African Journal of Biotechnology Vol. 6 (12), pp. 1469-1478, 18 June, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

*Full Length Research Paper*

# Isolation, characterization and identification of lactic acid bacteria involved in traditional fermentation of *borde*, an Ethiopian cereal beverage

**Kebede Abegaz**

Department of Food Science and Post-harvest Technology, Awassa College of Agriculture, Debub University, P. O. Box 5, Awassa, Ethiopia. E-mail: abegaz2005@yahoo.co.uk.

Accepted 13 November, 2006

**Changes in pH, titratable acidity, *Enterobacteriaceae*, aerobic mesophiles, lactic acid bacteria (LAB) and yeast counts were investigated during *borde* fermentation. A rapid decrease in pH was associated with accelerated growth rate of LAB and inhibition of *Enterobacteriaceae*. Wide diversities of LAB strains were present at early stage of *borde* fermentation. The number of species reduced as the fermentation progressed. Representatives of LAB involved in *borde* fermentation were isolated at 6 h intervals, characterized and identified using physiological and biochemical methods including API 50CH kit. The LAB strains were grouped into 3 major and 2 minor clusters. Most of the LAB strains were heterofermentative lactobacillus (79.4%), while pediococcus species dominating the cocci (20.6%). *Weissella confusa*, *Lactobacillus brevis*, *Lactobacillus viridescens*, *Pediococcus pentosaceus* and *P. pentosaceus* subsp. *intermedius* were identified throughout *borde* fermentation as dominant LAB. The frequency of dominating strains were: *W. confusa* (30.9%), *Lb. viridescens* (26.5%), *Lb. brevis* (10.3%) and *P. pentosaceus* (7.4%) and *P. pentosaceus* subsp. *intermedius* (8.8%), while sporadically isolated species of *Lactobacillus curvatus*, *P. acidilactici*, *Lactobacillus collinoides*, *Lactobacillus sanfrancisco*, *Lactobacillus pontis* and *Lactobacillus delbrueckii* subsp. *delbrueckii* were 16.3%. The dominating LAB strains can be potential starter cultures for *borde* fermentation.**

**Key words:** Beverage, cereal fermentation, Ethiopian *borde*, identification, lactic acid bacteria.

## INTRODUCTION

Fermentation has been used as a means of improving the keeping quality of food for more than 6000 years (Holzapfel, 1997). Beer brewed by the Babylonians and exported to Egypt around 3000 B. C. was most likely the product of both alcoholic and lactic fermentations. Present day sorghum, maize and millet beers in Africa possess similar features in which the lactic fermentation plays a key role in safety and acceptability of these products in tropical climates (Haggblade and Holzapfel, 1989). Many African foods that are prepared by the action of diverse species of fungi, bacteria and yeasts on plant materials are little known outside their native countries. Otherwise, foods are considered as habitats for microbial growth. The origin, development and succession of a particular microbial community in any food are governed by its ecological factors (Deak and Beuchat, 1996), which influence the physiological expression of microbial cells. Thus, strategies for food processing and preservation can be developed on the basis of ecological factors associated with specific foods and beverages (Gould, 1992).

Ethiopian *borde* is a spontaneously fermented, low or non-alcoholic cereal beverage. It is produced from a variety of locally available cereal ingredients using traditional techniques. The adjunct (all unmalted cereal ingredients) and malt could each be from one type or a mixture of cereals. *Borde* is an opaque, effervescent, whitish-grey to brown colour and has a thick consistency and sweet-sour taste. It must be consumed while actively fermenting within a day due to its poor keeping quality. It is widely consumed by adults and children mainly in southern and western parts of Ethiopia. *Borde* is often consumed as a low-cost meal replacement for many poor people.

The traditional technology of *borde* preparation is time-consuming, unhygienic and complex, which involves grinding, souring, roasting, steam cooking, boiling, cooling, mashing, wet-milling and wet-sieving operations (Abegaz et al., 2002a). This may create a variable bio-physico-chemical environment which select for involved microflora that overcome technological hurdles in the natural fermentation of *borde*. Although cooking of the adjuncts at about 90°C killed vegetative microbial cells (Abegaz et al., 2002b), addition of malt serves as a source of microorganisms (Sahle and Gashe, 1991; Ketema et al., 1998) and endogenous amylolytic enzymes. This would result in the hydrolysis of starch and thus create an environment suitable for proliferation of microorganisms during traditional ("four phases") fermentation of *borde*. However, the spontaneous fermentation resulted in unpredictable quality of *borde* with a low pH, high counts of aerobic mesophiles, lactic acid bacteria (LAB) and yeast (Abegaz et al. 2002b).

In general, a wide spectrum of microorganisms is involved during fermentation processes but a few types usually determine quality of the end product. Given adequate environmental conditions, a particular microbial community will determine the quality of a specific food. Therefore, isolation, characterization and identification of the involved microorganisms with a prospective selection of starter cultures that are adapted to *borde* production would be important to support the technical process and to obtain a predictable end product with a desired quality. This may help in development of starter culture and devising appropriate and affordable technology that could modernize *borde* production. Thus, there is a need of information on systematic isolation and identification of LAB involved in *borde* fermentation. The objective of this work is, therefore, to isolate LAB during the course of traditional *borde* fermentation, to characterize and identify them using biochemical, morphological and physiological techniques with the aim of starter culture development.

## MATERIALS AND METHODS

*Borde* was prepared by an experienced brewer from a mixture of cereal ingredients following the traditional recipe and four phases of its fermentation (Abegaz et al., 2002a). Appropriate equipment and raw materials for *borde* production were purchased locally. For convenience of sample analysis and isolation of LAB at 6 h intervals during the course of traditional *borde* fermentation, the experiment was carried out at Awassa College of Agriculture, Awassa (272 km south from Ethiopian capital Addis Ababa).

### Equipment and preparation of ingredients

The main equipments used in this study were plastic jars, plate and pan made from metal, grinding stones for wet-milling, bowls, grass baskets and sieve (1 mm pore). The adjuncts used were whole flour (Abegaz et al., 2004) from maize (*Zea mays*), sorghum (*Sorghum bicolor*) and from a mixture of wheat (*Triticum sativum*), finger millet (*Eleusine coracana*) and *tef* (*Eragrostis tef*). Barley (*Hordeum vulgare*) malt (*bikil*) was prepared following the traditional malting technique and 3% malt flour on weight basis of maize flour fermented at Phase I was used. The adjuncts were cooked at about 90°C (Abegaz et al., 2002b) into various forms (flat bread, steamed dough balls and thick porridge) and cooled to 23 - 25°C. The cooled flat bread, steamed dough balls and thick porridge were separately added into the plastic jar at Phase II, III and IV of *borde* fermentation, respectively; and blended with appropriate malt flour and/or the fermenting mash.

The proportions of adjuncts (on weight basis) were 3 kg maize flour for Phase I fermentation, 3 kg mixed flour (1 kg wheat, 1 kg finger millet, 1 kg *tef*) for dough balls, and 1.5 kg sorghum flour for porridge. The maize flour was fermented with equal amount (3 L) of water (w/v) for 24 h before baking a portion of it (40%) into flat bread on a hot plate. The remaining portion of dough (60%) was fermented for a further 24 h before steam cooking 40% of it with 3 kg mixed flour into dough balls or boiling 20% of it with 1.5 kg sorghum flour into a thick porridge. On the other hand, the proportion of total adjunct flour (on weight basis) before cooking was 14, 56 and 28% according to the adding time of cooked adjuncts at Phase II (flat bread), III (steamed dough balls) and IV (thick porridge) into the main fermentation jar, respectively (Abegaz et al., 2002b). Malt was added at Phase II (80% of the required malt) and Phase IV (the remaining 20%).

### Steps in traditional fermentation of *borde*

The traditional fermentation of *borde* has four major phases marked by the addition of fresh ingredients into the fermentation jar at different time. The full details of the process and ingredients used in each phase are described by Abegaz et al. (2002a, b). However, the four phases are described briefly as follow:

**Phase I:** Fermentation of maize flour for 24 to 48 h before various types of cooking.

**Phase II:** Blending 80% of the required malt with pancake-like flat bread (*kita*) prepared from 24 h fermented maize dough into a thick mash (*tinsis*) and the *tinsis* left for 24 h fermentation/

**Phase III:** Addition of steamed dough balls (*gafuma*), prepared from 48 h fermented maize dough and mixed flour, into the 24 h fermented *tinsis*, blending the mix to a thick mash (*difdif*) and the *difdif* left for a further 18 h fermentation.

**Phase IV:** Addition of thick porridge from 48 h fermented maize dough and sorghum flour along with 20% of the required malt into the 18 h fermented *difdif*. After homogenization of the mix to thick slurry (*mulet*), it was wet-sieved using a sieve (*wonfit*) of 1 mm pore. The filtrate was poured back into the jar and left for 4 h final fermentation.

The traditional fermentation of *borde* was done in duplicate and repeated three times at room temperature (20- 23°C). During fermentation, appropriate samples were taken at 6 h intervals for determination of pH, titratable acidity (TA), microbial counts and isolation of LAB.

### Microbiological enumeration and isolation

Samples (25 g or mL) were taken aseptically at 6 h intervals during traditional fermentation of *borde* and transferred separately to a Stomacher (Lab Blender 400, Seward Medical, London, England)

with 225 mL sterile 0.1% peptone water and then homogenized for 30 s, 'normal' speed. The homogenate was then serially diluted and aliquots of 0.1 mL from appropriate dilutions were spread-plated in duplicate on pre-dried agar plates of violet red bile dextrose (VRBD), plate count agar (PCA), MRS, and yeast extract glucose chloramphenicol bromophenol blue (YGC). All the culture media are from Merck (KGaA, 64271 Darmstadt, Germany) except YGC, which consisted of (gram $L^{-1}$): yeast extract, 5.0; glucose, 20.0; chloramphenicol, 0.1; bromophenol blue, 0.01; agar, 15; pH, 6.0 to 6.2. After incubation at 30°C for 24 h, purple-red colonies on VRBD agar plates were counted as *Enterobacteriaceae* (EB). The total aerobic mesophilic count (AMC) was enumerated on PCA plates after incubation at 30°C for 48 h. Colonies of LAB were counted on MRS agar plates after anaerobic incubation in GasPak jars (GasPak System, BBL) at 30°C for 72 h. Yeast and mould colonies were counted on YGC plates after incubation at 28°C for 3 to 5 days. The numbers of EB, AMC, LAB or yeasts from their respective duplicate countable plates are reported as log CFU $g^{-1}$ calculated from the arithmetic mean of three replicates of *borde* preparation. Three hundred fifty eight (358) representative colonies with distinct morphological differences such as colour, size and shape were randomly picked from countable MRS agar plates as presumably LAB isolates. Each LAB isolates were purified by repeated streak-plating on MRS agar for three times; maintained on appropriate slants at 4°C and sub-cultured every four weeks until required for characterization.

**Characterization and identification**

All the LAB isolates were characterized using phase-contrast microscopy and conventional biochemical and physiological tests. All isolates under examination were separately propagated twice in appropriate MRS broth and an overnight culture (inoculum) was used for all tests and incubated anaerobically (GasPak System, BBL) at 30°C. Each strain was initially examined for colony and cell morphologies, cell grouping, presence or absence of spores and motility using microscopy; Gram-reaction using the KOH test of Gregersen (1978); catalase production with 3% $H_2O_2$ (v/v); and separation into phenotypic groups was undertaken. The vigor of colony and broth culture in the growing media was also examined. Only the Gram-positive, catalase-negative and non-spore former isolates were characterized. Growth at different temperatures was observed in MRS broth after incubation at 15, 37 and 45°C for 5 days, and at 4°C and 10°C for 12 days. Growth in the presence of 4, 6, 8 and 10% NaCl was performed in MRS broth and incubated at 30°C for 5 days. Gas ($CO_2$) production from glucose was determined in modified MRS broth containing inverted Durham tubes, with diammonium citrate replaced by ammonium sulphate (Samelis et al., 1994). Growth of isolates was also assessed after inoculating 1% of 6 log CFU $mL^{-1}$ in sterilized *tinsis* (100 g) for *borde* production. Eighty seven (87) representatives of the isolates from the best *borde* that showed vigorous growth (increased by 3 to 4 log cycles in 24 h) in the sterilized *tinsis* were selected for further characterization as potential starter culture.

Gas ($CO_2$) production was determined after vigorously shaking of sealed headspace vials that contained 10.0 mL modified MRS broth culture (24 h) for 2 min and injecting 0.5 mL headspace gas twice using a sterile 1mL disposable syringe (Terumo Europe N.V., Leuven, Belgium) directly into the rubber tubing supplying nitrogen to the Infra-Red carbon dioxide analyzer (ADC 225 MK3, Analytical Development Co. Ltd., Hoddesdon, Hertfordshire, England) connected to a Chromatopac (C-R3A, Shimadzu corporation Analytical Instruments, Kyoto, Japan). Growth in pH 3.9, adjusted with HCl, was tested in MRS broth. Production of ammonia from arginine was tested according to the method described by Harrigan and McCance (1976) and also in modified MRS broth (Samelis et al., 1994).

The LAB isolates were tested for fermentation of the following 14 sugars (Merck): L-arabinose, ribose, D-xylose, galactose, fructose, maltose, lactose, saccharose, trehalose, raffinose, mannltol, sorbitol, salicin and esculin. In this test, phenol-red broth base was used as basal medium. A 1% filter-sterilized sugar solution, using 0.2 µm filter (Minisart-plus, sartorius, Germany), was added aseptically into autoclaved phenol-red broth base before inoculation with overnight culture of each LAB strain. The results were assessed with reference to the control after anaerobic incubation at 30°C for 5 days. Furthermore, to determine the biochemical profiles of LAB strains, 68 isolates were tested for fermentation of 50 carbohydrates, using API 50CH Lactobacillus Identification System (Bio Merieux, 69280 Marcy l'Etoile, France). The API 50 CHL medium was used according to the manufacturer's instructions. Anaerobiosis in the inoculated strips was obtained by overlaying with sterile paraffin oil and incubated at 30°C and then observations were made after 24 and 48 h. Results from conventional method of sugar test and the API 50 CHL system were compared for curiosity.

After identification, 24 h broth cultures at 30°C were centrifuged (Kubota 2010, Kubota Corporation, Tokyo, Japan) at 3000 rpm for 15 min and the pellets were re-suspended in MRS broth containing 10% (v/v) glycerol (Merck). The suspension was aseptically transferred into sterile cryo-tubes containing acid-washed glass beads and stored at -80°C for further investigation aimed at development of starter culture for *borde* fermentation.

**Statistical analysis**

The data from biochemical, morphological and physiological tests were analyzed with Principal Component Analysis using the Unscrambler® Programme (Unscrambler® v7.5, CAMO ASA, N-0115 Oslo, Norway). The positive and negative responses (Table 2) that are made by all the LAB strains were not included to this statistical analysis. Interpretation of the data was made by inspection of the scores and loadings plots. Identification of LAB strains was facilitated by the use of a computer programme, APILAB PLUS, version 3.2.2 (Bio Merieux), Bergey's Manual of Systemic Bacteriology (Sneath et al., 1986) and The Genera of Lactic Acid Bacteria (Wood and Holzapfel, 1995).

## RESULTS

The initial counts of LAB at Phase I were low ($10^4$ CFU $mL^{-1}$) but a rapid increase was observed within 12 h and then reached to about $10^9$ CFU $mL^{-1}$ after 24 h fermentation. The changes in pH, TA and microbial load during fermentation of *borde* are shown at Figure 1. The growth of AMC was comparable to that of LAB though the AMC were somewhat higher until 12 h from the initiation of fermentation. A rapid decrease in pH was in agreement with accelerated growth rate of LAB and then followed by reduction of *Enterobacteriaceae*. Despite the AMC and LAB enter stationary growth phase, the yeasts were constantly increasing. Moulds were declined as the fermentation progressed and reached at non-detectable level after 12 h at Phase I and after 6 h at Phase II (data not shown). There were no moulds at Phase III and IV fermentation. Representatives of LAB from each phase of *borde* fermentation were isolated, characterized and identified using physiological and biochemical methods

**Table 1.** Morphological and physiological properties of LAB isolated during *borde* fermentation.

| Characteristics | Percentage of strains with positive reactions | | | | | |
|---|---|---|---|---|---|---|
| | Cluster No.[a] | I | II | III | IV | V |
| | No. of strains | 31 | 21 | 5 | 10 | 1 |
| Cell morphology[b] | | R | R | 4C/1R | C | R |
| $CO_2$ from glucose | | 100 | 100 | 0 | 0 | 0 |
| Growth at 4°C | | 39 (48)[c] | 81 (19) | 20 (80) | 90 (10) | 100 |
| 10°C | | 100 | 100 | 100 | 100 | 100 |
| 15°C | | 100 | 100 | 100 | 100 | 100 |
| 37°C | | 100 | 100 | 100 | 100 | 100 |
| 45°C | | (39) | 48 (43) | 60 | 90 | 0 |
| Growth in 4% | | 100 | 100 | 100 | 100 | 100 |
| 6% | | 100 | 100 | 100 | 100 | 100 |
| 8% NaCl | | 84 (13) | 86 (5) | 100 | 100 | 100 |
| 10% NaCl | | 0 (3) | 0 | 0 | 30 | 0 |
| Growth at pH 3.9 | | 42 (58) | 100 | 60 (40) | 100 | 100 |

[a]Cluster No. I = *Lb. viridescens* and *Lb. brevis*; Cluster No. II = *W. confusa*; Cluster No. III = *P. acidilactici*, and *Ent. Flavenscens*; Cluster No. IV = *P. pentosaceus* and *P. pentosaceus* subsp. i*ntermedius*; Cluster No. V = *Lb. delbrueckii* subsp. *delbrueckii*.
[b]R, rod shape; C, cocci (spherical shape);
[c]the numbers in parenthesis indicate percent of strains that showed weak or delayed reactions.

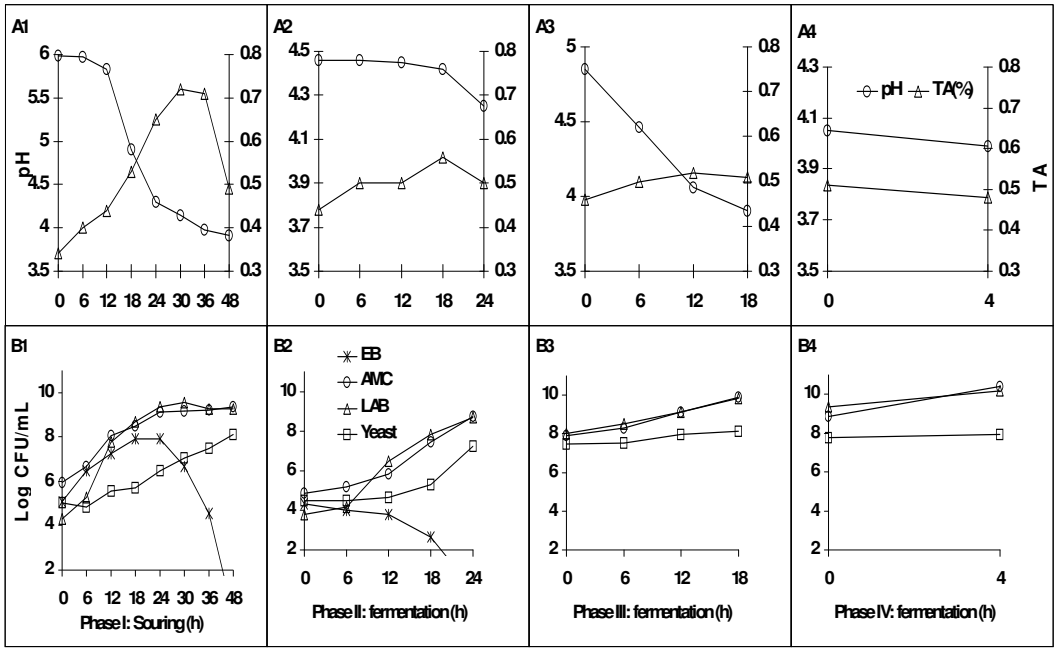


**Figure 1.** Changes in pH and TA and microbial growth during fermentation of *borde*. TA, titratable acidity expressed as lactic acid (%); EB, *Enterobacteriaceae*; AMC, aerobic mesophilic count; LAB, lactic acid bacteria; **A1-A4**, changes in pH and TA; **B1-B4**, changes in microbial growth.

aiming at selection and development of starter culture.

All the isolates were Gram-positive and catalase-negative rods or cocci except two of them. Basic data including Gram-reaction; morphology; gas production from glucose; growth at 4, 10, 15, 37 and 45°C; and growth in 2, 4, 6, 8 and 10% NaCl were essential (Table 1) in addition to the pattern of carbohydrate fermentation using API 50CH strips (Table 2). All the strains showed

**Table 2.** Differences in fermentation of carbohydrates by LAB strains isolated from *borde*.

| Carbohydrates[a] | Percentage of strains with positive reactions to API 50 CH | | | | | |
|---|---|---|---|---|---|---|
| | Cluster No.[b] | I | II | III | IV | V |
| | No. of strains | 31 | 21 | 5 | 10 | 1 |
| Arabinose | | 65 (13)[c] | 57 | 80 | 40 | 0 |
| Ribose | | 42 | 52 (5) | 60 | 100 | 100 |
| Xylose | | 3 | 100 | 100 | 10 | 0 |
| Galactose | | 3 (65) | 76 (19) | 100 | 100 | 100 |
| Fructose | | 94 | 100 | 100 | 100 | 100 |
| Mannose | | 94 | 100 | 100 | 100 | 100 |
| Rhamnose | | 0 | 0 | 40 (20) | 50 | 0 |
| Mannitol | | 0 | 0 (5) | 40 | 0 | 0 |
| Methyl-D-mannoside | | 0 | 0 | 0 (20) | 0 (10) | 0 |
| Methyl-D-glucoside | | 3 | 0 | 0 | 0 | 0 |
| Amygdalin | | 3 | 86 (10) | 100 | 100 | 0 |
| Arbutin | | 3 (6) | 76 (14) | 100 | 100 | 0 |
| Esculin | | 3 (3) | 100 | 100 | 100 | 0 |
| Salicin | | 3 (3) | 81 (14) | 100 | 100 | 0 |
| Cellobiose | | 0 | 95 (5) | 100 | 100 | 0 |
| Maltose | | 100 | 100 | 80 | 100 | 100 |
| Lactose | | 0 | 14 (10) | 40 (20) | 80 (20) | 0 |
| Melibiose | | 3 | 0 | 0 (20) | 40 (10) | 0 |
| Sucrose | | 94 | 90 | 0 | 0 | 100 |
| Trehalose | | 0 | 0 | 80 | 90 | 100 |
| Starch | | 0 | 0 | 40 | 0 | 0 |
| Gentiobiose | | 0 | 95 (5) | 100 | 100 | 0 |
| D-Togatose | | 0 | 5 | 40 | 90 | 0 |
| Gluconate | | 55 (10) | 81 (5) | 20 | 0 (30) | 0 |
| 5-Keto-gluconate | | 3 | 0 | 0 | 0 | 0 |

[a]All strains fermented glucose and N-acetyl glucosamine, but none of the strains fermented glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl xyloside, sorbose, dulcitol, inositol, sorbitol, inulin, melezitose, raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, and 2-keto-gluconate.
[b,c] See [a] and [c] under Table 1, respectively.

growth at 10, 15 and 37°C incubations and in 4 and 6% NaCl; but growth at 45°C and growth in 8 and 10% NaCl were important for differentiation. When the strains inoculated into API 50CHL medium were further incubated for 24 to 72 h, their responses were similar to that of the conventional method of sugar test carried out in test tubes for 5 days. Although the data on response of LAB strains in API 50CH strips was taken after 24 and 48 h incubation (according to manufacturer's instruction), some of the strains showed slow positive reaction after 24 to 48 h further incubation. However, except for curiosity on the response of wild-type LAB strains on API 50CHL compared to the conventional method, the data after 48 h incubation of API 50CHL were not utilized for identification of the isolates. Results from arginine test were not included for identification. Because, some of the strains were repeatedly showed positive responses both on treatment and control tests for ammonia production that could be from hydrolysis of arginine or other compounds.

Most of the strains appeared to be lactobacillus species (79.4%), while pediococcus species were dominating the cocci (20.6%). However, the strains of *Lactobacillus* were found to be heterofermentative, which is in agreement with reports elsewhere (Hounhouigan et al., 1993; Ketema et al., 1998), except one incidence of homofermentative species identified from the end product. As a result of Principal Component Analysis, the LAB strains were grouped into 3 major and 2 minor clusters (Figure 2) based on similarities in biochemical, morphological and physiological characteristics. A total of 68 strains of LAB isolates were identified.

Cluster I consisted of *Lb. viridescens*, *Lb. brevis*, *Lb. curvatus*, *Lb. collinoides* (fructose negative and grew at

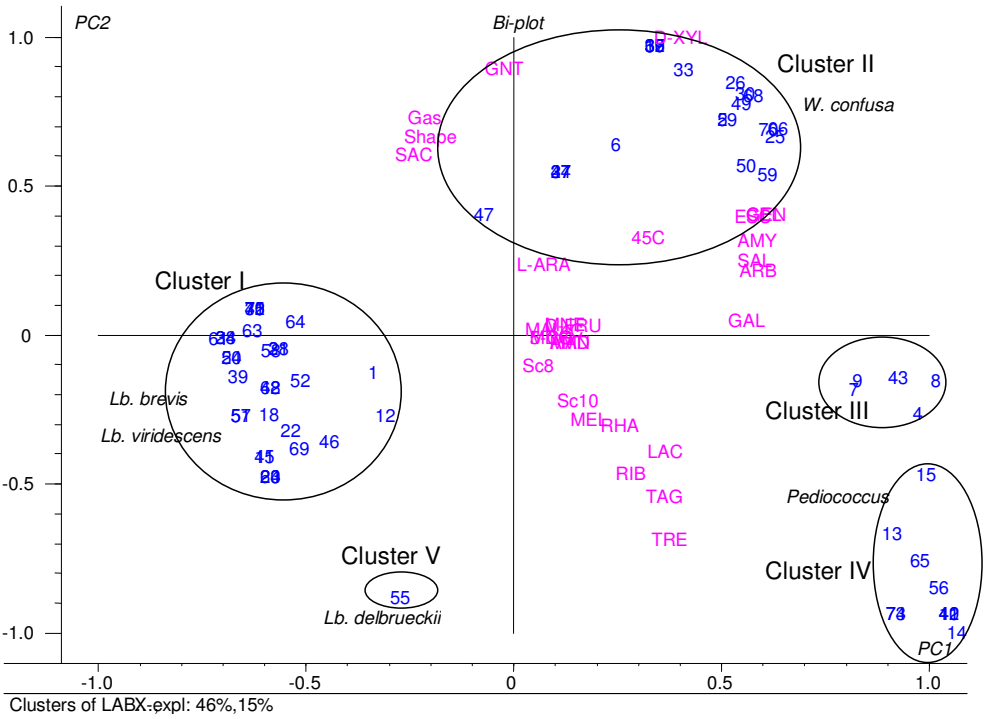


**Figure 2.** Principal component analysis of characterization tests on LAB isolated during traditional fermentation of *borde*. Number 1 to 74 are reference no. of strains; Cluster I includes also sporadic strains: *Lb. collinoides* (1), *Lb. carvatus* (45, 46 and 60), *Lb. sanfrancisco* (18), *Lb. pontis* (22); Cluster III includes *P. acidilactici* (4), *E. flavescens* (7 and 9); *P. pentosaceus* (8), unidentified (43); gas, $CO_2$ production from glucose; 15C or 45C, growth at 15 or 45°C; Sc8 or Sc10, growth in 8 or 10% salt concentration; carbohydrates, three letter abbreviations from API 50 CHL medium.

45°C), *Lb. pontis* (L-arabinose positive) and *Lb. sanfrancisco* (mannose positive). The latter three strains were isolated only at Phase I, while *Lb. curvatus* (cellobiose negative) was isolated from Phase II and III fermentation. In contrast, *Lb. viridescens* and *Lb. brevis* were found throughout the four phases of *borde* fermentation. However, differentiation between some of the *Lb. viridescens* (L-arabinose, ribose and gluconate positives)and *Lb. brevis* (ribose and gluconate negatives but grown at 45°C) strains may require further studies using tech-niques of molecular genetics. There are also one salicin positive *Lb. brevis* and another amygdalin positive *Lb. viridescens* strains. Otherwise, all the *Lb. brevis* and *Lb. viridescens* strains in this cluster are showed positive (fructose, mannose and sucrose) and negative (xylose and melibiose) responses on these tests. In general, *Lb. viridescens* strains showed weak growth both in pH 3.9 and at 4°C incubation tests.

Cluster II contained *W. confusa* where some of the 21 strains were arabinose positive and ribose negative (8), arabinose positive (2), lactose positive (2), amygdalin and salicin negative (1). Four of the above 8 strains were not fermented gluconate and another one was sucrose negative. Cluster III consisted of *Enterococcus flavenscens*, *P. acidilactici*, *P. pentosaceus* that were isolated during fermentation of maize dough at Phase I. This cluster also included an unidentified short rod LAB strain isolated from Phase III fermentation. In this study, *Ent. Flavenscens* strains were the only isolates that fermented mannitol and starch. The *Ent. flavenscens* grew weakly at pH 3.9 but not grew at 45°C. Cluster IV included *P. pentosaceus* and *P. pentosaceus* subsp. i*ntermedius* (Wood and Holzapfel, 1995), which are isolated throughout the fermentation. Fermentation of arabinose and rhamnose by *P. pentosaceus* was important to discriminate the subspecies. But one of the *P. pentosaceus* subsp. i*ntermedius* strains is rhamnose positive and D-togatose negative. In addition, one *P. pentosaceus* strain is xylose positive and trehalose negative. Cluster V contained *Lb. delbrueckii* subsp. *delbrueckii*, the only homofermentative lactobacillus strain in this report. It is galactose and ribose positive and also does not grow at 45°C.

The frequencies of dominating LAB strains were as follows: *W. confusa* (30.9%), *Lb. viridescens* (26.5%), *Lb. brevis* (10.3%) and *P. pentosaceus* (7.4%) and *P. pentosaceus* subsp. *intermedius* (8.8%). In addition, *P. acidila-*

**Table 3.** Distribution of the dominating LAB strains in the four phases of *borde* fermentation.

| Phases and Major activities | LAB[a] counts (log cfu/g) | Reference No. of LAB strains | Dominating strains of LAB |
|---|---|---|---|
| **Phase I** Fermentation (souring) of maize dough before cooking | 0 h (4.3) 24 h (9.4) 48 h (9.3) | 1, 4, 5, 6, 7, 8, 9, 10 11, 12, 13, 14, 15 | *W. confusa*, *L. brevis*, *L. viridescens* and *P. pentosaceus* |
| **Phase II** Blending of flat bread and malt flour into *tinsis* | 0 h (3.8) 24 h (8.7) | 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 | *W. confusa*, *L. brevis*, *L. viridescens* and *P. pentosaceus* |
| **Phase III** Blending of steamed dough balls and *tinsis* into *difdif* | 0 h (8.0) 18 h (9.9) | 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 73 | *W. confusa*, *L. brevis*, *L. viridescens* and *P. pentosaceus* |
| **Phase IV** Blending of porridge and malt with *difdif*, wet-sieving into *mulet* | 0 h (9.4) 4 h (10.2) | 52, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 74 | *W. confusa*, *L. brevis*, *L. viridescens* and *P. pentosaceus* |

[a] LAB (lactic acid bacteria) counts at the beginning (0 h) and the end of each phase.

*ctici* (1.5%), *Ent. flavescens* (2.9%) and *Lb. collinoides* (1.5%) at Phase I fermentation; *Lb. sanfrancisco* (1.5%) and *Lb. pontis* (1.5%) at Phase II fermentation; non-identified isolate (1.5%) at Phase III fermentation; and *Lb. delbrueckii* subsp. *delbrueckii* (1.5%) at Phase IV fermentation; *Lb. curvatus* (4.4%) both at Phase III and IV fermentation were sporadically found as part of the LAB population during the fermentation of *borde*. Succession of the dominating LAB strains in *borde* fermentation is shown in Table 3. A wider range of sporadic species of LAB including *E. flavescens* was also present at early period of the fermentation. In general, heterofermentative *Lactobacillus* strains and homofermentative *Pediococcus* spp were found to be involved from the beginning to the end of *borde* fermentation. However, *W. confusa*, *Lb. viridescens*, *Lb. brevis* and *P. pentosaceus* and *P. pentosaceus* subsp. *intermedius* appeared to be the most important LAB population in *borde* fermentation.

## DISCUSSION

The natural fermentation of *borde* is unpredictable. Spontaneous fermentation typically results from the competitive activities of different microorganisms whereby strains best adapted and with the highest growth rate will dominate during particular stages of the process. Among the bacteria associated with food fermentation, LAB are of predominant importance. The LAB are known to be associated with many natural and man-made environments. It was reported elsewhere (Damelin et al., 1995) that samples from plant material showed the greatest diversity of LAB with *Lactobacillus* strains being predominant in food-related ecosystems.

It appeared that *Enterobacteriaceae* pioneered *borde* fermentation at Phase I as shown by their early leading rate of growth that was followed by the succession of LAB (Figure 1 B1). The initial high pH 6.0 of the dough fermentation at Phase I (Figure 1 A1) would explain the growth of *Enterobacteriaceae* (Figure 1 B1), including *Enterococcus* spp which is indicated by the isolation of *Ent. Flavescens*, while the lower initial pH 4.4 at Phase II fermentation inhibited their growth (Figure 1 A2). The high number of LAB attained after 12 h dough fermentation in Phase I was responsible for a marked reduction of pH and increase in TA (Figure 1 A1) probably thereby the inhibition of *Enterobacteriaceae* (Nout et al., 1989; Lorri, 1993). In Phase I, dough fermentation for 24 h appeared to be a turning point for an accelerated reduction in number of *Enterobacteriaceae* and stabilizing the maximum numbers of aciduric bacteria that are involved in the fermentation. This would be the maximum aciduric bacterial population attainable in this dough fermentation. The cooking of fermented dough at about 90°C would kill vegetative microbial cells in it. Otherwise, the cooked cereal ingredients showed no LAB and yeast except bacterial spores (Abegaz et al., 2002b), which cannot grow in acidic condition. However, the addition of malt flour to unmalted and cooked ingredients at Phase II is the source of both starter microorganisms and endogenous enzymes that initiate the main fermentation of *borde*. The microflora on the malt would comprise the normal flora of

the grains and also contaminants from threshing and equipment associated with storage and malting process (Sahle and Gashe, 1991; Ketema et al., 1998). Processing utensils, water and the brewing personnel constitute additional sources of microorganisms that could initiate the fermentation at Phase II.

A balance between effects of low pH on selection of aciduric microorganisms and on activity of malt enzymes at Phase II would be vital for the subsequent stages of *borde* fermentation. The addition of pancake-like flat bread from 24 h fermented maize dough was responsible for a low initial pH at Phase II and also partially affected the pH at the start of Phase III and IV. In fact, the bulk of unmalted mixed cereal ingredients (56%, on weight basis) and high microbial inoculum that had been built up in Phase II (*tinsis*) were blended at the start of Phase III. Thus, the fermentation at Phase II and Phase III can be considered as starter culture building up and bulk fermentation phases, respectively. The high microbial load observed in *borde* is in agreement with Zimbabwean *mangisi* (Zvauya et al., 1997). Since the number of fermenting microorganisms at the start of Phase IV is very high, the small amount of malt added in Phase IV is mainly to hydrolyse the newly added unmalted adjuncts and sweeten the *borde*. A few hours fermentation at Phase IV meant for attainment of optimal sensory attributes of a sweet-sour *borde*.

Finally, since further holding renders *borde* too sour, it should be consumed as a sweet-sour product while actively fermenting. Despite unhygienic wet-milling and wet-sieving process (Abegaz et al., 2002a, b), the low pH of *borde* would hopefully make it safe for consumption. The acidic fermentation and LAB metabolites are also responsible for inactivation of *Enterobacteriaceae* including toxin-producing and foodborne infectious pathogens (Nout et al., 1989; Lorri, 1993, Dessie et al., 1997; Byaruhanga et al., 1999; Kunene et al., 1999; Ana et al., 2006).

Although the possible sources of chance inocula for fermentation of dough at Phase I from maize flour and main fermentation at Phase II from malt flour appeared to be different due to the effects of cooking and malting processes, the dominating LAB were similar in all the four phases of *borde* fermentation. The LAB were in the minority at start of Phase II fermentation with the addition of malt flour but they were the dominating flora as the fermentation progressed. This indicates the significant role of LAB in *borde* fermentation. The growth of both LAB and yeast populations during fermentation of *borde* would be indicative for their involvement. However, yeasts are known to facilitate alcoholic fermentation, while *borde* is supposed to be non-alcoholic beverage. Hence the present work focused on characterization and identification of LAB aimed at selection of potential starter culture for controlled fermentation of *borde*.

The group of LAB includes several genera of bacteria that differ considerably in morphological, physiological and functional properties. The API LAB program distinguishes between species on the basis of pattern-matching principle and assesses the entire group of LAB as a whole (Figure 2). The differentiation efficiency of commercially available identification test kits such as API 50 CHL System is based on testing the organisms' ability to assimilate or ferment a fixed number of specific substrates. The resulting fermentation patterns of wild-type LAB strains in the present work were compared with patterns obtained from recognized test organisms from known source. In a group as heterogeneous as LAB, this approach may lead to results with wrong species and even wrong genus particularly for those wild-type strains isolated from peculiar niche. According to API 50 CHL System, it was particularly difficult to distinguish between some of the *Lb. brevis* and *Lb. viridescens* strains isolated from *borde*. However, this investigation is aimed at identification of wild-type LAB strains to select suitable starter cultures that are adapted to the ecological niche of *borde* fermentation. Accordingly, the identified *W. confusa*, *Lb. brevis*, *Lb. viridescens*, *P. pentosaceus* and *P. pentosaceus* subsp. *intermedius* are adapted to the ecological niche of *borde* fermentation and overcome its production technology hurdles. Thus, they could be suitable candidate starter cultures for *borde* production in the near future.

Several of the LAB isolated from *borde* have also been isolated from various fermented foods. Hounhouigan et al. (1993) isolated *W. confusa*, *Lb. brevis*, *Lb. curvatus*, *P. pentosaceus* and *P. acidilactici* during spontaneous fermentation of mawe from maize. Uchimura et al. (1991a) reported that the principal coccus in Indonesian ragi was *P. pentosaceus*, which is in agreement with the present report. Hancioglu and Karapinar (1997) and Getcheva et al. (2000) isolated *W. confusa*, *Lb. brevis* and *Lb. sanfrancisco* from *boza*, a thick beverage related to *borde* (the investigator's personal observation). Paludan-Muller et al. (1999) isolated *W. confusa*, *Lb. brevis*, *P. pentosaceus* and *Lb. curvatus* during fermentation of *som-fak* prepared from minced fish fillet, ground boiled rice and garlic. Many other investigators also isolated *W. confusa*, *Lb. brevis*, *Lb. viridescens* and *Pediococcus* spp. (Nout, 1980; Sakai and Caldo, 1985b; Samelis et al., 1994; Johansson et al., 1995; Olasupo et al., 1997; Sanchez et al., 2000; Corsetti et al., 2001; Leisner et al., 2001; Gobbetti et al., 2005), *P. pentosaceus* and *P. acidilactici* (Halem et al., 1993), *Lb. collinoides* (Leisner et al., 2001) and *Lb. delbrueckii* subsp. *delbrueckii* (Olasupo et al., 1997; Corsetti et al., 2001) as part of the LAB population in different fermented foods. *Ent. flavescens* has been isolated from human clinical specimens and its habitat and pathogenic significance are unknown (Wood and Holzapfel, 1995). However, the *Ent. flavescens* was not detected after 18 h of fermentation at Phase I, even if, cooking of the dough could safeguard from further contamination of the product. The presence of *Ent. flavenscens* strains that fermented starch at Phase I may

also be an advantage for hydrolysis of starch and thus create a suitable ecosystem for the proliferation of microbes, like that of the malt enzymes at Phase II.

It was reported elsewhere (Nout, 1980; Johansson et al., 1995; Olasupo et al., 1997; Paludan-Muller et al., 1999; Getcheva et al., 2000; Sanchez et al., 2000; Leisner et al., 2001) that *Lb. plantarum* in fermented plant materials was dominating the LAB flora. However, there was no *Lb. plantarium* observed during the acidic fermentation of *borde* in the present work. Among the dominating strains, *Lb. viridescens* may not be a potential starter culture for acidic fermentation of *borde* due to its weak growth in pH 3.9. Moreover, there was no accessible publication on use of *Lb. viridescens* as starter culture in fermentation of plant material. If any of the strains appeared to be important starter culture for *borde* fermentation, necessary investigation would be carried out for further details. Otherwise, the evaluation of *W. confusa*, *Lb. brevis* and *P. pentosaceus* as starter culture for *borde* fermentation is underway.

In conclusion, the results of this study indicated an association of LAB, composed of heterofermentative *Lactobacillus* strains and homofermentative *Pediococcus* spp, involved from the beginning to the end of *borde* fermentation. The least initial counts, at Phase I and II fermentation, and the highest final counts of LAB indicate their important role in *borde* fermentation. A wider diversity of LAB strains (Cluster I and III), both rods and cocci, were present at the early period of maize dough fermentation at Phase I than any other phases of *borde* fermentation. The number of species reduced as the fermentation progressed and finally, *W. confusa*, *Lb. brevis*, *Lb. viridescens* and *P. pentosaceous* spp were the most significance species. Despite these species of LAB are adapted to the substrates and overcome the possible physiological, technological and biochemical hurdles during the four phases of *borde* fermentation, it will also be important to assess their performance on the actual ingredients of *borde* under controlled fermentation and then select for the best starter culture. This approach would be a paramount strategy (among others) to develop starter culture, to produce microbiologically stable and predictable end product aimed at upgrading and commercial production of *borde*.

## ACKNOWLEDGEMENTS

The author would like to thank the Norwegian Universities Committee for Development Research and Education (NUFU), for sponsoring this work; and Mrs Warite Alambo for the traditional brewing of borde.

### REFERENCE

Abegaz K, Beyene F, Langsrud T, Narvhus JA (2002a). Indigenous processing methods and raw materials of *borde*, an Ethiopian traditional fermented beverage. J. Food Technol. Afr. 7: 59-64.

Abegaz K, Beyene F, Langsrud T, Narvhus JA (2002b). Parameters of processing and microbial changes during fermentation of *borde*, a traditional Ethiopian beverage. J. Food Technol. Afr. 7: 85-92.

Abegaz K, Langsrud T, Beyene F, Narvhus JA (2004). The effects of technological modifications on the fermentation of *borde*, an Ethiopian traditional fermented cereal beverage. J. Food Technol. Afr. 9: 3-12.

Ana ATC, Rosinea AP, Hilario CM, Celia AM (2006). Inhibition of *Listeria monocytogenes* by lactic acid bacterium isolated from Italian salami. Food Microbiol. 23: 213-219.

Byaruhanga YB, Bester BH, Watson TG (1999). Growth and survival of *Bacillus cereus* in mageu, a sour maize beverage. World J. Microbiol. Biotechnol. 15: 329-333.

Corsetti A, Lavermicocca P, Mprea M, Baruzzi F, Tosti N, Gobbetti M (2001). Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy. Int. J. Food Microbiol. 64: 95-104.

Damelin LH, Dykes GA, von Holy A (1995). Biodiversity of lactic acid bacteria from food-related ecosyst.. Microbios 83: 13-22.

Deak T, Beuchat LR (1996). Handbook of Spoilage Yeasts. CRC Press, Inc. Boca Raton. pp 1-36, 61-154.

Dessie G, Abegaz K, Ashenafi M (1997). Effect of “siljo” fementation on growth of *Stahpylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes*. Ethiop. Med. J. 73: 432-434.

Getcheva V, Pandiella SS, Angelov A, Roshkova ZG, Webb C (2000). Microflora identification of the Bulgarian cereal-based fermented beverage boza. Process Biochem. 36: 127-130.

Gobbetti M, De Angelis M, Corsettti A, Di Cagno R (2005). Biochemistry and physiology of sourdough lactic acid bacteria. Trends Food Sci. technol. 16: 57-69.

Gregersen T (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. Eur. J. Appl. Microbiol, 5:123-127.

Gould GW (1992). Ecosystem approaches to food preservation. J. Appl. Bacteriol. (Symp. Suppl.) 73: S58-S68.

Haggblade S, Holzapfel WH (1989). Industrialization of Africa’s indigenous beer brewing. *In*: Industrialization of indigenous fermented foods (K.H. Steinkraus, ed.). Marcel Dekker, New York.

Halem M, Lillie A, Sørensen AK, Jakobsen M (1993). Microbiological and aromatic characteristics of fermented maize doughs for kenkey production in Ghana. Int. J. Food Microbiol. 19: 135-143.

Hancioglu O, Karapinar M (1997). Microflora of Bouza, a traditional fermented Turkish beverage. Int. J. Food Microbiol. 35: 271-274.

Harrigan WF, McCance ME (1976). Laboratory methods in food and dairy microbiology. Academic Press, London.

Hounhouigan DJ, Nout MJR, Nago CM, Houben JH, Rombouts FM (1993). Characterization and frequency distribution of species of lactic acid bacteria involved in the processing of mawe, a fermented maize dough from Benin. Int. J. Food Microbiol. 18: 279-287.

Holzapfel W (1997). Use of starter cultures in fermentation on a household scale. Food Control 8: 241-258.

Johansson ML, Sanni A, Lonner C, Molin G (1995). Phenotypically based taxonomy using API 50CH of Lactobacilli from Nigerian ogi, and the occurrence of starch fermenting strains. Int. J. Food Microbiol. 25: 159-168.

Ketema B, Tetemke M, Mogessie A (1998). The microbial dynamics of *borde* fermentation, a traditional Ethiopian fermented beverage. Ethiop. J. Sci. 21: 195-205.

Kunene NF, Hastings JW, von Holy A (1999). Bacterial populations associated with a sorghum-based fermented weaning cereal. Int. J. Food Microbiol. 49: 75-83.

Lorri WSM (1993). Nutritional and Microbiological Evaluation of Fermented Cereal Weaning Foods. Ph.D. Thesis. Dept. of Food Science, Chammers University of Technology, Goteborg, Sweden.

Leisner JI, Vancanneyt M, Rusul G, Pot B, Lefebvre K, Fresi A, Tee LK (2001). Identification of lactic acid bacteria constituting the predominating microflora in an acid-fermented condiment (tempoyak) popular in Malaysia. Int. J. Food Microbiol. 63: 149-157.

Nout MJR (1980). Microbiological aspects of the traditional manufacture of Bussa, a Kenyan opaque maize beer. Chem. Microbiol. Technol.

Lebensm. 6: 137-142.
Nout MJR, Rombouts FM, Havelaar A (1989). Effect of accelerated natural lactic acid fermentation of infant food ingredients on some pathogenic mocroorganisms. Int. J. Food Microbiol. 8: 351-361.
Olasupo NA, Olukoya DK, Odunfa SA (1997). Identification of *Lactobacillus* species associated with selected Aftrican fermented foods. Z. Naturforsch. 52: 105-108.
Paludan-Muller C, Huss HH, Gram L (1999). Characterization of lactic acid bacteria from a Thai low-salt fermented fish product and the role of garlic as substrate for fermentation. Int. J. Food Microbiol. 46:219-229.
Sahle S, Gashe BA (1991). The microbiology of *tella* fermentation. SINET: Ethiop. J. Sci. 14: 81-92.
Sakai H, Caldo GA (1985b). Microbiological and chemical changes in Tapuy fermentation. J. Ferm. Technol. 63: 11-16.
Samelis J, Maurogenakis F, Metaxopoulos J (1994). Characterization of lactic acid bacteria isolated from naturally fermented Greek dry salami. Int. J. Food Microbiol. 23: 179-196.
Sanchez I, Palop L, Ballesteros C (2000). Biochemical characterization of lactic acid bacteria isolated from spontaneous fermentation of 'Almagro* egg plants. Int. J. Food Microbiol. 59: 9-17.
Sneath PHA, Mair NS, Sharpe ME, Holt JG (1986). Bergey's Manual of Systematic Bacteriology, vol 2, Williams, Wilkins, Baltimore.
Uchimura T, Okada S, Kozaki M (1991a). Identification of lactic acid bacteria isolated from Indonesian Chinese starter, ragi. J. Brew. Soc. Jpn. 86: 55-61.
Wood BJB, Holzapfel WH (1995). The Genera of Lactic Acid Bacteria, vol. 2, Blackie Academic and Professional, Chapman and Hall, Glasgow.
Zvauya R, Mygochi T, Parawira W (1997). Microbiological and biochemical changes occurring during production of *masvusvu* and *mangisi*, traditional Zimbabwean beverages. Plant Foods Human Nutr. 51: 43-51.