International Food Research Journal 18(3): 1189-1194 (2011)

Antagonism of Lactic Acid Bacteria against foodborne pathogens during fermentation and storage of *borde* and *shamita*, traditional Ethiopian fermented beverages

[1]Anteneh, T., [2]Tetemke, M. and [3*]Mogessie, A.

[1]Department of Biology, [2]Department of Chemistry, [3]Institute of Pathobiology, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia

Abstract: The inhibitory property of nine pure or mixed cultures of potentially probiotic lactic acid bacteria (LAB) was tested against *Escherichia coli*, *Salmonella* Typhimurium DT104, and *Staphylococcus aureus* during fermentation and storage of *borde* and *shamita* at ambient temperatures. Pure LAB cultures reduced in average the count of test pathogens by 5-6 and 4 log cycles at 24 h during fermentation of *borde* and *shamita,* respectively. Mixed LAB cultures reduced the counts of pathogens by 7 and 5 log units after 24 h of fermentation in *borde* and *shamita*, respectively. During storage of both products at ambient temperature, the test pathogens were reduced by 4 log units at 12 h and totally eliminated at 24 h. The LAB strains survived at levels around log 9 cfu/ml at 24 h during storage. The results strongly suggest that the isolates are possible candidates for the formulation of starter cultures that can be used to produce safe and bioprotective products. Moreover, the study also indicated the possible use of the products as carrier for potential probiotic cultures .

Keywords: *Borde*, *shamita*, lactic acid bacteria, probiotic quality, foodborne pathogens

Introduction

Borde and *shamita* are very popular traditional fermented beverages mainly prepared in central and southern Ethiopia, and mainly consumed as meal replacement by low–income groups (Ashenafi and Mehari, 1995; Ashenafi, 2002).

Previous studies indicated that the fermentation processes of *borde* and *shamita* mainly involved lactic acid bacteria (Ashenafi and Mehari, 1995; Bacha *et al*., 1998, 1999; Abegaz *et al*., 2004; Abegaz, 2007). Basically, in addition to different species of lactic acid bacteria, various non-lactic acid bacterial species such as aerobic mesophilic bacteria (*Bacillus* spp. and micrococci), coliforms, other members of *Enterobacteriaceae*, and yeasts were shown to be present in fermenting *borde* (Bacha *et al*., 1998; Abegaz *et al*., 2007) and *shamita* (Bacha *et al*., 1999). Heterofermentative LAB were indicated as dominant type and reached high count at 24 h in both fermentations, and resulted in dropping of the pH of fermenting *borde* and *shamita* from 5.2 and 5.8 to 3.8 and 4.2 within 12 h, respectively (Ashenafi and Mehari, 1995; Bacha *et al*., 1998). The microbial sources of both *borde* and *shamita* included ingredients, fermentation utensils, fermenting pot, and back slopping (Bacha *et al*. 1999; Abegaz *et al*., 2002).

Lactic acid fermentation is a traditional household-level technique, reported as effective in reducing or eliminating the growth of foodborne pathogens. Generally, the addition of LAB to various foods including milk has been believed to be a biopreservation measure to inhibit and probably eliminate food spoilage and pathogenic microorganisms (Stiles, 1996). Various workers indicated microbial antagonism to be the basis for preservation and enhancement of microbiological safety of fermented products (Gänzle *et al*., 2000; Callewaert and De Vuyst, 2000). Possible antagonistic effects of lactic acid-producing bacteria against pathogens have been proposed to include organic acid production, competition for nutrients, hydrogen-peroxide formation and production of bacteriocins and antibiotic-like substances (Gibbs, 1987).

The inhibitory effects of mixed-LAB cultures against foodborne pathogens during fermenting *borde* were shown by Tadesse *et al*. (2005a). Similarly, the inhibitory effect of extracts of LAB isolates recovered from *borde* and *shamita* against food borne pathogens on laboratory medium was also studied by Tadesse *et al.* (2005b). The *in vitro* and *in vivo* probiotic qualities of the nine LAB strains considered in this study is reported elsewhere (Tesfaye *et al*., 2011). The objectives of this study were to assess the antagonistic effects of these potentially probiotic LAB in the form of pure or defined mixed-cultures against some foodborne pathogens during fermentation and storage of *borde* and *shamita*.

*Corresponding author.
Email:*mogessie@gmail.com*

© All Rights Reserved

Materials and Methods

Bacterial strains and preparation of borde *and* shamita

All LAB strains used in this study were recovered from locally fermented dairy products (*ayib* and *ergo*) and low-alcoholic beverages (*borde* and *shamita*). The LAB were tentatively identified to species and subspecies level using API 50CHL kit, and nine isolates were selected based on their *in vitro* and *in vivo* probiotic qualities (data not included). *S.* Typhimurium DT104, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as target test strains. The LAB isolates were used either as pure or mixed LAB starter cultures. The mixed cultures were formulated based on their homofermentative or heterofermentative characteristics (Table 1).

Borde was prepared at laboratory scale following the protocol given by Bacha *et al*.(1998) as shown in Figure 1. Maize flour (833 g) was soaked in excess water and deeply roasted on a hot metallic pan. After cooling, the roasted maize was mixed with 8.33 g ground malted maize in 1000 ml of boiling water and left to ferment overnight. Similarly, *shamita* was prepared at a laboratory scale as indicated in Figure 2 following the protocol specified by Bacha *et al.* (1999). For preparation of *shamita* barely was lightly roasted on metallic pan and ground to fine powder. Linseed was also ground to fine powder. The roasted and ground barely (150 g), ground linseed (9 g), spices (0.04 g) and salt (4 g) were mixed in 2000 ml beaker with 1000 ml of sterile water. The mix was left to ferment overnight.

Table 1. Pure and mixed LAB cultures used as starter culturesduering preparation of *borde* and *shamita*

Starter LAB cultures		
Pure LAB cultures	Mixed LAB cultures (MLC)	
Lb. acidophilus 1*, *Lb. brevis* 1†, *Lb. cellobiosus*†, *Lb. delbrueckii* ssp *delbrueckii**, *Lb. paracasei* ssp *paracasei* 3†, *Lb. plantarum 1*†, *Lb. plantarum 2*†, *Lac. lactis* ssp *lactis* 1* and *Ped. pentosaceus 1*†	MLC 1	*Lac. lactis ssp lactis* 1, *Lb. paracasei ssp paracasei* 3, and *Lb. brevis* 1
	MLC 2	*Lb. acidophilus* 1, Lb. *cellobiosus and Lb. plantarum 1*
	MLC 3	*Lb. delbrueckii ssp delbrueckii, Lb. plantarum 2* and *Ped. pentosaceus 1*

*- homofermentative, † -heterofermentative

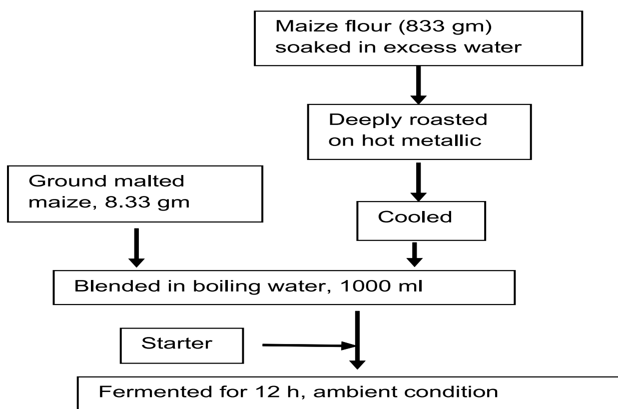

Figure 1. Laboratory scale preparation of *borde*

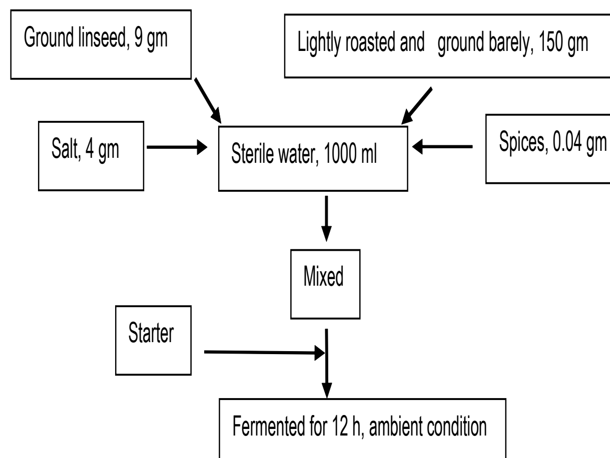

Figure 2. Laboratory scale preparation of *shamita*

Analyses of antagonism of LAB during products preparations and storage

Ingredients of *borde* and *shamita* were separately blended and pasteurized at 80°C for 10 min in 200 ml amounts in 250 ml bottles and cooled to room temperatures. Each pure LAB culture was grown overnight at 32°C in 10 ml MRS broth (Oxoid). The culture was further diluted in 90 ml sterile peptone water to give log 7 cfu/ml. Similarly, culture of each test pathogen was grown overnight at 32°C in 10 ml Tryptose Soya broth (TSB). The growth suspension was serially diluted in 90 ml sterile peptone water to give log 4 cfu/ml. To separate pairs of cooled 200 ml blended ingredients, each pure LAB culture was inoculated to give an initial inoculum level of log 6 cfu/ml. Then, the inoculated blend was further co-inoculated with each test pathogen to give initial inoculum level of log 3 cfu/ml. The same procedure was followed for all pure and mixed starter cultures. The enumeration of the test pathogens in the experimental and control fermenting blends was done at 0, 6, 12 and 24 h by plating 0.1 ml of an appropriate dilution on duplicate Plate Count (PC) plates. After 30 minutes, PC plates were overlayed with Violet Red Bile (VRB) agar, Xylose Lysine Desoxycholate agar (XLD) and Mannitol Salt agar (MSA) for detection of *E. coli*, *S.* Typhimurium and *S. aureus*, respectively (all media were from Oxoid.) All plates were incubated at 32°C for 24/48 h. Enumeration of LAB isolates was done on MRS agar plates after incubation at 32°C for 24/48 h in anaerobic jar (Oxoid). During each sampling, pH of each sample was determined using a pH meter. For storage studies, *borde* and *shamita* were prepared by separately using different mixed starter cultures. A volume of 200 ml of ready-to-consume *borde* and *shamita* were separately inoculated with each of the test pathogens to give an initial inoculum level of log 6 cfu/ml. The products were maintained at

ambient temperatures. Enumeration of LAB and the test pathogens and determination of pH were done at 0, 6, 12 and 24 h. When counts of test pathogens were <log 1 cfu/ml, samples were enriched in Tryptic Soya broth and spread plated on the respective media. Experiments were conducted in triplicates. Results indicated are averages values, and were described by descriptive statistics.

Results and Discussion

Pure and mixed LAB strains grew to > log8 cfu/ml at 24 h in fermenting *borde* and *shamita* (Figure 3). Pure cultures reduced the pH to 3.41 and 4.19 at 24 h of fermentation of *borde* and *shamita*, respectively. Correspondingly, mixed cultures reduced the pH of fermenting *borde* and *shamita* to 3.31 and 3.65 (Figure 4). Naturally, LAB grow to large numbers during the fermentation of the products (Abegaz, 2007; Ashenafi and Mehari, 1995; Bacha *et al*., 1998, 1999). Increase in the count of LAB in the presence of foodborne pathogens is particularly important to lower the pH and produce and accumulate sufficient antimicrobial metabolites to exert their inhibitory effect against foodborne pathogens.

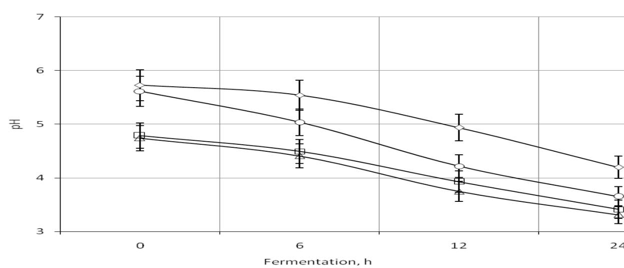

Figure 3. Changes in pH during *borde* and *shamita* fermentation with various pure and mixed LAB cultures
(□ Average pH of pure cultures in *borde*, ◊ average pH of pure cultures in *shamita*, Δ average pH of mixed cultures in *borde*, and ○average pH of mixed cultures in *shamita*)

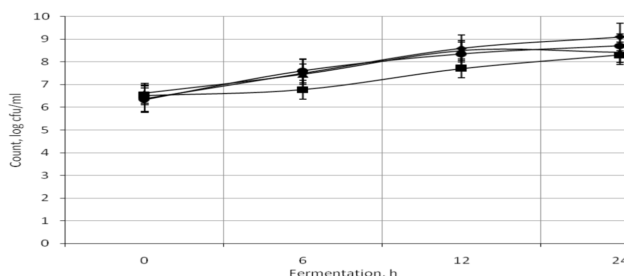

Figure 4. Counts of various pure and mixed LAB cultures during *borde* and *shamita* fermentation
(■ Average counts of pure cultures in *borde*, ♦ average counts of pure cultures in *shamita*, ▲average counts of mixed cultures in borde, ● average counts of mixed cultures in *shamita*)

Slight increase of the test pathogens by up to 1.5 log units was seen at 12 h of fermentation of *borde* by the various pure cultures. The counts, however declined to log 2.4 cfu/ml at 24 h (Figure 5). Similarly, during *shamita* fermentation, the count of the test pathogens increased by about 2 log units at 12 h and further dropped to log 3.65 cfu/ml at 24 h (Figure 7). Correspondingly, the pH of both products fell by about 1.5 units at 24 h. (Figures 6 and 8). In both *borde* and *shamita* ingredient blends without lactic starter cultures, the count of the test pathogens reached >log7 cfu/ml at 24 h and the pH remained over 4.5.

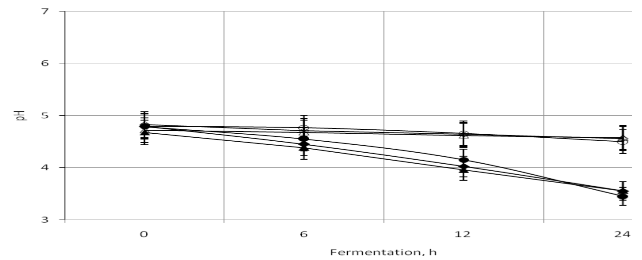

Figure 5. Changes in pH of fermenting *borde* into which test pathogens were inoculated in the presence (open symbols) and absence (closed symbols) of pure LAB culture; *E. coli* (triangle), *S.* Typhimurium (diamond), and *S. aureus* (circle)

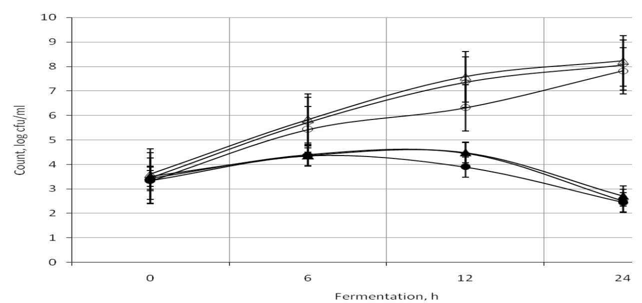

Figure 6. Mean counts of test pathogens during fermenting *borde* in the absence of pure LAB culture (open symbols) and presence (closed symbols); *E. coli* (triangle), *S.* Typhimurium (diamond), and *S. aureus* (circle)

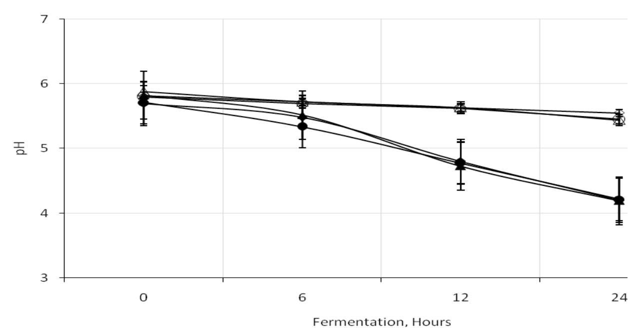

Figure 7. Changes in pH of fermenting *shamita* into which test pathogens were inoculated in the presence (open symbols) and absence (closed symbols) of pure LAB cultures; *E. coli* (triangle), *S.* Typhimurium (diamond). and *S. aureus* (circle)

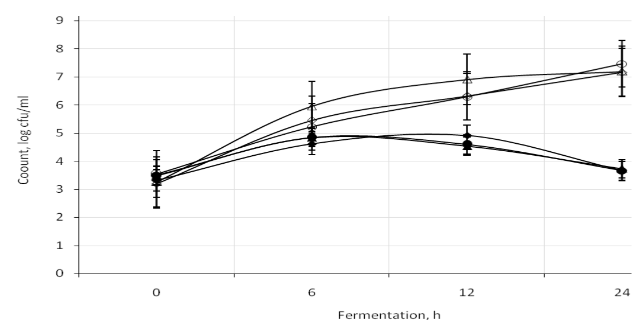

Figure 8. Mean counts of test pathogens during fermenting *shamita* in the presence (open symbols) and absence (closed symbols) of pure LAB cultures; *E. coli* (triangle), *S.* Typhimurium (diamond), and *S. aureus* (circle)

Fermentation of *borde* by the mixed LAB cultures resulted in the reduction of test pathogens to levels as low as log 1 cfu/ml at 24 h (Figure 9). Whereas in fermenting *shamita*, the average count of the test pathogens showed a slight increase at 6 h but decreased to log 2.02 cfu/ml at 24 h (Figure 11).

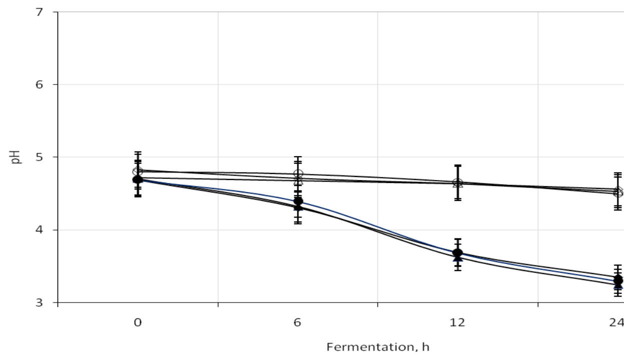

Figure 9. Changes in pH during fermenting borde into which test pathogens were inoculated in the presence (open symbols) and absence (closed symbols) of mixed LAB cultures; *E. coli* (triangle), *S.* Typhimurium (diamond), and *S. aureus* (circle)

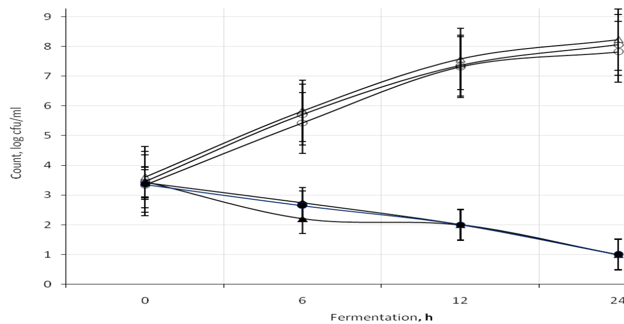

Figure10. Mean counts of the test pathogens during fermenting *borde* in the presence (open symbols) and absence (closed symbols) of mixed LAB cultures; *E. coli* (triangle), *S.* Typhimurium (diamond), and *S. aureus* (circle)

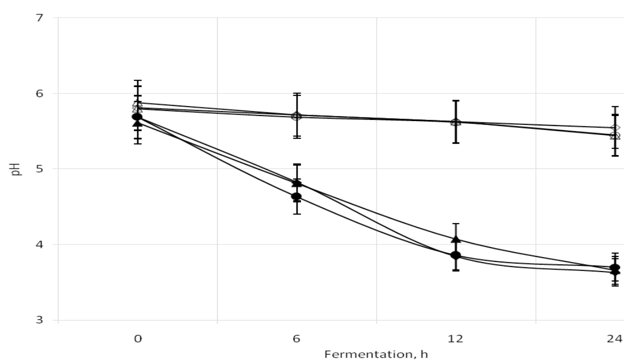

Figure 11. Changes in pH during fermenting *shamita* into which test pathogens were inoculated in the presence (open symbols) and absence (closed symbols) of mixed LAB cultures; *E. coli* (triangle), *S.* Typhimurium (diamond), and *S. aureus* (circle)

Final pH of *borde* fermented by mixed cultures was 3.29 (Figure 10) and that of *shamita* was 3.70 (Figure 12). The difference in the level of reduction in the count of the test pathogens in *borde* and *shamita* could be related to differences in the pH values of the two products, particularly during the early stage of fermentation.

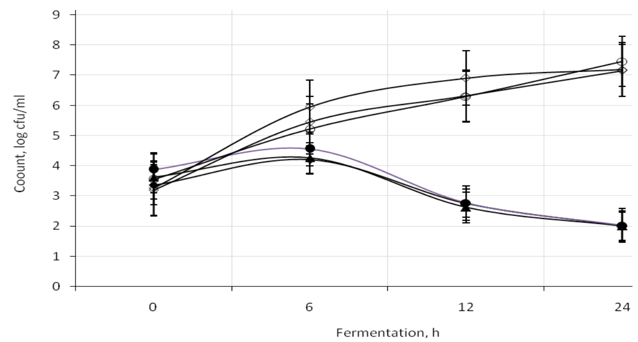

Figure 12. Mean counts of test pathogens during fermenting *shamita* in the presence (open symbols) and absence (closed symbols) of mixed LAB cultures; *E. coli* (triangle), *S.* Typhimurium (diamond), and *S. aureus* (circle)

Unlike the report given by Tadesse *et al*. (2005a) in which *E. coli* 0157:H7 was reduced only by 4 log units, our results demonstrated reduction of *E. coli* by 6 log factors during fermentation not only by mixed cultures but also by pure lactic cultures, too**.** In a similar study, Dineen *et al*. (1998) reported the inhibition of *E. coli* O157:H7 by thermophilic mixed-culture rather than by single-culture in fermenting milk. Significant level of reduction of Gram-negative intestinal pathogenic bacteria, enterotoxigenic *Escherichia coli*, *Campylobacter jejuni*, *Shigella flexneri* and *Salmonella* Typhimurium by natural lactic fermenting mixed-culture as a result of lowered pH was reported by Svanberg *et al*. (1992).

Unlike our result, in which *S.* Typhimurium was significantly reduced but not completely eliminated, Tadesse *et al*. (2005a) reported the gradual reduction in the count of *Salmonella* spp. to complete elimination at 24 h during fermenting *borde*. The difference in the two studies could be related to the initial inoculum level of lactic cultures employed, in which it was log 6 cfu/ml in this study but log 8 cfu/ml in the study of Tadesse *et al.* (2005a). The decrease in the count of *Salmonella* during controlled pig feed fermentation using pure culture of *Lb. plantarum* was reported by van Winsen *et al*. (2000). Similar to our observations, several studies suggested that mixed cultures had relatively stronger inhibitory effect against foodborne pathogens than pure cultures. Van der Wielen *et al*. (2002) reported inhibition of the growth of *Salmonella enterica* serovar Enteritidis by a mixed culture of *Lb. crispatus* and *Clostridium lactatifermentans* but not by a monoculture of *Lb. crispatus* at pH 5.8. The inhibition of the growth of *E. coli*, *S*. Typhimurium, and *C. perfringens* by probiotic *Lb. salivarius* and *Lb. plantarum* from starter and grower diets of broiler chickens was demonstrated by Murry *et al*. (2004). The inhibition of Gram-positive and Gram-negative bacteria during the initial stage of fermenting maize dough with *Lb. plantarum* and *Lb. fermentum/reuteri*

was reported by Olsen *et al*. (1995).

Despite, early pH drop during fermentation of *borde* with mixed cultures, complete elimination of the test pathogens was not achieved. This may be related to the fact that brief exposure of enteric pathogens such as *E. coli* to mild acidic pH could contribute to the development of acid-tolerance (Bearson *et al*., 1997).

When the ready-to-consume products were inoculated with test pathogens and maintained at ambient temperature, the mean counts of the test pathogens were reduced by 4 log units at 12 h and completely eliminated from both ready-to-consume products at 24 h (Figures 13 and 14). The pH dropped to 3.2 and 3.4 at the end of storage of ready-consume *borde* and *shamita*.

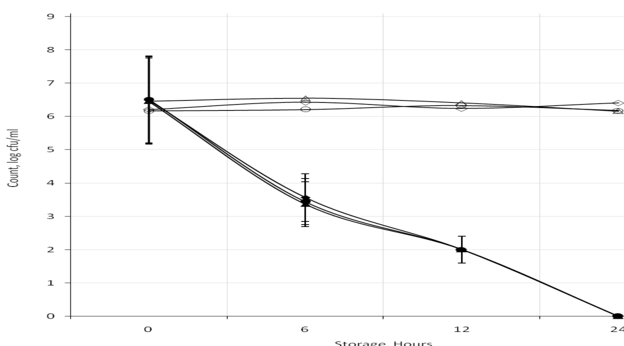

Figure 13. Changes in mean counts of *E. coli* (triangle), *S*. Typhimurium (diamond), and *S. aureus* (circle) in ready-to-consume borde fermented with various mixed LAB cultures (closed symbols) and in PBS (open symbols) stored at ambient condition

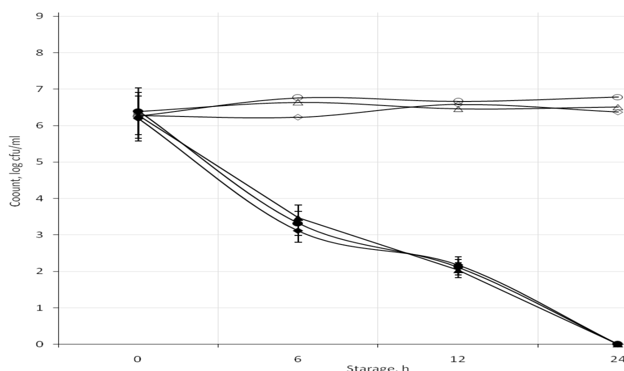

Figure 14. Changes in mean counts of *E. coli* (triangle), *S*. Typhimurium (diamond), and *S. aureus* (circle) in ready-to-consume shamita fermented with various mixed LAB cultures (closed symbols) and in PBS (open symbols) stored at ambient condition

In similar study, *S*. Typhimurium was completely inhibited within 12 h (in 24 and 48 h fermented finger millet flour) and 48 h (in 12 and 18 h fermented finger millet flour) as demonstrated by Yang *et al*. (2008). Ephrem and Ashenafi (2005) did not detect *S*. Typhimurium DT104 after keeping ready-to-consume *siljo*, fermented legume gruel, for 3 days at ambient temperature.

Tadesse *et al.* (2005a) detected a substantial reduction, but not complete inhibition, of *S. aureus* after 24 h of maintaining *borde* at ambient temperatures. During cereal gruel fermentation with lactic acid bacterial culture, Kingamkono *et al*. (1995) reported that *Staphylococcus* was not detected after 12 h. In our study, the test pathogens were totally eliminated during keeping of both products at ambient condition at 24 h.

The LAB strains survived in both ready-to-consume *borde* and *shamita* at an average count of ≥ log 8 cfu/ml at 24 h during storage at ambient condition (Table 2).

In fact, both *borde* and *shamita* are overnight fermented products with limited acid and alcohol content. They are consumed within 4 h of an overnight fermentation. Thus, pre- or post-fermentation contaminations of the products could pose a health hazard (Tadesse *et al*., 2005a and b). However, the survival of the antagonistic LAB strains at high levels for up to 24 h could be important if the shelf life of the products could be increased to about a day or two.

Our defined mixed starter cultures, on top of improving the safety and keeping qualities of the products, are potentially probiotic cultures as observed in another experiment (data not included). Considering the impacts of mixed cultures and longer survival of the LAB strains in the products, we suggest that the isolates are possible good candidate starters and both products can be employed as vehicles for provisions of health promoting strains.

Acknowledgements

The financial assistance obtained from the Graduate Program of Addis Ababa University is acknowledged.

References

Abegaz, K., Beyene, F., Langsrud, L. and Narvhus, J.A. 2002. Indigenous processing methods and raw materials of *borde*, an Ethiopian traditional fermented beverage. Journal of Food Technology in Africa 7: 59-64.

Abegaz, K. 2007. Isolation, characterization and identification of lactic acid bacteria involved in traditional fermentation of *borde*, an Ethiopian cereal beverage. African Journal of Biotechnology 6: 1469-1478.

Abegaz, K., Langsrud, T., Beyene, F. and Narvhus, J. A. 2004. The effect of technological modification on the fermentation of *borde*, an Ethiopian traditional fermented cereal beverage. Journal of Food Technology in Africa 9: 3-12.

Ashenafi, M. 2002. The microbiology of Ethiopian foods and beverages: a review. *SINET*: Ethiopian Journal of Science 25 (1): 97 – 140.

Ashenafi, M. and Mehari, T. (1995). Some microbiological and nutritional properties of "*borde*" and "*shamita*", traditional Ethiopian fermented beverages. Ethiopian Journal of Health Development 9 : 105 - 110.

Bacha, K., Tetemke, M. and Ashenafi, M. (1998). The microbial dynamics of "*Borde*" fermentation, a traditional Ethiopian fermented beverage. *SINET*: Ethiopian Journal of Science, 21: 195 - 205.

Bacha, K., Mehari, T. and Ashenafi, M. 1999. Microbiology of the fermentation of '*Shamita*', a traditional Ethiopian fermented beverage. *SINET*: Ethiopian Journal of Science 22: 113 – 126.

Bearson, B., B. Bearson, and J. W. Foster. 1997. Acid stress responses in enterobacteria. FEMS Microbiology Letters 147:173-180.

Callewaert, R. and De Vuyst, L. (2000). Bacteriocin production with *Lactobacillus amylovorus* DCE 471 is improved and stabilized by fed-batch fermentation. Applied and Environmental Microbiology 66: 606 – 613.

Dineen, S.S., Takeuchi, K., Soudah, J.E. and Boor, K.J. 1998. Persistence of *Escherichia coli* 0157:H7 in dairy fermentation system. Journal of Food Protection 61:1602-1608.

Ephrem, E. and Ashenafi, M. 2005. Fate of *Salmonella typhimurium* DT104 during the Fermentation of 'Siljo', a Traditional Ethiopian Fermented Legume Condiment, and during Product Storage at Ambient and Refrigeration Temperatures. World Journal of Microbiology and Biotechnology 21: 1259-1265.

Gänzle, M.G., Holtzel, A., Walter, J., Jung, G. and Hammes, W. P. (2000). Characterization of reutericycline produced by *Lactobacillus reuteri* LTH2584. Applied and Environmental Microbiology 66: 4325 – 4333.

Gibbs PA. 1987. Novel uses for lactic acid fermentation in food preservation. Journal of Applied Bacteriology Symposium Supplement 16:515-85.

Kingamkono , R., Sjögren, E., Svanberg U. and Kaijser, B. 1995.Inhibition of different strains of enteropathogens in a lactic-fermenting cereal gruel. World Journal of Microbiology and Biotechnology 11: 299-303.

Murry, A.C. Jr ., Hinton, A. Jr. and H. Morrison, H. 2004. Inhibition of Growth of *Escherichia coli*, *Salmonella typhimurium*, and *Clostridia perfringens* on Chicken Feed Media by *Lactobacillus salivarius* and *Lactobacillus plantarum*. International Journal of Poultry Science 3: 603-607.

Olsen A., Halm, M. and Jakobsen, M. 1995. The antimicrobial activity of lactic acid bacteria from fermented maize (kenkey) and their interaction during fermentation. Journal of Applied Bacteriology 79: 506-512.

Stiles M E 1996 Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70: 331–345.

Svanberg, U., Sjögren, E., Lorri, W., Svennerholm, A.M. and Kaijser, B. 1992. Inhibited growth of common enteropathogenic bacteria in lactic-fermented cereal gruels. World Journal of Microbiology and Biotechnology 8: 601-606.

Tadesse, G., Ashenafi, M. and Ephraim. E. 2005a. Survival of *E. coli* O157:H7 *Staphylococcus aureus*, *Shigella flexneri* and *Salmonella* spp. in fermenting '*Borde*', a traditional Ethiopian beverage. Food Control, 16: 189-196.

Tadesse, G., Ephraim, E. and Ashenafi, M. 2005b. Assessment of the antimicrobial activity of lactic acid bacteria isolated from *Borde* and *Shamita*, traditional Ethiopian fermented beverages, on some foodborne pathogens and effect of growth medium on the inhibitory activity. Internet Journal of Food Safety 5: 13-20.

Tesfaye, A., Mehari, T., and Ashenafi, M.. 2011. Evaluation of the *in vitro* and *in vivo* probiotic qualities of lactic acid bacteria (LAB) recovered from locally fermented products. International Journal of Probiotics and Prebiotics. (in press)

van der Wielen, P.W.J.J., Lipman, L.J.A., van Knapen, F. and Biesterveld, S. 2002. Competitive Exclusion of *Salmonella enterica* serovar Enteritidis by *Lactobacillus crispatus* and *Clostridium lactatifermentans* in a Sequencing Fed-Batch Culture. Applied and Environmental Microbiology 68: 555-559.

van Winsen, R.L., Lipman,L.J.A., Biesterveld, S., Urlings, B.A.P., Snijders, J.M.A. and van Knapen, F. 2000. Mechanism of *Salmonella* reduction in fermented pig feed. Journal of the Science of Food and Agriculture 81: 342-346.

Yang, Y., Tao, W., Liu, Y. and Zhu, F. 2008. Inhibition of *Bacillus cereus* by lactic acid bacteria starter cultures in rice fermentation. Food Control 19: 159-161.