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Research Paper

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

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ABSTRACT

The effect of fermentation on the production of lactic acid, pH-development an on the growth of *Escherichia coli* strain NG7C in gruels made of whole grain wheat and whole grain tef under different conditions was studied. The results show that the number of *E. coli* decreased rapidly in both 1% and 10% back-slopping quicker than in control that was fermented without back-slopping. The growth of *E. coli* in the gruels was found to depend not only on the amount of lactic acid and pH but also on the amount of undissociated lactic acid both in whole wheat gruel and whole tef gruel. At the lowest pH (3.5) obtained in all fermentation experiments, the number of *E. coli* was found to be reduced to > 6-log-unit. 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. The sample with the highest amount of undissociated lactic acid had the fastest reduction of viable *E. coli* during fermentation.

Keywords: *fermentation, latobacilli,* E. coli, *lactic acid*

Food-borne diseases are a major cause of morbidity and mortality especially among children in low income communities where sanitation and hygiene are of very low standard. Poverty, lack of knowledge, poor methods of food preparation and storage, scarcity of potable water and absence of health facilities also contribute to the case. Hence, the control of food-spoilage organisms and food-borne pathogens is not easily attainable in such communities.

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, diarrhea diseases are the most common illness and have the greatest

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negative impact upon the growth of infants and young children. The causes of diarrhea have traditionally been ascribed to water supply and sanitation. Based on studies reported in the literature , weaning foods prepared under unhygienic conditions are frequently heavily contaminated with pathogens and thus a major role in child mortality is played by combinations of diarrhea diseases, nutrient malabsorption, and malnutrition. Although the genera *Salmonella, Campylobacter, Shigella, Vibrio, Yersinia,* and *Escherichia* are mostly associated with bacterial diarrhoeal diseases, other enterotoxigenic organisms, including *Pseudomonas, Enterobacter, Klebsiella, Serratia, Proteus, Providencia, Aeromonas, Achromobacter,* and *Flavobacterium,* have also been implicated .

Fermentation is an efficient and simple technique of food preservation which can increase the shelf-life and reduce the need for refrigeration. It is less expensive, easily acceptable, adaptable and applicable specially to local households of low income communities.

Traditional lactic acid fermentation of (LAB-fermented) foods use spontaneous fermentation which is usually initiated by the microflora which is originally present in the raw material and which is further selected during the course of fermentation by the physical, chemical and biological conditions of the process is used. The addition of inoculum from a previous batch (back-slopping) is a common practice by which the desired qualities of the product is regulated. By adjusting the environmental factors such as partial or total anaerobiosis, temperature and water activity of the process is controlled to a certain extend both in temperate and tropical regions .

A number of studies on fermentation of foods have demonstrated that it can improve not only the shelf life but also the nutritional qualities such as increased zinc bioavailability and reduced phytate content in fermented cereal-legume mixtures significant reduction of trypsin inhibitors in fermented green beans , improvement of functional properties, digestibility and overall nutritional value of the finished products.

During fermentation lactic acid bacteria can produce a variety of metabolites including lactic and acetic acids which lower pH, that are inhibitory to competing bacteria, including psychrotrophic pathogen. The inhibition by organic acids has been attributed to the protonated form of these acids, which are uncharged and may therefore cross biological membranes. The resulting inhibition of growth may be due to acidification of the cytoplasm and/or accumulation of anions inside the cell . 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).

Nout *et al.* found that the recycling technique can be employed successfully to

enhance the pre-fermentation of soybeans for tempe manufacture, resulting in improved bacteriological safety and acceptability of tempe. In addition, they investigated that there appeared to be no significant difference between their behavior 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 did not play a detectable role. On the other hand, Adams 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. Several papers reported that lactic acid bacteria are able to inhibit growth of pathogenic bacteria such as *Clostridium* in cheese-making , *Yersinia enterocolitica* and some *Enterobacteriaceae* species in yogurt , *Bacillus cereus, Escherichia coli* and *Staphylococcus aureus* in kadhi (an Indian milk-based fermented product).

Benthin S. and Villadsen J. studied the different 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 different inhibitory effects of D- and L-lactic acid have implications for yogurt manufacture and potential for improved food preservation .

This present study was designed to determine the inhibitory action of fermenting whole grain wheat flour slurry and whole grain tef flour slurry under different conditions including back-slopping (1% and 10%) as well as normal control without back-slopping. Furthermore, using lactic acid at lower pH instead of back-slopping technique against encountered food-borne pathogen would be done. The study on fermenting whole grain wheat and whole grain tef flour slurry proposed a model in order to be probably useful in the future studies.

Materials and methods

Whole grain wheat and whole grain tef:

Wheat grains of the Kosack variety was kindly provided by Svalöv/Weibulls AB, Svalöv, Sweden while tef grains (*Eragrostis tef*) variety DZ-01-196 was obtained from the Bio-Diversity Institute of Federal Government of Ethiopia, Addis Ababa, Ethiopia.

Test bacteria

The test bacteria, *Escherichia coli* strain NG7C, was obtained from the division of Medical Microbiology, Lund University. The bacteria was isolated from a child with diarrhea in Papua New Guinea.

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. All chemicals used in this study were of analytical grade.

Sample preparation

The seeds were cleaned and washed first with distilled water and then dried in an oven at 50 °C over night. The dry seeds were then milled using a laboratory sample mill (Tecator, Cyclotec 1093) equiped with a sieve of pore size 1mm.

Fermentation

The flour (300g) was mixed with warm (40 °C) deionized water (600ml) in a 1000ml beaker. To get a well mixed slurry, the water was divided into 3 portions (250,150 and 200ml) and each portion was poured and slightly stirred until flour was thoroughly mixed with the water. The mixture was allowed to ferment spontaneously at 25 °C in a water bath for 3 and a half days (84 hours) without stirring.

Before the experiment was started, the fermentation process was carried out with repeated (3 to 4 times) back-slopping until a stable final pH of 3.5 was registered. For backslopping, about 1% gruel (~10g) of a previous fermentation was used to start the next fermentation.

Measurement of pH

The pH of the slurry during fermentation was measured at different intervals by dipping an electrode connected to a pH meter (Orion, Model 8103 Ross) at every 1cm from the top surface of the mixture.

Measurement of the growth of lactic acid bacteria

For determination of the number of lactic acid bacteria in the fermenting slurry, samples (~5 ml) were taken at different intervals from the top (1cm below the surface) and bottom (1cm above) of the mixture. The samples (~1g) were diluted with saline solution (9 ml) for pour plate count on Rogosa agar (Oxoid, PM221) medium. The incubation at 37^0 was done for a period of 3days in an anaerobic jar.

Measurement of the growth of *E. coli*

For determination of the number of *E. coli* in the fermenting slurry, samples (~5 ml) were taken at different intervals from the top (1cm below the surface) and bottom (1cm above) of the mixture. The samples (~1g) were diluted with saline solution (9 ml) for pour plate count on violet red bile glucose agar (Oxoid, CM485). The incubation at 37 °C was done in an incubator for a period of 18-24 hours.

Design of experiments

The study contains three set 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 is any difference between top surface and bottom layer.

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

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

The details of the layout of the experiments are shown in Table I.

Results and discussion

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 in Table 3 and Figures 1, 3 and 5 . 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. 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 Figures 1-6. 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

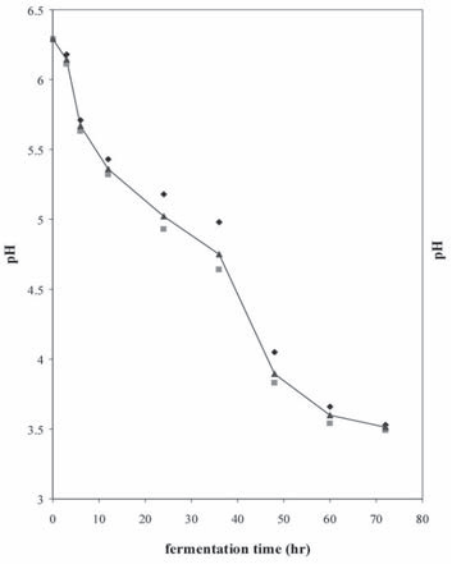

Figure 1: pH development in fermenting whole grain wheat flour slurry without back-slopping

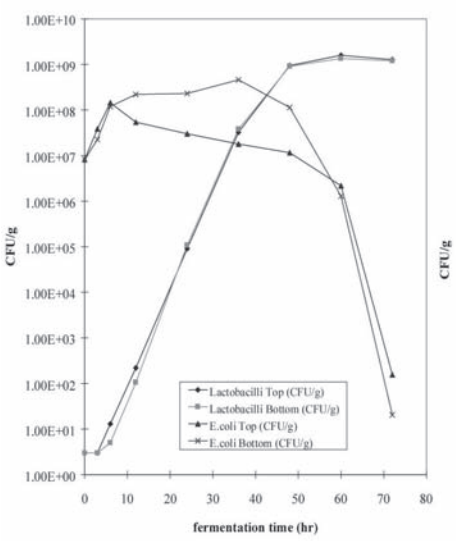

Figure 2: Growth of Lactobacilli and E.coli in fermenting whole grain wheat flour slurry without back-slopping

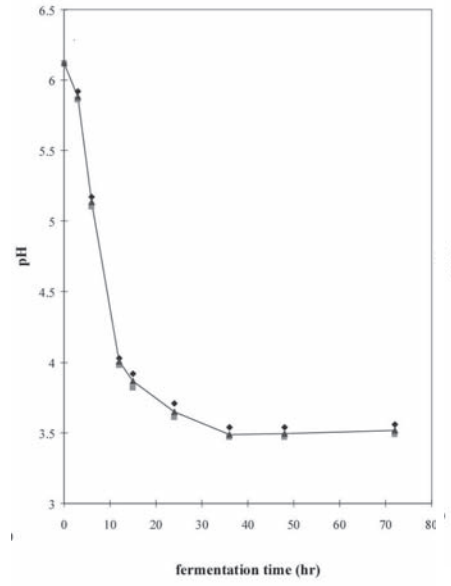

Figure 3: pH development in fermenting whole grain wheat flour slurry with 1% back-slopping

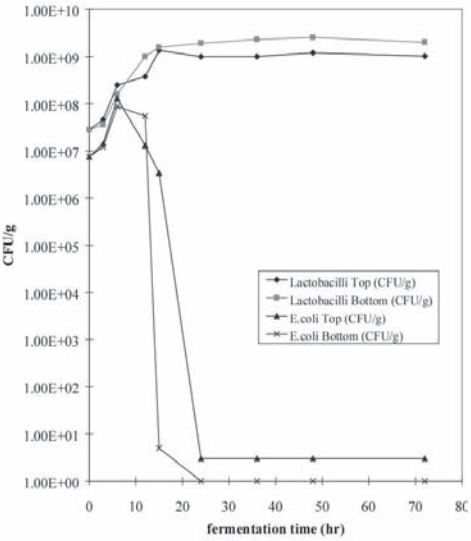

Figure 4: Growth of Lactobacilli and E.coli in fermenting whole grain wheat flour slurry with 1% back-slopping

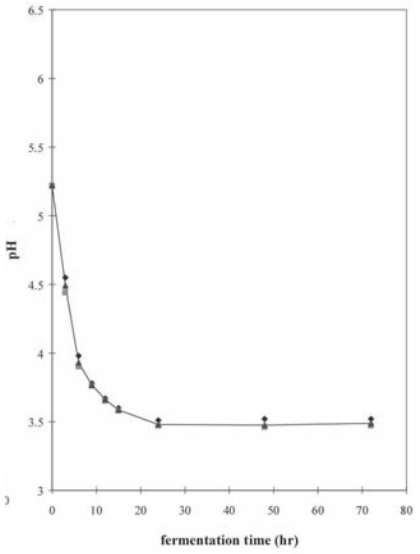

Figure 5: pH development in fermenting whole grain wheat flour slurry with 10%back-slopping

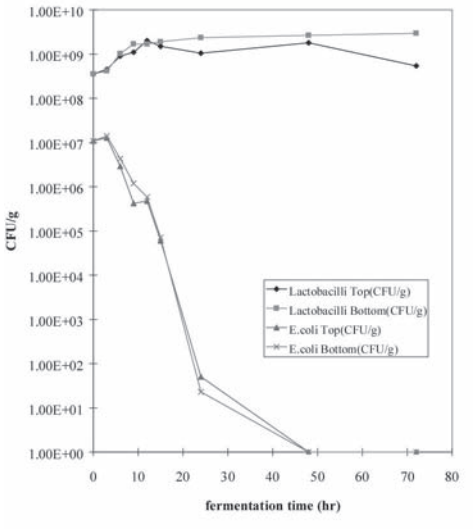

Figure 6: Growth of Lactobacilli and E.coli in fermenting whole grain wheat flour slurry with 10% back-slopping

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 (Figures. 2, 4, 6, 8, 10 and12). 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. 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

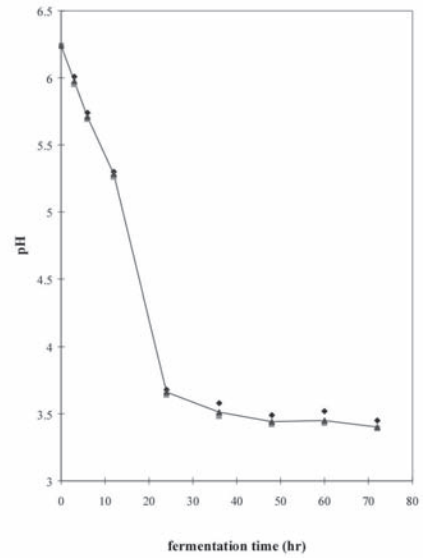

Figure 7: pH development in fermenting whole grain Tef flour slurry without back-slopping

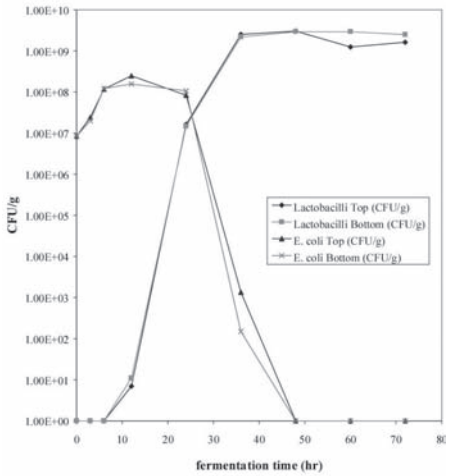

Figure 8: Growth of lactobacilli and *E. coli* in fermenting whole grain tef flour slurry without back-slopping

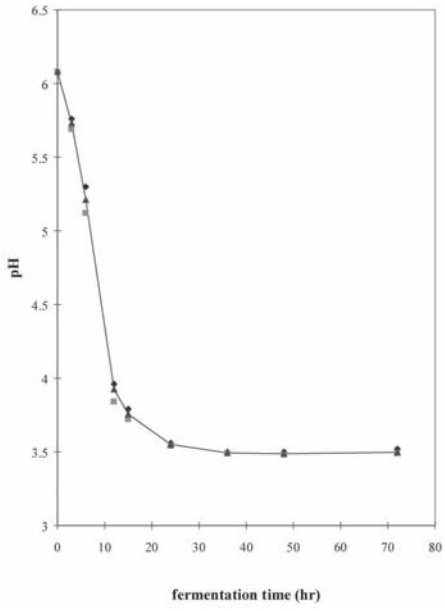

Figure 9: pH development in fermenting whole grain Tef flour slurry with 1% back-slopping

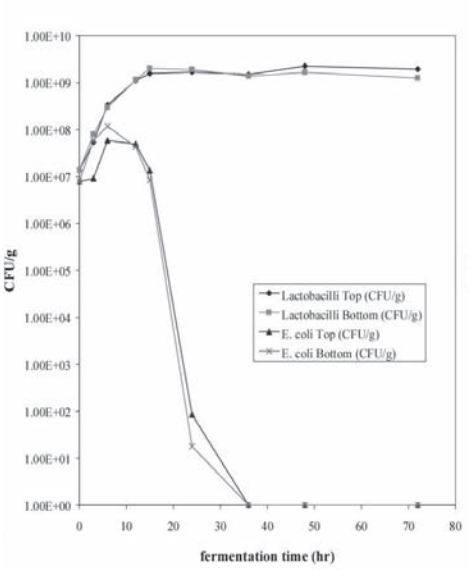

Figure 10: Growth of lactobacilli and E.coli in fermenting whole grain tef flour slurry with 1% back-slopping

growth rate of lactobacilli in fermentation experiments show strong relation to with drop in pH both in wheat flour and in tef.

In the Figures 13-18 the survival rate of *E. coli* in fermenting slurry at different conditions of pH, 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 coil* 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 hr or till the pH dropped to 4.7. Thereafter, growth was inhibited and decreases in

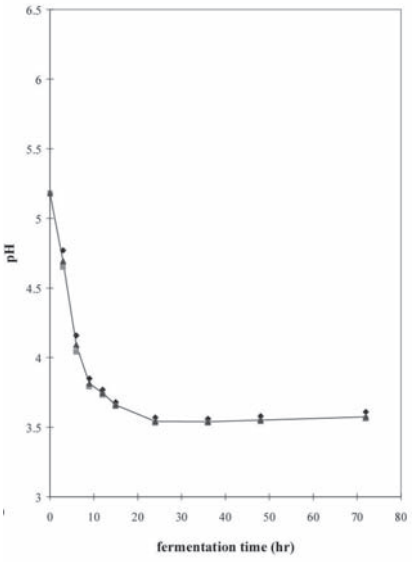

Figure 11: pH development in fermenting whole grain Tef flour slurry with 10% back-slopping

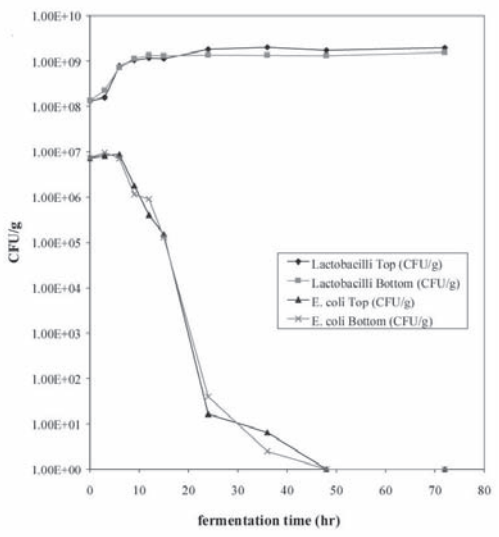

Figure 12: Growth of lactobacilli and E.coli in fermenting whole grain tef flour slurry with 10% back-slopping

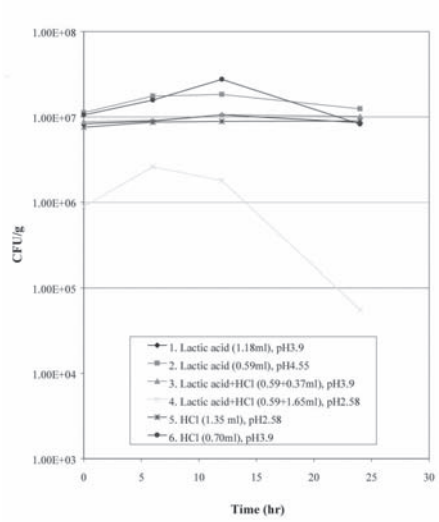

Figure 13: Growth of E.coli in whole grain wheat flour slurry with added lactic acid and HCl to adjust the initial pH

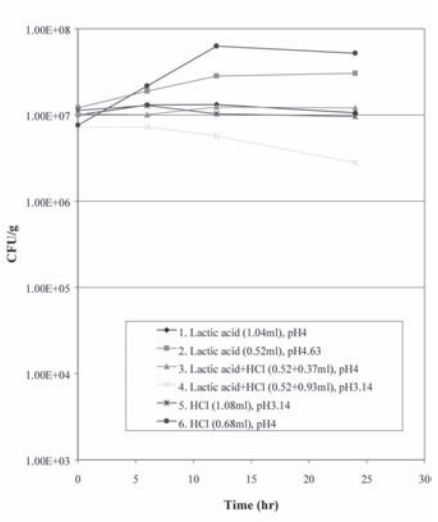

Figure 14: Growth of E.coli in whole grain wheat flour slurry with added lactic acid and HCl to adjust the initial pH

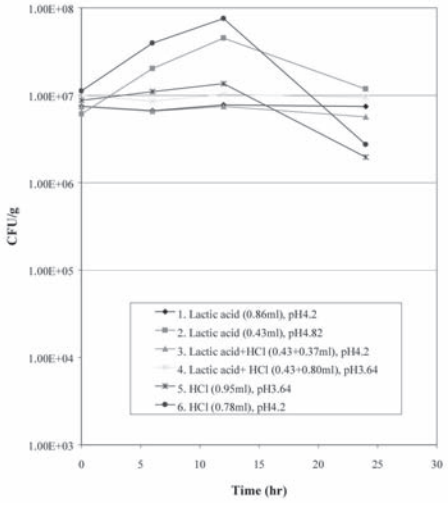

Figure 15: Growth of E.coli in whole grain wheat flour slurry with added lactic acid and HCl to adjust the initial pH

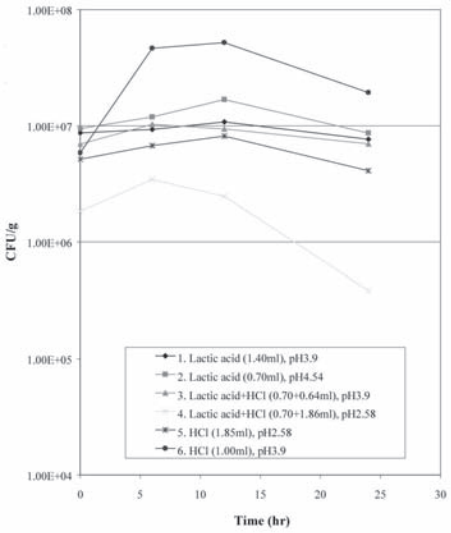

Figure 16: Growth of E.coli in whole grain tef flour slurry with added lactic and HCl to adjust the initial pH

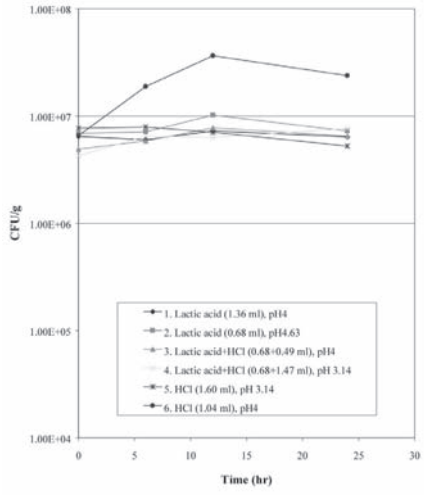

Figure 17: Growth of E.coli in whole grain tef flour slurry with added lactic acid and HCl to adjust the initial pH

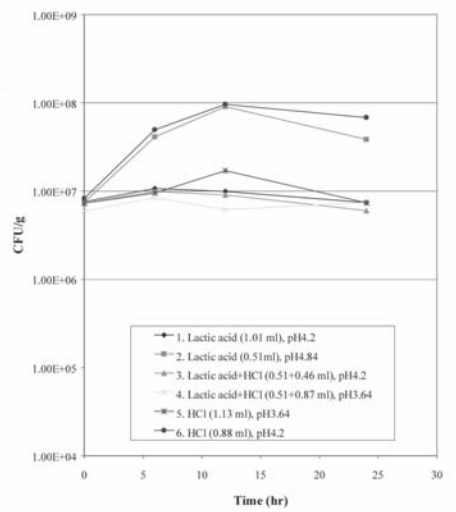

Figure 18: Grwoth of E.coli in whole grain tef flour slurry with added lactic acid and HCl to adjust the initial pH

population were apparent.

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

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

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

Figures 13-18 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 amount 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 grain tef flour slurry.

Some earlier studies were found to show that the inhibitory effect of lactic acid bacteria could be due to some substances other than lactic acid. Brkic B. *et al*. (1995) used the agar spot test and disc assay method to study the inhibitory effects of lactic acid bacteria on the growth of enteropathogenic microorganisms. Cell-free supernatants of the lactic acid bacteria was found to inhibit growth of *Staphylococcus aureus, Salmonella mumum, Escherichia coli, Bacillus cereus* and *B. subtilis* which shows that inhibitory substances other than lactic acid were present in supernatant preparations. In addition, *Lactobacillus sake* CTC494, isolated from a naturally fermented sausage was found to produce an antibacterial agent active against selected strains of *Listeria monocytogenes* and *L. innocua*. This agent was identified as a bacteriocin and designated to be sakacin-K showing effective inhibition of *Listeria* growth according to Hugas.

Leyer 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.

Conclusion

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. May be 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. May be other agents produced during fermentation have also an effect on the growth of *E. coli.*

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