MICROBIAL GROWTH RESPONSES IN FERMENTED MAIZE DOUGH SYSTEMS

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A DOCTORIAL THESIS SUBMITTED TO THE DEPARTMENT OF NUTRITION AND FOOD SCIENCE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS OF THE AWARD OF THE DOCTOR OF PHILOSOPHY DEGREE IN FOOD SCIENCE

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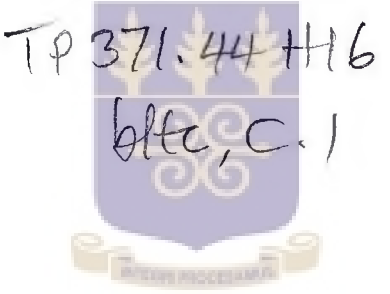

DECLARATION

This dissertation is the result of research work undertaken by Mary Halm at the University of Ghana, Legon and the Royal Veterinary and Agricultural University, Denmark under supervision.

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ABSTRACT

Food safety and the occurrence of diarrhoea are a challenge in the management of food systems in Africa. Infections arising from diarrhoea can be very devastating on the population, especially children. The traditional fermented maize dough systems have been identified as being able to reduce considerably the growth of diarrhoeal causing organisms and improve safety

The study was set up to investigate: 1. The survival of selected diarrhoeal causing bacteria (*Salmonella, Shigella* and *Escherichia coli*) in maize dough fermenting systems (steeping water, maize dough, Ga kenkey water and maize dough porridge koko) to determine the safety of the products; 2. The survival of four *Escherichia coli* strains in synthetic medium containing lactic and acetic acids; 3. Tolerance of the dominant yeasts (*Candida krusei* and *Saccharomyces cerevisiae)* involved in maize fermentation for lactic acid. 4. Changes in short-term intracellular pH of single cells of *Candida krusei* and *Saccharomyces cerevisiae* in the presence of high and low concentrations of lactic acid to explain their tolerance for lactic acid.

Five *Salmonella* species, three *Shigella* species, five pathogenic *Escherichia coli* strains and two non-pathogenic strains were inoculated into fermented maize dough systems at a concentration of $10^6 - 10^7$ cfu/ml at 28 °C. Viable cells were recovered on selective and non-selective media. Almost all the bacteria survived in maize steeping water for 48 h without reduction in numbers. All the salmonellae and shigellae and two *E. coli* strains were completely inhibited in fermenting maize dough after 48 hours whilst three *E. coli* strains (O26 (VTEC), O157 (VTEC II) and O3 EAggEC) survived for 48 h but

were significantly reduced by more than 3 log units. None of the *Salmonella* strains survived in koko for 24 h but *Sh. flexneri* 2a II and four pathogenic *E. coli* strains (O157 (VTEC II), O26 (VTEC), O3 (EAggEC), O111 (EPEC)) and one laboratory strain *E. coli* K12, survived in koko for 48 h with less than 2 log reductions. All the bacteria were completely inhibited in kenkey water after 24 h. The decrease in populations of the bacteria in the different fermentation systems was observed at pH of ≤ 4.

Greater numbers of survivors were recovered with non-selective medium than with a selective medium for Gram-negative bacteria.

In Trypticase Soy Broth Yeast Extract (TSBYE) supplemented with lactic and acetic acids in concentrations found in fermented maize dough systems, *E. coli* strains O111 (EPEC), O3 (EAggEC), K12 and M23 were inhibited to various extents. Concentrations of 31 - 62 mM, undissociated lactic acid had only a bacteriostatic effect on the four pathogens, while above 62 mM, a bactericidal effect was noted after 24 h. Concentrations of > 17 - 33 mM undissociated acetic acid were required to completely inhibit the four *E. coli* strains. These results confirm that fermented maize dough systems have antimicrobial properties which may inhibit the survival of some pathogenic and non-pathogenic bacteria. The extent of inhibition varied among the species investigated, namely *Salmonella, Shigella* and *Escherichia coli;* and also among the maize dough systems. The presence of lactic acid at low pH was found to be the main anti-microbial property of the fermented maize dough systems.

The influence of different lactic acid concentrations (0.2, 0.4, 0.8 and 1.2% V/V), within a pH range of 3.5 to 4.2 on the survival kinetics of *E. coli* 0111: H2 (EPEC) was determined in TSBYE at 30 °C. Survival data were analysed and fitted with the model of Peleg and Cole (1998). The model parameters b and n were estimated and used to calculate the time to one log decrease in bacterial population. The model of Peleg and Cole gave a good description of the survival of *E. coli* under the experimental conditions tested. A strong correlation of the time to one log reduction in bacterial numbers with the undissociated lactic acid concentration was demonstrated.

Also using the Number Cruncher Statistical Sytems (NCSS), a multiple regression analysis was performed on the data and a model was obtained which relates the death rate (time to one log reduction) to the lactic acid concentration and pH. The proposed model for the death rate of *E. coli* 0111 (EPEC) in response to pH and total lactic acid concentration in TSBYE provided a good description of the data. Except for the low pH of 3.5 and 1.2% lactic acid, where the model predicted a negative value for the death rate, all other predicted values were in agreement with actual values obtained in the broth studies.

Growth responses of two strains each of *Candida krusei* and *Saccharomyces cerevisiae* singly and as mixed cultures were determined in MYGP broth, pH 2.5 and pH 3.5 with or without lactic acid at 30 °C. At pH 2.5, in the presence of 106.4 mM undissociated lactic acid, *C. krusei* strains grew within 48 h from 4.0 $\log_{10}$ cfu/ ml to 7.0 $\log_{10}$ cfu/ ml irrespective of whether cultured singly or combined as mixed

cultures with either strain of *Sacch. cerevisiae*. But *Sacch. cerevisiae* strains did not grow when cultured individually in combination with either strain of the two *C. krusei* investigated. When the *Sacch. cerevisiae* strains were cultured individually as single cultures only one grew at pH 2.5 in the presence of 106.4 mM undissociated lactic acid. At pH 3.5, irrespective of the presence or absence of 77 mM lactic acid, *C. krusei* 29 grew from 4.0 $\log_{10}$ cfu/ml to about 8.0 $\log_{10}$ cfu/ml whether cultured singly or in combination with either strain of *Sacch. cerevisiae*. In contrast, both strains of *Sacch. cerevisiae* showed good growth as single cultures but reduced growth when cultured with *C. krusei* as mixed cultures and the reduced growth was greater in the presence of lactic acid. These results indicate that *C. krusei* is more tolerant to lactic acid at low pH than *Sacch. cerevisiae*.

To explain the differences in lactic acid tolerance of the two yeast species, fluorescence-ratio-imaging microscopy and a perfusion system were used to determine the short-term intracellular pH (pH_i) changes in single cells of *C. krusei* and *S. cerevisiae*. The changes were investigated both in the presence of low (20.7 mM) and high (106.4 mM) concentrations of undissociated lactic acid. For both the investigated species 20.7 mM undissociated lactic acid did not seem to influence the initial pH_i which for *C. krusei* was found to be approx. 8.0 and for *S. cerevisiae* 6.9-7.5. For both *C. krusei* strains, perfusion with 106.4 mM undissociated lactic acid induced only weak short-term pH_i responses with a decrease in pH_i of less than one pH unit. Contrary for both strains of *Sacch. cerevisiae,* perfusion with 106.4 mM undissociated lactic acid resulted in a significant decrease in pH_i from initially 6.9 -

7.5 to 6.2 - 6.4 after 1 min and further to a pH_i of ≤ 5.5 after 3 min after which it remained constant. The results obtained show that *C. krusei* is more resistant to short-term pH_i changes caused by lactic acid than *S. cerevisiae*, and this, in turn, may explian why *C. krusei* is more tolerant to lactic acid than *S. cerevisiae*.

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1.0 INTRODUCTION

1.1 Maize in the Ghanaian Diet

Maize is the principal cultivated cereal crop grown all over Ghana. With a total annual production of 1,013,000 metric tons, it accounts for 4 % of the Gross Domestic Product with a per capita consumption of 42 5 kg (Ministry of Food and Agriculture, 2002). As a cereal crop most widely used in Ghana, maize contributes significantly to the total calorie and protein content of the Ghanaian diet, furnishing between 90-95% of the total calorie in the diet of the people in the coastal plains. However, the consumption of maize is less extensive in the northern part of the country where sorghum and millet are the main staples (Dovlo, 1970).

In Ghana, maize is processed by a variety of indigenous methods, which transform the crop into an intermediate or finished product with a stable shelf life, more digestible, improved nutritive quality and desirable organoleptic properties. Processing may also include improvement of the protein quantity and quality of the product by fortification with legumes such as cowpeas and soybeans (Plahar and Leung, 1983; Nche *et al.*, 1994; Afoakwa, 1996; Obiri-Danso *et al.*, 1997; Plahar *et al.*, 1997; Sefa-Dedeh *et al.*, 2000). Maize processing methods include a combination of different unit operations, which comprise soaking or steeping, dehulling, roasting, milling, fermentation, *aflatalization*, cooking and packaging. In the southern part of Ghana, most of the maize is processed by fermentation into an intermediate product (fermented maize dough), which is used for the preparation of a wide variety of dishes such as *kenkey, nsiho*, *banku* and *koko*. *Kenkey* is a stiff gruel or dumpling made from a mixture of cooked and non-cooked

whole fermented maize dough which is moulded into balls, wrapped in leaves or maize husks and cooked into *kenkey*. The water obtained after cooking the maize dough into *kenkey* is known as *kenkey water*. *Nsiho* is a stiff porridge or dumpling similar in consistency and method of preparation to *kenkey* but the dough is made from dehulled maize. *Banku* is a dumpling made by cooking a slurry of fermented maize dough into a semi-solid consistency and moulded into balls without packaging. *Koko* is a porridge prepared by boiling a thin slurry of fermented maize dough used mainly as infant food and as breakfast cereal for adults (Dovlo, 1970).

Fermented maize dough is prepared by steeping or soaking of clean maize kernels in water for 24 to 48 h; milling the steeped maize into a smooth meal; addition of water to form a dough and spontaneous fermentation of dough for 24 to 72 h. Fermentation as a unit operation in food processing offers many advantages, including food preservation, improved food safety, enhanced flavour and acceptability, increased variety in the diet, improved nutritional value, reduction of antinutrition factors and in some cases improved functional properties (Afoakwa, 1996; Nout and Motarjemi, 1997; Plahar *et al.*, 1997; Sefa-Dedeh and Plange, 1989; Sefa-Dedeh *et al.,* 2000).

1.2 Spontaneous Fermentation of Maize

Spontaneous fermentation of maize in Ghana like most other cereal and root crops fermentations in Africa is known to be lactic acid fermentation (Mbugua, 1987; Halm *et al.,* 1993; Hounhouigan *et al.,* 1994; Amoa-Awua *et al.*, 1996; Odunfa and Oyewole, 1998). According to Oyewole (1997), lactic acid fermentation has survived in Africa

because of the many benefits of this technology: It serves as a household technology for improving food safety; it serves as a low-cost method of food preservation in Africa; it contributes to the improvement of the nutritional value and digestibility of food raw materials. In addition, lactic acid bacteria fermentations have survived in Africa due to traditional beliefs, good taste and appearance of product as well as long shelf life.

1.3 Microbial Successions During Maize Fermentation

Studies carried out on the microbiology of fermented maize dough in Ghana have revealed that a series of microbial successions take place during maize fermentation which lead to the selection of a defined stable flora comprising *Lactobacillus fermentum* and the yeasts *Candida krusei* and *Saccharomyces cerevisiae* (Halm *et al.*, 1993; Jespersen *et al.*, 1994; Obiri-Danso, 1994; Olsen *et al.*, 1995; Hayford *et al.*, 1999; Hayford and Jakobsen, 1999; Hayford and Jespersen, 1999). These studies showed that at the start of maize steeping, a mixed group of microorganisms are present including lactic acid bacteria, Gram-positive catalase positive bacteria, Gram-negative bacteria, yeast and moulds. During steeping, a strong selection takes place leading to a significant increase in the population of the lactic acid bacteria. After milling the steeped maize and addition of water to form the dough, the main fermentation starts and within 0 to 72 hours the lactic acid bacteria multiply to levels of more than 10^9 cfu/g. Yeast growth reaches a maximum level of 10^6 cfu/g after 24.to 48 hours, followed by a decline in their numbers. Filamentous fungi and moulds present reduce significantly and after 24 hours of dough fermentation, moulds and Gram-positive catalase-positive and Gram-negative bacteria are not detected. At the advanced stage of fermentation, the microbial population mainly consists of

Lactobacillus fermentum and the yeasts *Candida krusei* and *Saccharomyces cerevisiae* (Halm et al., 1993).

Other lactic acid bacteria that have been associated with maize dough fermentation include *Lactobacillus plantarum, Pediococcus pentosaceus, Pediococcus acidilactici, Lactobacillus brevis, Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus casei,* and *Lactobacillus cellobiosus* (Fields *et al.*, 1981; Halm *et al.*, 1993; Olasupo *et al.*, 1997; Hounhouigan *et al.*, 1993a; Olsen *et al.*, 1995).

The microbial successions that take place during maize fermentation are believed to be due to interactions between the various microorganisms. Olsen *et al.* (1995) investigated the microbial interactions involving the lactic acid bacteria, Gram-positive, catalase-positive bacteria and Gram-negative bacteria isolated from various processing stages of maize dough fermentation. Their results indicated that each processing stage has its own microenvironment with strong antimicrobial activity. About half of the *Lactobacillus plantarum* and practically all the *Lactobacillus fermentum* investigated were shown to inhibit other Gram-positive and Gram-negative bacteria, explaining the elimination of these organisms during the initial processing stages. Further, they showed widespread inhibitory reactions between lactic acid bacteria isolated from maize, amounting to 85% of all the combinations tested. This decreased to 41% for isolates from steep water then to 35% for fresh dough and 18% for the final fermented product. These observations seemed to explain the microbial succession that took place amongst lactic acid bacteria during maize fermentation. These apparent strong antimicrobial activities of the dominant lactic acid bacteria were explained by the pronounced decrease of pH from 6.5 to 3.7 within 24 h of

maize fermentation (Halm *et al.*, 1993) and specific effects of organic acids, with other compounds like hydrogen peroxide and bacteriocins produced by lactic acid bacteria playing a minor role (Olsen *et al.*, 1995). These findings are in support of earlier work carried out by Mensah *et al.* (1991), which showed significant reductions in numbers of *Shigella flexneri*, and enterotoxigenic *Escherichia coli* strains inoculated into fermented maize dough and fermented maize dough porridge. Investigations by Annan-Prah and Agyeman (1997) also showed significant reductions in numbers of enterotoxigenic *Escherichia coli J955* and *101685, Staphylococcus aureus* and *Klebsiella pneumoniae* inoculated into Ga *kenkey* of pH 4 and post-cooking *kenkey* water. Several investigators have also reported the inhibition of enteric pathogens in many other African fermented cereal products (Simango and Rukure, 1992; Mbugua and Njenga, 1991; Nout *et al.*, 1989; Svanberg *et al.*, 1992). The antimicrobial activities observed against enteric pathogens in these products have been related to the lactic acid bacteria involved in the fermentations. The preservation of fermented foods by lactic acid bacteria is due primarily to sugars being converted to organic acids (lactic and acetic) causing a reduction in pH and removal of carbohydrates as nutrient sources (Gilliland, 1985; Daeschel, 1989). In addition, lactic acid bacteria produce substances including hydrogen peroxide, diacetyl, secondary reaction products and bacteriocins, which have the potential to inhibit a variety of other microorganisms (Daeschel, 1989). Strains of *Lactobacillus* isolates from some selected African fermented products including *kenkey* have been reported to produce bacteriocins (Olukoya *et al.*, 1993; Olasupo *et al.*, 1994, 1995).

Apart from the lactic acid bacteria, a succession is also observed amongst the yeasts, which also form an important part of the microbial flora in fermented maize dough (Halm *et al.*, 1993; Hounhouigan *et al.*, 1993b; Jespersen *et al.*, 1994). In maize dough fermentation, a mixed population of yeasts comprising *Candida, Saccharomyces, Trichosporon, Kluyveromyces* and *Debaryomyces* spp. are present on the raw maize during steeping and early stages of fermentation. After 24 h of fermentation when the dough has reached a pH of 3.7, the yeast flora consist of 5 to 78 % *C. krusei* and 6 to 65 % *S. cerevisiae*. After 72 h of dough fermentation, the yeast population comprises 62 to 71 % *C. krusei* and 14 to 18 % *S. cerevisiae* (Jespersen *et al.*, 1994). As lactic acid is the major metabolite of lactic acid bacteria, the concentration of lactic acid during maize fermentation may reach as high levels as 14 g kg^{-1} dough (Halm *et al.*, 1993), which at a pH of about 3.7 according to the Henderson-Hasselbalch equation is equivalent to 93.3 mM undissociated lactic acid. The reasons why *C. krusei* is dominant in the later stages of maize dough fermentation are not known but one possible explanation could be that *C. krusei* at low pH values is more tolerant to high concentrations of lactic acid than *S. cerevisiae* (Spicher and Schröder, 1980). However, the mechanisms underlying the different lactic acid tolerances of these two yeasts are, as yet, unknown.

With respect to the elimination of moulds during maize fermentation, inhibitory effects of *C. krusei* and *S. cerevisiae* have been demonstrated against isolates of *Penicillium citrinum, Aspergillus flavus* and *Aspergillus parasiticus* isolated from maize as well as 15 known mycotoxin producing strains of the same mould species (Halm and Olsen, 1996; Lei, 1996). The *Candida* isolates showed greater inhibitory potential than the

Saccharomyces isolates and the *Penicillium* species were more sensitive to the yeasts than the *Aspergillus* species. The mould growth was not affected by either yeast supernatant or cell free extracts suggesting that the inhibitions were not due to compounds produced by the yeasts and were attributed to competition for nutrients between the yeasts and the moulds (Halm and Olsen, 1996; Lei, 1996).

Interactions between lactic acid bacteria in fermented foods and Gram-negative pathogens have generally been concluded but the range of Gram-negative bacteria studied is narrow. Recent research has shown that verocytotoxin-producing *Escherichia coli* (VTEC) particularly VTEC O157 can be very acid tolerant (Conner and Kotrola, 1995; Leyer *et al.*, 1995; Diez-Gonzalez and Russell, 1997) and have been responsible for food borne disease outbreaks associated with acidic foods such as yoghurt (Morgan *et al.*, 1993), apple juice (Besser *et al.*, 1993), mayonnaise (Weagent *et al.*, 1994), and dry fermented salami (Centres for Disease Control and Prevention, 1995). It is known that the infective dose of the organism is quite low, less than 100 viable cells (Doyle *et al.*, 1997). The incidence of *E. coli* VTEC O157 in Africa is not known as infections may not always be reported possibly because of inadequate health care facilities and national epidemiological and microbiological surveillance systems. However, in many rural communities in developing countries including Ghana, processing and preparation of foods are carried out under unhygienic and poor sanitary conditions such that if there is cross contamination of cooked fermented products the organism may survive and pose a risk to consumers. This is an area that calls for investigation. The purpose of this study was to determine the survival of selected diarrhoeal bacteria, including strains of reported high acid and low pH

tolerance, such as verotoxigenic *E. coli* (VTEC) serotype O157: H7, in maize dough fermenting systems (maize steeping water, maize dough, maize dough porridge-koko and Ga kenkey water) to determine the safety of these products. Furthermore, to determine whether the observed dominance of *Candida krusei* over *Saccharomyces cerevisiae* at the advanced stage of maize fermentation is due to the reported higher lactic acid tolerance of *Candida krusei* and to determine the mechanisms underlying the different lactic acid tolerances of the two yeasts.

1.4 Objectives of the Study

The objectives of the study are:

1. To determine the survival of selected diarrhoeal bacteria including strains of reported high acid and low pH tolerance, verotoxigenic *E. coli* (VTEC) serotype O157: H7, enteropathogenic (EPEC) and enteroaggregative (EAggEC) *E. coli* in addition to strains of *Sh. flexneri* and *Salmonella* species, in maize steeping water, maize dough, maize dough porridge-koko and Ga kenkey water.

2. To determine the survival of *Escherichia coli* in culture medium at different combinations of lactic acid concentrations and pH values, and to develop a predictive model for predicting the death rate of *Escherichia coli* as a function of pH and total lactic acid concentration.

3. To compare the growth performance of *Candida krusei* and *Saccharomyces cerevisiae* when grown as single or mixed cultures in the presence of lactic acid and low pH to determine their tolerance for lactic acid.

4. To investigate the short-term intracellular pH (pH_i) responses in single cells of *Candida krusei* and *Saccharomyces cerevisiae* to lactic acid to explain differences in their tolerance for lactic acid.

2.0 LITERATURE REVIEW

2.1 Traditional Fermentation of Maize in Ghana

2.1.1 *General*

The bulk of the maize produced in Ghana is consumed in the form of fermented products such as *kenkey, nsiho, fomfom, banku* or *koko*, all indigenous foods prepared from an intermediary fermented maize dough.

Fermentation of maize in Ghana can be described as an indigenous or traditional technology that has been handed down from generations. It is practiced mainly by women as a family acquired art either as individuals or as a family business in the household using simple techniques (Sefa-Dedeh, 1993). Maize fermentation like most other cereal and root crops fermentations in Africa is known to be lactic acid fermentation (Mbugua, 1987; Halm *et al.*, 1993; Hounhouigan *et al.*, 1994; Amoa-Awua *et al.*, 1996; Odunfa and Oyewole, 1998). The importance of lactic acid bacteria in food

fermentation has been to effect preservation by converting sugars to organic acids thus causing a reduction in pH, by removing carbohydrates as nutrient sources and by producing antimicrobial compounds like acids, hydrogen peroxide, bacteriocins, diacetyl and other secondary reaction products. Aside their preservative role, they produce diversity in foods by altering flavour, texture and appearance of raw commodities in a desirable way. The sour aromatic flavours imparted by lactic acid fermentation are desirable traits in fermentation products and give a natural image to the products (Gilliland, 1985; Daeschel, 1989). Lactic acid bacteria are also able to colonize the human intestinal mucosa leading to beneficial effects (Fuller, 1992).

Household technology for improving food safety in Africa.

Diarrhoea due to poor hygienic conditions has long been recognized as a major health hazard for infants in Africa. Many studies have shown that contamination of infant weaning foods constitutes a potential source of diarrhoeal diseases in African children (Roland *et al.*, 1978; Barrel and Roland, 1979). The role of lactic acid bacteria in health and disease control has been documented (Sandine, 1979). Yoghurt and other milk ferments have been reported to be effective in the treatment of a variety of disorders, including colitis, constipation and diarrhoea (Sanders, 1993). The *in vitro* inhibition of growth of pathogenic microorganisms by lactic acid bacteria has encouraged investigations into its use as a prophylactic and therapeutic means of treating gastrointestinal and other diseases. *Lactobacillus acidophilus* is reported to be effective in the treatment of different types of diarrhoea in humans and chicks (Watkins and Miller, 1983). Acidophilus milk, which is yoghurt produced by fermenting milk with

Lactobacillus acidophilus, is being used to treat *Escherichia coli, Salmonella* and *Shigella*-mediated diarrhoea and dysenteries in infants in certain parts of Europe (Alms, 1983).

Other investigations have also been carried out on some lactic African foods used for feeding infants. Odugbemi *et al.* (1991) confirmed that enteropathogenic *Escherichia coli, Salmonella typhi* and *Salmonella paratyphi* were incapable of multiplying in *ogi*, a lactic fermented maize product used for feeding infants in West Africa. In other studies on South African *mahewu* (Simango and Rukure, 1991), disease-causing *Campylobacter*, *Escherichia coli* and *Shigella* spp. were unable to survive or multiply in the lactic fermented products on inoculation. Mbugua and Njenga (1991) reported the inhibition of the growth of *Staphylococcus aureus, Salmonella typhi,* enteropathogenic *Escherichia coli and Shigella dysenteriae* in *uji* a traditional weaning food of Kenya. Earlier work carried out by Mensah *et al.* (1991) on the antimicrobuial effect of fermented maize dough on the pathogen *Shigella flexneri*, and enterotoxigenic *Escherichia coli* showed that although unfermented maize dough could not inhibit growth of any of these pathogens, fermented maize dough inhibited half the strains tested when examined 8 h after inoculation. The cooking of the fermented maize dough into porridge reduced the antimicrobial effect despite the acidic pH of 3.3, and it was suggested that the antimicrobial effect of fermented maize dough is not due to pH per se but involved some unique heat-labile antimicrobial compound. Nout *et al.* (1989) also showed that lactic fermented sorghum gruels of pH less than 4 inhibited some food borne pathogens inoculated into them.

The antimicrobial properties observed for lactic fermented foods have been explained to be largely due to acid production and the consequent decrease in pH by lactic acid bacteria involved in the fermentations (Adams and Nicolaides, 1997). Other anti-microbial factors produced by lactic acid bacteria may also be involved. These include bacteriocins, low pH, carbon dioxide, hydrogen peroxide, ethanol, diacetyl, low redox potential, nutrient depletion and crowding (Adams and Nicolaides, 1997). Studies by Olasupo *et al.* (1994 and 1995) have shown that some lactic acid bacteria isolated from African fermented foods including kenkey produce bacteriocins. which have some plasmid-borne characteristics.

Low-cost method of food preservation in Africa.

The costs and infrastructural requirements of many advanced food preservation methods such as refrigeration, freezing, canning and irradiation, which are common in industrialized countries, greatly reduce their applications in the developing world (Cooke *et al.*, 1987). The inhibitory effects of lactic acid bacteria, which are used in the production of many fermented foods in Africa, have made this technology a low–cost means of preserving food in Africa (Gibbs, 1987).

In Ghana, maize is fermented as solid-state fermentation from whole maize grains or dehulled maize grains. According to Aidoo *et al.* (1982), solid-state or solid substrate fermentation is any fermentation process in which the substrate is not a free liquid and may take place on a solid or semi-solid substrate or in a nutritionally inert solid support which provides some advantage to the microorganisms with respect to access to

nutrients. Fermented maize dough so produced forms the basis for many products in Ghana such as *kenkey, nsiho, fomfom, banku, koko* and many other foods as reviewed by several authors (Whitby, 1968; Dovlo, 1970; Sefa-Dedeh, 1993). The production of *nsiho, fomfom, banku* and *koko* will be briefly described but a more detailed description of *kenkey* which is the most popular product prepared from fermented whole maize dough will be given.

2.1.2 *Nsiho*

The traditional methods for processing maize into *nsiho* and *fomfom* are shown in Fig 1. *Nsiho* is a dumpling similar in consistency to kenkey. It is a popular product in the Western and Central regions of Ghana made from dehulled maize. In the processing of *nsiho* dry maize is cleaned of foreign matter and tempered with a little quantity of water. The conditioned maize is then dehulled and winnowed to remove the hulls and chaff. The dehulled maize is washed and steeped in water for 24 h. The steep water is drained off and the maize is milled into a smooth meal in a disc attrition mill, mixed with water to form a dough and allowed to ferment for 6 to 24h. The period of fermentation can be reduced to 6 hours when the steep water is used in forming the dough. After fermentation, one half of the dough is cooked into a paste known as *aflata*, which is mixed with the remaining half uncooked dough, and moulded into balls. The balls are packaged in plantain leaves and cooked for up to 2 h or more to give *nsiho* (Whitby, 1968; Dovlo, 1970; Sefa-Dedeh, 1993; Johnson and Halm, 1998).

2.1.3 *Fomfom*

The traditional procedure for processing maize into *fomfom* is shown in Fig 1. *Fomfom* is a stiff porridge similar to *kenkey* in consistency but made from dehulled maize. Maize is dehulled and milled as for *nsiho*. The meal is mixed with water and an inoculum of old dough is added to shorten fermentation period from 24 h to 6 – 9 h. The fermented dough is shaped into balls with holes made in the centre. The balls are cooked twice in boiling water. After each boiling, the balls are pounded in a mortar. After the second pounding, the mass is moulded into balls and packaged in banana leaves ready for consumption. This product is popular in the Western region of Ghana (Johnson and Halm, 1998).

2.1.4 *Kenkey*

Kenkey is a sour tasting cooked solid dumpling of elastic consistency made from fermented maize dough shaped into balls or cylindrical forms and wrapped in maize husks or plantain leaves. It has a moisture content of between 62 to 68 %, a pH of 3.7 and a shelf life of about 3 to 4 days.

There are two main types of kenkey, the Ga kenkey locally referred to as *Komi* and Fanti kenkey known as *Dokono*. The main differences consist in the materials for packaging the balls of kenkey and the addition of salt to Ga kenkey. Fanti kenkey is wrapped in several layers of plantain leaves, which gives it a longer shelf life than the Ga kenkey.

Kenkey is a staple for most of the peoples in the coastal regions of Ghana, principally the Gas, Fantis and Ewes. The type of kenkey produced depends on the ethnic group.

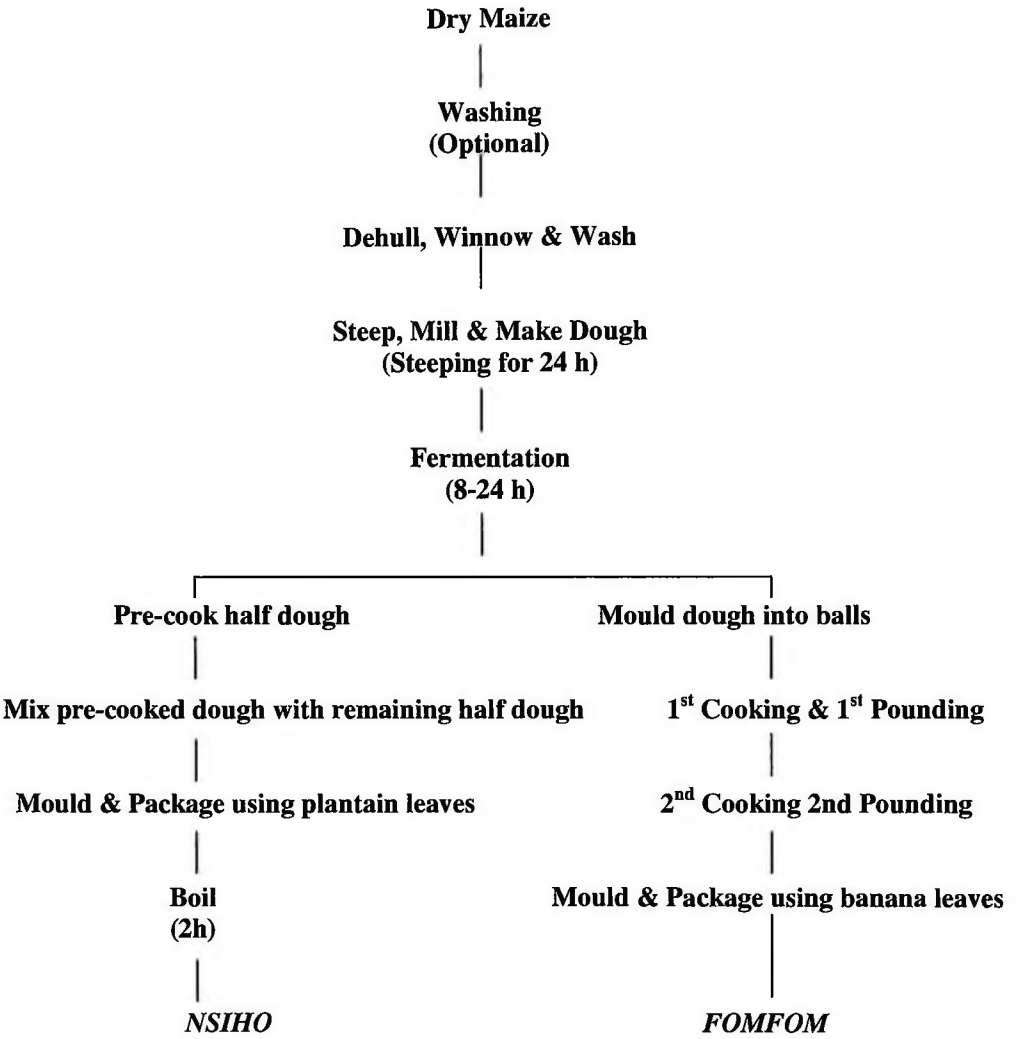

Figure 1. Flow diagram for the production of *Nsiho* and *Fomfom*. (Source: Johnson and Halm, 1998)

Ghanaians in the Central and Western Regions of Ghana produce Fanti kenkey whilst those from in and around Accra produce the Ga kenkey. However, as a result of urbanisation and movement of people from one place to the other, kenkey has assumed an important place in the diets of most workers hence it can be found in all the urban centres in Ghana as a ready-to-eat street food.

The traditional method of kenkey production has earlier been described by various authors including Christian (1966, 1970), Whitby (1968), Dovlo (1970), Nyarko-Mensah and Muller (1972), Plahar and Leung (1982), Sefa-Dedeh and Plange (1989), Halm *et al.* (1993, 1996 and 2004). It involves the following processes as illustrated in Fig 2: cleaning; steeping; milling and doughing; dough fermentation; *aflata* preparation; mixing of *aflata* and raw dough; moulding and packaging and cooking into kenkey.

Cleaning

Cleaning is done by one or a combination of several processes including, winnowing, hand picking, sieving and sedimentation. These operations remove dust, chaff, stones, insect damaged grains and other debris. The sedimentation process involves pouring the grains into a big basin of clean water, stirring with a wooden ladle to allow the mature and good quality grains to settle at the bottom whilst the less dense immature, some insect holed and diseased grains float on the surface. The latter are collected with small baskets or sieves and used as animal feed. The good maize is then washed again in water before steeping.

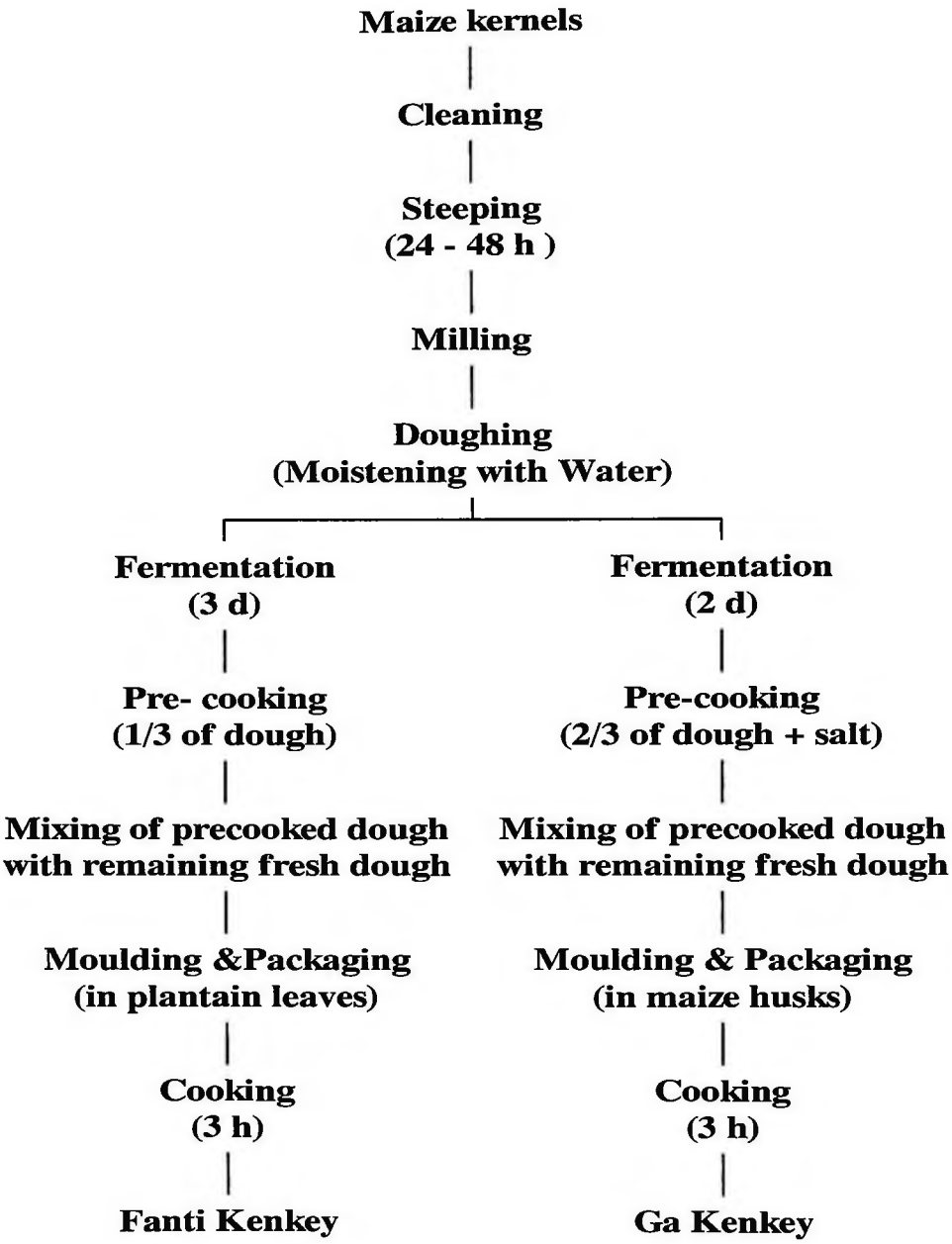

Figure 2. Flow diagram for kenkey production in Ghana. (Source: Halm *et al.*, 1996)

Steeping

Cleaned maize grains are steeped in tap water in steeping tanks for one to three days depending on the initial moisture content and hardiness of the grains. Most local varieties are steeped for one day whilst some hybrid maize with very hard kennels and high portion of testa are steeped for up to three days to soften the kernels and facilitate milling. The steep water is drained off and the maize is washed before milling. Steeping allows imbibition of water into the grains; facilitates size reduction and conditions the grains for smooth milling (Akingbala *et al.*, 1987; Sefa-Dedeh, 1993). Steeping of maize is also crucial to the production of dough with high pasting and setback viscosities (Bediako-Amoa and Austin, 1976) necessary for making aflata with the desired binding potential. It has been established that soaking of whole maize before milling remains the best option for developing the necessary dough textural characteristics (Akingbala *et al.*, 1987; Nche *et al.*, 1996).

Milling and doughing

The steeped maize is milled in a plate mill popularly known in Ghana as corn mill into a smooth meal which is mixed with water to form a dough with moisture content of about 50 to 55 %. The amount of water used to form the dough is very important as this affects the rate of fermentation as well as the quality and shelf life of the dough. This amount varies widely from one producer to another, between 17.3 and 44 litres of water to 100 kg maize (Allotey, 1996).

Fermentation

The dough is packed tightly into wooden vats, aluminium pots, enamel or aluminium basins, plastic containers etc and allowed to ferment spontaneously for up to three days. Normally after two days of fermentation, the dough is ready for use in making different products including kenkey, *banku* and *koko*. Dough fermented for twenty four hours does not give a good textured product but this can be mixed with older dough to obtain the desired texture. Sometimes the traditional processors hasten the fermentation by back-slopping with old dough. With back-slopping, fermentation can be shortened to 24 hours. Usually the processors use up all the fermenting dough between the second and third day of fermentation but if fermentation is prolonged beyond the third day it might lead to the development of undesirable flavours and high acidity. However according to some commercial producers the shelf life of the dough can be extended without adverse effect on consumer acceptance if less water is used in making the dough. The dough may also be sun dried after the desired acidity has been obtained within 2 days (Halm *et al.*, 2004).

Aflata Preparation and Mixing

A portion of the fermented dough is made into a slurry by adding 2 or 3 parts of water and cooked with continuous stirring into a sticky gelatinous paste known as *aflata.* During this process, salt is added in the case of Ga kenkey. The *aflata* is mixed thoroughly with a portion of the uncooked fermented dough using wooden ladles and allowed to cool. The ratio of *aflata* mixed with uncooked dough depends upon the type of kenkey being produced and the preference of the consumers. Amongst the indigenous

Ga people who consume kenkey as a major staple, the ratio of *aflata* to the uncooked dough is usually 1 to 1. However some producers mix two thirds of *aflata* with one-third of the uncooked dough whilst others mix one-third *aflata* with two-thirds uncooked dough. The ratio of *aflata* mixed with the uncooked dough determines the texture of the kenkey that will be produced. In a sensory evaluation of the texture of kenkey, the highest score of 8.02 was obtained by panellists for kenkey prepared from a 50/50 *aflata* to uncooked dough mixture (Bediako-Amoa and Austin, 1976). The study confirmed that '*aflatalisation*' (the process of mixing the *aflata* with the uncooked dough) is necessary to produce kenkey of the desired texture. *Aflata* is reported to act as a binding agent when mixed with uncooked fermented dough and enables the product to be moulded into balls and other shapes (Sefa-Dedeh and Plange, 1989).

Moulding and Packaging

For Ga kenkey the mixture is moulded into balls of uniform sizes of about 300 g weights and wrapped in clean pre-wetted maize husks. Ga kenkey may also be moulded into rectangular shapes and wrapped with plantain leaves just as Fanti kenkey.

Cooking

Some clean maize husks or plantain leaves are placed at the bottom of large aluminium cooking pots and the balls of kenkey are packed on top to prevent the balls from sticking to the pots during cooking. Boiling water is poured into the pot to cover the balls and the top covered with a piece of cloth or polythene sheet to conserve steam. The kenkey is cooked for about 3 to 3½ hours. The length of cooking depends on the ratio of *aflata* to

the uncooked dough and on how well the *aflata* was cooked. For kenkey containing less *aflata* boiling lasts longer. After the balls are well cooked, they are removed from the pots and placed in large bowls lined with polythene sheets that are also used to cover the balls of kenkey to keep them hot until sold for consumption. The cook water left in the pot (Ga kenkey), referred to as 'kenkey water' is collected and drunk as a thin porridge and is believed to have curative properties against malaria, diarrhoea and jaundice. It has been reported that the carbohydrate and electrolyte levels of 'kenkey water' are comparable to the UNICEF/WHO Oral Rehydration Salts and therefore suitable for use in oral rehydration in Ghana (Yartey *et al.*, 1993).

2.1.5 *Banku/Etsew*

Banku and *Etsew* are consumed along the coastal regions of Ghana. The processing steps as far as the dough preparation is concerned are similar to Ga kenkey. Fermented whole maize dough is made into a slurry with water and stirred till cooked into a semi-solid consistency. This is then moulded into balls and packaged in plantain leaves (*Etsew*) ready to be eaten with a sauce. *Banku* is the same product without any packaging (Whitby, 1968).

2.1.6 *Koko*

This is a thin gruel used as breakfast porridge consumed all over the country but especially as infant food. This is also referred to as *Akasa* in certain parts of Ghana. It is made by adding a large quantity of water to fermented whole maize dough to form a slurry which is cooked by boiling into a thin gelatinous porridge. It can also be made by

adding a large quantity of water to milled steeped maize. The mixture is strained using fine sieves to remove all chaff and the slurry is left overnight to ferment and settle. The supernatant water is decanted off leaving behind a slurry mash which is used for cooking *koko*. The flow diagram for *koko* preparation is presented in Figure 3 (Halm *et al.*, 1996).

2.2 Microbiology of Ghanaian Fermented Maize Dough

2.2.1 *Microbial Successions in Maize Fermentation*

Microorganisms develop during both the soaking of maize and dough fermentation. Investigations by various authors (Christian 1966, 1970; Halm *et al.*, 1993; Jespersen *et al.*, 1994; Olsen *et al.*, 1995) have revealed that on the maize kernels and during maize steeping, the flora consists of a complex mixed population of lactic acid bacteria, catalase-positive Gram-positive bacteria, Gram-negative bacteria, yeasts and moulds. During dough fermentation the flora changes towards the selection of a defined stable flora comprising heterofermentative lactic acid bacteria and yeasts by the end of 48 h.

2.2.2 *Gram-negative, Gram-positive catalase-positive bacteria*

On the maize grains at the start of steeping, Gram-negative and Gram-positive catalase-positive bacteria occur in levels of 1.4×10^6 cfu/g. But at the initiation of dough fermentation, the Gram-negative and Gram-positive catalase-positive bacteria begin to decrease in numbers and after 1 to 2 days of main fermentation colony-forming units of these organisms ($<10^2$/g) are not detected (Christian, 1966; Halm *et al.*, 1993; Olsen *et al.*, 1995). Fields *et al.* (1981) also found that viable coliforms disappeared from the

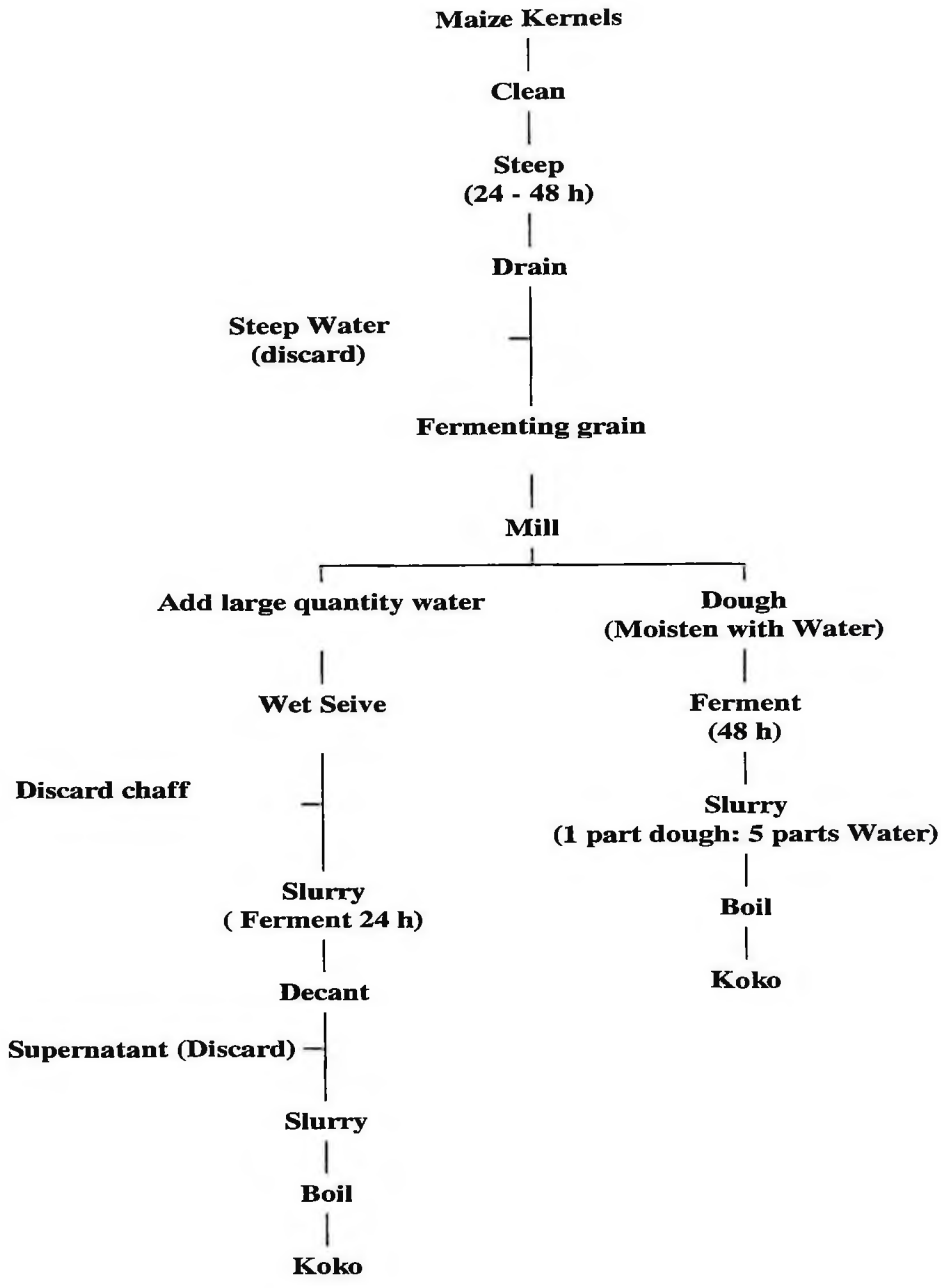

Figure 3. Flow diagram for Koko preparation. (Source: Halm *et al.*, 1996)

fermentation of corn meal during the second day. In support of these findings, Mensah *et al.* (1991) reported no significant reductions in numbers of *Shigella flexneri,* and enterotoxigenic *Escherichia coli* strains inoculated into soaked maize. But the numbers of these enteric pathogens were markedly reduced when inoculated into fermented maize dough (Mensah *et al.*, 1991). Olsen *et al.* (1995) screened a total of 241 lactic acid bacteria belonging to *Lactobacillus plantarum, Pediococus pentosaceus, Lactobacillus fermentum/reuteri* and *Lactobacillus brevis* isolated from various stages of maize dough fermentation. They found that each processing stage has its own micro- environment with strong antimicrobial activity. About half of the *L. plantarum* and practically all of the *Lactobacillus fermentum/reuteri* investigated were shown to inhibit other Gram-positive and Gram-negative bacteria, explaining the elimination of these organisms during maize dough fermentation. These observations were attributed to antimicrobial properties in fermenting maize dough and it is not surprising that enteric pathogens are inhibited in many fermented maize products (Mensah *et al.*, 1991; Simango and Rukure, 1992; Mbugua and Njenga, 1991; Annan-Prah and Agyeman, 1997).

2.2.3 *Moulds*

The mould flora forming part of the surface micro flora of maize kernels comprises mainly the genera *Penicillium, Aspergillus,* and *Fusarium* (Akinrele, 1970; Fields *et al.*, 1981; Halm *et al.*, 1993; Jespersen *et al.*, 1994). The dominating species of these genera include *Penicillium citrinum, Aspergillus flavus, Aspergillus parasiticus, Aspergillus wenttii,* and *Fusarium subglutinans* (Jespersen *et al.*, 1994). Initial high counts of 10^5 cfu

/g were reduced to less than 10^2 cfu /g within 24 h of dough fermentation. The decrease in viable mould counts during the early stage of fermentation was distinct and in agreement with other investigations on fermented maize products. Akinrele (1970) reported that moulds are eliminated early during the steeping period in ogi fermentation. Fields *et al.* (1981) also found that moulds disappeared from the fermentation of corn meal during the second day.

2.2.4 *Lactic Acid Bacteria*

Fields *et al.* (1981) identified the dominating lactic acid bacteria in spontaneous fermentation of corn meal under laboratory conditions as heterofermentative *L. fermentum, L. cellobiosus* and *Pediococcus acidilactici*. Christian (1966, 1970) reported that lactic acid bacteria involved in maize fermentation consisted of homofermentative *Pediococcus cerevisiae* and the heterofermentative species *Leuconostoc mesenteroides* and *Lactobacillus fermentum*. Halm *et al.* (1993), Jespersen *et al.* (1994) and Olsen *et al.* (1995) carried out systematic studies on the microbial ecology of the kenkey process in two large commercial production sites in Accra which extended and confirmed the findings by earlier workers. They showed that at the start of maize steeping, a mixed group of microorganisms were present including lactic acid bacteria in counts of about10^6 cfu/g of dry maize. During steeping, a significant increase in the number of the lactic acid bacteria took place reaching counts up to 10^8 cfu/g of maize. After milling and addition of water, the main fermentation started and within 0 to 72 h, growth of lactic acid bacteria took place reaching counts of > 10^9 cfu/g. By the advanced stage of fermentation, the micro-population consisted of lactic acid bacteria and yeast. For the obligately heterofermentative

lactobacilli, this pattern of fermentation was found to be closely related to *Lactobacillus fermentum* and *Lactobacillus reuteri* (Kandler and Weiss, 1986). Halm *et al.* (1993) could not differentiate between *Lactobacillus fermentum* and *Lactobacillus reuteri* due to the conventional methods of identification employed, i.e. assimilation profiles, morphological, nutritional, growth requirements and fermentation characteristics. Hayford and Jakobsen (1999) subsequently identified the strains referred to as *L. fermentum/ reuteri* as *L. fermentum* by use of Random Amplified Polymorphic DNA (RAPD).

The dominance of *Lactobacillus plantarum* in the latter stages of maize dough fermentation has been reported. Nche *et al.* (1994) identified *Lactobacillus plantarum, Lb. confusus, Lb. brevis*, and *Pediococcus pentosaceus* as the main lactic acid bacteria present in fermenting maize and maize-cowpea doughs. Olasupo *et al.* (1997) in their studies on selected African fermented foods obtained 48 *Lactobacillus* isolates from kenkey (fermented maize) which they identified as *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus delbrueckii, Lactobacillus acidophilus, Lactobacillus casei,* and *Lactobacillus cellobiosus.*

Olsen *et al.* (1995) found *L. plantarum* at the initial stages of maize fermentation where it dominated the homofermentative lactic acid bacteria present. The differences observed in the microorganisms during the latter stages of fermentation could be due to many factors such as the microbiota of the raw material maize, the indigenous microorganisms of the environment and also the spontaneous nature of the fermentations. Hounhouigan *et al.* (1993a, b, 1994) also reported that a number of lactic acid bacteria were isolated in the

initial stages of *mawé* fermentation but *Lactobacillus fermentum* or *L. reuteri* and *L. fermentum* biotype *cellobiosus* rapidly attained dominance. *Mawé* is a fermented maize dough from Benin similar to Ghanaian fermented maize dough.

Olsen *et al.* (1995) screened a total of 241 lactic acid bacteria belonging to *Lactobacillus plantarum, Pediococus pentosaceus, Lactobacillus fermentum/reuteri* and *Lactobacillus brevis* isolated from various stages of maize dough fermentation. They found that each processing stage has its own micro- environment with strong antimicrobial activity. About half of the *L. plantarum* and practically all of the *Lactobacillus fermentum/reuteri* investigated were shown to inhibit other Gram-positive and Gram-negative bacteria, explaining the elimination of these organisms during the initial processing stages. It was observed that *L. plantarum strains* from initial stages of fermentation were less inhibitory towards Gram-positive and Gram- negative organisms and two closely related species *L. plantarum* (K965) and *Lb. delbrueckii subsp.lactis* (K33) than *Lactobacillus fermentum/reuteri* strains from the advanced stage of fermentation. In addition, isolates belonging to the *Lactobacillus fermentum/reuteri* group showed a wide antimicrobial spectrum. About half of the strains from late stage of fermentation inhibited *L. plantarum* (K 965) and almost all strains inhibited *Lb. delbrueckii subsp. lactis* (K 33). Several of the strains also inhibited *Staphylococcus carnosus, Staphylococcus caseolyticus, Enterobacter cloacae, Klebsiella pneumoniae* and *Proteus mirabilis.* Widespread microbial interactions were further seen between the maize isolates amounting to 85% of all combinations tested. This decreased to 41% for the steep water then to 35% for fresh dough and 18% for the final fermented product. These observations seem to explain the microbial succession, which took place amongst lactic

acid bacteria during maize dough fermentation. The antimicrobial effect was explained by the combined effects of acids, compounds sensitive to proteolytic enzymes and other compounds with antimicrobial activity, with the acid production being the most important factor.

The antimicrobial properties observed for lactic fermented cereal foods have been explained to be largely due to acid production and the consequent decrease in pH by the lactic acid bacteria involved in the fermentations (Adams and Nicolaides, 1997). Other anti-microbial factors produced by lactic acid bacteria may also be involved. These include bacteriocins, low pH, carbon dioxide, hydrogen peroxide, ethanol, diacetyl, low redox potential, nutrient depletion and crowding (Adams and Nicolaides, 1997). Studies by Olasupo *et al.* (1994, 1995) have shown that lactic acid bacteria isolated from African fermented foods including kenkey produce bacteriocins which have some plasmid-borne characteristics. They found 4 isolates of *Lactobacillus plantarum* which produce bacteriocins. All four isolates inhibited *Lactobacillus plantarum* while only one isolate produced bacteriocin against some selected food-borne pathogens, *Pseudomonas sp., Aeromonas sorbria* and *Aeromonas cavice.*

2.2.5 *Yeasts*

Dominant Yeasts in Maize Fermentation

Halm *et al.* (1993) found *Candida* and *Saccharomyces spp.* as the most frequently isolated yeasts and therefore considered them to play a role in the aroma of the product. Further investigations by Jespersen *et al.* (1994) indicated a mixed flora comprising *Candida,*

Saccharomyces, Trichosporon, Kluveromyces and *Debaryomyces species* from raw maize during steeping and early stages of fermentation. A selection was observed during steeping and maize dough fermentation with *Candida krusei* and *Saccharomyces cerevisiae* becoming the dominating yeasts with counts exceeding 10^6 cfu /g. There was a limited increase in yeast numbers but a significant multiplication of *C. krusei* and *S. cerevisiae* was observed in all the fermentations investigated leading to a flora consisting of 62 to 71 % *C. krusei* and 14 to 18 % *S. cerevisiae* (Halm *et al.*, 1993; Jespersen *et al.*, 1994; Hayford and Jakobsen, 1999). Obiri-Danso (1994) also found that the yeast microflora of fermented maize dough consisted of *S. cerevisaie, C. tropicalis,* C. *kefyr* and *C. krusei.*

The dominant *Saccharomyces* spp. and *Candida* spp. involved in maize fermentation have been confirmed by molecular methods as *Saccharomyces cerevisiae* and *Candida krusei*, respectively (Hayford and Jespersen, 1999; Hayford and Jakobsen, 1999).

It has been suggested that the presence of large numbers of yeasts in maize dough fermentation is likely to influence the organoleptic and structural quality of the dough (Jespersen *et al.*, 1994). Nyarko and Obiri-Danso (1992) also reported that inoculation of maize dough with 10^6 cfu/g of *Saccharomyces cerevisiae* in pure cultures or in combination with various *Candida spp.* increased the organoleptic scores of the dough significantly. Yeasts are known to produce a wide range of aromatic compounds, such as organic acids, esters, alcohols, aldehydes, lactones and terpenes as reviewed by Stam *et al.* (1998). However the role of yeasts in the formation of aroma compounds in

indigenous African fermented foods and beverages has not been extensively investigated until now.

Recently, Annan *et al.* (2003a), identified a total of 76 compounds in spontaneously fermented maize dough by gas chromatography/mass spectrometry. The compounds included 21 carbonyls, 19 alcohols, 17 esters, 12 acids, a furan, 2 phenolic compounds, an alkene and 4 unidentified compounds. The majority of the compounds showed a sharp increase after 24 h of fermentation reaching a peak after 48 h. In general, alcohols and esters were produced in much larger amounts than other aroma compounds and ethanol was the most abundant alcohol. The most abundant esters were ethyl acetate and ethyl lactate. The development of alcohols and some esters coincided with high levels of yeasts. The esters found in fermented maize dough have also been reported as a result of yeast activity during beer fermentation and during spontaneous fermentation of Danish sour dough (Hammond, 1993; Hansen *et al.,* 1989). In order to identify volatile compounds produced by the microorganisms associated with maize dough fermentation, samples obtained by spontaneous fermentation and prepared by use of starter cultures were compared (Annan *et al.*, 2003b). The starter cultures examined were *Saccharomyces cerevisiae, Candida krusei* and *Lactobacillus fermentum*. Of the total of 64 volatile compounds, 51 were detected by GC-Olfactometry as contributing to the aroma of the different fermented dough samples. Spontaneously fermented maize dough was characterised by higher levels of carbonyl compounds while fermentations with *Lactobacillus fermentum* recorded the highest concentration of acetic acid. *S. cerevisiae* produced higher amounts of fusel alcohols and increasing levels of esters with

fermentation time and *C. krusei* showed similarity to *L. fermentum* with lower levels of most volatiles. Maize dough prepared with lactic acid bacteria had a lower pH and higher levels of lactic acid than doughs prepared with yeasts or spontaneously fermented. Dough fermented with starter culture of *Saccharomyces cerevisiae* had increased levels of alcohols and esters while doughs fermented with lactic acid bacteria and *Candida krusei* in general had lower levels of most volatiles (Annan *et al.*, 2003b and c).

The contribution of yeasts to the sensory characteristics of other traditional African fermented foods has also been reported. Hamad *et al.* (1992) found that fermented sorghum dough with high numbers of *Candida krusei* (10^4 cfu/g) had a more pleasant aroma than dough with less yeast. Mixed cultures of *S. cerevisiae* and *L. brevis* used for fermentation of Nigerian *ogi* resulted in a product with an aroma profile quite similar to that of traditionally produced *ogi* (Teniola and Odunfa, 2001).

It has also been reported that yeast growing in association with lactic acid bacteria stimulate the growth of the lactic acid bacteria as well as their lactic acid production by release of nutrients from autolysis, vitamin production and utilization of organic acids (Leroi and Pidoux, 1993a; b; Gobetti *et al.*, 1994). In support of this, Halm *et al.* (1993) found an early increase in acid production when mixed cultures of *Saccharomyces cerevisiae* and *L. fermentum* other than single cultures of *L. fermentum* were used as starter cultures for production of fermented maize dough. However, Houghuigan *et al.* (1996) observed that a starter culture comprising *C. krusei* in combination with lactobacilli had no significant effect on the acidity of *mawé*, a fermented maize product

from Benin, but favoured a fast growth of lactic acid bacteria such as *L. fermentum* and *L. brevis*.

Saccharomyces cerevisiae

The yeast *S. cerevisiae* belongs to the Genus *Saccharomyces,* a member of the group of teleomorphic (perfect) yeasts of the phylum Ascomycota. Members of this genus reproduce vegetatively by multilateral budding. Cells may be globose, ellipsoidal or cylindrical, psuedomycelium maybe formed but true mycelium is absent. The vegetative state is predominantly diploid or can be of higher ploidy. Nitrate is not assimilated and lactose is not utilized, while sugars are vigorously fermented (Kurtzman and Fell, 1998). Pellicles are not formed in liquid media. Early classification allocated to the genus *Saccharomyces,* a large number of species which constituted a somewhat heterogeneous mixture. In current classification system, baker's yeasts, brewer's yeasts, wine yeasts, champagne yeast are all considered to be strains of *Saccharomyces cerevisiae* (Kurtzman and Fell, 1998), with the exception of larger yeast which is now classified as *Saccharomyces pastorianus* (Vaughan-Martini and Martini, 1998).

The fermentation and assimilation profiles for *Saccharomyces cerevisiae* vary greatly and there are about eight sugars that may or may not be utilized by this species as a whole. These include galactose, melibiose, melezitose, a-Methyl- D-glucoside, D-Gluconate, DL-lactate and succinate.

Hayford and Jespersen (1999) examined the assimilation profiles of 48 isolates of *S. cerevisiae* from different stages of maize fermentation. They demonstrated 8

assimilation profiles with 50% of the isolates having the same profile i.e. being able to assimilate galactose, saccharose, DL-lactate, raffinose, maltose and glucose. Almost all 40 isolates were able to assimilate DL-lactate and 19 were able to assimilate melibiose. In addition, Hayford and Jakobsen (1999) used Pulse Field Gel Electrophoresis (PFGE), PCR and MAL genotyping techniques to determine the chromosome profiles of the 48 isolates. Based on the above techniques all 48 isolates were identified as *S. cerevisiae.* Clustering correlation analysis further grouped the strains into 5 clusters and isolates from different stages of fermentation were found in all the 5 clusters. Four different *MAL* genotypes were observed with *MAL*11 and *MAL*31 predominating. *MAL*11 was seen for all isolates whereas no evidence of *MAL*21 and *MAL*41 was observed. *MAL* refers to a locus of the genome responsible for the regulation of the uptake and hydrolysis of maltose, the main fermentable carbohydrate of maize dough. A total of five different *MAL* loci are described for the genome of *S. cerevisiae* (Hayford and Jespersen, 1999). Phylogenetic studies of *S. cerevisiae* isolated from different foods and beverage fermentations including fermented maize show characteristic patterns of *MAL* loci (i.e. *MAL* genotypes). Hayford and Jespersen (1999) demonstrated that a high number of *S. cerevisiae* isolates were involved throughout the spontaneous fermentation of maize dough.

Candida krusei

Candida krusei belongs to the Genus *Candida* a member of the group of imperfect yeasts (anarmorphic) of the phylum Ascomycota. Vegetative reproduction is by multilateral budding and the cells occur singly, budding and in chains. The cells are spheroidal,

ellipsoidal and elongate and pseudohyphae and septate hyphae may be present (Kurztman and Fell, 1998). *C. krusei* ferments and assimilates only few carbohydrates and carbon compounds. Glucose is the only carbohydrate fermented and the carbon compounds assimilated are glucose, D-glucoseamine, N-acetyl-D-glucoseamine, glycerol, DL-lactate, succinate, L-sorbate (weak) and citrate (weak). C. *krusei* is unable to assimilate nitrate (Kreger-van Rij, 1984). Pellicle is formed in liquid media.

C. krusei can grow in vitamin-free medium, but not on 50% (w/w) glucose-yeast extract agar. Furthermore, C. *krusei* grows at 37°C and has a pH optimum between 4 to 5 (Kreger-van Rij, 1984).

With respect to *C. krusei,* chromosome profiles, PCR profiles, restriction endonuclease analysis (REA), and Southern blot hybridization techniques, revealed DNA polymorphism that enabled subspecies typing of 48 isolates of *C. krusei.* On the basis of PCR and REA profiles, isolates were grouped into clusters. The results showed that several strains of *C. krusei* were involved in the fermentation of maize dough from the onset and remain dominant throughout the fermentation (Hayford and Jakobsen, 1999). Of 48 *C. krusei* isolates obtained from fermented maize dough for kenkey production in Ghana, 82% had the same assimilation profiles being able to assimilate N-acetyl-glucoseamine, DL-lactate, glycerol and ferment glucose (Hayford and Jakobsen, 1999).

In the fourth edition of 'The Yeasts, A Taxonomic Study', *C. krusei* is considered to represent the anamorphic form of *Issatchenkia orientalis* because the type strains as well as other isolates of both species showed significant (93-100%) DNA base complimentarity (Kurztman and Fell, 1998; Kurztman *et al.*, 1980b). *Issatchenkia*

orientalis and its anamorph *C. krusei* are found in a wide variety of habitats including humans and animals. Hurley *et al.* (1987) summarised clinical evidence supporting evidence that *C. krusei* should be considered a pathogen rather than a transient saprophyte. However, considering the predominance of *C. krusei* in many African fermented foods, it is almost impossible to assign it a pathogenic status (Goldman *et al.*, 1993).

C. krusei has been found dominant in many African fermented cereals such as maize and sorghum (Halm *et al.*, 1993; Jespersen *et al.*, 1994; Hayford and Jakobsen, 1999; Hounhouigan *et al.*, 1994; Hamad *et al.*, 1992), root crops like cassava (Amoa-Awua *et al.*, 1997; Oyewole, 1990) and alcoholic beverages such as palm wine in Ghana, sorghum beer in West Africa, Busua beer in East Africa (Nout, 1980, Konlani *et al.*, 1996a). C. *krusei* appears to be capable of tolerating a low oxygen tension and an increasing acidity (Kreger-van Rij, 1984). Perhaps it is the acid resistant nature of this yeast that allows it to survive in acid fermented products (Spicher and Schröder, 1980).

2.2.6 *Interactions Between Yeasts and Moulds*

Studies conducted by Halm and Olsen (1996) and Lei (1996) determined the inhibitory effects in a laboratory medium of the two dominating yeast species *C. krusei* and *S. cerevisiae* from fermented maize dough against *Penicillium citrinum, Aspergillus flavus* and *Aspergillus parasiticus* isolates from maize and known mycotoxin producing strains of the same species. Distinct growth inhibitory effects of the two yeasts were demonstrated against 12 isolates of the 3 mould species from maize as well as 15

mycotoxin producing strains of the same mould species. The *Candida* isolates were found to have a greater inhibitory potential than the *Saccharomyces* isolates and the *Penicillium spp.* were more sensitive to the yeasts than the *Aspergillus spp.*

The types and numbers of microorganisms growing with the mould and the volatile metabolites from these organisms, affect mould growth and sporulation (Barr, 1976; More-Landecker and Stotzky, 1972, 1973 and 1974). Reports can be found on the inhibitory and stimulatory growth effects of associative growth of bacteria and moulds on mycotoxin production (Barr, 1976; Coallier-Ascha and Idziak, 1985; Cueroo *et al.*, 1987; Nout, 1989; Wechbach and Marth, 1977). In addition, yeasts produce a variety of compounds including organic acids, esters, alcohols and aldehydes (Janssens *et al.*, 1992). Some of these e.g. ethyl acetate is known to reduce mould growth and spore germination (Saksena and Tripathi, 1987) and killer toxins (extracellular glycoproteins) can inhibit both yeasts (Rosinii, 1983) and moulds (Polonelli *et al.,* 1990).

Preliminary investigations conducted to determine the mechanism of inhibition of moulds by the yeasts isolated from maize fermentation, indicated that mould growth was not affected by either yeast supernatant or cell free extracts suggesting that the inhibitions were not due to compounds produced by the yeasts (Halm and Olsen, 1996; Lei, 1996). They concluded that the inhibitions observed against the moulds were due to competition for nutrients between the yeasts and the moulds.

2.2.7 *Yeast-Yeast Interactions*

Yeast-yeast interactions can be typified by antagonism, where killer toxins produced by one species or strain inhibits or eliminates another species or strain (Young, 1987; Shimizu, 1993). Killer activity has been found in more than 100 species representing about 20 genera among both ascomycetous and basidiomycetous yeasts. Some of these genera include, *Saccharomyces, Hansenula, Pichia, Candida, Kluyveromyces, Debaryomyces, Cryptococcus* and *Torulopsis* (Golubev, 1998). Yeast- yeast interactions have not been previously reported in maize fermentations.

2.3 Anti-Microbial Principles Associated with Lactic Acid Bacteria

Lactic acid bacteria are often inhibitory to other microorganisms and this is the basis of their ability to improve the keeping quality and safety of many fermented foods. The generally known factors that contribute to this inhibition include the following: low pH; organic acids; hydrogen peroxide; diacetyl; bacteriocins; reuterin; ethanol; carbon dioxide; nutrient depletion and crowding and low redox potential.

2.3.1 *Low pH*

The production of organic acids by lactic acid bacteria and the consequent decrease in pH is the most important factor controlling and inhibiting microbial growth (Adams and Moss, 1995). pH is not only important in determining the fate of bacteria in foods, their survival and growth in manufacturing environments may also be affected by pH (Brown and Booth, 1991).

The pH limits for microbial growth are in the range of 1 to 11 but most microorganisms associated with foods have optimum growth pH in the range of 5 to 8. In general, yeasts and moulds can grow at more acidic pH than the bacteria.

Many factors reduce the pH range for the growth of microorganisms including implicit factors such as competing microbial flora, reduced oxygen tension or heat damage during processing, reduced water activity or the presence of acid-potentiated preservatives which can all influence pH tolerance. Adverse pH will influence all factors used to characterise microbial growth: lag time, growth rate and yield (biomass). Above and below the pH optimum, lag time is extended, while growth rate and yield are reduced (Brown and Booth, 1991).

Microbial cells are said to be surviving or in the stationary phase when growth stops. Cells may be metabolically active if growth has stopped because of adverse conditions such as low pH e.g. in the post souring of yoghurt (Robinson and Tamime, 1981) or during repair after sub-lethal injury caused by a combination of heat and low pH (Ray, 1986). As the external pH drops, the maintenance energy requirement of microbial cells increases, because energy- driven pumps are used to control the internal environment by transporting protons across the cell membranes (Kashket, 1985; Brown and Booth, 1991; Russell, 1992).

When net flow of protons H^+ to and from the cell becomes uncontrolled, then the internal pH eventually changes to an extent that the synthesis of cellular components and the cell's ability to divide are inhibited. Under these conditions there is no growth, and the length of time that the cells remain viable becomes critical to their ability to cause

spoilage or infection or to grow if conditions alter to within their growth limits (Booth, 1985; Brown and Booth, 1991). Cells may also be injured or killed by pH values below the minimum for growth (Ahamad and Marth, 1989) and this may reduce the range of conditions under which growth can be initiated subsequently (Ray, 1986).

Increasingly, acidic conditions ultimately preclude the growth of microorganisms, interfering with the synthesis of cellular components, and inducing cell death as a result of damage to the outer membrane (Rowbury, 1995), disruption of cytoplasmic pH homeostasis, and subsequent damage to DNA and enzymes (Raja *et al.*, 1991a).

Many factors such as storage temperature and water activity can affect the survival times of non-growing cells at adverse pH values. Low temperatures and low water activity can extend survival times and there are marked differences both between species and between cells of the same species produced under different growth conditions (Brown and Booth, 1991). Gram-positive bacteria usually survive for longer periods than Gram-negative bacteria but the viability of spores is unaffected by storage at low pH (Gould *et al.*, 1983). Bacteria tend to be more fastidious in their relationships to pH than moulds and yeasts, with the pathogenic bacteria being the most fastidious (Table 1).

2.3.2 Organic Acids

Organic acids are present in food either as an end product of fermentation or from direct addition. The most common classical preservative agents are the weak organic acids, e.g. acetic, lactic, benzoic and sorbic acids. These molecules inhibit the outgrowth of both bacterial and fungal cells and sorbic acid is also reported to inhibit the germination and

Table 1. Minimum pH values for the growth of food-poisoning bacteria

Species	Minimum pH	Temperature	Reference
Gram-negative			
Aeromonas hydrophila	5.5	-	Brown & Booth, 1991
Escherichia coli	4.4	37°C	Lin *et al.*, 1995
Escherichia coli	4.1	30°C	Ostling & Lindgren., 1993
Salmonella typhimurium	4.0	37°C	Lin *et al.*, 1995
Shigella flexneri	4.8	37°C	Lin *et al.*, 1995
Campylobacter spp	5.0		Stern & Kazmi, 1989
Gram- positive			
Bacillus cereus	4.9	30°C	Brown & Booth, 1991
Clostridium botulinum	4.8-5.0		Hauschild, 1989
Staphylococcus aureus	4.5	37°C	Bergdoll, 1989
	5.15*		Bergdoll, 1989
Listeria monocytogenes	4.4	30°C	George *et al.*, 1988
	5.23	4°C	George *et al.*, 1988

2.3.2.1 Mode of Action of Weak Organic Acids

In solution, weak acids exist in a dynamic equilibrium between the undissociated uncharged molecules and their respective dissociated charged anions for example lactic acid/lactate. The proportion of undissociated acid increases as pH declines; the pH value

at which there exist equal proportions of molecular acids and charged anions, is termed the pK_a. Weak organic acids have optimal inhibitory activity at low pH because this favours the uncharged, undissociated state of the molecule, which freely diffuses across the bulk lipids of the plasma membrane because of its lipid solubility and is thus able to enter the cell (Stratford and Rose, 1986). Therefore, the inhibitory action is classically believed to be due to the compound crossing the plasma membrane in the undissociated state. Subsequently, upon encountering the higher pH inside the cell (pH_i), the molecule dissociates resulting in the release of charged anions and protons, which cannot cross the plasma membrane. Bacteria maintain internal pH near neutrality to prevent conformational changes to the cell structural proteins, enzymes, nucleic acids and phospholipids. Protons generated from intracellular dissociation of the organic acid acidify the cytoplasm. Acidification of the cytoplasm may prevent growth by inhibition of glycolysis and essential metabolic reactions (Krebs *et al.*, 1983), membrane disruption and prevention of active transport (Freese *et al.*, 1973; Stratford and Anslow, 1998) or by interference with signal transduction; pH_i is increasingly recognised as having a role in signalling (Thevelein, 1994); stress on intracellular pH homeostasis (Salmond *et al.*, 1984; Cole and Keenan, 1987) and accumulation of toxic anions (Eklund, 1985; Russell, 1992). Affected microorganisms are rarely killed but growth is prevented. After

extended lag periods lasting days or even weeks, growth is poor and cell yields are greatly reduced (Lambert and Stratford, 1999). Cherrington *et al* (1990) reported that the inhibitory effects of formic and propionic acids on the growth and metabolism of *E. coli* were related to reduction in rates of RNA, DNA, protein, lipid and cell wall synthesis.

According to Mitchell's chemiosmotic theory (Mitchell, 1966), the cytoplasmic membrane is impermeable to protons and the protons must be transported to the exterior. This proton extrusion creates an electrochemical potential across the membrane called the proton motive force (PMF). The PMF is a function of the differences in membrane potential ($\Delta\psi$) and pH (ΔpH) and is defined as $\Delta p = \Delta\psi - Z\Delta pH$, where $Z = 2.3\ RT/F$ (R = gas constant; T = absolute temperature; F = Faraday constant). The extrusion of protons generated by the organic acid inside the cell uses energy in the form of ATP, therefore the constant influx of protons will eventually deplete cellular energy as illustrated in Figure 4. As the energy reserve is limited, proton extrusion through proton pumps stops, causing a lowering of pH_i, which in turn denatures proteins and destabilizes other structural and functional components (enzymes, nucleic acids and phospholipids) of the cells and interferes with viability and growth (Booth, 1985; Booth and Kroll, 1989). In addition, the cell will accumulate the acid anion that can disrupt intracellular processes (Russell, 1992). Thus the antimicrobial potential of a weak organic acid will be determined in part by its pK_a, which describes how much undissociated, and therefore permeant acid is present at a given pH. It will also be determined by the intrinsic toxicity of the acid anion, differences which account for the differing antimicrobial effect of organic acids with similar pK_a values.

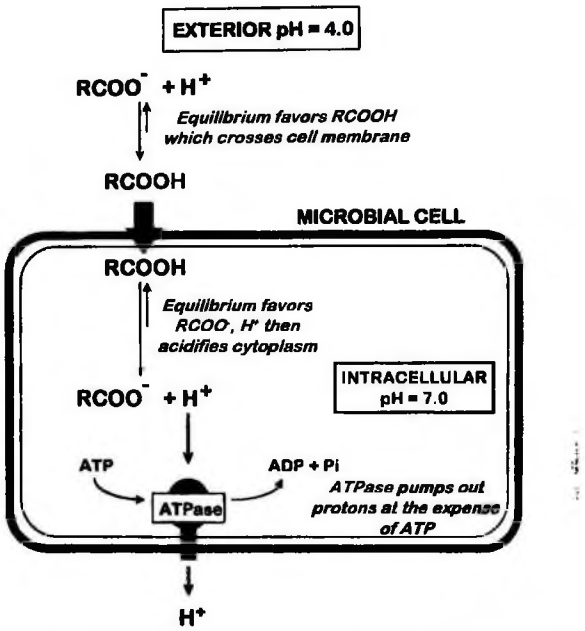

Figure 4. Fate of an organic acid (RCOOH) in a low-pH environment in the presence of a microbial cell. (Source: Davidson, 1997).

At one time the antimicrobial effectiveness of weak organic acids was considered only to be due to undissociated molecules. Thus lactic acid with a lower pK_a was thought to be of less value as an antimicrobial agent than acetic or propionic acid in food systems with pH 5.0 and above. Recent studies have shown that the combined effects of dissociated and undissociated molecules are responsible for the total antimicrobial effects of weak acids. These molecules have significant effects on bacterial cytoplasmic membranes, interfering with metabolite transport and maintenance of membrane potential (Ray and Sandine, 1992).

In yeasts, it has been proposed that the actual inhibitory action of weak organic acids could be due to the induction of an energetically expensive protective mechanism stress response that attempts to restore homeostasis and results in the reduction of available energy pools for growth and other essential metabolic functions (Holyoak *et al.*, 1996).

According to Ray and Sandine (1992) the antimicrobial effectiveness of weak organic acids (such as acetic, propionic and lactic acids) is influenced by many different factors. Therefore to effectively use these acids to control growth of spoilage and pathogenic microorganisms in foods, the following factors should be considered:

a. The particular acid, its concentration and pK_a and pH of the food system- these factors influence the proportion of undissociated acid molecules such that an acid with higher pK_a used at a high concentration and at a lower pH will be most effective. However regulatory agencies control the maximum concentrations of an acid that can be incorporated into a food. In most food systems with a pH above 5.0 where spoilage and pathogenic bacteria are of concern, acetic and propionic acids are more effective than lactic acid on an equimolar basis because of the lower pK_a of lactic acid.

b. Lipophilic property and solubility of the acid- an acid that is highly lipophilic can pass through the cell membrane more easily and is more soluble in the aqueous phase will have the greatest antimicrobial effect. Lactic acid is less lipophilic than acetic or propionic acids.

c. Temperature of exposure- the antimicrobial effect of an acid is increased at high temperature as compared to low temperature.

d. Resistance of microorganisms- there are differences in the abilities of microorganisms to survive and or grow at the pH of their environment. Yeast and moulds are able to grow at lower pH than most bacteria and among bacteria Gram-negative bacteria are more sensitive to lower pH than Gram-positive bacteria. Similarly fermentative bacteria are more resistant to lower pH than respiratory bacteria. Spores are also more resistant to lower pH than vegetative cells.

e. Microbial load in the food- at a higher microbial load a given concentration of acid will be less effective.

f. Food composition- food components can act as a physical barrier or by neutralising or buffering can reduce the antimicrobial effectiveness of an acid.

g. Other factors – anaerobic conditions increase the antimicrobial effects of an acid over that of aerobic conditions. For example, propionic acid will have a higher efficiency with CO_2. Acetic acid and lactic acid have a synergistic effect when used together. The addition of lactic acid or ethanol alone or in combination collapsed cytoplasmic pH and contributed to cell death of *Escherichia coli* O157:H7 (Jordan *et al.*, 1999).

2.3.2.2 Specific Anti-Microbial Properties

Lactic acid and salts

Lactic acid ($CH_3CHOHCOOH$; $pK_a = 3.86$) is the characteristic fermentation product of lactic acid bacteria and may cause a pH change in the growth medium sufficient to antagonise microorganisms (Piard and Desmazeaud, 1991). Lactic acid bacteria can produce up to 1.0 to 1.5% lactic acid. In fermented products the acid production is the

main key controlling manufacturing processes. While lactic acid and its salts act as preservatives in food products, they are used primarily for acidification and flavour enhancement.

Lactic acid may reduce pH to a level where putrefactive (e.g. clostridia and pseudomonads), pathogenic (e.g. salmonellae and *Listeria* spp.) and toxigenic bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*) will either be inhibited or destroyed (Holzapfel *et al.*, 1995). Lactic acid inhibited spore-forming bacteria at pH 5.0 but was more effective against yeast and moulds (Woolford, 1975a) Lactic acid is about four times more effective than malic, citric, propionic, or acetic acid in limiting growth of *Bacillus coagulans* in tomato juice (Rice and Pederson, 1954).

Cold-pack cheese normally containing lactic acid was formulated with acetic acid. The cheese was then inoculated with *Salmonella typhimurium* and destruction of cells was monitored over time. Destruction rates for *S. typhimurium* were similar to those with acetic and lactic acids (Park *et al.*, 1970). Smulders *et al.* (1986) and Snijders *et al.* (1985) found that lactic acid at 1 to 2% reduces *Enterobacteriaceae* and aerobic mesophillic microorganisms on beef, veal, pork, and poultry and delays growth of spoilage microflora during long-term storage of products. Sodium lactate (2.5 to 5.0%) inhibits *Clostridium botulinum*, *Clostridium sporogenes*, *Listeria monocytogenes*, and spoilage bacteria in various meat products (Maas *et al.*, 1989; Unda *et al.*, 1991; Chen and Shelef, 1992; Pelroy *et al.*, 1994). Growth of *Escherichia coli* is inhibited at pH 5.1 by lactic acid as compared to pH 4.5 in presence of hydrochloric acid (Gudkow, 1987).

Glass *et al.* (1992) demonstrated that lactic acid had a greater inhibitory effect on *Escherichia coli* O157:H7 at an equivalent pH than did TSB acidified with HCl. At pH levels of 4.0 and 3.5, the population of *Escherichia coli* O157:H7 was inactivated (4-log10 cfu/ml reduction) within 17 and 10 days, respectively where as no viable cells were detected within 7 days or 24 h in broth acidified with lactic acid to pH 4.0 or 3.5, respectively (Glass *et al.*, 1992). The rapid reduction of pH below 5.3 during raw sausage fermentation has been shown to be sufficient to inhibit growth of salmonellae (Schillinger and Lücke, 1988), and *Staphylococcus aureus* (Hechelmann *et al.*, 1988).

Lactic acid and other monocarboxylic acids might, in high concentrations, be potential inhibitors of yeast growth and survival (Arneborg *et al.*, 1995). In aerobically grown cultures the anionic form of lactic acid can be taken up actively by an electroneutral proton-symport with a proton-acid stoichiometry of 1:1. The permease has been reported both for *Saccharomyces cerevisiae* (Cássio *et al.*, 1987; Narendranath *et al.*, 2001) as well as for other yeasts such as *Candida utilis* (Leão and van Uden, 1986).

Very little research has been done specifically on the mechanism of action of lactic acid against food borne microorganisms. It is presumed that the mechanism is similar to other organic acids and has a primary mechanism involving disruption of the cytoplasmic membrane PMF (Eklund, 1989). It has also been suggested that the undissociated acid on account of its fat solubility (Brown and Booth, 1991), will diffuse into the bacterial cell thereby reduce the intracellular pH and slow down metabolic activities. Organic

acids such as lactic acid are also able to chelate elements essential for growth, such as iron, which may be a possible mechanism of inhibition (Shelef, 1994).

There have been some speculations concerning the mechanism of action of lactate salts used at concentrations of 2.5% and higher. It is known that lactate salts have minimal effects on product pH as most of the lactate remains in the anionic form, which is less effective. Some evidence exists that high concentrations of lactate salts reduce water activity sufficiently to inhibit microorganisms (Debevere, 1989). However, Chen and Shelef (1992) measured the water activity of cooked meat model systems and liver sausage, respectively, containing lactate salts up to 4% and concluded that water activity is not sufficient to inhibit *L. monocytogenes*. They suggested that the inhibition could be due to the combined effect of sufficient undissociated lactic acid obtained from the high concentration of lactate used together with a slightly reduced pH and water activity.

Acetic acid

Acetic acid (CH_3COOH), because of its higher dissociation constant (pK_a 4.75) shows stronger inhibition than lactic acid (pK_a 3.86) at a given molar concentration and pH. Acetic acid is produced by heterofermentative lactic acid bacteria (*Leuconostoc* spp. and some lactobacilli) in equimolar amounts with lactic acid from hexoses and is usually present in small concentrations as a result of lactic acid bacteria metabolism where it may constitute a vital factor for the establishment of the initial lactic acid bacteria population (Piard and Desmazeaud, 1991).

It has been confirmed that acetic acid blocks the uptake of amino acids (Sheu and Freese, 1972). Acetic acid was shown to inhibit amino acid uptake non-competitively in membrane vesicles of *Bacillus subtilis* but it did not inhibit NADH reduction. It was also demonstrated that sugar uptake in cells was unaffected. Denaturation of protein by acetic acid has been shown as a mechanism of action (Reynolds, 1975) and lowering of internal pH by acetate in bacteria has been demonstrated by Huang *et al.* (1986). Acetic acid has also been shown to cause lowering of internal pH in yeasts (Carmelo *et al.*, 1997; Guldfeldt and Arneborg, 1998; Arneborg *et al.*, 2000).

Woolford (1975b) confirmed the effectiveness of acetic acid at pH 6, 5, and 4. The author reported that *Bacillus* species and Gram-negative bacteria were more inhibited than lactic acid bacteria, *Clostridium*, yeasts and moulds. As the pH decreased to 4, however, the latter four groups were similarly affected. It has been shown through studies with *Listeria monocytogenes* that acetic acid caused greater inhibition than lactic acid and citric acids, and inhibition increased as the temperature of incubation increased (Ahamad and Marth, 1989, 1990).

Sorrells *et al.* (1989) compared acetic, citric, malic, lactic and hydrochloric acids for their antimicrobial action on *L. monocytogenes*. At equal pH values, acetic was superior to lactic, citric, malic and hydrochloric acids at all incubation times and temperatures. Acetic acid is applied in meat decontamination, salad dressing, pickled products and infant food formulas (Doores, 1993).

2.3.2.3 Resistance Mechanisms of Bacteria against Organic Acids

As reviewed by Russel (1991), resistance mechanisms of bacteria against weak organic acids are essentially of two types:

1. Intrinsic resistance, which is a natural (innate) chromosomally, controlled property of an organism.
2. Acquired resistance resulting from genetic changes in a bacterial cell and arising either by mutation or by the acquisition of genetic material from another cell (usually via a plasmid).

Gram-positive bacteria lack an outer membrane in their cell walls. Consequently preservatives can easily enter these cells and their intrinsic resistance is therefore low. In contrast, the cell walls of Gram-negative bacteria possess an inner and an outer membrane. The outer membrane of Gram-negative bacteria plays an important role in regulating the accessibility of a cell to preservatives and small molecules (Nikaido and Vaara, 1985; Helander *et al.*, 1997). The lipopolysaccharide layer acts as a barrier to the entry of long chain fatty acids (Helander *et al.*, 1996).

Another type of intrinsic resistance is the possession of specific enzymes that enable bacteria degrade preservatives. An example of this is degradation of methyl *para* (4)-hydroxybenzoate by *Pseudomonas aeruginosa*. However, this mechanism is rare at in-use preservative concentrations (Hugo and Foster, 1964).

Recently, inducible resistance mechanisms in microorganisms have been more extensively studied. It is known that members of the family *Enterobacteriaceae* can encounter many potential stress factors in their 'natural' habitat e.g. the extremely low pH in the stomach or large amounts of weak organic acids in the intestine. In *E. coli* three distinct low-pH-induced acid resistance (AR) systems have been identified (Lin *et al.*, 1995). These include:

(1) Acid-induced oxidative system, which is expressed in oxidatively metabolising bacteria grown in complex media, but will protect cells in minimal medium to pH 2.5. This system is not apparent in fermentatively metabolising cells (cells grown in complex media containing glucose)

(2) Acid-induced arginine-dependent system, which is expressed in fermentatively metabolising cells which will also protect cells in minimal medium to pH 2.5 but only if the medium is supplemented with arginine (arginine-dependent acid resistance);

(3) A glutamate-dependent system, which is expressed in fermentatively metabolising cells which will also protect cells in minimal medium to pH 2.5 but only if the medium is supplemented with glutamate (glutamate-dependent acid resistance). This system is not induced by low pH in *E. coli.*

Studies with 11 strains of enterohemorrhagic *E. coli* (O157:H7) and 4 commensal strains of *E. coli,* have shown that the arginine and glutamate dependent acid resistance systems were both effective in protecting all the *E. coli* strains against the bactericidal effects of 20 mM benzoic acid at pH 4.0 (Lin *et al.*, 1996). It was however found that the oxidative acid resistance system was much less effective. Lin *et al.* (1996) also characterized the

genetic aspects of *E. coli* acid resistance and showed that the alternate sigma factor RpoS is required for the oxidative resistance system but only partially involved with the arginine- and glutamate-dependent acid resistance systems. RpoS is an alternate sigma factor (σ^S) involved in regulating the expression of a variety of stress response genes (Hengge-Aronis, 1993). These genes enable the cell to transport glutamate from the acidified media to the interior of the cell where it is converted by glutamate decarboxylase to γ-amino butyric acid. This basic amine presumably provides a buffering effect and maintains the internal pH homeostasis of the cell (Waterman and Small, 1996a, b). The arginine dependent acid resistance system was thought to utilize an inducible arginine decarboxylase encoded by *adiA* and the regulator *cysB* while the glutamate dependent acid resistance system utilizes an inducible glutamate decarboxylase.

Castanie-Cornet *et al.* (1999) have further defined the genetics and control of the three acid resistance systems in *E. coli*. They showed the oxidative resistance system to be also dependent in many situations on the cyclic AMP receptor protein. They confirmed earlier reports on the dependence of arginine, the structural gene for arginine decarboxylase *adiA* and the regulator *cysB* by the arginine dependent AR system. The third AR system required glutamate for protection at pH 2.5, one of two genes encoding glutamate decarboxylase (*gadA or gadB*), and the gene encoding the putative glutamate:γ-aminobutyric acid antiporter (*gadC*). Only one of the two glutamate decarboxylases was needed for protection at pH 2.5. However, survival at pH 2 required both glutamate decarboxylase isozymes. Stationary phase and acid pH regulation of the

gad genes proved separable. Stationary phase induction of the *gadA* and *gadB* required the alternative sigma factor σ^S encoded by *rpoS*. However, acid induction of these enzymes, which was demonstrated to occur in exponential- and stationary-phase cells, proved to be σ^S independent. Neither *gad* gene required the presence of volatile fatty acid for induction. The data also indicated that AR via the amino acid decarboxylase systems requires more than an inducible decarboxylase and antiporter. They also found that the σ^S –dependent oxidative system, originally thought to be acid induced, actually proved to be induced following entry into stationary phase regardless of the pH . However, an inhibitor produced at pH 8 somehow interferes with the activity of this system, giving an illusion of acid induction. The results also revealed that that the AR system affording the most effective protection at pH 2 in complex medium (either Luria-Bertani broth or brain heart infusion broth plus 0.4% glucose) is the glutamate dependent GAD system. Thus, *E. coli* possesses three overlapping acid survival systems whose various levels of control and differing requirements for activity ensure that at least one system will be available to protect the stationary-phase cell under naturally occurring acidic environments.

Salmonella typhimurium is also known to possess two low-pH-inducible acid tolerance response (ATR) systems that function in minimal or complex medium to protect cells to pH 3.0 and depend on the physiological status of the cell. The two systems are designated log-phase ATR and stationary-phase ATR (Lin *et al.*, 1995). Log-phase ATR can be induced in two ways, adaptation at pH 5.8 for one doubling or a 20- to 90-min acid shock at pH 4.3. Acid survival studies with *S. typhimurium* using minimal medium

have shown two acid-inducible log-phase ATR systems, one of which is RpoS independent and the other is RpoS dependent (Lin *et al.*, 1995). The *S. typhimurium* studies also revealed two stationary–phase ATR systems, one that is acid inducible and RpoS independent, and one that is unresponsive to pH but RpoS dependent. The RpoS-dependent system appears to be the stationary-phase general stress resistance system (Hengge-Aronis, 1993), which includes resistance to low pH (Lee *et al.*, 1994). These systems have also been found in *E. coli* but *S. typhimurium* does not possess the amino acid-based inducible AR systems found in *E. coli* (Small *et al.*, 1994; Lin *et al.*, 1995).

Shigella flexneri also possess similar acid survival systems (AR) as *E. coli.* These are the oxidative system and the glutamate-dependent system but not the arginine- dependent AR system. The glutamate system in *S. flexneri* depends upon RpoS. *S. flexneri* does not possess the low pH or acid-inducible log-phase and stationary-phase acid tolerance response(ATR) systems found in *S. typhymurium* and *E. coli* (Small *et al.*, 1994; Lin *et al.*, 1995).

2.3.2.4 Resistance Mechanisms of Fungi against Organic Acids

Similar inducible resistance mechanisms have been documented in fungi. Extensive studies have been carried out on the resistance of spoilage yeasts to weak organic acid preservatives and this is known to depend on the H^+ -pumping P-type membrane ATPase (Holyoak *et al.*, 1996). Studies by Kubo and Lee (1998) have shown that compounds that inhibit the plasma membrane H^+-ATPase synergistically enhance the activity of sorbic acid. Piper *et al.* (1997) showed that the long-term stress response of yeasts to

weak organic acids also involves the induction of the intergral membrane protein, Hsp30, which down regulates the increased activity of the membrane ATPase. From these observations it was deduced that Hsp30 acts as a molecular 'switch' that down regulates the activity of the membrane ATPase in order to conserve cellular energy pools which the enzyme would consume in attempt to restore homeostasis (Braley and Piper, 1997). Studies by Henriques *et al.* (1997) have shown that *Saccharomyces cerevisiae* is able to extrude C-14 labelled benzoic acid, suggesting that there is an efflux system, which may be membrane localized that removes accumulated anions from inside the cell. This view is supported by data gathered by Piper *et al.* (1998) that demonstrated the existence of a multidrug resistance pump, the ATP binding cassette transporter, Pdr12, that actively extrudes preservative anions from the cell. The current understandings of the mechanisms that are involved in weak acid resistance in yeasts are summarized in Figure 5. Shown are a glucose transporter, the membrane located Pdr12 multidrug resistance pump active against anions of acetic, sorbic and benzoic acid and the plasma membrane P-type H^+ -ATPase (Piper *et al.,* 1998).

As shown in the diagram, the pumping of preservative anions out of the cell could create a futile cycle where the anions re-associate at the lower external pH and re-enter the cell. This would be under the assumption that any rate of diffusion across the plasma membrane remains the same and that the cell makes no effort to alter membrane composition or structure to reduce the access of the toxic compound. Studies by Loureiro-Dias (1998) have shown that adapted yeasts reduce the diffusion coefficient of preservatives across the plasma membrane such that passage of weak acid preservatives into the cell is reduced. Therefore, efflux of protons and anions by the H^+-ATPase and

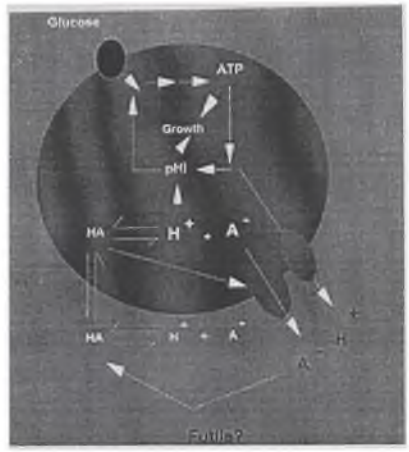

Figure 5. A schematic diagram of the stress response of a yeast cell challenged with weak organic acids (Piper *et al.*, 1998).

Pdr12, respectively, would not create a futile cycle if there were a concurrent reduction in the ability of the compounds to diffuse across the cell membrane and enter the cytosol (Brul and Coote, 1999).

2.3.3 Hydrogen Peroxide

A number of lactic acid bacteria produce hydrogen peroxide in the presence of molecular oxygen together with lactate, pyruvate and NADH by the activity of flavoprotein oxidases (Earnshaw, 1992). Since lactic acid bacteria are catalase-negative, H_2O_2 can accumulate in the medium and it's inhibitory effects are well documented (Earnshaw, 1992; DeVuyst and Vandamme, 1994b). Lactic acid bacteria appear to be more resistant to the effect of H_2O_2 than many other bacteria. The minimum inhibitory concentration of

H_2O_2 for *Pseudomonas* was found to be 2 to 8 mg/ml (Price and Lee, 1970), and 23-35 mg/ml (Gudkow, 1987). The minimum inhibitory concentration for *Staphylococcus aureus* is 125 mg/ml (Wheater *et al.*, 1952; Dahiya and Speck, 1968).

The amounts of H_2O_2 accumulated by lactic acid bacteria are quite variable and will critically depend on the degree to which the culture medium is oxygenated. Improved H_2O_2 production has been noted at low temperatures (Dahiya and Speck, 1968), when the solubility of oxygen is higher, and when shaken cultures are used (Collins and Aramiki, 1980). Levels below 1 mg/ml have been reported for lactococci grown in static cultures and for the starter, *Pediococcus cerevisiae* (Ross, 1981; Raccach and Baker, 1978).

Temperature is an extremely important parameter in determining the sporicidal efficacy of hydrogen peroxide. H_2O_2 was found to be weakly sporicidal at room temperature but very potent at higher temperatures (Block, 1991).

2.3.4 Diacetyl

Diacetyl (2,3-butanedione) is produced by strains of several species of lactic acid bacteria, *Leuconostoc cremoris* and *Lactococcus lactis* ssp. *Lactis* bv. *diacetylactis*. It is produced from pyruvate, little of which is available when lactic acid bacteria are growing actively on a readily fermentable carbohydrate since most is needed to regenerate NAD from NADH. It can accumulate when cells reach stationary phase or

when an alternate source of pyruvate such as citrate is available. It is an important flavour volatile in a number of fermented dairy products (Varnam and Sutherland, 1994). Many *in vitro* studies have indicated that diacetyl deactivates enzymes from several microorganisms e.g. alcohol dehydrogenase and adenylase from yeasts (Varmio and Londesborough, 1979), glutamate dehydrogenase (Hemmila and Mantsala, 1978) and transketolase in the pentose cycle (Kremer *et al.*, 1980). It has been postulated that the carbonyl group in the diacetyl molecule (CH_3-CO-CO-CH_3) reacts with the guanido portions of the amino acid arginine (H_2N-C$(NH)_2$-$(CH_2)_3$-CH(NH_2)COOH) in the microbial enzymes and hence deactivates the enzymes either by blocking or modifying the catalytic sites (Yankeelov, 1966).

Jay (1982) reported that yeasts and Gram-negative bacteria were more sensitive than the non-lactic acid bacteria and Gram-positive bacteria to diacetyl. Lactic acid bacteria and the clostridia seemed to be insensitive to its inhibitory activity. Jay (1982) showed that 200 µg/mL diacetyl inhibited yeasts and Gram-negative bacteria, 300 µg/mL inhibited non-lactic Gram-positive bacteria and >350 µg/mL was required to inhibit lactic acid bacteria.

Motlagh *et al.* (1991) reported that 344 µg/mL diacetyl treatment increased the viability loss of strains of *Listeria monocytogenes, L. ivanovii, L. innocua, Salmonella spp., Yersinia enterocolitica, Escherichia coli* and *Aeromonas hydrophila* in Trptone Soy Broth from 0 to >95% during a 24 h incubation at 4° C.

2.3.5 Bacteriocins

Bacteriocins are antimicrobial, proteinaceous compounds with a bactericidal mode of action against bacteria closely related to the producer strain (Tagg *et al.*, 1976). Both Gram-negative and Gram-positive bacteria, including the lactic acid bacteria, produce bacteriocins. The genera of lactic acid bacteria involved include, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Pediococcus*, and *Carnobacterium* as reviewed by De Vuyst and Vandamme (1994a). Many bacteriocins are active against food-borne pathogens such as *Listeria monocytogenes, Clostridium perfrigens, Bacillus cereus* and *Staphylococcus aureus* (Okereke and Montville, 1991; Schllinger *et al.*, 1995) and are therefore considered as natural food preservatives (Daeschel, 1993; De Vuyst and Vandamme, 1994a).

The definition of Tagg *et al.* (1976) is thought to be too narrow to encompass all of the bacteriocins produced by lactic acid bacteria, since many of them that are chromosomally encoded have broad spectrum activity (Klaenhammer, 1993), and have a bacteriostatic rather than a bactericidal effect on the sensitive (indicator) strains (Klaenhammer, 1988, Nettles and Barefoot, 1993; De Vuyst and Vandamme, 1994a; Desmazeaud, 1996). In addition, some of them are lipo- or glyco-protein. Therefore a broader definition has been given. Bacteriocins are biologically active ribosomally synthesized proteins (which may be post-translationally modified) with inhibitory properties against other bacterial species but not the producer organism (Montville and Kaiser, 1993). They constitute a large and heterogeneous group of antimicrobials, which vary in spectrum of activity, molecular weight, genetic determinants and biochemical

characteristics such as thermostability and pH range of activity (Stiles and Hastings, 1991).

Bacteriocins of most lactic acid bacteria are small cationic molecules containing 30-60 amino acid residues forming amphiphilic helices and being thermo stable at 100^0 C for 10 minutes (Holzapfel *et al.*, 1995).

The target of bacteriocins is the cytoplasmic membrane and because of the protective barrier provided by the lipopolysacharide layer of the outer membrane of gram-negative bacteria, they are generally only active against gram-positive cells (Abee *et al.*, 1995; Sahl *et al.*, 1995). However, chelating agents such as EDTA or citrate can be used to bind magnesium ions in the lipopolysaccharide layer of the outer membrane of gram-negative bacteria to render them susceptible to nisin and other bacteriocins (Stevens *et al.*, 1992; Kalchayanand *et al.*, 1992). Also, the permeability of Gram-negative bacteria can be increased through sub lethal injury including that caused when using ultra high hydrostatic pressure (UHP) and pulsed electric field (PEF) as nonthermal methods of preservation, to render gram-negative bacteria bacteriocin sensitive (Kalchayanand *et al.*, 1994).

Since bacteriocins are proteinaceous compounds, they are generally inactivated by an array of proteolytic enzymes including: trypsin, α-chymotrypsin, pepsin and proteinase K (Piard and Desmazeaud, 1992; Ray, 1992; Desmazeaud, 1996).

Over the past decade, the interest in bacteriocins produced by lactic acid bacteria has grown dramatically. One reason for this interest is that many bacteriocins inhibit food spoilage and pathogenic organisms such as *Listeria monocytogenes*, which are recalcitrant to traditional food preservation methods. In addition, bacteriocinogenic lactic acid bacteria are associated with and are used as starter cultures. The use of bacteriocins, the organisms which produce them, or both is attractive to the food industry because it is facing both increasing consumer demand for natural products i.e. preservative free foods and increasing concern about food borne disease (Montville and Winkowski, 1997).

2.3.6 Reuterin

Reuterin or ß-hydroxypropionaldehyde is a non-peptide antimicrobial substance produced as an intermediate during anaerobic metabolism of glycerol by strains of *Lactobacillus reuteri* (Axelsson *et al.*, 1989; El-Ziney, 1997; El-Ziney *et al.*, 1998). *L. reuteri* is a heterofermentative *Lactobacillus* inhabiting the gastrointestinal tract of man and animals (Kandler *et al.*, 1980). *L. reuteri* is found in a range of foods particularly dairy products, meats and sourdough (Dellagio *et al.*, 1981; Kandler and Weiss, 1986; Vogel *et al.*, 1994).

Other lactobacilli have been shown to synthesize reuterin from glycerol in the presence of glucose and glycerol (Sobolov and Smilley, 1960; Schutz and Radler, 1984) but the reuterin is immediately converted to equimolar amounts of β-hydroxypropionic acid and 1,3 propanediol, or used as a hydrogen acceptor, resulting in accumulation of 1,3 propanediol (Sobolov and Smilley, 1960; Schutz and Radler, 1984). The synthesis of

reuterin from glycerol is also reported to occur in *Aerobacter aerogenes* (Paweekiewicz and Zagalak, 1965), *Klebsiella pneumoniae* (Forage and Foster, 1982; Forage and Lin, 1982;-Ahrens *et al.*, 1998), and *Clostridium* spp. (Forsberg, 1987).

Chung *et al.* (1989) and El-Ziney (1997) determined the sensitivity of different microorganisms to reuterin and observed that 4 to 5 AU/ml of reuterin was sufficient to inhibit the growth of various species tested (Table 3).

2.3.7 Ethanol

Ethanol is a well-established antimicrobial and is produced in low concentrations by heterofermentative lactic acid bacteria. The amounts of ethanol and ethanoic acid produced are inversely related since both compounds represent alternative fates of acetyl phosphate in the heterofermentative pathway.

2.3.8 Carbon dioxide (CO_2)

Fermentation of sugars by heterofermentative lactic acid bacteria produces 1 mol of CO_2 per mole of hexose dissimilated. This can contribute to the inhibition of other organisms in two ways. Firstly, in conjunction with residual respiration of the food tissues, it can create anaerobic conditions that will preclude the growth of obligate aerobes such as moulds. Secondly, an increase partial pressure of carbon dioxide has its own specific antimicrobial activity (Adams and Nicolaides, 1997). Microorganisms vary in their sensitivity to CO_2; moulds and oxidative Gram-negative bacteria are most susceptible

Table 3. Minimum inhibitory concentration of reuterin against different bacterial, yeast, fungal and protozoan species (Chung *et al.*, 1989; El-Ziney, 1997).

Species	*Tested strain*	MIC (AU/ml)
Gram-positive bacteria		
Bacillus cereus	3	2
Bacillus megaterium	1	5
Staphylococcus aureus	2	2
Staphylococcus epidermidis	1	5
Listeria monocytogenes	6	4-8
Lactobacillus bulgaricus	1	9
Lactobacillus plantarum	1	12
Lactobacillus acidophilus	6	12-40
Leuconostoc mesenteriodes	1	16
Pediococcus cerevisiae	1	12
Gram-negative bacteria		
Escherichia coli	ETEC (1), VTEC (1), K12 (2), EHEC (2)	4
Pseudomonas aeruginosa	1	2
Pseudomonas alcaligenes	1	4
Pseudomonas fluorescens	2	2-4
Shewanella putrefacines	1	2
Yersinia enterocolitica	0:3 (1), 0:8 (1), 0:9 (1)	2-4
Salmonella typhimurium	1	4
Yeast		
Candida albicans	2	2
Sacharomyces cerevisiae	1	12
Sacharomyces fibuligera	1	16
Torulopsis glograta	1	4
Fungi		
Aspergillus flavus	1	8
Fusarium samfucienum	1	8
Protozoa		
Trypnosoma cruzi	1	5

while lactobacilli and some yeast show high tolerance. The mechanism of inhibition is not well understood but is thought to involve combination of decreased intracellular pH, inhibition of enzymic reactions by a mass action effect and interaction with the cell membrane to disrupt solute transport (ICMSF, 1980). At low concentration it may be stimulatory to the growth of some bacteria (Lindgren and Dobrogosz ,1990).

Carbon dioxide inhibition in microorganisms is widely exploited on a commercial scale in modified atmosphere packing of foods. Its role in lactic acid fermentation is thought to be at the start of fermentation when the less acid tolerant heterofermenters can grow, and when CO_2 can inhibit the large numbers of aerobes present and set the fermentation on it desired course.

2.3.9 Competition and nutrient depletion

According to Adams and Nicolaides (1997) the bacterial cell requires an environment that is free from inhibitory agents as well as sufficient space and nutrients to grow and multiply. It is possible that the rapid growth of a large population of lactic acid bacteria in fermented foods could restrict the growth of other organisms by competition for available space and nutrients. This factor could act in concert with other antimicrobial principles to produce the overall level of inhibition observed for lactic acid bacteria against spoilage and pathogenic organisms in fermented foods.

2.4 pH homeostasis and acid stress in bacteria and yeast.

Biological and chemical systems depend on an interaction between acid and base systems. The microbial cell normally reflects the equilibrium by attempting to maintain an internal pH (pH_i) near neutrality (Biard-Parker, 1980). Homeostasis is the tendency of a cell to sustain chemical equilibrium despite a fluctuation in the acid-base environment. Through the interaction of a series of chemical mechanisms this delicate balance is maintained and alteration of this balance causes destruction of the microbial cell. Proteins, nucleic acids, and phospholipids can be structurally altered by pH_i changes. The availability of metallic ions to the organism is also altered and becomes a function of membrane permeability, because membranes are less permeable to charged molecules than to uncharged molecules.

As a result of work on a wide range of organisms a number of generalizations can be made about bacterial pH homeostasis. (1) It is recognised that acidophiles exhibit pH_i values in the range of 6.5 to 7.0, neutrophiles have pH_i values of 7.5 to 8.0 and alkalophiles have pH_i values of 8.4 to 9.0 (Booth, 1985). The differences in pHi values of these organisms reflect different enzyme systems which operate in these ranges. (2) Organisms exhibit different capacities to regulate their cytoplasmic pH. Some organisms (e.g. *Escherichia coli* and other respiratory organisms) show relatively tight control over cytoplasmic pH such that as the external pH (pH_o) is varied there is little variation in the internal pH (for example, a variation of less than 0.1 pH unit in pH_i for every 1 pH unit change in external pH would be considered tight regulation).

Other organisms exert less control over their cytoplasmic pH, and much larger changes in pH_i have been observed e.g. *Enterococcus faecalis* and other fermentative bacteria (Ray and Sandine, 1992). But there is always a trend towards maintaining progressively larger pH gradient, as the external pH becomes more acid. (3) Fermentative organisms such as lactic acid bacteria exhibit a greater range of values of pH_i over which growth will occur than do organisms which have a respiratory mode of metabolism (Kobayashi and Unemoto, 1980; ten Brink and Konings, 1982). (4) In one respect, alkalophiles differ from acidophiles and neutrophiles namely, the regulated value of pH_i is lower than the external pH (McLaggan *et al.*, 1984). In these organisms it appears to be generally true that the generation of a transmembrane pH gradient, interior acid, is dependent upon the cycling of sodium ions across the cell membrane (McLaggan *et al.*, 1984).

The regulation of cytoplasmic pH implies control over the permeability of the cell membrane to protons. In the absence of lipid-permeable organic anions, the permeability of biological membranes is low (Raven and Beardall, 1980). Since the cytoplasmic membrane is not significantly permeable to protons, it is valuable to consider what factors act to perturb the cytoplasmic pH and how microbial cells counter them.

Raven and Smith (1973) proposed that the two principal factors that cause perturbations of pH_i are the passive movement of protons across the cytoplasmic membrane and the production of acids and bases in the cytoplasm. It is known that most bacteria possess membrane bound proton pumps that extrude protons from the cytoplasm to generate the transmembrane electrochemical gradient of protons, the proton motive force (Mitchell, 1966 and 1973). The recorded values of the proton motive force are about 200 mV in

respiring organisms and somewhat less in fermenting organisms (Booth, 1985). The passive influx of protons in response to the PMF could be a major problem for cells attempting to regulate their cytoplasmic pH. However, biological membranes exhibit low proton permeability due to the intrinsic impermeability of the lipid bilayer and the very specific control of ion flux through the protein complexes inserted in the membrane. Thus, dramatic shifts of external pH cause only slight changes in pHi, which are usually transient (Slonczewski *et al.*, 1982). For example, in *E. coli* a drop in the external pH of 1.0 to 1.6 units due to the addition of HCl provokes only a fall of 0.10 to 0.15 units in internal pH. The change in internal pH has been found to be rapid, taking less than 30 seconds, and recovery takes place over several minutes and requires the presence of potassium ions (Brown and Booth, 1991). It is generally assumed that this rapid acidification is a consequence of increased proton influx through the ATPase as a result of the transient rise in the proton motive force consequent upon an increase in the transmembrane pH gradient. The fact that the observed pH_i changes are small indicates that the passive influx of protons is readily countered by the pH homeostatic capacity of the organism. Booth and Kroll (1983) referred to this as the stress or 'load' against which the pH-regulating systems must act.

Other major sources of cytoplasmic pH perturbations are essential metabolic processes associated with growth and metabolism that can lead to either acid production or consumption (Raven and Smith, 1973).

Raven and Smith (1976) identified acid production consequent on ammonia assimilation as a major problem for cells attempting to maintain a constant intracellular pH during

growth on neutral molecules such as glucose and carbon dioxide. They showed that in *Penicillium cyclopium* net acid production was equimolar with ammonia assimilation.

Intracellular pH must be maintained above some critical pH at which intracellular proteins become irreversibly denatured. In *Salmonella typhimurium*, where it has been studied most extensively, there are three progressively more stringent mechanisms to maintain a pH_i consistent with viability (Foster and Hall, 1991; Foster *et al.*, 1994). These three mechanisms are the homeostatic response, the acid tolerance response, and the synthesis of acid shock proteins (Montville, 1997).

At $pH_0 > 6.0$, salmonella cells adjust their pH_i through the homeostatic response. The homeostatic response maintains pH_i by allosterically modulating the activity of proton pumps, antiports, and symports to increase the rate at which protons are expelled from the cytoplasm. The homeostatic mechanism is constitutive and functions in the presence of protein synthesis inhibitors (Foster and Hall, 1991; Foster *et al.*, 1994).

The acid tolerance response (ATR) is triggered by pH_o of 5.5 to 6.0 (Foster and Hall, 1991). This mechanism is sensitive to protein synthesis inhibitors; at least 18 ATR-induced proteins have been identified. ATR appears to involve the membrane-bound ATPase proton pump and maintains $pH_i > 5.0$ at pH_o values as low as 4.0. The loss of ATPase activity caused by gene disruption mutations or metabolic inhibitors abolishes the ATR but not the pH homeostatic mechanism. The ATR may confer cross protection to other environmental stressors. The exposure of *S. typhimurium* cells to pH 5.8 for a

few doublings induces 12 proteins, represses 6 proteins, and renders the cells less sensitive to salt and heat (Leyer and Johnson, 1993).

The synthesis of acid shock proteins is the third way that cells regulate pH_i. The synthesis of these proteins is triggered by pH_o from 3.0 to 5.0. They constitute a set of trans-acting regulatory proteins distinct from the ATR proteins. They may be similar to cold shock proteins, which help confer acid resistance in *Listeria monocytogenes* (Foster *et al.*, 1994).

In yeasts, the proton translocating ATPase in the plasma membrane creates the trans-membrane electrochemical proton gradient for active transport of nutrients and is also involved in the regulation of intracellular pH by pumping protons out of the cells. (Serrano, 1984). This enzyme is essential for growth (Serrano *et al.*, 1986) and can consume up to 60% of cellular ATP (Serrano, 1991). Glucose (Serrano, 1983) and several environmental stressors such as ethanol (Rosa and Sá-Correia, 1991), weak organic acids (Viegas and Sá-Correia, 1991) acid pH (Eraso and Gancedo, 1987), deprivation of a nitrogen source (Benito *et al.*, 1992) and supraoptimal temperatures (Viegas *et al.*, 1995) stimulate in vivo the activity of this membrane enzyme. This activation constitutes a response that helps the cell to counteract the dissipation of the proton motive force across the plasma membrane and or the decrease of internal pH taking place in cells exposed to liposoluble compounds, external acidification or supraoptimal temperatures (Eraso and Gancedo, 1987; Rosa and Sá-Correia, 1991; Viegas *et al.*, 1995).

2.4.1 Fluorescence microscopy and ratio-imaging for measurements of pH homeostasis

Several approaches have been used to determine the pH_i of yeast cells. These approaches include distribution of radio labelled weak acids (Rottenberg, 1979; Burlini *et al.*, 1993), the ^{31}P nuclear magnetic resonance spectroscopy technique (Rabaste *et al.*, 1995; Coote *et al.*, 1994 and Carmelo *et al.*, 1997) and the application of pH-dependent fluorescence probes (Cimprich *et al.*, 1995; Imai and Ohno, 1995; Guldfeldt and Arneborg, 1998; Calahorra *et al*,. 1998; Breeuwer and Abee, 2000; Arneborg *et al.*, 2000). Among these techniques, the fluorescence technique has several advantages as it gives the researcher the possibility to investigate rapid intracellular pH changes and to monitor the pH of single cells.

The fluorescence technique most commonly used for determination of intracellular pH in yeasts includes staining of cells with a fluorescent precursor that penetrates the cell membrane and subsequently is cleaved by intracellular enzymes (esterases) resulting in liberation of the fluorescent compound. Using this technique, the intracellular pH can be measured by different methodologies including fluorescence spectroscopy (Haworth and Fliegel, 1993), flow cytometry (Preston *et al.*, 1989; Guldfeldt and Arneborg, 1998) and fluorescence microscopy (Cimprich *et al.*, 1995, Imai and Ohno, 1995, Guldfeldt and Arneborg, 1998, Breeuwer and Abee, 2000; Arneborg *et al.*, 2000).

Fluorescence microscopy is well suited for studying single cell physiology of living cells for many reasons (Herman, 1998). First, it is specific since excitation and emission spectra allow the selective detection of a given fluorescent probe among other probes. Second, it is sensitive since only a few fluorescent probes are needed to obtain detectable responses. Third, it has high temporal resolution since the excitation lifetime of fluorescent probes is very short. Fourth, it has high spatial resolution, since single molecules and fine structures can be tagged with fluorescent probes, and since images of high contrast can be obtained. Finally, the fluorescence excitation and emission spectra of some probes are very sensitive to the environmental conditions, allowing the design of specific probes for monitoring physiological changes in e.g. intracellular pH, ion concentrations, and redox potential.

Fluorescent probes can be divided into probes useful at near neutral pH and acidic pH (Haugland, 1992). The most widely used probes for determination of intracellular pH in yeasts are the probes useful at near neutral pH including fluorescein and fluorescein derivatives, SNARF and SNAFL (Haugland, 1992; Breeuwer and Abee, 2000).
Fluorescein and fluorescein derivatives are ratiometric pH probes that exhibit no pH sensitivity when excited at 435 nm and maximal sensitivity when excited at 490 nm. After a fluorescent signal is obtained at each excitation wavelength, a concentration-independent ratio between pH-sensitive and pH-insensitive signals is calculated.

Breeuwer *et al.* (1996) reported the use of a pH probe, 5 (and-6)-carboxyfluorescein succinimidyl ester (cFSE) for determination of the pH_i in gram-positive bacteria. cFSE

can easily be taken up by bacteria following incubation with its diacetate ester cFDASE. Once incorporated the succinimidyl group of cFSE conjugates with aliphatic amines (Haugland, 1992). In yeast cells this approach is expected to limit the sequestration of fluorescent probe in the vacuole and the active extrusion to the extracellular environment. But the transport of cFDASE into the yeast cells at normal growth temperatures (25-30 °C) is insufficient for fluorescent labelling of the cells. Bracey *et al.* (1998) reported that labelling of yeast cells could be obtained after 24 h growth at 37 °C and pH 4. Breeuwer and Abee (2000) however thought that these conditions are not optimal for growth of *Saccharomyces cerevisiae* and might affect the physiology of the cells. They successfully labelled *Saccharomyces cerevisiae* cells after incubation for 15 minutes in 50 mM potassium citrate buffer pH 4 containing 0.11 mM cFDASE at 30 °C, or in 50 mM potassium phosphate buffer pH 7 containing 0.07 mM cFDASE at 35 °C.

Fluorescence ratio imaging microscopy (FRIM) involves five basic steps: (1) An image of the cell and background is recorded at each of the two wavelengths i.e. the pH-sensitive and pH-insensitive; (2) the background images are subtracted from cell images; (3) image restoration algorithms for correction for shading, nonlinearity, etc, are applied to all images; (4) the ratio of the two resultant images is calculated; (5) the ratio images are calibrated in terms of the parameter of interest under investigation. In this case the pH (Bright *et al.*, 1989).

The basic elements of a fluorescence ratio imaging microscope system include a light source, an excitation light control device (e.g. Shutter), a wavelength tuning device (e.g.

filter wheel), microscope, low light level camera, image processor, with computer coordinated control over all aspects of the system (Bright *et al.*, 1989).

The basic function of the fluorescence microscope is to deliver excitation energy to the fluorescing species in the specimen and to separate the much weaker emitted fluorescence light from the brighter excitation light. In doing so, only the emitted light reaches the detector and a high contrast image is generated. Two different types of optical paths have been employed in fluorescence microscopy: light illumination and incident light illumination (epi-illumination).

The objective lens of an epi-illumination system functions both as a condenser, delivering the emission light to the specimen surface, and a collector of reflected fluorescence. The dual function is made possible by a dichroic mirror that reflects short wavelengths but allows long wavelengths to pass. Short wavelength excitation light is reflected by the dichroic mirror, through the objective, towards the specimen in one direction and fluorescence reflected in the opposite direction, collected by the objective is allowed to pass through the dichroic mirror towards the detector. Epi-illumination is the preferred optical configuration for fluorescence microscopy today, because the optical path of epi-illumination effectively separates the excitation light from the fluorescence (Bright *et al.*, 1989).

Quantitative fluorescence microscopy is a difficult task because of many factors affecting measurements. Although a specific probe may be sensitive to a parameter of interest, there may be many other influencing factors such as instrumentation, sample geometry and probe chemistry (Bright *et al.*, 1989). Fluorescence ratio imaging is a

powerful technique that eliminates some of the influencing factors including optical path length, differences in local probe concentrations, unequal distribution of the excitation light, photo bleaching, leakage of the indicator and slight changes in focus. Ratio imaging is the relationship between the collected emissions at a fixed wavelength after excitation at two different wavelengths, or between emission signals collected at two different wavelengths after excitation at a specific wavelength. The first option is often used in fluorescence microscopy, whereas the latter is well adapted to flow cytometry.

To study the response of individual yeast cells to changing environmental conditions and over a period of time the cells need to be immobilized on a support, e.g. glass slides. Various authors have used different methods for immobilization of the cells on glass slides. Guldfeldt and Arneborg (1998) coated the glass slides with poly-L-lysine, but Breeuwer and Abee (2000) reported that this method is not generally applicable for all *Saccharomyces cerevisiae* strains. Immobilization by using carrageenan (Imai and Ohno, 1995) has also been applied but Breeuwer and Abee (2000) reported that this method does not allow continuous perfusion. Breeuwer and Abee (2000) successfully immobilized yeast cells using ferric nitrate treated glass slides. Concanavalin–A has also been successfully used for immobilization of yeast cells (Roberts *et al.,* 1991 and Vindeløv, 2001). In this thesis, Concanavalin–A was successfully used for immobilization of *Candida krusei* and *Saccharomyces cerevisiae* cells.

3.0 MATERIALS AND METHODS

3.1 Materials

The 'Local 'variety of normal dent white maize (*Zea mays*) grains were purchased from a retail market in Accra, Ghana. They were cleaned and stored in a wooden silo under shade at ambient temperatures (28 to 30^0 C) until used.

3..2 Microorganisms, Source and Maintenance

Sixteen Gram-negative bacterial strains were included in the investigations as listed in Table 4. All the organisms had been isolated and characterized biochemically for virulence genes at the State Serum Institute, Denmark. During the experiments the bacteria were grown at 37 °C and maintained at 4 °C on slopes of nutrient agar (Difco, Detroit, USA). *Escherichia coli* K12 (MG1655), a wild type K12 strain (Guyer *et al.* 1981) and *Escherichia coli* M23 were maintained as frozen stock cultures in 20% (v/v) glycerol.

Twenty strains each of the yeasts *Candida krusei and Saccharomyces cerevisiae* (Tables 5 and 6) previously isolated from maize fermentation and identified by pheno- and genotyping by Halm *et al.* (1993); Hayford and Jespersen (1999); Hayford and Jakobsen (1999) were used in the yeast experiments. The yeast strains were maintained on MYGP agar (pH 5.6) containing g/l distilled water; 3, malt extract (Difco, Detroit, USA), 3, yeast extract (OXOID, Hampshire, England), 10, glucose (Merck, Darmstadt, Germany), 5, peptone (Difco) and agar (Difco), 20, at 4 °C.

Table 4 Bacterial strains used in the study

Bacterial species	Serotype (pathogenic type)	Strain designation used in text
Salmonella	*enteritidis*	*S. enteritidis*
Salmonella	*typhimurium*	*S. typhimurium*
Salmonella	*typhi*	*S. typhi*
Salmonella	*stanleyville*	*S. stanleyville*
Salmonella	*durban*	*S. durban*
Shigella flexneri	2a	*Sh. flexneri* 2a I
Shigella flexneri	2a	*Sh. flexneri* 2a II
Shigella dysenteriae	1	*Sh. dysenteriae* 1
Escherichia coli	O157: H7 (VTEC)	*E. coli* O157 (VTEC I)
Escherichia coli	O157: K: H7 (VTEC)	*E. coli* O157 (VTEC II)
Escherichia coli	O26: H11 (VTEC)	*E. coli* O26 (VTEC)
Escherichia coli	O111: H2 (EPEC)	*E. coli* O111 (EPEC)
Escherichia coli	O3 K: H2 (EAggEC)	*E. coli* O3 (EAggEC)
Escherichia coli	Laboratory strain	*E. coli* K12
Escherichia coli	Laboratory strain	*E. coli* M23

TABLE 5. *Candida krusei* strains used in the study (Cluster analysis of chromosome profiles are from Hayford and Jakobsen, 1999.)

Species /strain	Source/ origin	Cluster analysis of Chromosome Profiles
Candida krusei		
18A-3	Fermented maize (72 h)	Cluster 2
19A-3	Fermented maize (72 h)	Cluster 1
19B-4	Fermented maize (72 h)	Cluster 2
20B-2	Fermented maize (72 h)	Cluster 1
9	Fermented maize (72h)	Cluster 1
29	Fermented maize (72 h)	Cluster 5
44	Fermented maize (72 h)	Cluster 4
63	Fermented maize (72 h)	Cluster 4
64	Fermented maize (72 h)	Cluster 1
65	Fermented maize (72 h)	Cluster 4
75	Fermented maize (72h)	Cluster 1
76	Fermented maize (72h)	Cluster 4
79	Steep water	Cluster 1
80	Fermented maize (72h)	Cluster 4
119	Fermented maize (48h)	Cluster 4
173	Fermented maize (24 h)	Cluster 1
183	Corn mill	Cluster 1
191	Corn mill	Cluster 4
121	Fermented maize (48 h)	Cluster 1
199	Corn mill	Cluster 1

TABLE 6. *Saccharomyces cerevisiae* strains used in the study (Assimilation profiles and cluster analysis of chromosome profiles are from Hayford and Jespersen, 1999).

Species /strain	Source/ origin	Assimilation Profiles*	Cluster analysis of Chromosome Profiles
Saccharomyces cerevisiae			
31	Fermented maize (72 h)	**A**	Cluster **g**
37	Fermented maize (72 h)	A	Cluster a
38	Fermented maize (72 h)	D	Cluster j
120	Fermented maize (48 h)	C	Cluster j
123	Fermented maize (48 h)	B	Cluster d
127	Fermented maize (48 h)	C	Cluster e
136	Fermented maize (48 h)	A	Cluster g
142	Fermented maize (48 h)	A	Cluster g
143	Fermented maize (48 h)	A	Cluster d
144	Fermented maize (48 h)	A	Cluster g
149	Fermented maize (24h)	B	Cluster b
150	Fermented maize (24h)	B	Cluster c
157	Fermented maize (24h)	A	Cluster j
163	Fermented maize (24h)	A	Cluster h
167	Fermented maize (24h)	A	Cluster c
25-1-3	Fermented maize (72 h)	A	Cluster c
26-1-0	Fermented maize (72 h)	A	Cluster f
26-1-7	Fermented maize (48 h)	**A**	Cluster **b**
26-1-8	Fermented maize (72 h)	A	Cluster c
26-1-e	Fermented maize (72 h)	A	Cluster c

*A: galactose, saccharose, DL-lactate, raffinose, maltose and glucose; B: galactose, saccharose, DL-lactate, raffinose, maltose, glucose and melibiose; C: galactose, saccharose, raffinose, maltose, glucose and melibiose; D: galactose, saccharose, raffinose, maltose and glucose.

3.3 Experimental Design

Survival studies with Gram-negative bacteria in maize steep water, fermenting maize dough, koko and kenkey water were conducted in duplicate on two separate occasions. Results of all analysis represent means of four replicate trials with duplicate determinations.

The experiments on inactivation of *Escherichia coli* in Trypticase Soy Broth Yeast Extract were performed as a complete factor experiment with two variables, namely pH (3.5, 3.7, 4.0 and 4.2) and lactic acid (0, 0.2, 0.4, 0.8 and 1.2%) and each combination was performed in duplicate (4×5×2 = 40 experiments).

Yeast experiments were performed on a minimum of two separate occasions in duplicate determinations.

3.4 Survival of Gram-negative Bacteria in Maize Steep water, Fermenting Maize Dough, Koko and Kenkey Water

3.4.1 Preparation of Inocula

The Gram-negative bacteria used in maize steeping, fermentation and products studies were produced by growing pure cultures of each pathogen on nutrient agar plates at 37 °C for 16 h (stationary phase cells). The colonies were washed off (using a sterile inoculation needle to lightly remove the colonies) with sterile diluent (pH 7.2) containing g/l distilled water; NaCl 8 and Bacto Peptone (OXOID), 1, to give approximately 10^{10} colony forming units (cfu/ ml) as determined by spread plate count on nutrient agar following incubation at 37 °C for 24 h. The required volume of freshly prepared bacterial suspension was added to

maize steep water, maize dough, koko and kenkey water at different stages of preparation to give approximately 10^7 cfu/g.

For the bacterial pathogens used in broth studies, the inocula were frozen batch cultures prepared from frozen stock cultures of individual bacteria in 20% (v/v) glycerol. Frozen batch cultures of test bacteria were prepared from a subculture of stock by transferring one colony into 50 ml Trypticase Soy Broth (TSB, pH 7.3 ± 0.05, OXOID) and incubated at 37 °C for 14 h. Thereafter the cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C, the pellet resuspended in 2.75 ml fresh TSB broth and 1.75 ml 50% glycerol, vortexed and distributed in 250 µl portions in eppendorf tubes and kept frozen at –40 °C until use. Required volumes from a culture of the frozen batch (2 x 250 µl) in 15 ml TSB grown statically at 30 °C to a stationary phase population ca. 2.0×10^9 cfu/ml were used as inoculum to give an initial contamination level of 10^7 cfu/ml.

Yeasts were grown as single cultures to late exponential phase (8 h fermentation in MYGP broth pH 5.6 containing g/l distilled water; 3, malt extract (Difco), 3, yeast extract (OXOID), 10, glucose (Merck) and 5, peptone (Difco), at 30 °C statically. The cells were harvested by centrifugation (7000 × g for 3 min at 4 °C) and resuspended in diluent pH 7.2.

3.4.2 Inoculation Studies in Maize Steep Water and Fermenting Maize Dough

Five kilograms maize were added to 7.5 l of water in a 10 l plastic bucket. The test bacteria were added and the mixture stirred manually and left to steep at ambient temperature (about 28°C) for 48 h.

For dough fermentation, steeped maize was milled in a disc attrition mill (Disc attrition mill, Rajan Universal, Madras, India) to a smooth meal. The test bacteria were added to

300 ml potable tap water and used to knead 1 kg of maize meal into dough. The dough was thoroughly kneaded to ensure an even distribution of the bacteria, smoothed at the surface and allowed to ferment spontaneously at ambient temperature for 48 h. For each experiment, a non-inoculated batch served as control.

3.4.3 Inoculation Studies in Ga Kenkey Water and Koko

Ga Kenkey water was prepared as follows:

Thirty three grams of cooking salt (NaCl) and 0.75 l of water were added to 1 kg of 48 h fermented maize dough. The fermented maize dough was prepared as described by Halm et al. (1993). The slurry obtained was cooked into *aflata* and added to 1 kg of uncooked dough, mixed well and moulded into balls of about 300 g weight, wrapped with maize husks and cooked in 2.8 l water for 3.5 h following the traditional method of cooking Ga kenkey. After cooking, the water (kenkey water) was collected and allowed to cool to ambient temperature. Three hundred grams portions of kenkey water were aseptically distributed into sterile stomacher bags. The test bacteria were added to the kenkey water and homogenized in a stomacher (Lab Blender, Model 4001, Seward Medical, London, England) at normal speed for 30 s. The bagged kenkey water samples were placed in a basket and left at ambient temperature for 48 h.

Koko was prepared by adding one part of fermented maize dough to five parts of water, made into a slurry, heated until boiling and allowed to cook for 20 min with constant stirring. The porridge was left to cool to ambient temperature and aseptically distributed in 300 g portions into sterile stomacher bags. The test bacteria were added to the koko portions and homogenized in a stomacher as described above. The bagged koko samples were placed in a basket and left at ambient temperature for 48 h.

3.4.4 Inoculation Studies in Ga Kenkey Water and Koko adjusted to pH 6.0

Koko and kenkey water samples were adjusted to pH 6.0 with a 1 N NaOH solution to investigate whether it is the low pH of the products that is responsible for the inhibition of the test bacteria. The pH adjusted koko and kenkey water samples were aseptically distributed in 300 g portions into sterile stomacher bags. Each product was then inoculated with the test bacteria and homogenized as described for kenkey water and koko and kept at ambient temperature for 48 h.

3.4.5 Inoculation Studies in Laboratory Media Supplemented with Organic Acids

Trypticase Soy Broth plus 0.6% Yeast extract containing 4 levels of lactic (0.4%, 0.8%, 1.0%, and1.4%) and acetic acids (0.05%, 0. 1%, 0.2%, and 0.3%) respectively were all adjusted to pH 3.5 corresponding to the lowest pH of dough after 24 to 48 h fermentation, were prepared as follows: Trypticase Soy Broth was prepared according to the manufacturers specifications but with added 0.6% Yeast Extract, pH adjusted to 3.5 with 5 M HCl and sterilized by autoclaving at 121 °C for 15 min. A 10% solution of lactic acid adjusted to pH 3.5 with 10 M NaOH was prepared and sterilized by filtration with 0.2 μm filter membranes. Adequate volumes of the filter sterilised 10% solutions of lactic acid and acetic acid solutions (pH 3.5) were aseptically added to portions of Trypticase Soy Broth Yeast Extract at the same pH to achieve the required lactic or acetic acid concentration. The pH was checked again to ensure there was no change. Test bacteria were added to 50 ml portions acidified broth to give an initial level of 10^6 to 10^7 cfu/ml, and incubated at 30 °C for 48 hours.

3.4.6 Recovery of Inoculated Bacteria

Samples were taken immediately after addition of test bacteria to the steep water, maize dough, *koko* and *kenkey water*, respectively and subsequently at 4, 8, 24, and 48 h for analysis. For fermenting dough samples, the surface layer was removed aseptically and 10 g sample was taken from within the dough. For liquid samples, 10 g samples were taken after thorough mixing by stirring the maize steep water, *koko* and *kenkey* water under aseptic conditions. Each sample was then homogenized as described earlier with 90 ml of sterile diluent (0.1% peptone (Bacto), 0.8% NaCl, pH 7.2). Serial tenfold dilutions were prepared in diluent and 0.1 ml of appropriate dilutions were spread plated onto Trypticase Soy Agar Yeast Extract (TSAYE, pH 7.2 ± 0.05) containing Trypticase Soy Agar (Oxoid) plus 0.6% Yeast Extract (Oxoid)) and State Serum Institute (SSI) enteric medium (SSI enteric medium, State Serum Institute, Copenhagen Denmark) as described by Blom *et al.* (1999). The inoculated agar plates were incubated at 37 °C and typical colonies were counted after 24 and 48 h.

On SSI enteric medium, salmonellae appear with a black precipitate located centrally and deeply in the more anaerobic parts of the colony, whilst shigellae, (except *Shigella sonnei*), appear as flat irregular translucent colonies with a slightly pinkish centre. *Escherichia coli* appear as red colonies due to lactate production. The identity of the test pathogens was confirmed in slide agglutination tests using specific *Salmonella, Shigella and E. coli* commercial antisera (*Salmonella* and *E. coli* antisera were obtained from The State Serum Institute, Denmark; *Shigella* antisera were obtained from Denka Seiken Co. Ltd. Tokyo, Japan). When growth of the test organisms was not observed after direct culture on the agar plates, resuscitation of 1 g in 9 ml of Trypticase Soy Broth Yeast Extract (TSBYE) was done for 20 h at 37 °C followed by surface streaking onto SSI enteric medium and

incubation for 24 h at 37 °C for *Shigella* and *E. coli*. For *Salmonella*, 25 g samples were pre-enriched in 225 ml Buffered Peptone Water (BPW, Oxoid) at 37 °C for 21 ± 3 h. Thereafter, selective enrichment of 0.1 ml BPW culture was done in 10 ml Rappaport Vassiliadis medium (RV, Oxoid) at 41.5 ± 0.5 °C for 48 h with subsequent surface streaking onto Xylose Lysine Deoxycholate Agar (XLD, Oxoid) and SSI enteric medium agar plates.

For the recovery of *E. coli* strains from Trypticase Soy Broth plus 0.6% Yeast Extract and organic acids, 1 ml samples were withdrawn from each culture, tenfold serial dilutions prepared in sterile diluent and 0.1 ml of appropriate dilutions surface plated onto TSAYE. Colony forming units were enumerated following incubation of the plates at 30 °C for 24 hours.

3.5 Inactivation Studies of *Escherichia coli* in Laboratory Media Supplemented with Organic Acids

The pathogenic *Escherichia coli* strain 0111(EPEC) was used in modelling studies.

On the basis of initial experiments with Gram-negative bacteria in maize steep water, maize dough, koko and kenkey water, *E. coli* was evaluated as the less acid sensitive and was therefore used for the model building. The survival of *E. coli* 0111(EPEC) at 30 °C in acidified TSBYE with different lactic acid concentrations and pH levels was monitored with time by the plate count method. The experiments were performed as a complete factor experiment with two variables, namely pH (3.5, 3.7, 4.0 and 4.2) and lactic acid (0, 0.2, 0.4, 0.8 and 1.2%) and each combination was performed in duplicate (4×5×2 = 40 experiments).

3.5.1 Culturing Techniques

Experiments were conducted in 100 ml screw-cap bottles. Trypticase Soy Broth and Trypticase Soy Agar with 0.6% Yeast Extract respectively were prepared according to the manufacturer's specifications plus 0.6% yeast extract. The pH of TSBYE was adjusted to the desired pH by drop wise addition of 5 N HCl followed by autoclaving at 121 °C for 15 min. Acidified TSBYE containing lactic acid at various pH levels were made by adding sufficient amounts of filter sterilized 10% solution of lactic acid (Merck, Darmstadt, Germany) previously adjusted to the required pH with 10 N NaOH, to autoclaved TSBYE at the same pH level to achieve the required final total concentration of lactic acid. After addition of lactic acid, the pH was checked again and a change of not more or less than 0.05 was observed. Fifty ml portions of acidified TSBYE in 100 ml screw cap bottles were inoculated with 500 µl of stationary phase culture (8 h) of *E. coli* 0111(EPEC) to give an initial concentration of ca. $\log_{10}$ 6-7 cfu/ml. Following inoculation, the number of cfu/ml was determined immediately and after 2, 4, 6, 8, 10, 12, 14, 16, 24, 36 and 48 h of incubation at 30 °C. Aliquots (1.0 ml) were removed from the test cultures at appropriate intervals and diluted in sterile diluent (0.1% peptone (Oxoid) and 0.8% NaCl; pH 7.2). Appropriate dilutions of samples were surface plated, with replication, onto TSAYE. Plates were incubated at 30 °C for 24 to 36 h. Colonies were enumerated and converted to Log_{10} surviving cfu/ml. Each experiment was performed twice.

3.5.2 Undissociated Fractions of Lactic Acid

Proportions of dissociated and undissociated forms of lactic acid at each pH were calculated using the Henderson-Hasselbalch equation (Barrow, 1981) with a pK_a value of 3.86 for lactic acid: $pH = pK_a + \log [A^-]/[HA]$. The applied concentrations of dissociated,

undissociated and total lactic acid at pH values used in survival studies with *E. coli* O111 (EPEC) in acidified Trypticase Soy Broth Yeast Extract are shown in Table 7.

3.5.3 Model Development

At each concentration of lactic acid and pH, 12 bacterial count points were modelled as a function of time. The survival curves were characterised by having a downward concavity, and therefore the data was fitted with the model of Peleg and Cole (1998) that is able to describe this particular behaviour.

$$\log S(t) = -bt^n \qquad (1)$$

where S is the survival ratio ($S=N/N_0$, $0 = S = 1$), N is the momentary number of cells, N_0 is the initial number of cells, b and n are parameters and t is time. The value of n determines the shape of the curve. If n = 1 the semi-logarithmic survival curve is linear, whereas the curve has an upward or downward concavity for $n < 1$ and $n > 1$, respectively (Peleg and Cole, 1998). The parameters b and n were estimated using the PROC NLIN of the SAS version 8 statistical programme (SAS Institute, Cary, NC, USA) employing the Gauss Newton algorithm. To compare the survival of *E. coli* the parameters b and n found for each treatment were used to calculate the time to one log decrease in bacterial population by rearrangement of equation 1.

Also using the Number Cruncher Statistical Sytems (NCSS), a multiple regression analysis was performed on the data and a model was obtained which relates the death rate (time to one log reduction) to the lactic acid concentration and pH.

Table 7. Applied concentrations of dissociated, undissociated and total lactic acid at pH values from 3.5 to 4.2 used in survival studies with *E. coli* O111 (EPEC) in acidified Trypticase Soy Broth Yeast Extract. Values were calculated using the Henderson-Hasselbalch equation with pK_a value of lactic acid as 3.86.

pH	% Lactic acid	Total lactic acid (mM)	Dissociated lactic acid (mM)	Undissociated lactic acid (mM)
3.5	1.2	133.0	40.4	92.6
3.5	0.8	88.8	27.0	61.8
3.5	0.4	44.4	13.5	30.9
3.5	0.2	22.2	6.7	15.5
3.77	1.2	133.0	59.6	73.4
3.77	0.8	88.8	39.8	49.0
3.77	0.4	44.4	19.9	24.5
3.77	0.2	22.2	9.9	12.2
4.0	1.2	133.0	77.1	55.9
4.0	0.8	88.8	51.5	37.3
4.0	0.4	44.4	25.7	18.7
4.0	0.2	22.2	12.9	9.3
4.2	1.2	133.0	91.3	41.7
4.2	0.8	88.8	60.9	27.9
4.2	0.4	44.4	30.5	13.9
4.2	0.2	22.2	15.2	7.0

3.6 Effect of Low pH and Lactic Acid on growth of *Candida krusei* and *Saccharomyces cerevisiae*

3.6.1 Screening of Strains of *Candida krusei* and *Saccharomyces cerevisiae* for Tolerance to Low pH

Twenty strains each of *C. krusei* and *S. cerevisiae* as listed in Tables 8 and 9 were tested for growth in MYGP medium containing 1% lactic acid at pH 2.5. Growth in MYGP broth at pH 5.6 without lactic acid acted as control. The strains were inoculated into the growth medium at a level of 10^5 cfu/ml and incubated at 30 °C for 48 h. Growth was determined by OD measurements at 620 nm after 8 h intervals up to 48 h (0, 8, 16, 24, 32, 40 and 48 h). Eight replicates were made for each treatment in micro titre plates. Selected strains showing high and low tolerance to lactic acid were used in growth studies in single and mixed cultures to determine growth performance. Further experiments with single cells of the selected yeasts were conducted to investigate the short-term intracellular pH (pH_i) responses to lactic acid.

3.6.2 Growth of Single and Mixed Cultures of *C. krusei* and *S. cerevisiae* in MYGP Broth.

Yeast cells were grown as single cultures to late exponential phase in MYGP broth containing per litre: 3 g malt extract (Difco), 3 g yeast extract (OXOID), 10 g glucose (Merck) and 5 g peptone (Difco), pH 5.6 at 30 °C statically. The cells were harvested by centrifugation (5000 × g for 3 min at 4 °C) and resuspended in sterile saline peptone water containing 0.1% peptone and 0.8% NaCl, pH 7.2. Subsequently both yeast species were inoculated singly or as mixed cultures into 100 ml MYGP broth with or without 1% total lactic acid at two pH levels of 2.5 and 3.5. (111 mM total lactic acid corresponding to

106.4 mM undissociated lactic acid at pH 2.5 and 77.3 at pH 3.5). The cultures were grown without shaking in 300 ml Erlenmeyer flasks inoculated to an initial concentration of 2 $\times 10^4$ cells/ml respectively, determined by cell count using a Neubauer counting chamber. The cultures were incubated at 30 °C for 48 h. Growth was monitored by determination of colony forming units after spread plating appropriate dilutions of samples taken after 0, 8, 16, 24, 32, 40 and 48 h, on MYGP agar with incubation at 30 °C for 48h. Two replicates were made for each treatment. The two species were distinguished by their macro-morphological characteristics on MYGP agar. Colonies of *C. krusei* were irregular, rough, slightly raised with margins fringed with pseudomycelium in agreement with the description by Kurtzman (1998) whilst colonies of *S. cerevisiae* were smooth and glistening, circular, raised with entire margins in agreement with Vaughan-Martini and Martini (1998).

3.6.3 *Determination of intracellular pH (pH_i) of single cells exposed to lactic acid by fluorescence-ratio-imaging microscopy*

Fluorescent Staining of Yeast Cells

Yeast cells were propagated as pure cultures in 300 ml Erlenmeyer flasks with 100 ml MYGP broth, pH 5.6, to late exponential phase at 30 °C corresponding to a cell count of 10^7 cells/ml. One milliliter of cell culture was taken out and the cells were harvested by centrifugation at 7000 × g for 3 min at 4 °C and washed twice in cold potassium phosphate buffer (PPB) containing per litre: 12.52 g KH_2PO_4 and 1.39 g K_2HPO_4, pH 5.6 adjusted with 1M HCl, and finally resuspended with PPB to I ml. Twenty µl of a stock solution of 4.5 mM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (C-FDA-se) (Molecular Probes Inc., Eugene, Oreg.) in 3 mM dimethyl sulfoxide was added per 1.0 ml of cell

suspension. The suspension was mixed thoroughly for 10 seconds, incubated at 40 °C for 20 min, and then immediately centrifuged at 7000 × g for 3 min at 4 °C and the supernatant discarded. The harvested cells were resuspended in 1.0 ml PPB buffer, pH 5.6 and kept on ice until analysed.

Immobilization of Cells for Microscopical Analysis

Yeast cells were immobilized on concanavalin A (CON A, Sigma)-coated bottom cover slips which were prepared by placing 10 µl of a 1.0 g per litre CON A solution on a clean cover slip and left to dry overnight as previously described by Roberts *et al.* (1991). Two hundred and fifty microlitres of the stained yeast cell suspension were placed in a perfusion chamber (RC-21A cell culture perfusion chamber, Warner Instrument, Ham, Conn, USA) as previously described by Guldfeldt and Arneborg (1998). The cells were allowed to immobilize on the CON A-coated bottom cover slip during centrifugation of the chamber for 5 min at 5000 × g.

Fluorescence-Ratio-Imaging Microscopy.

The set-up for fluorescence-.ratio-imaging microscopy (FRIM) was as previously described by Guldfeldt and Arneborg (1998) and consisted of a monochromator with a 75 W xenon lamp (Monochromator B, TILL Photonics, Planegg, Germany) to provide the two excitation wavelengths (490 nm and 435 nm). The inverted epifluorescent microscope (Zeiss Axiovert 135 TV, Carl Zeiss, Oberkochen, Germany) was equipped with a Zeiss Fluar 100 × objective (numerical aperture 1.3), a dichroic mirror (510 nm) and an emission bandpass filter (515-565 nm). Fluorescence emission was recorded using a cooled (-40 °C)

CCD camera (EEV 512 × 1024, 12 bit frame transfer camera, Princeton Instruments, Trenton, NJ, USA).

To expose the cells to undissociated lactic acid, the perfusion chamber was mounted on the stage of the microscope. Solutions of buffer and acids were perfused through the inlet of the chamber at a rate of 500 µl /min using a modified Alitea XV pump (Microlab Aarhus A/S, Aarhus, Denmark) and removed from the outlet of the chamber using a 101U pump (Watson Marlow, Wilmington, Mass. USA).

The following perfusion solutions were used: (1) PPB 111 mM (total) lactic acid adjusted to pH 4.5 with 5 N KOH (2) PPB, 111mM (total) lactic acid adjusted to pH 2.5 with 5 N HCL. According to Henderson-Hasselbalch equation, the perfusion solutions contained 20.7 and 106.4 mM undissociated lactic acid respectively.

During perfusion, stained cells were excited at 490 nm and 435 nm with exposure times of 3 s for each wavelength. A 6 % neutral-density filter was used to prevent photo bleaching. Perfusion was initiated at time zero and images were recorded at approximately 60 s intervals for 7 min. The images were stored on a Pentium PC using Metafluor 3.5 (Universal Imaging, West Chester, PA) and were later analysed. Single cells were randomly selected as regions of interest on the 490-nm image, and background intensity (representative region with no cells) was subtracted before calculating the 490 nm/435 nm ratios. Twenty cells were analysed in each experiment.

Preparation of Calibration Curves and Calculation of pH_i

Calibration curves for the two strains each of *C. krusei* and *Sacch. cerevisiae* were determined in PPB with pH ranging from 5.5 to 8.5. Following the normal staining procedure, the cells were permeabilized by treatment with 70 % ethanol for 30 min at 30 °C. The cells were then centrifuged (7000 × g, 3 min, 4 °C) and the pellet resuspended in 1 ml PPB adjusted to the required pH value. After 5 min incubation at room temperature (for equilibration) the cells were immobilized on the cover slips as previously described and images acquired on the fluorescence microscope. The 490 nm/435 nm ratios were calculated as described above.

Calculation of pH_i from the ratio images was based on the pH-equilibrated cells of all four individual strains of *C. krusei* and *Sacch. cerevisiae* used in the investigations. Approximately 20 cells were used for every calibration point. Piecewise linear equations describing the relationship between $R_{490/435}$ and pH_i were derived as described by Siegumfeldt *et al.* (2000). Conversion was automatically performed with Microsoft Excel, and the ratio value ($R_{490/435}$) for every cell at every time point converted to pH_i before the average and standard deviations were calculated. The lower sensitivity limit of the method was pH_i 5.5.

3.7 Determination of pH and Titratable Acidity

pH

The pH of all samples was determined with a pH meter (Radiometer, Copenhagen, Denmark). For solid dough samples, 40 g were homogenized with 40 ml of distilled water in a stomacher as described above before pH determination.

Determination of Titratable Acidity

Titratable acidity was determined by titration of 100 ml filtrate obtained from 10 g of dough, kenkey water and koko each mixed with 250 ml distilled water added 2-3 drops of 1% phenolphthalein solution against 0.1M NaOH. One ml of 0.1M NaOH was taken as equivalent to 9.008×10^{-3} g lactic acid.

The titratable acidity was expressed as percent lactic acid using the following formula:

$$\text{TTA (v/v)} = \frac{100 \text{ ml} \times \text{Titre} \times 0.009998 \text{ g} \times 250}{10 \text{ g} \times 100 \text{ ml}}$$

3.8 Data Analysis

Means of log bacterial numbers obtained from survival studies in maize steep water, fermenting maize dough, koko and kenkey water were calculated from a minimum of two experimental replications. Statistical difference between mean values were determined by the least significance difference using the General Linear Models procedure of the Statistical Analysis System version 8.1 statistical programme (SAS Institute, Inc., Cary, NC, USA). The results are reported as significant with a *P*-value of less than or equal to 0.05.

4.0 RESULTS AND DISCUSSIONS

4.1 Survival of Gram-Negative Pathogens in Fermenting Maize Dough Systems

4.1.1 Survival of Gram-Negative Pathogens in Maize Steeping Water and Fermenting Maize Dough

The changes in bacterial numbers of thirteen enteric pathogens inoculated into maize steep water and maize dough at a concentration of 10^6 - 10^7 cfu/g are shown in Tables 8, 9, 10. Also shown are the changes in pH during maize steeping and maize dough fermentation. There was significant ($P < 0.05$) drop in pH of maize steep water from 5.6 – 6.6 to 4.1 5.3 after 48 h of steeping. Generally the pathogens were only partially inhibited in maize steeping water when the pH had dropped to about 5.2 and below (Table 8, 9, 10). However, the reduction in numbers was not more than 1 to 2 $\log_{10}$ cfu/ ml of the initial populations. Differences in the sensitivities of the strains were also observed. *Salmonella stanleyville*, *Salm. typhi* and *Salm. typhimurium* were reduced significantly ($P < 0.05$) by 1 - 2 $\log_{10}$ cfu/ ml whilst there were no significant reductions in numbers of *Salm. durban* and *Salm. enteritidis* after 48 h of inoculation into maize steep water (Table 8). Only *Sh. flexneri* 2A I was partially inhibited in maize steeping water after 24 h (Table 9). Three of the five *E. coli* strains O111 (EPEC), O157 VTEC II and O3 (EAggEC) were also partially inhibited in maize steeping water after 8 h and beyond (Table 10).

Table 8. pH development and changes in numbers of *Salmonella* serotypes inoculated into maize steep water and maize dough incubated at ambient temperature of 28° C and plated on State Serum Institute (SSI) Enteric Medium.

	Maize steep water		Maize dough	
Time after inoculation (h)	pH	Log_{10} CFU/ ml	pH	Log_{10} CFU/ g
Salm. durban				
0	6.30 a	7.43 a	6.00 a	6.73 a
4	5.65 b	7.31 a	5.00 b	6.25 a
8	5.00 c	7.46 a	4.50 c	6.25 a
24	4.89 c	8.29 a	4.20 d	–
48	5.10 c	7.90 a	3.80 e	–
Salm. enteritidis				
0	6.00 a	6.45 b	5.80 a	6.97 a
4	5.25 b	6.58 b	5.51 a	6.54 b
8	4.55 c	7.18 a b	4.47 b	5.76 c
24	4.50 c	6.51 b	3.92 c	–
48	4.60 c	7.92 a	3.96 b c	–
Salm. stanleyville				
0	6.50 a	7.53 a	6.40 a	7.25 a
4	5.25 b	7.42 a	6.20 b	7.24 a
8	4.80 c	7.20 a	4.90 c	6.78 b
24	4.55 c d	6.67 b	4.20 d	3.50 c
48	4.35 d	6.59 b	3.80 e	–
Salm. typhi				
0	6.06 a	8.40 a	6.14 a	7.29 a
4	5.03 b	7.31 b	5.73 b	6.80 b
8	4.85 b	6.34 c	4.45 c	7.03 a b
24	4.55 c	6.50 c	4.32 c	–
48	4.40 c	6.35 c	3.90 d	–
Salm. Typhimurium				
0	5.63 a	7.43 a b	6.10 a	7.59 a b
4	4.69 b	7.19 b	5.62 b	7.91 a
8	4.53 b c	7.22 b	4.35 c	7.42 b
24	4.15 d	7.83 a	3.83 d	–
48	4.40 c	5.35 c	3.72 d	–

–Not detected in 25 g after resuscitation in buffered peptone water, selective enrichment in Rappaport Vassiliadis (RV) medium with subsequent streaking onto Xylose Lysine Deoxycholate Agar (XLD) and SSI enteric medium agar plates. Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P < 0.05$).
The results are shown as the mean of two independent experiments carried out on two separate occasions.

Table 9. pH development and changes in numbers of *Shigella dysenteriae* and *Shigella flexneri* strains inoculated into maize steep water and maize dough incubated at ambient temperature of 28° C and plated on State Serum Institute (SSI) Enteric Medium.

Time after inoculation (h)	Maize steep water pH	Log_{10} CFU/ ml	Maize dough pH	Log_{10} CFU/ g
Sh. dysenteriae 1				
0	6.00 a	6.56 a	5.97 a	6.17 a
4	5.40 a b	6.20 a	5.41 b	6.83 a
8	4.68 c	7.11 a	4.40 c	6.40 a
24	4.72 c	6.97 a	4.10 d	2.90 b
48	5.02 b c	7.80 a	4.05 d	–
Sh. flexneri 2a I				
0	6.45 a	6.78 a	6.18 a	7.06 a
4	5.18 b	6.71 a	5.65 b	7.31 a
8	4.82 c	6.65 a b	4.38 c	7.34 a
24	4.59 d	5.98 b	3.93 c d	2.75 b
48	4.50 d	6.60 a b	3.70 d	–
Sh. flexneri 2a II				
0	6.45 a	7.06 a	6.06 a	6.51 a
4	5.5 b	6.56 a	5.56 b	6.19 a
8	5.0 c	6.65 a	4.44 c	6.05 a
24	4.9 c	7.09 a	4.03 d	–
48	5.18 b c	7.05 a	3.89 d	–

–Less than 1 CFU/ ml confirmed by resuscitation in Trypticase Soy Broth plus 0.6% Yeast Extract followed by surface streaking on SSI enteric medium agar plates.

Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P < 0.05$).

The results are shown as the mean of two independent experiments carried out on two separate occasions.

Table 10. pH development and changes in numbers of *Escherichia coli* serotypes inoculated into maize steep water and maize dough incubated at ambient temperature of 28° C and plated on State Serum Institute (SSI) Enteric Medium.

Time after inoculation (h)	Maize steep water pH	Log_{10} CFU/ ml	Maize dough pH	Log_{10} CFU/ g
E. coli O111(EPEC)				
0	6.41 a	7.15 a	6.30 a	6.20 b
4	5.48 b	6.90 a b	6.11 a	7.30 a
8	4.83 c	7.10 a	4.95 b	7.00 a
24	4.59 d	6.70 b c	4.23 c	4.63 c
48	4.56 d	6.57 c	3.78 d	–
E. coli O157 (VTEC I)				
0	6.65 a	7.56 a	6.24 a	7.18 a
4	5.80 b	6.86 a	5.65 b	6.63 b
8	5.32 c	7.57 a	4.54 c	6.78 a b
24	4.95 d	7.80 a	4.20 d	–
48	5.30 c	7.62 a	3.80 e	–
E. coli O157 (VTEC II)				
0	6.18 a	8.30 a	6.15 a	7.54 a
4	4.94 b	8.04 a	5.73 a	8.08 a
8	4.79 c	7.46 b	4.44 b	8.18 a
24	4.46 d	7.09 b	3.90 c	3.46 b
48	4.55 d	7.34 b	3.84 c	3.62 b
E. coli O26 (VTEC)				
0	6.04 a	7.42 a b	6.12 a	6.53 a
4	5.38 b	6.83 b	5.51 b	6.26 a
8	4.63 c	7.33 a b	4.47 c	7.18 a
24	4.56 c	6.69 b	3.91 d	5.21 a
48	4.60 c	8.29 a	3.96 d	3.06 b
E. coli O3 (EAggEC)				
0	6.14 a	7.60 a	6.10 a	7.42 a
4	4.97 b	6.84 b c	5.15 b	7.82 a
8	4.76 c	7.18 a b	4.26 c	7.48 a
24	4.37 d	6.87 b c	3.92 c	3.74 b
48	4.10 e	6.41 c	3.88 c	3.06 b

– Less than 1 CFU/ ml (g) confirmed by resuscitation in Trypticase Soy Broth plus 0.6% Yeast Extract followed by surface streaking on SSI enteric medium agar plates.

Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P < 0.05$).

The results are shown as the mean of two independent experiments carried out on two separate occasions.

In maize steeping experiments without added bacteria, the pH of steep water dropped sharply from an initial value of 6.25 ± 0.35 to 5.1 ± 0.24 after 4 h (Fig 6) then more gradually to 4.78 ± 0.35 after 24 h. A slight increase in pH was observed after 48 h when the experiment was terminated.

The natural flora of Gram-negative bacteria present at the beginning of steeping increased significantly by more than 4 $\log_{10}$ cfu/ml to 8.76 $\log_{10}$ cfu/ml after 48 h of steeping (Fig.6). Three main types of bacteria were recovered on the SSI enteric agar plates being the normal Gram-negative bacteria found during maize steeping and dough fermentation. The State Serum Institute in Denmark identified these as *Klebsiella pneumonia*, *Enterobacter cloaceae* and *Erwinia herbicula*.

The results obtained in the present study agree with those obtained by Mensah *et al.* (1988 and 1991) who evaluated the anti-microbial effects of the different processes involved in the preparation of fermented maize dough porridge and found that the soaking process reduced the pH to 3.8 ± 0.1 but no anti-microbial effect against *Shigella* and entero toxigenic *Escherichia coli* (ETEC) was noted. Similar to the findings of Mensah *et al* (1991), the *E. coli* and *Shigella* strains used in the present study were only partially inhibited in the maize steep water. The studies of Mensah *et al.* (1991) however, did not include *Salmonella* species.

It was not surprising that the inoculated pathogens were generally not inhibited in maize steeping water. This reflects what happens during the traditional maize steeping process where the natural flora of Gram-negative bacteria present at the

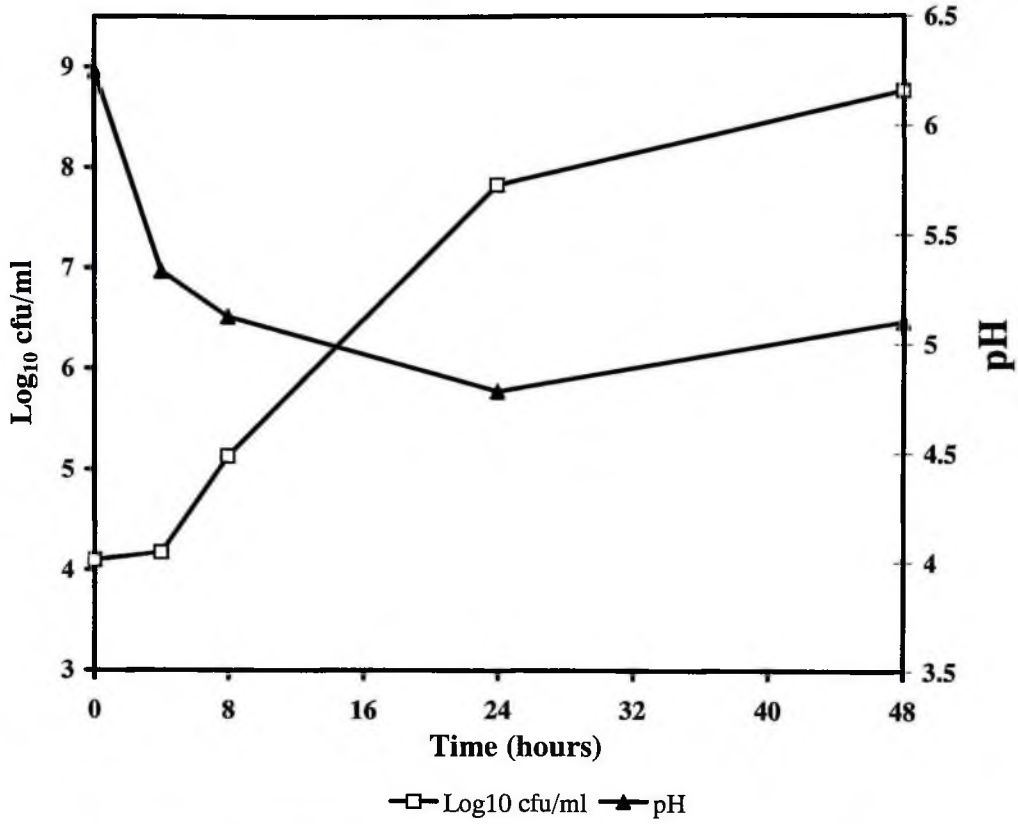

Figure 6. Changes in pH and numbers of coliform bacteria during maize steeping at ambient temperature of 28 °C.

beginning of steeping increase in numbers. This was confirmed in the maize steeping experiments run alongside the inoculation experiments in the present study.

During maize fermentation (Tables 8, 9 and 10), the initial pH of dough dropped rapidly to 4.4 – 4.9 after 8 h. The subsequent drop in pH was much slower, till it reached 3.8 to 4.3 at 24 h. No significant drop in pH was observed thereafter except for the fermentations in which the pH was more than 4.0 at 24 h where there was further drop to less than 4.0 after 48 h. Of the five *Salmonella* species (Table 9), only *Salm. enteritidis* and *Salm. stanleyville* were significantly ($P < 0.0001$) reduced after 8 h of fermentation but after 24 h when the pH of the dough had dropped to below 4.0, significant reductions in numbers were observed for all the *Salmonella* species such that four were completely inhibited whilst *Salm. stanleyville* was only partially inhibited. *Salm. stanleyville* was however, not detected in 25 g of dough after 48 h of fermentation when the pH had dropped to 3.8.

There were no significant reductions in numbers of the shigellae after 8 h of dough fermentation but significant reductions were seen after 24 h when the pH was about 4.0 and viable bacteria were not detected in 1 g of dough after 48 h (Table 9).

As observed for the *Shigella* species, significant reductions in numbers of *E. coli* were seen only after 24 h when the pH of the dough had dropped to 3.9 – 4.3 (Table 10). *E. coli* strains O157 (VTEC I) and O111 (EPEC) were not detected in dough

after 24 and 48 h of fermentation. But *E. coli* O26 (VTEC), O157 (VTEC II) and O3 (EAggEC) survived in the dough for 48 h having reduced significantly by about 3 to 4 $\log_{10}$ cfu/ g of the original concentration.

During spontaneous fermentation of maize dough without added Gram-negative bacteria, coliform bacteria present at the beginning of fermentation showed no reduction in numbers until after 8 h when the pH of dough had dropped to 4.6 then their numbers reduced significantly ($P< 0.0001$) from 6.21 ± 0.99 $\log_{10}$ cfu/g of dough to less than 2.0 $\log_{10}$ cfu/g after 24 to 48 hours at pH of ≤ 4 (Fig.7).

The complete inhibition of the pathogens in the fermenting maize dough after 24 to 48 h coincided with the drop in pH to ≤ 4, which is the critical level necessary for growth inhibition of enteropathogenic bacteria (Nout *et al.*, 1989; Kingamkono *et al.*, 1994, 1995; Olsen *et al.*, 1995). The salmonellae, shigellae and *E. coli* strains were all susceptible to the inhibitory effect of fermenting maize dough but *E. coli* survived longer. Whereas all the salmonellae, and shigellae were completely inhibited in the fermenting maize dough after 48 h, some of the *E. coli* strains survived in the dough after 48 h. Mensah *et al.* (1988, 1991) also observed differences in the susceptibilities of the *Shigella flexneri* and ETEC strains inoculated into unfermented maize dough (pH 6.1 ± 0.1), fermented maize dough (pH 3.3 ± 0.1) and fermented maize dough porridge (pH 3.3) as found in the present investigations. Fourteen strains of *Shigella flexneri* showed complete inhibition whereas six showed partial inhibition after 48 h

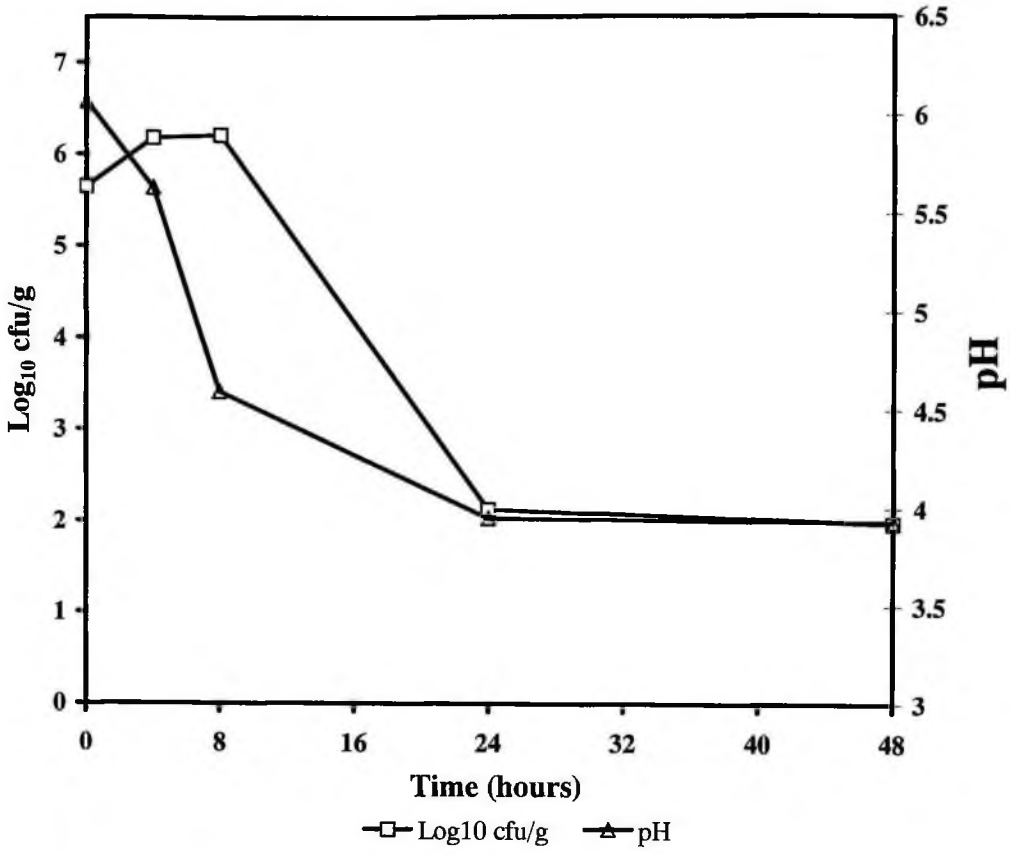

Figure 7. Changes in pH and numbers of coliform bacteria during spontaneous fermentation of maize dough at ambient temperature of 28 °C.

when the fermentation process had become established. Similarly, fifteen strains of ETEC were completely inhibited while five were partially inhibited. Annan-Prah and Agyeman (1997) also showed that the populations of enterotoxigenic *Escherichia coli* J955 and 101685, *Staphylococcus aureus*, and *Klebsiella pneumoniae* were reduced by 1-3 log units in 30 min in Ga-kenkey of pH 4 and kenkey water but the rate of decline was variable for different organisms.

4.1.2 Survival of Gram-Negative Pathogens in Koko and Ga Kenkey Water.

The pH of batches of koko and kenkey water used for the survival studies was 3.6, and only minor changes were observed during the 48 h period of the experiments (Tables 11, 12, and 13). The amounts of titratable acidity expressed as lactic acid, in koko and kenkey water used in the inoculation experiments were 0.36 ± 0.03% and 0.45 ± 0.13% (w/v) respectively. All the *Salmonella* species were inhibited within 24 h of inoculation into both koko and kenkey water (Table 11), but there was strain to strain variability in the rate of inhibition. Within 4 to 8 h after inoculation into the products, three strains *Salm. enteritidis*, *Salm. typhi,* and *Salm. typhimurium* were completely inhibited whilst two strains *Salm. durban* and *Salm. stanleyville* were not completely inhibited until after 24 h.

Some strains of *Shigella flexneri* and *E. coli* survived longer than 24 h in koko and kenkey water but were more strongly inhibited in kenkey water than in koko. *Sh. flexneri* 2a II survived in kenkey water for 24 h with the initial counts having been reduced from $\log_{10}$ 7.01 to $\log_{10}$ 5.18 and was not completely inhibited until after 48 h

Table 11. pH and changes in numbers of *Salmonella* serotypes inoculated into koko and kenkey water incubated at ambient temperature of 28° C and plated on Trypticase Soy Yeast Extract Agar.

Time after inoculation (h)	Koko pH	Log_{10} CFU/ ml	Kenkey water pH	Log_{10} CFU/ ml
Salm. durban				
0	3.64 a	7.28 a	3.60 a	7.34 a
4	3.64 a	7.06 b	3.57 a	6.92 b
8	3.62 a	5.04 c	3.57 a	5.98 c
24	3.54 a	–	3.59 a	–
48	3.58 a	–	3.59 a	–
Salm. enteritidis				
0	3.66 a	7.38 a	ND	ND
4	3.63 a b	4.69 b	ND	ND
8	3.63 a b	–	ND	ND
24	3.52 c	–	ND	ND
48	3.54 b c	–	ND	ND
Salm. stanleyville				
0	3.65 a	7.46 a	3.60 a	7.14 a
4	3.64 a	7.03 b	3.58 a	6.78 b
8	3.62 a b	5.44 c	3.56 a	6.12 c
24	3.50 b c	–	3.59 a	–
48	3.50 c	–	3.58 a	–
Salm. Typhi				
0	3.65 a	7.12 a	3.60 a	7.00 a
4	3.64 a	–	3.58 a	7.00 a
8	3.60 a b	–	3.57 a	–
24	3.55 b c	–	3.65 a	–
48	3.51 c	–	3.53 a	–
Salm. typhimurium				
0	3.64 a	7.55 a	3.60 a	7.16 a
4	3.63 a	5.16 b	3.60 a	3.65 b
8	3.62 a	–	3.56 a	–
24	3.58 a	–	3.62 a	–
48	3.56 a	–	3.55 a	–

– Not detected in 25 g after resuscitation in buffered peptone water, selective enrichment in Rappaport Vassiliadis (RV) medium with subsequent streaking onto Xylose Lysine Deoxycholate Agar (XLD) and SSI enteric medium agar plates.
ND = Not determined.
Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P < 0.05$). The results are shown as the mean of two independent experiments carried out on two separate occasions.

Table 12. pH and changes in numbers of *Shigella dysenteriae* and *Shigella flexneri* strains inoculated into koko and kenkey water incubated at ambient temperature of 28° C and plated on Trypticase Soy Yeast Extract Agar.

Time after inoculation (h)	Koko pH	Log_{10} CFU ml^{-1}	Kenkey water pH	Log_{10} CFU ml^{-1}
Sh. dysenteriae				
0	3.64 a	7.13 a	ND	ND
4	3.64 a	6.73 b	ND	ND
8	3.61 a	6.01 c	ND	ND
24	3.57 a	2.78 d	ND	ND
48	3.53 a	–	ND	ND
Sh. flexneri 2a I				
0	3.64 a	7.12 a	3.60 a	7.05 a
4	3.64 a	6.13 b	3.60 a	4.41 b
8	3.59 a	5.48 c	3.56 a	–
24	3.59 a	–	3.62 a	–
48	3.56 a	–	3.52 a	–
Sh. flexneri 2a II				
0	3.63 a	7.16 a	3.59 a	7.01 a
4	3.64 a	7.15 a b	3.58 a	6.84 b
8	3.60 a	6.98 b c	3.56 a	6.82 b
24	3.59 a	6.85 c	3.62 a	5.18 c
48	3.58 a	5.65 d	3.54 a	–

ND = Not determined.
– Less than 1 CFU/ml confirmed by resuscitation in Trypticase Soy Broth plus 0.6% Yeast Extract followed by surface streaking on SSI enteric medium agar plates.

Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P < 0.05$). The results are shown as the mean of two independent experiments carried out on two separate occasions.

Table 13. pH and changes in numbers of *Escherichia coli* serotypes inoculated into koko and kenkey water incubated at ambient temperature of 28° C and plated on Trypticase Soy Yeast Extract Agar.

Time after inoculation (h)	Koko pH	Log_{10} CFU/ ml	Kenkey water pH	Log_{10} CFU/ ml
E. coli O111 (EPEC)				
0	3.65 a	7.24 a	3.62 a	7.08 a
4	3.68 a	7.16 a	3.63 a	6.62 a
8	3.60 a	6.56 b	3.63 a	5.07 b
24	3.60 a	5.83 c	3.64 a	2.91 c
48	3.58 a	3.55 d	3.60 a	–
E. coli O157 (VTEC I)				
0	3.63 a	7.36 a	3.54 a	7.18 a
4	3.62 a	5.88 b	3.55 a	6.13 b
8	3.63 a	4.00 c	3.54 a	4.90 c
24	3.59 a	–	3.59 a	–
48	3.63 a	–	3.56 a	–
E. coli O157 (VTEC II)				
0	3.62 a	7.27 a	3.63 a	7.28 a
4	3.67 a	7.27 a	3.64 a	7.24 a
8	3.60 a	7.22 a	3.60 a	7.03 b
24	3.59 a	6.60 b	3.64 a	4.52 c
48	3.56 a	6.36 b	3.59 a	–
E. coli O26 (VTEC)				
0	3.64 a	7.32 a	3.60 a	7.19 a
4	3.64 a	7.19 a	3.59 a	7.14 a b
8	3.63 a	7.15 a	3.58 a	6.90 b
24	3.53 a	6.30 b	3.62 a	4.12 c
48	3.64 a	5.62 c	3.55 a	–
E. coli O3 (EAggEC)				
0	3.68 a b	7.35 a	3.64 a	7.29 a
4	3.69 a	7.14 a	3.64 a	7.13 a b
8	3.60 a b	7.10 a	3.64 a	6.89 b
24	3.56 c	6.27 b	3.66 a	3.10 c
48	3.58 b c	5.20 c	3.59 a	–
E. coli M23				
0	3.64 a b	7.27 a	3.62 a	7.25 a
4	3.64 a b	5.49 b	3.60 a	6.75 a
8	3.68 a	4.25 c	3.59 a	5.96 b
24	3.61 b	–	3.60 a	–
48	3.62 b	–	3.59 a	–
E. coli K12				
0	3.66 a	7.30 a	3.62 a	7.25 a
4	3.68 a	7.13 b	3.60 a	7.10 a b
8	3.65 a	6.97 b	3.60 a	6.97 b
24	3.60 a	6.18 c	3.63 a	2.91 c
48	3.56 a	5.45 d	3.59 a	–

–Less than 1 CFU/ ml confirmed by resuscitation in Trypticase Soy Broth plus 0.6% Yeast Extract followed by surface streaking on SSI enteric medium agar plates.
Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P < 0.05$).
The results are shown as the mean of two independent experiments carried out on two separate occasions.

(Table 12). However, *Sh. flexneri* 2a I was completely inhibited in kenkey water after 8 h of inoculation. *Sh. flexneri* strain 2a II was also only partially inhibited in koko after 48 h with significant reduction in numbers from $\log_{10}$ 7.16 to $\log_{10}$ 5.65 whilst *Sh. flexneri* 2a I and *Sh. dysenteriae* were completely inhibited after 48 h. Table 13 shows the survival of 5 pathogenic *E coli* strains and 2 laboratory strains tested in koko and kenkey water. Four pathogenic strains i.e. O157 (VTEC II), O26 (VTEC), O3 (EAggEC), O111 (EPEC) and one laboratory strain *E. coli* K12, survived in koko for 48 h by which time their numbers had reduced by only 1 – 2 log cfu/ ml. Only pathogenic *E. coli* strain O157 (VTEC I) and the non-pathogenic *E coli* M23 were completely inhibited in koko after 24 h. The same strains were also completely inhibited in kenkey water after 24 h. All the five strains of *E. coli* that survived in koko for 48 h were completely inhibited in kenkey water after 48 h of inoculation.

The longer survival of the *E. coli* strains in koko was not limited to the O157 (VTEC II) strain as two other pathogenic *E. coli* strains O26 (VTEC), and O3 (EAggEC) as well as the non-pathogenic *E. coli* K12 strain, showed similar resistance as O157 VTEC II in koko. Also, one of the O157 VTEC strains (O157 VTEC I) showed similar sensitivity in fermenting maize dough, koko and kenkey water as the sensitive *Salmonella* and *Shigella* strains. It was observed that of the two non-pathogenic *E. coli* strains tested, strain K12 was as resistant as the pathogenic *E. coli* strains in koko whilst *E. coli* M23, which is reported to react more like the virulent *E. coli* VTEC O157 strains (Shadbolt *et al.* 1999) was inhibited within 24 h of inoculation into koko.

The pH of koko (3.64 ± 0.01) and kenkey water (3.60 ± 0.02) used in these experiments was lower than the pH of 48 h fermented dough (3.85 ± 0.10), but the pathogens were more inhibited in fermented dough and in kenkey water than in koko. This could be attributed to the differences in the organic acid contents of the products. The amount of lactic acid in kenkey water used in the inoculation experiments with the Gram-negative bacteria was higher (0.45 ±0.13%) than that of koko (0.36 ±0.03%). The salt (NaCl) content of about 6% (w/w) in kenkey water could also enhance the inhibition of the pathogens. Salt was not added to koko used in these experiments.

The different sensitivities observed amongst the various pathogens tested in the systems in the present studies are in agreement with results obtained by other investigators using different fermented food systems. Nout *et al.* (1989) found differences in survival rates of *Salmonella* species, *Shigella* species and *Escherichia coli* strains inoculated into sorghum/pigeon pea porridge of pH ≤ 4.0. Their results indicated that the most resistant *Salmonella* sp. died at a faster rate (1.2 log cycle/h) than the most resistant *Shigella* (0.9 log cycle/h) and *Escherichia coli* (0.6 log cycle/h) strains. Simango and Rukure (1992) also found none of the *Aeromonas* and *Campylobacter* spp. 20 minutes after inoculation into *mahewu* and sour porridge. The *Salmonella* species were not found 4 h after inoculation in either food but some *Shigella* species and pathogenic *E. coli* strains survived for 24 h but showed sharp decreases in numbers.

Many fermented food systems contain metabolites produced by lactic acid bacteria and yeasts during the fermentation processes principally, lactic acid and acetic acid that have been shown to be bactericidal to some organisms such as *E. coli, Salmonella, Listeria* and yeast (Lindgren and Dobrogosz, 1990; Piard and Desmazeaud, 1991; Earnshaw, 1992). The main organic acids in fermented maize dough were reported to be lactic, acetic, butyric and propionic acids (Plahar and Leung, 1982; Halm *et al.*, 1993). Also many aroma compounds such as, alcohols, carbonyls, esters, acids and phenolic compounds have been detected in spontaneously fermented maize dough (Halm *et al.*, 1993; Annan *et al.,* 2003a). Amongst these, alcohols and esters were produced in much larger amounts and ethanol was the most abundant alcohol whilst ethyl acetate and ethyl lactate were the most abundant esters (Annan *et al.,* 2003a). It has been shown that ethanol can act in synergy with lactic acid to inhibit the survival of *E. coli* O157:H7 (Jordan *et al.,* 1999).

That some strains of *E. coli* and *Shigella flexneri* survived in koko pH 3.6 for 48 h with less than 2.0 $\log_{10}$ cfu/ml in the present investigations is not surprising. The unusual acid tolerance of verotoxin producing *E. coli* particularly *E. coli* O157:H7 has been previously documented. Glass *et al.* (1992) showed that *E. coli* O157:H7 can survive fermentation, drying, and storage of fermented sausage (pH 4.8) for up to 2 months at 4 °C, with only 100-fold reduction in cell populations. Brackette *et al.* (1994) showed that neither acetic, citric nor lactic acid at concentrations up to 1.5% applied to beef at 20 or 55 °C appreciably reduced *E. coli* O157:H7. Conner and Kotrola (1995) showed that *E. coli* O157:H7 has the ability to survive in acidic

conditions (pH $\geq$ 4.0) for up to 56 days, but survival is affected by type of acidulant and temperature. Zhao and Doyle (1994) showed that *E. coli* O157:H7 inoculated into commercial mayonnaise pH 3.6 – 3.9 and incubated at 5 or 20 °C did not grow at either temperature but survived for 34 to 55 days at 5 °C and 8 to 21 days at 20 °C.

It is known that enteric microorganisms have evolved several mechanisms for handling acid stress. *Escherichia coli* O157:H7 and *Sh. flexneri* can survive extreme acid conditions of pH 2.5 or less for a number of hours in vitro (Arnold and Kaspar, 1995; Gordon and Small, 1993; Lin *et al.*, 1996; Waterman and Small, 1996a, 1996b). This acid resistance is induced in stationary phase under starvation conditions and is dependent upon the alternate sigma factor, σ^S, encoded by *rpoS* (Cheville *et al.*, 1996; Small *et al.*, 1994; Waterman and Small, 1996a, 1996b).

It has been shown that commensal and O157 strains of *E. coli* possess three overlapping acid survival systems whose various levels of control and differing requirements for activity ensure that at least one system will be available to protect the stationary-phase cell under naturally occurring acidic environments (Castanie-Cornet *et al.*, 1999; Lin *et al.*, 1995, 1996). These systems include (1) σ^S –dependent oxidative system, originally thought to be acid induced, but found to be induced following entry into stationary phase regardless of the pH of the medium. This system, is expressed in oxidatively metabolising bacteria grown in complex media, but will protect cells in minimal medium to pH 2.5. (2) Acid-induced arginine-dependent system, which is expressed in fermentatively metabolising cells which will also protect cells in minimal medium to pH 2.5 but only if the medium is

supplemented with arginine (arginine-dependent acid resistance); (3) A glutamate-dependent system, which is expressed in fermentatively metabolising cells which will also protect cells in minimal medium to pH 2.5 but only if the medium is supplemented with glutamate. *Shigella flexneri* also possess similar acid survival systems as *E. coli i.e.* the oxidative system and the glutamate-dependent system but not the arginine- dependent system. The mechanism of acid tolerance appears to be associated with proteins that can be induced following entry into the stationary phase and by pre-exposing the bacteria to acidic conditions.

4.1.3 Effect of Selective and Non-Selective Media on Recovery of Pathogens from Koko and Kenkey water.

To adequately quantify the surviving bacteria in koko and kenkey water, the recovery from the use of selective (SSI enteric medium) and non-selective (Trypticase Soy Yeast Extract Agar) agars was compared. Bacterial numbers of *Salmonella, Shigella* and *E. coli* serotypes recovered from inoculated koko on SSI enteric medium and Trypticase Soy Yeast Extract Agar are listed in Table 14. Generally, but not always significant, more organisms were recovered from direct plating on Trypticase Soy Yeast Extract Agar than on the SSI enteric medium for all the species tested. A change with time was observed. As storage time increased, the difference between the numbers of organisms detected on selective and non-selective medium increased. Up to and more than 3 $\log_{10}$ cfu /g differences between counts on SSI enteric medium and Trypticase Soy Yeast Extract Agar were observed for the survival studies with koko.

Table 14. Mean populations of Gram-negative bacteria inoculated into koko incubated at ambient temperature of 28° C and recovered on Trypticase Soy Yeast Extract Agar (non-selective medium) and on State Serum Institute Enteric Medium ((SSI) selective medium).

		Log_{10} CFU/ ml				
	Enumeration medium*	0 h	4 h	8 h	24 h	48 h
E. coli O157 (VTEC II)	TSAYE	7.27 a	7.27 a	7.23 a	6.77 a	6.36 a
	SSI	7.26 a	7.01 a	6.77 a	6.61 a	3.21 b
E. coli M23	TSAYE	7.27 a	5.49 a	4.25 a	<2.0 a	<2.0 a
	SSI	6.12 a	2.80 b	<2.0 b	<2.0 a	<2.0 a
Salm. stanleyville	TSAYE	7.47 a	7.03 a	5.44 a	<2.0 a	<2.0 a
	SSI	7.43 a	5.36 b	2.39 b	<2.0 a	<2.0 a
Salm. typhi	TSAYE	7.12 a	<2.0 a	<2.0 a	<2.0 a	<2.0 a
	SSI	5.65 b	<2.0 a	<2.0 a	<2.0 a	<2.0 a
Sh. flexneri 2a II	TSAYE	7.15 a	7.01 a	6.94 a	6.97 a	5.65 a
	SSI	7.10 a	7.01 a	6.97 a	6.85 a	3.22 b
Sh. dysenteriae	TSAYE	7.13 a	6.73 a	6.01 a	2.70 a	<2.0 a
	SSI	6.98 a	5.28 b	3.53 b	<2.0 b	<2.0 a

* TSAYE, Trypticase Soy Agar plus 0.6% Yeast Extract; SSI, State Serum Institute Enteric Medium.

Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P < 0.05$).

The results are shown as the mean of two independent experiments carried out on two separate occasions.

Because the use of selective agar underestimated the numbers of the test organisms that survived in koko and kenkey water as compared to the recovery from the non-selective medium, the non-selective medium was used for the recovery of inoculated bacteria from the cooked products koko and kenkey water in order to obtain the true lethality. The ability to detect sub lethally injured cells in acidic foods is particularly important due to the fact that stressed cells retain their virulence and are still considered to be a hazard (McCarthy *et al.*, 1998). This is especially important in the case of *E. coli* (VTEC) O157: H7 that has a low infective dose of less than a few hundred cells per g. (Doyle *et al.*, 1997).

4.1.4 Survival of Gram-Negative Pathogens in pH-Adjusted Koko and Kenkey Water

It was observed that the pathogens were not inhibited in fermenting maize dough until the pH had dropped to below 4.0 and the pathogens were partially or completely inhibited in koko and kenkey water (pH 3.6) indicating that it was the low pH/acidity that was responsible for the inhibition of the pathogens. This necessitated the challenging of some of the test pathogens in koko and kenkey water adjusted from pH of 3.6 to near neutral (pH 6.0). None of the inoculated pathogens was inhibited in the pH adjusted koko and kenkey water. Rather, some bacteria showed significant ($P < 0.05$) increases in numbers over the 48 h period of incubation (Tables 15 and 16). By raising the pH of koko and kenkey water it was confirmed that the low pH/acidity is a critical factor in determining the survival of each enteric pathogen.

Table 15. Changes in numbers of Gram-negative bacteria after inoculation into koko at neutral pH kept at ambient temperature (28 °C). [1]

	Time after inoculation (h)	*Salm. typhimurium*	*Salm.typhi*	*Salm. enteritidis*	*Sh. dysenteriae* 1	*Sh. flexneri* 2a I	*Sh. flexneri* 2aII	*E. coli* O111 (EPEC)	*E. coli* O157 (VTEC I)	*E. coli* O26 (VTEC)
Koko										
Log_{10} cfu/g	0	7.22 (0.05) a	7.20 (0.04) a	6.97 (0.04) a	6.72 (0.45) a	6.99 (0.08) a	6.99 (0.13) a	6.83 (0.25) a	7.00 (0.07) a	6.69 (0.02)a
	4	7.39 (0.07) a	7.32 (0.09) a	7.00 (0.06) a	6.72 (0.17) a	7.20 (0.08) a	7.04 (0.06) a	6.80 (0.14) ab	7.15 (0.15) ab	7.04 (0.27)ab
	8	7.26 (0.21) a	7.42 (0.24) a	6.98 (0.19) a	6.72 (0.17) a	7.23 (0.07) a	6.89 (0.16) a	6.91 (0.29) ab	7.14 (0.09) ab	7.14 (0.20) ab
	24	7.38 (0.25) a	7.68 (0.60) a	7.00 (0.21) a	7.10 (0.70) a	7.19 (0.02) a	7.03 (0.11) a	7.87 (0.16) bc	7.63 (0.09) bc	7.43 (0.67) ab
	48	7.91 (0.19) b	8.22 (0.92) a	7.94 (0.14) b	7.46 (0.54) a	7.39 (0.55) a	7.75 (0.21) b	8.71 (0.23) c	7.83 (0.40) c	8.00 (0.64) b
pH	0	6.1 (0.0)	6.1 (0.0)	6.0 (0.0)	6.1 (0.0)	6.0 (0.0)	6.1 (0.0)	6.0 (0.0)	6.1 (0.0)	6.1 (0.0)
	4	5.8 (0.1)	5.7 (0.0)	5.8 (0.0)	5.7 (0.0)	5.8 (0.0)	5.8 (0.0)	5.8 (0.0)	5.8 (0.0)	5.7 (0.0)
	8	5.6 (0.1)	5.5 (0.0)	5.6 (0.0)	5.5 (0.0)	5.6 (0.0)	5.3 (0.1)	5.6 (0.0)	5.3 (0.1)	5.5 (0.0)
	24	5.3 (0.1)	5.2 (0.1)	5.4 (0.1)	5.2 (0.1)	5.3 (0.1)	5.3 (0.1)	5.4 (0.1)	5.3 (0.1)	5.2 (0.1)
	48	5.3 (0.1)	5.1 (0.1)	5.3 (0.1)	5.1 (0.1)	5.3 (0.1)	5.3 (0.1)	5.3 (0.1)	5.3 (0.1)	5.1 (0.2)

[1] Results are means of two independent experiments.
[2] Means with the same letter in a column are not significantly different ($P< 0.05$).

Table 16. Changes in numbers of Gram-negative bacteria after inoculation into kenkey water at neutral pH kept at ambient temperature (28 °C).[1]

	Time after inoculation (h)	*Salm. typhimurium*	*Salm .typhi*	*Salm. enteritidis*	*Sh. dysenteriae* 1	*Sh. flexneri* 2a I	*Sh. flexneri* 2a II	*E. coli* O111 (EPEC)	*E. coli* O157 (VTEC I)	*E. coli* O26 (VTEC)
u/g (SD)	0	7.32 (0.03) a	7.37 (0.19) a[2]	7.11 (0.10) a	7.04 (0.0) a	7.08 (0.08) a	7.00 (0.09) a	7.20 (0.09) a	7.16 (0.03) a	6.99 (0.01) a
	4	7.50 (0.07) a	7.67 (0.06) a	7.35 (0.10)ab	7.43 (0.15)ab	7.36 (0.03) ab	7.43 (0.02) ab	7.31 (0.11) a	7.47 (0.07) b	7.22 (0.02) a
	8	7.85 (0.10) b	7.77 (0.07) ab	7.85 (0.05) c	7.60 (0.06) ab	7.78 (0.03) b	7.65 (0.13) b	8.00 (0.02) b	7.70 (0.05) bc	7.32 (0.09) a
	24	7.96 (0.16) b	7.80 (0.40) ab	7.78 (0.09) bc	7.62 (0.59) ab	7.62 (0.16) ab	7.56 (0.31) b	8.14 (0.04) b	7.84 (0.19) c	7.39 (0.50)ab
	48	8.07 (0.07) b	8.20 (0.04) b	7.52 (0.37) abc	7.39 (0.05) b	7.39 (0.44) ab	7.92 (0.32) b	8.17 (0.09) b	8.18 (0.0) d	7.96 (0.08) b
	0	6.0 (0.0)	6.1 (0.0)	6.2 (0.0)	6.1 (0.0)	6.2 (0.0)	6.0 (0.0)	6.2 (0.0)	6.0 (0.0)	6.1 (0.0)
	4	5.9 (0.1)	5.7 (0.1)	5.9 (0.0)	5.7 (0.1)	5.9 (0.0)	5.9 (0.1)	5.9 (0.0)	5.9 (0.1)	5.7 (0.1)
	8	5.2 (0.1)	5.2 (0.2)	5.3 (0.1)	5.2 (0.2)	5.3 (0.1)	5.2 (0.1)	5.3 (0.1)	5.2 (0.1)	5.2 (0.2)
	24	5.0 (0.2)	5.1 (0.0)	5.1 (0.1)	5.1 (0.0)	5.1 (0.1)	5.0 (0.2)	5.1 (0.1)	5.0 (0.2)	5.1 (0.0)
	48	5.1 (0.1)	5.2 (0.1)	5.1 (0.2)	5.2 (0.1)	5.1 (0.2)	5.1 (0.1)	5.1 (0.2)	5.1 (0.1)	5.2 (0.1)

s are means of two independent experiments.
; with same letter in a column are not significantly different ($P < 0.05$).

4.1.5 Survival of *E. coli* in Laboratory Media Supplemented with Organic Acids

Model experiments in Trypticase Soy Broth plus 0.6% yeast extract were conducted to determine the effect of lactic and acetic acids at the range of concentrations found in fermented maize dough and fermented maize dough products in combination with low pH of 3.5 on inactivation of four *E. coli* strains. *E. coli* strains were used for the broth studies because they were the least inactivated in the products. Because of the highly virulent property of the VTEC O157 strains, they were not included in the broth studies. Instead, a non-pathogenic laboratory strain *E. coli* M23, which has been reported to respond similarly to acid stress as some of the most virulent pathogenic *E coli* O157:H7 strains (Shadbolt *et al.*, 1999), and another non-pathogenic *E. coli* K12 in addition to two pathogenic *E. coli* O111 (EPEC) and *E. coli* O3 (EAggEC) strains were used for the broth studies. *E. coli* M23 and K12 were earlier tested in koko and kenkey water for comparison with the verotoxigenic *E. coli* strains. *E. coli* K12 showed equal sensitivity as the resistant VTEC O157 strains tested whilst *E. coli* M23 was found to be very sensitive in koko and kenkey water.

Figure 8 shows the inhibitory effect of undissociated lactic acid (0 – 110 mM) at pH 3.5 on four selected *E. coli* strains after 24 h exposure at 30°C. The extent of inhibition on the four strains was dependent on the concentration of the acid and the strain. At a pH of 3.5 adjusted with HCl no appreciable inhibitory effect was observed on all four *E. coli* strains as indicated by less than one log unit decrease in the initial numbers inoculated. At concentrations between 31 mM (equivalent to 0.4% total

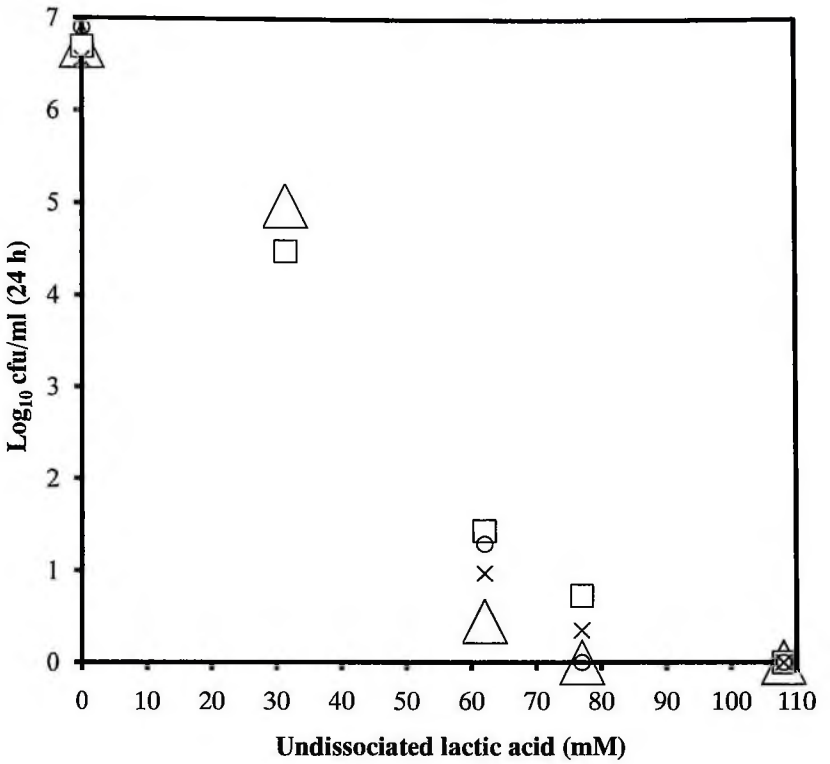

Figure 8. Effect of undissociated lactic acid concentration in Trypticase Soy Broth supplemented with 0.6% Yeast Extract at pH 3.5 on the survival of *Escherichia coli* strains (Δ) O111 (EPEC), (□)O3 (EAggEC), (o)K12 and (×) M23.

lactic acid at pH 3.5) and 62 mM (0.8% total lactic acid at pH 3.5), undissociated lactic acid showed a bacteriostatic effect on the four strains and above 62 mM a bacteriocidal effect on all four *E. coli* strains was observed after 24 h exposure (Fig. 8). A similar trend was observed with acetic acid as shown in Fig. 9 but lower concentration of undissociated acetic acid i.e. > 17 mM - 33 mM (equivalent to > 0.1% - 0.2% total acetic acid at pH 3.5) at the same pH was required to completely inhibit the test organisms (Fig. 9). These results indicate that the concentrations of lactic and acetic acids found in fermented maize dough are sufficient to cause the inhibitions observed against the pathogens tested. They also confirm the earlier observation by Olsen *et al.* (1995) that the inhibitory effects of *Lactobacillus plantarum* and *Lactobacillus fermentum* isolated from fermented maize against Gram-positive and Gram-negative bacteria (*Staphylococcus carnosus, Staphylococcus caseolyticus, Enterobacter cloacae, Klebsiella pneumoniae* and *Proteus mirabilis*) in agar diffusion tests are largely due to acid production and the consequent decrease in pH during maize fermentation and that other antimicrobial metabolites do not play a significant role.

The present study has shown that koko and kenkey water of pH $\leq$ 4.0 made with lactic fermented maize dough have anti-microbial properties which may contribute to the reduction of food borne infection. However, the risk posed by the prolonged survival of some *E coli* and *Shigella flexneri* strains for up to 24 to 48 h in koko has not been emphasized in previous studies. The potential of lactic acid fermentation for controlling food contamination depends on many factors, such as initial level of contamination,

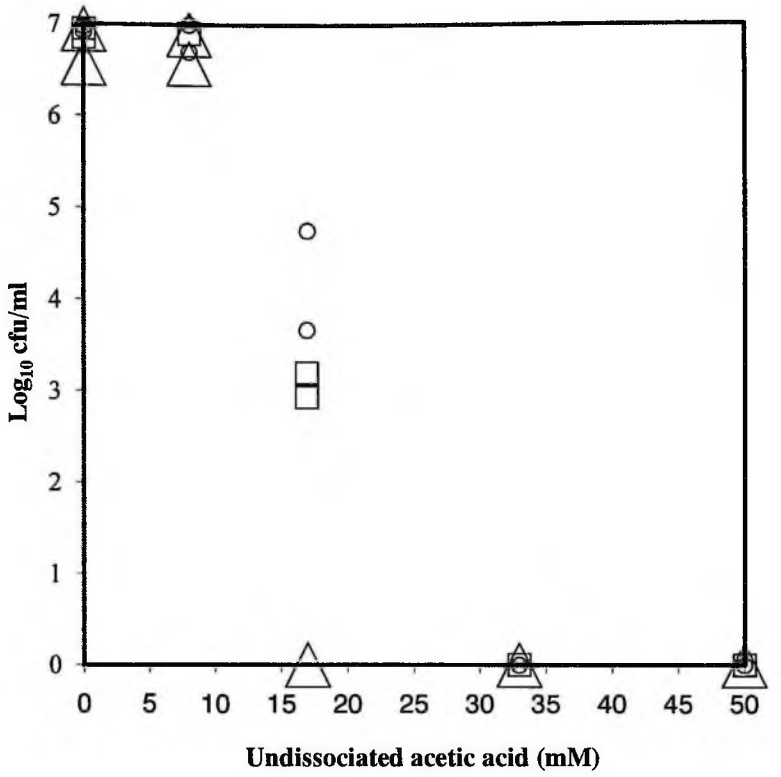

Figure 9. Effect of undissociated acetic acid concentration in Trypticase Soy Broth supplemented with 0.6% Yeast Extract at pH 3.5 on the survival of *Escherichia coli* strains (Δ) O111 (EPEC), (□)O3 (EAggEC) and (o)K12.

levels of hygiene and sanitation, which in turn depend on local conditions and on the degree of acidification. It should be noted that fermentation cannot replace the need for observing rules of food hygiene, and minimising the risk of post-processing contaminations. This is particularly important since some pathogens may be acid resistant and may survive in fermented products as shown for some of the *E coli* and *Shigella flexneri* strains in koko. The need for adequate acidification to obtain the desired pH $\leq$ 4.0 in fermented products, and the risk involved in keeping koko for 24 h or more should be emphasized. It is recommended that the Hazard Analysis Critical Control Point (HACCP) system be applied in production of fermented foods to ensure safety of products. It is also important that the monitoring of Critical Control Point (CCP) in HACCP system for fermented products should not be limited to pH. Proper storage and handling of the cooked fermented products should be ensured by defined guidelines to prevent contamination.

4.2 Modelling the Effect of Lactic Acid on Inactivation of *E. coli*

The influence of different lactic acid concentrations (0.2, 0.4, 0.8 and 1.2% V/V), within pH range of 3.5 to 4.2 on the survival kinetics of *Escherichia coli* strain *E. coli* O111 (EPEC), was determined in Trypticase Soy Broth with 0.6% Yeast Extract held at 30 °C. Survival data were analysed and fitted with the model of Peleg and Cole (1998). This model gave a good description of the survival of *E. coli* under the experimental conditions tested. Figure 10 is an example of the survival curves obtained at pH 3.5 with various concentrations of lactic acid. Both experimental data (symbols) and survival data as fitted by the model of Peleg and Cole are shown. This

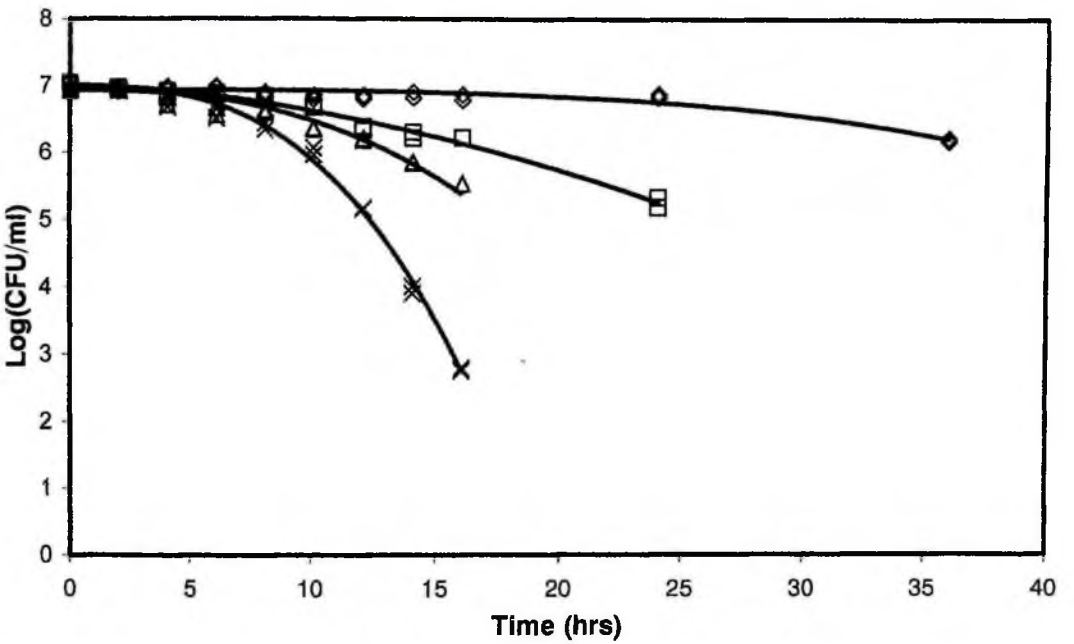

Figure10. Survival of *Escherichia coli* O111 (EPEC) in Tryptone Soy Broth supplemented with 0.6% yeast extract at pH 3.5 and 0.2% lactic acid (◊), 0.4% lactic acid (□), 0.8% lactic acid (Δ) and 1.2% lactic acid (×).

plot is characteristic of all the survival curves obtained from pH 3.5 to pH 4.2. The curves were characterised by having a downward concavity. This concavity can mean that the surviving bacteria are increasingly weakened by the exposure and therefore it takes progressively shorter time to destroy them (Peleg and Penchina, 2000).

The results of adding various concentrations of lactic acid at set pH values established that the rate at which lactic acid inactivates *E. coli* is dependent on the concentration of the acid and the pH of the system. At set pH levels, exposure to higher lactic acid concentration resulted in a greater decline in numbers. As shown in Figure 10, *E. coli* cells survived for 36 h when exposed to 0.2% total lactic acid at pH 3.5 with less than one log unit decrease, while exposure to 1.2% total lactic acid at the same pH resulted in a decline of more than 4 log units in 16 h. To compare the survival of *E. coli* under the different experimental conditions, the model parameters b and n found for each treatment were used to calculate the time to one log decrease in bacterial population by rearrangement of the model.

Table 17 shows the survival data for *E. coli* 0111 (EPEC) at different pH and lactic acid concentrations and the equivalent concentrations of undissociated lactic acid calculated using the Henderson-Hasselbalch equation with a pK_a value of 3.86 for lactic acid. Generally, a rise in total lactic acid concentration at set pH levels increased the death rate as seen by the reduction in time to one log decrease in numbers. It was also observed that at set total lactic acid concentrations the death rate was greatest and more pronounced at low pH (3.5).

Table 17. Survival data for *Escherichia coli* 0111 (EPEC) at different pH and concentrations of lactic acid.

pH	% lactic acid	Undissociated lactic acid (mM)	b	n	Time to one log reduction (h)
3.5	0.2	15.5	0.0000026	3.504	39.34
			0.000048	2.691	40.27
3.5	0.4	30.9	0.0078	1.707	17.24
			0.0075	1.654	19.33
3.5	0.8	61.8	0.0012	2.574	13.46
			0.0038	2.174	12.98
3.5	1.2	92.6	0.0016	2.847	9.64
			0.020	1.825	8.55
3.77	0.2	12.2			
3.77	0.4	24.5	0.000027	2.509	66.21
			0.00080	1.694	67.31
3.77	0.8	49.0	0.0039	1.938	17.62
			0.0035	2.046	15.90
3.77	1.2	73.4	0.0014	2.338	16.78
			0.00038	2.847	15.92
4	0.2	9.3			
4	0.4	18.7			
			-0.0224	0.136	
4	0.8	37.3	0.000027	2.919	36.75
			0.000026	3.045	32.06
4	1.2	55.9	0.0092	1.742	14.73
			0.00281	2.192	14.59
4.2	0.2	7.0	-		
4.2	0.4	13.9	0.00000081	2.982	110.16
4.2	0.8	27.9	0.0030	1.246	105.47
4.2	1.2	41.7	0.0000072	3.239	38.79
			0.000012	3.155	36.27

- parameters not able to converge

The time to one log decrease in bacterial population was also related to the level of undissociated lactic acid and it was observed that the time decreased consistently as the concentration of undissociated lactic acid increased (Table 17). The time to one log reduction was approximately equal for equal undissociated lactic acid concentrations, regardless of the pH or total lactic acid concentration. This implies that the most significant factor under the conditions tested was the undissociated acid, which in turn is determined by the total lactic acid concentration and pH. As can be seen from Table 17, undissociated lactic acid had a significant effect on rates of survival reducing the time as concentration increased e.g. 38.7 hours for a one log decrease was observed with 41.7 mM (pH 4.2, 1.2% total lactic acid at 30 °C) but 9.6 hours for the same decrease with 92.6 mM (pH 3.5, 1.2% total lactic acid at 30 °C).

The relationship between the time to one log reduction and the concentration of undissociated lactic acid is shown in Fig. 11. The strong correlation observed supports the concept that the undissociated acid is the active form of the acid. This also implies that increases in antimicrobial activity can be expected if the pH of a lactate containing system was decreased to a small extent such that it was closer to the acids pK_a.

Using multiple regression analysis on the data derived from the second stage of modelling, it was possible to generate a model predicting the time to one log reduction in numbers of *E. coli* 0111 EPEC in response to pH and total lactic acid concentration in TSBYE.

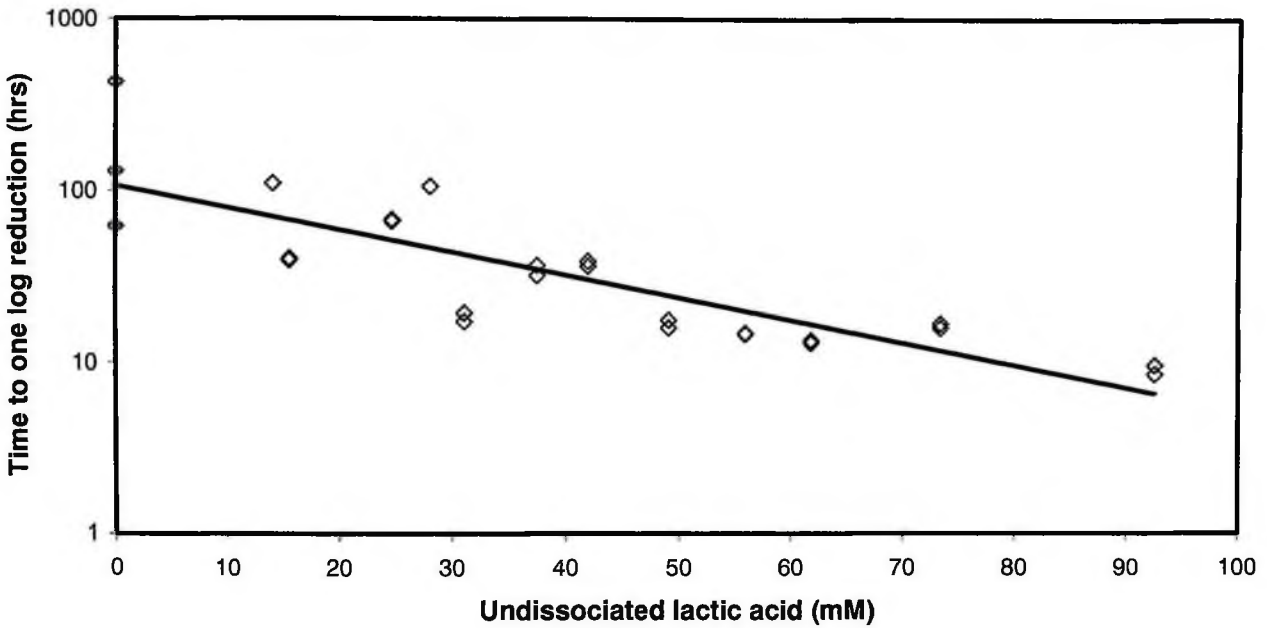

Figure 11. Correlation between concentration of undissociated lactic acid and time to one log reduction for *Escherichia coli* O111 (EPEC) at conditions listed in Table 21.

This model was: $Y(h) = -233.93(X_1) + 18.99(X_2) + 48.8(X_1 \times X_2)$ where Y, X_1 and X_2 are time to one log reduction in numbers of *E. coli* 0111 (EPEC), total lactic acid concentration and pH, respectively.

The analysis of variance section of the multiple regression report is shown in Table 18. It was observed from the probability levels that the three terms i.e. pH, lactic acid concentration and the interaction between lactic acid and pH all had significant ($p < 0.05$) effect on the model. The model could explain 81.45% ($R^2 = 0.8145$) of the variations in death rate of *E. coli* 0111 EPEC in TSBYE. Thus only 18.55% of the variation was due to other factors not included in the model. From the R^2 values of the three terms, the pH contributes the highest (40.53%) to the total R^2 value. It can be seen from the model that the death rate is negatively correlated to lactic acid concentration whilst pH is positively correlated and the interaction between lactic acid concentration and pH correlates positively with the death rate. Therefore a high lactic acid concentration and a low pH are required to increase the death rate of the pathogen. Since there is interaction between lactic acid concentration and pH it shows that there is an optimum pH and lactic acid concentration at which the death rate will not be significantly affected. The agreements between observed values from the experimental data as shown in Table 17 and the predicted values using the model are shown in Table 19. The proposed model for the death rate of *E. coli* 0111 EPEC in response to pH and total lactic acid concentration in TSBYE provided a good description of the data (Table 19). Except for the low pH of 3.5 and total lactic acid

Table 18. Analysis of variance section of the multiple regression report on response of the time to one log reduction in numbers of *Escherichia coli* 0111 EPEC to pH and lactic acid concentration in Tryptone Soy Broth supplemented with 0.6% Yeast Extract at 30 °C.

Model Term	DF	R^2	Sum of Square	Mean Square	F-Ratio	Probability Level	Power (5%)
Model	3	0.8145	35243	11747.67	27.806	0.0000	1.0000
Lactic acid	1	0.0941	4069.961	4069.961	9.634	0.0058	0.8374
pH	1	0.4053	17539.48	17539.48	41.516	0.0000	1.0000
Lactic acid*pH	1	0.0623	2695.434	2695.434	6.380	0.0206	0.6689
Error	19	0.1855	8027.115	422.4797			
Total	22	1.0000	43270.12	1966.823			

Table 19.

Comparison of observed time to one log reduction in numbers (T (h)) for the survival of *Escherichia coli* O111 (EPEC) in the presence of lactic acid and various combinations of pH versus values predicted using the model T (h) = -233.93*LA + 18.99*pH + 48.8*LA*pH

pH	% Lactic acid	Actual Time (h)	Predicted Time (h)	Standard Error of Predicted Time
3.5	0.2	39.8	53.9	8.1
3.5	0.4	18.3	41.3	6.5
3.5	0.8	13.2	16.0	6.8
3.5	1.2	9.1	-9.2	10.7
3.77	0.2	-	61.6	8.8
3.77	0.4	66.8	51.7	6.7
3.77	0.8	16.8	31.7	4.5
3.77	1.2	16.4	11.7	6.9
4.0	0.2		68.3	9.5
4.0	0.4	-	60.5	7.4
4.0	0.8	34.4	45.0	5.2
4.0	1.2	14.7	29.6	7.4
4.2	0.2		74.0	10.2
4.2	0.4	110.2	68.2	8.4
4.2	0.8	105.5	56.6	7.4
4.2	1.2	37.6	45.1	10.3

concentration of 1.2% where the model predicted a negative value for the death rate all the other predicted values were in agreement with the actual values obtained in the broth studies.

The observation that a high lactic acid concentration and a low pH are required to increase the death rate of the pathogen is in relation to the fact that lactic acid like other weak organic acids has optimal inhibitory activity at low pH because this favours the uncharged, undissociated state of the molecule which freely diffuses across the bulk lipids of the plasma membrane because of its lipid solubility and is thus able to enter the bacterial cell (Stratford and Rose, 1986). Subsequently, upon encountering the higher pH inside the cell (pH_i), the molecule dissociates resulting in the release of charged anions and protons, which cannot cross the plasma membrane. The extrusion of protons generated by the organic acid inside the cell uses energy in the form of ATP, therefore the constant influx of protons will eventually deplete cellular energy.

As the energy reserve is limited, proton extrusion through proton pumps (proton translocating ATPase) stops, causing a lowering of pH_i, which in turn denatures proteins and destabilizes other structural and functional components (enzymes, nucleic acids and phospholipids) of the cells and interferes with viability and growth (Booth, 1985; Booth and Kroll, 1989). In addition, the cell will accumulate the acid anion that can disrupt intracellular processes (Russell, 1992). Cherrington *et al.*

(1990) reported that the inhibitory effects of formic and propionic acids on the growth and metabolism of *E. coli* were related to reduction in rates of RNA, DNA, protein, lipid and cell wall synthesis. Organic acids such as lactic acid are also able to chelate elements essential for growth, such as iron, which may be a possible mechanism of inhibition (Shelef, 1994).

4.3 Growth Responses of *Candida krusei* and *Saccharomyces cerevisiae* to Low pH and Lactic Acid

In order to study the lactic acid tolerance of *C. krusei* and *Sacch. cerevisiae* in a system that partly mimics fermeted maize dough, single and mixed culture growth experiments with the two yeast species were carried out in MYGP broth, pH 2.5 with 106.4 mM undissociated lactic acid at 30 °C (Tables 20 – 23). As can be seen from Tables 20 and 21, *C. krusei* 29 grew from initial counts of approximately 4.0 $\log_{10}$ cfu/ ml to 7.0 $\log_{10}$ cfu/ ml irrespective of whether it was grown as a single culture or combined as a mixed culture with either strain of *Sacch. cerevisiae*. But *Sacch. cerevisiae* 31 performed better when grown as a single culture than as a mixed culture with *C. krusei* 29. In mixed cultures of the two yeasts, *C. krusei* achieved a viable count of about 7.0 $\log_{10}$ cfu/ ml after 24 h, whereas viable count of *Sacch. cerevisiae* 31 was less than 5.0 $\log_{10}$ cfu/ ml. The second strain of *Sacch. cerevisiae* 26-1-7 which in preliminary screening experiments was shown to be more acid sensitive than *Sacch. cerevisiae* 31, failed to grow over the 48 h period whether cultured singly or as a mixed culture with *C. krusei* at the low pH of 2.5 in the presence of 106.4 mM

Table 20. Changes in numbers of *Candida krusei* 29 and *Saccharomyces cerevisiae* 31 grown as single cultures and as mixed cultures in MYGP broth plus 1 % lactic acid pH 2.5 at 30 °C

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 29		*Saccharomyces cerevisiae 31*	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	4.00 (0.08)	4.45 (0.01)	3.93 (0.11)	4.85 (0.05)
8	4.72 (0.30)	4.62 (0.17)	4.00 (0.09)	4.61 (0.02)
16	6.76 (0.26)	6.52 (0.06)	4.61 (0.24)	4.30 (0.43)
24	7.72 (0.13)	7.17 (0.04)	5.52 (0.26)	ND
32	7.59 (0.10)	7.41 (0.06)	5.59 (0.16)	ND
40	7.51 (0.15)	7.36 (0.07)	6.07 (0.26)	ND
50	7.18 (0.19)	6.73 (0.09)	6.10 (0.26)	ND

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.
ND, no cells were detected in 10^{-5} dilution

Table 21. Changes in numbers of *Candida krusei* 29 and *Saccharomyces cerevisiae* 26-1-7 grown as single cultures and as mixed cultures in MYGP broth plus 1 % lactic acid pH 2.5 at 30 °C

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 29		*Saccharomyces cerevisiae* 26-1-7	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	4.00 (0.08)	4.51 (0.05)	3.86 (0.06)	4.69 (0.02)
8	4.72 (0.30)	4.45 (0.02)	3.78 (0.15)	4.50 (0.05)
16	6.76 (0.26)	6.65 (0.10)	3.52 (0.18)	4.0 (0.00)
24	7.72 (0.13)	7.12 (0.12)	3.51 (0.10)	ND
32	7.59 (0.10)	7.38 (0.04)	3.32 (0.21)	ND
40	7.51 (0.15)	7.36 (0.03)	3.14 (0.54)	ND
50	7.18 (0.19)	6.73 (0.09)	2.68 (0.17)	ND

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.
ND = not detected in 10^{-5} dilution

Table 22. Changes in numbers of *Candida krusei* 65 and *Saccharomyces cerevisiae* 31 grown as single cultures and as mixed cultures in MYGP broth plus 1 % lactic acid pH 2.5 at 30 °C

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 65		*Saccharomyces cerevisiae* 31	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	3.90 (0.09)	4.06 (0.03)	3.93 (0.11)	4.74 (0.01)
8	4.56 (0.35)	3.15 (0.21)	4.00 (0.09)	4.62 (0.04)
16	5.55 (0.22)	4.62 (0.18)	4.61 (0.24)	4.53 (0.04)
24	6.17 (0.28)	5.78 (0.04)	5.52 (0.26)	4.74 (0.06)
32	6.88 (0.20)	6.56 (0.01)	5.59 (0.16)	ND
40	7.21 (0.11)	7.05 (0.06)	6.07 (0.26)	ND
50	7.29 (0.28)	7.44 (0.06)	6.10 (0.26)	ND

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.
ND = not detected in 10^{-5} dilution

Table 23. Changes in numbers of *Candida krusei* 65 and *Saccharomyces cerevisiae* 26-1-7 grown as single cultures and as mixed cultures in MYGP broth plus 1 % lactic acid pH 2.5 at 30 °C

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 65		*Saccharomyces cerevisiae 26-1-7*	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	3.90 (0.09)	4.11 (0.05)	3.86 (0.06)	4.51 (0.09)
8	4.56 (0.35)	3.00 (0.00)	3.78 (0.15)	4.10 (0.02)
16	5.55 (0.22)	4.08 (0.05)	3.52 (0.18)	3.80 (0.14)
24	6.17 (0.28)	5.43 (0.10)	3.51 (0.10)	4.00 (0.00)
32	6.88 (0.20)	6.31 (0.04)	3.32 (0.21)	ND
40	7.21 (0.11)	7.47 (0.65)	3.14 (0.54)	ND
50	7.29 (0.28)	7.39 (0.02)	2.68 (0.17)	ND

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.
ND = not detected in 10^{-5} dilution

undissociated lactic acid (Table 21). A similar trend of growth performance was observed with *C. krusei* 65 (Tables 22 and 23) as shown for *C. krusei* 29 even though this strain had been found to be less tolerant to low pH and lactic acid than *C. krusei* 29 in preliminary screening experiments. The two strains of *Sacch. cerevisiae* showed similar growth responses as observed with *C. krusei* 29 when each strain was cultured as mixed cultures with *C. krusei* 65. The pH of MYGP broth was increased to 3.5 by addition of NaOH in the presence of 1% lactic acid in order to simulate conditions in maize dough at an advanced stage of fermentation. At the pH of 3.5 with total lactic acid concentration of 1% the amount of undissociated lactic acid present in the MYGP broth was 77 mM.

The growth of *C. krusei* 29 and two strains of *Sacch. cerevisiae* was monitored individually as single cultures and together as mixed cultures in MYGP broth pH 3.5, with or without 77 mM undissociated lactic acid at 30° C. The results as shown in Tables 24- 27 indicate that, irrespective of the presence or absence of lactic acid, *C. krusei* 29 grew from initial counts of about 4.0 log10 cfu/ml to about 8.0 log10 cfu/ml whether cultured singly or in combination with either strain of *Sacch. cerevisiae*. The two strains of *Sacch. cerevisiae* showed good growth as single cultures as indicated by increases of more than 3.0 log10 cfu/ml after 48 h in the presence or absence of lactic acid. But the strains of *Sacch. cerevisiae* showed reduced growth when cultured with *C. krusei* as mixed cultures and the reduced growth was greater in the presence of lactic acid. When the less lactic acid sensitive strain *Sacch. cerevisiae* 31 was grown in mixed culture with *C. krusei* 29 (Table 24), yeast counts of more than one log cycle lower were obtained after 48 h in MYGP broth pH 3.5 with 77 mM

Table 24. Changes in numbers of *Candida krusei* 29 and *Saccharomyces cerevisiae* 31 grown as single cultures and as mixed cultures in MYGP broth plus 1 % lactic acid pH 3.5 at 30 °C

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 29		*Saccharomyces cerevisiae* 31	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	4.08 (0.10)	4.07 (0.10)	4.23 (0.15)	4.00 (0.06)
4	4.35 (0.05)	4.38 (0.10)	4.50 (0.12)	4.50 (0.13)
8	6.00 (0.15)	5.96 (0.08)	5.80 (0.07)	5.70 (0.02)
16	7.75 (0.10)	7.57 (0.08)	7.69 (0.02)	7.47 (0.06)
24	7.77 (0.08)	7.62 (0.09)	7.76 (0.05)	7.19 (0.16)
32	7.64 (0.0.7)	7.61 (0.11)	7.39 (0.07)	6.71 (0.29)
40	7.78 (0.11)	7.80 (0.09)	7.24 (0.05)	6.00 (0.26)
48	7.82 (0.17)	7.90 (0.05)	7.28 (0.08)	6.13 (0.16)

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.

Table 25. Changes in numbers of *Candida krusei* 29 and *Saccharomyces cerevisiae* 31 grown as single cultures and as mixed cultures in MYGP broth pH 3.5 at 30 °C (without lactic acid)

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 29		*Saccharomyces cerevisiae* 31	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	4.05 (0.09)	4.07 (0.02)	4.22 (0.03)	4.07 (0.04)
4	4.36 (0.20)	4.39 (0.17)	4.55 (0.23)	4.42 (0.08)
8	6.05 (0.12)	5.98 (0.09)	5.95 (0.04)	5.73 (0.11)
16	7.79 (0.07)	7.57 (0.05)	7.82 (0.10)	7.57 (0.10)
24	8.06 (0.12)	7.89 (0.05)	7.95 (0.05)	7.43 (0.17)
32	7.97 (0.07)	7.84 (0.13)	7.86 (0.08)	7.41 (0.15)
40	8.10 (0.04)	8.02 (0.09)	7.89 (0.03)	7.25 (0.28)
48	8.12 (0.06)	8.00 (0.05)	7.92 (0.05)	7.38 (0.12)

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.

Table 26. Changes in numbers of *Candida krusei* 29 and *Saccharomyces cerevisiae* 26-1-7 grown as single cultures and as mixed cultures in MYGP broth plus 1 % lactic acid pH 3.5 at 30 °C

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 29		*Saccharomyces cerevisiae* 26-1-7	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	4.08 (0.10)	4.14 (0.07)	4.28 (0.04)	4.10 (0.04)
4	4.35 (0.05)	4.34 (0.06)	4.59 (0.07)	4.33 (0.09)
8	6.00 (0.15)	5.98 (0.08)	5.26 (0.14)	5.07 (0.14)
16	7.75 (0.10)	7.69 (0.09)	6.37 (0.08)	5.53 (0.29)
24	7.77 (0.08)	7.60 (0.09)	7.19 (0.12)	5.56 (0.23)
32	7.64 (0.0.7)	7.61 (0.04)	7.60 (0.05)	5.20 (0.17)
40	7.78 (0.11)	7.80 (0.05)	7.32 (0.09)	5.15 (0.17)
48	7.82 (0.17)	7.76 (0.11)	7.05 (0.03)	5.25 (0.33)

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.

Table 27. Changes in numbers of *Candida krusei* 29 and *Saccharomyces cerevisiae* 26-1-7 grown as single cultures and as mixed cultures in MYGP broth pH 3.5 at 30 °C (without lactic acid).

	Cell numbers [Log_{10} cfu/ml (SD)][a]			
	Candida krusei 29		*Saccharomyces cerevisiae* 26-1-7	
Time (hour)	Single culture	Mixed culture	Single culture	Mixed culture
0	4.05 (0.09)	4.04 (0.08)	4.25 (0.04)	4.12 (0.02)
4	4.36 (0.20)	4.31 (0.11)	4.58 (0.04)	4.43 (0.11)
8	6.05 (0.12)	6.02 (0.07)	5.40 (0.07)	5.25 (0.13)
16	7.79 (0.07)	7.72 (0.04)	6.75 (0.08)	6.00 (0.12)
24	8.06 (0.12)	8.06 (0.05)	7.82 (0.10)	6.06 (0.29)
32	7.97 (0.07)	7.98 (0.05)	7.72 (0.10)	5.69 (0.36)
40	8.10 (0.04)	8.09 (0.04)	7.70 (0.09)	6.00 (0.30)
48	8.12 (0.06)	8.06 (0.16)	7.72 (0.13)	6.05 (0.54)

[a] Values represent mean and Standard Deviations (in parentheses) of two experiments carried out in duplicates.

undissociated lactic acid. With the more lactic acid sensitive strain *Sacch. cerevisiae* 26-1-7, counts of more than two log cycles lower were obtained when grown in mixed culture with *C. krusei* 29 under similar conditions (Table 26). These results indicate that *C. krusei* is more tolerant to lactic acid and low pH than *Sacch. cerevisiae*, which agree with previously reported results obtained with strains isolated from sourdough (Spicher and Schröder, 1980). However, it cannot be excluded that other factors are involved in the succession of the yeast species as well.

4.4 Intracellular pH Responses of *Candida krusei* and *Saccharomyces cerevisiae* to Low pH and Lactic Acid

The calibration curves of ratio values of fluorescence intensity between the pH-dependent point (490 nm) and the pH-independent point (435 nm) plotted against pH are shown in Figures 12 and 13 for the two strains of *C. krusei* and Figures 14 and 15 for the two trains of *Sacch. cerevisiae*. The curves were used for calculating the pH_i of the two yeasts as described by Siegumfeldt *et al.* (2000).

The results of experiments conducted to investigate the differences in lactic acid tolerance between *C. krusei* and *Sacch. cerevisiae* by determining the short-term intracellular pH_i responses in the presence of undissociated lactic acid in concentrations of 20.7 mM and 106.4 mM are shown in Figs.16 and 17. Even the highest concentration of undissociated lactic acid only induced weak short-term pH_i responses in cells of the two *C. krusei* strains (Fig. 16A and B). A decrease in pH_i of less than 1 pH unit (i.e. from a pH_i of approx. 8.0 to a pH_i of 7.1 for strain 29 and 7.4

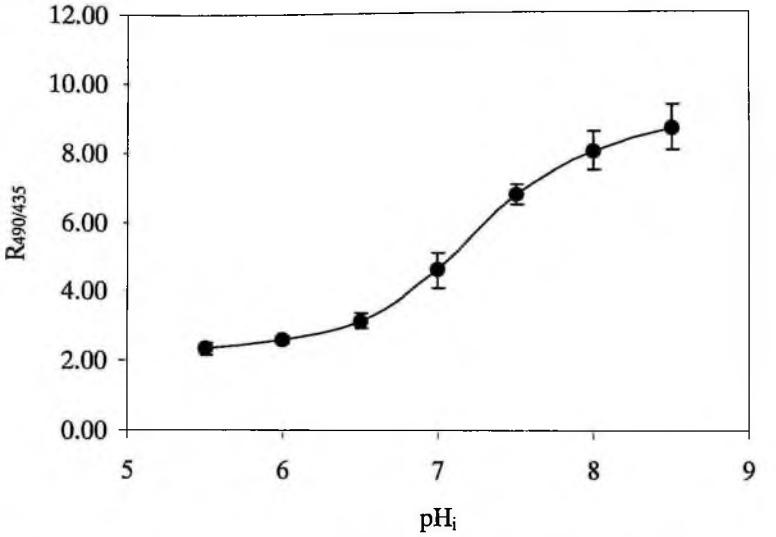

Figure 12. Relationship between excitation ratio 490 nm/ 435 nm ($R_{490/435}$) and pH-equilibrated cells of *Candida krusei* 29

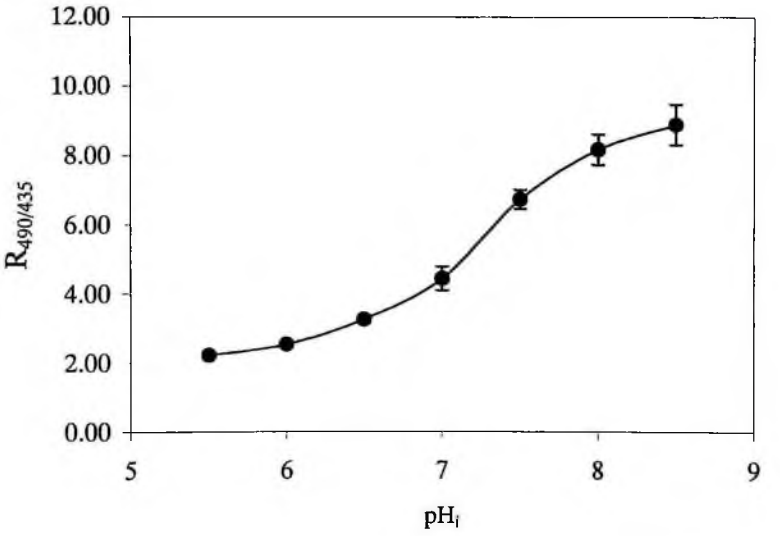

Fig.13. Relationship between excitation ratio 490 nm/ 435 nm ($R_{490/435}$) and pH-equilibrated cells of *Candida krusei* 65

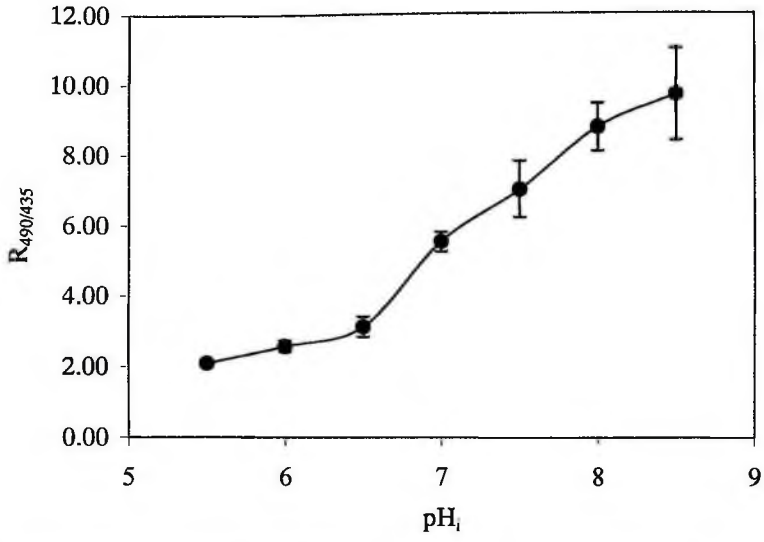

Figure 14. Relationship between excitation ratio 490 nm/ 435 nm ($R_{490/435}$) and pH-equilibrated cells of *Saccharomyces cerevisiae* 31

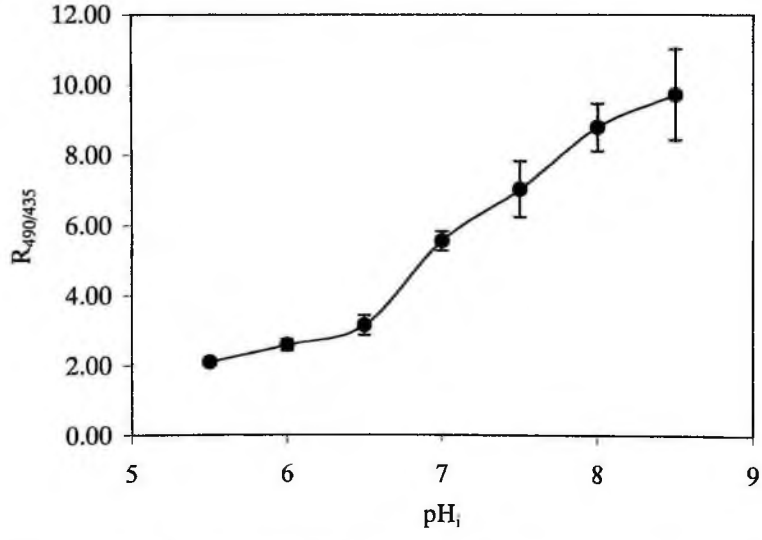

Fig.15. Relationship between excitation ratio 490 nm/ 435 nm ($R_{490/435}$) and pH-equilibrated cells of *Saccharomyces cerevisiae* 26-1-7

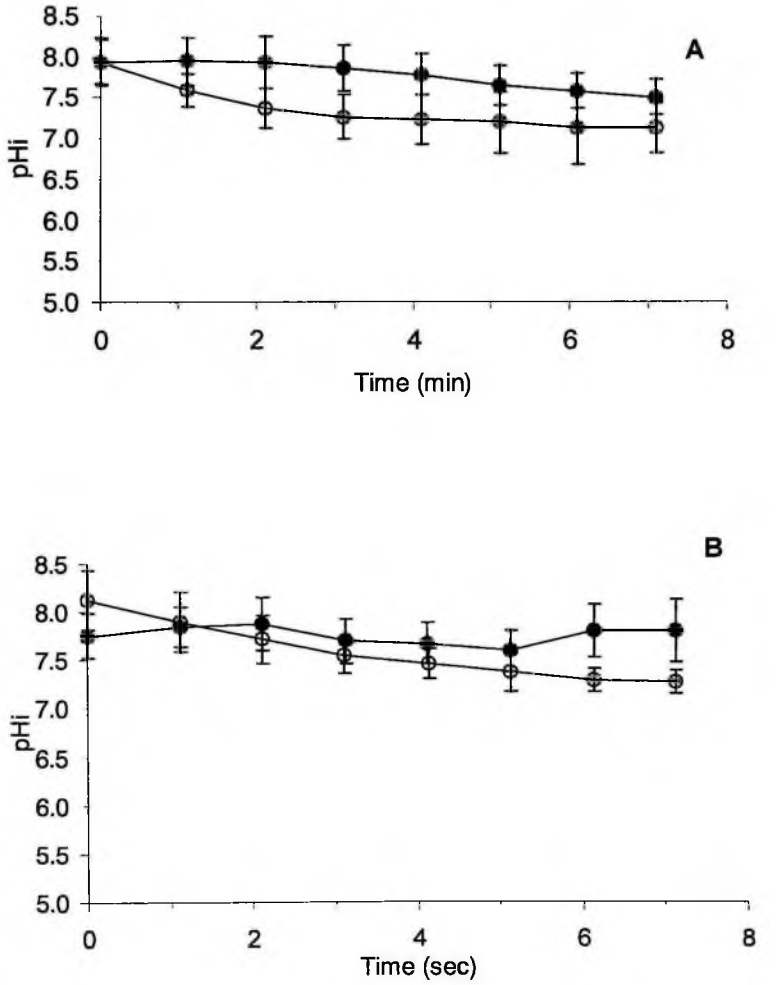

Figure 16. Intracellular pH of single cells of (A) *Candida krusei* 29 and (B) *Candida krusei* 65 during perfusion at a rate of 500 µl / min with (•) 20.7 mM, pH 4.5 and (o) 106.4 Mm, pH 2.5, undissociated lactic acid with potassium phosphate buffer. Perfussion was initiated at time zero. The values are means of approx. 20 cells from a representative growth experiment. Vertical bars represent standard deviations.

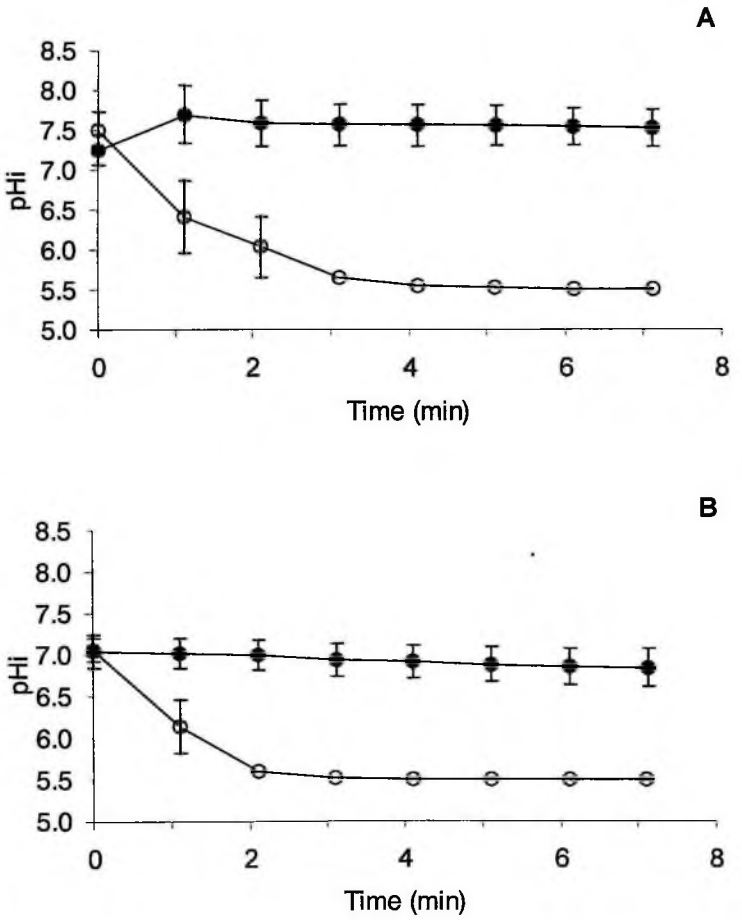

Figure 17. Intracellular pH of single cells of (A) *Saccharomyces cerevisiae* 31 and (B) *Saccharomyces cerevisiae* 26-1-7 during perfusion at a rate of 500 µl / min with (•) 20.7 mM, pH 4.5 and (o) 106.4 Mm, pH 2.5, undissociated lactic acid with potassium phosphate buffer. Perfussion was initiated at time zero. The values are means of approx. 20 cells from a representative growth experiment. Vertical bars represent standard deviations. For pH_i values ≤ 5.5 standard deviations are not included.

for strain 65) over the 7 min period was observed. The two strains of *Sacch. cerevisiae* were found to be very sensitive especially to high concentrations of undissociated lactic acid as seen in Fig. 17A and B. Perfusion with 106.4 mM lactic acid caused a considerable intracellular acidification after about one minute with a decrease in pH_i from 7.5 to 6.4 and after 3 min pH_i had dropped to $\leq$ 5.5 where it remained throughout the experiment. When cells of *S. cerevisiae* were perfused with 20.7 mM lactic acid the initial pH_i of 7.2 remained more or less constant over the 7 min period.

No pronounced differences were observed in the pH_i responses of the two strains of *Sacch. cerevisiae* used in the experiments (Fig. 17A and B). These results indicate that *C. krusei* is more resistant to short-term pH_i changes caused by lactic acid than *S. cerevisiae*.

Similar pronounced short-term pH_i changes have been observed with acetic acid in *Sacch. cerevisiae* cells but much lower concentrations of total acetic acid (33 mM) were required at a given pH to decrease the pH_i (Arneborg *et al.*, 2000). The reason why acetic acid is inhibitory to yeasts at a much lower concentration than lactic acid is probably due to the higher pK_a value of acetic acid (pK_a = 4.74) in comparison to that of lactic acid (pK_a = 3.86) which means that at a given pH there is more undissociated acetic acid present than would be found with an equal concentration of lactic acid. Also acetic acid is more lipophilic and hence can diffuse through the cell membrane more easily than lactic acid (Ray and Sandine, 1992).

As seen from Figs. 16 and 17 the initial pH_i of *C. krusei* was up to one pH unit higher than for *Sacch. cerevisiae* which should make it even more demanding for *C. krusei* to withstand acidic environments. In the presence of 106.4 mM undissociated lactic acid (pH 2.5) *C. krusei* experienced a pH gradient between the extracellular pH and its pH_i of 5.0 to 5.5 pH units. The explanation why this particular yeast can handle such a hostile environment and maintain a condition of almost pH homeostasis could be explained by the following factors: the plasma membrane of *C. krusei* could be less permeable to lactic acid than that of *Sacch. cerevisiae*, *C. krusei* might have a higher buffering capacity of the cytosol or *C. krusei* might have a more immediate response in H^+-ATPase activity and a higher amount of endogenous energy reserves. However, no specific information on this issue is available for *C. krusei* and the factors described have only been reported for *Sacch. cerevisiae* (Holyoak *et al.*, 1996; Brul and Coote, 1999; Thomas *et al.*, 2002).

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1. CONCLUSIONS

There were differences in the extent of inhibition of Gram-negative food borne bacterial pathogens *Salmonella, Shigella* and *Escherichia coli* when inoculated into different maize dough systems (fermenting maize dough, Ga kenkey water and fermented maize dough porridge koko). *Salmonella* species were inhibited at a faster rate than *Shigella* and *E. coli*; and *Shigella* and *E. coli* strains survived longer in fermented maize dough porridge koko than in Ga kenkey water and fermented maize dough.

Inhibition of the pathogens was observed at pH of ≤ 4.0.

For the Gram negative pathogens investigated, greater numbers of viable cells were recovered on non-selective medium than on selective medium for Gram negative bacterial pathogens.

Concentrations of 31 to 62 mM undissociated lactic acid had only a bacteriostatic effect on the four pathogenic *E. coli* strains tested while above 62 mM, a bactericidal effect was noted after 24 h.

The results of the study have confirmed that the fermented maize dough systems have anti-microbial properties which may inhibit the survival of some food borne pathogenic and non-pathogenic bacteria. The anti-microbial properties of the fermented maize dough systems are due mainly to the presence of lactic and acetic acids at low pH.

The kinetics of the survival data generated for *E. coli* O111 (EPEC) in Tryptone Soy Broth Yeast Extract with different concentrations of lactic acid (0.2, 0.4, 0.8 and 1.2% V/V) within a pH range of 3.5 to 4.2 at 30^0 C, were variable, depending on the test conditions. Modelling using the model of Peleg and Cole (1998) provided a good description of the data generated. The predicted values obtained for the death rate of *E. coli* O111 (EPEC) from the regression model derived from the whole data set were in agreement with the actual values obtained in broth studies.

Growth responses of *Candida krusei* and *Saccharomyces cerevisiae,* to lactic acid showed that *C. krusei* is more tolerant to lactic acid at low pH than *Sacch. cerevisiae.*

The application of fluorescence-ratio-imaging microscopy and a perfusion system to determine intracellular pH of single cells of *C. krusei* and *Sacch. cerevisiae* showed that *C. krusei* is more resistant to short-term intracellular pH changes caused by lactic acid than *Sacch. cerevisiae*. This may explain the observed differences in lactic acid tolerance of the two yeasts.

5.2. RECOMMENDATIONS

Though fermentation of maize dough leads to a reduction of most food borne pathogens which may be present in the system, the present study has shown that some *E coli* and *Shigella flexneri* strains can survive in koko for 24 to 48 hours. There is therefore the need for greater control of the maize dough fermentation process to achieve the desired pH of less than 4 to ensure the safety of the product. This can be facilitated through the use of

high acid producing strains of lactic acid bacteria as starter cultures. Such starter cultures have been developed (Halm *et al.*, 1996; Amoa-Awua, 1996; Hayford and Jakobsen, 1999; Hayford and Jespersen, 1999; Hayford *et al.*, 1999) and it is recommended that the technology of using these starter cultures is transferred to commercial kenkey producers in order to ensure the safety of the product.

It is also recommended that the Hazard Analysis Critical Control Point (HACCP) system be applied in the production of fermented foods to ensure the quality and safety of products. An HACCP system that is suitable for kenkey production at both traditional and upgraded commercial production sites has been developed by Amoa-Awua *et al.* (1998). This system should be transferred to household and cottage industries.

It is also recommended that the monitoring of Critical Control Point (CCP) in HACCP system for fermented products should not be limited to pH and acidity but, proper storage and handling of the cooked fermented products should be ensured by observation of good hygiene and sanitation practices to prevent contamination.

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