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# The effect of fermentation on the growth and survival of *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* in fermenting tef (*Eragrostis tef*)

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## Introduction

Tef injera, a pancake-like fermented bread is the mainstay of the majority of Ethiopians. It is prepared after the flour of tef (*Eragrostis tef*) has been fermented for 2–3 days. Few studies on the microbiology of fermented tef have been published. Gashe *et al.* (1982) identified *Enterobacter*, *Klebsiella*, *Proteus* and *Citrobacter* as the most predominant microorganisms during the first 18 to 24 h of fermentation. *Leuconostoc mesenteroides* and *Streptococcus faecalis*, followed by *Pediococcus cereviceae* and *Lactobacillus* sp., were also isolated at a latter stage of the fermentation. According to Gifawesen and Besrat (1982) the yeasts isolated from fermenting tef were *Saccharomyces*, *Torulopsis*, *Candida* and *Pichia* species. Gashe (1985) has also pointed out that members of the Enterobacteriaceae are responsible for the pH falling to 5.8 during the first 18 h of fermentation. The lactic acid bacteria then reduced the pH to 3.8 at a latter stage of the fermentation.

Acidic pHs will inhibit the development of most bacteria (Pederson 1977). However, contamination of acidic foods by food poisoning- and spoilage-microorganisms has been reported by several workers. Outbreaks due to *Salmonella typhimurium* and *Staphylococcus aureus* were reported by Hupper (1975) and Reller *et al.* (1970).

*Salmonella typhimurium*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Yersinia enterocolitica* introduced into cottage cheese varieties (pH 4.8–5.2) and maintained at 20°C survived for as long as 15 days (Girma 1986). However, similar work has not been documented on fermenting tef. The survival and/or growth

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of microorganisms, which could be a cause of spoilage or toxicity in fermenting tef, has implications on both the keeping quality and health aspects.

In the present study we investigated the influence of the lactic acid bacteria of fermenting tef on the survival and/or growth of *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa*. The first three species used in this study are responsible for gastrointestinal disturbances. The last is a spoilage organism as well as an opportunistic pathogen.

## Materials and methods

Tef was purchased from the open market in Addis Ababa. It was ground in a local flour mill. The dough was prepared by adding enough tap water to give a 1:1.6 (w/v) ratio. The fermentation was carried out in jars for up to 96 h.

### *Microorganisms*

*Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus* or *Pseudomonas aeruginosa* was introduced into the dough at zero time. A population of about $10^3$ cells/g of dough was used for such purposes. Samples of bacteriological analysis were taken at different intervals during the fermentation. When appropriate, a series of 10-fold dilutions of the dough was prepared using sterile peptone water to obtain countable numbers of colonies on the plates. To enumerate the test organisms, 0.5 ml of the diluted sample was spread on solidified media. The plates were then incubated at 37°C for 24–48h. The media used for isolating the organisms from the fermenting dough were xylose/lysine/desoxycholate (XLD) agar for *Salm. typhimurium*, KG agar for *B. cereus*, mannitol/salts/agar (MSA) for *Staph. aureus* and Pseudomonas isolation agar (PIA) for *P. aeruginosa*. Nutrient broth with and without 0.6% (w/v) NaCl and brilliant green bile broth were used as enrichment media. All media were purchased from Oxoid, England.

### *Influence of pH on the growth of the test microorganisms*

Nutrient broth, whose pH was adjusted to 4.0, 4.2, 4.5, 5.0, 5.5, 5.8, 6.0, 6.2, or 6.5 before sterilization, was used to check for growth of the test microorganisms (*Staph. aureus*, *Salm. typhimurium*, *B. cereus*, *P. aeruginosa*). A loopful of 24-h broth culture of the test microorganism served as inoculum. Incubation of the inoculated tubes was at 37°C. Growth in the broth was monitored for over a week. Increase in turbidity was used as a measure of growth at any particular pH. Where growth was not observed, the broth sample was transferred into enrichment medium or onto agar medium to check for the presence of survivors or viable but non-growing cells.

### *Effect of baking temperature on the test microorganisms*

Sufficient of each of the test microorganisms ($10^5$ to $10^6$/ml of dough) was introduced into fermentation jars containing tef dough and left for 24 h. About 10% of the fermenting dough was boiled for 2–5 min, and then mixed with the rest in the fermentation vat. The dough rising was noted within 30–60 min. This then was baked into injera on an earthen pan. The baked injera was mixed with a quarter strength Ringer's solution (Collins & Lyne 1976) in a 1:1 (w/v) ratio and homogenized using

aseptic techniques. The homogenate was diluted as necessary in sterile peptone water. Diluted samples were then spread plated on the different media and incubated as mentioned earlier either directly or after enrichment.

*Assay for antimicrobial substances*

The agar diffusion method described by Tramer (1966) was used to detect for antimicrobial substances. The antimicrobial sensitivity tests were carried out on *Salm. typhimurium*, *B. cereus*, *Staph. aureus* and *P. aeruginosa*.

## Results and discussion

*Growth in fermenting tef*

As shown in Table 1, *Staph. aureus* grew well until the pH dropped to 5.5. Thereafter the viability decreased but even after 72 h when the pH was 4.2, viable cells were still present. *Bacillus cereus* on the other hand grew poorly below pH 5.8. Below this pH most vegetative cells reverted to spores and these remained resistant to the acid produced. As a result, the spores were recovered from fermented tef dough after 96 h when pH was about 4.0. *Salmonella typhimurium* also grew until the pH dropped to 5.5; thereafter its viability significantly declined and at pH 4.0 only a few viable cells were detected. *Pseudomonas aeruginosa* also grew well until the pH of the dough was reduced to 5.5 and thereafter the population decreased and at pH 4.0 only few viable organisms were isolated from the dough.

**Table 1** Growth of the test bacteria in fermenting tef

| | | Viable count/ml of dough | | | |
|---|---|---|---|---|---|
| Fermentation time (h) | pH | *Staph. aureus* | *B. cereus* | *S. typhimurium* | *P. aeruginosa* |
| 0 | 6.5 | $1 \times 10^3$ | $1 \times 10^3$ | $1 \times 10^3$ | $1 \times 10^3$ |
| 12 | 6.2 | $2 \times 10^4$ | $2 \times 10^3$ | $1 \times 10^6$ | $5 \times 10^5$ |
| 18 | 6.0 | $1 \times 10^5$ | $2 \times 10^3$ | $1 \times 10^5$ | $3 \times 10^6$ |
| 24 | 5.8 | $6 \times 10^5$ | $3 \times 10^3$ | $3 \times 10^7$ | $4 \times 10^6$ |
| 36 | 5.5 | $8 \times 10^5$ | $9 \times 10^2$ | $6 \times 10^7$ | $5 \times 10^6$ |
| 40 | 5.0 | $6 \times 10^5$ | $6 \times 10^2$ | $6 \times 10^6$ | $5 \times 10^6$ |
| 48 | 4.5 | $1 \times 10^4$ | $6 \times 10^2$ | $1 \times 10^6$ | $3 \times 10^5$ |
| 72 | 4.2 | $1 \times 10^3$ | $6 \times 10^3$ | $7 \times 10^4$ | $4 \times 10^3$ |
| 96 | 4.0 | <10 | $6 \times 10^2$ | <10 | <10 |

*Growth in acidified broth*

In fermenting tef the test organisms displayed variable pH tolerance (Table 2) but as can be seen from Table 2, they tolerated far more acidic conditions when grown in broth. Previous studies have indicated that the high acidic conditions in fermenting tef were due to the activities of lactic acid bacteria (Gashe *et al.* 1982; Gashe 1985). The short-chain acids produced by the fermenting organisms have been identified and their concentrations determined: the concentrations of lactic and propionic acids at 48 h, when the pH was about 4.4, were 400 to 425 mg and 1.1 to 3.2 mg/100 g dry weight of dough, respectively (Umeta 1986). The incorporation of these acids into the acidified broth separately or mixed at concentrations equivalent to that found in fermenting tef

**Table 2** Inhibitory pH for growth of the test microorganisms in broth and fermenting tef

| Test microorganism | Growth inhibitory pH | |
|---|---|---|
| | fermenting tef | nutrient broth |
| *Staph. aureus* | 5.5 | 4.5 |
| *B. cereus* | 5.8 | 5.0 |
| *Salm. typhimurium* | 5.5 | 4.4 |
| *P. aeruginosa* | 5.8 | 4.5 |

did not inhibit growth of the test organisms. However some lactic acid bacteria also produce antibacterial or bacteriostatic agents (Kodoma *et al.* 1952; Sabine 1963; Tramer 1966) and several investigators have claimed that lactic acid, $H_2O_2$ and other heat-labile substances are the cause for the inhibitory effects that are manifested by lactobacilli (Wheater et al. 1952). Moreover, Dahiya & Speck (1968) have also reported that certain species of lactobacilli produce sufficient $H_2O_2$ to inhibit various microorganisms such as salmonellae and staphylococci. We were of the opinion that the dough would enhance the capacity of the test organisms to tolerate very acidic pHs or conditions. However that was not the case in this study. We therefore checked the culture supernatant for antimicrobial activity after growing the lactic acid bacteria in nutrient broth. The methods of Tramer (1966) concerning the use of spent medium on agar plates to detect antibiotic activity was employed. Spent medium obtained after the growth of *Lactobacillus* spp. (isolated from fermenting tef) in nutrient broth for 96 h showed 1 to 2-cm diameter zone of inhibition indicating that an antimicrobial substance(s) existed in it. Culture supernatants obtained by growing the rest of the lactic acid bacteria of tef in nutrient broth from the rest of the lactic acid bacteria of tef, were not as inhibitory as that from the *Lactobacillus* species. We suspect that some inhibitory substances are produced by at least the *Lactobacillus* spp. in fermenting tef and that the substances are responsible for inhibition of growth at less acidic pH in fermenting tef than in broth.

*Effect of heat on the inactivation of the test organisms*

Since the spores of *B. cereus* and some of the other test organisms were consistently isolated from both fermenting dough and nutrient broth whose pH was 4.0, we decided to check the survival of these bacteria during the baking of the dough. The temperature of the dough after it was spread on the hot oven run from 90 to 93°C. The baking process took about 1–2 min. The process killed all the asporogenous cells but *B. cereus* survived. Since study on toxin production by *B. cereus* or *Staph. aureus* has not been conducted by us, it would be difficult to speculate what heat-stable toxins could survive the baking process. The survival of *B. cereus* spores during the baking process has great significance to food hygiene. Foods with very high moisture contents ($a_w > 0.80$) which are kept at ambient temperature for several hours could allow the growth of microorganisms on or in them. If *B. cereus* was present initially in the injera in large numbers, it will grow and produce toxins and that will affect the health of the consumers. Since our study is of a preliminary nature, we recommend that detailed studies be carried out using such types of foods.

Fermentation not only serves as means of food preservation but also as a mechanism of inhibiting the growth of undesirable or disease-causing microorganisms.

Recent research has shown that starter cultures do exert marked antagonism against many food-borne pathogens as well as food spoilage microorganisms (Daly *et al.* 1972). This inhibition is realized if starter culture of lactic acid bacteria are introduced initially. As a result of their activities, acidic conditions will be created in a short time span and inhibit the growth of the undesirable organisms. Because of these advantages, we are now exploring ways of preparing starter cultures not only for tef but for other indigenously fermented foods.

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**Summary**

Growth of *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* was inhibited when the pH of fermenting tef approached 5.0, 5.0, 5.5 and 5.0,

respectively. However the test organisms grew in far more acidic conditions in broth than in fermenting tef and this is due to antimicrobial substance(s) being produced by some of the lactic acid bacteria. Except for *Bacillus cereus* spores, all the test organisms were heat-inactivated during the baking process of the final tef injera.

**Résumé**

*Effet de la fermentation sur la croissance et la survie de* Salmonella typhimurium, Staphylococcus aureus, Bacillus cereus *et* Pseudomonas aeruginosa *dans le tef (*Eragrostis tef*) en fermentation*

La croissance de *Salmonella typhimurium*, de *Staphylococcus aureus*, de *Bacillus cereus* et de *Pseudomonas aeruginosa* est inhibée lorsque les pH du tef en fermentation approchent respectivement 5:0, 5.0, 5.5 et 5.0. Toutefois, les organismes tests croissent dans des conditiones bien plus acides que dans le tef en fermentation. Ceci est dû à des substances antimicrobiennes produites par certaines bactéries lactiques. A l'exception des spores de *Bacillus cereus*, tous les organismes tests sont inactivés par la chaleur durant le processus de cuisson.