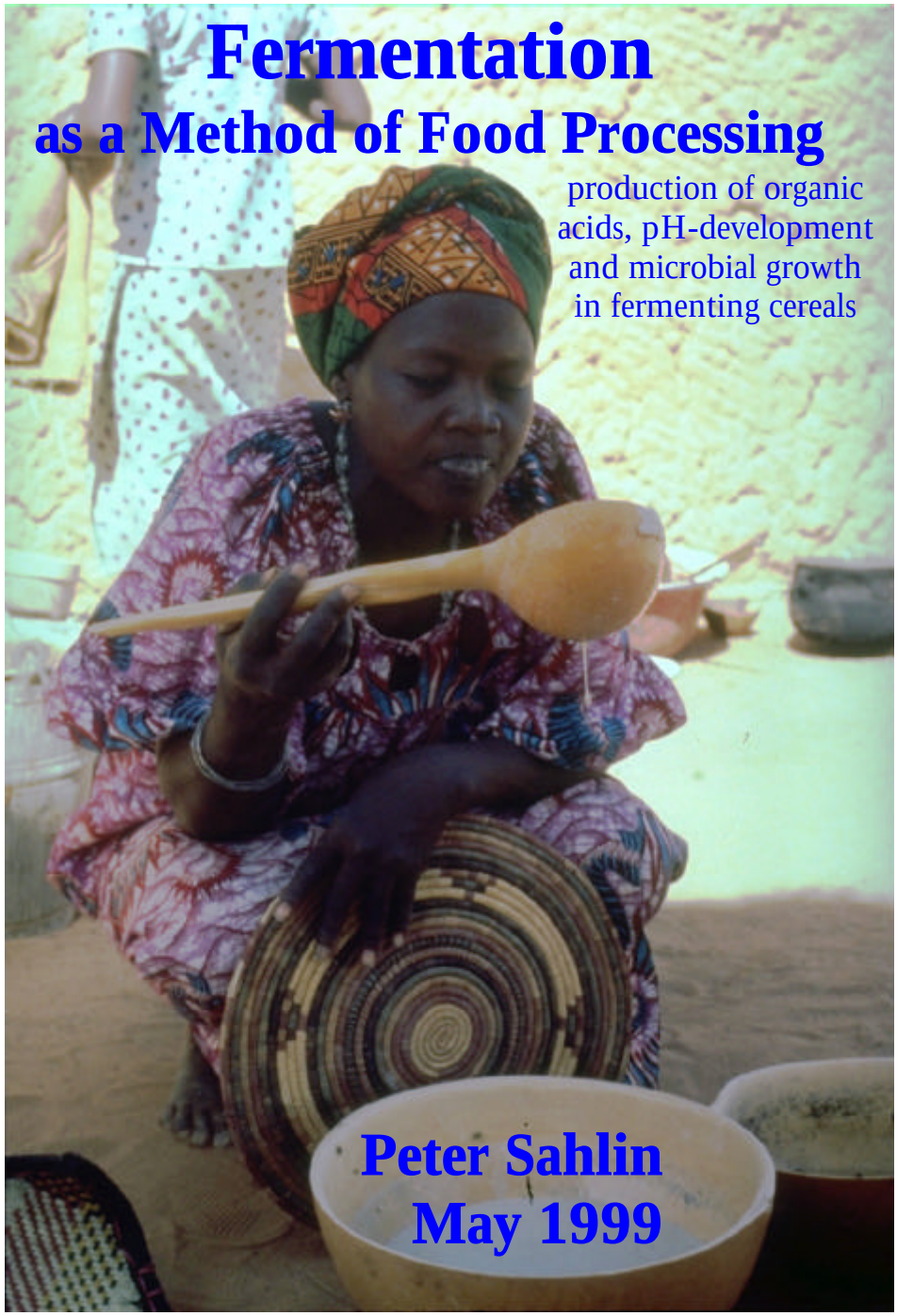
Fermentation
as a Method of Food Processing

production of organic acids, pH-development and microbial growth in fermenting cereals



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Preface

In developing countries, one tenth of the children under five years of age dies due to dehydration. The dehydration is mainly caused by too many of severe incidences of diarrhoea. The main cause for getting diarrhoea is the ingestion of food not having the appropriate standard regarding the hygienic condition. The hygienic standard of a food is based on the processing and handling of the food, as well as on the conditions of the raw materials. A food item prepared with water contaminated with pathogenic microorganisms will successively become contaminated, and a health risk.

It is known that pathogenic microorganisms normally found in food will not be able to grow in an acid environment, that is at pH below four. This acidity is normally found in lactic acid fermented food.

This thesis deals with the production and properties of lactic acid fermented food. At the beginning of the fermentation step, the food is vulnerable to contamination since it does not have any acidity. This work has followed the development of the acidity by measuring the rise in lactic acid content during the process. In addition, the ability of the acid environment to suppress pathogenic bacteria has been studied. The studies have been made on cereal-water slurries, a common base for the production of gruels, pancakes, porridges, puddings and other food items.

It takes 12 to 24 hours for the type of food studied to reach an acidity level that is safe regarding common pathogenic microorganisms. It is also shown that a strain of enterotoxinogenic *Escherichia coli* can not withstand the acidic environment produced in this process.

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Contents

This licentiate thesis is based on the following papers:

1. Fermentation as a method of food preservation - a literature review

Part I - Nutrition and health effects

Peter Sahlin

Manuscript

2. Fermentation as a method of food preservation - a literature review

Part II - Food safety

Peter Sahlin

Manuscript

3. Production of organic acids, titratable acidity and pH-development during fermentation of cereal flours

Peter Sahlin and Baboo M. Nair

Submitted for publication

4. Effect of fermentation on the growth of *Escherichia coli* - strain NG7C in gruels made from whole grain flours of wheat and tef.

Apiradee Wangsakan, Peter Sahlin and Baboo M. Nair

Manuscript

Background

Introduction

The WHO food safety unit has given high priority to the research area of fermentation as a technique for preparation/storage of food. One main reason for this is that in developing countries, one tenth of the children under five years of age dies due to dehydration. The dehydration is mainly caused by incidences of diarrhoea. The main cause for getting diarrhoea is the ingestion of food not having the appropriate standard regarding the hygienic condition. The hygienic standard of a food is based on the processing and handling of the food, as well as on the conditions of the raw materials. A food item prepared from water contaminated with pathogenic microorganisms will successively be contaminated, and a health risk.

Lactic acid fermentation of food has been found to reduce the risk of having pathogenic microorganisms grow in the food.

Definition of fermented food

Campbell-Platt (1987) has defined fermented foods as those foods which have been subjected to the action of micro-organisms or enzymes so that desirable biochemical changes cause significant modification to the food. However, to the microbiologist, the term ”fermentation” describes a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is incompletely oxidised, and an organic carbohydrate acts as the electron acceptor (Adams, 1990). This definition means that processes involving ethanol production by yeasts or organic acids by lactic acid bacteria are considered as fermentations, but not the production of fish sauces in Southeast Asia, that still has not been shown to have a significant role for microorganisms, and not the tempe production since the metabolism of the fungi is not fermentative according to Adams definition.

Whichever definition used, foods submitted to the influence of lactic acid producing microorganisms is considered a fermented food.

Classification of fermented foods

Fermented foods can be classified in many different ways, see Table 1. Dirar (1993) says that in Southeast Asia the classification often is according to the kind of

microorganism involved (Yokotsuka, 1982). Other classifications are based on commodity (Campbell-Platt, 1987)(Odunfa, 1988)(Kuboye, 1985). Dirar presents the traditional Sudanese classification that is based on the function of the food.

Table 1. Different classification of fermented foods. Adapted from Dirar (1993)

Yokotsuka (1982)	Campbell-Platt (1987)	Odunfa (1988)	Kuboye (1985)	Sudanese (Dirar, 1993)
1 alcoholic beverages (yeast)	1 beverages	1 starchy roots	1 cassava-based	1 *kissar* – staples
2 vinegars (*Acetobacter*)	2 cereal products	2 cereals	2 cereals	2 *milhat* – sauces and relishes for the staples
3 milk products (*Lactobacilli*)	3 dairy products	3 alcoholic beverages	3 legumes	3 *marayiss* – beers and other alcoholic drinks
4 pickles (*Lactobacilli*)	4 fish products	4 vegetable proteins	4 beverages	4 *akil-munasabat* – food for special occasions
5 fish or meat (enzymes and *Lactobacilli*)	5 fruit and vegetable products	5 animal protein		
6 plant protein (moulds, with or without *Lactobacilli* and yeasts)	6 legumes			
	7 meat products			
	8 starch crop products			
	9 miscellaneous products			

The different classifications show the different viewpoints of the authors, and often a classification that works very well in one part of the world is not suitable in other parts. Once the classification scheme is made up, it can be difficult to distribute the foods, e.g. is sorghum gruel a beverage or a cereal product? To add to the flora of classification systems other possibilities are mentioned in Table 2.

Table 2. Other classifications of fermented foods.

1 ready for consumption, ex yoghurt, salami, bread	1 containing viable microorganisms, ex yoghurt, cheese	1 LAB-fermentation
2 ready for consumption but mostly used as ingredient, ex crème fraîche	2 not containing viable microorganisms, ex soy sauce, bread, beer, wine	2 mould-fermentation
3 only used as ingredient, ex soy sauce, dawadawa	3 microorganisms used in an early step of the production, ex cocoa, coffee, cassava products	3 yeast-fermentation
		4 other bacteria
		5 enzymatic

Benefits of fermenting food

The benefits of food fermentation as compiled by Adams, is shown in Table 3.

Table 3. The benefits of food fermentation (from Adams 1990)

Raw material	Stability	Safety	Nutritive value	Acceptability
Meat	++	+	-	(+)
Fish	++	+	-	(+)
Milk	++	+	(+)	(+)
Vegetables	+	(+)	-	(+)
Fruits	+	-	-	++
Legumes	-	(+)	(+)	+
Cereals	-	-	(+)	+
++ definite improvement	+ usually some improvement	(+) some cases of improvement	- no improvement	

Many fermented milk products, which are eaten as they are, contain living microorganisms. *Acidofilus milk*, *filmjölk*, *yoghurt*, *junket* and *kefir* are fermented milks containing either Lactic Acid Bacteria (LAB) alone or both LAB and yeast or mixed cultures producing mainly lactic acid or a combination of lactic acid and small amounts of alcohol. *Kumiss* is fermented milk made of mare's milk using a mixed culture. *Lassi* in India, a fermented milk consumed as a beverage after dilution with water, and *Yakult* in Japan and China are typical fermented milk products made of mixed culture by spontaneous fermentation. Other milk based products which are fermented with some cereals are *flummery* which is a fermented yoghurt like product containing boiled whole grains and *prokllada* which is mainly fermented whey with addition of taste enhancing substances. *Lao-chao*, a fermented, glutinous, slightly alcoholic, steam cooked rice, *maheu* a non-alcoholic beverage from maize, sorghum or millet, *pozol* which is either a thick porridge like food or a thin beverage made of maize flour, a thick alcoholic beverage similar to beer made of sorghum, and *tapé* a thick pasty fermented food containing alcohol made from millet or maize but also some times from cassava are typical examples of fermented foods made of cereals. Foods like i*njera* from tef, and *kisra* from sorghum are commonly made after fermenting dough for two or three days with or without starter. The common fermented legume products include *hama-natto* which is a soybean paste, used for

flavouring, *oncom* made of groundnut presscake, or soybean presscake used as a relish, fermented soy milk and *sufu* made of soybean curd, mould, salt and alcohol. *Kimchi* is a popular fermented food made mainly of vegetables in Korea. Pickled fruits and vegetable are common in many countries and *sauerkraut* is a well known product made by fermenting cabbage. *German salami* (smoked), *Italian salami*, *Lebanon bologna* (sausage), *Longaniza* (sausage), and *Teewurst* are typical fermented meat products of Europe. While *paak* made of fish and cereal by lactic acid fermentation and *pin dang* and *tarama* made of fermented roe are typical fermented fish products of the Far Eastern countries.

Microflora in fermented foods

By tradition, lactic acid bacteria (LAB) are the most commonly used microorganisms for preservation of foods. Their importance is associated mainly with their safe metabolic activity while growing in foods utilising available sugar for the production of organic acids and other metabolites. Their common occurrence in foods and feeds coupled with their long-lived use contributes to their natural acceptance as GRAS (Generally Recognised As Safe) for human consumption (Aguirre & Collins, 1993). However, there are many kinds of fermented foods in which the dominating processes and end products are contributed by a mixture of endogenous enzymes and other microorganisms like yeast and mould. Very often, a mixed culture originating from the native microflora of the raw materials is in action in most of the food fermentation processes. However, in an industrial scale a particular defined starter culture, which has been developed under controlled conditions, is of first preference so that the qualities of the finished product could be consistently maintained day after day. Moreover, modern methods of gene-technology makes it possible for the microbiologists to design and develop starter cultures with specific qualities.

Many microbiological studies deal with identification of organisms isolated from various fermented foods. Lactic acid bacteria isolated from tomatoes that were naturally fermented under partial anaerobic conditions were found to *be Leuconostoc mesenteroides, Lactobacillus brevis* and *Streptococcus* sp. (Beltrán-Edeza & Hernández-Sánchez, 1989). In Asia mainly moulds of the genera *Aspergillus, Rhizopus, Mucor, Actinomucor, Amylomyces, Neurospora* and *Monascus* are used in the manufacture of fermented foods. In Europe, mould-ripened foods are primarily cheeses and meats, usually using a *Penicillium*-species (Leistner, 1990). Gari made by fermenting cassava slurry was found to contain *Bacillus, Aspergillus* and *Penicillium* spp. as the predominant organisms (Ofuya & Akpoti, 1988). The micro-organisms present in a

fermented food made in Ghana called dawadawa after 24 h of fermentation, predominantly were *Bacillus* sp. with small numbers of (0,3%) *Staphylococcus* sp., after 36 h 60% *Bacillus* sp., 34% *Staphylococcus* sp. and after 48 h 56% *Bacillus* sp. and 42% *Staphylococcus* sp. (Odunfa & Komolafe, 1989). Indonesian tapé ketan, a sweet, sour and alcoholic rice product, is produced using a starter culture containing moulds, yeasts and bacteria. After 72 h of fermentation, the pH was 3,5 while the biomass of the hyphae of the moulds was 15,3 mg/g and of the yeast 3,3 mg/g. (Cook *et al.*, 1991). In Okpiye, which is a food condiment prepared by the fermentation of *Prosopis africana* seeds, several species of bacteria especially *Bacillus subtilis, B. licheniformis, B. megaterium, Staphylococcus epidermis* and *Micrococcus* spp. were found to be the most active organisms (Achi, 1992). In trahanas, a fermented food prepared in Greece from a mixture of milk and wheat flour, *Streptococcus lactis, Streptococcus diacetylactis, Leuconostoc cremoris, Lactobacillus lactis, Lactobacillus casei, Lactobacillus bulgaricus* and *Lactobacillus acidophilus* were found to play the major role in producing acid and aroma (Lazos *et al.*, 1993).

Nutritional value of fermented foods

Generally, a significant increase in the soluble fraction of a food is observed during fermentation. The quantity as well as quality of the food proteins as expressed by biological value, and often the content of watersoluble vitamins is generally increased, while the antinutritional factors show a decline during fermentation (Paredes-López & Harry, 1988). Fermentation results in a lower proportion of dry matter in the food and the concentrations of vitamins, minerals and protein appear to increase when measured on a dry weight basis (Adams, 1990). Single as well as mixed culture fermentation of pearl millet flour with yeast and lactobacilli significantly increased the total amount of soluble sugars, reducing and non-reducing sugar content, with a simultaneous decrease in its starch content (Khetarpaul & Chauhan, 1990). Combination of cooking and fermentation improved the nutrient quality of all tested sorghum seeds and reduced the content of antinutritional factors to a safe level in comparison with other methods of processing (Obizoba & Atii, 1991). Mixed culture fermentation of pearl millet flour with *Saccharomyces diastaticus, Saccharomyces cerevisiae, Lactobacillus brevis* and *Lactobacillus fermentum* was found to improve its biological utilisation in rats (Khetarpaul & Chauhan, 1991). Fermentation induced a significant decrease in lipid and lignin contents of okara, which is an insoluble residue obtained as a by-product in the manufacture of soybean milk. The fermented okara on the other hand neither increased PER nor the

weight gain in rats (Guermani *et al.*, 1992) compared to non-fermented samples. The digestibility of starch in bengal gram, cowpea and green gram was increased by fermentation. Cooking of these fermented legumes further increased the starch digestibility (Urooj & Puttaraj, 1994).

Proteins

The protein efficiency ratio (PER) of wheat was found to increase on fermentation, partly due to the increase in availability of lysine. A mixture of wheat and soybeans in equal amounts would provide an improved pattern of amino acids. The fermentation process raised the PER value of the mixture to a level which was comparable to that of casein (Hesseltine & Wang, 1980). Fermentation may not increase the content of protein and amino acids unless ammonia or urea is added as a nitrogen source to the fermentation media (Reed, 1981). The relative nutritional value (RNV) of maize increased from 65% to 81% when it was germinated, and fermentation of the flour made of the germinated maize gave a further increase in RNV to 87% (Lay & Fields, 1981). Fermentation of legumes for making dhokla and fermentation of millet for making ambali did not show any improvement in the values reported for PER, TD, BV and NPU in relation to the unfermented products (Aliya & Geervani, 1981). The soaked, washed and steamed seeds of *Lathyrus sativus*, had a score of 14 with cystine and methionine as the limiting amino acids. On tempe fermentation the score was raised to 16 and autoclaving followed by tempe fermentation raised the score to 21 (Moslehuddin & Hang, 1987). Solid substrate fermentation of cassava with added urea increased the protein content from 1% to 10,7%, together with a dry matter loss of 32% (Daubresse *et al.*, 1987). Fermentation of cassava improved the utilisation of the diets, measured as protein efficiency ratio and biological value (Aletor, 1993). The protein content of cassava decreased from 2,36 g/100g to 1,61 g/100g during fermentation (Padmaja *et al.*, 1994). Coagulation of protein in leaf extract by natural fermentation gave a higher yield of leaf protein concentrate compared to heat coagulation. Biological value of the leaf protein concentrate obtained through fermentation was also significantly higher. Leaf protein concentrate coagulated by fermentation was free from grassy odour and is generally more acceptable by human consumers as compared with LPC coagulated by heat at isoelectric pH or natural pH of leaf extract (Pandey & Srivastava, 1993).

Vitamins

During fermentation certain micro-organisms produce vitamins at a higher rate than others do. The content of thiamine and riboflavin in dhokla and ambali was about 50% higher after fermentation. Fermented milk products in general showed an increase in folic acid content and a slight decrease in vitamin B12 while other B-vitamins were affected only slightly (Alm, 1982) in comparison to raw milk. The levels of vitamin B12, riboflavin and folacin were increased by lactic acid fermentation of maize flour, while the level of pyridoxine was decreased (Murdock & Fields, 1984). Fermented whole onion plant retained 97% of vitamin A activity, while fermented egg plant only retained 34% of the vitamin A activity (Speek *et al.*, 1988). Kefir made from ten different kefir grain cultures showed significant (>20%) increase for pyridoxine, cobalamin, folic acid and biotin and reduction exceeding 20% for thiamine, riboflavin, nicotinic acid, and pantothenic acid depending on the culture used. There was a 40% increase in thiamine content in two of the cultures. While riboflavin showed a small increase in two cultures, pyridoxine increased more than 120% in 3 cultures (Kneifel & Mayer, 1991). During tempe fermentation, *Rhizopus* strains were found to produce riboflavin, nicotinic acid, nicotinamide and vitamin B_6, but not vitamin B_{12}. The addition of cobalt and 5,6-dimethylbenzimidazole were found to increase the vitamin B12 content of tempe (Keuth & Bisping, 1994).

Minerals

The mineral content is not affected by fermentation unless some salts are added to the product during fermentation or by leaching when the liquid portion is separated from the fermented food. Sometimes, when fermentation is carried out in metal containers, some minerals are solubilised by the fermented product, which may cause an increase in mineral content. Phytate content in bread was lowered when the amount of yeast or the fermentation time was raised (Harland & Harland, 1980). Phytate content in locust bean seeds was lowered from 0,51 mg/g to 0,31 mg/g by fermentation (Eka, 1980). Natural lactic fermentation of maize meal decreased phytate phosphorus by 78% (Chompreeda & Fields, 1984). The reduction of phytate content during dough fermentation for whole grain flour was about 50% (Roos *et al.*, 1990). Phytic acid could be reduced during fermentation of pearl millet in an increasing rate with increase in fermentation temperature (Kheterpaul & Chauhan, 1991). Fermentation by *Saccharomyces diastaticus* followed by *Lactobacillus brevis* completely eliminated phytic acid from pearl millet flour (Khetarpaul &

Chauhan, 1991). In bambara nut milk (Obizoba & Egbuna, 1992), tannin content could be reduced by fermentation. There was a marked increase in protein availability and concentration during fermentation of siljo, a traditional Ethiopian fermented food. A study on the effect of fermentation of cowpea (*Vigna unguiculata*) on the nutritional quality of the cowpea meal showed that 72h fermentation increased the content of protein, ash and lipid levels while decreasing the levels of tannin and phytate (Nnam, 1995). Trypsin inhibitors, thiamine and riboflavin were reduced significantly during fermentation. A decrease in protein content was observed during the first 2 days of fermentation and thereafter the decrease was not significant. (Gupta *et al.*, 1998). Vaishali et al (1997) who studied effect of natural fermentation on *in vitro* zinc bioavailability in cereal-legume mixtures found that fermentation increased the zinc solubility (2-28%) and the zinc uptake by intestinal segment (1-16%) to a significant level.

Health effects of fermented foods

Probiotic effect

One of the reasons for the increasing interest in fermented foods is its ability to promote the functions of the human digestive system in a number of positive ways. This particular contribution is called probiotic effect. Already early in 1900, Metchnikoff pointed out the use of fermented milks in the diet for prevention of certain diseases of the gastrointestinal tract and promotion of healthy day to day life. Since then a number of studies have now shown that the fermented food products do have a positive effect on health status in many ways. The human intestinal microbial flora is estimated to weigh about 1000 grams and may contain 10^{16} – 10^{17} colony forming units representing more than 500 strains. For physiological purposes, it can be considered to be a specialised organ of the body with a wide variety of functions in nutrition, immunology and metabolism (Gustafsson, 1983). Studies on mice have shown that the indigenous microorganisms in the stomach are *Lactobacillus, Streptococcus* and *Torulopsis*, while in the small intestine, ceacum and colon several different species (*Bacteroides, Fusobacterium, Eubacterium, Clostridium,* etc.) coexist (Savage, 1983). The gastrointestinal microflora in humans are also known to contain hundreds of species. Even though there is a wide variation among individuals, the number of species and size of the population are usually kept stable in normal healthy subjects. There is a constant struggle in maintaining the desirable balance

and a dynamic equilibrium between microbial populations within the intestinal flora (Robinson & Samona, 1992). The anaerobic organisms, which outnumber the gram negative enteric bacteria by about 10 000 : 1, are associated with the intestinal epithelium limiting adherence of potential pathogens by effective colonosation (Van der Waaij *et al.*, 1972; Nord & kager, 1984; Swank & Dietch, 1996). The stability of the intestinal microflora is affected by many factors including dietary habits. Decrease in the number of anerobic bacteria is associated with increase in the number of gram negative pathogens in the intestinal tract and their translocation to extraintestinal tissues. Under normal conditions the intestinal wall prevents translocation of organisms both dead and living as well as microbial products like toxins from the gut to the blood. However, in patients with systemic insult like starvation, shock, injury and infection or specific insult of the gastrointestinal canal through inflammation, chemotherapy or radiation, the gut mucosal permeability will be increased leading to translocation of microbes (Carrico & Meakin, 1986; Alexander *et al.*, 1990; Wells, 1990; Kasravi *et al.*, 1997). A fermented food product or live microbial food supplement which has beneficial effects on the host by improving intestinal microbial balance is generally understood to have probiotic effect (Fuller, 1989).

Flatulence reducing effect

During fermentation of the beans for preparation of tempe, the trypsin inhibitor is inactivated, and the amount of several oligosacharides which usually cause flatulence are significantly reduced (Hesseltine, 1983). Bean flour inoculated with *Lactobacillus* and fermented with 20% moisture content, showed a reduction of the stachyose content (Duszkiewicz-Reinhard *et al.*, 1994)

Anticholesterolemic effect

Hepner et al. (1979) reported hypercholesteremic effect of yoghurt in human subjects receiving a one-week dietary supplement. Studies on supplementation of infant formula with *Lb. acidophilus* showed that the serum cholesterol in infants was reduced from 147 mg/ml to 119mg/100 ml (Harrison & Peat, 1975). In an *in vitro* study the ability of 23 strains of lactic acid bacteria isolated from various fermented milk products the bacterial cells to bind cholesterol was investigated. No cholesterol was found inside the cells (Taranto *et al.*, 1997). Poppel and Schafsma (1996) have also reported the ability of yoghurt to lower the cholesterol in serum by controlled human trials. Possible role of lactic acid bactera in lowering cholesterol concentration

and various mechanisms by which it may be possible has been discussed by Haberer et al (1997). Brigidi *et al* (1993) have cloned a gene encoding cholesterol oxidase from *Streptomyces lividans* into *Bacillus , Lactobacillus* and *E. coli*.

Effect on transit time, bowel function and glycemic index

The transit time for 50% (t50) of the gastric content was significantly reduced for regular unfermented milk (42+-10 min) in comparison with a fermented milk product indegenous to Sweden called "långfil" or ropy milk (62+-14 min). Another study (Wilhelm, 1993) reports increase in transport time and improved bowel function in patients with habitual constipation. The number of defeacations per week increased from three during control period to seven using conventional fermented milk and fifteen when acidophilus milk was served. Regular unfermented milk also gave significantly higher increase in glycemic index curve than fermented milk product called långfil (Strandhagen *et al.*, 1994). Liljeberg *et al* (1995) have shown that presence of acid, specially acetic or lactic acid, would lower the glycemic index in breads to a significant level. Koji which is prepared from *Aspergillus oryzae* and beni-koji made from *Monascus pilosus* were found to express rises in blood pressure (Tsuji *et al.*, 1992).

Anticancerogenic effect

Apart from this, there are interesting data on anticarcinogenic effect of fermented foods showing potential role of lactobacilli in reducing or eliminating procarcinogens and carcinogens in the alimentary canal (Reddy *et al.*, 1983; Shahani, 1983; Mital & Garg, 1995). The enzymes β-glucuronidase, azoreductase and nitroreductase, which are present in the intestinal canal, are known to convert procarcinogens to carcinogens (Goldin & Gorbach, 1984). Oral administration of *Lb rhamnosus* GG was shown to lower the faecal concentration of β-glucuronidase in humans (Salminen *et al.*, 1993) implying a decrease in the conversion of procarcinogens to cancinogens. Fermented milk containing *Lactobacillus acidophilus* given together with fried meat patties significantly lowered the excretion of mutagenic substances compared to ordinary fermented milk with *Lactococcus* fed together with fried meat patties (Lidbeck *et al.*, 1992). The process of fermentation of foods are also reported to reduce the mutagenicity of foods by degrading the mutagenic substances during the process.

Lactic acid bacteria isolated from dadih, a traditional Indonesian fermented milk, were found to be able to bind mutagens and inhibit mutagenic nitrosamines. Milk

fermented with *Lactobacillus acidophilus LA-2* was demonstrated to suppress faecal mutagenicity in the human intestine. Studies on antimutagenic activity of milk fermented with mixed-cultures of various lactic acid bacteria and yeast, showed that the fermented milks produced with mixed cultures of lactic acid bacteria had a wider a wider range of activity against mutagens than those produced with a single strain of lactic acid bacteria [Tamai, 1995). However, a review by McIntosh (1996) concludes that there is only limited data to support the hypothesis that probiotic bacteria are effective in cancer prevention. On the other hand, a study by Hosono & Hisamatsu (1995) on the ability of the probiotic bacteria to bind cancerogenic substances have reported that *E feacalis* was able to bind aflatoxin B1, B2, G1 and G2 as well as some pyrolytic products of tryptophan.

Immunoactive effects

Some lactic acid bacteria which are present in fermented milk products, are found to play an important role in the immune system of the host after colonisation in the gut (De Simone, 1986). Oral administration of *Lactbacillus casei* caused an improvement of the function of the peritoneal macrophages and increased the production of IgA (Sato *et al.*, 1988). The mechanism of this effect is not clearly known, but it is speculated that the lactobacilli, their enzymes or the metabolic products present in the fermented food product may act as antigens, activating production of antibodies. Marin *et al*. (1997) have studied the influence of lactobacilli used in fermented dairy products on the production of cytokines by macrophages. The results indicated that for most strains, direct interaction with macrophages caused a concentration dependent increase in tumour necrosis factor and interleukin. A study by Perdigon *et al* (1995) showed that the *Lactobacillus casei* could prevent enteric infections and stimulate secretory IgA in malnourished animals but also translocate bacteria, while yoghurt could inhibit growth of intestinal carcinoma through increased activity of IgA, T cells and macrophages. In a review by Marteau & Rambaud (1993) the authors concluded that there is a potential of using lactic acid bacteria for therapy and immunomodulation in mucosal diseases, especially in the gastrointestinal tract. Isolauri (1996) have presented a study suggesting that *Lactobacillus* sp. strain GG could be used in the prevention of food allergy. It is suggested that dietary antigens induce immunoinflamatory response that impairs the intestine's barrier function and that probiotic organisms could be a means of introducing a tool to reinforce the barrier effect of the gut.

Food safety aspects of fermented foods

It has been estimated that more than 13 million infants and children under five years of age die annually in the tropical regions of the world. After respiratory infections, diarrhoea diseases are the commonest illnesses and have the greatest negative impact upon the growth of infants and young children. The causes of diarrhoea have traditionally been ascribed to water supply and sanitation (Motarjemi *et al.*, 1993). Foods prepared under unhygienic conditions and frequently heavily contaminated with pathogenic organisms play a major role in child mortality through a combination of diarrhoea diseases, nutrient malabsorption, and malnutrition. All food items contain microorganisms of different types and in different amounts. Which microorganisms that will dominate depends on several factors, and sometimes microorganisms initially present in very low numbers in the food, for example lactic acid bacteria (LAB), will outnumber the other organisms inhibiting their growth. In contrast to fermented meat, fish, dairy and cereal products, fermented vegetables have not been recorded as a significant source of microbial food poisoning (Fleming & McFeeters, 1981).

Effect of fermentation on pathogenic organisms

Over a study period of nine months, a group of children fed with lactic acid fermented gruel had a mean number of 2,1 diarrhoea episodes compared to 3,5 for the group fed with unfermented gruel (Lorri & Svanberg, 1994). Although *Salmonella, Campylobacter, Shigella, Vibrio, Yersinia* and *Escherichia* are the most common organisms associated with bacterial diarrhoea diseases, other enterotoxigenic genera, including *Pseudomonas, Enterobacter, Klebsiella, Serratia, Proteus, Providencia, Aeromonas, Achromobacter* and *Flavobacterium,* have also been reported (Nout *et al.*, 1989). In addition, it was found that there was no significant difference between the behaviour of the pathogens in fermented porridge or acid-supplemented non-fermented porridge, which implies that the anti-microbial effect is due to presence of lactic and acetic acids at reduced pH, and that other anti-microbial substances do not play a detectable role (Nout *et al.*, 1989). Similarly, Adams (1990) suggested that lactic acid bacteria are inhibitory to many other microorganisms when they are cultured together, and this is the basis of the extended shelf life and improved microbiological safety of lactic-fermented foods. Lactobacillus species can produce a variety of metabolites, including lactic and acetic acids which lower pH, that are inhibitory to competing bacteria, including psychrotrophic pathogen (Breidt &

Fleming, 1997). This effect could be due to a combination of many factors as shown in Table 4.

Table 4. Metabolites of lactic acid bacteria which may be inhibitory to other pathogenic and food spoilage organisms (Breidt & Fleming, 1997)

Product	Main target organisms
Organic acids	
Lactic acid	Putrefactive and Gram-negative bacteria, some fungi
Acetic acid	Putrefactive bacteria, clostridia, some yeasts and some fungi
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein-rich foods
Enzymes	
Lactoperoxidase system with hydrogen peroxide	Pathogens and spoilage bacteria (milk and diary products)
Lysozyme (by recombinant DNA)	Undesired Gram-positive bacteria
Low-molecular-weight metabolites	
Reuterin	Wide spectrum of bacteria, yeasts, and molds
Diacetyl	Gram-negative bacteria
Fatty acids	Different bacteria
Bacteriocins	
Nisin	Some LAB and Gram-positive bacteria, notably endospore-formers
Other	Gram-positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type

The inhibition by organic acids has been attributed to the protonated form of these acids, which are uncharged and may therefore cross biological membranes (Figure 1). The resulting inhibition of growth may be due to acidification of the cytoplasm and/or accumulation of anions inside the cell (Adams, 1990; Russel, 1992; Breidt & Fleming, 1997).

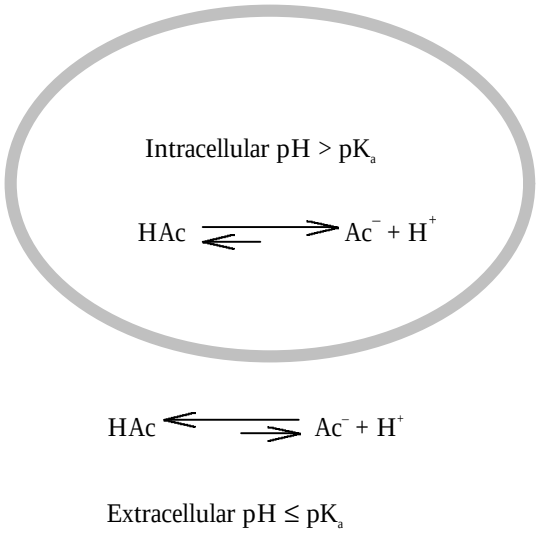

Figure 1. The diffusion of a weak organic acid into a microbial cell, and its dissociation yielding protons (H^+) and potentially toxic anions (A^-) (Adams, 1990).

The ability of an acid to inhibit bacteria depends principally on the pKa of the acid: the higher the pKa of the acid, the greater the proportion of undissociated acid, and the more inhibitory the acid is likely to be. On this basis, one would expect acetic acid (pKa = 4.75) to be a more effective antimicrobial agent than lactic acid (pKa = 3.86) (Adams, 1990). *Lactobacillus acidophilus* and *L. bulgaricus* inhibit activities of a wide variety of Gram-positive and Gram-negative organisms (Shahani, 1983). Mould growth was prevented in high-moisture maize samples (27% moisture) that were inoculated with *Lactobacillus plantarum* or *Propionibacterium shermanii* and stored for 60 days at 26°C and the initial yeast population was drastically reduced in samples inoculated with *Propionibacterium shermanii* while samples inoculated with *Lactobacillus plantarum* had an accelerated acid production in the early stage of fermentation (Flores-Galarza *et al.*, 1985). Studies with porridges inoculated with pathogenic bacteria (*Salmonella* spp., *Shigella* spp., *Staphylococcus aureus, Yersinia enterocolitica, Escherichia coli, Citrobacter* 3465/69, *Enterobacter cloacae* etc.) showed that acidification, either by adding acids or by fermentation, prevented the bacterial growth. The most resistant *Salmonella* died at a rate of 1,2 log cycle/h, the most resistant *Shigella* at 0,9 log cycle/h, the most resistant *Escherichia coli* at 0,6 log cycle/h. Drum dried and reconstituted porridge also showed the same characteristics.

The fermentation was accelerated by inoculum recycling, which was necessary to obtain a pH low enough to prevent the growth of the pathogenic bacteria (Nout *et al.*, 1989).

During fermentation of soybeans for tempe production, a growth reduction of the inoculated microorganisms *Salmonella infantis*, *Enterobacter aerogenes* and *Escherichia coli* by 6 – 7 log units in 40 h was found. A similar pattern was also found with acidified beans. Inoculation with *Lactobacillus plantarum* at a level of 10^6 /g resulted in a complete inhibition of the test organisms. Higher inoculum levels with acidified beans resulted in a marked drop in pH which gave complete inhibition of mould growth and hence no tempe production (Ashenafi & Busse, 1989), because for tempe production successful growth of mould is a requirement. *Staphylococcus aureus* and *Escherichia coli* introduced into nham, which is a Thai-style fermented pork sausage without any starter culture, showed little change in growth of *E. coli* and slow growth of *S. aureus*. With 0,75% starter culture added to the sausage, after 48 h *S. aureus* was not detectable and after 96 h *E. coli* had decreased by 1 log. With 1,5% starter culture, after 36 h *S. aureus* was not detectable and after 96 h *E. coli* was not detectable (Petchsing & Woodburn, 1990). Maize dough weaning foods prepared by mothers in a Ghanian village were examined for Gram-negative bacilli (GNB). The extent of contamination was higher in unfermented dough (5,9 log cfu/g) than in fermented dough (4,0 log cfu/g), and all 51 samples of unfermented dough contained CNB compared to only 16 of 51 samples of fermented dough. Cooking reduced the number of contaminated samples to 10 of unfermented and 6 of fermented dough, but after 6 h of storage, 45 of unfermented compared to 22 of fermented contained GNB (Mensah *et al.*, 1990). Inoculation of maize dough and porridge with *Shigella Flexneri* and enterotoxinogenic *Escherichia coli* (ETEC) showed that if the dough was fermented, it inhibited the inoculated bacterias to a greater extent. Even after cooking, the porridge from fermented maize dough showed some inhibition of the inoculated bacterias (Mensah *et al.*, 1991).

Three strains of *Escherichia coli* inoculated in milk which were fermented in a traditional manner usually followed in Zimbabwe multiplied to 10^7–10^9 /ml, while from Lacto a fermented milk using a starter culture from Denmark, two strains could not be recovered and the third strain survived only in very low numbers (Feresu & Nyati, 1990). Ayib is an Ethiopian cottage cheese made by heating skimmed, fermented milk. Commercially bought ayib was found to be largely contaminated with various microorganisms (Ashenafi & Busse, 1992). Rabadi is a fermented food popular in north-western semi-arid regions of India which is prepared by fermenting

a mixture of a flour of wheat, pearl millet, barley or maize with buttermilk. Resulting product show a lowered pH and raised titratable acidity specially in summer months (Gupta *et al.*, 1992). Neutralised extracellular culture filtrate obtained from isolates of *Lactobacillus acidophilus, Lactobacilus delbruecki ssp. bulgaricus, Lactobacillus salivarius* and *Lactococcus lactis ssp. lactis* from a fermented milk called "dahi" indigenous to India showed weak to moderate inhibition of *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Bacillus brevis, Bacilus circulans, Bacillus coagulans, Bacillus aterosporus, Bacillus subtilis* and *Pseuomonas aeruginosa*. The effective minimum quantity of lactic culture filtrates required to obtain complete inhibition of an inoculum of 10^3 cfu/ml of the bacteria tested was between 20 and 26% (Varadaraj *et al.*, 1993).

A study on the capacity of *Lactococcus lactis L1A* to inhibit growth of pathogenic bacteria by an *in vitro* method showed that between 92-100% of all 453 strains of gramnegative facultative aerobic rods were inhibited, as well as all 100 strains of *Group B streptococci*, but only about 10% of *Bacteroides* and *Clostiridium* (Grahn *et al.*, 1994). *Escherichia coli* O157:H7 was found to grow well in trypticase soy broth (TSB) with 6.5% NaCl, between pH 4,5 and 9,0 but in TSB acidified with lactic acid, the organism grew at pH 4,6 but not at pH 4,5 (Glass *et al.*, 1992). Minimal inhibitory concentrations (MIC) of lactic acid against *Listeria monocytogenes* was 5 mg/ml (Oh & Marshall, 1993). Strains of *Staphylococcus aureus, Listeria monocytogenes, Brochothrix thermospacta* that were able to grow at water activities of 0,95 and below in the presence of NaCl were inhibited by sodium lactate (Houtsma *et al.*, 1993). *Lactococcus lactis* which produces the physiological L-lactate isomer, thus avoiding the risk of D-lactate acidosis, was not as effective as strong acid producers such as the DL lactate producing *Lactobacillus plantarum*, and it only caused significant inibition of pathogens when present in a large numerical superiority ($>10^5$:1). Prefermentation of weaning food with *Lactococcus lactis* for 24 h produced a final product with a pH of 3,7-3,8 containing 0,25% lactate (>96% L-lactate) which was bactericidal to pathogenic organisms introduced subsequently. Despite the production of 100-150 international units of nisin per gram during fermentation, the inhibition of pathogens could be ascribed to acid production alone (Yusof *et al.*, 1993). Several papers published recently also report that lactic acid bacteria are able to inhibit growth of pathogenic bacteria such as *Clostridium* in cheese (Mayra Makinen & Suomalainen, 1996), *Yersinia enterocolitica* and some *Enterobacteriaceae* species in yogurt (Akbulut *et al.*, 1995), *Bacillus cereus, Escherichia*

coli and *Staphylococcus aureus* in kadhi which is a milk-based fermented product found in India (Balasubramanyam & Varadaraj, 1995).

Effect of lactic fermentation on survival of enteropathogens in cereal gruels, commonly used as weaning foods, were investigated by Kingamkono *et al.*, (1996) who added 28 enteropathogens to cereal gruels prepared from low tannin sorghum and inoculated them with a lactic acid starter culture. After 6 h of fermentation, *Campylobacter* strains were not detectable and after 12 h, *Salmonella*, *Shigella* and *Staphylococcus* strains were also not detectable. No viable *Bacillus* strain were found after 16 h and ETEC strains were completely inhibited after 24 h. On the other hand, in gruels prepared without using the lactic acid starter culture, all enteropathogens increased in number during incubation at 32°C, except *Campylobacter* strains which showed a decrease after 12 h.

The staple food in most parts of Ethiopia called injera is a fermented product prepared from tef (*Eragrostis tef*). The inhibitory effect of fermenting tef and the lactic acid bacteria isolated from fermenting tef dough on *Salmonella spp.*, *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Bacillus cereus* and *Staphylococcus aureus* was investigated (Nigatu & Gashe, 1994). The test bacteria was found to grow well in the fermenting tef until 30 h when the pH dropped to 4,7 followed by a significant decrease in the population.

Kocho, which is a traditional fermented food in Ethiopia prepared from ensete (*Ensete ventricosum)* with a pH of about 4,3, was also investigated in the same way with same test bacterial strains (Nigatu & Gashe, 1994). This shows that most of the test bacteria except *B. cereus* failed to grow during the first 30 h of incubation.

Toxins and toxin producing organisms in fermented foods

Lactic starter culture were found to be effective in preventing the formation of botulin toxin, even in the absence of nitrate (Shahani, 1983). No aflatoxin production was reported in tempe and miso prepared using *Rhizopus oligosporus* and *Aspergillus oryzae* on soya bean, chickpea and horsebean (Paredes-López & Harry, 1988). *Aspergillus flavus* grown in broth had a lower aflatoxin production when 10% cell free supernatant culture fluid from lactobacilli was added. This effect could not be explained on the basis of pH or competition (Karunaratne *et al.*, 1990).

Studies, mainly with *Aspergillus oryzae*, have shown no traces of aflatoxin production in traditional mould-fermented products (Wang & Hesseltine, 1981). However when an aflatoxin producing strain was inoculated at the same time, large amounts

of aflatoxin was found. The aflatoxin production of *Aspergillus parasiticus* was studied and found to increase in the presence of *Lactococus lactis* (Luchese & Harrigan, 1990). In contaminated peanut press-cake, *Rhizopus oligosporus* and *Neurospora sitophila* were found to reduce the aflatoxin content by 50 and 60% (Paredes-López & Harry, 1988).

The length of fermentation of cassava roots, the type of gari flour and the source of the flour affected the levels of HCN detected (Ukhun & Nkwocha, 1989). During the fermentation of melon seeds for the preparation ogiri, the aflatoxin content was reduced, and after 4 days of the 7 days of fermentation no aflatoxin was found (Ogunsanwo *et al.*, 1989). These results were sustained by the analysis of 26 market samples that were found negative in aflatoxin. Still the microorganism *Aspergillus flavus* was found in the fermenting ogiri at all stages. Apricot seeds were tested for the production of tempe. Amygdalin, a toxic, cyanogenic substance in particularly the bitter seeds, was reduced by 70% during the tempe process (Tunçel *et al.*, 1990). More than 90% of 488 cassava products from villages in Africa were fermented. Some microorganisms can hydrolyse linamarin, 52% of isolates from gari had the ability, but studies have shown that in grated products, like gari, it is primarily edogenous linamarase that hydrolyses the linamarin (Westby, 1991). The cyanide levels in blood of humans in Nigeria was found to be 0,294 µmol/l, which is higher than the cyanide level of a non-tropic population, 0,13 µmol/l. This could be attributed to a higher degree of exposure to cyanide since the staple diet in this part of Nigeria is cassava based meals (Uwakwe *et al.*, 1991). Grating prior to fermentation, fermentation, and the garrification process itself are all three components that together decides the residual cyanide content of garri produced from cassava (Aletor, 1993).

The results indicate that consumtion of fermented dairy products may provide protection against the development of tumors. The underlying machanism is unknown, but may involve stimulation of the immune system (Schaafsma, 1992). In a *Salmonella typhimurium* mutagenicity assay using nitrosated beef extract, eight of ten isolated *Lactobacillus* strains reduced the number of revertants back to the levels of untreated controls (Pool-Zobel *et al.*, 1993). Rats given *Lactobacillus casei* before a genotoxic carcinogen, had higher levels of undamaged colon cells than rats who did not recieve *Lactobacillus casei* before the genotoxic carcinogen (Pool-Zobel *et al.*, 1993).

Aflatoxins, secondary metabolites, have been demonstrated to be carcinogenic, teratogenic, and mutagenic. Lactic acid bacteria such as *Lactobacillus* spp. were found

to inhibit aflatoxin biosynthesis. The studies showed that the aflatoxin inhibition was probably due to an inhibitory metabolite other than hydrogen peroxide and low pH. However, other lactic bacteria such as *Lactococcus lactis* were found to stimulate growth and aflatoxin production of *Aspergillus parasiticus* and at the same time promote the transformation of aflatoxin B_1 which is highly mutagenic to nontaxic aflatoxinB_{2a} and less toxic aflatoxicol (R_0) (Gourama & Bullerman, 1995). Gourama who studied ability of *Lactobacillus spp* to inhibit the growth of *Penicillium* and its production of mycotoxin, found that two of lactic acid bacteria isolates which was identified as *Lactobacillus casei spp* showing high inhibitory activity against *Penicillium citrinum* and *P. expansum.* Further, the cell-free supernatants of the two Lactobacilli was shown to have inhibitory activity, independent of their production of lactic acid or hydrogen peroxide, which was sensitive to proteolytic enzymes like trypsin and pepsin as well as to higher temperatures (100^{o}C). This could be an indication that the antimycotic and antimycotoxigenic activity could be due to LAB metabolites that are proteinaceous in nature. (Gourama, 1997).

In vitro experiments with lactic acid bacteria isolated from the fermented non-dairy food called idly (idli), made of rice and legume flour, showed that they were effective in binding products of amino acid pyrolysis but other mutagens such as aflatoxins to a significant level (Thyagaraja & Hosono, 1994). Lactic acid bacteria isolated from dadih, a traditional Indonesian fermented milk were found to be able to bind mutagens and inhibit mutagenic nitrosamines. By using the Ames test, it was shown that highest antimutagenicity was displayed by milk cultured with *Lactobacillus casei subsp. casei R-52,* which showed 96.93% inhibition, while the lowest antimutagenicity (29.39% inhibition) was observed for milk cultured with *Lactococcus lactis subsp. lactis* R-63 (Surono & Hosono, 1996).

Milk fermented with *Lactobacillus acidophilus LA-2* was demonstrated to suppress faecal mutagenicity in the human intestine. Faecal mutagenicity and bacterial composition of 6 healthy subjects consuming their regular diet were investigated before and during the administration of milk fermented with *Lactobacillus acidophilus LA-2.* The result has shown that administration of fermented milk caused a remarkable decrease (71.9% on average; with a range of 19.4-90.6%) in faecal mutagenicity compared to that before the administration as well as an increase in population of *Lactobacillus* spp. and *Bifidobacterium* spp. in the faeces of all subjects (Hosoda *et al.*, 1996).

Studies on the antimutagenic activity of the milk fermented with mixed-culture with various lactic acid bacteria and yeast, showed that the fermented milks produced with

mixed cultures of lactic acid bacteria had a wider range of activity against mutagens than those produced with a single strain of lactic acid bacteria (Tamai *et al.*, 1995). Antimutagenic properties of the methanol extract of kimchi, a fermented food from Korea were evaluated by using Ames test for its antimutagenic activities towards aflatoxin B_1(AFB_1). Mutagenicity of AFB_1 was reduced by 35-75% upon addition of kimchi methanol extracts and the inhibition of mutagenicity was highest in those samples of kimchi which were fermented for 3 weeks (Kun Young *et al.*, 1995).

Birk et al (1996) studied effects of fermentation of cassava by *Aspergillus niger B-1* on the cyanide and protein contents of cassava. It was shown that the fermentation process reduced the cyanide content of cassava by 95% to 2 mg/kg, and increased its total protein content by 50%. A significant decrease in cyanogenic glycosides was detected after 3 days of fermentation.

Production of antimicrobial substances

Inhibitory effects of lactic acid bacteria against enteropathogenic microorganisms were measured (Brkic *et al.*, 1995) using the agar spot test and disc assay method. Cell-free supernatants of the lactic acid bacteria inhibited growth of *Staphylococcus aureus, Salmonella mumum, Escherichia coli, Bacillus cereus* and *B. subtilis.* It is suggested that inhibitory substances other than lactic acid were present in supernatant preparations, and that production of these antibacterial substances may be plasmid directed. Antimicrobial activity of 241 lactic acid bacteria belonging to *Lactobacillus plantarum, Pediococcus pentosaceus, L. fermentum/reuteri* and *L. brevis* isolated from various processing stages of maize (corn) dough fermentation (for kenkey) were shown to inhibit other Gram-positive and Gram-negative bacteria (Olsen *et al.*, 1995). The antimicrobial effects were explained by the combined influence of acids, compounds sensitive to proteolytic enzymes and other compounds with antimicrobial activity, with acid production being the most important factor. The pattern of antimicrobial factors was not species-specific as well as the safety and storage stability of fermented maize is suggested to depend on a mixed population of lactic acid bacteria with different types of antimicrobial characteristics. It is concluded that the introduction of pure cultures as starters may, therefore, impose a risk to the final product.

Fermented meats have caused food-borne illness due to enterohemorrhagic *Escherichia coli*. Consumption of Lebanon bologna, a moist fermented sausage manufactured from lean beef (final moisture-to-protein ratio is ~3.5:1 wt/wt), was

epidemiologically associated with outbreak of salmonellosis. A study was conducted to determine the effects of pH (after the fermentation step), final heating temperature, and time on destruction of *E. coli* O157:H7 and *Salmonella tryhimurium* in Lebanon bologna. The results showed that fermentation alone reduced populations of both pathogens by <2 log units and heating alone reduced populations of *E. coli* O157:H7 by <3 log units. A combination of fermenting to either pH 5,2 or 4,7, followed by heating at 110°F (43.3°C) for 20 h, 115°F (46,1°C) for 10 h, or 120°F (48.9°C) for 3 h reduced populations of both pathogens by >7 log units (Kameswar *et al.*, 1998).

Lactobacillus sake CTC494, isolated from a naturally fermented sausage, produced an antibacterial agent active against selected strains of *Listeria monocytogenes* and *L. innocua.* The agent was identified as a bacteriocin and designated sakacin K. Results indicated the effectiveness of sakacin K in inhibiting *Listeria* growth. It is concluded that *L. sake* CTC494 is a good starter culture, providing good organoleptic and sensory qualities to the products, and can also be employed as a bioprotective starter culture in fermented meat products (Hugas *et al.*, 1995).

Benthin S. and Villadsen J. studied the inhibition of *Lactobacillus delbrueckii subsp. bulgaricus* by D- and L-lactic acid: effects on lag phase, growth rate and cell yield. They showed that inhibition of *L. bulgaricus* by lactic acids at constant pH is governed by 2 factors: the total concentration of lactic acids and the D/L ratio of lactic acid stereoisomers. L-Lactic acid was more inhibitory to the bacterium than the D-isomer, the latter being the only isomer produced by *L. bulgaricus.* It is suggested that the difference in the inhibitory effects of D- and L-lactic acid have implications for yogurt manufacture and potential for improved food preservation (Benthin & Villadsen, 1995). Recently, there was a study about survival of acid-adapted and unadapted *E. coli* in lactic acid, during sausage fermentation and in shredded dry salami (pH 5.0) and apple cider (pH 3.4). Acid-adapted cells, *E. coli* O157: H7, showed increased resistance in lactic acid compared to unadapted cells. It is suggested that acid adaptation of *E. coli* must be taken into account in laboratory food challenge studies (Leyer *et al.*, 1995).

Nisin most probably inactivates sulfhydryl groups in the cytoplasmic membrane, thereby acting as an inhibitor of both spore outgrowth (at the stage of swelling) and vegetative growth. From a number of toxicological studies the nontoxic and nonimmunogenic character of nisin has become clear (Rayman *et al.*, 1981). *Streptococcus lactis* produces the polypeptide nisin, active against gram-positive organisms including *Streptococcus cremoris* which, in turn, produces ”diplococcin”

active against gram-positive organisms including *Streptococcus lactis*. Thus these microorganisms compete in the fermentation of milk products while inhibiting growth of other gram-positive bacteria (Steinkraus, 1983). Klaenhammer (1988) has made a review on bacteriocins of lactic acid bacteria. Bacteriocins are bactericidal proteins that can have either a narrow spectrum (inhibit closely related bacteria) or a wide spectrum (inhibit a diverse group of Gram-positive bacteria). Bacteriocins mentioned are: nisin, pediocin A, diplococcin, lactacin B, lactacin F, lactocin 27, helveticin J, lactostrepcins. These compounds are important in the preservation of fermented foods, and helps the producing microorganism to dominate the flora of the substrate. There are numerous reports on natural antimicrobials produced by lactic acid bacteria: nisin, acidoline, acidophiline, lactacine, lactocidine, lactocine, helveticine, bulgarican, plantaricin, reuterin, diplococcin, lactostrepcin etc. (Huis In'T Veld *et al.*, 1990). The bacteriocin produced by *Lactococcus lactis* SIK-83 is extremely heat stable and survives 170°C for 10 minutes (Andersson & Åkesson, 1990). There are two major classes of bacteriocins produced by LAB, those bacteriocins (e.g. lactacin B and F, and lactocin 27) that are cidal to a narrow range of target organisms, usually closely related to the producer organisms, and those bacteriocins (e.g. nisin, pediocin A and pedioin PA-1) that inhibit a broad spectrum of (gram-positive) organisms. The latter include many species or strains of spoilage and pathogenic bacteria associated with food, such as *Listeria monocytogenes* and *Clostridium botulinum*. Studies on several bacteriocins have indicated that they are nontoxic and nonimmunogenic (Marugg, 1991). Lactococcus lactis produces the bacteriocin nisin. Despite the production of 100-150 international units nisin per g during fermentation, the inhibition of pathogens could be ascribed to acid production alone (Yusof *et al.*, 1993).

Present study

The objective of the present experiments was to follow the changes in the production of organic acids, development of pH, amount of tritratable acidity. Fermentation was carried out with bakery wheat flour, whole grain wheat flour and tef flour, at temperature 25°C and 35°C and different amounts of starter for initiating the fermentation process. A study was made on the effect of fermentation on the growth of *Escherichia coli* - strain NG7C in slurries made from whole grain flours of wheat and tef.

The processing conditions applied in fermenting food under household conditions varies from one part of the world to the other to a great extend regarding the water to

solids ratio, type of microbial flora present or added, the type of raw material used and the type of finished product expected. Very often a spontaneous fermentation utilising the microflora present on the raw materials is used for initiating fermentation. Some times a kind of starter, which is a previously fermented product is used not only to initiate the fermentation but also to keep a uniform quality from one day to another. In addition to cereals fish, meat, dairy products, fruits and vegetables are also fermented either alone or in combinations. Sometimes fermented foods are given some characteristic sensory properties by addition of spices, condiments and salts.

The present study deals with the changes that are taking place during fermentation of a slurry made of cereal flour and water. This is a common method for producing fermented food not only for adults but also for infants in many African countries. A common Ethiopian food item called “injera”, a circular, flat, spongy, pancake like, bread that is made of fermented slurry of the flour of the cereal tef (*Egragrostis tef*) was chosen as a model for our investigation. Tef is a major cereal in Ethiopia having very small grains, cultivated on more than 50% of Ethiopia’s total cereal area in 1980 (Lost crops of Africa, 1996). A recipe taken from an Amharic cookbook was scaled 1/10 to make a laboratory scale, see appendix 1.

Materials and methods

Materials

Cereal grains

Commercial quality wheat flour of 70% extraction rate designated as "bagerivetemjöl" was obtained from the industrial flourmill Skånemöllan AB, Sweden. Wheat grains of the Kosack variety was kindly provided by the seed company Svalöv/Weibulls AB, Svalöv, Sweden. Tef grains (*Eragrostis tef*) of the DZ-01-196 variety was bought from the Bio-Diversity Institute of the Federal Government of Ethiopia, Addis Ababa, Ethiopia.

Test bacteria

The test bacteria, *Escherichia coli* strain NG7C, was obtained from the Medical Microbiology Department, Lund University. The bacteria was isolated from a child with diarrhea in Papua New Guinea (Ljungh *et al.*, 1991; Dael *et al.*, 1993).

Media

The media used for isolation of the bacteria from the fermenting whole grain wheat and whole grain tef flour slurry were Violet Red Bile Glucose Agar (Oxoid, CM485) for *E. coli* and Rogosa agar (Oxoid, PM221) for lactobacilli. Brain Heart Infusion Broth (Oxiod, CM225) was employed to maintain the test bacteria used for assaying.

Chemicals

Lactate standard 40 mg/dl (ref no. 826-10) was obtained from Sigma. Organic acid analysis standard with 0,8 µmol sodium oxalate, 4,0 µmol sodium citrate, 8,0 µmol sodium malate, 20,0 µmol sodium succinate, 20 µmol sodium formate and 20,0 µmol sodium acetate was obtained from Bio-Rad (no. 125-0586). All other chemicals used in this study were of analytical grade.

Methods

Preparation of flour

To obtain the tef and whole wheat flour, the grains were cleaned by rinsing three times in distilled water, dried in a ventilated oven at 50°C and milled in a Tecator Cyclotec mill using a 1-mm sieve. The flour was stored in a closed plastic container at 4°C until it was used for further experiments.

Fermentation

Fermentation was performed in a slurry made with 300 g of flour mixed with 600 ml of 40°C warm water in a 1-l beaker. To get a homogenous mixture, 250 ml of water was first added to the flour and mixed, then two subsequent portions of 75 ml water was added with mixing in between. Finally, the remaining 200 ml was added together with the inoculum and thoroughly mixed. The beaker was placed in a water bath with a constant temperature of 25°C or 35°C and the fermentation was carried out without stirring, in accordance with the usual household practise.

Three different amounts were used, 1 g (≈0,1%), 10 g (≈1%) and 100 g (≈10%) for backslopping. The fermentation was started without inoculum as a spontaneous fermentation, and then backslopping was performed at 84-h intervals. Three consecutive backsloppings were made before the microbial flora was adapted and the system was considered to be consistent (Nout *et al.*, 1989) to allow samples being taken for analysis. The inoculum was taken from the previous batch after the liquid top layer above the sediment has been decanted.

Sampling

Samples were withdrawn at the beginning of fermentation and after every 3, 6, 9, 12, 18, 24, 48 and 84 hours respectively using a syringe like sampling device, sucking about 10 ml of the sample about 1 cm above the bottom surface of the beaker. The samples for organic acids were quickly frozen by placing each of them in a plastic container directly on a refrigerated shelf of a freezer. The frozen samples were kept at -18°C until they were used for further analysis.

For testing which type of lactic acid bacteria was present in the tef fermentation, samples were taken at the end of the fermentation process. These samples were kept refrigerated at 4°C until they were used for further analysis.

Measurement of pH

The pH of the samples was measured using an Orion expandable ion analyzer EA 920 and an Orion Sure-Flow Ross pH-electrode.

Sample preparation for analysis of organic acids

The frozen sample was thawed to room temperature, and approximately 1 to 2 g in duplicates were weighed in to a centrifuge tube to which 200 µl of fumaric acid (12,1 mg/ml) was added as internal standard together with 7 ml of distilled water. The content was homogenised and placed in a water bath of 65°C for 5 minutes to prevent the lactic acid bacteria from continuing the fermentation process during the analyses.

The centrifuge tube with its contents was cooled to room temperature in an ice bath, neutralised to pH 7 with 0,1 M sodium hydroxide, and water was added to adjust the volume to 10 ml. The tube was then placed in an ultrasonic bath for 5 min to facilitate the extraction of the organic acids, and then centrifuged at 4000 rpm (Johansson *et al.*, 1995). The supernatant in the centrifuge tube was filtered through a 45-µm filter (Millipore HAWP 02500) and 20 µl of the filtrate was injected in the HPLC column.

Analysis of organic acids

Organic acids were analysed using an HPLC-apparatus consisting of a Pharmacia Pump P-3500, an Aminex® HPX-87H column from Bio-Rad Laboratories and a Pharmacia Liquid Chromatography Controller LCC 500. As the mobile phase 0,005 M sulphuric acid was used at a flow rate of 0,6 ml/min. The column was kept immersed in a water bath kept at 35°C. For detection a Varian 2550 UV-detector at

410 nm set at range 0,16 was used. Recordings were made on a Pharmacia Two-channel Recorder REC-482 and on the LCC 500. Formic acid was used as internal standard and it did not interfere with the separation of other acids. Standard curves for lactic acid were plotted using peak height and peak area given by the LCC 500, and peak height was chosen to be used as it showed the best correlation. All analysis was done in duplicates.

Titratable acidity

The titratable acidity was measured by titrating a mixture of 3 g of sample and 27 ml of distilled water to pH 8.5 using 0.1 M sodium hydroxide solution (Kingamkono *et al.*, 1994). The result was expressed as g lactic acid/100 g sample, and plotted against the results from HPLC-analysis. The trendline for the plot was established using Microsoft Excel.

Measurement of the growth of bacteria

For determination of the number of bacteria in the fermenting slurry, samples (~5 ml) were taken at different intervals from the top (1 cm below the surface) and bottom (1 cm above) of the mixture. The samples (~1 g) were diluted with saline solution (9 ml) for pour plate count on Rogosa agar (Oxoid, PM221) medium for lactic acid bacteria, and on violet red bile glucose agar (Oxoid, CM485) for *E. coli*. Lactic acid bacteria was incubated at 37°C for a period of 3 days in an anaerobic jar. *E. coli* was incubated at 37°C for a period of 18-24 h.

Typing of the flora in the fermented slurry

For typing the flora, samples were taken from the final fermented tef slurries and plated on Rogosa agar. Colonies were randomly picked, purified and stored in freezing buffer (Ahrné *et al.*, 1989). From each of these samples, five isolates were drawn for identification and subtyping.

Identification and subtyping of *Lactobacillus plantarum* was done by Randomly Amplied Polymorphic DNA (RAPD) according to the method described by Johansson et al, (Johansson *et al.*, 1995) and by API 50 CH (API System, Montalieu, Vercieu, France).

Design of *E. coli* experiments

The study contains three sets of experiments. The first set of fermentation experiments were done to compare the pH development and growth of lactic acid bacteria in spontaneous fermentation to back-slopping (1% and 10%) in whole grain

flour slurry made of tef and wheat. The pH of the fermentation was measured at different levels to see if there was any difference between top surface and bottom layer.

The second set of experiments was done to study the effect of LAB fermentation on the growth of *E. coli* added to the sample. The pH development and numbers of LAB and *E. coli* were determined at different intervals.

In the third set of experiments the pH was adjusted by adding lactic acid and hydrochloric acid to study the effect of pH, amount of lactic acid, and degree of dissociation of lactic acid.

Results and discussion

Organic acids

Figure 2 shows the chromatogram with separation of the standard organic acids which may be found in fermenting organic material.

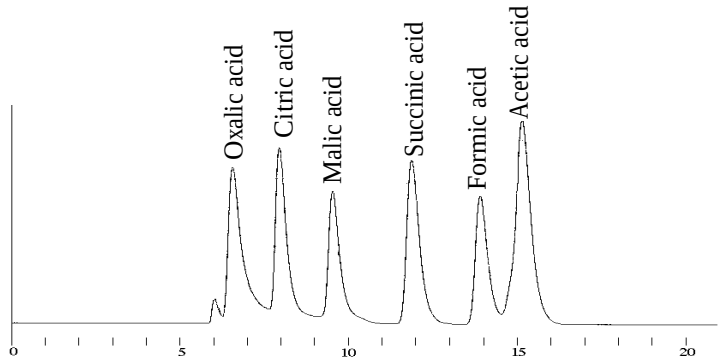

Figure 2. HPLC standard curve

In Figure 3 the chromatogram of standard lactic acid with internal standard formic acid is shown. The response for lactic acid was linear between the values corresponding to 0,06 and 0,96 g/100 g with an R^2 value of 0,9999. Formic acid used as internal standard separated well from the other acids and under normal circumstances it is not likely to be present in fermenting cereals.

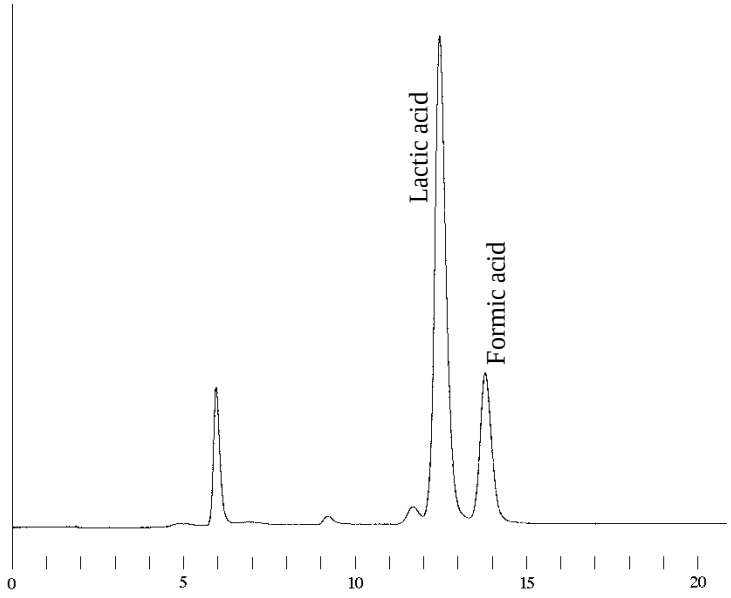

Figure 3. HPLC-curve with lactic acid, formic acid as internal standard

Figure 4 a-d shows the separation of organic acids from nonfermented samples and fully fermented samples of bagerivetemjöl and tef. The detection level at the used setup was found to be equivalent to 0.01 g lactic acid/100 g sample and 0,005 g acetic acid/100 g sample. Only the amount of lactic acid was quantified and presented since no peaks were found that could be attributed to other acids.

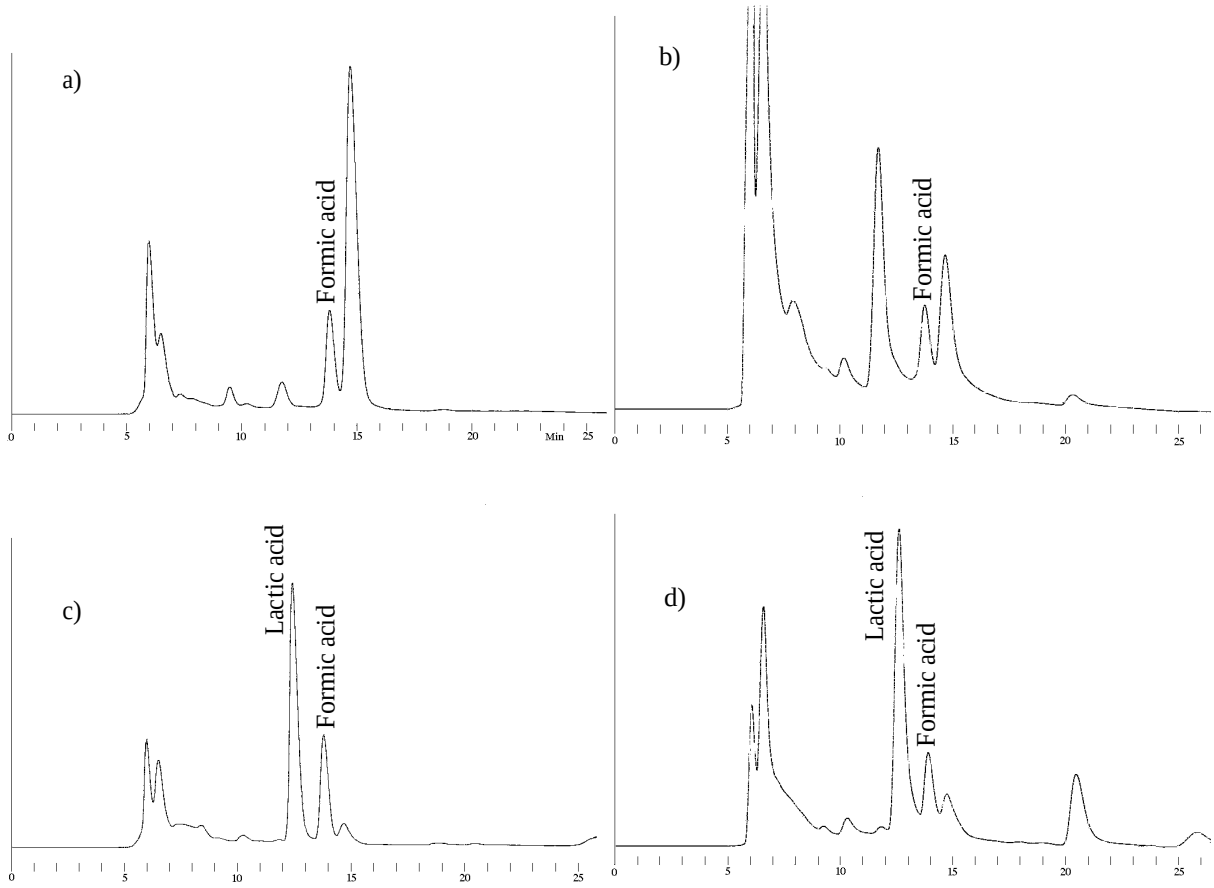

Figure 4. HPLC-curves, a) nonfermented bagerivetemjöl, b) nonfermented tef, c) fermented bagerivetemjöl, d) fermented tef.

Final concentration of lactic acid

The total amount of lactic acid at the end of the fermentation was for tef slurry fermented at 25°C approximately 1,0 g per 100 g (Figure 5). At the higher temperature, 35°C, the amount was slightly lower. The whole grain wheat flour slurry had a different pattern, where at 25°C the final amount was 0,6–0,8 g per 100 g while at 35°C it was 1,0–1,2 g per 100 g (Figure 6). For bagerivetemjöl the pattern was similar, but not as accentuated, and the final amounts were all below 0,9 g per 100 g (Figure 7). Although the lactic acid amounts were not high, the pH reached using bagerivetemjöl was the lowest.

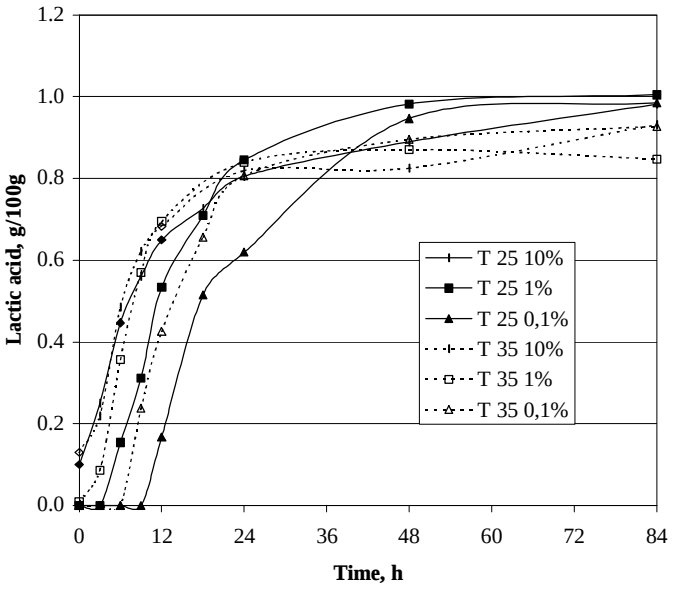

Figure 5. Lactic acid content in fermenting tef slurry.

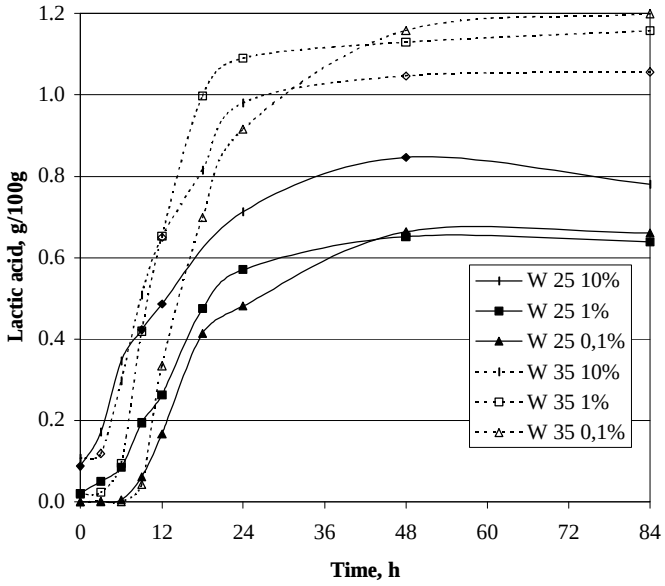

Figure 6. Lactic acid content in fermenting whole wheat slurry.

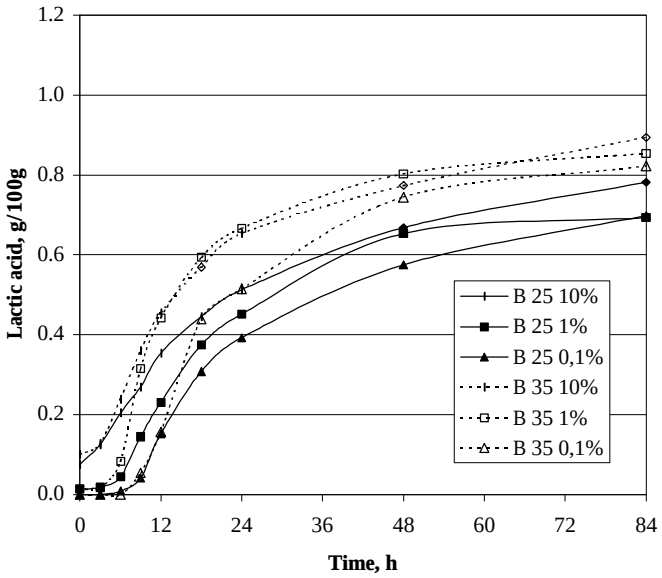

Figure 7. Lactic acid content in fermenting bagerivetemjöl slurry.

Lactic acid production rate

The production rate of lactic acid was higher at higher temperature in all cases. The results show a difference in buffering capacity for the different raw materials. For bagerivetemjöl a great portion of the outer parts of the grain are removed. In this case, only 70% of the grain are retained in the milling process of bagerivetemjöl. The production rate of lactic acid in the bagerivetemjöl slurry was lower that in the other slurries, but the change in pH was about the same (Figure 8).

The production rate of lactic acid was also influenced by the amount of inoculum. At 35°C, the initial production rate (just after the lag phase) was higher with 1% backslopping than with 10% backslopping. This can be seen in Figure 5, Figure 6 and Figure 7, where the curves for 1% backslopping closes in on, and after about 12 hours passes, the 10% backslopping curves.

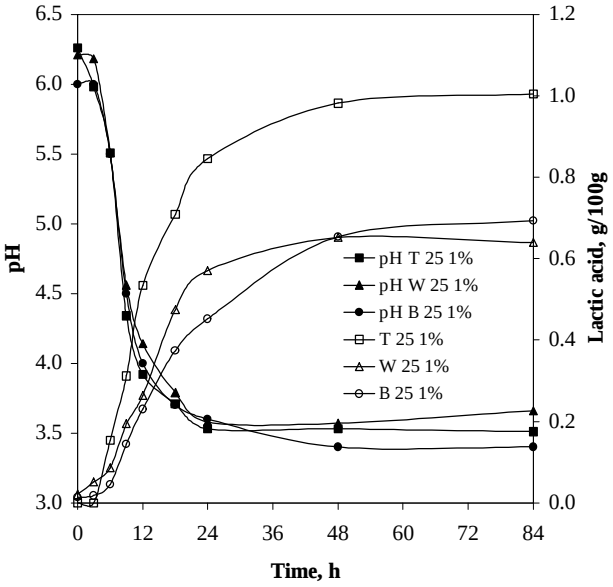

Figure 8. Lactic acid production and pH-development at 25°C, 1% backslopping, using tef, whole grain wheat and bagerivetemjöl.

Inoculum amount for backslopping

To test the importance of the amount of inoculum in the fermentation, three different amounts were used in the experiments. The original recipe's ladle was estimated to contain 100 g, which gave 10 g as normal inoculum for the 900-g laboratory scale slurry (approx. 1%). This amount was 10-folded and divided by 10, giving 1 g and 100 g respectively as the other assay inoculum amounts.

A higher amount of inoculum gave a quicker lowering of the pH, Figure 9 and Figure 10. This can be explained by the fact that the number of bacteria put into the slurry was higher, giving a higher lactic acid production. In addition, the initial pH was lower since the amount of inoculum put into the slurry was considerable. A 10% inoculum into the slurry can also be considered as a 10-folded dilution of the inoculum. This could theoretically mean a rising of the pH in the inoculum (approximately 3,5) with one unit (to approximately 4,5). In practise, the rising of the pH was higher. The bagerivetemjöl fermentation started at pH 4,8 while the flour of whole wheat and tef started at pH 5,4. At 35°C the 10% inoculum slurry had a short lag phase, while at 25°C the acid production started almost immediately.

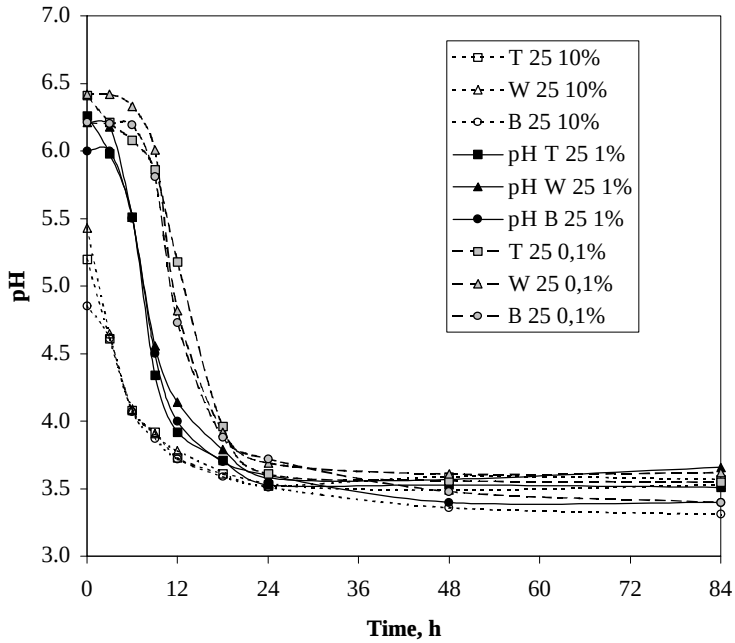

Figure 9. Development of pH at 25°C, using tef, whole grain wheat and bagerivetemjöl.

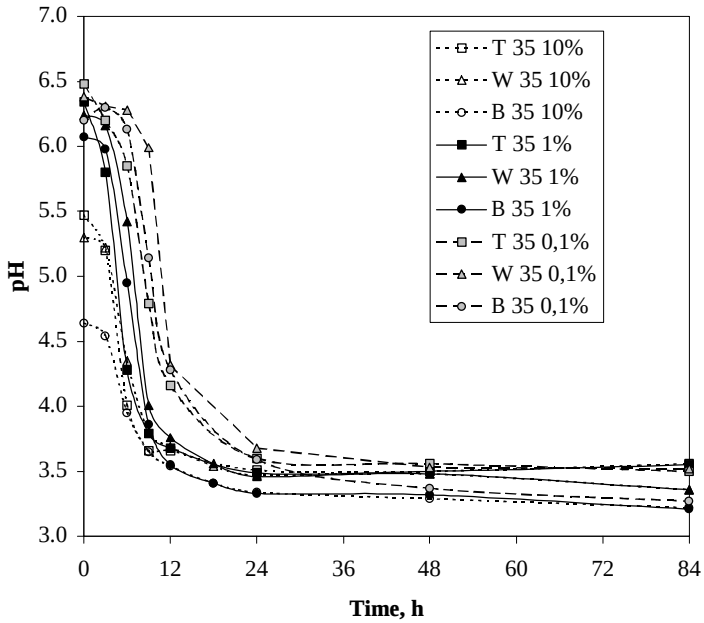

Figure 10. Development of pH at 35°C, using tef, whole grain wheat and bagerivetemjöl.

A low inoculum (0,1%) gave a longer lag phase, and almost no change in initial pH compared to a slurry without inoculum.

Temperature dependence

All assays were made at two different temperatures: 25°C and 35°C.

Higher temperature raised the production rate of lactic acid. It also had an impact on the lactic acid bacteria in that the strains found were somewhat different.

With a high inoculum, the temperature difference was not very important; the high starting number of lactic acid bacteria compensated for differences in temperature.

In whole grain wheat flour, there was a clear difference between the two temperatures used, the higher temperature giving higher concentrations of lactic acid. For tef the final difference was opposite, the final concentrations of lactic acid was higher at the lower temperature, but here the difference was much smaller.

Influence of raw material

At 25°C and 1% backslopping the difference between the pH-development of the fermentations was very small (Figure 8), but the lactic acid production was very different with lowest production in bakery flour slurry and highest production in tef slurry. The food safety depends on the amount of undissociated lactic acid in the slurry. In Figure 11 it is shown that the concentration of undissociated lactic acid is rising much slower than the total amount. The pH has to go down to 4 before the concentration of undissociated lactic acid starts to rise significantly.

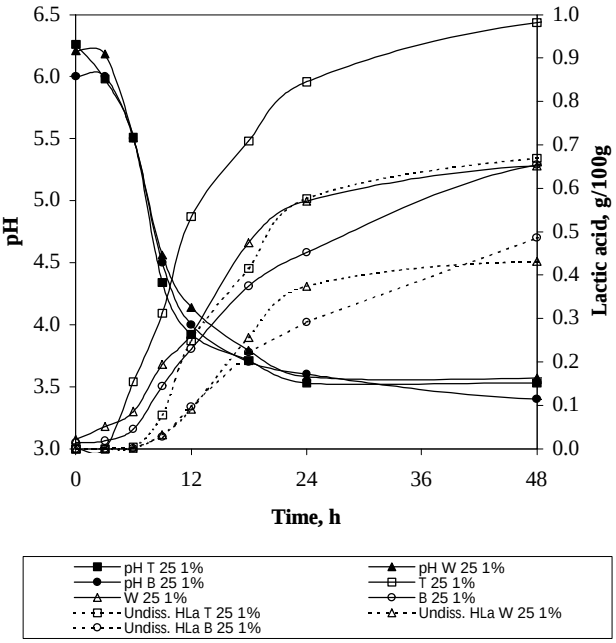

Figure 11. Lactic acid content (total and undissociated) and pH-development at 25°C, 1% backslopping, using tef (T), whole grain wheat (W) and bagerivetemjöl (B).

Titratable acidity

The amounts of lactic acid derived from titrations corresponded to the values found using HPLC according to the results in Table 5. Titratable acidity gave higher values for lactic acid than HPLC-determination.

Table 5. Titratable acidity compared to HPLC-determination of lactic acid in slurry of bagerivetemjöl (B), whole wheat flour (W) and tef (T).

Cereal flour, backslopping amount	Temperature					
	25°C			35°C		
	Slope	Zero	R^2	Slope	Zero	R^2
B, 0,1%	1,1643	0,0574	0,9911	1,3160	0,0608	0,9877
B, 1%	1,1493	0,0772	0,9934	1,2357	0,0736	0,9956
B, 10%	1,1414	0,0669	0,9976	1,2267	0,0673	0,999
W, 0,1%	1,5619	0,0742	0,9842	1,2228	0,1039	0,9939
W, 1%	1,5317	0,1184	0,9835	1,1836	0,1209	0,9781
W, 10%	1,4097	0,1443	0,9859	1,2410	0,1489	0,9912
T, 0,1%	1,5301	0,0751	0,9838	1,5531	0,1190	0,9917
T, 1%	1,5439	0,0923	0,9927	1,4981	0,0976	0,9811
T, 10%	1,6212	0,0027	0,9698	1,5582	0,1083	0,9470

Relation pH – lactic acid

A combination of all the results regarding the relation between pH and lactic acid for the different raw materials (at all temperatures and back-slopping amounts) is given in Figure 12 . The diagram shows that it was much easier to lower the pH in the bagerivetemjöl, and that tef flour was more resisting to changes in pH. At 25°C and with 1% back-slopping (normal injera production) the development of pH was very similar for the three different raw materials (Figure 8), although the lactic acid amounts were different with a much higher end concentration of lactic acid in the tef slurry.

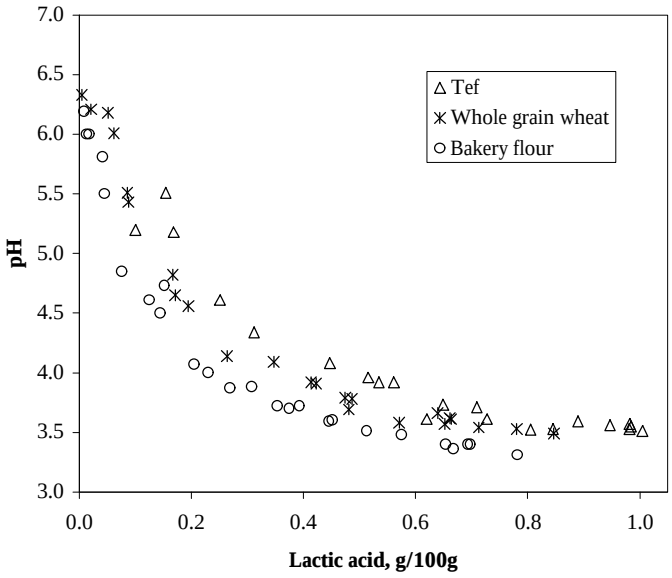

Figure 12. Lactic acid content and pH in fermenting slurry.

Lactic acid bacteria at the final stage

The dominant species in the tef fermentation was *Lactobacillus plantarum* at a final level of approximately 10^8 cfu/g (Table 6). At 25°C only *L. plantarum* was found, of two different strains (1 and 2). Using the low amount of inoculum only 20% of the lactic acid bacteria belonged to strain 1, with normal inoculum amount 40% belonged to strain 1, and with high inoculum amount only strain 1 was present.

Table 6. Lactobacilli count in final fermented tef slurry, cfu/g.

	Backslopping amount		
Temperature	0,1%	1%	10%
25°C	$2{,}2 \cdot 10^8$	$2{,}6 \cdot 10^8$	$2{,}6 \cdot 10^8$
35°C	$1{,}7 \cdot 10^8$	$1{,}0 \cdot 10^8$	$3{,}4 \cdot 10^8$

At the higher temperature (35°C), *L. plantarum* was also the dominant species, but other *Lactobacillus* species were also present. Low inoculum amount contained 40% *L. plantarum* strain 1. Three other strains were present, two were obligate homofermentative lactobacilli strain 4 and strain 5, while strain 6 seems to be an

obligate heterofermentative lactobacilli. Normal amount of inoculum contained 80% of *L. plantarum*, 20% strain 1, 40% strain 2 and 20% strain 8. Additionally strain 7 was found that resembles strain 6 but not being completely identical. At high inoculum 40% of the isolates contained *L plantarum* (20% strain 1, 20% strain 2). The rest of the isolates contained obligate homofermentative lactobacilli of three different strains (strain 3, 4 and 5) that resemble each other genetically but have different phenotypes. These results are compiled in Table 7.

Table 7. Lactobacilli found in fermented tef slurry.

		Temperature					
		25°C			35°C		
Bacteria		0,1%	1%	10%	0,1%	1%	10%
Lactobacillus plantarum	Strain 1	X	XX	XXXXX	XX	X	X
	Strain 2	XXXX	XXX			XX	X
	Strain 8					X	
Obligate homo-fermentative	Strain 3						X
	Strain 4				X		X
	Strain 5				X		X
Obligate hetero-fermentative	Strain 6				X		
	Strain7					X	

E. coli study

The results of the measurement of pH in the fermenting whole grain wheat flour slurry without back-slopping and with back-slopping at 1% and 10% level are presented at top of Figure 13. It clearly shows, as could be expected, that the rate of pH development as well as the final pH was significantly favoured by back-slopping. The pH of the mixture during the initial periods of the fermentation process is of great importance in respect to the final quality of the fermented product regarding safety and sensory properties. A low pH would promote the growth of lactic acid bacteria in a higher rate causing similar drop in pH and resultant inhibition of the growth of many undesirable organisms.

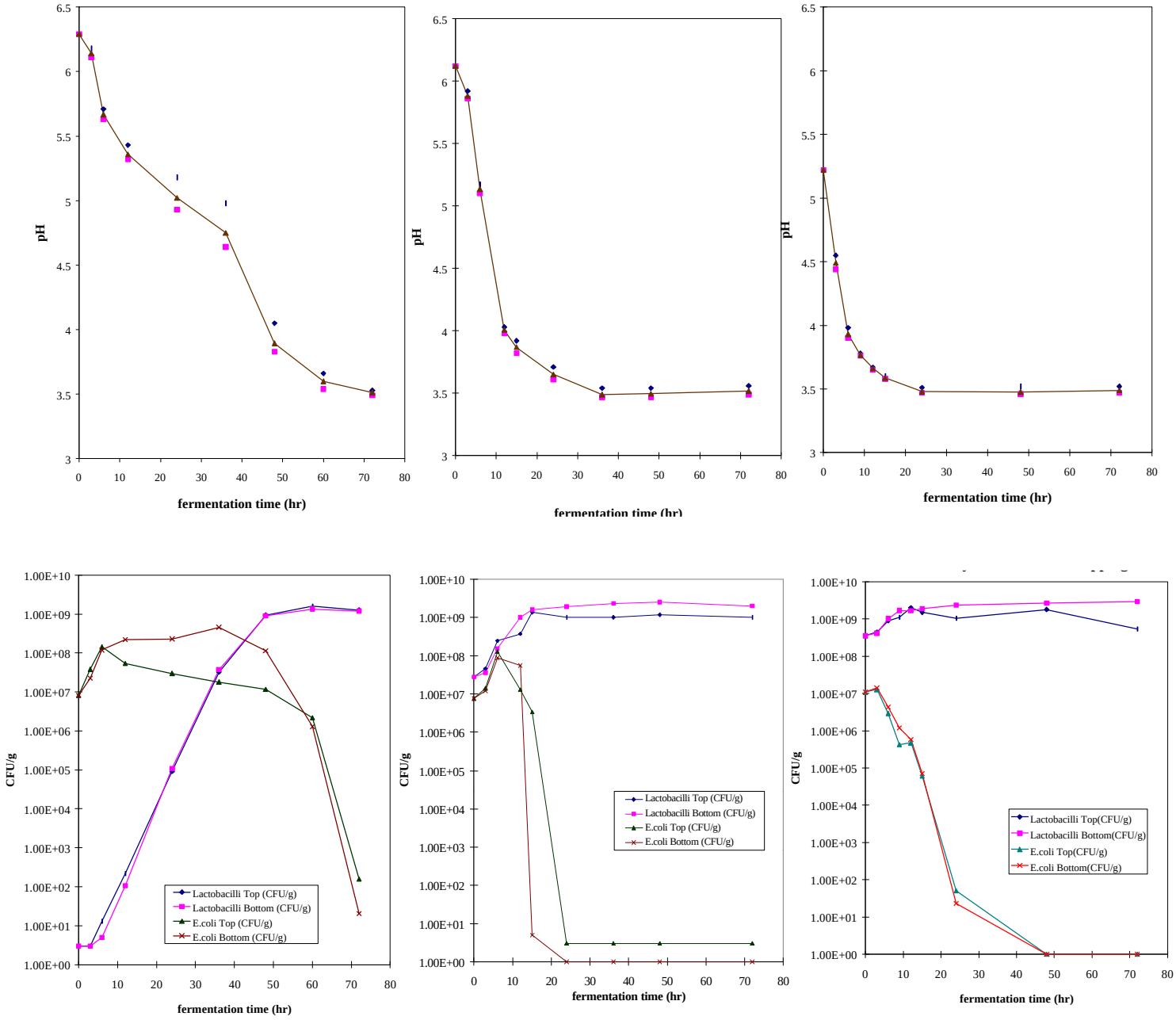

Figure 13. Whole grain wheat flour slurry, pH-development and growth of bacteria. Backslopping: left - no; middle - 1%; right - 10%.

In the present study, the pH of the wheat flour slurry without back-slopping takes 60 hours to reach the final pH of 3,5. But the duration of this period is reduced to 30 hours in 1% back-slopping and to 20 hours in 10% back-slopping. The rate of pH development was -0,015 unit/h in 10% back-slopping compared to -0,039 unit/h in

fermentation without back-slopping. In general there were no significant difference between the pH-values, registered at different levels of the slurry during fermentation without stirring. However, there were small differences of pH at measured at 24, 36 and 48 h in the fermentation without back-slopping.

A strong relation of pH development to the growth of lactobacilli could be found in all cases. However, the growth of lactobacilli began to be stable when pH dropped to 4 and no significant (P>0,05) growth between the top and bottom of the fermenters was observed in any fermentation experiments.

The determination of the growth of lactobacilli and *E. coli* as well as pH development in fermenting whole grain wheat flour slurry without back-slopping, with 1% and 10% back-slopping is shown in Figure 13. The initial number as well as the rate of growth of lactic acid bacteria in the gruel is important in relation to the rate of production of lactic acid and development of pH. Therefore the use of sufficient amount of fermented gruel from an earlier batch or addition of a starter culture would be a better way of facilitating the initial growth of lactic acid bacteria in the fermenting gruels. The results of the present experiment show that the growth of lactic acid bacteria as well as the pH development in fermenting tef followed a pattern quite similar to that of fermenting wheat flour. But the rate of initial drop of pH in tef flour fermented without back-slopping was higher than that in fermenting wheat flour without backslopping. This could be due to the presence of easily fermentable carbohydrates in the tef flour or a large number of viable lactic acid bacteria or both.

The growth of pathogenic *E. coli* inoculated in the whole grain flour slurry of wheat and tef was strongly inhibited by the fermentation process (Figure 13, Figure 14). The decrease in the number of *E. coli* follows the increase in the number of lactic acid bacteria and subsequent reduction in pH. Below pH 4, the growth of *E. coli* is significantly reduced. In the experiments where the fermentation was done without back-slopping an initial delay in the reduction of the number of *E. coli* was noticed. This followed a corresponding delay in the growth of lactic acid bacteria and change of pH. Back-slopping on the other hand significantly facilitates the growth of lactic acid, drop of pH and reduction in the number of *E. coli*. The initial reduction in the number of viable *E. coli* in the fermenting slurry could be controlled by increased amount of back-slopping.

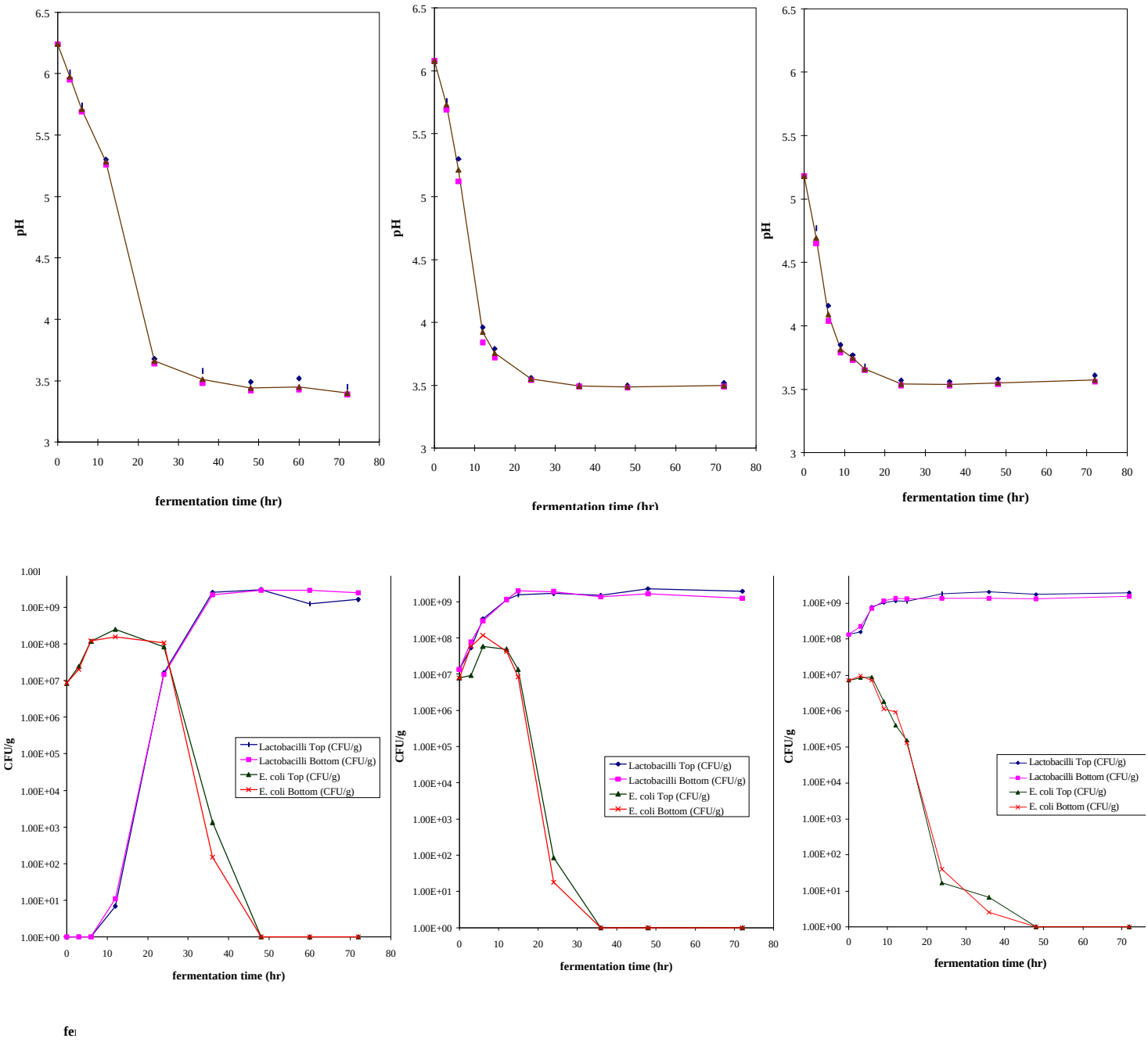

Figure 14. Tef flour slurry, pH-development and growth of bacteria. Backslopping: left - no; middle - 1%; right - 10%.

In 10% back-slopping a significant reduction of the number of *E. coli* could be noticed already after a couple of hours incubation, both in tef and in wheat. From the data on pH development and the number of lactobacilli the rates of pH development and growth rate of lactobacilli in both fermenting whole grain wheat and whole grain tef flour slurry were calculated. The growth rate of lactobacilli in

fermentation experiments show strong relation to drop in pH both in wheat flour and in tef.

In Figure 15 the survival rate of *E. coli* in fermenting slurry at different conditions of pH and lactic acid content is plotted against time. The initial pH of the slurry was adjusted to different levels by adding lactic acid alone or by adding hydrochloric acid. It shows that the lower pH have an effect on the growth of *E. coli*. However a lower pH caused by the addition of lactic acid is more effective in reducing the number of *E. coli* than addition of HCl. It also shows that the effect of fermentation of a system on the reduction of the number of viable cells of unwanted micro-organisms is found not only to depend on the pH and lactic acid content but also to the degree of dissociation of the lactic acid.

The sample with the highest amount of undissociated lactic acid had the fastest reduction of viable *E. coli* during fermentation. The reduction could not be explained only on the basis of pH reduction and amount of undissociated lactic acid. Therefore it could be expected that lactic acid bacteria produce some other growth inhibiting substances in its effort to over power the competing strains. In 1994, Nigatu and Gashe also studied the effect of fermenting tef and the lactic acid bacteria isolated from fermenting tef dough on *Salmonella spp.*, *Pseudomonas aeruginosa*, *Klebsiella spp.*, *Bacillus cereus* and *Staphylococcus aureus.* The test bacteria grew in the fermenting tef until 30 h or till the pH dropped to 4,7. Thereafter, growth was inhibited and decreases in population were apparent.

Kingamkono *et al.*, (1996) added several enteropathogens to cereal gruels prepared from sorghum and inoculated with a lactic acid starter culture. On fermentation, *Campylobacter* strains were not detectable after 6 h, while *Salmonella, Shigella* and *Staphylococcus* strains were not detectable after 12 h. After 16 h, no viable *Bacillus* strain were found, and ETEC strains were completely inhibited after 24 h. On the other hand, in the gruels prepared without the lactic acid starter culture, all enteropathogens increased in number during incubation at 32^{o}C except for *Campylobacter* strains which decreased after 12 h.

According to the results of the present study, the number of *E. coli* decreased rapidly in both 1% and 10% backslopping, quicker than in the fermentation without back-slopping. However, reduction of *E. coli* was quicker in fermenting whole grain wheat flour slurry compared to whole grain tef flour slurry. The survival of *E. coli* not only depends on the amount of lactobacilli but also on the lowering of pH. The lowest pH about 3.5 in all fermentations could reduce the amount of *E. coli* > 6-log-unit.

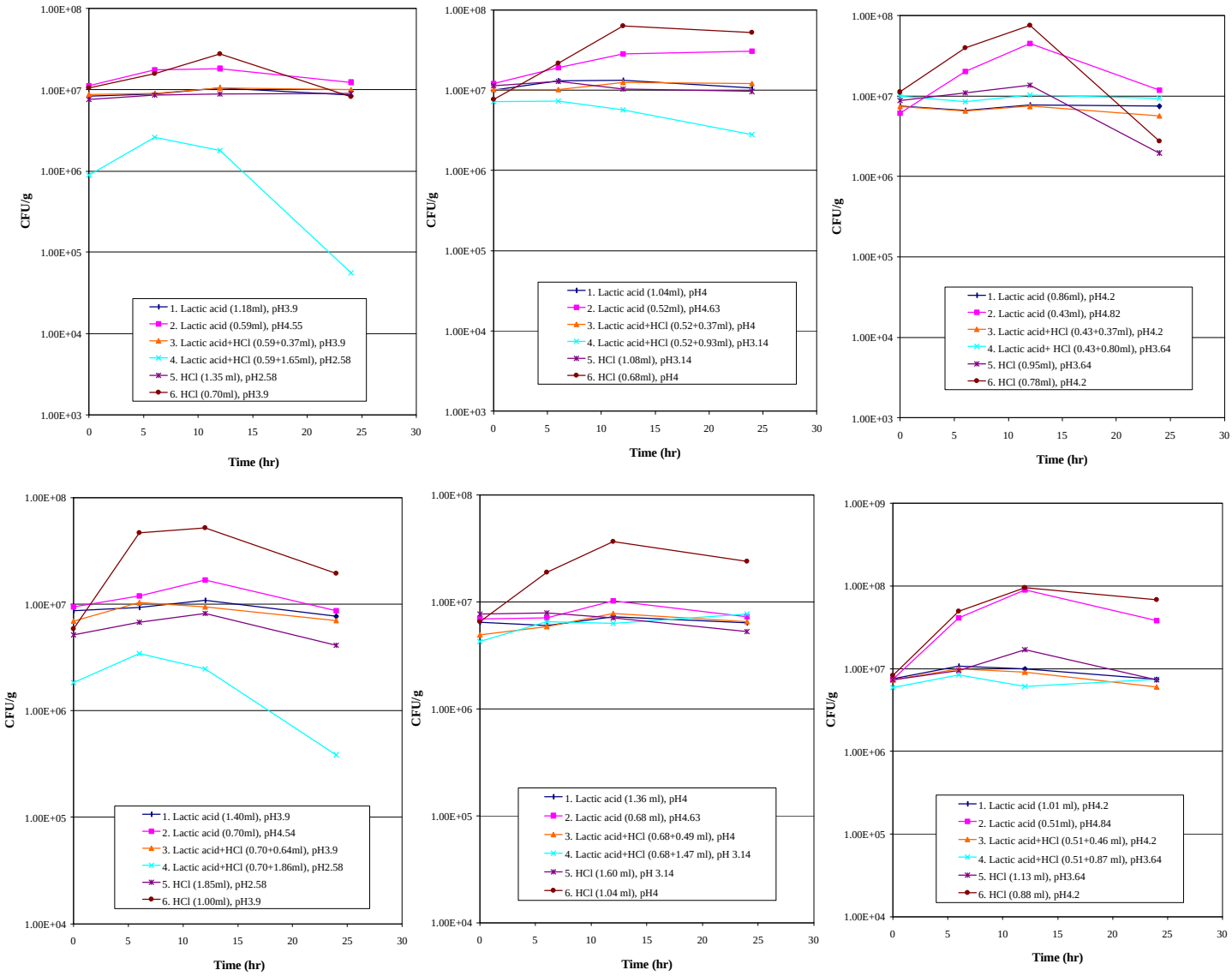

Figure 15. Growth of E. coli in slurry with added lactic acid and HCl to adjust initial pH. Top whole grain wheat slurry, bottom tef slurry.

In an experiment conducted to determine the effects of pH after the fermentation step, final heating temperature, and time on destruction of *E. coli* O157:H7 and *Salmonella tryhimurium* in Lebanon bologna, Kameswar *et al.* 1998 found that fermentation alone reduced populations of both pathogens by <2 log units and heating alone reduced populations of *E. coli* O157:H7 by <3 log units. A combination of fermenting to either pH 5,2 or 4,7, followed by heating at 110°F

(43,3°C) for 20 h, 115°F (46.1°C) for 10 h, or 120°F (48.9°C) for 3 h reduced populations of both pathogens by >7 log units.

Figure 15 show the growth of *E. coli* in fermented gruel with different initial pH adjusted with lactic acid alone, lactic acid and HCl, and HCl alone in the present study. The results show that pH have an inhibitory effect directly on the growth of *E. coli* and the amout of undissociated lactic acid adds to the inhibitory effect on *E. coli.* Note that the amount of undissociated lactic acid is 0,03M, 0,02M and 0,01M at pH 3,9, 4,0 and 4,2, respectively. Further, the effect of acid on *E. coli* up to pH 3,9 was not significant. The use of HCl alone had less inhibitory effect compared to the use of lactic acid alone or both lactic acid and HCl. The different amount of lactic acid at different pH showed to have similar effect on *E. coli* in whole grain tef gruel and whole grain wheat gruel. It seems that the acid itself could not reduce the number of *E. coli* to a safe level suitable for human consumption. There may be other agents which may have had some inhibitory effect on *E. coli.* In addition, the growth of *E. coli* did not show any decrease at 24h in the fermentation without back-slopping either in whole grain wheat or in tef flour slurry.

Leyer *et al*. (1995) reported of survival of acid-adapted and unadapted *E. coli* in lactic acid, during sausage fermentation and in shredded dry salami (pH 5,0) and apple cider (pH 3,4). Acid-adapted cells of *E. coli* O157: H7, showed increased resistance in lactic acid compared to unadapted cells. One of the problems in this connection could be the eventual adaptation of *E. coli* in acidic medium and it is suggested that acid adaptation of *E. coli* is taken into consideration in connection with food challenge studies.

Comments to *E. coli* study

The general trend and characteristics of pH development, the growth of lactobacilli and the number of *E. coli* were similar both in wheat & tef but the initial pH drop was quicker in tef than in wheat. Maybe tef contains more easily fermentable carbohydrates and/or higher amount of natural microflora.

The growth rate of *E. coli* decreased rapidly when the pH was below 4 in all cases. In addition, the rate of decrease in the number of *E. coli* was significantly higher in the fermentations with back-slopping (1% and 10%) than without back-slopping.

pH have an inhibitory effect directly on the growth of *E. coli* in the fermenting slurry as well as pH through its effect on the degree of dissociation of lactic acid adds to the inhibitory effect on *E. coli* in a cumulative manner. However, the acid itself could

not reduce the number of *E. coli* to a safe level. Maybe other agents produced during fermentation also have an effect on the growth of *E. coli*.

Two different temperatures

The temperatures 25°C and 35°C were chosen for the experiments. The lower temperature was chosen since this is the temperature mainly found in Ethiopia. The high altitude at most parts of the country gives this comparatively low temperature for a tropical country, and it is also fairly stable around the year. The higher temperature was chosen, as it is a temperature that often can be found in tropical countries during the hot time of the year.

The pH dropped quicker at the higher temperature.

At 25 C *Lactobacillus plantarum* was the only species found in the tef fermentation, while at the higher temperature other lactobacilli was also found. This indicates that the end product might be different, so even though a higher temperature can give a better food safety, the food item might not be exactly the expected.

Inoculum amount

A high inoculum amount gives a faster entry to the safe area where pathogenic bacteria can not survive. However, the high inoculum also changes the strains involved, and thus the changes on the product provoked by the bacteria will not be the same.

The optimum inoculum amount for fermentation needs to be established using sensoric criteria. The results of this study can be used to estimate the food safety during the process. A higher inoculum amount will make the entry into the safe area come quicker, but since the lactic acid bacteria strains involved changes, the final product will not be the same.

Lactic acid content

The lactic acid production rate at 35°C was higher with the normal inoculum amount than with a high inoculum amount. This could be explained by the fact that these bacteria is continuously in an acid environment, while the bacteria in a normal inoculum gets refreshed for every backslopping.

The content of lactic acid at a certain pH is very much dependent on the raw material.

Buffering effect

Due to buffering agents in the flours, the pH will rise more than expected at the backslopping. The different raw materials had different buffering capacity, indicating that they contained different amounts of buffering agents. The bagerivetemjöl had the lowest buffering capacity, while tef had the highest buffering capacity. An explanation to this can be that the outer parts of the grains contains compounds reacting as buffering agents. Tef has very small seeds which gives a high portion of outer parts, while in bagerivetemjöl the outer parts of the grain has been removed. The difference in buffering capacity is so big that to reach pH 4 you need more than the double amount of lactic acid in tef flour than in bagerivetemjöl.

With less buffering agents a low pH will be reached with less acid produced, and the low pH will then hindering the lactic acid bacteria to continue the production of lactic acid.

A rise in the production rate of lactic acid will secure the food safety quicker, which is desirable under circumstances that other factors like taste and functional properties of the final food also will be considered.

Effect of lactic acid on pathogenic organisms

Lactic acid inhibits many pathogenic bacteria, and the undissociated form of the acid is considered to be the active component (Robinson & Samona, 1992). The amount of undissociated lactic acid depends on both the concentration of lactic acid and the pH. The pKa of lactic acid is 3,86, giving that at pH=4,8 only 10% while at pH=3,86 half of the lactic acid is in the undissociated form (Figure 16). In contrast to the lactate ion, the uncharged undissociated form of lactic acid can penetrate the cell membrane of the bacteria. The cytoplasm of the bacteria has a much higher pH than the surrounding, which provokes the dissociation of the lactic acid molecule inside the bacteria, thus liberating H^+. This will lower the pH of the cytoplasm. The amount of undissociated lactic acid passing through the cell membrane will at a point near pH 4 be to high for the mechanisms in the cell dealing with the regulation of pH in the cytoplasm. The pH inside the bacteria will drop and eventually cause the destruction of the pathogen.

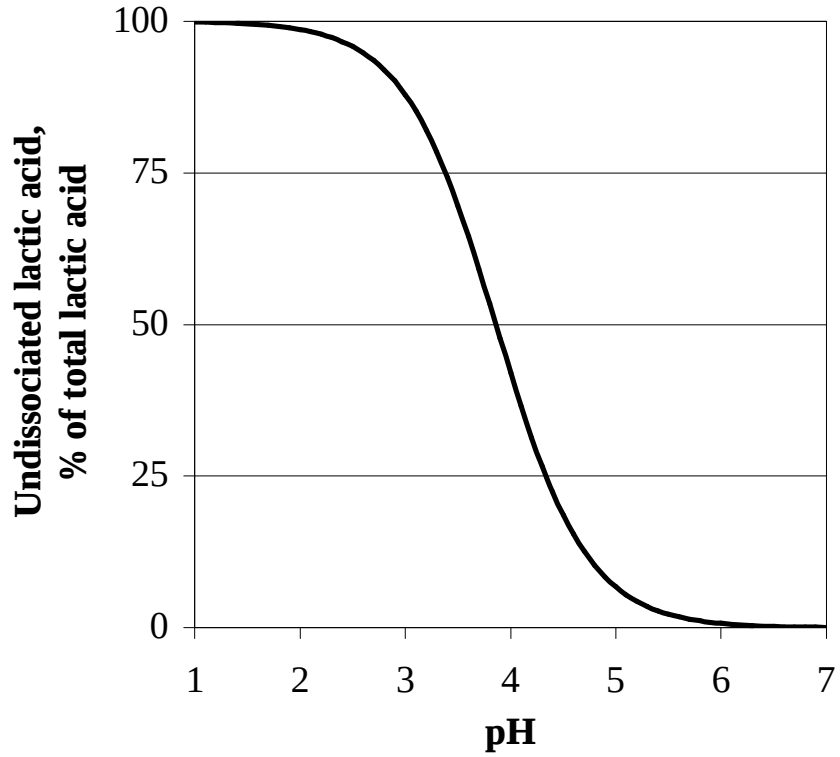

Figure 16. Portion undissociated lactic acid at different pH.

At the “normal” injera fermentation (tef 25°C 1%), it took twelve hours before the lactic acid content reached a level that gave some safety to the product, and another twelve hours to reach high levels of lactic acid (Figure 11). Therefore, the first twelve to twentyfour hours is the vulnerable period of the fermentation, where an infection by pathogenic bacteria can harm the safety of the product.

Regarding pathogenic bacteria, the final product in this type of fermentation, having its normal sourness, can be considered as a safe product (FDA 1992). No pathogens should be able to withstand the environment in the sour slurry for the length of time involved in the process. If the vulnerable period is to be shortened, a solution could be to increase the inoculum amount. But this will probably also change the properties of the product.

Final remarks

Lactic acid fermentation is a well-established method of treating foods. It is used as a household practise in many areas of the world. For most fermentations some kind of starter culture is used. This can be very simple, like using the same container as always, with its microbial flora on the inside walls. It can be made by using

commercially produced starter culture specified for the fermentation. It can also be using a portion from the former fermentation made – backslopping.

Nout (1989) has shown that after a few backsloppings the system can be considered stable. In this trial, three consecutive backsloppings were made before the system was considered stable. The starting fermentation had a slow development as could be expected, while the second fermentation was very quick having active bacteria that has just entered the final static zone. The third fermentation was close to normal backslopping fermentation, and from the forth fermentation the behaviour was consistent.

A rise in the production rate of lactic acid will secure the food safety quicker, which is desirable under circumstances that other factors like taste and functional properties of the final food also will be considered.

The circumstances influence the development of the bacteria in the slurry. Inoculum amount, temperature and raw material influence the types of bacteria found in the fermentation.

Commercial bakery flour showed a comparatively low buffering capacity, less than 0,1 g lactic acid/100 g was needed to reduce pH to <5. Tef, a cereal with very small seeds contributing a larger portion of outer parts of the seeds in the flour, had the highest buffering capacity. In the region pH 6 to 4, approximately double the amount of lactic acid was needed compared to commercial bakery flour in order to attain the same pH. This indicates that at the same pH-level, a fermented food item made of tef, and also of whole grain wheat, probably has a higher food safety than a food item made of commercial bakery flour. Thus, the use of whole grain flour improves not only the nutritional quality, but also the food safety of a fermented food, which is of importance under household conditions of low-income countries.

At normal injera fermentation (tef, 1% backslopping at 25°C) it took 12 h to reach a pH of 4 that could be regarded safe with respect to the prevention of the growth of pathogenic organisms.

The fermentation step in injera production, due to the prolonged (>48 h) processing at a pH <4, should give the product a high food safety regarding pathogenic microorganisms common in food.

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