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Title:

Survival of Gram-negative bacterial pathogens during maize fermentation and in fermented maize weaning food

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SUMMARY

AIMS: The behaviour of some important food borne bacterial pathogens was studied during maize fermentation and in two fermented maize products.

METHODS AND RESULTS: Five *Salmonella* serotypes, two *Shigella flexneri,* one *Shigella dysenteriae* and three VTEC serotypes, one each of enteropathogenic (EPEC) and entero- aggregative (EAggEC) *Escherichia coli* were inoculated into maize steep water, maize dough, koko and kenkey water, respectively, at a concentration of $10^6 - 10^7$ cfu ml^{-1} at ambient temperature of about 28 °C. Enumeration was carried out using selective and nonselective media. Almost all the bacteria survived in steep water for 48 h although there was a reduction in pH. No *Salmonella* serotypes were detected in dough after 48 h of maize fermentation, when pH fell below 4.0. Some resistant *Shigella* and *E. coli* strains survived in the dough after 48 h, but reduced in numbers by more than 3.4 log units. None of the salmonellae was detected after 24 h in koko (pH 3.6) and kenkey water (pH 3.6). *Shigella* and *E. coli* were inactivated in kenkey water after 24 h. However, *Sh. flexneri* II and four *E. coli* survived in koko for 48 h with less than 2 log reductions. Greater numbers of survivors were obtained with non-selective than with a newly developed selective medium designed to recover Gram-negative bacterial pathogens.

CONCLUSION: Significant differences in the ability to survive maize fermentation and in koko and kenkey water were observed for the species of *Salmonella, Shigella* and *E. coli.* The most resistant were *Shigella* and *E. coli.*

SIGNIFICANCE AND IMPACT OF THE STUDY: The risk posed by survival of some acid resistant *Shigella* and *E. coli* strains was demonstrated for fermented maize products particularly koko.

KEY WORDS: maize fermentation; koko, kenkey water; *Salmonella*; *Shigella*; *Escherichia coli*; survival.

RUNNING TITLE: Survival of Gram-negative pathogens in fermented maize products.

INTRODUCTION

Diarrhoea is one of the common illnesses in children, and one of the major causes of infant and childhood mortality in developing countries. It was estimated that the number of deaths among under five year olds was 1.5 million in 1999 (Victora *et al.* 2000). Diarrhoea in developing countries is poorly reported in terms of incidences and causative organisms. However, it is often caused by consumption of contaminated food with major Gram-negative bacterial pathogens, in particular *Escherichia coli, Salmonella, Shigella, Campylobacter and Vibrio cholerae* (Black *et al.* 1982 and 1989; Motarjemi *et al.* 1993).

In Ghana, the traditional weaning food is a fermented maize porridge called koko. It is made from spontaneously fermented maize dough. Traditionally, fermented maize dough is made by steeping cleaned whole maize grains in water for 24 – 48 h after which the steep water is decanted and the maize milled into a smooth meal. The meal is mixed with water into a dough which is left to ferment spontaneously for 24 – 72 h. During fermentation of the dough, a succession of naturally occurring microorganisms results in a population dominated by lactic acid bacteria and yeast (Halm *et al.* 1993; Jespersen *et al.* 1994; Hayford *et al.* 1999; Hayford and Jakobsen 1999; Hayford and Jespersen 1999). At the end of the fermentation period of 48 h, the dough has a pH of 3.7 – 3.9 and a titratable acidity of 0.8 –1.4% calculated as lactic acid with a volatile non-volatile acid ratio of about 0.16 (Halm *et al.* 1993). Koko is prepared by mixing fermented maize dough with water in the ratio of 1:5 to form a slurry which is boiled with continuous stirring to produce a gelatinous porridge. Koko has a pH of about 3.4 – 3.7 and a titratable acidity of 0.3 – 0.4% calculated as lactic acid. Koko for infant feeding is normally prepared in bulk in the morning for use at intervals during the day. It is often stored at ambient temperature under conditions where contaminations can easily occur. Another important product from fermented maize dough is kenkey. Kenkey is a sour tasting stiff porridge or dumpling made from a mixture of raw fermented maize dough and a partially

cooked portion of dough. The mixture known as *aflata* is moulded into balls, wrapped with maize husks or plantain leaves and cooked for 3 hours into kenkey. The resultant cook water known as 'kenkey water' is used as a rehydration fluid in Ghana for treatment of diarrhoea (Yartey *et al.* 1993). Kenkey water has a pH of about 3.4 – 3.7 and a titratable acidity of 0.4 – 0.6% as lactic acid. Because of the labour involved and time spent in preparing kenkey, it is not produced at the household level but obtained from commercial processors. The kenkey water is also collected from the commercial processors as a by-product from kenkey production. It can be kept under refrigeration and used over a period of 24 h or more. But among the poor, who have no access to refrigeration, it is usually stored at ambient temperatures and under unhygienic conditions with a high risk of contamination from the household environment. Many researchers (Mensah *et al.* 1988 and 1991; Nout *et al.* 1989a and 1989b; Mbugua and Njenga, 1991; Simango and Rukure; 1992, Svanberg *et al.* 1992; Kingamkono *et al.* 1994 and 1995; Annan-Prah and Agyeman, 1997) have established the inability of various pathogens to survive or grow in lactic fermented African foods. Mensah *et al.* 1991 simulated the unhygienic conditions of a typical rural community in a developing country by inoculating fermented maize dough porridge (koko) and their unfermented controls with *Shigella flexneri* and enterotoxigenic *Escherichia coli* (ETEC). The antimicrobial effects of the different processes involved in the preparation of fermented maize dough porridge were also assessed. They showed that even though the soaking process reduced the pH no antimicrobial effect against *Shigella* and ETEC was noted, but fermented maize dough inhibited half of the strains tested. They also found that cooking the fermented dough into koko reduced the antimicrobial effect considerably despite the low pH. The antimicrobial effect of kenkey water has however not been extensively studied.

The purpose of the present study, was to assess the survival of a wide range of strains of diarrheagenic bacteria and strains of reported high acid and low pH tolerance including

verotoxigenic *E. coli* (VTEC) serotype O157: H7, enteropathogenic (EPEC) and enteroaggregative (EAggEC) *E. coli* in addition to *Sh. flexneri* and *Salmonella* serotypes during maize steeping and fermentation and in koko and kenkey water. The study also considered recovery of acid stressed cells by application of an enriched non-selective medium in comparison with a newly developed selective medium for Gram-negative bacteria.

MATERIALS AND METHODS

Microorganisms and inoculation experiments

The bacterial pathogens included in the investigations are listed in Table 1. All organisms had been isolated and characterized biochemically and for virulence genes at the State Serum Institute, Denmark (unpublished information). During the experiments the bacteria were grown at 37 °C and maintained at 4 °C on slopes of nutrient agar (Difco, Detroit, USA). *Escherichia coli* K12 (MG1655), a wild type K12 strain. (Guyer *et al.* 1981) was maintained as a frozen stock culture in 20% (v/v) glycerol.

Pure cultures of the individual pathogens were grown on nutrient agar plates at 37 °C for 16 h. The colonies were washed off with sterile diluent (pH 7.2; 8 g l^{-1} NaCl; 1 g l^{-1} Bacto Peptone (Oxoid, Hampshire, UK) to give approximately 10^{10} colony forming units (cfu) ml^{-1} as determined by spread plate count on nutrient agar following incubation at 37 °C for 24 h. The required volume of freshly prepared bacterial suspension was added to maize steep water, maize dough, koko and kenkey water, respectively, at the beginning of each experiment to give approximately 10^7 cfu g^{-1}. For each experiment described in the following, a non-inoculated batch served as control. For each test bacterium, the experiments were carried out twice on two separate occasions. Determination of viable bacteria was carried out immediately after inoculation and after 4, 8, 24 and 48 h of incubation as described below.

Maize steeping, dough fermentation, kenkey water and koko

All trials were carried out according to local practices at the pilot plant of Food Research Institute, Accra. For maize steeping, five kg maize was added to 7.5 l of water in a 10 l plastic bucket. The pathogens were added and the mixture stirred manually and left at ambient temperature (about 28°C) for 48 h. For dough fermentation, steeped maize was milled in a disc attrition mill (Disc attrition mill, Rajan Universal, Madras, India) to an average particle size of about 0.3 mm. The pathogens were added to 300 ml potable tap water used to knead 1 kg of the maize meal into dough. The dough was thoroughly kneaded to ensure an even distribution of the pathogen and left to ferment spontaneously at ambient temperature for 48 h.

Kenkey water was obtained from cooking 2 kg of fermented maize dough into kenkey by the following method. One kg of fermented dough was made into a slurry by adding 0.75 l of water and partially cooked into a stiff porridge with 33 g cooking salt (NaCl) added. The partially cooked porridge was then mixed with one kg of uncooked fermented dough and moulded into balls of about 300 g, wrapped with maize husks and cooked in 2.8 l water for 3.5 h following the traditional method. After cooking, the balls of kenkey were taken out and the cook water (kenkey water) was collected and allowed to cool at ambient temperature. Three hundred gram portions of kenkey water were aseptically distributed into sterile stomacher bags. The pathogens were added and the kenkey water homogenized in a stomacher (Lab Blender, Model 4001, Seward Medical, London, England) at normal speed for 30 s.

For preparation of koko, one kg of fermented maize dough was made into slurry with five litres of water, heated until boiling and allowed to cook for 20 min with constant stirring. The koko was left to cool to ambient temperature and aseptically distributed in 300 g portions

into sterile stomacher bags. The pathogens were added and the koko homogenized as described for kenkey water.

The pH of individual batches of koko and kenkey water were adjusted to pH 6.0 with a 1 mol l^{-1} NaOH solution and aseptically distributed in 300 g portions into sterile stomacher bags. Each product was then inoculated with pathogens and homogenized as described above.

pH and titratable acidity determination

The pH of all samples was determined with a pH meter (pH M92, Radiometer, Copenhagen, Denmark). For solid dough samples, 40 g were homogenized with 40 ml of distilled water in a stomacher as described above before pH determination.

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

Enumeration of pathogens

For fermenting dough samples, the surface layer was removed aseptically and 10 g sample was taken. For liquid samples, 10 g samples were taken after thorough mixing under aseptic conditions of maize steep water, koko and kenkey water, respectively, by stirring. Each sample was then homogenized as described earlier with 90 ml of diluent (0.1% peptone (Bacto), 0.8% NaCl, pH 7.2). Serial tenfold dilutions were prepared in diluents and 0.1 ml of appropriate dilutions were spread plated onto Tryptone Soy Yeast Extract Agar (TSYEA) plus 0.6% Yeast Extract (Oxoid)) and State Serum Institute (SSI) enteric medium (SSI enteric medium, State Serum Institute, Copenhagen Denmark) as described by Blom *et al.* 1999.

The agar plates were incubated at 37 °C and typical colonies were counted after 24 and 48 h. On SSI enteric medium, salmonellas appear with a black precipitate located centrally and deeply in the more anaerobic parts of the colony, *Shigella* species, (except *Sh. sonnei*), appear as flat irregular translucent colonies with a slightly pinkish centre. *Escherichia coli* appear as red colonies due to lactate production. The identities of the test pathogens were confirmed in slide agglutination tests using specific *Salmonella, Shigella and E. coli* commercial antisera (*Salmonella* and *E. coli* antisera were obtained from The State Serum Institute, Denmark; *Shigella* antisera were obtained from Denka Seiken Co. Ltd. Tokyo, Japan). When growth of the test organisms was not observed after direct culture on the agar plates, resuscitation of 1 g in 9 ml of Tryptone Soy Yeast Extract Broth (TSYEB) was carried out at 37 °C for 20 h followed by surface streaking onto SSI enteric medium and incubation at 37 °C for 24 h for *Shigella* and *E. coli*. For the salmonellas, 25 g samples were pre-enriched in 225 ml Buffered Peptone Water (BPW, Oxoid) at 37 °C for 21 ± 3 h followed by selective enrichment of 0.1 ml BPW culture in 10 ml Rappaport Vassiliadis medium (RV, Oxoid) at 41.5 ± 0.5 °C for 48 h with subsequent surface streaking onto Xylose Lysine Deoxycholate Agar (XLD, Oxoid) and SSI enteric medium agar plates.

Data analysis

The number of colony forming units of bacteria per gram of food was expressed as a logarithm to the base ten ($\log_{10}$ cfu g^{-1}). Means of log bacterial numbers were calculated from two experimental replications. Statistical data analysis was performed using the General Linear Models procedure of the Statistical Analysis System version 8.1 statistical programme (SAS Institute, Inc., Cary, NC, USA). The results are reported as significant with a *P*-value of less than or equal to 0.05.

RESULTS

Survival of pathogens during maize steeping and maize dough fermentation

The pH development and changes in bacterial numbers during maize steeping and maize dough fermentation are shown in Tables 2, 3 and 4. The pH of steep water fell to pH 4.4 - 5.1 after 48 h of steeping (Table 2). Three *Salmonella* serotypes i.e. *Salm. stanleyville*, *Salm. typhi* and *Salm. typhimurium* showed reduction in numbers of 1 to 2 $\log_{10}$ cfu ml^{-1} whilst there was no significant reduction in numbers of *Salm. durban* and *Salm. enteritidis* after 48 h of inoculation into maize steep water. During maize fermentation, the initial pH of dough decreased to 4.4 – 4.9 after 8 h of fermentation. During this period, only *Salm. enteritidis* and *Salm. stanleyville* were significantly reduced ($P < 0.0001$). However, after 24 h of fermentation when the pH decreased below 4.0, significant reductions in numbers were observed for all salmonellas and only *Salm. stanleyville* survived, but was not detected in 25 g of dough after 48 h of fermentation.

None of the three shigellas showed significant reduction in numbers during maize steeping (Table 3). They showed no significant reductions in numbers after 8 h of dough fermentation but numbers decreased significantly after 24 h and viable bacteria were not detected in 1 g after 48 h (Table 3).

Like the shigellas, there was no significant reduction in *E. coli* numbers after 48 h of inoculation into maize steep water (Table 4). Table 4 also shows that *E. coli* serotypes survived longer in the fermenting dough than the salmonellas (Table 2) and shigellas (Table 3) although reductions in pH were similar. During dough fermentation, significant reductions in numbers of *E.coli* were seen only after 24 h. *E. coli* strains O157 (VTEC I) and O111 (EPEC) were not detected in dough after 24 and 48 h fermentation, respectively. But *E. coli* O26 (VTEC), O157 (VTEC II) and EAggEC survived in the dough for 48 h having reduced by about 3 to 4 $\log_{10}$ cfu g^{-1} of the original concentration.

None of the test bacteria was detected from the non-inoculated controls for maize steeping and fermentation experiments (results not shown).

Koko and kenkey water

The pH of batches of koko and kenkey water used for the survival studies was about 3.6, and only minor changes were observed during the 48 h period of the experiments. (Tables 5, 6 and 7). The titratable acidity expressed as lactic acid, in koko and kenkey water used in the inoculation experiments were 0.3 – 0.4% and 0.4 – 6% (w/v), respectively (data not shown). All the *Salmonella* spp. were inactivated within 24 h of inoculation into both koko and kenkey water (Table 5). None of the *Shigella* strains was detected in kenkey water (Table 6) after 48 h of inoculation, but *Sh. flexneri* strain 2a II survived in koko for 48 h. Table 7 shows the survival of 5 pathogenic *E coli* strains and 2 laboratory strains tested in koko and kenkey water. Four strains i.e. O157 (VTEC II), O26 (VTEC), O3 (EAggEC) and one laboratory strain *E. coli* K12, survived in numbers of more than 10^5 cfu ml^{-1} after 48 h of inoculation into koko. None of the *E coli* strains was detected in kenkey water after 48 h of inoculation.

Effect of selective and non-selective media on recovery of pathogens from koko

Bacterial numbers of *Salmonella, Shigella* and *E. coli* serotypes recovered from inoculated koko on SSI enteric medium (selective) and TSYEA (non-selective) are listed in Table 8. Generally, but not always significantly, more organisms were recovered from direct plating on TSYEA than on the selective SSI enteric medium for all the species tested. A change with time was observed. As storage time increased, the difference between the numbers of organisms detected using selective medium and non-selective medium increased. Up to and more than 3 $\log_{10}$ cfu g^{-1}differences between counts on SSI enteric medium and TSYEA were observed in the survival studies with koko.

pH neutralization experiments and pathogen survival

When the pH of koko and kenkey water was adjusted to pH 6 by addition of NaOH, the pathogens survived in the products for 48 h with no significant decrease in numbers counted on TSYEA over the 48 h period (results not shown). All the uninoculated portions of koko and kenkey water were assayed and found negative for all the pathogens tested (data not shown).

DISCUSSION

The pathogens were inactivated in the maize steeping and fermentation processes and in koko and kenkey water to various degrees varying between and within genera and species. In general, the *Salmonella* strains were the most sensitive in all the survival experiments. The pathogens were not inactivated in the maize steep water except for some of the *Salmonella* species that showed ≤ 2 $\log_{10}$ reductions after 48 h. *Salmonella* species were also strongly inactivated within 24 h of inoculation into dough, kenkey water and koko. The strains of *Shigella flexneri* and *E. coli* were more resistant than *Salmonella.* In general they were more inactivated in dough fermentation and kenkey water than in koko. The most resistant *Shigella* and *E.coli* strains survived in koko for up to 48 h. Of the three VTEC O157 strains tested, one was very sensitive whilst two were more resistant. One of the two non-pathogenic *E. coli* strains tested in koko and kenkey water, *E. coli* K12 reacted like the resistant pathogenic strains whilst *E. coli* M23, which is reported, to react more like the virulent *E. coli* O157 strains (Shadbolt *et al.* 1999) was more sensitive.

The pH of koko (3.64 ± 0.01) and kenkey water (3.60 ± 0.02) used in these experiments was lower than that of fermented dough (3.85 ± 0.10), but the pathogens were more inhibited during dough fermentation and in kenkey water than in koko. This could be

explained by the differences in amounts of organic acids i.e. lactic and acetic acids (Halm *et al.* 1993; Plahar and Leung 1982, Olsen *et al.* 1995) in the products. Cooking of maize dough into koko and kenkey water could cause the removal of volatile compounds e.g. acetic acid. The dilution of acids in koko by addition of five parts water to dough could also play a role. The NaCl content of about 6% in kenkey water could enhance the inhibitory activity on the pathogens. Salt is not normally added to koko.

A range of antimicrobial substances is produced by lactic acid bacteria namely, bacteriocins, carbon dioxide, hydrogen peroxide, ethanol, diacetyl, reuterin, etc., but the production of organic acids and the consequent decrease in pH in a previous study (Olsen *et al.* 1995) was found to be the most important factor controlling microbial contaminants in fermented maize. It was found that more than half of the *Lactobacillus plantarum* and practically all the *Lactobacillus fermentum* strains isolated from various stages of maize fermentation, inhibited other Gram-positive and Gram-negative bacteria including *Staphylococcus carnosus, Staphylococcus caseolyticus, Enterobacter cloacae, Klebsiella pneumoniae* and *Proteus mirabilis* in agar diffusion tests (Olsen *et al.* 1995). Studies of the mechanism of inhibition for selected isolates, which showed pronounced inhibition against the Gram-positive and Gram-negative bacteria, showed that for 70% of the isolates the inhibitory effect was partly or fully eliminated by pH neutralization (Olsen *et al.* 1995). In the present investigations, all the pathogens survived when the pH of koko and kenkey water was adjusted to pH 6.0, indicating the major role of low pH in addition to the effect of organic acids in the products. Nout *et al.* 1989a also established that the inhibition of several intestinal pathogenic bacteria i.e. *Salmonella typhimurium, Yersinia enterocolitica, Escherichia coli, Citrobacter and Enterobacter cloacae* by sour sorghum and pigeon pea porridge, was mainly due to the presence of lactic and acetic acid at reduced pH.

The use of selective agar underestimated the numbers of the test organisms recovered from koko as compared to the recovery from non-selective medium suggesting the presence of sub lethally injured cells. A number of investigations have been carried out on the survival of some pathogenic bacteria in lactic fermented African cereal foods (Mensah *et al.* 1988 and 1991; Nout *et al.* 1989a and 1989b; Mbugua and Njenga, 1991; Simango and Rukure; 1992, Svanberg *et al.* 1992; Kingamkono *et al.* 1994 and 1995; Annan-Prah and Agyeman, 1997). Some of these studies used only selective media, which may have underestimated the numbers of surviving cells. The ability to detect sub lethally injured cells is particularly important due to the fact that stressed cells retain their virulence and are still considered to be a hazard (McCarthy *et al.* 1998). This is especially important in the case of *E. coli* (VTEC) O157: H7 that has a low infective dose (Doyle *et al.* 1997).

In agreement with other investigations, the present studies have shown that koko and kenkey water of pH below 4.0 made with lactic fermented maize dough have pronounced anti-microbial properties through which they might contribute to the reduction of food borne infection. However, the risk indicated by the lack of effect on some *E coli* strains for up to 24 - 48 h in koko has not been considered in previous studies. Not only the need for adequate acidification to obtain the desired pH of below 4.0 in fermented products, but also the risk involved in keeping the koko and the kenkey water for 24 h or more should be emphasized. There are instances of poor fermentation when the desired pH and acidity are not attained in fermented dough within the normal 48 h of fermentation (unpublished results). Therefore unless there is proper control of the fermentation process, products made from such doughs in particular will pose a health risk upon post processing contamination. It will be obvious to apply the Hazard Analysis Critical Control Point (HACCP) system to ensure safety of fermented products. It is also important that the monitoring of Critical Control Point (CCP) in HACCP

system for fermented products should not be limited to pH. Proper storage and handling of the fermented products to prevent contamination should be ensured by defined guidelines.

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Table 1 Bacterial enteric pathogens used in the study

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

Table 2 pH development and changes in numbers of *Salmonella* inoculated into maize steep water and maize dough incubated at ambient temperature of about 28° C and plated on State Serum Institute (SSI) Enteric Medium. The results are shown as the mean of two independent experiments carried out in duplicate

Time after inoculation (h)	Maize steep water pH	Maize steep water Log_{10} CFU ml^{-1}	Maize dough pH	Maize dough Log_{10} CFU g^{-1}
Salm. durban				
0	6.30 a	7.43 a	6.00 a	6.73 a
4	5.65 b	7.31 a	5.00 b	6.25 a
8	5.00 c	7.46 a	4.50 c	6.25 a
24	4.89 c	8.29 a	4.20 d	_ *
48	5.10 c	7.90 a	3.80 e	_
Salm. enteritidis				
0	6.00 a	6.45 b	5.80 a	6.97 a
4	5.25 b	6.58 b	5.51 a	6.54 b
8	4.55 c	7.18 a b	4.47 b	5.76 c
24	4.50 c	6.51 b	3.92 c	_
48	4.60 c	7.92 a	3.96 b c	_
Salm. stanleyville				
0	6.50 a	7.53 a	6.40 a	7.25 a
4	5.25 b	7.42 a	6.20 b	7.24 a
8	4.80 c	7.20 a	4.90 c	6.78 b
24	4.55 c d	6.67 b	4.20 d	3.50 c
48	4.35 d	6.59 b	3.80 e	_
Salm. typhi				
0	6.06 a	8.40 a	6.14 a	7.29 a
4	5.03 b	7.31 b	5.73 b	6.80 b
8	4.85 b	6.34 c	4.45 c	7.03 a b
24	4.55 c	6.50 c	4.32 c	_
48	4.40 c	6.35 c	3.90 d	_
Salm. typhimurium				
0	5.63 a	7.43 a b	6.10 a	7.59 a b
4	4.69 b	7.19 b	5.62 b	7.91 a
8	4.53 b c	7.22 b	4.35 c	7.42 b
24	4.15 d	7.83 a	3.83 d	_
48	4.40 c	5.35 c	3.72 d	_

* Not detected in 25 g after resuscitation in buffered peptone water, selective enrichment in Rappaport Vassiliadis (RV) medium with subsequent streaking onto Xylose Lysine Deoxycholate Agar (XLD) and SSI enteric medium agar plates.
Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P \leq 0.05$).

Table 3 pH development and changes in numbers of *Shigella dysenteriae* and *Shigella flexneri* strains inoculated into maize steep water and maize dough incubated at ambient temperature of about 28° C and plated on State Serum Institute (SSI) Enteric Medium. The results are shown as the mean of two independent experiments carried out in duplicate.

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

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

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

Table 4 pH development and changes in numbers of *Escherichia coli* serotypes inoculated into maize steep water and maize dough incubated at ambient temperature of about 28° C and plated on State Serum Institute (SSI) Enteric Medium. The results are shown as the mean of two independent experiments carried out in duplicate

Time after inoculation (h)	Maize steep water pH	Log_{10} CFU ml^{-1}	Maize dough pH	Log_{10} CFU g^{-1}
E. coli O111(EPEC)				
0	6.41 a	7.15 a	6.30 a	6.20 b
4	5.48 b	6.90 a b	6.11 a	7.30 a
8	4.83 c	7.10 a	4.95 b	7.00 a
24	4.59 d	6.70 b c	4.23 c	4.63 c
48	4.56 d	6.57 c	3.78 d	_ *
E. coli O157 (VTEC I)				
0	6.65 a	7.56 a	6.24 a	7.18 a
4	5.80 b	6.86 a	5.65 b	6.63 b
8	5.32 c	7.57 a	4.54 c	6.78 a b
24	4.95 d	7.80 a	4.20 d	_
48	5.30 c	7.62 a	3.80 e	_
E. coli O157 (VTEC II)				
0	6.18 a	8.30 a	6.15 a	7.54 a
4	4.94 b	8.04 a	5.73 a	8.08 a
8	4.79 c	7.46 b	4.44 b	8.18 a
24	4.46 d	7.09 b	3.90 c	3.46 b
48	4.55 d	7.34 b	3.84 c	3.62 b
E. coli O26 (VTEC)				
0	6.04 a	7.42 a b	6.12 a	6.53 a
4	5.38 b	6.83 b	5.51 b	6.26 a
8	4.63 c	7.33 a b	4.47 c	7.18 a
24	4.56 c	6.69 b	3.91 d	5.21 a
48	4.60 c	8.29 a	3.96 d	3.06 b
E. coli O3 (EAggEC)				
0	6.14 a	7.60 a	6.10 a	7.42 a
4	4.97 b	6.84 b c	5.15 b	7.82 a
8	4.76 c	7.18 a b	4.26 c	7.48 a
24	4.37 d	6.87 b c	3.92 c	3.74 b
48	4.10 e	6.41 c	3.88 c	3.06 b

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

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

Table 5 pH and changes in numbers of *Salmonella* serotypes inoculated into koko and kenkey water incubated at ambient temperature of about 28° C and plated on Tryptone Soy Yeast Extract Agar. The results are shown as the mean of two independent experiments carried out in duplicate

Time after inoculation (h)	Koko pH	Log_{10} CFU ml^{-1}	Kenkey water pH	Log_{10} CFU ml^{-1}
Salm. durban				
0	3.64 a	7.28 a	3.60 a	7.34 a
4	3.64 a	7.06 b	3.57 a	6.92 b
8	3.62 a	5.04 c	3.57 a	5.98 c
24	3.54 a	_*	3.59 a	_
48	3.58 a	_	3.59 a	_
Salm. enteritidis				
0	3.66 a	7.38 a	ND†	ND
4	3.63 a b	4.69 b	ND	ND
8	3.63 a b	_	ND	ND
24	3.52 c	_	ND	ND
48	3.54 b c	_	ND	ND
Salm. stanleyville				
0	3.65 a	7.46 a	3.60 a	7.14 a
4	3.64 a	7.03 b	3.58 a	6.78 b
8	3.62 a b	5.44 c	3.56 a	6.12 c
24	3.50 b c	_	3.59 a	_
48	3.50 c	_	3.58 a	_
Salm. typhi				
0	3.65 a	7.12 a	3.60 a	7.00 a
4	3.64 a	_	3.58 a	7.00 a
8	3.60 a b	_	3.57 a	_
24	3.55 b c	_	3.65 a	_
48	3.51 c	_	3.53 a	_
Salm. typhimurium				
0	3.64 a	7.55 a	3.60 a	7.16 a
4	3.63 a	5.16 b	3.60 a	3.65 b
8	3.62 a	_	3.56 a	_
24	3.58 a	_	3.62 a	_
48	3.56 a	_	3.55 a	_

* Not detected in 25 g after resuscitation in buffered peptone water, selective enrichment in Rappaport Vassiliadis (RV) medium with subsequent streaking onto Xylose Lysine Deoxycholate Agar (XLD) and SSI enteric medium agar plates.
† ND = Not determined.
Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P \leq 0.05$).

Table 6 pH and changes in numbers of *Shigella dysenteriae* and *Shigella flexneri* strains inoculated into koko and kenkey water incubated at ambient temperature of about 28° C and plated on Tryptone Soy Yeast Extract Agar. The results are shown as the mean of two independent experiments carried out in duplicate

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

*ND = Not determined.

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

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

Table 7 pH and changes in numbers of *Escherichia coli* serotypes inoculated into koko and kenkey water incubated at ambient temperature of about 28° C and plated on Tryptone Soy Yeast Extract Agar. The results are shown as the mean of two independent experiments carried out in duplicate

Time after inoculation (h)	Koko pH	Log_{10} CFU ml^{-1}	Kenkey water pH	Log_{10} CFU ml^{-1}
E. coli O111 (EPEC)				
0	3.65 a	7.24 a	3.62 a	7.08 a
4	3.68 a	7.16 a	3.63 a	6.62 a
8	3.60 a	6.56 b	3.63 a	5.07 b
24	3.60 a	5.83 c	3.64 a	2.91 c
48	3.58 a	3.55 d	3.60 a	_ *
E. coli O157 (VTEC I)				
0	3.63 a	7.36 a	3.54 a	7.18 a
4	3.62 a	5.88 b	3.55 a	6.13 b
8	3.63 a	4.00 c	3.54 a	4.90 c
24	3.59 a	_	3.59 a	_
48	3.63 a	_	3.56 a	_
E. coli O157 (VTEC II)				
0	3.62 a	7.27 a	3.63 a	7.28 a
4	3.67 a	7.27 a	3.64 a	7.24 a
8	3.60 a	7.22 a	3.60 a	7.03 b
24	3.59 a	6.60 b	3.64 a	4.52 c
48	3.56 a	6.36 b	3.59 a	_
E. coli O26 (VTEC)				
0	3.64 a	7.32 a	3.60 a	7.19 a
4	3.64 a	7.19 a	3.59 a	7.14 a b
8	3.63 a	7.15 a	3.58 a	6.90 b
24	3.53 a	6.30 b	3.62 a	4.12 c
48	3.64 a	5.62 c	3.55 a	_

(*Continued*)

Table7. (Continued)

Time after inoculation (h)	Koko pH	Log_{10} CFU ml^{-1}	Kenkey water pH	Log_{10} CFU ml^{-1}
E. coli O3 (EAggEC)				
0	3.68 a b	7.35 a	3.64 a	7.29 a
4	3.69 a	7.14 a	3.64 a	7.13 a b
8	3.60 a b	7.10 a	3.64 a	6.89 b
24	3.56 c	6.27 b	3.66 a	3.10 c
48	3.58 b c	5.20 c	3.59 a	_
E. coli M23				
0	3.64 a b	7.27 a	3.62 a	7.25 a
4	3.64 a b	5.49 b	3.60 a	6.75 a
8	3.68 a	4.25 c	3.59 a	5.96 b
24	3.61 b	_	3.60 a	_
48	3.62 b	_	3.59 a	_
E. coli K12				
0	3.66 a	7.30 a	3.62 a	7.25 a
4	3.68 a	7.13 b	3.60 a	7.10 a b
8	3.65 a	6.97 b	3.60 a	6.97 b
24	3.60 a	6.18 c	3.63 a	2.91 c
48	3.56 a	5.45 d	3.59 a	_

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

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

Table 8 Mean populations of 6 test strains inoculated into koko incubated at ambient temperature of about 28° C and recovered on Tryptone Soy Yeast Extract Agar (non-selective medium) and on State Serum Institute Enteric Medium ((SSI) selective medium). The results are shown as the mean of two independent experiments carried out in duplicate

		Log_{10} CFU ml^{-1}				
	Enumeration medium*	0 h	4 h	8 h	24 h	48 h
E. coli O157 (VTEC II)	TSAYE	7.27 a	7.27 a	7.23 a	6.77 a	6.36 a
	SSI	7.26 a	7.01 a	6.77 a	6.61 a	3.21 b
E. coli M23	TSAYE	7.27 a	5.49 a	4.25 a	<2.0 a	<2.0 a
	SSI	6.12 a	2.80 b	<2.0 b	<2.0 a	<2.0 a
Salm. stanleyville	TSAYE	7.47 a	7.03 a	5.44 a	<2.0 a	<2.0 a
	SSI	7.43 a	5.36 b	2.39 b	<2.0 a	<2.0 a
Salm. typhi	TSAYE	7.12 a	<2.0 a	<2.0 a	<2.0 a	<2.0 a
	SSI	5.65 b	<2.0 a	<2.0 a	<2.0 a	<2.0 a
Sh. flexneri 2a II	TSAYE	7.15 a	7.01 a	6.94 a	6.97 a	5.65 a
	SSI	7.10 a	7.01 a	6.97 a	6.85 a	3.22 b
Sh. dysenteriae	TSAYE	7.13 a	6.73 a	6.01 a	2.70 a	<2.0 a
	SSI	6.98 a	5.28 b	3.53 b	<2.0 b	<2.0 a

* TSAYE, Tryptone Soy Agar plus 0.6% Yeast Extract; SSI, State Serum Institute Enteric Medium.
Note: Numbers that are not followed by the same letter in a column for each organism are significantly different ($P \leq 0.05$).

LIST OF TABLES

Tryptone Soy Yeast Extract Agar. The results are shown as the mean of two independent experiments

Table 8 Mean populations of 6 test strains inoculated into koko incubated at ambient temperature of about 28° C and recovered on Tryptone Soy Yeast Extract Agar (non-selective medium) and on State Serum Institute Enteric Medium ((SSI) selective medium). The results are shown as the mean of two independent experiments.