion. After such a wash-out period the probiotic culture can not be recovered from faeces samples, indicating the inability of the probiotic culture to permanently colonise the GIT.

It must also be noted that non-adhesive probiotics may still influence the adhesion of pathogens and the composition and adhesion of the intestinal microflora, e.g. through production of metabolites and coaggregation, and thereby influencing the health of the host (Ouwehand *et al*., 1999b).

3.4 Lactic acid bacteria marketed as probiotics

An example of specific LAB strains used for probiotic products are presented in Table 3.1. However, it must be stressed again, that extrapolation of results from one strain to even closely related strains should be avoided, since results indicate that probiotic effect is very strain dependant (Apostolou *et al*., 2001; Ouwehand *et al*., 2001; Gardiner *et al*., 2002; Saxelin *et al*., 2005).

Table 3.1 Specific strains of lactic acid bacteria tested and manufactured for their probiotic properties (modified from Saxelin *et al.*, 2005; Santosa *et al.*, 2006; www.usprobiotics.org).

Strain	Source
L. acidophilus NCFM®	Danisco (Madison, USA)
B. infantis 35264	Procter & Gamble (Mason, USA)
L. rhamnosus R0011 *L. acidophilus* R0052	Institut Rosell (Montreal, Canada)
L. acidophilus LA-1 *L. paracasei* CRL 431 *L. reuteri* RC-14™ *B. lactis* Bb-12	Chr. Hansen (Hørsholm, DK)
L. casei Shirota *B. breve* strain Yakult	Yakult (Tokyo, Japan)
L. casei DN114001 (“L. casei Defensis™”)	Danone (Paris, France)
B. animalis DN173 010 (“Bifidis regularis™”)	Dannon (Tarrytown, USA)
L. rhamnosus GR-1™	Urex Biotech (London, Ontario, Canada)
L. johnsonii Lj-1 (same as NCC533 and formerly *L. acidophilus* La-1)	Nestlé (Lausanne, Switzerland)
L. plantarum 299v *L. plantarum* 299 *L. rhamnosus* 271	Probi AB (Lund, Sweden)
L. reuteri SD2112	Biogaia (Stockholm, Sweden)
L. rhamnosus GG (“LGG”)	Valio Dairy (Helsinki, Finland)
L. rhamnosus LB21 *Lactococcus lactis* L1A	Essum AB (Umeå, Sweden)
L. salivarius UCC118	University College (Cork, Ireland)
B. longum BB536	Morinaga Milk Industry Co., Ltd. (Zama-City, Japan)
B. lactis HN019 (DR10)	Danisco (Madison, USA)
L. rhamnosus HN001 (DR20)	Fonterra (Wellington, New Zealand)
L. acidophilus LB	Lacteol Laboratory (Houdan, France)
L. paracasei F19	Medipharm (Des Moines, USA)
L. fermentum KLD	LaneLabs (Allendale, USA)
L. fermentum PCC®	Probiomics (Eveleigh, Australia)

When comparing the species presented in Table 3.1 to the LAB species predominant in traditional fermented products, *L. plantarum* and *L. fermentum* are found to overlap. An outline is given below of the outcome of the investigations carried out for specific strains of *L. plantarum* and *L. fermentum* used as probiotics.

Studies of probiotic effect of *L. plantarum* are many. Haller *et al*. (2001) investigated *in vitro* the probiotic potential of *L. plantarum* strains isolated from both fermented foods and the human GIT. They investigated the ability of the isolates to survive low pH and bile, the metabolic activity in the presence of bile salts and mucins, as well as their potential to adhere to enterocyte-like Caco-2 cells. The results showed no significant difference in probiotic potential of the *L. plantarum* strains isolated from the GIT compared to the strains from the fermented food origin. On the other hand, Cebeci and Gurakan (2003) studied fifteen strains of *L. plantarum* from different origin for their tolerance to acid and bile salts, ability to ferment fructooligo-saccharides, as well as their beta-galactosidase activity. The results of the strains varied, and of the fifteen strains, three strains were concluded as potential probiotics, indicating a great strain variance.

Especially the specific *L. plantarum* 299v strain has gained attention as a human probiotic though the multiple studies carried out with positive outcome. *In vitro* studies have found this strain capable of reducing the secretory response of intestinal epithelial cells to enteropathogenic *E. coli* infection (Michail and Abernathy, 2002), inhibit adherence of *E. coli* (Mack *et al*., 1999), as well as down regulate interleukin-8 secretion (McCracken *et al*., 2002). *Lactobacillus plantarum* 299v has *in vivo* been found to inhibit *E. coli*-induced intestinal permeability (Mangell *et al*., 2002),

In human studies, *L. plantarum* 299v has shown to reduce low-density lipoprotein (LDL)-cholesterol and fibrinogen in healthy volunteers (Bukowska *et al*., 1998; Naruszewicz *et al*., 2002) and reduce symptoms in patients with irritable bowel syndrome (Nobaek *et al*., 2000; Niedzielin *et al*., 2001). It has further been observed that *L. plantarum* 299v markedly decrease the rate of post-operative infection (Rayes *et al*., 2002a,b). Finally, it has been observed that the strain is unable to colonise the GIT more than one week after cessation of intake (Goossens *et al*., 2003).

Likewise for the strain *L. fermentum* KLD, *in vitro*, *in vivo* and human studies of its probiotic effect have been published. Charteris *et al*. (1998a) mimicked the human upper GIT and tested survival of 15 potential probiotics. Only *L. fermentum* KLD was considered intrinsically resistant. Another *in vitro* study showed the strain to have better adhesion properties to mice tissue compared to two other lactobacilli (*L. fermentum* and *L. casei*)(Plant and Conway, 2002). *Lactobacillus fermentum* KLD persisted in the GIT of mice longer than the two other lactobacilli (Plant and Conway, 2002). In addition, the strain was capable of affecting the indigenous microbiota of mice and inducing significant shifts in the indigenous microbiota of specific pathogen-free mice (Plant *et al*., 2003). In contrast, a human study showed no effect of *L. fermentum* KLD compared to a placebo with respect to, stool frequency, number of defecations per week and symptom scores in patients with long-standing bacterial overgrowth (Stolzer *et al*., 1996). In addition to this negative finding, Vesa *et al*. (2000) found a lower and shorter ileal survival of the *L. fermentum* KLD strain in six healthy subjects compared to a *L. plantarum* strain.

Lactobacillus fermentum was found to be predominant in fermented millet and sorghum (Lei and Jakobsen, 2004 - Appendix I; Sawadogo-Lingani *et al*., Appendix III). The preliminary *in vitro* investigations of probiotic potential (antimicrobial activity) of the *L. fermentum* isolates from fermented millet did, however, not show a more pronounced probiotic effect than the other LAB species found in the product, i.e. *Weissella confusa*, *L. salivarius* and *Pediococcus* spp. (Lei and Jakobsen, 2004 - Appendix I). In addition, the intervention study carried out (Lei *et al*., 2006 - Appendix II) using KSW as treatment for diarrhoea also showed no probiotic effect of the product. These results indicate that the species found in this spontaneously fermented millet product possess little probiotic potential short term. In addition it should also be pointed out that there is no commercially available probiotic from the genera *Weissella*, indicating a possible low probiotic potential of this species.

3.5 Safety of lactic acid bacteria as probiotics

As mentioned earlier (Section 2.5.1) EFSA (2005) has proposed the QPS concept (Qualified Presumption of Safety) which consists of recommendations for harmonisation of approaches for the safety assessment of microorganisms used in food and

feed production. Presumptions are being defined as "*a belief or assumption based on reasonable evidence*" and qualified to allow certain restrictions to apply. However, the QPS concept is not applicable to traditional, undefined microbial mixtures originating from spontaneous fermentations, or back-slopping processes, until the mixture becomes defined (EFSA, 2005). The KSW used in the intervention study of the present Thesis was spontaneously fermented, however the predominant LAB were identified and preliminary investigations of their probiotic potential were carried out. This Section describes the evaluation procedure for defined probiotic cultures.

Overall, the QPS concept should permit the identification of what is required to make an adequate safety assessment, in order to ensure a better use of assessment resources by focussing on those organisms which represent the greatest risk or level of uncertainty. For example, in the case of many of the live organisms currently used in the manufacture of, or added to, dairy products, this may simply be a requirement to demonstrate the absence of acquired antibiotic resistance factors (EFSA, 2005). Figure 3.2 shows the suggested decision tree for the QPS concept.

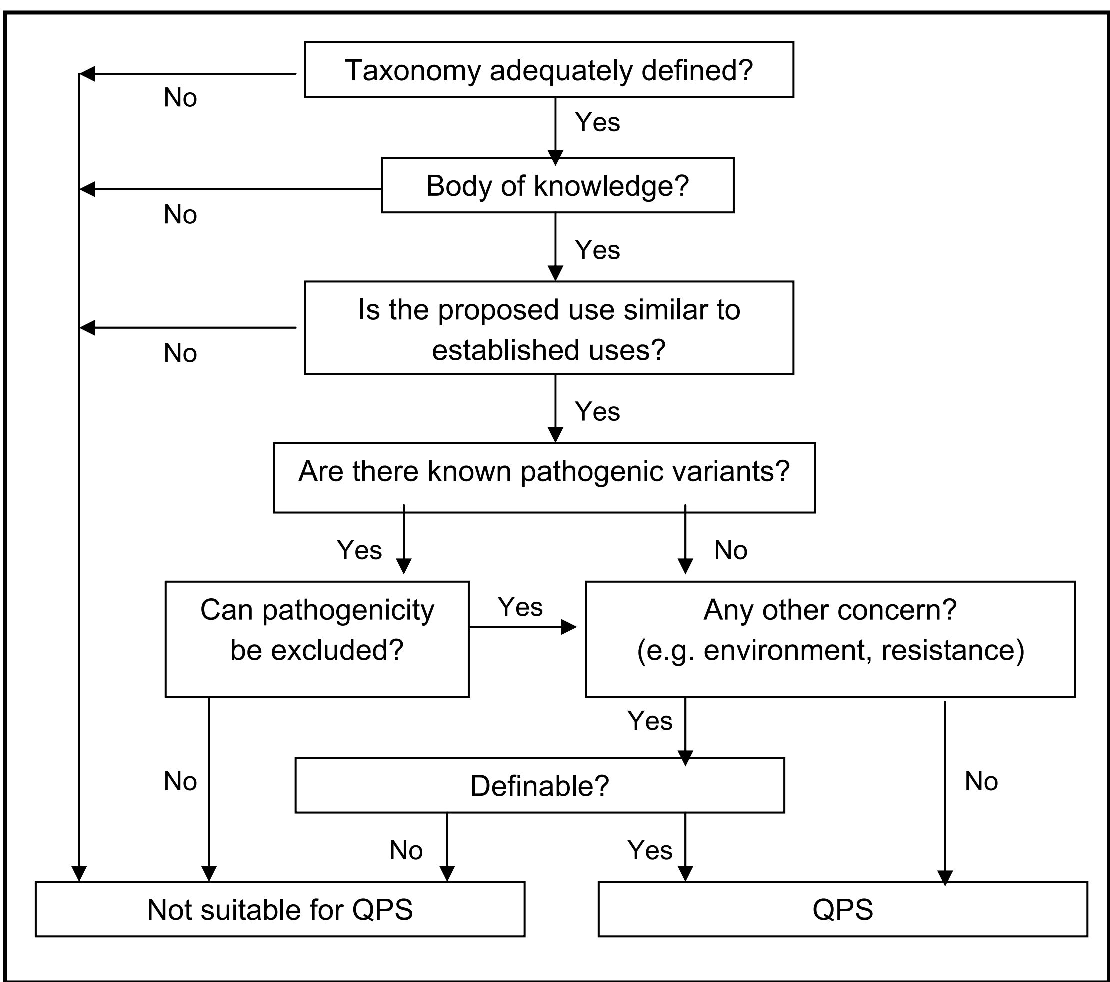

Figure 3.2 Suggested decision tree for Qualified Presumption of Safety (QPS) of microorganisms in food and feed (modified from EFSA (2005)).

If following the QPS concept when evaluating the safety of a probiotic lactic acid bacterium, the first point would be to define its taxonomy (as reviewed in Section 2.5.1) and second the "body of knowledge" (described below) (Figure 3.2). With regard to the level of identity required for a QPS risk assessment, EFSA (2005) propose that QPS should use the identity of an isolate set at the highest taxonomic unit that is appropriate for the purpose for which the evaluation is intended. This will depend upon the "body of knowledge" available for the microorganism to be assessed, and upon the nature of the microorganism being assessed. It is considered that for lactobacilli, assessment at the genus level could be appropriate (EFSA, 2005). This is due to the large "body of knowledge" for lactobacilli: Lactobacilli have a long history of use in fermented foods and dairy products without significant problems and are considered commensal microorganisms with no

pathogenic potential. Furthermore, the ubiquitous presence of lactobacilli in the human GIT adds to their safety (Aguirre and Collins, 1993; Donohue and Salminen, 1996; Collins *et al.*, 2000).

Components comprising to the “body of knowledge” (Figure 3.2) include peer-reviewed scientific literature, understanding of history of use of a microorganism, its industrial applications, its ecology, any clinical reports concerning the micro-organism and entries in public databases (EFSA, 2005).

An example of knowledge to be obtained is the potential ability of the probiotic, by metabolic activity, to produce harmful substances from either physiological secretions or from food components. Secondary bile acids are an example of harmful substances created from physiological secretion after bacterial deconjugation of bile salts in the GIT (Cheah, 1990). Toxic food components produced by certain bacteria either during production of fermented foods or in the intestines include ammonia, indole, phenols, ethyl carbamate and amines after degradation of proteins and deconjugation of amino acids (Westby *et al.*, 1997; Silla-Santos, 2001).

Biogenic amines, especially histamine and tyramine, are examples of such toxic compounds frequently produced by amino acid decarboxylase positive microorganisms during fermentation (Silla-Santos, 2001). The total amount of different amines formed depends strongly on the nature of the food and the microorganisms present (TenBrink *et al.*, 1990). The occurrence of biogenic amines in traditional food has been reported by Nout *et al.* (1994). However, Mathara (2004) observed that none of the tested LAB isolates from traditional fermented milk showed any biogenic amine production from the amino acid tested. In addition, Bover-Cid and Holzapfel (1999) did not find any biogenic amine producers among the few strains tested of *L. fermentum*, *L. salivarius*, *Leuconostoc mesenteroides*, *Weissella* spp. and pediococci from different origin. These species were the LAB found to be predominant in ‘koko’ and KSW (Lei and Jakobsen, 2004 – Appendix I). Even though the above mentioned toxic compounds are a possibility in fermented foods, they are unlikely to pose a general food safety risk due to the low concentrations involved. They should however be considered in the overall assessment (Westby *et al.*, 1997).

When the body of knowledge is obtained, the next step is to define the proposed use of the microorganism, and to establish whether this use is similar to how the micro-organism previously has been used (Figure 3.2). Implying a significant level of use, in terms of either the period for which the microorganisms has been used, or the number of individuals exposed to the microorganism, or a combination of both (EFSA, 2005).

Next, after establishing the intended use of the probiotic, is to evaluate whether there are known pathogenic variants (Figure 3.2; EFSA, 2005). Pathogenicity varies within LAB. As mentioned, lactobacilli are generally recognised as safe, whilst members of the genera *Streptococcus* and *Enterococcus* contain opportunistic pathogens (Moellering, 1992; Donohue and Salminen, 1996; Collins *et al*., 2000; Ishibashi and Yamazaki, 2001). In recent years LAB have occasionally been isolated in connection with clinical infections and a debate has been initiated, as to whether the bacteria are actually infective (Adams and Marteau, 1995; Donohue and Salminen, 1996; Husni *et al*., 1997). However, isolation of LAB from infections is likely to be the result of opportunistic infections. It is believed that for LAB to invade the host, by translocation or other routes, and cause bacteremia, both bacterial factors and host factors need to be involved. Host factors could be chronic diseases leading to a suppressed immune defence and hence an enhanced possibility of the bacteria to invade (Gasser, 1994; Ishibashi and Yamazaki, 2001).

As a part of investigating pathogenicity, the ability to adhere should also be considered. As mentioned in Section 3.3 adhesion is considered a prerequisite for colonisation. This is not only true for probiotics, but also for pathogens. The ability to adhere is often regarded as a virulence factor for pathogens. Therefore, the ability of probiotic microorganisms to adhere could be a potential risk factor (Ouwehand *et al*., 1999b). However, Kirjavainen *et al*. (1999a) found that high adhesion levels were not associated with clinical lactobacilli alone; indicating that adhesion to the intestinal mucosa is not a virulence factor for probiotic LAB. On the other hand, Apostolou *et al*. (2001) found that blood culture isolates of LAB adhered better to human intestinal mucus, than the fecal and dairy isolates tested. Although this indicated a trend for blood culture isolates to bind to intestinal mucus in higher numbers than strains of

dairy and human fecal origin, other factors are also likely to be involved in the etiology of bacteremia since some of the clinical *Lactobacillus* isolates exhibited a relatively low level of adhesion (Apostolou *et al*., 2001).

If it is determined, that there are known pathogenic variants of the assessed probiotic, the next step is to establish if pathogenicity of the specific probiotic can be excluded (Figure 3.2). This would, among other aspects, include the possibility of the probiotic, by gene transfer, to take in pathogenicity islands from other bacteria, which could lead to potentially hazardous situations, which again could lead to dissemination of virulence factors through fermented products. Transfer of pathogenicity islands have been shown for *Enterococcus faecium* to *Enterococcus feacalis* (Roberts *et al*., 2006).

If, however, there are no known pathogenic variants of the probiotic being assessed, any other concerns should be considered (Figure 3.2). The main concern to be reviewed is the resistance towards antibiotics and the ability to transfer this resistance to other bacteria.

3.5.1 Resistance to antibiotics

Antibiotics are one of the great discoveries against bacterial infections. Unfortunately bacteria can fight back by being resistant towards antibiotics. From a human perspective, resistance to antibiotics is an un-dersirable ability of microorganisms, including probiotics. A probiotic with antibiotic resistance that becomes an opportunistic pathogen could be highly detrimental to the infected host. Furthermore, a probiotic with antibiotic resistance and the ability to transfer the resistance to pathogenic bacteria could cause great harm to humans and animals (Teuber *et al*., 1999; Saarela *et al*., 2000). A probiotic should preferably not possess any antibiotic resistance and if it does, it should be unable to transfer the antibiotic resistance genes. These considerations are also valid for the LAB of the KSW used for the intervention study (Lei *et al*., 2006 – Appendix II).

Antibiotic resistance in bacteria may be intrinsic or acquired. Intrinsic resistance is a naturally occurring trait and may be considered as a species characteristic, whereas

acquired resistance derives either from genetic mutation or acquisition of foreign DNA from other bacteria (Teuber *et al*., 1999; Saarela *et al*., 2000). Recently the relationship between antibiotic use and resistance was reviewed by Singer *et al*. (2006). In this overview the authors argued that antibiotic resistance must be viewed as an ecological problem. It is commonly believed that resistance will decrease when the corresponding antibiotic is removed; however, Singer *et al*. (2006) challenged this, as well as claiming that the large background pool of resistance of bacteria where no antibiotics are used, probably results from the fact that many resistance genes protect the bacterial cell in such a way, that they are likely to be functional against other compounds in the environment. Hence in order to understand the complex problem of antibiotic resistance, there is a mammoth task in elucidating the diversity of selection pressures and routes of transmission that are influencing the evolution, dissemination and persistence of antibiotic resistance.

The QPS concept requires a determination of the nature of any antibiotic resistance determinant present in a candidate microorganism. Antibiotic resistance is not a safety issue *per se*, it only becomes a safety issue when horizontal transfer is concerned. Intrinsic resistance may arise because the candidate microorganism may lack the target for the antibiotic. This will raise less concern in a safety evaluation, than a microorganism that has acquired mobile DNA encoding for an enzyme that modifies or destroys an antibiotic. Furthermore, it is also important to recognise that antibiotics differ in their clinical and veterinary importance (EFSA, 2005).

Lactobacilli possess naturally a wide range of antibiotic resistance and in most cases antibiotic resistance is not of the transmissible type. Several species of *Lactobacillus* are intrinsically resistant to vancomycin, an antibiotic against Gram-positive bacteria (Charteris *et al*., 1998b; Klein *et al*., 2000; Charteris *et al*., 2001; Cebeci and Gurakan, 2003; Danielsen and Wind, 2003; Zhou *et al*., 2005; Ouoba *et al*., Appendix IV). Many intrinsically vancomycin resistant strains of lactobacilli have a long history of safe use as probiotics and there is no indication that vancomycin resistant lactobacilli could transfer the resistance to other bacteria (Salminen *et al*., 1998). Tynkkynen *et al*. (1998) have demonstrated that the vancomycin resistance factor of a probiotic strain (*L. rhamnosus* GG) is not closely related to those of enterococci,

and they could not observe the transfer of antibiotic resistance between the probiotic strain and enterococci. On the other hand, horizontal transfer of resistance factor from *L. reuteri* to *Enterococcus faecalis* of a gene involved in the resistance towards erythromycin (Ouoba *et al.*, Appendix IV), as well as from *L. plantarum* to *E. faecalis* of genes involved in the resistance towards erythromycin and tetracykline has been shown (Jacobsen *et al.*, 2007).

Olukoya *et al*. (1993) and Ouoba *et al*. (Appendix IV) investigated the resistance to antibiotics of LAB isolated from traditional fermented products. Of the antibiotics tested Olukoya *et al*. (1993) found the indigenous lactobacilli resistant to cloxacillin, penicillin and streptomycin. Ouoba *et al*. (Appendix IV) tested eight LAB from millet 'koko' and KSW, i.e. one *L. paraplantarum*, two *L. salivarius*, two *W. confusa* and three *L. fermentum* (isolated by Lei and Jakobsen, 2004 – Appendix I) towards 24 antibiotics. The isolates were found <u>resistant</u> to vancomycin, colistin, spectinomycin, ciprofloxacin, apramycin, trimethoprim, nalidixan, neomycin and sulphamethoxazole. Furthermore the isolates were found <u>sensitive</u> towards gentamycin, penicillin, chlor-amphenicol, florfenicol and cephalothin. Great variation between species as well as strains was found for the antibiotics kanamycin and tetracykline. Chloramphenicol was one of the antibiotics given to the children in the intervention study (Lei *et al.*, 2006 – Appendix II) and this antibiotic was also used in the study by Ouoba *et al*. (Appendix IV). The results showed that all eight 'koko' LAB tested, were sensitive towards chloramphenicol, indicating that the LAB from the KSW could have been affected by the prescense of the antibiotic. However, only very few (3%) of the children enrolled were treated with chloramphenicol (un-published results).

In addition, it was attempted to determine the resistance gene for the LAB that had shown a resistance towards any of the 24 antibiotics tested (Ouoba *et al.*, Appendix IV). This was done by PCR reactions using specific primers for well-known deter-minants for the individual antibiotics (Ouoba *et al.*, Appendix IV). None of the eight 'koko' isolates showed positive PCR amplicons for investigated resistance genes. This indicates that even though these isolates are resistant towards a number of antibiotics, they are not likely to transfer these resistance genes to other bacteria.

In the intervention study by Lei *et al.* (2006 – Appendix II) 72% of the 184 children enrolled were treated with antibiotics. The most common antibiotics given were metronidazol and cotrimoxazole, and to a lesser extent amoxicillin and chloramphenicol. It was investigated whether there was a difference in the group of children having received antibiotics compared to those who had not. This was carried out since it was speculated from the investigations by Lei *et al.* (2006) that one of the reasons for the lack of diarrhoea reducing effect from the KSW, was due to the high number of children treated with broad spectrum antibiotics, which could also have affected the potential probiotic LAB. It was also postulated that the greater reported well-being associated with KSW 14 days after the start of the intervention could be due to a preventing effect of KSW on antibiotic-associated diarrhoea. However, the only significant differences found when comparing the group of children having received antibiotics with the group having received none, was that the children receiving antibiotics had a significantly more solid stool consistency on Day 1, Day 2 and Day 5 ($P = 0.002$, $P = 0.001$, $P = 0.02$, respectively), indicating the positive effect of the antibiotics (Lei *et al.*, 2006 – Appendix II (unpublished results)).

The great variation in antibiotic resistance patterns of different species of lactobacilli, as well as individual strains, indicate the necessity for resistance testing of each potential probiotic strain, as also recognised by the QPS concept (Charteris *et al.*, 1998b; Klein *et al.*, 2000; Saarela *et al.*, 2000; Cebeci and Gurakan, 2003; Danielsen and Wind, 2003; EFSA, 2005; Ouoba *et al.*, Appendix IV).

An objective evaluation of the risk of the KSW given to the children in the intervention study (Lei *et al.*, 2006 – Appendix II) is that it can be regarded as safe. This is derived from the fact that; the ‘body of knowledge’ for LAB in general is large and that LAB in general are recognised as safe. None of the predominant LAB isolated from KSW have known pathogenic variants, and there is a long time safe use of ‘koko’ in Northern Ghana. Even though the LAB were shown to have a resistance towards certain antibiotics, the resistance seems to be inherent and not transferable.

4. Probiotic properties of indigenously fermented cereals on childhood diarrhoea

Chapter 2 gave and overview of traditional fermented cereal foods, including weaning foods and their benefits. Whilst Chapter 3 gave an introduction to the possible beneficial effects of LAB. This final Chapter will report on human *in vivo* studies to evaluate the potential of traditional fermented foods to act as a probiotic product. First, the effect of defined probiotic cultures of childhood diarrhoea will be discussed.

4.1 Probiotics and childhood diarrhoea

Diarrhoea is defined as three or more watery stools in 24 hours (WHO, 1990) and is estimated to be the second biggest cause of mortality in children below the age of five, only surpassed by pneumonia (UNICEF, 2006). As mentioned, 18% of the estimated 10.6 million yearly deaths in children below five are dying from diarrhoea (Bryce *et al.*, 2005). This amounts to approx. 2 million children yearly world-wide. Forty-two percent occur in the African region alone (Bryce *et al.*, 2005).

Diarrhoea due to poor hygienic condition has long been recognised as a major health hazard for infants and children in developing countries. It is estimated that up to 70% of the diarrhoea cases from developing countries are of foodborne origin (Mortarjemi *et al.*, 1993; Ribeiro, 2000). In addition, the level of malnutrition in a population is regarded as an indicator for diarrhoea-related mortality (Thapar and Sanderson, 2004).

Sazawal *et al.* (2006) recently made a meta-analysis of masked, randomised, placebo-controlled trials of probiotics and diarrhoea. They concluded from evaluation of the studies that defined probiotics significantly reduced antibiotic-associated diarrhoea by 52%, reduced the risk of traveller's diarrhoea by 8%, and that of acute diarrhoea of diverse causes by 34%. They further concluded that probiotics reduced the associated risk of acute diarrhoea among children by 57% and by 26% among adults. With reference to such findings the use of probiotics in association with children's diarrhoea seems obvious. Many more human studies have been carried out to evaluate the effect of probiotic LAB on childhood diarrhoea and Table 4.1 gives a comprehensive overview of these investigations.

Table 4.1 Overview of human intervention studies carried out to evaluate probiotic lactobacilli and their effect on childhood (0 – 5 years) diarrhoea.

Reference	Probiotic(s)	Diarrhoea type	Study design	Conclusion
Pearce *et al*. (1974)	*Strep. thermophilus* + *L. acidophilus* + *L. bulgaricus*	Acute	Placebo-controlled	No significant difference was found between placebo and treatment group.
Tankanow *et al*. (1990)	*L. acidophilus* + *L. bulgaricus*	Antibiotic associated	Masked, randomised, placebo-controlled	The *Lactobacillus* preparation did not appear to consistently prevent diarrhoea in the patient population.
Isolauri *et al*. (1991)	*L. casei* GG	Acute	Randomised, placebo-controlled	*L. casei* GG in the form of fermented milk or freeze-dried powder is effective in shortening the course of acute diarrhoea.
Boulloche *et al*. (1994)	*L. acidophilus* LB	Acute	Randomised, double-blind, placebo-controlled	Recovery from diarrhoea was significantly faster in the group receiving *L. acidophilus* LB.
Isolauri *et al*. (1994)	*L. casei* GG	Acute	Randomised, placebo-controlled	The duration of diarrhoea was significantly shortened in the group receiving *L. casei* GG.
Majamaa *et al*. (1995)	*L. casei* GG	Acute	Randomised, double-blind	*L. casei* GG significantly reduced the duration of diarrhoea.
Raza *et al*. (1995)	*L. casei* GG	Acute	Randomised, placebo-controlled, triple-blind	On day 2 the frequency of both vomiting and diarrhoea was less in the *L. casei* GG group. Of the children with non-bloody diarrhoea the percentage of children with persistent watery diarrhoea at 48 hours was significantly less in the *L. casei* GG group. No significant difference was observed by 48 hours in the children with bloody diarrhoea.

Table 4.1 *-continued*

Reference	**Probiotic(s)**	**Diarrhoea type**	**Study design**	**Conclusion**
Pant *et al*. (1996)	*L. casei* GG	Acute	Randomised, placebo-controlled, triple-blind	*L. casei* GG accelerates recovery from acute watery diarrhoea in young children in a tropical setting.
Guarino *et al*. (1997)	*L. casei* GG	Acute	Randomised, placebo-controlled	*L. casei* GG was found effective reducing duration of rotavirus-positive and rotavirus-negative diarrhoea in children. Further, it reduced the duration of rotavirus excretion.
Shornikova *et al*. (1997a)	*L. reuteri*	Acute	Randomised, double-blind, placebo-controlled	*L. reuteri* significantly reduced duration of diarrhoea compared to the placebo group.
Shornikova *et al*. (1997b)	*L. reuteri*	Acute	Randomised, placebo-controlled	*L. reuteri* effectively colonised the gastrointestinal tract after administration and significantly shortened the duration of watery diarrhoea associated with rotavirus. There was a correlation between the dosage of *L. reuteri* and the clinical effect.
Shornikova *et al*. (1997c)	*L. rhamnosous* GG	Acute	Randomised, double-blind	*L. rhamnosous* GG significantly shortened the duration of rotavirus diarrhoea, but not diarrhoea with confirmed bacterial aetiology.
Rautanen *et al*. (1998)	*L. casei* GG	Acute	Double-blind	Early administration of *L. casei* GG at the start of oral rehydration resulted in the shortest duration of diarrhoea, best weight gain, and fastest correction of acidosis.

Table 4.1 *-continued*

Reference	**Probiotic(s)**	**Diarrhoea type**	**Study design**	**Conclusion**
Arvola *et al*. (1999)	*L. rhamnosous* GG	Antibiotic associated	Masked, randomised, placebo-controlled	Administration of *L. rhamnosous* GG to children receiving antimicrobial therapy for respiratory infection reduced the incidence of antibiotic- associated diarrhoea to one third.
Oberhelman *et al*. (1999)	*L. rhamnosous* GG	General	Masked, randomised, placebo-controlled	Subjects in the *L. rhamnosous* GG group had significantly fewer episodes of diarrhoea.
Pedone *et al*. (1999)	*L. casei*	Acute	Randomised	The incidence of diarrhoea was not shown to be different between the groups; however, the severity of diarrhoea over the six-month study was significantly decreased with the supplementation of *L. casei* fermented milk compared to placebo.
Vanderhoof *et al*. (1999)	*L. rhamnosous* GG	Antibiotic associated	Masked, randomised, placebo-controlled	*L. rhamnosous* GG reduced the incidence of antibiotic-associated diarrhoea in children treated with oral antibiotics for common childhood infections.
Guandalini *et al*. (2000)	*L. rhamnosous* GG	General	Double-blind, placebo-controlled	Administering oral rehydration solution containing *L. rhamnosous* GG to children with acute diarrhoea resulted in shorter duration of diarrhoea, less chance of a protracted course, and faster discharge from the hospital.
Pedone *et al*. (2000)	*L. casei*	General	Randomised, double-blind	*L. casei* fermented milk significantly reduced the incidence of diarrhoea compared to standard yoghurt.

Table 4.1 *-continued*

Reference	**Probiotic(s)**	**Diarrhoea type**	**Study design**	**Conclusion**
Simakachorn *et al*. (2000)	*L. acidophilus* LB	Acute	Randomised, placebo-controlled	Addition of *L. acidophilus* LB to oral rehydration therapy was effective in the treatment of children with acute diarrhoea by decreasing the duration of diarrhoea.
Boudraa *et al*. (2001)	*L. bulgaricus* + *Strep. thermophilus*	Acute	Randomised, placebo-controlled	Duration of diarrhoea and number of stools were significantly less in group receiving *L. bulgaricus* and *Strep. thermophilus* compared with placebo.
Szajewska *et al*. (2001)	*L. rhamnosous* GG	Antibiotic associated	Masked, randomised, placebo-controlled	Prophylactic use of *L. rhamnosous* GG significantly reduced the risk of nosocomial diarrhoea in infants, parti-cularly nosocomial rotavirus gastroenteritis
Rosenfeldt *et al* (2002a)	*L. rhamnosus* + *L. reuteri*	Acute	Randomised, placebo-controlled	The two probiotics ameliorated acute diarrhoea in hospitalised children and reduced the period of rotavirus excretion. Oral bacteriotherapy was associated with a re-duced length of hospital stay. The beneficial effects were most prominent in children treated early in the diarrhoeal phase.
Rosenfeldt *et al* (2002b)	*L. rhamnosus* + *L. reuteri*	Acute	Randomised, placebo-controlled	In children from day-care centres with mild gastroenteritis, the two probiotics was effective in reducing the duration of diarrhoea.
Weizman *et al*. (2005)	*L. reuteri*	Infectious	Masked, randomised, placebo-controlled	Child care infants fed a formula supplemented with *L reu-teri* or *B lactis* had fewer and shorter episodes of diarr-hoea, with no effect on respiratory illnesses. These effects were more prominent with *L reuteri*, which was also the only supplement to improve additional morbidity parameters.

The results given in Table 4.1 strongly suggest that defined probiotic cultures are capable of reducing and/or preventing diarrhoea among children. However, very few of the studies with probiotics and their capability to reduce/prevent diarrhoea among children have been carried out in less developed countries. Oberhelman *et al*. (1999) in Peruvian children, Pant *et al*. (1996) in Thai children and Raza *et al*. (1995) in children from Pakistan. All found a beneficial effect of the probiotic cultures tested. Morbidity and mortality from diarrhoea are first of all a matter of grave concern in developing countries; hence there is a need for further studies to be carried out in these countries and in particular in Africa, where no studies of the magnitude presented in this Thesis have previously nor subsequently to date been carried out.

4.2 Traditional fermented cereal foods and diarrhoea

Since lactic fermented foods contain a large number of viable or non-viable LAB, the probiotic potential of these foods is considerable. African fermented food products are a largely unexploited area with many different products that are accepted by the population as well as being low cost. The ability of African lactic fermented foods to inhibit pathogens has been extensively reviewed in Chapter 2; however additional beneficial effects towards diarrhoea should also be considered, including use in oral rehydration therapy.

Oral Rehydration Therapy (ORT) with glucose-electrolyte solutions is used world-wide as a means of regaining the water/electrolyte balance during and after diarrhoeal diseases. The most widely used Oral Rehydration Solution (ORS) world-wide is the one recommended by WHO (Farthing, 1994). UNICEF (2006) reported from a survey that 40% of Ghanaian children (0-4 years) with diarrhoea received either ORT (ORS or recommended homemade fluids) or increased fluids and continued feeding.

Since commercial ORS is not always available, cereal-based ORS has been evaluated. Investigations by Kenya *et al*. (1989), Pelleboer *et al*. (1990) and Meyers *et al*. (1997) show that use of non-fermented cereal-based ORS is as effective as standard ORS. Moreover, with respect to cereal-based ORS being equivalent to standard ORS, studies have found that non-fermented cereal-based ORS is capable

of reducing diarrhoea symptoms compared to standard ORS (Molla *et al.*, 1989; Mustafa *et al.*, 1995; Goepp *et al.*, 1997; Gore *et al.*, 1997; Ho and Yip, 2001). Furthermore, Mustafa *et al.* (1995) found the diarrhoea reducing effects to be higher with sorghum-based ORS compared to rice-based ORS. Additional studies found also the cereal-based ORS to be more accepted by the children (Molla *et al.*, 1989; Pelleboer *et al.*, 1990), which was contradictory to the finding of Meyers *et al.* (1997). These findings suggest that intake of cereal-based liquid in connection with diarrhoea could have a diarrhoea reducing effect.

Yartey *et al.* (1995) investigated the effect of fermented cereal-based ORS compared to standard ORS. They found the fermented cereal-based ORS to be as effective as standard ORS; however, they did not find a diarrhoea reducing effect of the fermented cereal-based ORS, as found for the non-fermented cereal-based ORS described above. In the intervention study by Lei *et al.* (2006 - Appendix II) KSW was offered to the children of the intervention group in addition to standard ORS. However, the children receiving KSW drank significantly less ORS than the control group, and at the same time recovered from their diarrhoea as fast as the control group. This result is supported by the findings of Yartey *et al.* (1995), indicating KSW to be at least as effective as ORS.

As indicated, Lei *et al.* (2006 - Appendix II) found no effect of KSW with respect to stool frequency, stool consistency and duration of diarrhoea. It was suggested that the lack of effect could be, because most children (69%) had a mild form of diarrhoea, and many were treated with antibiotics (72%). Either this could have affected the LAB, or the LAB in KSW had no probiotic effects *per se*.

There is a lack of studies of the direct impact of traditional fermented foods on diarrhoea. Darling *et al.* (1995) carried out a controlled clinical trial. Seventy-five children aged 6-25 months admitted to hospital with acute diarrhoea were rehydrated and randomly allocated to three corn porridge dietary groups, i.e. conventional, amylase-digested, and fermented and amylase-digested. Similar to the study by Lei *et al.* (2006 - Appendix II) Darling *et al.* (1995) found no significant differences between the groups for duration of diarrhoea, frequency of stooling or vomiting.

Even though the lack of studies investigating the direct impact of fermented foods on diarrhoea is evident, there are studies indicating that fermented foods could possess probiotic benefits towards diarrhoea. Willumsen *et al*. (1997) found that amylase digested porridge was more effective than conventional porridge in the treatment of acute diarrhoea, with respect to repair of mucosal damage. Rani and Khetarpaul (1998) investigated a gruel of millet, chickpea, skim milk and tomato pulp fermented with a probiotic strain and unfermented, respectively. The fermented product significantly reduced diarrhoea in mice compared to the non-fermented product. Rahman *et al*. (1994), Mitra *et al*. (1995) and Rahman *et al*. (1997a,b) evaluated an energy-dense diet liquefied with amylase of germinated wheat flour and found that the diet increased calorie intake and absorption of macronutrients in malnourished children with diarrhoea, and that the diet did not produce any adverse effects. Finally, Kingamkono *et al*. (1999) investigated the presence of enteropathogens (*Campylobacter* spp., entero-haemorrhagic and enterotoxigenic *E. coli*, *Salmonella* spp. and *Shigella* spp.) in healthy children consuming a lactic acid-fermented cereal gruel ‘togwa’. They found that the prevalence of enteropathogens in the children was significant lower during the intervention and in the follow up, compared to the level prior to the study. They concluded, that regular consumption of ‘togwa‘ with a pH less than or equal to 4, once a day, three times a week, may help to control intestinal colonisation with potential diarrhoea-causing pathogens in young children.

In addition to the above mentioned human *in vivo* studies, and the indications of beneficial effects of fermented foods on childhood diarrhoea, it should be repeated that many *in vitro* studies have shown that fermentation of cereals significantly reduces the ability of pathogens to survive in the product (Mensah *et al*., 1988 and 1991; Odugbemi *et al*., 1991; Simango and Rukure, 1991 and 1992; Svanberg *et al*., 1992; Odugbemi *et al*., 1993; Olukoya *et al*., 1994; Simango, 1995; Annan-Prah and Agyeman, 1997; Olasupo *et al*., 1997b; Antony *et al*., 1998; Bakare *et al*., 1998; Kingamkono *et al*., 1998; Kunene *et al*., 1999; Muyanja *et al*., 2002; Thaoge *et al*., 2003; Tetteh *et al*., 2004).

Prevention of diarrhoea using traditional fermented foods

It could be speculated that traditional lactic fermented foods could have a preventive effect of diarrhoea, since defined probiotic cultures have been shown to prevent diarrhoea (Chapter 3 and Table 4.1). In 1994 Lorri and Svanberg investigated two villages within close range in Tanzania. One major difference between the two villages was that the children in one village regularly consumed fermented cereal gruels, whereas the children from the other did not. Over a nine month period it was found, that the children living in the village eating fermented gruels regularly, had a 40% lower frequency of diarrhoea compared to the village where fermented cereal gruels were not eaten regularly. Lei *et al.* (2006 - Appendix II) also found a tendency towards a preventive effect of KSW, in that a significantly larger amount of the children in the KSW group was regarded as being well coupled with a tendency towards less diarrhoea and less illness compared to the control group at the 14-day follow-up. This outcome was, however, based on the objectivity of the parents and since the study was not blinded, the positive result could be due to bias.

This tendency to a long term effect of KSW found by Lei *et al.* (2006 - Appendix II) could also be related to a reduction in antibiotic-associated diarrhoea, as more than 70% of the children were treated with antibiotics. As described in Section 3.2.1 probiotics have proven effective in reducing antibiotic-associated diarrhoea. This type of diarrhoea commences approx. 5-7 days after onset of antibiotic treatment (Turck *et al.*, 2003; Yapar *et al.*, 2005). It is possible that the positive effect seen at the 14-day follow-up could be caused by the LAB from the KSW preventing antibiotic-associated diarrhoea (Lei *et al.*, 2006 - Appendix II). However, as indicated above it can not be excluded that the effect seen could be a bias from the group receiving KSW.

It can be summarised that in the intervention study by Lei *et al.* (2006 - Appendix II) that no short term effect of KSW was found with respect to stool frequency, stool consistency and duration of diarrhoea. It was however indicated that there was a long term effect of KSW, possibly due to prevention of new incidences of acute diarrhoea or prevention of antibiotic-associated diarrhoea. More African human studies are needed to establish the possible probiotic potential of fermented foods. This could include use of other fermented products; e.g. milk or cassava, as well as the possibility of other fermenting microorganisms; e.g. *Bacillus* spp.

5. Discussion

The hypothesis of this Thesis was that traditional African fermented foods possess a probiotic potential, which would alleviate and prevent diarrhoea in African children. To test this hypothesis the objectives of the study were to:

- study the occurrence of lactic acid bacteria in a selected spontaneously fermented millet ('koko') from the Northern Region of Ghana and to isolate and identify the pre-dominant LAB.
- estimate the ability of predominant lactic acid bacteria isolates to survive the passage through the gastro-intestinal tract by *in vitro* studies.
- screen isolates for antimicrobial activity.
- Investigate the ability of the spontaneously fermented millet drink to alleviate diarrhoea in children in Northern Ghana by a human *in vivo* study.

Pheno- and genotyping methods were used for identification of the predominant LAB in spontaneously fermented millet and sorghum. By initial grouping of the LAB using ITS-PCR RFLP followed by identification using sequencing of the 16S rRNA gene, the dominating LAB was found to be *Lactobacillus fermentum* (Lei and Jakobsen, 2004 – Appendix I; Sawadogo-Lingani *et al.*, Appendix III). In fermented millet, *Weissella confusa* was also found in high numbers together with the less dominant *Lactobacillus salivarius* and *Pediococcus* spp. (Lei and Jakobsen, 2004 – Appendix I). For fermented sorghum, other less dominating LAB were *Lactobacillus delbrueckii* and *Pediococcus acidilactici* (Sawadogo-Lingani *et al.*, Appendix III). From the literature dealing with spontaneous lactic acid fermentations of cereals, the most commonly associated LAB seem to be *Lactobacillus plantarum* (Nout, 1991; Boraam *et al.*, 1993; Olasupo *et al.*, 1997a; Kunene *et al.*, 2000), followed by *L. fermentum* (Akinrele, 1970; Odunfa and Adeyele, 1985; Achi, 1990; Elfaki *et al.*, 1991; Oyeyiola, 1991; Hamad *et al.*, 1992; Halm *et al.*, 1993, Hounhouigan *et al.*, 1993a,b; Hounhouigan *et al.*, 1994; Johansson *et al.*, 1995; Olasupo *et al.*, 1997a; El Nour *et al.*, 1999; Mugula *et al.*, 2002). *Weissella confusa* is a relatively new genus (Collins *et al.*, 1993) and only few authors have reported finding *W. confusa* in fermented cereal products and normally in lower numbers than found in the present study (Ampe *et al.* 1999b; Corsetti *et al.* 2001; Mugula *et al.* 2002; Muyanja *et al.*, 2002). However, *W.*

confusa is known from other types of fermented products (Paludan-Müller *et al.*, 1999; Leisner *et al.*, 2001; Lee *et al.*, 2005). Earlier microbiological studies of spontaneously fermented millet products have reported findings of *L. salivarius*, *L. casei*, *L. acidophilus*, *L. jensenii, L. cellobiosus*, *L. plantarum*, *Pediococcus* spp. and *Leuconostoc* spp. (Oyeyiola 1991; Antony and Chandra 1997; Olasupo *et al.* 1997a). *Lactobacillus salivarius* and *Pediococcus* spp. was also found in the present study; however the most predominant LAB found in this study, i.e. *L. fermentum* and *W. confusa* was not isolated from any of the above mentioned studies of fermented millet.

From the results of REA-PFGE a large strain diversity of the LAB species was found in the spontaneously fermented millet and sorghum products (Lei and Jakobsen, 2004 – Appendix I; Sawadogo-Lingani *et al.*, Appendix III). Even in the later stages, i.e. after the fermentation, no succession of single strains of the LAB leading to a more uniform micropopulation was seen. The development of a uniform micro-population has been claimed in other studies (Nout, 1991; Halm *et al.*, 1993; Olsen *et al.*, 1995; Hamad *et al.*, 1997; Mugula *et al.*, 2002). However, the few studies based upon genotyping at strain level of spontaneous fermentation of cereal are in agreement with the findings of the present study; Hayford *et al.* (1999) investigated dominant isolates of *L. fermentum* from Ghanaian fermented maize dough, and van der Aa Kühle *et al.* (2001) investigated dominant *Saccharomyces cerevisiae* from Ghanaian sorghum beer. Both studies found, that in the later stages of production several strains were involved in the fermentation.

There was a great variation in the distribution of predominant LAB between the millet 'koko' and KSW production sites; however a consistency in predominant LAB from production to production was seen within the individual production sites, indicating that from a probiotic point of view these spontaneous fermentations are consistent (Lei and Jakobsen, 2004 – Appendix I). The great variation in the distribution of LAB between the 'koko' production sites is believed to be due to the so called "house-flora" variation, where dominating LAB are found in high numbers in the processing water, on the vessels for production and/or on the hands of the food producer (Oyewole, 1997; Holzapfel, 2002). Sawadogo-Lingani *et al.* (Appendix III) observed

that cleaning between each production batch reduced the number of predominant species towards a uniform microflora.

Predominant LAB from fermented millet were tested by *in vitro* studies for their ability to survive the passage through the gastro-intestinal tract. Of the 215 LAB isolates tested, 150 (70%) were found capable of surviving four hours at physiological levels of the acids and bile (Lei and Jakobsen, 2004 – Appendix I). Hence, there is good reason to believe that the isolates would be able to reach the intestines and remain viable. This is in agreement with other authors that also have reported LAB, such as *L. fermentum* isolated from African fermented cereal, capable of surviving physiological levels of acid and bile (Sanni *et al*. 2002; Jacobsen *et al*. 1999).

In addition to the ability to survive the passage through the gastro-intestinal tract, the isolates were also screened for their antimicrobial activity. Both their own ability to exhibit antimicrobial activity towards indicator organisms, as well as their susceptibility towards antimicrobials was investigated (Lei and Jakobsen, 2004 – Appendix I); Ouoba *et al*., Appendix IV). To test the ability of the isolates to exhibit antimicrobial activity, a modified growth medium with low content of glucose was used, in order to minimize production of hydrogen peroxide and organic acids (Lei and Jakobsen, 2004 – Appendix I). This was done to obtain an inhibition solely from the production of other antimicrobial compounds and possible competition for nutrients (Schillinger and Lücke 1989; Jacobsen *et al*. 1999). Only few isolates showed inhibition towards the bacteriocin sensitive *Lactobacillus sakei* used as indicator organism, displaying that the LAB isolates are not producing bacteriocins. However, most of the isolates showed low levels of antimicrobial activity towards *Listeria innocua*, used as a model of the human pathogen *L. monocytogenes*. Overall the isolates did not seem to exhibit a pronounced antimicrobial activity (Lei and Jakobsen, 2004 – Appendix I).

The resistance towards 24 antibiotics was tested for eight isolates from fermented millet (Ouoba *et al*., Appendix IV). The isolates were found resistant to vancomycin, colistin, spectinomycin, ciprofloxacin, apramycin, trimethoprim, nalidixan, neomycin and sulphamethoxazole. The inherent resistance found for certain antimicrobials

were supported by the findings of Olukoya *et al*. (1993). Furthermore the isolates were found sensitive towards gentamycin, penicillin, chloramphenicol, florfenicol and cephalothin. Great variation between species as well as strains was found for the antibiotics kanamycin and tetracykline. Four different antibiotics were used in the intervention study by Lei *et al*. (2006 – Appendix II), they were metronidazol and cotrimoxazole, and to a lesser extent amoxicillin and chloramphenicol. Chloramphenicol was also used in the study by Ouoba *et al*. (Appendix IV). The results showed that all eight 'koko' LAB tested, were sensitive towards chloramphenicol.

It was also attempted to determine the resistance gene for the LAB, that had shown a resistance towards one or more of the 24 antibiotics tested (Ouoba *et al*., Appendix IV). This was done by PCR reactions using specific primers for well-known determinants for the individual antibiotics (Ouoba *et al*., Appendix IV). None of the eight isolates from 'koko' showed positive PCR amplicons for investigated resistance genes. This indicates that even though these isolates are resistant towards a number of antibiotics, they are not likely to transfer resistance genes to other bacteria.

The effect of the spontaneously fermented millet product koko sour water (KSW) as a therapeutic agent among Ghanaian children with diarrhoea was assessed (Lei *et al*., 2006 – Appendix II). KSW with its very low pH of 3.6 and a content in the order of 10^8 live LAB per ml being capable of surviving the pH 2.5 and 0.3% bile for several hours could have a potential as probiotic product (Lei and Jakobsen, 2004 – Appendix I; Lei *et al*., 2006 – Appendix II). A daily intake of 100 ml would correspond to a total of 10^{10} LAB, which is in line with the dose considered effective for probiotic bacteria (Shornikova *et al*. 1997b; Donnet-Huges *et al*. 1999). In addition, the predominant LAB showed an antimicrobial activity towards one indicator organism (*Listeria innocua*), which seemed to go beyond the effect of low pH and acid production (Lei and Jakobsen, 2004 – Appendix I). The taxonomic diversity was pronounced, however the studied probiotic characteristics, i.e. antimicrobial activity and ability to survive physiological levels of bile and acid, were rather uniform among the predominant LAB. In addition KSW is expected to contain prebiotics in the form of water-soluble fibres and oligosaccharides, giving the product a possible status as a synbiotic product.

The overall outcome of the intervention study was that no effects of KSW were found on stool frequency, stool consistency and duration of diarrhoea during the five days after commencement of KSW treatment. However, a moderate but yet significant protective effect of KSW was seen at the 14-day follow-up. This protective effect of KSW was seen as an overall improved well-being, as well as tendencies towards less diarrhoea and other illnesses of the children having consumed KSW two weeks prior to these findings.

It must be noted that the study did not interfere with the medical treatment given to the patients. Nearly three quarters of the patients were treated with antibiotics, i.e. metronidazol and cotrimoxazole, and to a lesser extent amoxicillin and chloramphenicol. It is speculated that a possible reason for not finding a short term effect of KSW could be due to a high susceptibility of the LAB towards the antibiotics used to treat the children's diarrhoea (Lei *et al.*, 2006 – Appendix II; Ouoba *et al.*, IV).

It is speculated that the effect found of KSW at the follow-up interview could be either a protective effect against new incidences of acute diarrhoea in the children, a prevention of antibiotic-associated diarrhoea or a combination of both.

Furthermore, probiotics are known to have a better effect the earlier the treatment is commenced from onset of diarrhoea (Rautanen *et al.*, 1998; Rosenfeldt *et al.*, 2002a,b). That any effect on diarrhoea by KSW was not shown, might be due to the fact that the children from both groups came to the health clinic, on average two days after onset of diarrhoea and most were only mildly sick and therefore quickly cured. The intervention study was conducted during the rainy season in Ghana, where the incidences of diarrhoea are highest. It is speculated that malaria was the most common reason for the diarrhoea observed, in that more than 90% of the enrolled children were treated for malaria (Lei *et al.*, 2006 – Appendix II). The possible effects of probiotics on diarrhoea caused by malaria have yet to be investigated. However, it is evident from the present study that the microflora of KSW was unable to alleviate this type of diarrhoea in the short term.

The tendency towards a preventive or long term effect seen for KSW is supported by the findings of Lorri & Svanberg (1994). Other *in vitro* or *in vivo* studies of different design investigating childhood diarrhoea in developing countries have been carried out, and found a positive effect of either the defined probiotic culture or the fermented product (Armar-Klemesu *et al*., 1991; Raza *et al*., 1995; Kingamkono *et al*., 1999; Oberhelman *et al*., 1999). Furthermore, an overall improvement in health and thriving in poor and undernourished children receiving probiotics was shown by Saran *et al*. (2002).

Studies indicate that defined probiotic cultures are capable of reducing antibiotic-associated diarrhoea (Vanderhoof *et al*., 1999; Bergogne-Bérézin, 2000; Cremonini *et al*., 2002b; D'Souza *et al*., 2002; Hawrelak et., 2005). The long term effect seen for the KSW treatment could be due to a preventive effect of antibiotic-associated diarrhoea, as well as a general improvement of health and well-being of the children. However, since the study was not blinded it is possible that the long term effect of KSW seen could be due to bias from the group receiving the KSW (Lei *et al*., 2006 – Appendix II).

Any effect in reducing diarrhoea by use of spontaneously fermented cereal foods is yet to be proven. Darling *et al*. (1995) found no significant effect of a fermented maize-sorghum porridge compared to a non-fermented on duration of diarrhoea, frequency of stools or vomiting. Yartey *et al*. (1995) investigated both fermented and un-fermented maize gruel as a substitute for standard ORS and found no significant effect on stool output, stool frequency and duration of diarrhoea between the three combinations. There are, however, some indications that fermented foods could possess probiotic potential. Willumsen *et al*. (1997) showed that an amylase digested and fermented porridge was more effective than conventional porridge in the treatment of acute diarrhoea, with respect to repair of mucosal damage. Kingamkono *et al*. (1999) investigated a lactic acid fermented maize gruel compared to a non-fermented maize gruel on the prevalence of faecal enteric bacteria such as Campylobacter, enterohaemorrhagic *Escherichia coli* (EHEC:O157), enterotoxigenic *Escherichia coli* (ETEC), *Salmonella* and *Shigella* in faecal swabs of young children and found a significant lower prevalence of these bacteria in the group receiving the

fermented product. Furthermore, the *in vitro* ability of fermented foods to reduce the presence of pathogenic bacteria is widely reported (Mensah *et al*., 1988; Nout *et al*., 1989; Mensah *et al*., 1990; Mensah *et al*., 1991; Odugbemi *et al*., 1991; Svanberg *et al*., 1992; Kingamkono *et al*., 1998; Kimmons *et al*., 1999; Tetteh *et al*., 2004).

There is convincing evidence that certain probiotic strains are effective in preventing and treating acute diarrhoea as well as exhibiting a protective effect. The potential of being able to use a locally fermented product as a probiotic treatment is immense. Because of the improved keeping qualities and the increased nutritional value, it is likely that traditionally fermented foods have an important role in preventing acute diarrhoea. With the low cost and widespread availability in certain populations with a high prevalence of diarrhoea, the effects of traditional fermented foods need to be further investigated.

6. Conclusions and perspectives

The main conclusions drawn from this PhD study can be summarised as follows:

- The predominant lactic acid bacteria of spontaneously fermented millet and sorghum was *Lactobacillus fermentum*.
- Other LAB isolated in significant numbers from spontaneously fermented millet were *Weissella confusa*, *Lactobacillus salivarius* and *Pediococcus* spp.
- The isolates from fermented millet and sorghum had pronounced taxonomic biodiversity at strain level.
- There was a great variation in the distribution of predominant LAB between the millet 'koko' production sites; however a consistency in predominant LAB from production to production was seen within the individual production sites.
- 70% of all isolates were capable of surviving four hours in an *in vitro* set-up of physiological levels of acid and bile.
- All isolates from fermented millet showed low, however uniform, levels of antimicrobial activity towards the indicator strain *Listeria innocua*.
- Eight isolates from fermented millet were found to have resistance towards the antibiotics: vancomycin, colistin, spectinomycin, ciprofloxacin, apramycin, tri-methoprim, nalidixan, neomycin and sulphamethoxazole. However, none of the well-known resistance genes of the above mentioned antibiotics were identified, indicating an inherent resistance.
- Based on the Qualified Presumption of Safety (QPS) concept it was assessed that the fermented millet product does not constitute a food safety risk.
- When assessing the fermented millet drink 'koko sour water' (KSW) as a therapeutic agent among Ghanaian children with diarrhoea, it was found that KSW had no effect with respect to stool frequency, stool consistency and duration of diarrhoea.
- There was a tendency to a long term effect of KSW. The children receiving KSW were associated with greater well-being 14 days after the start of the intervention, indicating a preventive effect.

Fermented cereal foods have many advantages over non-fermented foods: improved food safety, longer shelf-life, improved nutrition, improved digestibility and last but not least probiotic potential. Studies with defined probiotic cultures and diarrhoea have been carried out mainly in industrialised countries however; morbidity and mortality from diarrhoea are first and foremost a matter of grave concern in developing countries. The potential for using a locally produced indigenous product as a probiotic treatment is considered to be immense. With the low cost and their wide-spread availability in certain populations with a high prevalence of acute diarrhoea, the effects of such traditional foods must be investigated further in the African setting. Specifically, studies should include investigation of different products with different microbiota as well as investigations of both the treatment and prevention of diarrhoea.

References

Aa Kuhle, A., Jesperen, L., Glover, R. L. K., Diawara, B., & Jakobsen, M. (2001) Identification and characterization of *Saccharomyces cerevisiae* strains isolated from West African sorghum beer. *Yeast,* **18**, 1069-1079.

Abdalla, A. A., El Tinay, A. H., Mohamed, B. E., & Abdalla, A. H. (1998) Proximate composition, starch, phytate and mineral contents of 10 pearl millet genotypes. *Food Chemistry,* **63**, 243-246.

Abee, T., Krockel, L., & Hill, C. (1995) Bacteriocins: Modes of action and potentials in food preservation and control of food poisoning. *International Journal of Food Microbiology,* **28**, 169-185.

Achi, O. K. (1990) Microbiology of Obiolor – a Nigerian fermented non-alcoholic beverage. *Journal of Applied Bacteriology,* **69**, 321-325.

Adams, M.R. (2001) Why fermented foods can be safe. In: Fermentation and food safety. Adams, M.R. and Nout, M.J.R. (eds.). Aspen Publishers, Inc., Gaithersburg, Maryland, USA.

Adams, M.R. & Marteau, P. (1995) On the safety of lactic-acid bacteria from food. *International Journal of Food Microbiology*, **27**, 263-264.

Adams, M. R. & Nicolaides, L. (1997) Review of the sensitivity of different foodborne pathogens to fermentation. *Food control,* **8**, 227-239.

Adegoke, G. O., Otumu, E. J., & Akanni, A. O. (1994) Influence of grain quality, heat, and processing time on the reduction of aflatoxin B1 levels in 'tuwo' and 'ogi': two cereal-based products. *Plant Foods for Human Nutrition,* **45**, 113-117.

Afifi, Z. E. M., Nasser, S. S., Shalaby, S., & Atlam, S. A. E. (1998) Contamination of weaning foods: Organisms, channels, and sequelae. *Journal of Tropical Pediatrics,* **44**, 335-337.

Agerbaek, M., Gerdes, L. U., & Richelsen, B. (1995) Hypocholesterolemic effect of a new fermented milk product in healthy middle-aged men. *European Journal of Clinical Nutrition,* **49**, 346-352.

Agerholm-Larsen, L., Bell, M. L., Grunwald, G. K., & Astrup, A. (2000) The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. *European Journal of Clinical Nutrition,* **54**, 856-860.

Aguirre, M. & Collins, M. D. (1993) Lactic acid bacteria and human clinical infection. *Journal of Applied Bacteriology,* **75**, 95-107.

Akinrele, I.A. (1970). Fermentation studies on maize during the preparation of traditional African starch-cake food. *Journal of the Scence of Food and Agriculture*, **21**, 619-625.

Alander, M., Korpela, R., Saxelin, M., VilpponenSalmela, T., Mattila-Sandholm, T., & von Wright, A. (1997) Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. *Letters in Applied Microbiology,* **24**, 361-364.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., & von Wright, A. (1999) Persistence of colonization of human colonic mucosa by a

probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. *Applied and Environmental Microbiology,* **65**, 351-354.

Alberts, J.F., Engelbrecht, Y., Steyn, P.S., Holzapfel, W.H. & Zyl, W.H. (2006) Biological degradation of aflatoxin B_1 by *Rhodococcus erythropolis* cultures. *International Journal of Food Microbiology,* **109**, 121-126.

Ali, M. A. M., El Tinay, A. H., & Abdalla, A. H. (2003) Effect of fermentation on the in vitro protein digestibility of pearl millet. *Food Chemistry,* **80**, 51-54.

Alm, J. S., Swartz, J., Lilja, G., Scheynius, A., & Pershagen, G. (1999) Atopy in children of families with an anthroposophic lifestyle. *Lancet,* **353**, 1485-1488.

Ampe, F., Omar, N. B., & Guyot, J. P. (1998) Recovery of total microbial RNA from lactic acid fermented foods with a high starch content. *Letters in Applied Microbiology,* **27**, 270-274.

Ampe, F., ben Omar, N., & Guyot, J. P. (1999a) Culture-independent quantification of physiologically-active microbial groups in fermented foods using rRNA-targeted oligo-nucleotide probes: Application to pozol, a Mexican lactic acid fermented maize dough. *Journal of Applied Microbiology,* **87**, 131-140.

Ampe, F., ben Omar, N., Moizan, C., Wacher, C., & Guyot, J. P. (1999b) Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Applied and Environmental Microbiology,* **65**, 5464-5473.

Andah, A. & Muller, H.G. (1973) Studies on koko, a Ghanaian fermented maize porridge. *Ghana Journal of Science*, **6**, 103-108.

Annan, N.T., Poll, L., Sefa-Dedeh, S., Plahar, W.A. & Jakobsen, M. (2003a) Influence of starter culture combinations of *Lactobacillus fermentum*, *Saccharomyces cerevisiae* and *Candida krusei* on aroma in Ghanaian maize dough fermentation. *European Food Recearch and Technology*, **216**, 377-384.

Annan, N.T., Poll, L., Sefa-Dedeh, S., Plahar, W.A. & Jakobsen, M. (2003b) Volatile compounds produced by *Lactobacillus fermentum*, *Saccharomyces cerevisiae* and *Candida krusei* in single starter culture fermentations of Ghanaian maize dough. *Journal of Applied Microbiology*, **94**, 462-474.

Annan-Prah, A. and Agyeman, J.A. (1997) Nutrient content and survival of selected pathogenic bacteria in kenkey used as a weaning food in Ghana. *Acta Tropica*, **65**, 33-42.

Antony, U. & Chandra, T. S. (1997) Microbial population and biochemical changes in fermenting finger millet (*Eleusine coracana*). *World Journal of Microbiology & Biotechnology,* **13**, 533-537.

Antony, U., Moses, L. G., & Chandra, T. S. (1998) Inhibition of *Salmonella typhimurium* and *Escherichia coli* by fermented flour of finger millet (*Eleusine coracana*). *World Journal of Microbiology & Biotechnology,* **14**, 883-886.

Antony, U. & Chandra, T. S. (1998) Antinutrient reduction and enhancement in protein, starch, and mineral availability in fermented flour of finger millet (*Eleusine coracana*). *Journal of Agricultural and Food Chemistry,* **46**, 2578-2582.

Apostolou, E., Kirjavainen, P. V., Saxelin, M., Rautelin, H., Valtonen, V., Salminen, S. J., & Ouwehand, A. C. (2001) Good adhesion properties of probiotics: a potential risk for bacteremia? *FEMS Immunology and Medical Microbiology,* **31**, 35-39.

Armar-Klemesu, M. A., Wheeler, E. F., BrakohiapaA, L. A., & Yamamoto, S. (1991) Infant-feeding and growth in rural Ghana – is the use of the traditional fermented porridge a case for early supplementation?. *Journal of Tropical Pediatrics,* **37**, 111-115.

Armuzzi, A., Cremonini, F., Bartolozzi, F., *et al.* (2001) The effect of oral administration of *Lactobacillus* GG on antibiotic-associated gastrointestinal side-effects during *Helicobacter pylori* eradication therapy. *Alimentary Pharmacoloy and Therapeutics*, **15**, 163-169.

Arvola, T., Laiho, K., Torkkeli, S., Mykkanen, H., Salminen, S., Maunula, L., & Isolauri, E. (1999) Prophylactic *Lactobacillus* GG reduces antibiotic-associated diarrhea in children with respiratory infections: A randomized study. *Pediatrics,* **104**, L1-L4.

Ashturkar, P. B., Pande, V., & Reddy, S. (1992) Development and evaluation of weaning food formulations. *Journal of Food Science and Technology,* **29**, 197-198.

Ashworth, A. & Feachem, R. G. (1985) Interventions for the control of diarrheal diseases among young-children-weaning education. *Bulletin of the World Health Organization,* **63**, 1115-1127.

Atta-Quayson, J. (1999) In: Atlas for Ghana. Atta-Quayson, J. (ed.). Unimax Publishers Ltd.

Axelsson, L. (2004) Lactic acid bacteria: classification and physiology. In: Salminen, S., Von Wright A, eds. Lactic acid bacteria: microbiology and functional aspects. 3rd ed. New York: Marcel Dekker Inc.

Bakare, S., Smith, S. I., Olukoya, D. K., & Akpan, E. (1998) Comparison of survival of diarrhoeagenic agents in two local weaning foods (ogi and koko). *Journal of Tropical Pediatrics,* **44**, 332-334.

Barrangou, R., Yoon, S. S., Breidt, F., Fleming, H. P., & Klaenhammer, T. R. (2002) Identification and characterization of *Leuconostoc fallax* strains isolated from an industrial sauerkraut fermentation. *Applied and Environmental Microbiology,* **68**, 2877-2884.

Barry, T., Colleran, G., Glennon, M., Dunican, L.K. & Gannon, F. (1991) The 16S/23S ribosomal intergenic spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Applied*, **1**, 51-56.

ben Omar, N. & Ampe, F. (2000) Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Applied and Environmental Microbiology,* **66**, 3664-3673.

ben Omar, N., Ampe, F., Raimbault, M., Guyot, J. P., & Tailliez, P. (2000) Molecular diversity of lactic acid bacteria from cassava sour starch (Colombia). *Systematic and Applied Microbiology,* **23**, 285-291.

Bengmark, S. (2000) Colonic food: Pre- and probiotics. *American Journal of Gastroenterology,* **95**, S5-S7.

Bengmark, S. (2003) Use of some pre-, pro- and synbiotics in critically ill patients. *Best Practice & Research in Clinical Gastroenterology,* **17**, 833-848.

Bernet, M.-F., Brassart, D., Neeser, J.-R. & Servin, A.L. (1993) Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interaction. *Applied and Environmental Microbiology*, **59**, 4121-4128.

Berschneider, M. (1989) Development of a normal cultured small intestinal epithelial cell line which transport Na and Cl. *Gastroenterology*, **96**, Supplement part 2, A41.

Bielecka, M., Biedrzycka, E., Majkowska, A., Juskiewicz, J., & Wroblewska, M. (2002) Effect of non-digestible oligosaccharides on gut microecosystem in rats. *Food Research International,* **35**, 139-144.

Blum, S., Reniero, R., Schiffrin, E. J., Crittenden, R., Mattila-Sandholm, T., Ouwehand, A. C., Salminen, S., von Wright, A., Saarela, M., Saxelin, M., Collins, K., & Morelli, L. (1999) Adhesion studies for probiotics: need for validation and refinement. *Trends in Food Science & Technology,* **10**, 405-410.

Bomba, A., Nemcova, R., Gancarcikova, S., Herich, R., Guba, P., & Mudronova, D. (2002) Improvement of the probiotic effect of micro-organisms by their combination with maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. *British Journal of Nutrition,* **88**, S95-S99.

Boraam, F., Faid, M., Larpent, J. P., & Breton, A. (1993) Lactic acid bacteria and yeast associated with traditional Moroccan sour-dough bread fermentation. *Sciences des Aliments,* **13**, 501-509.

Boudraa, G., Benbouabdellah, M., Hachelaf, W., Boisset, M., Desjeux, J. F., & Touhami, M. (2001) Effect of feeding yogurt versus milk in children with acute diarrhea and carbohydrate malabsorption. *Journal of Pediatric Gastroenterology and Nutrition,* **33**, 307-313.

Boulloche, J., Mouterde, O., & Mallet, E. (1994) Management of acute diarrhea in infants and toddlers – controlled study of the antidiarrheal efficacy of killed *Lactobacillus acidophilus* (LB strain) versus a placebo and a reference agent (loperamide). *Annales de Pediatrie,* **41**, 457-463.

Bover-Cid, S. & Holzapfel, W. H. (1999) Improved screening procedure for biogenic amine production by lactic acid bacteria. *International Journal of Food Microbiology,* **53**, 33-41.

Boyle, R. J. & Tang, M. L. K. (2006) The role of probiotics in the management of allergic disease. *Clinical and Experimental Allergy,* **36**, 568-576.

Brauman, A., Keleke, S., Malonga, M., Miambi, E., & Ampe, F. (1996) Microbiological and biochemical characterization of cassava retting, a traditional lactic acid fermentation for foo-foo (cassava flour) production. *Applied and Environmental Microbiology*, 62, 2854-2858.

Bryce, J., Boschi-Pinto, C., Shibuya, K., Black, R.E., & the WHO Child Health Epidemiology Reference Group (2005) WHO estimates of the causes of death in children. *The Lancet*, **365**, 1147-1157.

Bukowska, H., Pieczul-Mroz, J., Jastrzebska, M., Chelstowski, K. & Naruszewicz, M. (1998) Decrease in fibrinogen and LDL-cholesterol levels upon supplementation of diet with *Lactobacillus plantarum* in subjects with moderately elevated cholesterol. *Atherosclerosis*, **137**, 437-438.

Calderon, M., Loiseau, G., & Guyot, J. P. (2003) Fermentation by *Lactobacillus fermentum* Ogi E1 of different combinations of carbohydrates occurring naturally in cereals: consequences on growth energetics and alpha-amylase production. *International Journal of Food Microbiology,* **80**, 161-169.

Campbell-Platt, G. (1994) Fermented foods – a world perspective. *Food Research International,* **27**, 253-257.

Caplice, E. & Fitzgerald, G. F. (1999) Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology,* **50**, 131-149.

Cebeci, A. & Gurakan, C. (2003) Properties of potential probiotic *Lactobacillus plantarum* strains. *Food Microbiology,* **20**, 511-518.

Charalampopoulos, D., Wang, R., Pandiella, S. S., & Webb, C. (2002) Application of cereals and cereal components in functional foods: a review. *International Journal of Food Microbiology,* **79**, 131-141.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998a) Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology,* **84**, 759-768.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998b) Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. *Journal of Food Protection,* **61**, 1636-1643.

Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (2001) Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. *Journal of Food Protection,* **64**, 2007-2014.

Chateau, N., Deschamps, A. M., & Sassi, A. H. (1994) Heterogeneity of bile-salts resistance in the *Lactobacillus* isolates of a probiotic consortium. *Letters in Applied Microbiology,* **18**, 42-44.

Cheah, P.Y. (1990) Hypotheses for the etiology of colorectal cancer – an overview. *Nutrition and Cancer*, **14**, 5-13.

Christian, W.F.K. (1970) Lactic acid bacteria in fermenting maize dough. *Ghana Journal of Science*, **10**, 22-28.

Chung, O.K. & Pomeranz V. (1985) Amino acids in cereal proteins fractions. In: J.W. Finely and D.T. Hopkins (Eds.), Digestibility and amino acid availability in cereals and oil seeds. St. Paul, MN: American Associations of cereal Chemists.

Coconnier, M. H., Klaenhammer, T. R., Kerneis, S., Bernet, M. F., & Servin, A. L. (1992) Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell-lines in culture. *Applied and Environmental Microbiology,* **58**, 2034-2039.

Collins, M.D., Williams, A.M. & Wallbanks, S. (1990) The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S ribosomal-RNA sequence-analysis – Description of *Tetragenococcus* gen *nov. FEMS Microbiology Letters*, **70**, 255-262.

Collins, M.D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M. & Wallbanks, S. (1991) Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiology Letters*, **77**, 5-12.

Collins, M. D., Samelis, J., Metaxopoulos, J., & Wallbanks, S. (1993) Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages – description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species *Journal of Applied Bacteriology,* **75**, 595-603.

Collins, J. K., Thornton, G., Sullivan, G. O., Klaenhammer, T. R., Connolly, J. F., Fitzgerald, R. J., Stanton, C., & Ross, R. P. (2000) Selection of probiotic strains for human applications. *Special Issue: Functional foods: designer foods for the future, Cork, Irish Republic, 30 September 2 October 1997,* **8**, 487-490.

Corsetti, A., Lavermicocca, P., Morea, M., Baruzzi, F., Tosti, N., & Gobbetti, M. (2001) Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy. *International Journal of Food Microbiology,* **64**, 95-104.

Couto, M. M. B., Vogels, J. T. W. E., Hofstra, H., Husiintveld, J. H. J., & Van der Vossen, J. M. B. M. (1995) Random amplified polymorphic DNA and restriction enzyme analysis of PCR amplified rDNA in taxonomy – 2 identification techniques for foodborn yeasts. *Journal of Applied Bacteriology,* **79**, 525-535.

Cremonini, F., Di Caro, S., Covino, M., Armuzzi, A., Gabrielli, M., Santarelli, L., Nista, E. C., Cammarota, G., Gasbarrini, G., & Gasbarrini, A. (2002a) Effect of different probiotic preparations on anti-*Helicobacter pylori* therapy-related side effects: A parallel group, triple blind, placebo-controlled study. *American Journal of Gastroenterology,* **97**, 2744-2749.

Cremonini, F., Di Caro, S., Nista, E. C., Bartolozzi, F., Capelli, G., Gasbarrini, G., & Gasbarrini, A. (2002b) Meta-analysis: the effect of probiotic administration on antibiotic-associated diarrhoea. *Alimentary Pharmacology & Therapeutics,* **16**, 1461-1467.

Crociani, J., Grill, J. P., Huppert, M., & Ballongue, J. (1995) Adhesion of different *Bifidobacteria* strain to human enterocyte-like Caco-2 cells and comparison with *in vivo* study. *Letters in Applied Microbiology,* **21**, 146-148.

D'Souza, A. L., Rajkumar, C., Cooke, J., & Bulpitt, C. J. (2002) Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis. *British Medical Journal,* **324**, 1361-1364.

Danielsen, M. & Wind, A. (2003) Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology,* **82**, 1-11.

Darling, J. C., Kitundu, J. A., Kingamkono, R. R., Msengi, A. E., Mduma, B., Sullivan, K. R., & Tomkins, A. M. (1995) Improved energy intakes using amylase-digested weaning foods in Tanzanian children with acute diarrhea. *Journal of Pediatric Gastroenterology and Nutrition,* **21**, 73-81.

de Vries, M. C., Vaughan, E. E., Kleerebezem, M., & de Vos, W. M. (2006) *Lactobacillus plantarum* - survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal,* **16**, 1018-1028.

Delzenne, N. M. & Kok, N. (2001) Effects of fructans-type prebiotics on lipid metabolism. *American Journal of Clinical Nutrition,* **73**, 456S-458S.

Dirar, H. (1993) The indigenous fermented foods of the Sudan: a study in African food and nutrition. Cab International, Wallingford, UK

Donohue, D.C. & Salminen, S. (1996) Safety of probiotic bacteria. *Asia Pacific Journal of Clinical Nutrition*, **5**, 25-28.

Donnet-Hughes, A., Rochat, F., Serrant, P., Aeschlimann, J. M., & Schiffrin, E. J. (1999) Modulation of nonspecific mechanisms of defense by lactic acid bacteria: Effective dose. *Journal of Dairy Science,* **82**, 863-869.

Dunne, C. & Shanahan, F. (2002) Role of probiotics in the treatment of intestinal infections and inflammation. *Current Opinion in Gastroenterology,* **18**, 40-45.

EFSA (2005) QPS – Qualified Presumption of SAFETY of microorganisms in food and feed. European Food Safety Authority (EFSA) scientific colloquium summary report 2, Brussels, Belgium.

Egounlety, M., Aworh, O. C., Akingbala, J. O., Houben, J. H., & Nago, M. C. (2002) Nutritional and sensory evaluation of tempe-fortified maize-based weaning foods. *International Journal of Food Sciences and Nutrition,* **53**, 15-27.

Eklund, Y. (1984) The effect of carbon dioxide on bacterial growth and on uptake processes in the bacterial membrane vesicles. *International Journal of Food Microbiology*, **1**, 179-185.

El-Nezami, H., Salminen, S. & Mykkanen, H. (2001) Binding of *Aspergillus* and *Fusarium* toxins by probiotic bacteria. *Toxicology*, **164**, 175.

El Nour, M. E. M., El Tigani, S., & Dirar, H. A. (1999) A microbiological study of Hussuwa: a traditional Sudanese fermented food from germinated *Sorghum bicolor* c.v. *feterita*. *World Journal of Microbiology & Biotechnology,* **15**, 305-308.

Elfaki, A. E., Dirar, H. A., Collins, M. A., & Harper, D. B. (1991) Biochemical and microbiological investigations of sigda – a Sudanese fermented food derived from sesame oilseed cake. *Journal of the Science of Food and Agriculture,* **57**, 351-365.

Elyas, S. H. A., El Tinay, A. H., Yousif, N. E., & Elshelkh, E. A. E. (2002) Effect of natural fermentation on nutritive value and in vitro protein digestibility of pearl millet. *Food Chemistry,* **78**, 75-79.

Erickson, K. L. & Hubbard, N. E. (2000) Probiotic immunomodulation in health and disease. *Journal of Nutrition,* **130**, 403S-409S.

Escalante, A., Wacher, C., & Farres, A. (2001) Lactic acid bacterial diversity in the traditional Mexican fermented dough pozol as determined by 16S rDNA sequence analysis. *International Journal of Food Microbiology,* **64**, 21-31.

Ezeji, C. & Ojimelukwe, P. C. (1993) Effect of fermentation on the nutritional quality and functional properties of infant food formulation prepared from bambarra-groundnut, fluted-pumpkin and millet seeds. *Plant Foods for Human Nutrition,* **44**, 267-276.

FAO (1995) Sorghum and millets in human nutrition. FAO Foods and Nutrition Series, 27.

Farber, J. M. (1996) An introduction to the hows and whys of molecular typing. *Journal of Food Protection,* **59**, 1091-1101.

Farthing, M. J. G. (1994) Oral rehydration therapy. *Pharmacology & Therapeutics,* **64**, 477-492.

Fedorak, R.N. & Madsen, K.M. (2004) Probiotics and prebiotics in gastrointestinal disorders. *Current Opinion in Gastroenterology*, **20**, 146-155.

Felley, C.P., Corthesy-Theulaz, I., Rivero, J.L.B., Sipponen, P., Kaufmann, M., Bauerfeind, P., Wiesel., P.H., Brassart, D., Pfeifer, A., Blum, A.L. & Michetti, P. (2001) Favourable effect of an acidified milk (LC-1) on *Helicobacter pylori* gastritis in man. *European Journal of Gastroenterology and Hepatology*, **13**, 25-29.

Fields, M.L., Hamad, A.M. & Smith, D.K (1981) Natural lactic acid fermentation of corn meal. *Journal of Food Science*, **46**, 900-902.

Fioramonti, J., Theodorou, V., & Bueno, L. (2003) Probiotics: what are they? What are their effects on gut physiology? *Best Practice & Research in Clinical Gastroenterology,* **17**, 711-724.

Fooks, L. J. & Gibson, G. R. (2002) Probiotics as modulators of the gut flora. *British Journal of Nutrition,* **88**, S39-S49.

Fujiwara, S., Seto, Y., Kimura, A., & Hashiba, H. (2001) Establishment of orally-administered *Lactobacillus gasseri* SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. *Journal of Applied Microbiology,* **90**, 343-352.

Fuller, R. (1989) Probiotics in man and animals. *Journal of Applied Bacteriology*, **66**, 365-378.

Gadaga, T. H., Mutukumira, A. N., Narvhus, J. A., & Feresu, S. B. (1999) A review of traditional fermented foods and beverages of Zimbabwe. *International Journal of Food Microbiology,* **53**, 1-11.

Gardiner, G. E., Heinemann, C., Baroja, M. L., Bruce, A. W., Beuerman, D., Madrenas, J., & Reid, G. (2002) Oral administration of the probiotic combination *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 for human intestinal applications. *International Dairy Journal,* **12**, 191-196.

Garriga, M., Pascual, M., Monfort, J. M., & Hugas, M. (1998) Selection of lactobacilli for chicken probiotic adjuncts. *Journal of Applied Microbiology,* **84**, 125-132.

Gasser, F. (1994) Safety of lactic acid bacteria and their occurrence in human clinical infections. *Bulletin de l Institut Pasteur,* **92**, 45-67.

Gendrel, D., Dupont, C., Richardlenoble, D., Gendrel, C., & Chaussain, M. (1990) Feeding lactose intolerant children with a powdered fermented milk. *Journal of Pediatric Gastroenterology and Nutrition,* **10**, 44-46.

Gevers, D., Huys, G., & Swings, J. (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiology Letters,* **205**, 31-36.

Gibson, G.R. & Roberfroid, M.B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition*, **125**, 1401-1412.

Gibson, G.R., Probert, H.M., Van Loo, J., Rastall, R.A. & Roberfriod, M. (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews*, **17**, 259-275.

Gill, H. S. (1998) Stimulation of the immune system by lactic cultures. *International Dairy Journal,* **8**, 535-544.

Gionchetti, P., Rizzello, F., & Campieri, M. (2002) Probiotics in gastroenterology. *Current Opinion in Gastroenterology,* **18**, 235-239.

Godward, G., Sultana, K., Kailasapathy, K., Peiris, P., Arumugaswamy, R., & Reynolds, N. (2000) The importance of strain selection on the viability and survival of probiotic bacteria in dairy foods. *Milchwissenschaft-Milk Science International,* **55**, 441-445.

Goepp, J. G., Katz, S., Cuervo, E., Reid, R., Moran, J. R., & Santosham, M. (1997) Comparison of two regimens of feeding and oral electrolyte solutions in infants with diarrhea. *Journal of Pediatric Gastroenterology and Nutrition,* **24**, 374-379.

Golden, B.E. (1993) Primary protein-energy malnutrition. In: Human nutrition and dietetics. Garrow, J.S. and James, W.P.T. (eds.). Churchill Livingstone, Edinburgh, UK.

Goldin, B. R., Gorbach, S. L., Saxelin, M., Barakat, S., Gualtieri, L., & Salminen, S. (1992) Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. *Digestive Diseases and Sciences,* **37**, 121-128.

Goossens, D., Jonkers, D., Russel, M., Stobberingh, E., van den Bogaard, A., & Stockbrugger, R. (2003) The effect of *Lactobacillus plantarum* 299v on the bacterial composition and metabolic activity in faeces of healthy volunteers: a placebo-controlled study on the onset and duration of effects. *Alimentary Pharmacology & Therapeutics,* **18**, 495-505.

Gore, S. M., Fontaine, O., & Pierce, N. F. (1992) Impact of rice based oral rehydration solution on stool output and duration of diarrhea - Metaanalysis of 13 Clinical-Trials. *British Medical Journal,* **304**, 287-291.

Graham, G. G., Maclean, W. C., Morales, E., Hamaker, B. R., Kirleis, A. W., Mertz, E. T., & Axtell, J. D. (1986) Digestibility and utilization of protein and energy from nasha, a traditional Sudanese fermented sorghum weaning food. *Journal of Nutrition,* **116**, 978-984.

Greene, J. D. & Klaenhammer, T. R. (1994) Factors involved in adherence of lactobacilli to human Caco-2 cells. *Applied and Environmental Microbiology,* **60**, 4487-4494.

Guandalini, S., Pensabene, L., Abu Zikri, M., Dias, J. A., Casali, L. G., Hoekstra, H., Kolacek, S., Massar, K., Micetic-Turk, D., Papadopoulou, A., de Sousa, J. S., Sandhu, B., Szajewska, H., & Weizman, Z. (2000) *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhea: A multicenter European trial. *Journal of Pediatric Gastroenterology and Nutrition,* **30**, 54-60.

Guarino, A., Canani, R. B., Spagnuolo, M. I., Albano, F., & DiBenedetto, L. (1997) Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with mild diarrhea. *Journal of Pediatric Gastroenterology and Nutrition,* **25**, 516-519.

Guarner, F., Casellas, F., Borruel, N., Antolin, M., Videla, S., Vilaseca, J., & Malagelada, J. R. (2002) Role of microecology in chronic inflammatory bowel diseases. *European Journal of Clinical Nutrition,* **56**, S34-S38.

Gupta, P., Andrew, H., Kirschner, B. S., & Guandalini, S. (2000) Is *Lactobacillus* GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *Journal of Pediatric Gastroenterology and Nutrition,* **31**, 453-457.

Guslandi, M. (2003) Probiotics for chronic intestinal disorders. *American Journal of Gastroenterology,* **98**, 520-521.

Haller, D., Colbus, H., Ganzle, M. G., Scherenbacher, P., Bode, C., & Hammes, W. P. (2001) Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: A comparative in vitro study between bacteria of intestinal and fermented food origin. *Systematic and Applied Microbiology,* **24**, 218-226.

Halm, M., Lillie, A., Sorensen, A. K., & Jakobsen, M. (1993) Microbiological and aromatic characteristics of fermented maize doughs for kenkey production in Ghana. *International Journal of Food Microbiology,* **19**, 135-143.

Halm, M., Osei, Y. A., Hayford, A., Kpodo, K. A., & Amoa-Awua, W.-K. A. (1996) Experiences with the use of a starter culture in the fermentation of maize for 'kenkey' production in Ghana. *World Journal of Microbiology & Biotechnology,* **12**, 531-536.

Halsted, C.H. (1993) Water-soluble vitamins. In: Human nutrition and dietetics. Garrow, J.S. and James, W.P.T. (eds.). Churchill Livingstone, Edinburgh, UK

Hamad, S. H., Boecker, G., Vogel, R. F., & Hammes, W. P. (1992) Microbiological and chemical analysis of fermented sorghum dough for Kisra production. *Applied Microbiology and Biotechnology,* **37**, 728-731.

Hamad, S. H., Dieng, M. C., Ehrmann, M. A., & Vogel, R. F. (1997) Characterization of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. *Journal of Applied Microbiology,* **83**, 764-770.

Hammes, W.P. & Vogel, R.F. (1995) The genus *Pediococcus* with notes on the genera *Tetragenococcus* and *Aerococcus*. In: The genera of lactic acid bacteria. Wood, B.J.B. and Holzapfel, W.H. (eds.), Chapman and Hall, Suffolk, UK.

Haskard, C., Binnion, C., & Ahokas, J. (2000) Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. *Chemico-Biological Interactions,* **128**, 39-49.

Hawrelak, J. A., Whitten, D. L., & Myers, S. P. (2005) Is *Lactobacillus rhamnosus* GG effective in preventing the onset of antibiotic-associated diarrhoea: A systematic review. *Digestion,* **72**, 51-56.

Hayford, A.E. (1998) Characterization of the dominant microbiota of Ghanaian maize fermentation (kenkey). PhD Thesis.

Hayford, A. E., Petersen, A., Vogensen, F. K., & Jakobsen, M. (1999) Use of conserved randomly amplified polymorphic DNA (RAPD) fragments and RAPD pattern for characterization of *Lactobacillus fermentum* in Ghanaian fermented maize dough. *Applied and Environmental Microbiology,* **65**, 3213-3221.

Helin, T., Haahtela, S., & Haahtela, T. (2002) No effect of oral treatment with an intestinal bacterial strain, *Lactobacillus rhamnosus* (ATCC 53103), on birch-pollen allergy: a placebo-controlled double-blind study. *Allergy,* **57**, 243-246.

Hesseltine, C. W. (1979) Some important fermented foods of mid-Asia, the Middle-East and Africa. *Journal of the American Oil Chemists Society,* **56**, 367-374.

Hesseltine, C. W. (1983) Microbiology of oriental fermented foods. *Annual Review of Microbiology,* **37**, 575-601.

Heyman, M. (2000) Effect of lactic acid bacteria on diarrheal diseases. *Journal of the American College of Nutrition,* **19**, 137S-146S.

Heyman, M. & Menard, S. (2002) Probiotic microorganisms: how they affect intestinal pathophysiology. *Cellular and Molecular Life Sciences,* **59**, 1151-1165.

Ho, T. F. & Yip, W. C. L. (2001) Oral rehydration solution - Rice water is cheap and effective. *British Medical Journal,* **323**, 1068.

Holzapfel, W. (1997) Use of starter cultures in fermentation on a household scale. *Food control,* **8**, 241-258.

Holzapfel, W. H. (2002) Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology,* **75**, 197-212.

Holzapfel, W. H. & Schillinger, U. (2002) Introduction to pre- and probiotics. *Food Research International,* **35**, 109-116.

Hounhouigan, D. J., Nout, M.-J. R., Nago, C. M., Houben, J. H., & Rombouts, F. M. (1993a) Characterization and frequency distribution of species of lactic acid bacteria involved in the processing of mawe', a fermented maize dough from Benin. *International Journal of Food Microbiology,* **18**, 279-287.

Hounhouigan, D. J., Nout, M. J. R., Nago, C. M., Houben, J. H., & Rombouts, F. M. (1993b) Composition and microbiological and physical attributes of mawe, a fermented maize dough from Benin. *International Journal of Food Science and Technology,* **28**, 513-517.

Hounhouigan, D. J., Nout, M.-J. R., Nago, C. M., Houben, J. H., & Rombouts, F. M. (1994) Microbiological changes in mawe during natural fermentation. *World Journal of Microbiology & Biotechnology,* **10**, 410-413.

Hounhouigan, D. J., Nout, M. J. R., Nago, C. M., Houben, J. H., & Rombouts, F. M. (1999) Use of starter cultures of lactobacilli and yeast in the fermentation of mawè, an African maize product. *Tropical Science*, **39**, 220-226.

Hove, H., Norgaard, H., & Mortensen, P. B. (1999) Lactic acid bacteria and the human gastrointestinal tract. *European Journal of Clinical Nutrition,* **53**, 339-350.

Hulse, J.H., Laing, E.M. & Pearson, O.E. (1980). Sorghum and the millets: Their composition and nutritive value. Academic Press, London.

Hunter, J. O., Tuffnell, Q., & Lee, A. J. (1999) Controlled trial of oligofructose in the management of irritable bowel syndrome. *Journal of Nutrition,* **129**, 1451S-1453S.

Husni, R.N., Gordon, S.M., Washington, J.A. & Longworth, D.L. (1997) *Lactobacillus* bacteremia and endocarditis: review of 45 cases. *Clinical Infectious Disease*, **25**, 1048-1055.

Infantes, M. & Tourneur, C. (1991) Survey on the lactic flora of natural sourdoughs located in various France areas. *Sciences des Aliments,* **11**, 527-545.

Ishibashi, N. & Yamazaki, S. (2001) Probiotics and safety. *American Journal of Clinical Nutrition,* **73**, 465S-470S.

Isolauri, E., Juntunen, M., Rautanen, T., Sillanaukee, P., & Koivula, T. (1991) A human *Lactobacillus* strain (*Lactobacillus casei* sp. Strain GG) promotes recovery from acute diarrhea in children. *Pediatrics,* **88**, 90-97.

Isolauri, E., KAILA, M., Mykkanen, H., LING, W. H., & Salminen, S. (1994) Oral bacteriotherapy for viral gastroenteritis. *Digestive Diseases and Sciences,* **39**, 2595-2600.

Isolauri, E., Arvola, T., Sutas, Y., Moilanen, E., & Salminen, S. (2000) Probiotics in the management of atopic eczema. *Clinical and Experimental Allergy,* **30**, 1604-1610.

Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, H., & Salminen, S. (2001) Probiotics: effects on immunity. *American Journal of Clinical Nutrition,* **73**, 444S-450S.

Iwuoha, C. I. & Eke, O. S. (1996) Nigerian indigenous fermented foods: Their traditional process operation, inherent problems, improvements and current status. *Food Research International,* **29**, 527-540.

Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Moller, P. L., Michaelsen, K. F., Paerregaard, A., Sandstrom, B., Tvede, M., & Jakobsen, M. (1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology,* **65**, 4949-4956.

Jacobsen, L., Wilcks, A., Hammer, K., Huys, G., Gevers, D. & Andersen, S.R (2007) Horizontal transfer of *tet*(M) and *erm*(B) resistance plasmids from food strains of *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in the gastrointestinal tract of gnobiotic rats. *FEMS Microbiology Ecology*, **59,** 158-166 (In press).

Jansen, G. R. (1992) Centrally processed weaning foods for use in developing countries. *Food Reviews International,* **8**, 307-345.

Jaya, P., Harsharanjit, G., Smart, J., Gopal, P. K., Prasad, J., & Gill, H. (1998) Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. *International Dairy Journal,* **8**, 993-1002.

Jespersen, L., Halm, M., Kpodo, K., & Jakobsen, M. (1994) Significance of yeast and moulds occurring in maize dough fermentation for 'kenkey' production. *International Journal of Food Microbiology,* **24**, 239-248.

Jespersen, L. (2003) Occurrence and taxonomic characteristics of strains *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. *FEMS Yeast Research*, **3**, 191-200.

Johansson, M. L., Molin, G., Jeppsson, B., Nobaek, S., Ahrne, S., & Bengmark, S. (1993) Administration of different *Lactobacillus* strains in fermented oatmeal soup: in vivo

colonization of human intestinal mucosa and effect on the indigenous flora. *Applied and Environmental Microbiology,* **59**, 15-20.

Johansson, M. L., Sanni, A., Lonner, C., & Molin, G. (1995) Phenotypically based taxonomy using API 50CH of lactobacilli from Nigerian Ogi, and the occurrence of stach fermenting strains. *International Journal of Food Microbiology,* **25**, 159-168.

Johnston, B. C., Supina, A. L., & Vohra, S. (2006) Probiotics for pediatric antibiotic-associated diarrhea: a meta-analysis of randomized placebo-controlled trials. *Canadian Medical Association Journal,* **175**, 377-383.

Joshi, N., Godbole, S. H., & Kanekar, P. (1989) Microbial and biochemical changes during dhokla fermentation with special reference to flavor compounds. *Journal of Food Science and Technology,* **26**, 113-115.

Kabir, A.M.A., Aiba, Y., Takagi, A., Kamiya, S., Miwa, T. & Koga, Y. (1997) Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. *Gut*, **41**, 49-55.

Kalliomaki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., & Isolauri, E. (2001) Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet,* **357**, 1076-1079.

Kalliomaki, M., Salminen, S., Poussa, T., ARVILOMMI, H., & Isolauri, E. (2003) Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. *Lancet,* **361**, 1869-1871.

Kandler, O. & Weiss, N. (1986) Genus *Lactobacillus*. In: P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt, (eds.), Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams and Wilkins, Baltimore, MD, pp. 1209-1234.

Kenya, P. R., Odongo, H. W., Oundo, G., Waswa, K., Muttunga, J., Molla, A. M., Nath, S. K., Molla, A., Greenough, W. B., Juma, R., & Were, B. N. (1989) Cereal based oral rehydration solutions. *Archives of Disease in Childhood,* **64**, 1032-1035.

Khetarpaul, N. & Chauhan, B. M. (1989) Effect of fermentation by pure cultures of yeasts and lactobacilli on phytic acid and polyphenol content of pearl millet. *Journal of Food Science,* **54**, 780-781.

Khetarpaul, N. & Chauhan, B. M. (1990) Fermentation of pearl millet flour with yeasts and lactobacilli – *in vitro* digestibility and utilization of fermented flour for weaning mixtures. *Plant Foods for Human Nutrition,* **40**, 167-173.

Khetarpaul, N. & Chauhan, B.M. (1991) Sequential fermentation of pearl millet by yeasts and lactobacilli – effect on the antinutrients and *in vitro* digestibility. *Plant food for Human Nutrition*, **41**, 321-327.

Kiessling, G., Schneider, J., & Jahreis, G. (2002) Long-term consumption of fermented dairy products over 6 months increases HDL cholesterol. *European Journal of Clinical Nutrition,* **56**, 843-849.

Kimmons, J. E., Brown, K. H., Lartey, A., Collison, E., Mensah, P. P. A., & Dewey, K. G. (1999) The effects of fermentation and or vacuum flask storage on the presence of coliforms in complementary foods prepared for Ghanaian children. *International Journal of Food Sciences and Nutrition,* **50**, 195-201.

Kimoto, H., Kurisaki, J., Tsuji, N. M., Ohmomo, S., & Okamoto, T. (1999) Lactococci as probiotic strains: adhesion to human enterocyte- like Caco-2 cells and tolerance to low pH and bile. *Letters in Applied Microbiology,* **29**, 313-316.

Kingamkono, R. R., Sjogren, E., & Svanberg, U. (1998) Inhibition of enterotoxin production by, and growth of enteropathogens in a lactic acid-fermenting cereal gruel. *World Journal of Microbiology & Biotechnology,* **14**, 661-667.

Kingamkono, R., Sjogren, E., & Svanberg, U. (1999) Enteropathogenic bacteria in faecal swabs of young children fed on lactic acid-fermented cereal gruels. *Epidemiology and Infection,* **122**, 23-32.

Kirjavainen, P. V., Ouwehand, A. C., Isolauri, E., & Salminen, S. J. (1998) The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters,* **167**, 185-189.

Kirjavainen, P. V., El Nezami, H. S., Salminen, S. J., Ahokas, J. T., & Wright, P. F. A. (1999a) The effect of orally administered viable probiotic and dairy lactobacilli on mouse lymphocyte proliferation. FEMS Immunology and Medical Microbiology, 26, 131-135.

Kirjavainen, P.V.; Apostolou, E.; Salminen, S.J.; Isolauri, E. (1999b) New aspects of probiotics - a novel approach in the management of food allergy. Allergy, 54, 909-915.

Klein, G., Hallmann, C., Casas, I. A., Abad, J., Louwers, J., & Reuter, G. (2000) Exclusion of vanA, vanB and vanC type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. *Journal of Applied Microbiology,* **89**, 815-824.

Klingberg, T.D. & Budde, B.B. (2005) Application of measurements of transepithelial electrical resistance of intestinal epithelial cell monolayers to evaluate probiotic activity. *Applied and Environmental Microbiology*, **71**, 7528-7530.

Klingberg, T.D., Pedersen, M.H., Cencic, A. & Budde, B.B. (2005) Application of measurements of transepithelial electrical resistance of intestinal epithelial cell monolayers to evaluate probiotic activity. *Applied and Environmental Microbiology*, **71**, 7528-7530.

Klingberg, T. D. & Budde, B. B. (2006) The survival and persistence in the human gastrointestinal tract of five potential probiotic lactobacilli consumed as freeze-dried cultures or as probiotic sausage. *International Journal of Food Microbiology,* **109**, 157-159.

Kpodo, K., Sorensen, A. K., & Jakobsen, M. (1996) The occurrence of mycotoxins in fermented maize products. *Food Chemistry,* **56**, 147-153.

Kuisma, J., Mentula, S., Jarvinen, H., Kahri, A., Saxelin, M., & Farkkila, M. (2003) Effect of *Lactobacillus rhamnosus* GG on ileal pouch inflammation and microbial flora. *Alimentary Pharmacology & Therapeutics,* **17**, 509-515.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C., & Kilk, A. (2002) Two antioxidative lactobacilli strains as promising probiotics. *International Journal of Food Microbiology,* **72**, 215-224.

Kunene, N. F., Hastings, J. W., & von Holy, A. (1999) Bacterial populations associated with a sorghum-based fermented weaning cereal. *International Journal of Food Microbiology,* **49**, 75-83.

Kunene, N. F., Geornaras, I., von Holy, A., & Hastings, J. W. (2000) Characterization and determination of origin of lactic acid bacteria from a sorghum-based fermented weaning food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting. *Applied and Environmental Microbiology,* **66**, 1084-1092.

Kwaku, E. A., Omwega, A. M., Muroki, N. M., & Mwadime, R. K. N. (1998) Evaluation of weaning diets in peri urban Kumasi, Ghana. *East African Medical Journal,* **75**, 142-147.

Laiho, K., Hoppu, U., Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002a) Probiotics: on-going research on atopic individuals. *British Journal of Nutrition,* **88**, S19-S27.

Laiho, K., Ouwehand, A., Salminen, S., & Isolauri, E. (2002b) Inventing probiotic functional foods for patients with allergic disease. *Annals of Allergy Asthma & Immunology,* **89**, 75-82.

Lartey, A., Manu, A., Brown, K. H., Peerson, J. M., & Dewey, K. G. (1999) A randomized, community-based trial of the effects of improved, centrally processed complementary foods on growth and micronutrient status of Ghanaian infants from 6 to 12 mo of age. *American Journal of Clinical Nutrition,* **70**, 391-404.

Lay, C., Rigottier-Gois, L., Holmstrom, K., Rajilic, M., Vaughan, E. E., de Vos, W. M., Collins, M. D., Thiel, R., Namsolleck, P., Blaut, M., & Dore, J. (2005) Colonic microbiota signatures across five northern European countries. *Applied and Environmental Microbiology,* **71**, 4153-4155.

Lee, C. H. (1997) Lactic acid fermented foods and their benefits in Asia. *Food control,* **8**, 259-269.

Lee, J. S., Heo, G. Y., Lee, J. W., Oh, Y. J., Park, J. A., Park, Y. H., Pyun, Y. R., & Ahn, J. S. (2005) Analysis of kimchi microflora using denaturing gradient gel electrophoresis. *International Journal of Food Microbiology,* **102**, 143-150.

Lei, V. & Jakobsen, M. (2004) Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *Journal of Applied Microbiology,* **96**, 384-397.

Lei, V., Friis, H., & Michaelsen, K. F. (2006) Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: An intervention study in Northern Ghana. *International Journal of Food Microbiology,* **110**, 246-253.

Leisner, J. J., Vancanneyt, M., Rusul, G., Pot, B., Lefebvre, K., Fresi, A., & Tee, L. K. (2001) Identification of lactic acid bacteria constituting the predominating microflora in an acid-fermented condiment (tempoyak) popular in Malaysia. *International Journal of Food Microbiology,* **63**, 149-157.

Lilley, D.M. & Stillwell, R.H. (1965) Probiotics: growth promotion factors produced by *microorganisms, Science, 147, 747-748.*

Lindgren, S.E. & Dobrogosz, W.J. (1990) Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews,* **87**, 149-163.

Lorca, G.L., Wadstrom, T., Valdez, G.F. & Ljungh, A. (2001) *Lactobacillus acidophilus* autolysins inhibit *Helicobacter pylori in vitro. Current Microbiology*, **42**, 39-44.

Lorri, W. & Svanberg, U. (1994) Lower prevalence of diarrhoea in young children fed lactic acide-fermented cereal gruels. *Food and Nutrition Bulletin*, **15**, 57-63

Macfarlane, S., Macfarlane, G. T., & Cummings, J. H. (2006) Review article: prebiotics in the gastrointestinal tract. *Alimentary Pharmacology & Therapeutics,* **24**, 701-714.

Mack, D. R., Michail, S., Wei, S., McDougall, L., & Hollingsworth, M. A. (1999) Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* **39**, G941-G950.

Madden, J. A. J. & Hunter, J. O. (2002) A review of the role of the gut microflora in irritable bowel syndrome and the effects of probiotics. *British Journal of Nutrition,* **88**, S67-S72.

Majamaa, H., Isolauri, E., Saxelin, M., & Vesikari, T. (1995) Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. *Journal of Pediatric Gastroenterology and Nutrition,* **20**, 333-338.

Malkki, Y. & Virtanen, E. (2001) Gastrointestinal effects of oat bran and oat gum - A review. *Lebensmittel-Wissenschaft Und Technologie-Food Science and Technology,* **34**, 337-347.

Malleshi, N.G. & Hadimani, N.A. (1993) Nutritional and technological characteristics of small millets and preparation of value added products from them. In: Riley, K.W., Gupta, S.C., Seetharam. D.E. & Mushonga, J.N. (eds). Advances in small millets. Oxford Publishing, New Delhi, pp. 270-287.

Mangell, P., Nejdfors, P., Wang, M., Ahrne, S., Westrom, B., Thorlacius, H., & Jeppsson, B. (2002) *Lactobacillus plantarum* 299v inhibits *Escherichia coli*-induced intestinal permeability. *Digestive Diseases and Sciences,* **47**, 511-516.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., Roca, J., & Dore, J. (2006) Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut,* **55**, 205-211.

Marshall, V. M. (1979) Screening hydrogen peroxide-producing lactic acid bacteria using a non-toxic chromogen. *Journal of Applied Bacteriology,* **47**, 327-328.

Masoud, W., Cesar, L. B., Jespersen, L., & Jakobsen, M. (2004) Yeast involved in fermentation of Coffea arabica in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. *Yeast,* **21**, 549-556.

Matsuzaki, T. & Chin, J. (2000) Modulating immune responses with probiotic bacteria. *Immunology and Cell Biology,* **78**, 67-73.

Mathara, J.M. (2004) Isolation, identification and functional characterization of potential probiotic lactic acid bacteria from traditional Maasai fermented milk in Kenya, PhD Thesis.

Mattila-Sandholm, T., Blum, S., Collins, J. K., Crittenden, R., de Vos, W., Dunne, C., Fonden, R., Grenov, G., Isolauri, E., Kiely, B., Marteau, P., Morelli, L., Ouwehand, A., Reniero, R., Saarela, M., Salminen, S., Saxelin, M., Schiffrin, E., Shanahan, F., Vaughan, E., & von Wright, A. (1999) Probiotics: towards demonstrating efficacy. *Trends in Food Science & Technology,* **10**, 393-399.

Mbugua, S.K. (1981). Microbiological and biochemical aspects of *uji* (an East African sour cereal porridge) fermentation and its enhancement through applications of lactic acid bacteria. PhD Thesis, Cornell University, USA.

McCracken, V.J., Chun, T., Baldeon, M.E., Ahrne, S., Molin, G., Mackie, R.L. *et al.* (2002) TNF-alpha sensitizes HT-29 colonic epithelial cells to intestinal lactobacilli. *Experimental Biology and Medicine*, **227**, 665-670.

McLaren, D.S., Loveridge, N., Duthie, G. & Bolton-Smith, C. (1993) Fat-soluble vitamins. In: Human nutrition and dietetics. Garrow, J.S. and James, W.P.T. (eds). Churchill Livingstone, Edinburgh, UK

Mensah, P. P. A., Tomkins, A. M., Drasar, B. S., & Harrison, T. J. (1988) Effect of fermentation of Ghanaian maize dough on the survival and proliferation of 4 strains of *Shigella flexneri*. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **82**, 635-636.

Mensah, P., Tomkins, A. M., Drasar, B. S., & Harrison, T. J. (1991) Antimicrobial effect of fermented Ghanaian maize dough. *Journal of Applied Bacteriology,* **70**, 203-210.

Meyers, A., Sampson, A., Saladino, R., Dixit, S., Adams, W., & Mondolfi, A. (1997) Safety and effectiveness of homemade and reconstituted packet cereal-based oral rehydration solutions: A randomized clinical trial. *Pediatrics,* **100**, 1-7.

Michaelsen, K. F. & Friis, H. (1998) Complementary feeding: A global perspective. *Nutrition,* **14**, 763-766.

Michail, S. & Abernathy, F. (2002) *Lactobacillus plantarum* reduces the in vitro secretory response of intestinal epithelial cells to enteropathogenic *Escherichia coli* infection. *Journal of Pediatric Gastroenterology and Nutrition,* **35**, 350-355.

Michetti, P., Dorta, G., Wiesel, P.H., Brassart, D., Verdu, E., Herranz, M., Felley, C., Porta, N., Rouvet, M., Blum, A.L. & nd Corthesy-Theulaz, I. (1999) Effect of whey-based culture supernatant of *Lactobacillus acidophilus (johnsonii)* La-1 on *Helicobacter pylori* infection in humans. *Digestion*, **60**, 203-209.

Metchnikoff, E. (1907) The Prolongation of Life. New York: GP, Putman's Sons.

Mitra, A. K., Rahman, M. M., Mahalanabis, D., Patra, F. C., & Wahed, M. A. (1995) Evaluation of an energy-dense meal liquefied with amylase of germinated wheat in children with acute watery diarrhea – a randomized controlled clinical trial. *Nutrition Research,* **15**, 939-951.

Moellering, R.C. (1992) Emergence of *Enterococcus* as a significant pathogen. *Clinical Infectious Disease*, **14**, 1173-1176.

Molla, A. M., Nath, S. K., Molla, A., & Khatun, M. (1989) Food-Based Oral Rehydration Salt Solution for Acute Childhood Diarrhea. *Lancet,* **2**, 429-431.

Moore, N., Chao, C. W., Yang, L. P., Storm, H., Oliva-Hemker, M., & SAAVEDRA, J. M. (2003) Effects of fructo-oligosaccharide-supplemented infant cereal: a double-blind, randomized trial. *British Journal of Nutrition,* **90**, 581-587.

Morelli, L., Vogensen, F.K. & Von Wright, A. (2004) Genetics of lactic acid bacteria. In: Salminen, S., Von Wright A, eds. Lactic acid bacteria: microbiology and functional aspects. 3rd ed. New York: Marcel Dekker Inc.

Morita, H., He, F., Fuse, T., Ouwehand, A. C., Hashimoto, H., Hosoda, M., Mizumachi, K., & Kurisaki, J. I. (2002) Adhesion of lactic acid bacteria to Caco-2 cells and their effect on cytokine secretion. *Microbiology and Immunology,* **46**, 293-297.

Moss, M.O. (2001) Chemical hazards and their control: Toxins. In: Fermentation and food safety. Adams, M.R. and Nout, M.J.R. (eds.). Aspen Publishers, Inc., Gaithersburg, Maryland, USA.

Motarjemi, Y., Kaferstein, F., Moy, G., & Quevedo, F. (1993) Contaminated weaning food – a major risk factor for diarrhea and associated malnutrition. *Bulletin of the World Health Organization,* **71**, 79-92.

Motarjemi, Y. & Nout, M. J. R. (1996) Food fermentation: a safety and nutritional assessment. *Bulletin of the World Health Organization,* **74**, 553-559.

Mugula, J. K. & Lyimo, M. (1999) Evaluation of the nutritional quality and acceptability of fingermillet-based tempe as potential weaning foods in Tanzania. *International Journal of Food Sciences and Nutrition,* **50**, 275-282.

Mugula, J. K. & Lyimo, M. (2000) Evaluation of the nutritional quality and acceptability of sorghum-based tempe as potential weaning foods in Tanzania. *International Journal of Food Sciences and Nutrition,* **51**, 269-277.

Mugula, J. K., Nnko, S. A. M., Narvhus, J. A., & Sorhaug, T. (2002) Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology,* **80**, 187-199.

Mugula, J. K., Narvhus, J. A., & Sorhaug, T. (2003) Use of starter cultures of lactic acid bacteria and yeasts in the preparation of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology,* **83**, 307-318.

Muller, H.G. (1970) Traditional cereal processing in Nigeria and Ghana. *Ghana Journal of Agriculture and Science*, **3**, 187-195.

Muller, M. R. A., Ehrmann, M. A., & Vogel, R. F. (2000) Multiplex PCR for the detection of *Lactobacillus pontis* and two related species in a sourdough fermentation. *Applied and Environmental Microbiology,* **66**, 2113-2116.

Murali, A. & Kapoor, R. (2003) Effect of natural and pure culture fermentation of finger millet on zinc availability as predicted from HCl extractability and molar ratios. *Journal of Food Science and Technology-Mysore,* **40**, 112-114.

Mustafa, S. A., Karrar, Z. E. A., & Suliman, J. I. (1995) Cereal-Based Oral Rehydration Solutions in Sudanese Children with Diarrhea - A Comparative Clinical-Trial of Rice-Based and Sorghum-Based Oral Rehydration Solutions. *Annals of Tropical Paediatrics,* **15**, 313-319.

Muyanja, C. M. B. K., Narvhus, J. A., Treimo, J., & Langsrud, T. (2002) Isolation, characterisation and identification of lactic acid bacteria from bushera: a Ugandan traditional fermented beverage. *International Journal of Food Microbiology,* **80**, 201-210.

Naidu, A.S., Bidlac, W.R. & Clemens, R.A. (1999) Probiotic spectra of lactic acid bacteria. *Critical Reviews in Food Science and Nutrition*, **38**, 13-126.

Naruszewicz, M., Johansson, M.L., Zapolska-Downar, D. & Bukowska, H. (2002) Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. *American Journal for Clinical Nutrition*, **76**, 1249-1255.

Niedzielin, K., Kordecki, H. & Birkenfeld, B. (2001) A controlled, double-blind, randomized study on the efficacy of *Lactobacillus plantarum* 299v in patients with irritable bowel syndrome. *European Journal of Gastroenterology and Hepatology*, **13**, 1143-1147.

Nielsen, D. S., Honholt, S., Tano-Debrah, K., & Jespersen, L. (2005) Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). *Yeast,* **22**, 271-284.

Nigatu, A. (2000) Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate *Lactobacillus plantarum*, *Lact. fermentum*, *Lact. rhamnosus*, *Lact. sake*, *Lact. parabuchneri*, *Lact. gallinarum*, *Lact casei*, *Weissella minor* and related taxa isolated from kocho and tef. *Journal of Applied Microbiology,* **89**, 969-978.

Nkama, I., Abbo, E. S., & Igene, J. O. (1994) Traditional production and chemical composition of Ndaleyi, a Nigerian fermented pearl millet food. *Plant Foods for Human Nutrition,* **46**, 109-116.

Nobaek, S., Johansson, M. L., Molin, G., Ahrne, S., & Jeppsson, B. (2000) Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *American Journal of Gastroenterology,* **95**, 1231-1238.

Nour, M. (1998) 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. *Research in Microbiology,* **149**, 433-448.

Nout, M. J. R., Rombouts, F. M., & Havelaar, A. (1989) Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic microorganisms *International Journal of Food Microbiology,* **8**, 351-361.

Nout, M. J. R. (1991) Ecology of accelerated natural lactic fermentation of sorghum-based infant food formulas. *International Journal of Food Microbiology,* **12**, 217-224.

Nout, M. J. R. (1992) Accelerated natural lactic fermentation of cereal-based formulas at reduced water activity. *International Journal of Food Microbiology,* **16**, 313-322.

Nout, M. J. R., Nche, P. F., & Hollman, P. C. H. (1994) Investigation of the presence of biogenis amines and ethyl carbamate in kenkey made with maize and maize cowpea mixtures as influenced by process conditions. *Food Additives and Contaminants,* **11**, 397-402.

Nout, M. J. R., Kok, B., Vela, E., Nche, P. F., & Rombouts, F. M. (1995) Acceleration of the fermentation of kenkey, an indigenous fermented maize food of Ghana. *Food Research International,* **28**, 599-604.

Nout, M. J. R. & Motarjemi, Y. (1997) Assessment of fermentation as a household technology for improving food safety: a joint FAO/WHO workshop. *Food control,* **8**, 221-226.

Nout, R. (2005) Food fermentation: an introduction. In: Food fermentation. Nout, de Vos and Zwietering (eds.) Wageningen Academic Publishers.

Nyatoti, V. N., Mtero, S. S., & Rukure, G. (1997) Pathogenic *Escherichia coli* in traditional African weaning foods. *Food control,* **8**, 51-54.

Oberhelman, R. A., Gilman, E. H., Sheen, P., Taylor, D. N., Black, R. E., Cabrera, L., Lescano, A. G., Meza, R., & Madico, G. (1999) A placebo-controlled trial of *Lactobacillus* GG to prevent diarrhea in undernourished Peruvian children. *Journal of Pediatrics,* **134**, 15-20.

Obizoba, I. C. & Atii, J. V. (1994) Evaluation of the effect of processing techniques in the nutrient and antinutrient contents of pearl millet (*Pennisetum glaucum*) seeds. *Plant Foods for Human Nutrition,* **45**, 23-34.

Odunfa, S.A. (1985) African fermented foods. In: Microbiology of Fermented Foods. Vol. 2. (first edition), Wood, B.J.B. (ed.). Elsevier Applied Science, London, 155-191.

Odunfa, S.A. & Oyewole, O.B (1998) African fermented foods. In: Microbiology of Fermented Foods Vol 2. (second edition), Wood, B.J.B. (ed.), Blackie Academic & Professional, London, UK, pp 713-752.

Odugbemi, T., Odujinrin, O. M. T., Akitoye, C. O., Oyerinde, J. P. O., & Esumeh, F. I. (1991) Study on the pH of Ogi, Nigerian fermented weaning food, and its effect on enteropathogenic *Escherichia coli*, *Salmonella typhi* and *Salmonella paratyphi*. *Journal of Tropical Medicine and Hygiene,* **94**, 219-223.

Odugbmi, T., Oyerinde, J. P. O., Odujinrin, O. M. T., Akitoye, C. O., & Esumeh, F. I. (1993) Bacteriological study of cooked Ogi (fermented cereal weaning food) and its potential safety in a rural Nigerian community. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **87**, 234-235.

Ohashi, Y., Nakai, S., Tsukamoto, T., Masumori, N., Akaza, H., Miyanaga, N., Kitamura, T., Kawabe, K., Kotake, T., Kuroda, M., Naito, S., Koga, H., Saito, Y., Nomata, K., Kitagawa, M., & Aso, Y. (2002) Habitual intake of lactic acid bacteria and risk reduction of bladder cancer. *Urologia Internationalis,* **68**, 273-280.

Oksanen, P. J., Salminen, S., Saxelin, M., Hamalainen, P., Ihantolavormisto, A., Muurasniemiisoviita, L., Nikkari, S., Oksanen, T., Porsti, I., Salminen, E., Siitonen, S., Stuckey, H., Toppila, A., & Vapaatalo, H. (1990) Prevention of travelers diarrhea by *Lactobacillus* GG. *Annals of Medicine,* **22**, 53-56.

Olasupo, N. A., Olukoya, D. K., & Odunfa, S. A. (1995) Studies on bacteriocinogenic *Lactobacillus* isolates from selected Nigerian fermented foods. *Journal of Basic Microbiology,* **35**, 319-324.

Olasupo, N. A., Olukoya, D. K., & Odunfa, S. A. (1997a) Identification of *Lactobacillus* species associated with selected African fermented foods. *Zeitschrift fur Naturforschung C-A Journal of Biosciences,* **52**, 105-108.

Olasupo, N. A., Olukoya, D. K., & Odunfa, S. A. (1997b) Assessment of a bacteriocin-producing *Lactobacillus* strain in the control of spoilage of a cereal-based African fermented food. *Folia Microbiologica,* **42**, 31-34.

Olsen, A., Halm, M., & Jakobsen, M. (1995) The antimicrobial activity of lactic acid bacteria from fermented maize (kenkey) and their interactions during fermentation. *Journal of Applied Bacteriology,* **79**, 506-512.

Olukoya, D.K., Ebigwei, S.I., Adebawo, O.O. & Osiyemi, F.O. (1993) Plasmid profiles and antibiotic susceptibility patterns of *Lactobacillus* isolated from fermented foods in Nigeria. *Food Microbiology*, **10**, 279-285.

Olukoya, D. K., Ebigwei, S. I., Olasupo, N. A., & Ogunjimi, A. A. (1994) Production of DOGIK – an improved Ogi (Nigerian fermented weaning food) with potential for use in diarrhea control. *Journal of Tropical Pediatrics,* **40**, 108-113.

Onilude, A. A., Sanni, A. I., & Ighalo, M. I. (1999) Effect of process improvement on the physico-chemical properties of infant weaning food from fermented composite blends of cereal and soybeans. *Plant Foods for Human Nutrition,* **54**, 239-250.

Onofiok, N.O. & Nnanyelugo, D.O. (1998) Weaning foods in West Africa: Nutritional problems and possible solutions. *Food and Nutrition Bulletin*, **19**, 27-33.

Onyango, C., Noetzold, H., Bley, T., & Henle, T. (2004) Proximate composition and digestibility of fermented and extruded uji from maize-finger millet blend. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology,* **37**, 827-832.

Onyango, C., Noetzold, H., Ziems, A., Hofmann, T., Bley, T., & Henle, T. (2005) Digestibility and antinutrient properties of acidified and extruded maize-finger millet blend in the production of uji. *LWT-Food Science and Technology,* **38**, 697-707.

Ott, S.J. & Schreiber, S. (2006) Reduced microbial diversity in inflammatory bowel diseases. *Gut*, **55**, 1207.

Ouoba, L.I.I., Jensen, L., Lei, V. and Jakobsen, M. Resistance of lactic acid bacteria from African and European origins to antibiotics: determination and transferability of the resistance genes to other bacteria. *Journal of Applied Microbiology,* (In preparation).

Ouwehand, A. C. & Salminen, S. J. (1998) The health effects of cultured milk products with viable and non-viable bacteria. *International Dairy Journal,* **8**, 749-758.

Ouwehand, A. C., Kirjavainen, P. V., Shortt, C., & Salminen, S. (1999a) Probiotics: mechanisms and established effects. *International Dairy Journal,* **9**, 43-52.

Ouwehand, A. C., Kirjavainen, P. V., Gronlund, M. M., Isolauri, E., & Salminen, S. J. (1999b) Adhesion of probiotic micro-organisms to intestinal mucus. *International Dairy Journal,* **9**, 623-630.

Ouwehand, A. C., Tuomola, E. M., Tolkko, S., & Salminen, S. (2001) Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. *International Journal of Food Microbiology,* **64**, 119-126.

Oyewole, O. B. (1997) Lactic fermented foods in Africa and their benefits. *Food control,* **8**, 289-297.

Oyeyiola, G. P. (1991) Fermentation of millet to produce kamu, a Nigerian starch-cake food. *World Journal of Microbiology & Biotechnology,* **7**, 195-201.

Paludan-Muller, C., Huss, H. H., & Gram, L. (1999) Characterization of lactic acid bacteria isolated from a Thai low-salt fermented fish product and the role of garlic as substrate for fermentation. *International Journal of Food Microbiology,* **46**, 219-229.

Paludan-Muller, C., Valyasevi, R., Huss, H. H., & Gram, L. (2002) Genotypic and phenotypic characterization of garlic-fermenting lactic acid bacteria isolated from som-fak, a Thai low-salt fermented fish product. *Journal of Applied Microbiology,* **92**, 307-314.

Pant, A. R., Graham, S. M., Allen, S. J., Harikul, S., Sabchareon, A., Cuevas, L., & Hart, C. A. (1996) *Lactobacillus* GG and acute diarrhoea in young children in the tropics. *Journal of Tropical Pediatrics,* **42**, 162-165.

Parker, R.B. (1974) Probiotics, the other half of the antibiotic story. *Animal Nutrition and Health*, **29**, 4-8.

Pearce, J. L. & Hamilton, J. R. (1974) Controlled trial of prally-administered lactobacilli in acute infantile diarrhea. *Journal of Pediatrics,* **84**, 261-262.

Pedone, C. A., Bernabeu, A. O., Postaire, E. R., Bouley, C. F., & Reinert, P. (1999) The effect of supplementation with milk fermented by *Lactobacillus casei* (strain DN-114 001) on acute diarrhoea in children attending day care centres. *International Journal of Clinical Practice,* **53**, 179-184.

Pedone, C. A., Arnaud, C. C., Postaire, E. R., Bouley, C. F., & Reinert, P. (2000) Multicentric study of the effect of milk fermented by *Lactobacillus casei* on the incidence of diarrhoea. *International Journal of Clinical Practice,* **54**, 568-571.

Pelleboer, R. A. A., Felius, A., Goje, B. S., & Vangelderen, H. H. (1990) Sorghum-based oral rehydration solution in the treatment of acute diarrhea. *Tropical and Geographical Medicine,* **42**, 63-68.

Peltonen, K., El Nezami, H., Haskard, C., Ahokas, J., & Salminen, S. (2001) Aflatoxin B-1 binding by dairy strains of lactic acid bacteria and bifidobacteria. *Journal of Dairy Science,* **84**, 2152-2156.

Penner, R., Fedorak, R. N., & Madsen, K. L. (2005) Probiotics and nutraceuticals: non-medicinal treatments of gastrointestinal diseases. *Current Opinion in Pharmacology,* **5**, 596-603.

Perdigon, G., de LeBlanc, A. D., Valdez, J., & Rachid, M. (2002) Role of yoghurt in the prevention of colon cancer. *European Journal of Clinical Nutrition,* **56**, S65-S68.

Pessi, T., Sutas, Y., Saxelin, M., Kallioinen, H., & Isolauri, E. (1999) Antiproliferative effects of homogenates derived from five strains of candidate probiotic bacteria. *Applied and Environmental Microbiology,* **65**, 4725-4728.

Pessi, T., Sutas, Y., Hurme, H., & Isolauri, E. (2000) Interleukin-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG. *Clinical and Experimental Allergy,* **30**, 1804-1808.

Plant, L. J. & Conway, P. L. (2002) Adjuvant properties and colonization potential of adhering and non-adhering *Lactobacillus* spp. following oral administration to mice. *FEMS Immunology and Medical Microbiology,* **34**, 105-111.

Plant, L., Lam, C., Conway, P. L., & O'Riordan, K. (2003) Gastrointestinal microbial community shifts observed following oral administration of a *Lactobacillus fermentum* strain to mice. *FEMS Microbiology Ecology,* **43**, 133-140.

Priest, F. and Austin, B (1993) Modern bacterial taxonomy. Second edition, Chapman & Hall.

PROSAFE (2002). PROSAFE – Biosafety evaluation of lactic acid bacteria used for human consumption. European Commission's 5th Framework Programme (QLRT-2000-01273). Website: www.ua.ac.be/prosafe.

Rafter, J. (2003) Probiotics and colon cancer. *Best Practice & Research in Clinical Gastroenterology,* **17**, 849-859.

Rahman, M. M., Islam, M. A., MahalanabisS, D., Biswas, E., Majid, N., & Wahed, M. A. (1994) Intake from an energy-dense porridge liquefied by amylase of germinated wheat – a controlled trial in several malnourished children during convalescence from diarrhea. *European Journal of Clinical Nutrition,* **48**, 46-53.

Rahman, M. M., MahalanabisS, D., Ali, M., Mazumder, R. N., Wahed, M. A., & Fuchs, G. J. (1997a) Absorption of macronutrients and nitrogen balance in children with dysentery fed an amylase-treated energy-dense porridge. *Acta Paediatrica,* **86**, 1312-1316.

Rahman, M. M., Mitra, A. K., Mahalanabis, D., Wahed, M. A., Khatun, M., & Majid, N. (1997b) Absorption of nutrients from an energy-dense diet liquefied with amylase from germinated wheat in infants with acute diarrhea. *Journal of Pediatric Gastroenterology and Nutrition,* **24**, 119-123.

Rani, B & Khetarpaul, N (1998) Probiotic fermented food mixtures: Possible applications in clinical anti-diarrhoea usage. *Nutrition and Health*, **12**, 97-105.

Rautanen, T., Isolauri, E., Salo, E., & Vesikari, T. (1998) Management of acute diarrhoea with low osmolarity oral rehydration solutions and *Lactobacillus* strain GG. *Archives of Disease In Childhood,* **79**, 157-160.

Rayes, N., Hansen, S., Seehofer, D., Muller, A. R., Serke, S., Bengmark, S., & Neuhaus, P. (2002a) Early enteral supply of fiber and lactobacilli versus conventional nutrition: A controlled trial in patients with major abdominal surgery. *Nutrition,* **18**, 609-615.

Rayes, N., Seehofer, D., Hansen, S., Boucsein, K., Muller, A. R., Serke, S., Bengmark, S., & Neuhaus, P. (2002b) Early enteral supply of *Lactobacillus* and fiber versus selective bowel decontamination: A controlled trial in liver transplant recipients. *Transplantation,* **74**, 123-128.

Raza, S., Graham, S. M., Allen, S. J., Sultana, S., Cuevas, L., & Hart, C. A. (1995) *Lactobacillus* GG promotes recovery from acute non-bloody diarrhea in Pakistan. *Pediatric Infectious Disease Journal,* **14**, 107-111.

Reddy, B. S. (1998) Prevention of colon cancer by pre- and probiotics: evidence from laboratory studies. *British Journal of Nutrition,* **80**, S219-S223.

Reid, G. & Burton, J. (2002) Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbes and Infection,* **4**, 319-324.

Ribeiro, H. (2000) Diarrheal disease in a developing nation. *American Journal of Gastroenterology,* **95**, S14-S15.

Roberfroid, M. B. (1998) Prebiotics and synbiotics: concepts and nutritional properties. *British Journal of Nutrition,* **80**, S197-S202.

Roberts, A.P., Davis, I.J., Seville, L., Villedieu, A. & Mullany, P. (2006). Characterization of the end and target site of a novel tetracycline resistance-encoding conjugative transposon from Enterococcus faecium 664.1H1. *Journal of Bacteriology*, **188**, 4356-4361.

Rochet, V., Rigottier-Gois, L., Sutren, M., Krementscki, M. N., Andrieux, C., Furet, J. P., Tailliez, P., Levenez, F., Mogenet, A., Bresson, J. L., Meance, S., Cayuela, C., Leplingard, A., & Dore, J. (2006) Effects of orally administered *Lactobacillus casei* DN-114 001 on the composition or activities of the dominant faecal microbiota in healthy humans. *British Journal of Nutrition,* **95**, 421-429.

Rolfe, R. D. (2000) The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition,* **130**, 396S-402S.

Rosenfeldt, V., Michaelsen, K. F., Jakobsen, M., Larsen, C. N., Moller, P. L., Pedersen, P., Tvede, M., Weyrehter, H., Valerius, N. H., & Paerregaard, A. (2002a) Effect of probiotic *Lactobacillus* strains in young children hospitalized with acute diarrhea. *Pediatric Infectious Disease Journal,* **21**, 411-416.

Rosenfeldt, V., Michaelsen, K. F., Jakobsen, M., Larsen, C. N., Moller, P. L., Tvede, M., Weyrehter, H., Valerius, N. H., & Paerregaard, A. (2002b) Effect of probiotic *Lactobacillus* strains on acute diarrhea in a cohort of nonhospitalized children attending day-care centers. *Pediatric Infectious Disease Journal,* **21**, 417-419.

Rosenfeldt, V., Benfeldt, E., Nielsen, S. D., Michaelsen, K. F., Jeppesen, D. L., Valerius, N. H., & Paerregaard, A. (2003) Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *Journal of Allergy and Clinical Immunology,* **111**, 389-395.

Roy, D., Ward, P., Vincent, D., & Mondou, F. (2000) Molecular identification of potentially probiotic lactobacilli. *Current Microbiology,* **40**, 40-46.

Rutgeerts, P. (2002) A critical assessment of new therapies in inflammatory bowel disease. *Journal of Gastroenterology and Hepatology,* **17**, S176-S185.

Saarela, M., Mogensen, G., Fonden, R., Matto, J., & Mattila-Sandholm, T. (2000) Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology,* **84**, 197-215.

Saarela, M., Lahteenmaki, L., Crittenden, R., Salminen, S., & Mattila-Sandholm, T. (2002) Gut bacteria and health foods - the European perspective. *International Journal of Food Microbiology,* **78**, 99-117.

Saavedra, J. M., Bauman, N. A., Oung, I., Perman, J. A., & Yolken, R. H. (1994) Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhea and shedding of rotavirus. *Lancet,* **344**, 1046-1049.

Saiki, R.K., Gelfand, D.H., Stroffel, S., Scharf, S., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with termostable DNA polymerase. *Science*, **239**, 487-491.

Sakamoto, I., Igarashi, M., Kimura, K., Takagi, A.Q., Miwa, T. & Koga, Y. (2001) Suppressive effect of *Lactobacillus gasseri* OLL 2716 (LG21) on *Helicobacter pylori* infections in humans. *Journal of Antimicrobial Chemotheraphy*, **47**, 709-710.

Salminen, S., Wright, A. v., Morelli, L., Marteau, P., Brassart, D., Vos, W. d., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S. E., Mattila, S. T., von Wright, A., & de Vos, W. M. (1998) Demonstration of safety of probiotics - a review. *International Journal of Food Microbiology,* **44**, 93-106.

Salminen, S., Ouwehand, A., Benno, Y., & Lee, Y. K. (1999) Probiotics: how should they be defined? *Trends in Food Science & Technology,* **10**, 107-110.

Salovaara, H. (2004) Lactic acid bacteria in cereal-based products. In: Salminen, S., Von Wright A, eds. Lactic acid bacteria: microbiology and functional aspects. 3rd ed. New York: Marcel Dekker Inc.

Sanchez, I., Palop, L., & Ballesteros, C. (2000) Biochemical characterization of lactic acid bacteria isolated from spontaneous fermentation of 'Almagro' eggplants. *International Journal of Food Microbiology,* **59**, 9-17.

Sanger, F., Niclen, S. & Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceeding of the National Academy of Sciences*, **74**, 5463-5467.

Sanni, A.I., Lönner, C., Marklinder, I., Johansson, M.-L. & Molin, G. (1994) Starter cultures for the production of Ogi, a fermented infant food from maize and sorghum. *Chemie, Mikrobiologie, Technologie der Lebensmittel*, **16**, 29-33.

Sanni, A.I., Onilude, A.A. & d Fatungase, M.O. (1998) Production of sour maize bread using starter-cultures. *World Journal of Microbiology and Biotechnology*. **14**, 101-106.

Sanni, A. I., Onilude, A. A., & Ibidapo, O. T. (1999a) Physicochemical characteristics of weaning food formulated from different blends of cereal and soybean. *Zeitschrift fur Lebensmittel Untersuchung und Forschung,* **208**, 221-224.

Sanni, A. I., Onilude, A. A., & Ibidapo, O. T. (1999b) Biochemical composition of infant weaning food fabricated from fermented blends of cereal and soybean. *Food Chemistry,* **65**, 35-39.

Sanni, A. I., Morlon-Guyot, J., & Guyot, J. P. (2002) New efficient amylase-producing strains of *Lactobacillus plantarum* and *L. fermentum* isolated from different Nigerian traditional fermented foods. *International Journal of Food Microbiology,* **72**, 53-62.

Santosa, S., Farnworth, E., & Jones, P. J. H. (2006) Probiotics and their potential health claims. *Nutrition Reviews,* **64**, 265-274.

Saran, S., Gopalan, S., & Krishna, T. P. (2002) Use of fermented foods to combat stunting and failure to thrive. *Nutrition,* **18**, 393-396.

Sawadogo-Lingani, H., Lei, V., Diawara, B., Nielsen, D.S., Møller, P.L., Traoré, A.S. and Jakobsen, M. The biodiversity of predominant lactic acid bacteria in dolo and pito wort, for production of sorghum beer. *Journal of Applied Microbiology* (Accepted for publication).

Saxelin, M., Pessi, T., & Salminen, S. (1995) Fecal recovery following oral-administration of *Lactobacillus* strain GG (ATCC-53103) in gelatin capsules to healthy volunteers. *International Journal of Food Microbiology,* **25**, 199-203.

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., & de Vos, W. M. (2005) Probiotic and other functional microbes: from markets to mechanisms. *Current Opinion in Biotechnology,* **16**, 204-211.

Sazawal, S., Hiremath, G., Dhingra, U., Malik, P., Deb, S., & Black, R. E. (2006) Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. *Lancet Infectious Diseases,* **6**, 374-382.

Scheppach, W., Luehrs, H., & Menzel, T. (2001) Beneficial health effects of low-digestible carbohydrate consumption. *British Journal of Nutrition,* **85**, S23-S30.

Schillinger, U. & Lucke, F. K. (1989) Antibacterial activity of *Lactobacillus sakei* isolated from meat. *Applied and Environmental Microbiology,* **55**, 1901-1906.

Schleifer, K. H. & Ludwig, W. (1995) Phylogeny of the genus *Lactobacillus* and related genera. *Systematic and Applied Microbiology,* **18**, 461-467.

Shanahan, F. (2002) Probiotics and inflammatory bowel disease: from fads and fantasy to facts and future. *British Journal of Nutrition,* **88**, S5-S9.

Sharma, A. & Kapoor, A. C. (1996) Levels of antinutritional factors in pearl millet as affected by processing treatments and various types of fermentation. *Plant Foods for Human Nutrition,* **49**, 241-252.

Shetty, P.H. & Jespersen, L. (2006) *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science & Technology*, **17**, 48-55.

Sheu, B.-S., Wu, J.-J., Lo, C.-Y., Wu. H.W., Chen, J.H., Lin, Y.S. & Lin, M.D. (2002) Impact of supplement with *Lactobacillus*- and *Bifidobacterium* –containing yogurt on triple therapy for *Helicobacter pylori* eradication. *Alimentary Pharmacology and Therapeutics*, **16**, 1669-1675.

Shinoda, T., Kusuda, D., Ishida, Y., Ikeda, N., Kaneko, K., Masuda, O., & Yamamoto, N. (2001) Survival of *Lactobacillus helveticus* strain CP53 in the human gastrointestinal tract. *Letters in Applied Microbiology,* **32**, 108-113.

Shornikova, A. V., Casas, I. A., Isolauri, E., Mykkanen, H., & Vesikari, T. (1997a) *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. *Journal of Pediatric Gastroenterology and Nutrition,* **24**, 399-404.

Shornikova, A. V., Casas, I. A., Mykkanen, H., Salo, E., & Vesikari, T. (1997b) Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. *Pediatric Infectious Disease Journal,* **16**, 1103-1107.

Shornikova, A. V., Isolauri, E., Burkanova, L., Lukovnikova, S., & Vesikari, T. (1997c) A trial in the Karelian Republic of oral rehydration and *Lactobacillus* GG for treatment of acute diarrhoea. *Acta Paediatrica,* **86**, 460-465.

Silla-Santos, M.H. (2001) Toxic nitrogen compounds produced during processing: Biogenic amines, ethyl carbamides, nitrosamines. In: Fermentation and food safety. Adams, M.R. and Nout, M.J.R. (eds.). Aspen Publishers, Inc., Gaithersburg, Maryland, USA.

Simakachorn, N., Pichaipat, V., Rithipornpaisarn, P., Kongkaew, C., Tongpradit, P., & Varavithya, W. (2000) Clinical evaluation of the addition of lyophilized, heat-killed *Lactobacillus acidophilus* LB to oral rehydration therapy in the treatment of acute diarrhea in children. *Journal of Pediatric Gastroenterology and Nutrition,* **30**, 68-72.

Simango, C. & Rukure, G. (1991) Survival of *Campylobacter jejuni* and pathogenic *Escherichia coli* in Mahwu, a fermented cereal gruel. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **85**, 399-400.

Simango, C. & Rukure, G. (1992) Survival of bacterial enteric pathogens in traditional fermented foods. *Journal of Applied Bacteriology,* **73**, 37-40.

Simango, C. (1995) Effective acidification of traditional fermented foods. *Journal of Tropical Medicine and Hygiene,* **98**, 465-468.

Simango, C. (1997) Potential use of traditional fermented foods for weaning in Zimbabwe. *Social Science & Medicine,* **44**, 1065-1068.

Simpson, W.J. & Taguchi, H. (1995) The genus *Pediococcus* with notes on the genera *Tetragenococcus* and *Aerococcus*. In: The genera of lactic acid bacteria. Wood, B.J.B. and Holzapfel, W.H. (eds.), Chapman and Hall, Suffolk, UK.

Sindhu, S. C. & Khetarpaul, N. (2003) Effect of feeding probiotic fermented indigenous food mixture on serum cholesterol levels in mice. *Nutrition Research,* **23**, 1071-1080.

Singer, R.S., Ward, M.P. & Maldonado, G. (2006) Can landscape ecology untangle the complexity of antibiotic resistance? *Nature Reviews Microbiology*, **4**, 943-952.

Steinkraus, K.H. (1996) Handbook of indigenous fermented foods, 2. edition. Steinkraus, K.H. (ed). Marcel Dekker, Inc., New York, USA.

Steinkraus, K. H. (1997) Classification of fermented foods: worldwide review of household fermentation techniques. *Food control,* **8**, 311-317.

Stiles, M. E. & Holzapfel, W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology,* **36**, 1-29.

Stolzer, P.-O., Blomberg, L., Conway, P.L., Henriksson, A. & Abrahamsson, H. (1996) Probiotic treatment of small intestinal overgrowth by *Lactobacillus fermentum* KLD. *Scandinavian Journal of Infectious Diseases*, **28**, 615-619.

Sullivan, A. & Nord, C. E. (2002) The place of probiotics in human intestinal infections. *International Journal of Antimicrobial Agents,* **20**, 313-319.

Svanberg, U., Sjogren, E., Lorri, W., Svennerholm, A. M., & Kaijser, B. (1992) Inhibited Growth of Common Enteropathogenic Bacteria in Lactic-Fermented Cereal Gruels. *World Journal of Microbiology & Biotechnology,* **8**, 601-606.

Svanberg, U. & Lorri, W. (1997) Fermentation and nutrient availability. *Food control,* **8**, 319-327.

Szajewska, H., Kotowska, M., Mrukowicz, J. Z., Armanska, M., & Mikolajczyk, W. (2001) Efficacy of *Lactobacillus* GG in prevention of nosocomial diarrhea in infants. *Journal of Pediatrics,* **138**, 361-365.

Szajewska, H., Mrukowicz, J. Z., Stoinska, B., & Prochowska, A. (2004) Extensively and partially hydrolysed preterm formulas in the prevention of allergic diseases in preterm infants: a randomized, double-blind trial. *Acta Paediatrica,* **93**, 1159-1165.

Tankanow, R. M., Ross, M. B., Ertel, I. J., Dickinson, D. G., Mccormick, L. S., & Garfinkel, J. F. (1990) A Double-Blind, Placebo-Controlled Study of the Efficacy of Lactinex in the Prophylaxis of Amoxicillin-Induced Diarrhea. *DICP-the Annals of Pharmacotherapy,* **24**, 382-384.

Taylor, J. & Taylor, J. R. N. (2002) Alleviation of the adverse effect of cooking on sorghum protein digestibility through fermentation in traditional African porridges. *International Journal of Food Science and Technology,* **37**, 129-137.

TenBrink, B., Damink, C., Joosten, H.M.L.J. & Tveld, J.H.J.H.I. (1990) Occurrence and formation of biologically-active amines in foods. *International Journal of Food Microbiology*, **11**, 73-84.

Teniola, O.D., Addo, P.A., Brost, I.M., Färber, P., Jany, K.-D., Alberts, J.F., van Zyl, W.H., Steyn, P.S. & Holzapfel, W.H. (2005) Degradation of aflatoxin B_1 by cell-free extracts of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenivorans* sp. nov. DSM44556. *International Journal of Food Microbiology*, **105**, 111-117.

Tetteh, G. L., Sefa-Dedeh, S. K., Phillips, R. D., & Beuchat, L. R. (2004) Survival and growth of acid-adapted and unadapted Shigella flexneri in a traditional fermented Ghanaian weaning food as affected by fortification with cowpea. *International Journal of Food Microbiology,* **90**, 189-195.

Teuber, M., Meile, L., & Schwarz, F. (1999) Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* **76**, 115-137.

Thaoge, M. L., Adams, M. R., Sibara, M. M., Watson, T. G., Taylor, J. R. N., & Goyvaerts, E. M. (2003) Production of improved infant porridges from pearl millet using a lactic acid fermentation step and addition of sorghum malt to reduce viscosity of porridges with high protein, energy and solids (30%) content. *World Journal of Microbiology & Biotechnology,* **19**, 305-310.

Thapa, S. & Tamang, J. P. (2004) Product characterization of kodo ko jaanr: fermented finger millet beverage of the Himalayas. *Food Microbiology,* **21**, 617-622.

Thapar, N. & Sanderson, I. R. (2004) Diarrhoea in children: an interface between developing and developed countries. *Lancet,* **363**, 641-653.

Thomas, M.R., Litin, S.C., Osmon, D.R., Corr, A.P., Weaver, A.L. & Lohse, C.M. (2001) Lack of effect of *Lactobacillus* GG on antibiotic-associated diarrhea: a randomized, placebo-controlled trial. *Mayo Clinic Procedings*, **76**, 883-889.

Todoriki, K., Mukai, T., Sato, S., & TOBA, T. (2001) Inhibition of adhesion of food-borne pathogens to Caco-2 cells by *Lactobacillus* strains. *Journal of Applied Microbiology,* **91**, 154-159.

Tou, E. H., Guyot, J. P., Mouquet-Rivier, C., Rochette, I., Counil, E., Traore, A. S., & Treche, S. (2006) Study through surveys and fermentation kinetics of the traditional processing of pearl millet (*Pennisetum glaucum*) into ben-saalga, a fermented gruel from Burkina Faso. *International Journal of Food Microbiology,* **106**, 52-60.

Towner, J.K. & Cockayne, A (1993) Molecular Methods for Microbial Identification and Typing. Chapman & Hall, London, UK.

Tuomola, E. M. & Salminen, S. J. (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International Journal of Food Microbiology,* **41**, 45-51.

Tuomola, E., Crittenden, R., Playne, M., Isolauri, E., & Salminen, S. (2001) Quality assurance criteria for probiotic bacteria. *American Journal of Clinical Nutrition,* **73**, 393S-398S.

Turck, D., Bernet, J. P., Marx, J., Kempf, H., Giard, P., Walbaum, O., Lacombe, A., Rembert, F., Toursel, F., Bernasconi, P., Gottrand, F., McFarland, L. V., & Bloch, K. (2003) Incidence and risk factors of oral antibiotic-associated diarrhea in an outpatient pediatric population. *Journal of Pediatric Gastroenterology and Nutrition,* **37**, 22-26.

Tynkkynen, S., Singh, K.V. & Varmanen, P. (1998) Vancomycin resistance factor in *Lactobacillus rahmnosus* GG is not related to enterococcal vancomycin resistance (*van*) genes. *International Journal of Food Microbiology*, **41**, 195-204.

Ulisse, S., Gionchetti, P., D'Alo, S., Russo, F. P., Pesce, I., Ricci, G., Rizzello, F., Helwig, U., Cifone, M. G., Campieri, M., & De Simone, C. (2001) Expression of cytokines, inducible nitric oxide synthase, and matrix metalloproteinases in pouchitis: Effects of probiotic treatment. *American Journal of Gastroenterology,* **96**, 2691-2699.

UNICEF (2006) The United Nations Children's Fund (UNICEF). The state of the world's children 2006. New York, USA.

Vaarala, O. (2003) Immunological effects of probiotics with special reference to lactobacilli. *Clinical and Experimental Allergy,* **33**, 1634-1640.

Vanderhoof, J. A., Whitney, D. B., Antonson, D. L., Hanner, T. L., Lupo, J. V., & Young, R. J. (1999) *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea in children. *Journal of Pediatrics,* **135**, 564-568.

Vanderhoof, J. A. & Young, R. J. (2001) The role of probiotics in the treatment of intestinal infections and inflammation. *Current Opinion in Gastroenterology,* **17**, 58-62.

Vesa, T.H., Marteau, P., Zidi, S., Briet, F., Pochart, P. & Rambaud, J.C. (1996) Digestion and tolerance of lactose from yoghurt and different semi-solid fermented dairy products containing *Lactobacillus acidophilus* and bifidobacteria in lactose maldigesters – is bacterial lactase important? European Journal of Clinical Nutrition, 50, 730-733.

Vesa, T., Pochart, P., & Marteau, P. (2000) Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. *Alimentary Pharmacology & Therapeutics,* **14**, 823-828.

Viljanen, M., Savilahti, E., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T., & Kuitunen, M. (2005a) Probiotics in the treatment of atopic eczema / dermatitis syndrome in infants: a double-blind placebo-controlled trial. *Allergy,* **60**, 494-500.

Viljanen, M., Kuitunen, M., Haahtela, T., Juntunen-Backman, K., Korpela, R., & Savilahti, E. (2005b) Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants. *Pediatric Allergy and Immunology,* **16**, 65-71.

Walker, W. A. (2000) Role of nutrients and bacterial colonization in the development of intestinal host defense. *Journal of Pediatric Gastroenterology and Nutrition,* **30**, S2-S7.

Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., & Hammes, W. P. (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology,* **67**, 2578-2585.

Web of Science: http://portal.isiknowledge.com/

Weizman, Z., Asli, G. & Alsheikh, A. (2005) Effect of a probiotic infant formula on infections in child care centers: Comparison of two probiotic agents. Pediatrics, 115, 5-9.

Westby, A., Reilly, A., & Bainbridge, Z. (1997) Review of the effect of fermentation on naturally occurring toxins. *Food control,* **8**, 329-339.

WHO (1990) World Health Organization. Diarrhoeal diseases control programme. The treatment and prevention of acute diarrhoea. WHO/CDD/SER/80.2 Rev. 2, 8-45.

Wikstrom, P., Andersson, A. C., & Forsman, M. (1999) Biomonitoring complex microbial communities using random amplified polymorphic DNA and principal component analysis. *FEMS Microbiology Ecology,* **28**, 131-139.

Willumsen, J. F., Darling, J. C., Kitundu, J. A., Kingamkono, R. R., Msengi, A. E., Mduma, B., Sullivan, K. R., & Tomkins, A. M. (1997) Dietary management of acute diarrhoea in children: Effect of fermented and amylase-digested weaning foods on intestinal permeability. *Journal of Pediatric Gastroenterology and Nutrition,* **24**, 235-241.

Wood, B.J.B. (1991) Industrial evolution of fermented foods. *Food Biotechnology*, **5**, 279-291.

Wollowski, I., Rechkemmer, G., & Pool-Zobel, B. L. (2001) Protective role of probiotics and prebiotics in colon cancer. *American Journal of Clinical Nutrition,* **73**, 451S-455S.

Yapar, N., Sener, A., Karaca, B., Yucesoy, M., Tarakci, H., Cakir, N., & Yuce, A. (2005) Antibiotic-associated diarrhea in a Turkish outpatient population: Investigation of 288 cases. *Journal of Chemotherapy,* **17**, 77-81.

Yartey, J., Nkrumah, F., Hori, H., Harrison, K., & Armar, D. (1995) Clinical-trial of fermented maize-based oral rehydration solution in the management of acute diarrhea in children. *Annals of Tropical Paediatrics,* **15**, 61-68.

Zarate, G., Chaia, A. P., Gonzalez, S., & Oliver, G. (2000) Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. *Journal of Food Protection,* **63**, 1214-1221.

Zhou, J. S., Pillidge, C. J., Gopal, P. K., & Gill, H. S. (2005) Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *International Journal of Food Microbiology,* **98**, 211-217.

Appendices

I Lei, V. and Jakobsen, M. (2004). Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *Journal of Applied Microbiology,* 96, 384-397.

II Lei, V., Friis, H. and Michaelsen, K.F. (2006). Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: An intervention study in Northern Ghana. *International Journal of Food Microbiology,* 110, 246-253.

III Sawadogo-Lingani, H., Lei, V., Diawara, B., Nielsen, D.S., Møller, P.L., Traoré, A.S. and Jakobsen, M. The biodiversity of predominant lactic acid bacteria in dolo and pito wort, for production of sorghum beer. *Journal of Applied Microbiology* (Accepted for publication).

IV Ouoba, L.I.I., Lei, V, Jensen, L. and Jakobsen, M. Resistance of lactic acid bacteria from African and European origins to antibiotics: determination and transferability of the resistance genes to other bacteria. *Journal of Applied Microbiology,* (In preparation).

Due to restrictions of the publishers of the journals in which the articles in appendix I-IV have been published, these articles are not available in this PDF. The articles can be found in:

Appendix I:
Lei, M. J. (2004). Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *Journal of Applied Microbiology, 96*, 384-397.
DOI: 10.1046/j.1365-2672.2004.02162.x

Appendix II:
Lei, V., Friis, H., & Michaelsen, K. F. (2006). Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: An intervention study in Northern Ghana. *International Journal of Food Microbiology, 110*, 246-253.
DOI: 10.1016/j.ijfoodmicro.2006.04.022

Appendix III:
Sawadogo-Lingani, V. L. (2007). The biodiversity of predominant lactic acid bacteria in dolo and pito wort for the production of sorghum beer. *Journal of Applied Microbiology, 103*, 765-777.
DOI: 10.1111/j.1365-2672.2007.03306.x

Appendix IV:
Ouoba, L. I. I., Lei, V., & Jensen, L. B. (2008). Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: Determination and transferability of the resistance genes to other bacteria. *International Journal of Food Microbiology, 121*, 217-224.
DOI: 10.1016/j.ijfoodmicro.2007.11.018

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