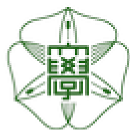
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1 **Title**

2 **Main microorganisms involved in the fermentation**

3 **of Ugandan ghee**

4

5

6 **Authors**

7 **Martin Patrick Ongol[a*] and Kozo Asano[b]**

8

9 **Authors Affiliations**

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12 [a] Institute for Technical Research, Glico Dairy Products Co., Ltd., 2-

13 14-1 Musashino, Akishima-Shi, Tokyo, 196-0021.

14 [b]Laboratory of Applied Microbiology, Graduate School of Agriculture,

15 Hokkaido University, Sapporo, 060-8589, Japan.

16

Abstract

Mashita, a traditional fermented butter-like product is widely produced in western Uganda. However, no detailed studies have been done to identify the microorganisms involved in *mashita* fermentation. Therefore, the aim of this study was to identify the microorganisms involved in *mashita* fermentation using culture-dependent and culture-independent techniques. The most commonly identified strains of lactic acid bacteria (LAB) in *mashita* using culture-dependent techniques were *Lactobacillus paracasei*, *Lactobacillus helveticus*, *Lactobacillus plantarum* and *Lactobacillus perolens* constituting 37.3%, 10.1%, 8.1% and 7.7% of total bacterial colonies isolated respectively. *L. paracasei* was the only bacterial species identified in all *mashita* samples. PCR-DGGE results indicated that acetic acid bacteria (AAB) and LAB were the dominant bacterial groups in *mashita*. *Acetobacter aceti*, *Acetobacter lovaniensis*, *Acetobacter orientalis* and *Acetobacter pasteurianus* were the main species of AAB identified in the *mashita* whereas *Bifidobacterium* sp., *Enterococcus faecium*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus acetotolerans*, *Lactobacillus* sp., *Lactococcus raffinolactis*, *Lactococcus* sp. and *Streptococcus salivarius* as the main strains of LAB in *mashita*. PCR-DGGE of the D1 region of yeasts showed that *Brettanomyces custersianus*, *Candida silvae*, *Geotrichum candidum*, *Issatchenkia occidentalis*, *Issatchenkia orientalis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, and *Trichosporon asahii* were part of the *mashita* microbial community. These results indicate that AAB, LAB and yeasts are involved in *mashita* fermentation.

40 *Key words*: Acetic acid bacteria; lactic acid bacteria; yeasts; ghee; PCR-DGGE;

41 traditional milk fermentation

42

43 **1.0 Introduction**

44 In Uganda ghee is processed by heat clarification of butter fat known as
45 *mashita* and is highly valued in the western part of Uganda. *Eshabwe*, a source
46 made by mixing ghee with warm water containing rock salt was used as a royal
47 pudding and is still used in Bahima/Banyankole cultural rituals. Meanwhile,
48 *obutahe,* a perfumed body cream is also made of out ghee. Ghee has an attractive
49 appearance, a grainy texture, a pleasant nutty aroma, a light yellow colour, and is
50 semisolid at room temperature (Sserunjogi, Abrahamsen & Narvhus, 1998; Gonfa,
51 Foster & Holzapfel, 2001). Its characteristic flavour serves as a major criterion for
52 acceptance (Sserunjogi, et al, 1998). A similar traditional ghee product referred to
53 as *neter kibe* and *desi* is also produced in Ethiopia and India respectively
54 (Abraham & Srinivasan 1980; Gonfa et al, 2001). Gourd (calabash), a dried fruit
55 wall of the plant *Lagenera peucantha* is used as the fermentation vessel.
56 Meanwhile the milk microflora and the microbial community established on the
57 inner surfaces of the gourd act as starter cultures.

58 Although ghee has been produced in Africa, Asia and the Middle East for
59 generations, it is manufacture is still largely based on indigenous traditional
60 methods and little is known about the origin of the desirable flavour components
61 of ghee (Abraham & Srinivasan, 1989; Sserunjogi, et al, 1998). Therefore, it is of
62 primary importance to obtain a reliable description of the physiologically active
63 microbial community in order to understand the role that different species of
64 lactic acid bacteria (LAB) play in dairy fermentations (Blaiotta, Pepe, Mauriello,
65 Villani, Andofi & Moschetti, 2002). Classically, such questions are addressed

66 through the enumeration of some microbial groups on a variety of culture media,
67 followed by identification through traditional microbiological methods (Blaiotta
68 et al, 2002). However, culture-based methods are time-consuming, limited in
69 terms of both discriminating ability and accuracy, and reveal a little portion of the
70 true populations in natural ecosystems (Giraffa & Neviani, 2001; Blaiotta, et al,
71 2002; Temmerman, Scheirlinck & Swings, 2003; Temmerman, Huys & Swings,
72 2004; Terzic-Vidojeviv, Jovcic, Begovic, Golic & Topisirovic, 2008). The
73 reasons essentially are the inability of detecting novel microorganisms, which
74 might not be cultivable with known existing media, inability of recovering known
75 microorganisms which are viable but enter a non-cultivable state (Giraffa &
76 Neviani, 2001). Selective enrichment cultures fail to mimic the conditions that
77 particular microorganisms require for proliferation in their natural habitat
78 (Muyzer, De Waal & Uitterlinden, 1993). More comprehensive insight into the
79 production process and the survival capacity of the introduced strains requires
80 analysis of both viable and nonviable bacteria (Temmerman, et al, 2003). Thus,
81 the description of bacterial diversity in traditional fermentations by culture-
82 dependent methods is difficult, cannot be accurately assessed by using a single
83 complex culture medium and these microbial ecosystems might be more complex
84 than believed until now (Miambi, Guyot & Ampe, 2003; Flórez & Mayo, 2006;
85 Parayre, et al, 2007).

86 Application of culture-independent methods such as denaturing gradient
87 gel electrophoresis (DGGE) in analysis of community profile of microbial species
88 involved in biotransformation of African traditional foods can enable

89 development of starter cultures, and identification of microorganisms that play a
90 major role in flavour development and spoilage. DGGE is a fast, reliable, and
91 reproducible culture-independent approach for analysis of probiotic products and
92 it has a greater detection and identification potential than conventional culture-
93 dependent analysis (Temmerman et al, 2003). DGGE is used to separate DNA
94 fragments of the same length but with different sequences (Pintado, Guyot &
95 Ampe, 2003). This allows determination of sequence variation in the
96 hypervariable sequence regions of the 16S rDNA gene, and can be used to study
97 microbial diversity and relative abundance in natural habitats (Muyzer et al, 1993).
98 Furthermore, using general bacterial 16S rDNA directed primers makes the
99 method not only widely applicable to any defined microbial mixed culture but
100 may also enable the detection of a wide range of contaminants (Pintado et al,
101 2003).

102 The fact that ghee made by indigenous methods has distinctively different
103 flavour seems to suggest that adventitious microorganisms participate in ghee
104 flavour biogenesis (Sserunjogi, et al, 1998). Of the methods used in the
105 preparation of ghee, namely indigenous (cream is subjected to natural souring by
106 indigenous microorganisms), direct cream, creamery butter and prestratification,
107 the indigenous method generally produces a product with the most desirable
108 flavour (Abraham & Srinivasan, 1980). Defining the microbial diversity in
109 fermented *mashita* is vital for starter culture development and to improve the
110 quality of traditionally fermented ghee. Therefore, the aim of this study was to
111 evaluate the microbial diversity in fermented *mashita* using the traditional culture-

112 based approach and DGGE in view of determining the major bacterial species that
113 might be involved in ghee fermentation, flavour development and spoilage.
114
115 **2. Materials and Methods**
116 *2.1. Brief description of traditional Mashita fermentation process*
117 Dry *Themeda triandra* (Red hood grass) locally known as *Emburara* is
118 burnt in a small fire pot referred to as *ekijunga*. The smoke is channelled inside
119 approximately 2 L gourds locally known as *ekyanzi* and covered tightly with the
120 lids prior to filling with milk. Milk is fermented in a cool place for about 12-48
121 and then poured into big gourds of about 20 to 28 L capacity locally known as
122 *ekisisi*. The big gourds are filled half way to enable easy churning. Churning starts
123 immediately and it involves rocking the gourd back and forth for 1 h followed by
124 sieving to drain out the watery (whey-like) milk meanwhile the *mashita* (cream)
125 remains inside the churning gourd. Clean water is then poured into the churning
126 gourd. The gourd is shaken and turned upside down so that the *mashita* can drain
127 into the collecting bucket. *Mashita* is then stored in a closed bucket for one month
128 as more cream is added each day or as often as it is being made. Impure salt
129 extracted from Lake Katwe in Uganda is mixed with water and poured into the
130 bucket. *Mashita* is kneaded with the salty water, washed and the water drained off.
131 Finally the *mashita* is scooped into the storage gourd. The *mashita* can then be
132 heat clarified to make ghee or it is packed into polythene bags and marketed.
133

134 *2.2.* Mashita *Sampling*

135 *Mashita* used in this study were randomly sampled from a makeshift
136 market adjacent to Kafu River Bridge on the Gulu-Kampala highway in Uganda.
137 Six *mashita* samples coded A, B, C, D, E, and F were obtained. All samples were
138 kept at 4ºC and aseptically handled. About 6 g of each sample was kept at -80ºC
139 freezer in 20% glycerol solution and DNA was later on extracted from these
140 samples for DGGE analysis.

141

142 *2.3. Isolation of bacteria*

143 *Mashita* was melted at 37ºC and 1 g of each sample was weighed into a
144 falcon tube followed by addition of 9 ml of sterile saline (0.85% NaCl) solution.
145 The samples were homogenised, 10 fold serial diluted in sterile saline and 0.1 ml
146 of each sample was spread plated on de Man Rogosa Sharpe (MRS) agar (Becton,
147 Dickinison Co., Sparks, Md., USA) and M17 agar (Becton, Dickinison Co.,
148 Sparks, Md., USA) containing glucose (10 g/l) as carbon source. Inoculated plates
149 were incubated at 37ºC for 48 h in an air tight jar (AnaeroPack Rectangular Jar™,
150 Mitsubishi Gas Chemical Co., Tokyo, Japan) containing an O_2 absorbing and CO_2
151 releasing pack (AnaeroPack™, Mitsubishi Gas Chemical Co., Tokyo, Japan).
152 Single colonies were picked and streaked on either fresh MRS or M17 agar plates
153 and incubated again as described above. This procedure was repeated at least
154 twice in order to purify the colonies. Isolated colonies were kept in 20% glycerol
155 solution at -80ºC.

156

157 *2.4. Extraction of DNA from bacterial cells*

158 Briefly cells were collected by centrifugation of 1 ml bacterial cultures at
159 10,000 x *g* for 10 min at 4ºC in an eppendorf tube. The cell pellets were washed
160 twice with sterile saline solution. Meanwhile bacterial cells in *mashita* were
161 collected by suspending about 1 g of *mashita* in 9 ml of sterile 2% (w/v)
162 trisodium citrate in a 50 ml falcon tube. The samples were warmed to 45ºC and
163 homogenised at 2500 rpm for 30 sec three times. The homogenates were then
164 centrifuged at 10,000 x *g* for 10 min at room temperature and the supernatant and
165 fat layers were removed. Pellets were again re-suspended in 9 ml of trisodium
166 citrate and centrifuged as described above. This process was repeated twice in
167 order to wash the cells and to remove residual fat. Bacterial cells were suspended
168 in 300 µl of bead solution. DNA was extracted using UltraClean™ Microbial
169 DNA isolation kit (MO BIO Laboratories Inc., Solana Beach, CA) according to
170 the manufacturer's instructions with minor modifications. Suspended bacterial
171 cells were incubated with lysozyme at a concentration of 20 mg/ml (Wako Pure
172 Chemical industries, Osaka Japan), *N*-acetylmuramidase (Seikagaku Tokyo,
173 Japan; final concentration 30 µg/ml) and Labiase (Seikagaku Tokyo, Japan; final
174 concentration 2.5 mg/ml) at 37ºC for 2 h to enable easy lysis of bacterial cell
175 walls. The tubes containing the cells, bead solution and beads were spun for 20 s
176 at 4 m/s on Fastprep™ FP120 (Qbiogene, Carlsbad, CA, USA.). The quantity of
177 DNA was determined by measuring absorbance at 260 nm with a Beckman DU
178 640 spectrophotometer (Beckman Coulter, Inc, Fullerton, CA). Isolated DNA
179 were either used immediately or stored at -20ºC.

180 *2.5. Extraction of DNA from yeast cells*

181 DNA from yeasts was isolated as described by Cocolin, Bisson & Mills,
182 (2000), with minor modifications. Briefly, the cell pellets were resuspended in
183 300 µl of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH
184 8, 1 mM EDTA pH 8). Three hundred µl of phenol/chloroform/isoamyl alcohol
185 (25:24:1; GIBCOBRL®, Canada) was added to the cell suspension. The cells were
186 mechanically disrupted in beater (FastPrep™, Bio101, USA) with 0.3 g of glass
187 beads (0.5 mm in diameter) at speed of 6.5 m/s for 30 s three times. About 300 µl
188 of TE buffer (10 mM Tris, 1 mM EDTA pH 7.6) was added and the mixture was
189 centrifuged at 15,000 x *g* for 15 min at 4ºC. The aqueous phase was transferred to
190 a 2 ml tube. DNA was precipitated with 2 volumes of 100% ice cold ethanol and
191 centrifuged at 15,000 x *g* for 15 min and the pellet was washed with 70% ethanol,
192 dried and resuspended in 50 µl of sterile distilled water.

193

194 *2.6. PCR amplification and partial sequencing of 16S rDNA gene*

195 The partial 16S rDNA gene sequences (*Escherichia coli* positions 27 to
196 518) were amplified using primers 27f and 518r (Table 1). The PCR for the 16S
197 rDNA gene was performed in 50 ul reaction mixture containing 1 ul of template
198 DNA (100 ng), 10 pmol of each primer, 5 ul of dNTP mixture (2.0 mM each), 1 X
199 PCR buffer, 3.5 ul of $MgCl_2$ solution (25 mM), 2.5 U of AmpliTaq Gold (Applied
200 Biosystems, Foster City, CA, USA). Amplification was performed on a
201 GeneAmp® PCR system 9700 (Applied Biosystems). The amplification program
202 was 95ºC for 2 min, 30 cycles of 95ºC for 0.5 min, 50ºC for 0.5 min and a final

203 extension of 72ºC for 5 min. The presence of amplified PCR products were
204 detected after electrophoresis on 1.5% agarose gel and staining for 15 min with
205 SYBR® Green I nucleic acid gel stain (Cambrex Bio Science, Inc, USA) in a UV
206 illuminator. PCR products were purified using SUPREC™ PCR (Takara Bio,
207 Otsu, Japan) and sequenced using the BigDye Primer Cycle Sequencing Reaction
208 Kit (Applied Biosystems). Sequences were analyzed with a 3100 Genetic
209 Analyzer (Applied Biosystems).
210
211 *2.7. PCR conditions for DGGE*
212 PCR conditions were as described by Minamida et al., (2004) with minor
213 modifications. All primers used in this study were synthesized by invitrogen,
214 Japan. Primers 338f-gc and 518r (Table 1) were used to amplify the V3 regions of
215 the bacterial 16S rDNA. For yeasts, primers NL1GC and LS2 (Table 1) designed
216 by Cocolin et al., (2000) were used to amplify the D1 region of the 26S rRNA
217 gene. The reaction mixture (50 µl) contained 50 pmol amounts of each primer, 5
218 µl of dNTP mixture (2.0 mM each), 1 X PCR reaction buffer, 3.5 µl of $MgCl_2$
219 solution (25 mM), 2.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA,
220 USA), 0.01% bovine serum albumin (Bio-Rad Laboratories, Hercules, CA, USA)
221 and 2 µl of DNA solution (100-200 ng). Amplification was performed on a
222 GeneAmp® PCR system 9700 (Applied Biosystems). The amplification program
223 was 95ºC for 5 min; 2 cycles of 94ºC for 1 min, 65º C for 1 min and 72ºC for 3
224 min; 18 cycles of 94ºC for 1 min, 64ºC for 1 min, 72ºC for 3 min; 11 cycles of
225 94ºC for 1 min, 55ºC for 1 min and 72ºC for 3 min; and a final extension at 72ºC

226 for 7 min. The above amplification program was used for both the V3 region of
227 the bacterial 16S rDNA and D1 region of 26S rDNA of yeasts. The amplification
228 was checked by running 5 μl of the amplicons on a 1.5% agarose gel, staining it
229 with cyber green and visualization of the DNA with a UV transilluminator.
230 .
231 *2.8. DGGE analysis*
232 The PCR products (50 μl) were concentrated by ethanol precipitation and
233 dissolved in 10 μl of distilled and sterilized water. Prior to DGGE, samples were
234 heated at 95ºC for 5 min and at 65ºC for 60 min, and were left at 37ºC for 4 to 5 h.
235 The DGGE was performed on the DCode system (Bio-Rad Laboratories, Hercules,
236 CA, USA) at 65 V, 60ºC in 1 X TAE for 14 h, on 10% polyacrylamide gels
237 containing 35% to 60%, 30% to 50% and 50% to 70% gradient of urea-
238 formamide, where 100% is defined as 7 M urea and 40% (vol/vol) formamide.
239 The gel was stained with SYBR Green 1 (Cambrex Bio Science Rockland, ME,
240 USA) for 45 min. The bands were excised with a sterile surgical blade and stored
241 at -20ºC until further analysis.
242
243 *2.9. Sequencing of DGGE fragments*
244 The excised DGGE bands were directly subjected to a PCR reaction. The
245 PCR was performed in a 50 μl reaction mixture containing small pieces of the gel
246 as the DNA template (equivalent to 2 μl), 50 pmol of each primer (for bacteria,
247 338f without incorporation of a GC clamp and 518r; for yeasts, (NL1 without
248 incorporation of a GC clamp and LS2), 1 X PCR buffer, 5 μl of dNTP mixture

249 (2.0 mM each), 3.0 µl of $MgCl_2$ solution (25 mM) and 2.5 U of AmpliTaq Gold.
250 PCR conditions were as follows; pre-denaturation for 5 min at 94ºC, followed by
251 30 cycles of denaturation for 30 s at 95ºC, annealing for 30 s at 53ºC, and
252 extension for 1.5 min at 72ºC. A final extension for 1.5 min at 72ºC was added.
253 PCR products were purified, sequenced and analyzed as described above.
254

255 *2.10. Analysis of the sequence*
256 Homology searches were performed in GeneBank database using the
257 BLAST program to identify the bacterial strains most closely related to isolates
258 and the DNA sequences of excised DGGE bands.
259

260 **3.0 Results**
261 *3.1. Identification of bacterial strains by partial 16S rDNA sequencing*
262 A total of 209 bacterial isolates were obtained from the 6 *mashita* samples.
263 The identities of the isolated strains are shown in Table 2. Identification of the
264 bacterial strains by partial 16S rDNA sequencing showed that 92.8% of the
265 isolates were strains of LAB. The most commonly isolated strains of LAB in
266 *mashita* were *Lactobacillus paracasei*, *Lactobacillus helveticus*, *Lactobacillus*
267 *plantarum* and *Lactobacillus perolens* constituting 37.3%, 10.1%, 8.1% and 7.7%
268 of total bacterial colonies isolated respectively (Table 3). About 4.78% of the
269 isolates were identified as *Enterococcus* sp. *L. paracasei* was the only bacterial
270 species identified in all the *mashita* samples. Meanwhile *L. plantarum* was
271 identified in 5 out of the 6 *mashita* samples. Strains identified as *Clostridium* sp.

272 constituted 5.26% of the total number of isolates. *Clostridium butryricum,* and
273 *Clostridium tyrobutryicum* consitituted 2.4% of the total number of isolates.
274 *Clostridium* sp. was isolated from all *mashita* samples except sample C. The
275 bacterial counts on MRS agar ranged from 3.1 X 10^7 to 2.2 X 10^9 CFU/ml and on
276 M17 agar ranged from 2.0 X 10^7 to 6.4 X 10^8 CFU/ml (data not shown).
277

278 *3.2. DGGE profiles of bacteria*

279 The bacterial community in *mashita* was further analyzed using PCR-
280 DGGE which is a culture independent-technique. The PCR-DGGE results
281 revealed that a wide diversity of bacterial species exist in *mashita* (Fig. 1). Each
282 *mashita* sample had a unique microbial profile. Many faint DGGE bands were
283 observed in all the six samples. Sequencing and identification of DNA sequences
284 of excised DGGE gel indicated that acetic acid bacteria (AAB) and LAB are the
285 dominant bacterial groups in *mashita* (Table 4). *Acetobacter aceti, Acetobacter*
286 *lovaniensis, Acetobacter orientalis* and *Acetobacter pasteurianus* were the main
287 species of AAB identified in the *mashita* samples. Bands corresponding to *A.*
288 *lovaniensis* were clearly observed in all the six *mashita* samples analyzed.
289 Meanwhile bands corresponding to *A. aceti* were clearly observed in samples
290 number 3, 4, and 6 and faintly in samples number 1, 2 and 3.

291 Out of the 20 bands analysed, the DNA sequences of 11 bands
292 corresponded to LAB, indicating that LAB is the main bacterial group involved in
293 *mashita* fermentation/ripening. The strains of LAB identified by PCR-DGGE
294 were *Bifidobacterium* sp.*, E. faecium*, *L. brevis, L. helveticus*, *L. acetotolerans,*

295 *Lactobacillus* sp., *Lactobacillus* sp., *L. raffinolactis, Lactococcus* sp. and *S.*
296 *salivarius.* Strains of *Bifidobacterium* sp., *L. acetotolerans, L. raffinolactis,*
297 *Lactococcus* sp. and *S. salivarius* could not be identified using culture-dependent
298 method. *L. brevis* and *L. helveticus* was identified both by culture-dependant and
299 culture-independent methods. *Prevotella buccae, Prevotella denticola*
300 ATCC33185 and Bacterium FLA21 were the main non-LAB species identified by
301 PCR-DGGE.
302
303 *3.3 DGGE profiles of yeasts*
304 Further PCR-DGGE analysis was performed to determine if yeast from
305 part of the *mashita* microbial community. Yeasts were identified in all the six
306 *mashita* samples (Fig. 2 and Fig. 3). Like in the bacterial community analysis,
307 each *mashita* sample had a unique fungal community profile. The sequence of the
308 excised DGGE bands were closely related to *Brettanomyces custersianus,*
309 *Candida silvae, Geotrichum candidum, Issatchenkia occidentalis* , *Issatchenkia*
310 *orientalis, Kluyveromyces marxianus, Saccharomyces cerevisiae,* and
311 *Trichosporon asahii* (Table 5 and Table 6). Bands corresponding to *Issatchenkia*
312 *orientalis* WL2002 and *Issatchenkia orientalis* QD15.1 were identified in all the
313 six *mashita* samples. Meanwhile *Brettanomyces custersianus, Geotrichum*
314 *candidum* and *Saccharomyces cerevisiae* were identified in 5 of the six *mashita*
315 samples. The most commonly identified yeasts species were *Issatchenkia* sp. and
316 *Brettanomyces* sp. However, *Brettanomyces* was mostly identified in sample
317 number 6.
318

319 **4.0 Discussion**

320 The results obtained in this study indicate that the microbial community in
321 *mashita* consists of AAB, LAB and yeasts. Although it has been reported that
322 yeasts and LAB play a role in ghee fermentation, so far there are no reports
323 indicating that AAB could be of importance in ghee fermentation and ripening.
324 To the best of our knowledge, this is one of the first scientific reports highlighting
325 the involvement of AAB in traditional African dairy fermentations. Furthermore,
326 *L. acetotolerans*, a difficult to culture LAB was detected for the first time in an
327 African traditionally fermented dairy product. Culture-dependent analysis
328 revealed the presence of many strains of LAB in *mashita*. Some of the strains
329 could be novel strains since the percentage identity to the closest relatives was
330 less than 97%. In this study we focused only on isolation and identification of
331 strains of LAB. However, there is a possibility that the bacterial community in
332 *mashita* could be more diverse with respect to the AAB group. The wide diversity
333 of strains of LAB isolated from *mashita*, does not enable accurate determination
334 of starter cultures for *mashita* fermentation. However, strains such as *L.*
335 *paracasei*, *L. helveticus*, and *L. plantarum* that were commonly isolated in
336 *mashita* could be potential targets for development of starter cultures. The inner
337 surface of the gourd is porous acting like a cell immobilization material and might
338 contain microorganisms that may form a stable microbial community. The
339 microbes inside the gourd could be the main source of starter cultures for *mashita*
340 fermentation and might explain why a wide diversity LAB was identified by
341 culture-dependent technique. About 5% of isolates were identified as *Clostridia*

342 spp. and could have been due to contamination of milk used in *mashita*
343 fermentation by cattle faeces. *Clostridium* is highly prevalent in dairy faecal
344 samples and constitutes 20% of the total microbial population (Dowd et al., 2008).
345 Gas production was observed in *Clostridium tyrobutryricum* cultures and it
346 occurred between 12 and 36 h of incubation. *Clostridium tyrobutricum* might be
347 one of the main spoilage bacterial species in *mashita.*

348 Culture-independent analysis of *mashita* bacterial community by PCR-
349 DGGE revealed that AAB and LAB were the main bacterial groups found in
350 *mashita*. *A*. *lovaniensis* and *A. aceti* were identified in all the 6 *mashita* samples.
351 It is most likely that these two strains play an important role in *mashita*
352 fermentation and ripening process and are potential strains for development of
353 adjunct cultures. Amongst the DGGE bands that were excised none was identified
354 to be closely related to either *Clostridia* sp. or other pathogenic strains. It is likely
355 that the *Clostridia* spp. identified by culture-dependent were contaminants,
356 existed in low numbers, and originated from the raw milk used in *mashita*
357 fermentation. In addition, PCR-DGGE results showed that *Prevotella denticola*,
358 *Prevotella buccae* and Bacterium FLA21 as the main contaminants in *mashita*.
359 The origin of the *Prevotella* spp, is likely to be cattle faces (Dowd et al., 2008).
360 *Bifidobacterium* sp*., L. raffinolactis, L. lactis* susbp. *lactis, Lactococcus* sp*., S.*
361 *salivarius* and *L. acetotolerans* were identified by culture-independent method but
362 not culture dependent method. It has been shown that both direct PCR-DGGE of
363 total community DNA and culture-dependent techniques yielded a different
364 description of microbial assemblages in fermented cassava dough, a biotope

365 thought to be mainly inhabited by cultivable microorganisms (Miambi, et al,
366 2003). *L. lactis* subsp. *lactis*, *Lactococcus* sp. and *L. raffinolactis* might could
367 have been involved in the earlier stages of fermentation and conditions such as
368 low pH might have affected their viability at the end of fermentation. Furthermore,
369 the incubation temperature used in culture-dependent method was 37ºC which is
370 not the optimum growth temperature for *Lactococcus* spp. These could be the
371 reasons why these strains were not detected using culture-dependent method. The
372 BLAST search for the DNA sequence of excised DGGE bands corresponding to *L.*
373 *acetotolerans* was the same as that of *Lactobacillus homohiochii* and *L.*
374 *fructivorans*. Both *L. acetotolerans* and *L. homohiochii* has been described as
375 difficult to culture strains of LAB (Kitahara, Kaneko & Goto, 1957; Nakayama et
376 al., 2007) and its presence in foods has mainly been detected using culture-
377 independent methods. Furthermore, the type strains of *Lactobacillus fructivorans*
378 and *L. homohiochii* showed a high degree of similarities (100%) on the basis of
379 *phe*S and *rpo*A gene sequences (Naser et al., 2007). Taxonomic studies are
380 needed to delineate the differences amongst *L. acetotolerans, L. fructivorans* and
381 *L. homohiochii*. *L. homohiochii* requires hiochic acid to grow, is resistant to
382 alcohol at concentrations ranging from 13-16 vol % and grows for 3-7 days in
383 liquid media (Kitahara et al., 1957). Absence of hiochic acid in the isolation
384 media might have prevented growth of *L. homohiochii* and attributed to its not
385 being detected by culture-independent technique. *Enterococcus* sp. was detected
386 by both culture dependent and culture independent methods. These results
387 indicates that entrococcus is part of the *mashita* microbial community. In *Kule*

388 *naoto*, Maasai traditional fermented, entrococccus was suggested to contribute to
389 some extent the fermentation of these products (Mathara, Schillinger, Kutima,
390 Mbugua & Holzapfel, 2004). However, further studies are needed to determine
391 the role of enterococcus in *mashita* fermentation in correlation to fermentation,
392 flavour development and safety.

393 Different bacterial and fungal community profile was observed in each
394 *mashita* sample, indicating lack of a standardised production process. The lack of
395 a standardized process might lead to wide variation in physico-chemical and
396 sensory attributes of *mashita*. A total of 9 strains of yeasts were identified by
397 PCR-DGGE, of which *Issatchenkia orientalis* WL2002 and *Issatchenkia*
398 *orientalis* QD15.1 were identified in all the six *mashita* samples. In addition,
399 strains of yeast closely related to *Brettanomyces custersianus*, *Geotrichum*
400 *candidum* and *Saccharomyces cerevisiae* were identified in 5 out of the 6 *mashita*
401 samples. These results indicate that yeasts are part of the microbial community in
402 *mashita* and could be of importance in developing of starter and adjunct cultures
403 for *mashita* fermentation and ripening respectively. A study by Gadaga,
404 Mutukumira & Narvhus (2001a) showed that *L. lactis* subsp. *lactis* biovar.
405 *diacetylactis* C1 and *Candida Kefyr* could grow mutually in a co-culture and
406 could have potential as mixed starter culture. In addition, the higher populations
407 of *L. paracasei* subsp. *paracasei* Lb11 recorded in co-culture with *Candida kefyr*
408 23 suggest that the yeast stimulated growth of the LAB (Gadaga, Mutukumira &
409 Narvhus, 2001b).

410 On the basis of culture-dependent and culture-independent results it can
411 be postulated that *mashita* fermentation occurs in three stages. In the first stage
412 strains of LAB such as *L. lactis* subsp. *lactis*, *L. raffinolactis* and *S. salivarius*
413 might initiate the fermentation process by the conversion of lactose to lactic acid.
414 The initiation of milk fermentation is typically associated with *L. lactis* followed
415 by *L. casei (paracasei*) and other Lactobacilli species during maturation
416 (Holzapfel, 2002). Most likely this process occurs simultaneously with the
417 conversion of milk carbohydrates to ethanol mainly by yeasts and takes place
418 during the first 12 to 24 h of fermentation when the milk is being fermented in
419 *ekiyanzi* (2L gourds). Milking cows and filling of *ekiyanzi* early in the morning
420 because of the prevailing cool temperatures might prevent disruption of initiation
421 of fermentation by organisms that grow at higher temperatures and allow
422 mesophilic LAB strains to proliferate and produce lactic acid. Production of lactic
423 acid might then offer the LAB strains a competitive advantage over other
424 microorganisms thereby allowing LAB to predominate first stage of *mashita*
425 fermentation. Further more, fermenting the milk in different 2L gourds in first
426 fermentation stage and then transferring the fermented milk into one big gourd in
427 the second stage of fermentation improves the diversity of the microbial flora and
428 stabilizes the fermentation process. Mixed strain cultures are less susceptible to
429 deterioration, relatively unaffected by fluctuation conditions of handling, storage,
430 applications and are, thus, better suited to small scale operations (Holzapfel,
431 2002). In addition they contribute to a more complex sensory quality, whilst
432 producing favourable synergistic effects, such as degradation of undesirable

433 factors, flavour production and accelerated ripening and maturation (Holzapfel,
434 2002). Fermenting milk in one container and then churning in another produce
435 *semin* (ghee) and rob (fermented milk) of good flavour (Abdelgadir et al., 1998).
436 Traditional *mashita* processors in Uganda smoke gourds to improve the flavour of
437 fermented milk, reduce fermentation time and to increase the yield of *mashita*.
438 Smoking might prevent the growth of spoilage microorgansims and improve the
439 chances of LAB dominating the initial fermentation stages. There is scientific
440 evidence indicating that LAB is more resistant to smoke compared to yeast and
441 other gram-positive bacteria (Holley & Patel, 2005). In the second stage there is
442 probably enhanced growth of AAB leading to conversion of ethanol to acetic acid.
443 At this stage acid and alcohol tolerant strains of LAB such as *L. acetotolerans*,
444 AAB and yeast might be the dominant organisms. *L. acetotolerans* has been
445 linked with vigorous acetate production, acid and alcohol tolerance and
446 persistence in food fermentations (Wang, Zhang, Zhao & Zu, 2008; Haruta et al.,
447 2006). It is during the second stage of fermentation that the milk is churned by
448 rocking to and fro. Churning probably increases the amount of oxygen getting
449 into the milk fermentation vessel leading to increased growth of strains of AAB.
450 Moreover, Kiryu, Kiso, Nakano, Ooe, Kimura and Murakami, (2009)
451 demonstrated that *A. orientalis* in Caspian Sea yoghurt oxidized lactose to
452 lactobionic acid especially with 10 mm from the surface where oxygen might
453 available. AAB are responsible for the oxidation of ethanol to acetic acid and
454 further oxidation of the latter to carbon dioxide and water (Schwan & Wheals,
455 2004). The conversion of ethanol to acetic acid by AAB leads to further decrease

456 in pH and this cause milk fat to coagulate. In cocoa fermentation, turning aerates
457 the fermenting mass quite well and favours *Acetobacter pasteurianus* growth and
458 metabolism, this species being more competitive in an acid and ethanol-rich
459 environment (Camu et al, 2008). Similarly, in *gariss* fermentation milk is shaken
460 by the jerky walk inherent to the camels, and yeasts and rod shaped bacteria that
461 were non-fermentative, strictly aerobic, catalase positive and heavy pellicle-
462 forming were frequently isolated (Abdelgadir, Ahmed, & Dirar, 1998). This suits
463 the description of *Acetobacter* strains provided by Lisdiyanti, Kawasaki, Seki,
464 Yamada, Uchimura & Komagata, (2000). Moreover, during *gariss* fermentation
465 ethanol concentration reaches a peak at one point and then invariably declines
466 sharply to a very low value with a concomitant rise in lactic acid (Abdelgadir et
467 al., 1998). The third stage might involve mainly processes related to development
468 of flavour and texture. The acidity developed in cream and butter during storage
469 appears to aid the removal of moisture in greater quantities during various heat
470 treatments (Abraham and Srinivasan, 1980). Unfortunately, we could not establish
471 a laboratory model of *mashita* fermentation process to enable dynamic monitoring
472 of microbial community profile and factors such as microbial metabolites, pH,
473 temperature, availability of nutrients and oxygen at the different phases. However,
474 on the basis of the microbial community profile it is likely that *mashita*
475 fermentation consists of aerobic and anaerobic biotransformation stages. The
476 existence of an anaerobic micro-environment in *mashita* fermentation process is
477 further reinforced by detection of the presence of *Clostridium* sp. and
478 *Bifidobacterium* sp. that are obligate anaerobes. There is a possibility that in the

later stages of fermentation AAB might reduce the oxygen concentration in the fermentation vessel leading to growth and survival of anaerobic microorganisms. In development of starter cultures it could be of considerable importance to consider how the alterations in oxygen supply will affect progress of the *mashita* fermentation process in correlation with flavour development and texture.

In conclusion, the results obtained in this study suggest that of AAB, LAB and yeasts play an important role in ghee fermentation. Strains such as *L. paracasei*, *L. helveticus*, *L. plantarum, A. lovaniensis, A. aceti, Issatchenkia orientalis*, and *Issatchenkia orientalis* are potential strains for development of starter and adjunct cultures. Furthers studies are need to develop an experimental model for determination of the biochemical and physiological characteristics of these strains during *mashita* fermentation.

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FIGURE CAPTIONS

Fig. 1.

PCR-DGGE profiles (35-60% denaturant gradient) of amplified 16S rDNA (V3 region) fragments of bacterial community in *mashita.* Lanes A, B, C, D, E, and F refers to the *mashita* samples as described in material and methods.

Fig. 2.

PCR-DGGE profiles (30-50% denaturant gradient) of amplified 26S rDNA (D1 region) fragments of the fungal community in *mashita.* Lanes A, B, C, D, E, and F refers to the *mashita* samples as described in material and methods.

Fig. 3.

PCR-DGGE profiles (50-70% denaturant gradient) of amplified 26S rDNA (D1 region) fragments of the fungal community in *mashita.* Lanes A, B, C, D, E, and F refers to the *mashita* samples as described in material and methods

.

Figure 1

Ongol & Asano

Fig. 1

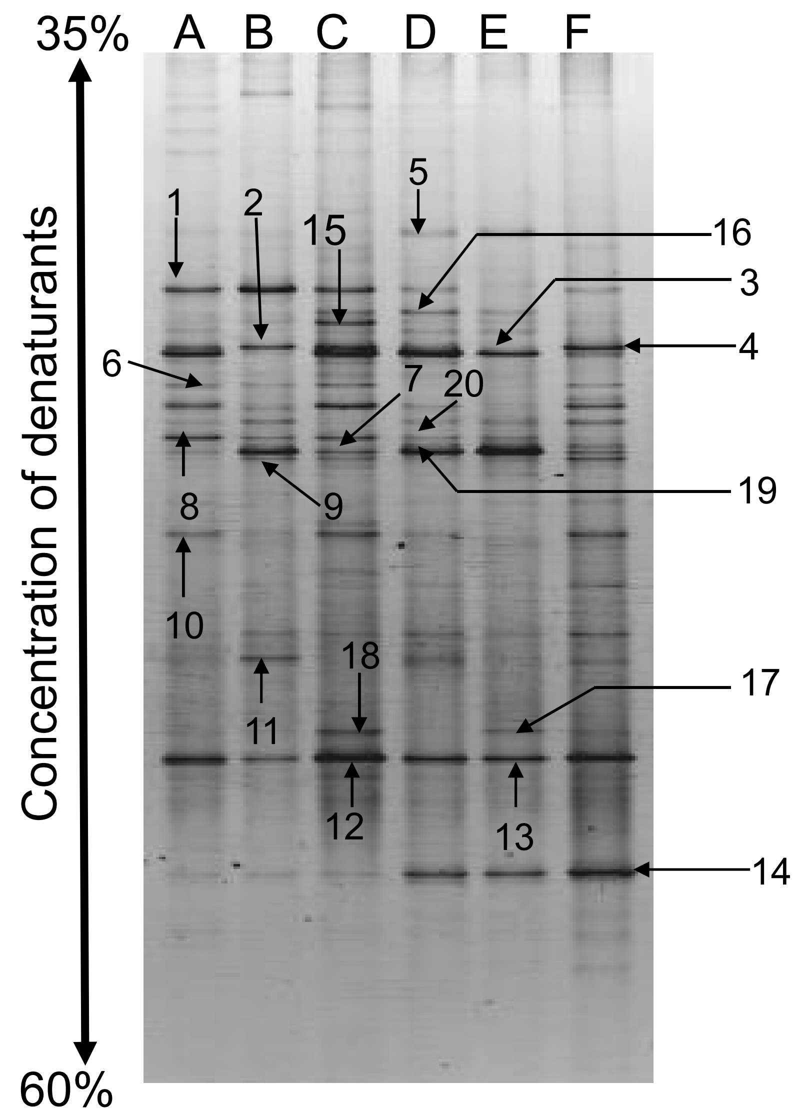

Figure 2

Fig. 3

Ongol and Asano

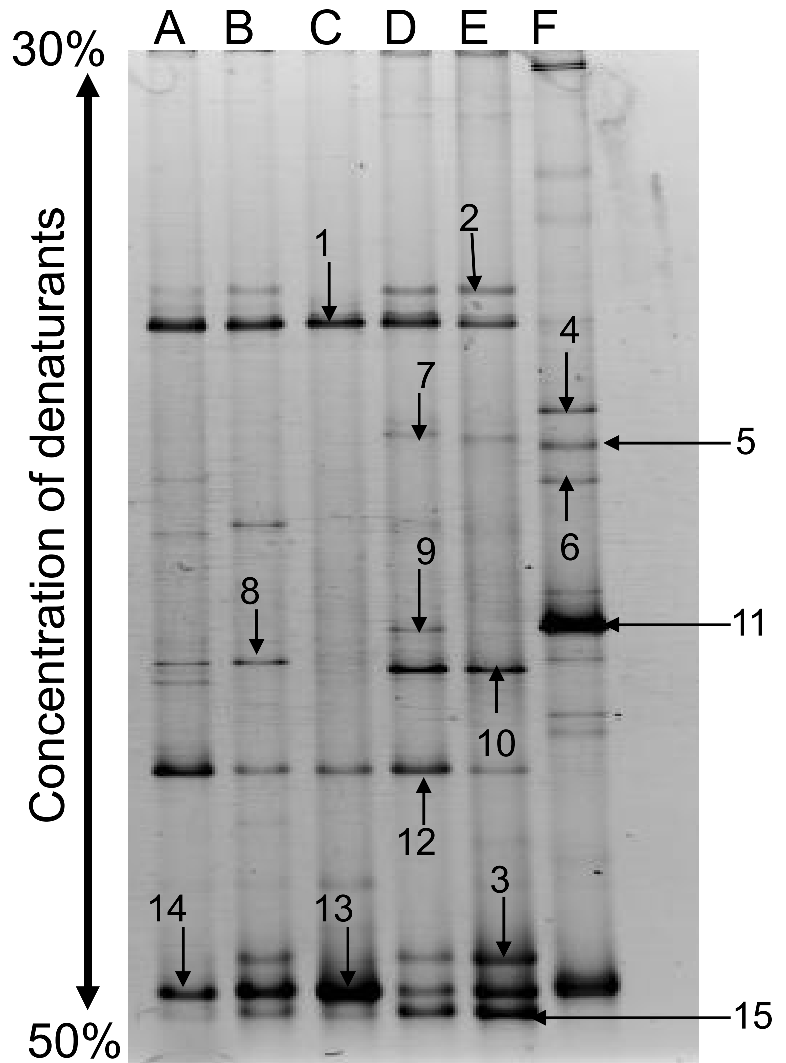

Figure 3

Ongol & Asano

Fig. 3

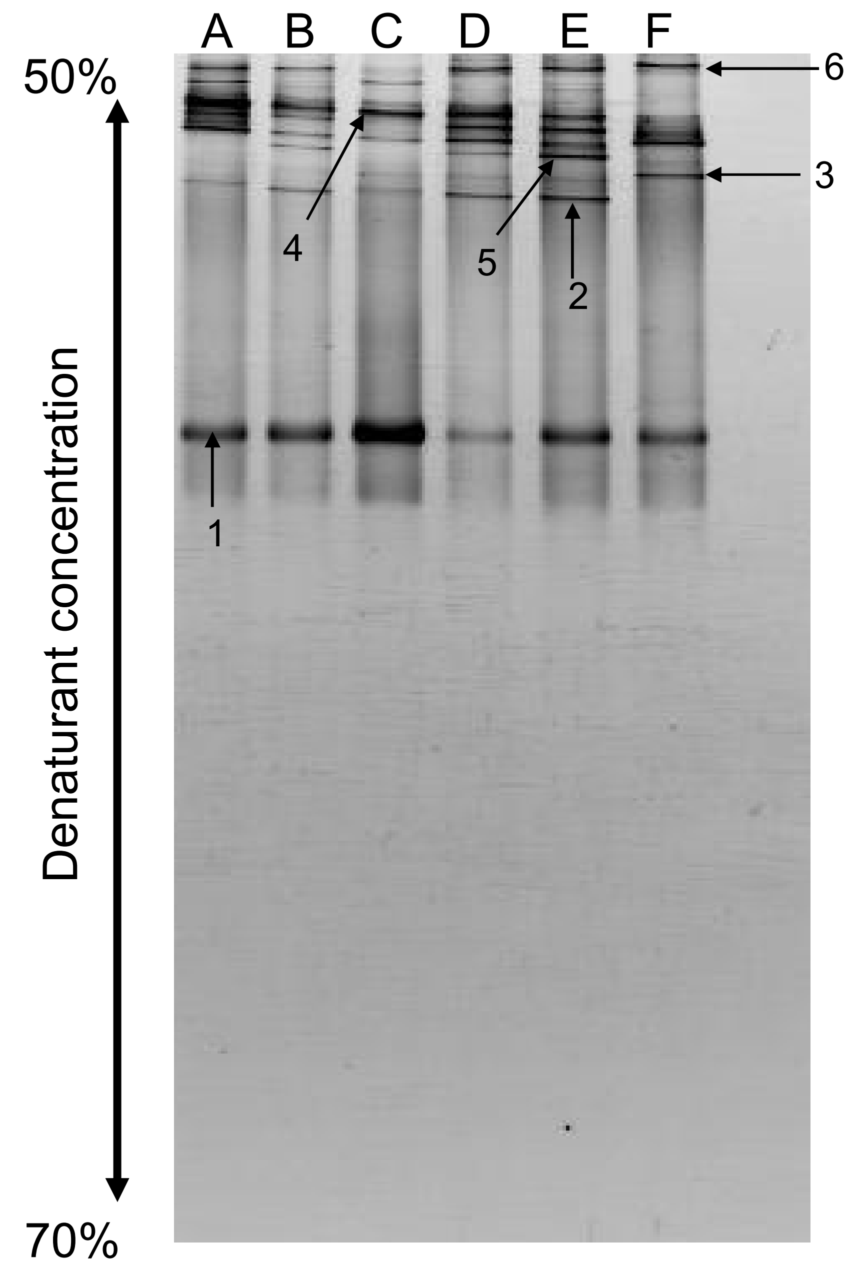

Table 1

PCR primers used in this study

Primer	Sequence (5´–3´)[a]	Target	Region	Reference
27f	AGAGTTTGATCCTGGCTCAG	Bacteria	16S rDNA	Lane, 1991
338f-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG	Bacteria	16S rDNA V3	Muyzer et al., 1993
518r	ATTACCGCGGCTGCTGG	Bacteria	16S rDNA V3	Muyzer et al., 1993
NL1-GC	GCGGGCCGCGCGACCGCCGGGACGCGCGAGCCGGCGGCGGGCCATATCAATAAGCGGAGGAAA	Eukarya	26S rDNA	Cocolin et al., 2000
LS2	ATTCCCAAACAACTCGACTC	Eukarya	26S rDNA	Cocolin et al., 2000

[a]GC-clamp sequence is underlined

Table 2

Identity of bacterial strains isolated from mashita

Code	Closest relative	% Identity	Acession No.	Isolation medium
UGA009	*Enterococcus hirae* F01959	93	DQ467844	MRS
UGA227	*Enterococcus italicus*	99	AB362595	M17
UGA074	*Enterococcus* sp. F157	95	EF204320	MRS
UGA116	*Lactobacillus brevis* ATCC14687	100	EF120367	MRS
UGA241	*Lactobacillus brevis* KLDS 1.0727	96	EU626012	M17
UGA151	*Lactobacillus buchneri*	99	AB205055	MRS
UGA051	*Lactobacillus camelliae*	95	AB257864	MRS
UGA201	*Lactobacillus casei* KLDS1.0720	99	EU626005	M17
UGA028	*Lactobacillus casei* ATCC334	100	CP000423	M17
UGA036	*Lactobacillus casei* KLDS1.0720	99	EU626005	MRS
UGA010	*Lactobacillus crustorum* LMG23701.	100	AM285454	MRS
UGA256	*Lactobacillus fermentum* L18	100	DQ523484	M17
UGA052	*Lactobacillus helveticus* ZL12-1	100	EF536361	MRS
UGA080	*Lactobacillus helveticus* DSM20075	100	AM113779	MRS
UGA108	*Lactobacillus helveticus* KLDS	97	EU419588	MRS
UGA096	*Lactobacillus helveticus* LLB	95	EU483108	MRS
UGA082	*Lactobacillus helveticus* LLB	100	EU483108	MRS
UGA001	*Lactobacillus hilgardii*	99	AB262962	MRS
UGA008	*Lactobacillus hilgardii* ATCC27305	98	AF429525	MRS
UGA017-1	*Lactobacillus parabuchneri*	99	AY026751	MRS
UGA158	*Lactobacillus paracasei* L7	97	EU526815	MRS
UGA004	*Lactobacillus paracasei* L7	99	EU526815	MRS
UGA018	*Lactobacillus paracasei* SFCB2-9c	100	DQ486146	MRS
UGA141	*Lactobacillus paraplantarum* NRIC1733	100	AB362736	MRS
UGA106	*Lactobacillus pentosus* C50-6	100	EU675926	MRS
UGA023	*Lactobacillus perolens* L534	100	Y19168	MRS
UGA083	*Lactobacillus plantarum* NRIC1594	99	AB362728	MRS
UGA081	*Lactobacillus plantarum* SFCB2-7c	97	DQ486145	MRS
UGA179	*Lactobacillus* sp. 123B	99	AJ514256	MRS
UGA181	*Lactobacillus* sp. 9C4	96	DQ682960	MRS
UGA017-2	*Lactobacillus* sp. MD-1	94	AY496039	MRS
UGA007	*Lactobacillus* sp. NBRC3231	99	NBRC00323101	MRS
UGA030	*Clostridium tyrobutyricum*	100	L08062	M17/MRS
UGA242	*Clostridium butyricum* (NCIMB8082)	99	X68178	M17
UGA253	*Clostridium sporogenes*	99	X68189	M17

Table 3

Bacterial communnity profile of mashita

Bacterial Strain	% of total isolates
Enterococcus sp.	4.78
Lactobacillus brevis	3.35
Lactobacillus buchneri	0.48
Lactobacillus camelliae	0.48
Lactobacillus casei	2.39
Lactobacillus crustorum	0.48
Lactobacillus fermentum	0.48
Lactobacillus helveticus	10.05
Lactobacillus hilgardii	4.78
Lactobacillus parabuchneri	5.74
Lactobacillus paracasei	37.32
Lactobacillus paraplantarum	0.48
Lactobacillus pentosus	0.48
Lactobacillus perolens	7.66
Lactobacillus plantarum	8.13
Lactobacillus sp.	5.74
Clostridia sp.	5.26

Table 4
Identities of bands obtained from bacterial community of *mashita*

Band No.	Closest relative(s)[a]	% Identity	Acession No.
1	*Lactobacillus helveticus* B2401	95	EU130905
	Lactobacillus sp. GTP5	95	AF157035
2	*Lactobacillus acetotolerans*	97	LBARR16S
	Lactobacillus homohiochii NBRC13121	97	NBRC-01312101
	Lactobacillus fructivorans	97	NBRC-01312001
	Lactobacillus sp. JCM 9717	97	AB289162
3	*Lactobacillus helveticus* NBRC3809	95	NBRC-00380901
	Lactobacillus sp. TV1018	95	Y18654
	Lactobacillus crispatus	95	X98052
	Lactobacillus gallinarum	95	X97898
4	*Lactobacillus acetotolerans*	94	LBARR16S
	Lactobacillus homohiochii	94	NBRC-01312101
	Lactobacillus fructivorans	94	NBRC-01312001
	Lactobacillus sp. JCM 9717	94	AB289162
5	*Lactobacillus brevis* H6	97	AY230232
6	*Prevotella denticola* ATCC 33185	94	L16466
7	*Prevotella buccae*	98	L16478
8	*Lactococcus raffinolactis*	96	AM490367
	Lactococcus lactis subsp. *lactis* NBRC100933	96	NBRC-11719101
9	*Streptococcus salivarius* NBRC13956	99	NBRC-01395601
10	Bacterium FLA21	93	AJ539235
11	*Bifidobacterium* sp. FR47	96	AY952450
12	*Acetobacter lovaniensis* NBRC103497	97	NBRC103497
13	*Acetobacter lovaniensis* NBRC103497	98	NBRC103497
14	*Acetobacter aceti*	98	X74066
15	*Lactobacillus* sp. L44	93	AF159022
16	*Enterococcus faecium*	96	AJ968593
17	*Acetobacter orentalis* 17BAM	96	EU676343
18	*Acetobacter pasteurianus*	95	X71863
19	*Streptococcus salivarius* AGLD1	96	M58839
20	*Lactococcus* sp. GM330	92	AB062558

[a]The DNA sequences of bands No. 1, 2, 3, 4, and 9 had similar matches to more than one bacterial strain. Therefore bacterial names and accession numbers of other of closely related strains have been included.

Table 5

Table 5

Identities of bands obtained from fungal community of *mashita* (30-50% gel)

Band No.	Closest relative	% Identity	Acession No.
1	*Geotrichum candidum*	96	NBRC460201
2	*Saccharomyces cerevisiae* V12	98	EU441887
	Kluyveromyces lactis NRRL Y-1140	98	CR382124
3	*Issatchenkia orientalis* WL1	97	EF644475
4	*Brettanomyces custersianus* CBS4805	99	DQ406717
5	*Brettanomyces custersianus* CBS4805	99	DQ406717
6	*Brettanomyces custersianus* CBS4805	98	DQ406717
7	*Saccharomyces cerevisiae* V12	94	EU441887
8	*Trichosporon asahii* YS124	94	AM900369
9	*Brettanomyces custersianus* CBS4805	97	DQ406717
10	*Kluyveromyces marxianus* 13MCHS	94	EU669470
11	*Brettanomyces custersianus* CBS4805	99	DQ406717
12	*Saccharomyces cerevisiae* V12	97	EU441887
13	*Issatchenkia orientalis* WL2002	100	AY707865
14	*Issatchenkia orientalis* WL2002	100	AY707865
15	*Candida silvae* VTT C-04527	96	DQ377641

[a]The DNA sequence of band No.2 had similar matches to more than one fungal bacterial strain. Therefore bacterial names and accession numbers of other of strains have been included.

Table 6

Ongol & Asano

Table 6

Identities of bands obtained from fungal community of *mashita* (50-70% gel)

Band No.	Closest relative	% Identity	Acession No.
1	*Issatchenkia orientalis* QD15.1	100	EU543688
2	*Candida silvae* VTT C-04527	96	DQ377641
3	*Issatchenkia occidentalis*	94	AB281316
4	*Geotrichum candidum* NBRC4602	98	NBRC460201
5	*Kluyveromyces marxianus* 13MCHS	98	EU669470
6	*Brettanomyces custersianus* CBS 4805	96	DQ406717