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ORIGINAL ARTICLE

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Survival and growth of foodborne bacterial pathogens in fermenting dough of *wadi*, a legume-based indigenous food

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Abstract *Wadi* is a hollow, brittle, ball- or cone-shaped popular traditional legume-based product of many countries in the Indian Subcontinent. To prepare *wadi*, blackgram (*Phaseolus mungo* L.) *dhal* (dehusked split seeds) was soaked, ground to a soft dough, fermented for 10 h in a closed container, moulded into balls or cones and dried for ~60 h (repeating a cycle of 8 h sun-drying at 29–33 °C and then 16 h shade-drying at 28–30 °C). This study aims at understanding the fate of some selected foodborne bacterial pathogens during a chance contamination of blackgram dough at the time of preparing *wadi*. Uninoculated dough, during the course of its processing to *wadi*, remained free from *Staphylococcus aureus* and *Escherichia coli*, but got contained by *Bacillus cereus* during the first 10 h of fermentation and also the next 24 h of drying *wadi*. *B. cereus*, when spiked into freshly prepared dough at a load of 5.2 log cfu/g, also diminished after 24 h of drying (detection limit (DL), 100 cfu/g). *S. aureus* (DL, 100 cfu/g) and *E. coli* (DL, 10 cfu/g) reached below the DL after 36 h of drying. After 10 h of fermentation and 36 h of drying, the moisture content decreased from initial 61.9 to 33.5%, and the pH declined from 6.0 to 4.8.

Keywords *Wadi* · *Bacillus cereus* · *Staphylococcus aureus* · *Escherichia coli* · Moisture · pH

Traditionally, *wadi* is prepared by soaking blackgram (*Phaseolus mungo*) *dhal* (dehusked split seeds) for 6–12 h, grinding to a smooth and soft mucilaginous dough, occasionally mixing with spices, salt and backslop, leaving at room temperature (20–27 °C) for 1–3 days, hand-moulding to balls or cones (3–8 mm diameter), depositing them on an oil-smeared bamboo or palm mat to sun dry for 4–8 days (Roy et al. 2007). Excepting the occasional cases when backslop is added, the inoculum is introduced from the environment and the substrate. The lactic acid bacteria (LAB) are mainly responsible for the acidification of dough, a condition favourable for the yeasts to grow and become active for leavening (Sandhu and Soni 1989). The surface of cones or balls becomes covered with a mucilaginous coating which helps to retain the gas formed during the fermentation. The *wadis* look hollow, with many air pockets and yeast spherules in the interior and a characteristic surface crust (Aidoo et al. 2006). Because of autofermentation, an inconsistent quality of the final product occurs on many occasions. The raw pulse is not processed prior to the preparation of *wadi* to reduce its microbial load. The cultivation, harvesting and storage methods of pulse give ample scope for contamination by undesirable bacteria. In addition, the prevailing condition of environmental and personal hygiene, poor sanitation, contaminated water used in preparation and drying environment may also introduce commonly occurring foodborne pathogenic bacteria into the product. Among the critical control points are raw pulse, water, beating of dough with bare hands, utensils, drying environment and post-preparative storage conditions.

Although a number of pulses are fermented, only a few reports are available on the antimicrobial effect of these fermenting substrates. The antibacterial activity of lactobacilli during Indonesia *tempe* production (Ashenafi and Busse 1991); complete inhibition of coliform bacteria in *uji*, a fermented Kenyan maize product (Mbugua 1984),

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inhibition of *Salmonella*, *Shigella* and *Escherichia coli* in fermented weaning mixtures made from sorghum, a Japanese millet and pigeon pea (Nout et al. 1989) and inhibition of *Shigella flexneri* and enterotoxigenic *E. coli* in Ghanaian fermented maize dough (Mensah et al. 1991) have been documented. However, Jama and Varadaraj (1999) found that foodborne pathogens occurring as contaminants in *idli* batter could survive and grow under conditions of natural fermentation.

B. cereus can cause both foodborne infection and intoxication, and *S. aureus* can produce several heat-stable enterotoxins that, after ingestion of the food, may cause food poisoning. *E. coli* is used as a hygiene indicator, and its strains are recognized as a cause of gastroenteritis (Beumer 2001). Considering the fact that no data are available on the fate of pathogenic bacteria during this legume fermentation, this study aims at evaluating the survival and growth of *B. cereus*, *S. aureus* and *E. coli* during the fermentation of *wadi* dough.

Materials and methods

The dehydrated culture media and reagents were obtained from HiMedia Laboratories Pvt Ltd, Mumbai, India.

Bacterial strains *B. cereus* 2-B1 and *E. coli* 7-E2 as indicator strains were isolated from market sample of *wadi*, while *S. aureus* 34-S1 was isolated from one market sample of *dhokla* (Roy et al. 2007). The cultures were maintained at 4 °C on nutrient agar slants with subculturing after every 6 months. Before use, 24 h-old cultures were suspended in sterile distilled water.

Preparation of wadi *Wadi* was prepared in the laboratory from blackgram (*Phaseolus mungo* L.) *dhal*, which was soaked in excess of tap water for 10 h and ground to a smooth mucilaginous dough using a wet grinder (Bajaj, India). The dough was incubated in a closed container at 32 °C for 10 h. After hand-beating continuously for 30 min, the dough was hand-moulded into small cones, which were deposited on a greased bamboo mat and sun-dried (29–33 °C) for 8 h daily on three successive days with an interval of 16 h shade-drying at room temperature (28–30 °C). Sampling was done at 0 and 10 h of fermentation, and at every 12 h interval during drying.

Determination of moisture content and pH The moisture content of dough and *wadi* was determined by drying ~ 10 g sample at 105±1 °C in a hot air oven to constant weight (Banerjee and Sarkar 2003). The pH was monitored using a pH meter (Type 335, Systronics, Naroda, India) of ~ 10 g sample blended with 20 ml of CO_2-free deionized water.

Microbiological analysis Samples (10 g) were homogenized with 90 ml sterile peptone-physiological saline (1 g neutral peptone/l, 8.5 g NaCl/l, pH 7.2) using a Stomacher lab-blender 400 (Seward Medical, London) at 'normal' speed for 2 min. Duplicate counting plates were prepared using appropriate dilutions. For pour-plating, 1 ml of the dilution was mixed with molten (45 °C) medium; for spread-plating, 0.1 ml of the dilution was spread on the surface of a dried plate. After incubation, the colonies appearing on the selected plates were counted and calculated as colony-forming units (cfu) per gram fresh weight sample. The representative colonies of each type were picked up and diluted by streaking out. After microscopic examination, the purified colonies were grown on slants or in broths of suitable media and stored at 4 °C.

Total aerobic mesophilic bacteria were isolated and enumerated by pour-plating using plate count agar (M091) and incubating at 35 °C for 18–24 h. Yeasts were isolated and enumerated on yeast malt agar (M424) supplemented with benzylpenicillin (10 IU/ml) and streptomycin sulphate (12 μg/ml), and incubated at 28 °C for 48 h. Presumptive LAB were isolated and enumerated by pour plate method using *Lactobacillus* MRS agar (M641) incubated at 35 °C for 48–72 h in an anaerobic jar with AnaeroHiGas pack (LE002A). These were confirmed after determining their Gram positive and catalase negative reactions and nonmotility and nonsporeformation. Isolation and enumeration of *B. cereus* were made on spread-plates of *B. cereus* selective agar (M833, FD003 and FD045), incubated at 35 °C for 24–48 h. Presumptive isolates were confirmed on the basis of motility, endospore formation, glucose fermentation, acetylmethylcarbinol production and nitrate reduction. Selective isolation and enumeration of *S. aureus* were carried out on spread-plates of Baird-Parker agar (M043, FD047 and D045), incubated at 35 °C for 24–48 h. Characteristic grey-black shiny colonies surrounded by a clear zone were confirmed by the production of coagulase and acid from mannitol using coagulase mannitol broth base (M277) with appropriate addition of sterile pre-tested coagulase plasma, thermostable DNase using DNase test agar with toluidine blue (MI041) and production of acetylmethylcarbinol. Selective isolation and enumeration of *E. coli* were made on spread-plates of MacConkey agar (M082) incubated at 35 °C for 24 h. The pink red colonies were confirmed by gas formation in brilliant green bile broth, 2% (M121) incubated at 44 °C for 24 h and indole production by using tryptone water (M463I) and Kovac's reagent strip (DD019) (Banerjee and Sarkar 2003).

Statistical analysis Data, generated from nine batches of fermentation, were analysed by determining correlation and analysis of variance after converting the microbial counts to a logarithmic (log_{10}) scale by using SPSS v. 12.0.

Results and discussion

During the first 10 h of fermentation moisture content remained unchanged ($p<0.05$), but the volume of the dough increased by 1.3 times (Fig. 1). During the subsequent drying for 60 h the moisture content decreased ($p<0.05$) at every 12 h interval. The pronounced effect of fermentation is the change in pH; it declined ($p<0.05$) during the first 10 h from 6.0 to 5.6, and then at every 12 h interval till 24 h of drying to reach 4.9. During this period, the change in pH was negatively correlated ($r=0.92$, $p<0.05$) with the changes in the counts of LAB, yeasts and total aerobic mesophilic bacteria. After 24 h of drying although each of the counts decreased ($p<0.05$) at every 12 h interval, the pH remained unchanged ($p<0.05$). The results indicate that *wadi* dough fermentation is possibly achieved due to the activities of both LAB and yeasts whose cell count increased by ~ 6 and 4 log cycles, respectively, till 24 h of drying. Both LAB and yeasts are found to occur in raw blackgram (Soni and Sandhu 1991).

It is noteworthy that the count (3 log cfu/g) of *B. cereus* decreased ($p<0.05$) during the first 10 h of fermentation and again during the first 12 h of drying; after 24 h of drying the count was below the DL (100 cfu/g). *S. aureus* (DL, 100 cfu/g) and *E. coli* (DL, 10 cfu/g) could not be detected at any stage of fermentation and drying.

Intentional inoculation of *wadi* dough at the onset of fermentation with *B. cereus*, *S. aureus* or *E. coli* cells had no apparent influence on the growth of inherent LAB, yeasts and total aerobic mesophilic bacteria, and changes in pH and dough volume (data not shown), which are the cause and consequence of this autofermentation (Fig. 2).

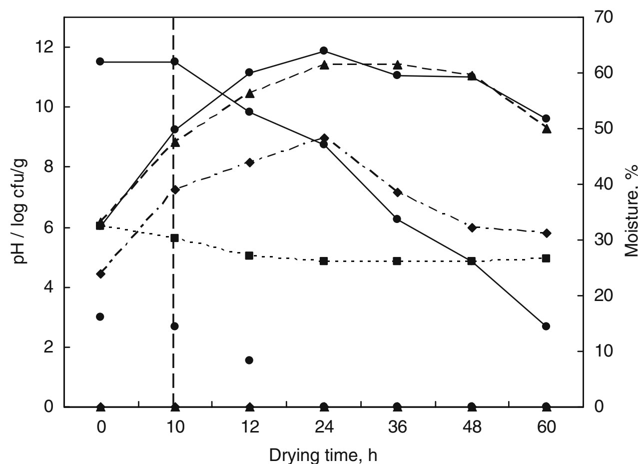

Fig. 1 Changes in pH, moisture and bacterial cell count of *wadi* dough during natural fermentation and drying ($n=9$). *TAMB* total aerobic mesophilic bacteria, *LAB* lactic acid bacteria, *Bc Bacillus cereus*, *Sa Staphylococcus aureus*, *Ec Escherichia coli*

None of the pathogenic bacteria, either inherent or introduced to the dough at the start of fermentation, survived after 36 h of drying (Figs. 1 and 2). When freshly prepared dough was spiked at a level of 5.2 log cfu/g (Fig. 2a), *B. cereus* could survive only for a while; the count reduced by 1-log cycle after 10 h of fermentation, and after 24 h of drying it could not be detected. After inoculation of dough with *S. aureus* at 5.4 log cfu/g (Fig. 2b), the count remained unchanged during the first 10 h of fermentation, but decreased ($p<0.05$) at every 12 h interval of drying, and went below the DL after 36 h. The count of *E. coli* increased ($p<0.05$) from 5.1 to 5.5 log cfu/

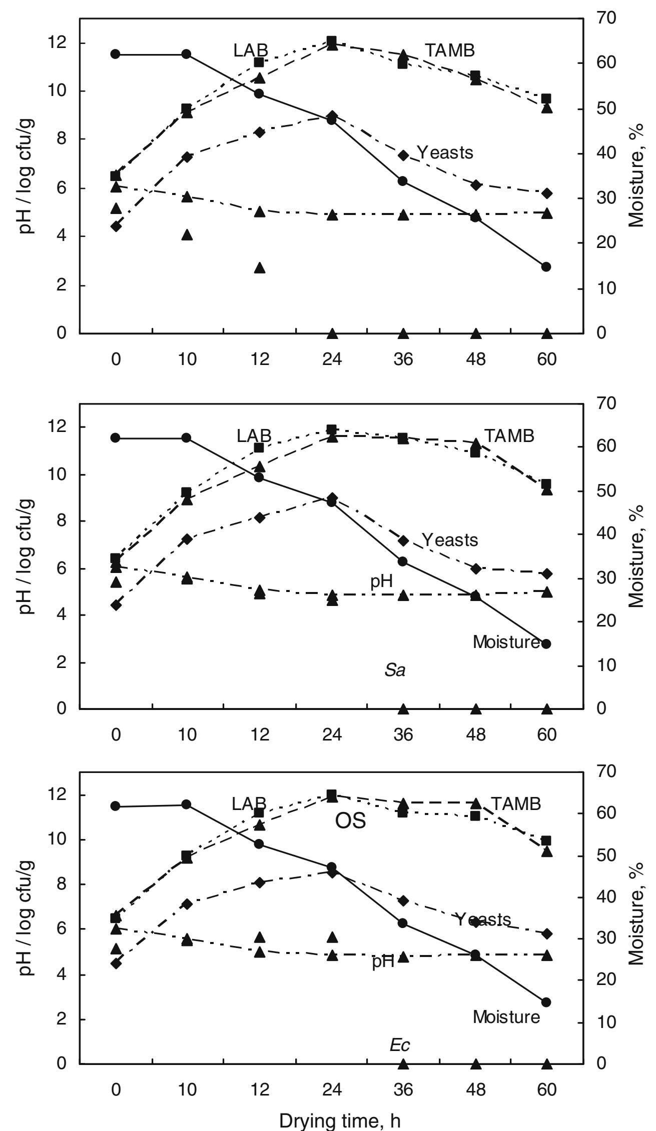

Fig. 2 Changes in pH, moisture and bacterial cell count during fermentation and drying of *wadi* dough which was inoculated at the start with Bc (**a**), Sa (**b**) or Ec (**c**). Values are the means of nine batches of fermentations. Abbreviations as in Fig. 1

g during the first 10 h of fermentation (Fig. 2c). Thereafter it remained unchanged till 24 h of drying, however *E. coli* could not be detected after 36 h. Considering the correlation between the rate of decrease in viable cell numbers of the three pathogens and that of pH and moisture content, the decrease in the cell count could mainly be attributed to the increase in the intensity of these two hurdles. According to Beumer (2001), the minimum pH for the growth of *B. cereus*, *S. aureus* and *E. coli* are 5.0, 4.0 and 4.4, respectively. In this study, the maximum pH of dough which did not support the survival of these three respective indicator organisms were 4.9, 4.9 and 4.8. The results indicate that reduction in moisture content of dough in combination with pH might have caused the death of the pathogens. None of these pathogens could grow in the fermenting dough having moisture content of 14–47%. This range of moisture, which is within the one for intermediate moisture foods, prohibits the growth of Gram negative as well as a large number of Gram positive bacteria, and yeasts (Adams and Moss 1995). It is also possible that the inhibition of pathogenic bacteria is due to the contribution of certain other less potent hurdles, like nutrient depletion and crowding, and the presence of starter-derived inhibitors such as bacteriocins, H_2O_2, ethanol, diacetyl and CO_2 (Adams and Nicolaides 1997). *S. aureus* is generally regarded as a poor competitor and its growth in fermented foods is generally associated with a failure of normal flora (Beumer 2001). The specific reason for holding the pathogens in check during this fermentation is an area requiring further study.

Conclusion

Fermenting dough of *wadi* may get contaminated by foodborne bacterial pathogens due to unhygienic preparation practices. But their growth is inhibited during simultaneous drying and fermentation with concomitant decrease in moisture and pH. However, good drying and storage environment are advised to ensure a longer shelf life of the product.

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