Fermentation of enset (*Ensete ventricosum*) in the Gamo highlands of Ethiopia: practices and microbial community dynamics

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Abstract

Enset (*Ensete ventricosum*) provides staple food for 15 million people in Ethiopia after fermentation into kocho. The fermentation process has hardly been investigated and is prone to optimization. The aim of this study was to investigate the physicochemical and microbial dynamics of fermentation practices in the Gamo highlands. These practices show local variation, but two steps were omnipresent: scraping of the pseudostem and fermenting it in a pit or a bamboo basket. Enset plants were fragmented and fermented for two months in order to investigate the physicochemical (temperature, moisture content, pH and titratable acidity) and microbial dynamics (total viable aerobic counts, counts of Enterobacteriaceae, lactic acid bacteria, yeasts and moulds and Clostridium spores counts, and Illumina Miseq sequencing). Samples were taken on days 1, 7, 15, 17, 31 and 60. The pH decreased, whereas the titratable acidity increased during fermentation. Of all counts those of lactic acid bacteria and Clostridium spores increased during fermentation. Leuconostoc mesenteroides initiated the fermentation. Later on, Prevotella paludivivens, Lactobacillus sp. and Bifidobacterium minimum dominated. These three species are potential candidates for the development of a starter culture.

Key words

Enset, kocho, fermentation, physicochemical analysis, metagenomics

1. Introduction

Enset (*Ensete ventricosum* (Welw.) Chees man, Musaceae) is an important food security crop for 15 million Ethiopian people (Yemataw et al., 2014), where it is cultivated as a food, forage and fiber crop (Brandt et al., 1997; Nurfeta et al., 2008). In the highlands of the southern, southwestern and central part of Ethiopia, where population density is high, enset products are the main staple food and are especially important in the diet of women and

children (Tsegaye and Struik, 2001; Olango et al., 2014). Moreover, due to high yield and drought tolerance, enset-based production systems are an interesting avenue to contribute to the United Nations sustainable development goals, especially in a context of global change.

Unlike other members of the Musaceae, enset does not bear edible fruit. Instead, the pseudostem and corm of the enset plant are traditionally processed into important starchy food products, being ‘kocho’ and ‘bulla’ (Pijls et al., 1995; Brandt et al., 1997; Atlabachew and Chandravanshi, 2008). To this end, the pseudostem and corm are pulverized manually and the solid fraction is fermented to obtain kocho. It is an age-old tradition, which is still used with little modification (Hunduma and Mogessie, 2011). Enset can take up to 8 years to mature but can be harvested at an earlier stage if necessary. The onset of flowering or the dry season are considered the best harvesting time for the production of kocho (Yemataw et al., 2014). The fermentation techniques and the tools used to process enset differ among regions and even among localities (Yirmaga, 2013; Karssa et al., 2014). Traditional methods of enset preparation are carried out in the backyard of the farmer's home (Figure 3.1A) and are time consuming and labor intensive. The fermentation is by far the slowest step, as fermentation time varies from a month to a year, depending on incubation temperature, which is partly dependent on the altitude of the production site (Gashe, 1987). The sensory quality of fermented enset is very variable and generally poor, which leads to a lower market price for kocho compared to other crops consumed in the country (Brandt et al., 1997; Ashenafi, 2006). It is also deficient in proteins and vitamin A (Tsegaye and Struik, 2001; Yirmaga, 2013). An accurate understanding of the microbial dynamics during the fermentation can help to optimize and standardize the process, but only a few studies have been carried out so far to characterize the microbiota involved in the traditional enset fermentation process. All of them exclusively use culture-dependent methods i.e. classical counts (Gashe, 1987; Yirmaga, 2013; Karssa et al., 2014). These studies indicate that lactic acid bacteria (LAB) are the dominant

micro-organisms responsible for enset fermentation. However, culture-based methods often fail to characterize less abundant organisms or those organisms that require selective enrichment. Additionally, organisms that cannot be cultivated under traditional laboratory conditions remain undetected. These limitations can be met by metagenetic analyses such as Illumina sequencing (Ercolini, 2013).

The aim of this study is therefore to contribute to an improved nutritional and economic revenue for enset-dependent households by gaining insight in the enset fermentation process in the Gamo highlands of Ethiopia, and its relation to fermentation practices. To this end, based on the survey among 60 households, three different enset varieties were selected, processed and fermented in bamboo baskets or so-called 'erosas' to obtain kocho. Physicochemical parameters and microbial numbers were monitored. Furthermore, the composition of the microbial community was analyzed using Illumina Miseq sequencing.

2. Materials and methods

2.1. Enset preparation practices and experimental setup

A survey of fermentation practices conducted among 60 households in the Gamo Highlands (districts Chencha, Dorze and Bonke) in 2016 was conducted to design the experimental setup. Based on the survey, three varieties were selected, i.e. Gena, Maze and Ketishe, which are preferred by over 90% of the respondents for kocho and bulla preparation for their superior product quality and their shorter fermentation time. Processing techniques in the study area showed considerable variation, but nevertheless a general process flow could be distinguished, which is presented in Fig. 2. The first phase of the general process flow involves scraping of the pseudostem and corm with a sharp-edged utensil to separate the pulp from the long fibres (subsequently used to produce ropes and packaging sacks). The pulp is subsequently squeezed using a clean cloth or sack. The squeezed liquid is decanted and used

to produce a starchy residue referred to as bulla. In the first phase of the fermentation, the pulp is placed loosely under wilted enset leaves pseudostem and corm with a sharp-edged utensil to separate the pulp from the long fibres (subsequently used to produce ropes and packaging sacks). The pulp is subsequently squeezed using a clean cloth or sack. The squeezed liquid is decanted and used to produce a starchy residue referred to as bulla. In the first phase of the fermentation, the pulp is placed loosely under wilted enset leaves and stored for fifteen to twenty-one days. During this first aerobic phase, the fermenting enset is opened and homogenized on days 7 and 13. Then, the mixture is compacted and placed in a fermentation basket (erosa), a pit or a package of enset leaves to obtain anaerobic conditions. A minimum of two months is required for complete fermentation. In the study area, there is no use of a starter culture or backslopping to aide fermentation. Therefore, it was also not included in the experimental set-up. For the experimental setup, four matured enset plants were purchased for each of these varieties (12 plants in total). In accordance with the general process flow described above, after scraping and squeezing of the pulp by local traditional enset processors (Fig. 1B), 48 kg of pulp was collected for each variety, mixed and divided into four equal parts. Each portion was wrapped and covered with wilted enset leaves to intiate the fermentation process and left in the enset farm for 15 days at ambient temperature. The farms were next to each other to ensure the same temperature and humidity conditions for the three varieties. The packages were opened, homogenized and covered again on days 7 and 13. On day 15, the fermenting mass from each wrap was into a separate erosa (four erosas per variety) and, according to the traditional practices, upon compaction covered with enset leaves, plastic bags and large stones to create anaerobic conditions (Fig. 1C). In this way the enset was fermented further for another 45 days to obtain kocho (Fig. 1D). The twelve erosas were all located in the same fermentation room to have identical environmental conditions.

2.2. Enset fermentation

Samples were taken in a sterilized beaker using sterilized spoons on days 1, 7, 15,17, 31 and 60. From each erosa, a sample was taken at the surface (about 80 g), at the middle (about 80 g) and at the bottom (about 80 g), and then pooled to one sample. Afterwards, the fermenting enset in the erosas was pressed to restore the anaerobic condition. All parameters were assessed on fresh samples, except for metagenetic analyses, that was performed on freeze-dried samples. The samples were freeze-dried for 24 h at -50 °C (Christ freeze-drier, Alpha 1e4 LD plus, Osterode am Harz, Germany).

2.3. Physicochemical parameters

The temperature in the fermenting enset was monitored using a disinfected digital thermometer (Traceable® Long-Stem Thermometers, Stainless steel probe, Bestone Meter Ltd, Shenzhen, China) at different depths in each of the 12 erosas at each sampling day. The room temperature was recorded during the study period using digital thermometer (HTC-1, Shenzhen Datronn Electronics, Guangdong, China).

The pH was measured according to the methods used by Assanvo et al. (2006); Moselhy et al. (2015) and Oguntoyinbo and Dodd (2010). Briefly, 10 g sample was taken from the pooled sample and homogenized for 60 s in 90ml of distilled water in a stomacher (StarBlender^{TM} LB 400, VWR International, Fontenay Sous Bois Cedex, France). The pH of the homogenate was measured using digital pH meter (PH 1100H, VWR International, Darmstadt, Germany). The titratable acidity of the homogenate was determined according to the methods used by Lefebvre (2002) and Oguntoyinbo and Dodd (2010). The moisture content was analyzed for three 10 g samples taken from the pooled sample using the oven drying method at 105 °C (AOAC, 1990). The average and standard deviation were calculated for the four erosas per variety.

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151 *2.4.* Microbiological analyses

152 Classical plate counts were determined according to the ISO standards assembled by Dijk et
153 al. (2015). Five grams of fermenting enset was transferred aseptically into a sterile stomacher
154 bag and 45 ml of peptone physiological salt solution (0.85% NaCl, 0.1% peptone, Biokar
155 Diagnostics, Beauvais, France) was added. The mixture was homogenized for 60 s
156 StarBlender^{TM} LB 400, VWR International, Fontenay Sous Bois Cedex, France). A ten-fold
157 serial dilution was plated on different media using the pour plate method. Total viable aerobic
158 bacterial counts were determined on Plate Count Agar (VWR International, Milano, Italy)
159 incubated at 30 °C for 3 days, Enterobacteriaceae on Violet Red Bile Glucose medium (VWR
160 International, Milano, Italy) incubated at 37 °C for 24 h, LAB on de Man Rogosa Sharpe
161 medium (VWR International, Milano, Italy) incubated at 30 °C for 3 days, yeasts and moulds
162 on Dichloran Glycerol (DG-18) agar (VWR International, Milano, Italy) supplemented with
163 0.1 g/l chloramphenicol, incubated at 25 °C for 5 days. *Clostridium* endospores were counted
164 by giving the 10^{-1} dilution a heat shock treatment (15 min at 75 °C), followed by serial
165 dilution, plating onto Reinforced Clostridium Agar (Biokar Diagnostics, Beauvais, France)
166 and anaerobic incubation at 37 °C for 24 h using anaerobic jars, gas generating kits and
167 indicator strips (IVD, Microbiology Anaerotest®, Merck KGaA, Darmstadt, Germany). All
168 microbial counts were expressed as log cfu/g. One analysis was performed for each pooled
169 sample per erosa. The average and standard deviation was calculated for the four erosas per
170 variety.

171 *2.5. Metagenetics*

172 In order to assess the bacterial community composition and its dynamics during fermentation,
173 enset samples were subjected to high-throughput 16S ribosomal RNA (rRNA) gene
174 sequencing via the Illumina MiSeq platform. For each variety, on day 1 and day 60, the

pooled samples obtained for each erosa were subjected as such to sequencing (four samples per variety). On days 7, 17 and 31, the pooled samples obtained from the four erosas were combined into one sample per variety for sequencing. For each sample, two DNA extractions were performed using the PowerSoil DNA Isolation Kit using 0.2 g starting material (Mo Bio Laboratories, Carlsbad, CA, USA). Subsequently, replicate DNA extracts were pooled and diluted ten times. PCR was used to amplify the V4 region of the 16S rRNA gene using barcode-tagged primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) with dual index strategy (Kozich et al., 2013; Table S1, Supporting Information). The PCR reaction (20 µl) was executed in duplicate, containing one unit of Titanium Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France), 1x Titanium Taq PCR buffer, 150 µM of each dNTP, 0.5 µM of each primer and 1 µl 1:10 diluted DNA. PCR amplification included initial denaturation for 2 min at 95 °C, followed by 30 cycles of denaturation for 45 s at 95 °C, annealing for 45 s at 60 °C and elongation for 45 s at 72 °C. Amplicons were purified by Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), quantified using the Qubit fluorometer (HS reaction kit, Invitrogen, Carlsbad, CA, USA) and combined into a library in equimolar concentrations. Finally, the library was diluted to 2 nM and sequenced at the Center of Medical Genetics Antwerp (University of Antwerp, Belgium) using an Illumina MiSeq sequencer (V2 500 cycle kit, Illumina, San Diego, CA, USA).

Sequences were received in the format of a demultiplexed FASTQ file (data deposited in a Sequence Read Archive; BioProject accession PRJNA392985). Paired-end reads were merged using USEARCH (v9.2.64) (Edgar, 2013) to form consensus sequences originating from each sample with a maximum number of 5 mismatches allowed in the overlap region. Subsequently, sequences were truncated at the 250th base. Shorter reads or reads with a total expected error threshold above 0.10 for all bases after truncation were discarded. Next, reads

were assigned taxonomic identities using the “classify. seqs” command in Mothur (v. 1.39.3; Schloss et al., 2009) against the Silva taxonomy database (release v1.2.3; Gurevich et al., 2013). DNA-sequences originating from chloroplasts or mitochondria were eliminated with Mothurs “remove lineage” command. Due to an uneven sequencing depth, the number of sequences was rarefied to 10,000 sequences per sample. Error-correction (denoising) was performed using the UNOISE algorithm (Edgar, 2016a) command implemented in USEARCH (Edgar, 2016b) as follows: (i) reads with sequencing errors were identified and corrected, (ii) chimeras were removed and (iii) PhiX were removed. Remaining sequences with a minimum abundance of two, were grouped into species-level operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off using the UPARSE greedy algorithm implemented in USEARCH (Edgar, 2013). As a result, during this step global singletons (i.e. OTUs representing only a single unique sequence in the entire dataset) were removed in order to minimize the risk of retaining sequences from sequencing errors (Brown et al., 2015; Waud et al., 2014). The taxonomic origin of each OTU was determined with the SINTAX algorithm in USEARCH, (Edgar, 2016b) based on the Silva Living Tree Project v123 (LTP v123) database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.80. Additionally, OTU representative sequences (selected by UPARSE) were subjected to a BLAST (Altschul et al., 1990) search against GenBank (Benson et al., 2008), excluding uncultured/environmental entries. Nonmetric multidimensional scaling (NMDS), Chao1 and Shannon-Wiener diversity indices calculations were performed on the microbial communities of the samples using R-packages (R Development Core Team, 2013) Vegan (v.2.41) and Phyloseq (v. 1.19.0).

223 *2.6. Statistical analysis*

224 All statistical analyses were performed using SPSS (IBM©SPSS Statistics v. 20, New York,
225 USA). One-way ANOVA was performed for the microbial counts to reveal significant
226 differences in counts across different fermentation days and between different enset varieties.
227 Multiple comparisons were performed by Tukey's post hoc test. For the statistical analysis of
228 the community diversity indices, cases with a coverage below 50% were omitted since they
229 do not represent diversity in a reliable way. Subsequently, One-way ANOVA with Tukey's
230 post hoc test was performed to compare microbial diversity between different varieties and
231 between different fermentation days. In cases of unequal variances, the Welch's ANOVA test
232 was used with the Games-Howell post hoc test. For all statistical analyses, differences were
233 considered to be significant at $p < 0.05$.

234

235 **3. Results and discussion**

236 *3.1. Physicochemical changes during enset fermentation*

237 Results obtained for the enset temperature, the moisture content, the pH and the titratable
238 acidity are presented in Fig. 3. The maximum temperatures within the fermenting mass were
239 recorded on day 7, being 21.9 ± 0.3 °C, 22.5 ± 2.9 °C and 20.4 ± 0.3 °C for Gena, Maze and
240 Ketishe, respectively (Figure 3A). The maximum temperatures were recorded during the first
241 phase in the packages, whereas the temperature during the second phase in the erosas was
242 generally lower. The minimum temperature for each of the varieties was recorded at different
243 fermentation days. The results are in agreement with Karssa et al. (2014), who reported the
244 temperature of the fermenting enset to be between 19.0 and 26.1 °C. The maximum external
245 environment or room temperature was recorded on day 15, being 19.1 ± 2.0 °C and the
246 minimum was recorded on day 31, being 13.1 ± 2.8 °C (Figure S1, Supporting Information).

247 The moisture content at the onset of fermentation was 82.36 ± 0.65% for Maze, followed by
248 80.94 ± 0.19% for Ketishe and 78.67% ± 1.81% for Gena (Fig. 3B). These differences can be
249 due to genetic differences between the varieties and/or to differences in crop management in
250 the field (Yirmaga, 2013). At day 60, the moisture contents were reduced to 69.18 ± 0.78%,
251 70.39 ± 0.24% and 72.74 ± 0.35%, for Gena, Maze and Ketishe, respectively. It is not clear
252 why in the local practice, the fermentation is usually first carried out in a heap covered by
253 wilted enset leaves (or in a package as in our study) which is opened and homogenized on
254 days 7 and 13. The homogenization allows oxygen to enter but it is assumed that the first
255 phase is included in the process to dry the material to some degree. In Western silage
256 processes, it is known that the moisture content of the material to be ensiled should not be too
257 high to avoid growth of unwanted microorganisms such as clostridia (Muck et al., 2003).
258 Considering the moisture content profile for the three varieties, the reduction is indeed faster
259 during the first 15 days compared to the moisture reduction during the rest of the
260 fermentation.
261 The pH of the enset on day 1 was 4.87 ± 0.13, 5.31 ± 0.08 and 5.29 ± 0.09 for Gena, Maze
262 and Ketishe, respectively (Figure 4C). On day 60, the pH values had steadily decreased to
263 4.06 ± 0.09, 3.95 ± 0.02 and 4.06 ± 0.04 for Gena, Maze and Ketishe, respectively. The pH at
264 the start and at the end of the fermentation showed significant differences for all varieties ($p = 0.000$). The reduction of pH coincided with an increase of titratable acidity from 0.36 ± 0.01,
265
266 0.32 ± 0.00 and 0.34 ± 0.01% to 0.57 ± 0.01, 0.59 ± 0.02 and 0.58 ± 0.01% from day 1 to 60
267 for Gena, Maze and Ketishe, respectively (Figure 3D). The reduction of pH and the increase
268 of titratable acid are likely to be related to the production of organic acids by the microbiota,
269 such as lactic, malic and acetic acid, which is reported to be common in vegetable and enset
270 fermentations (Bleve et al., 2015; Pereira et al., 2015; Tiruha et al., 2014; Xiong et al., 2012).
271 The changes were most pronounced during the first phase of the fermentation.

272 *3.2. Microbial counts during enset fermentation*

273 Microbial counts of fermenting enset are shown in Table 1. Total viable aerobic bacterial
274 count was 8.16 ± 0.03, 8.12 ± 0.12 and 8.35 ± 0.05 log cfu/g at the onset of the fermentation
275 for Gena, Maze and Ketishe, respectively. The counts attained a maximum of 9.66 ± 0.08,
276 9.95 ± 0.03 and 9.72 ± 0.01 log cfu/g for Gena, Maze and Ketishe, respectively. For the three
277 varieties, this maximum was attained at the same sampling point of 17 days. After that, the
278 total aerobic bacterial counts fell by 1.36 to 2.03 log cfu/g at the end of the fermentation.
279 That reduction may be due to a depletion of some nutrients for growth and/or a decreasing
280 pH. Whereas the total counts were not statistically different between Gena and Maze varieties
281 at the start of the fermentation (p=0.810), significant differences were found at the end of the
282 fermentation for the three varieties (p = 0.000, p = 0.000 and p = 0.022 for Gena, Maze and
283 Ketishe, respectively). On the first day of the fermentation, LAB counts were 6.17 ± 0.09,
284 6.06 ± 0.05 and 6.21 ± 0.05 log cfu/g, for Gena, Maze and Ketishe, respectively. The LAB
285 counts increased significantly (p = 0.000) from day 1 to day 7, from day 7 to day 15 and from
286 day 15 to day 17, resulting in a maximum value at day 17 for all varieties. Then a slight
287 decrease was seen on day 31, followed by a significant (p = 0.000) reduction towards day 60,
288 resulting in 8.07 ± 0.07, 7.91 ± 0.05 and 7.57 ± 0.15 log cfu/g for Gena, Maze and Ketishe,
289 respectively.The inhibitory effect of a low pH may have contributed to the decrease after day
290 17 (Gashe, 1987; Tiruha et al., 2014; Xiong et al., 2012).

291 Enterobacteriaceae counts were the highest at the start of the fermentation, being 7.98 ± 0.05,
292 8.09 ± 0.03 and 7.91 ± 0.06, for Gena, Maze and Ketishe, respectively. The counts decreased
293 below the detectable level after day 17 for all varieties. This can be explained by unfavourable
294 conditions created (in particular a low pH) during fermentation, and maybe also to the
295 production of antimicrobial substances (such as bacteriocins) by some LAB (Khedid et al.,
296 2009; Yu et al., 2013) and by exhaustion of nutrients. However, the minimum pH for *E. coli*

297 growth is 4.0 – 4.5 (Buchanan and Bagi, 1994) and pathogenic strains of *E. coli*, such as
298 *E. coli* O157:H7, have been shown to survive in a range of acidic foods as reviewed by
299 Buchanan and Doyle (1997).
300 On the first day of the fermentation, the yeast and mould counts were 4.76 ± 0.01, 4.72 ± 0.02
301 and 4.62 ± 0.03 cfu/g for Gena, Maze and Ketishe. For the three varieties, the yeast and
302 mould count increased with about 2 log cycles during the first phase of the fermentation and
303 in all cases the yeast and mould counts differed statistically between day 1 and day 15 ($p = 0.000$). At the end of the fermentation, the yeast and mould counts had decreased to 3.57 ±
305 0.02, 3.62 ± 0.02 and 3.34 ± 0.03 for Gena, Maze and Ketishe. In addition to drying, a second
306 reason for including the first phase with aeration (i.e. homogenizing) may be to allow yeasts
307 to grow. It is very plausible that some of the yeasts hydrolyze the starch into simple sugars
308 which then can be converted into organic acids by LAB. Likewise, Gashe (1987) and Karssa
309 et al. (2014) also reported the co-occurrence of yeasts and LAB in enset fermentation. The
310 simultaneous occurrence of yeasts and LAB was also reported in fermented milk from Kenya
311 (Nyambane et al., 2014), corn kernel silage from Brazil (Carvalho et al., 2017), unsulfited
312 Tannat grape from Uruguay (Muñoz et al., 2014) and in different fermented foods from
313 Zimbabwe (Gadaga, 1999). Whereas yeasts may have grown in the first aerobic phase, it
314 cannot be excluded that moulds have grown as well, since the counts presented are a
315 combination of yeasts and moulds. Mould growth is unwanted, however, as it can be
316 accompanied by nutrient utilization, a negative impact on taste, but above all with mycotoxin
317 production.
318 Anaerobic *Clostridium* spore counts increased from 4.08 ± 0.07, 4.02 ± 0.03 and 4.02 ± 0.03
319 on day 1 to a maximum of 7.78 ± 0.07, 7.83 ± 0.04 and 7.93 ± 0.05 at day 17 for Gena, Maze
320 and Ketishe. Similarly, Gashe (1987) reported anaerobic spore-forming bacteria isolated from
321 traditionally fermenting enset mash, dominated by *Clostridium* spp. The fermenting enset also

had a characteristic butyrous smell, which was suggested to be the result of metabolic activity of clostridial species(Urga et al., 1997). In Western silage processes, it is known that starting the fermentation with a material with a high moisture content can lead to unwanted growth of Clostridiaceae, due to a high water activity and to the dilution of organic acids (Muck et al., 2003).

As indicated by the survey, local kocho producers claimed the first aerobic phase to be necessary to obtain good kocho. Currently, after scraping, the enset is pressed manually and hence only a limited pressure can be applied, yielding a moisture content of about 80%. It is not known yet how the enset fermentation would progress (pH reduction and microbial profile), when the enset mass would be pressed mechanically to obtain a much lower initial moisture content and when the mass would then be brought immediately into anaerobic conditions, in the same way as the silage of crops for feed purposes. A lower moisture content than values currently obtained (and concomitantly lower water activities) may inhibit the growth of undesirable microorganisms to a larger extent than in the current conditions and enhance silage fermentation (Danner et al., 2003; Inoue et al., 2010). Omitting the aerobic phase might affect the microbial dynamics and in particular the mycoflora.

3.3. Bacterial community composition during enset fermentation

A visual comparison between bacterial community compositions from different fermentation days (1, 7, 17, 31 or 60) per enset variety (Gena, Maze, Ketishe) is shown by non-metric multidimensional scaling in Figure 5. The distance between two symbols, which represent samples, illustrates the similarity of those samples in terms of bacterial community composition. On day 1, samples were highly dissimilar, both between and within different varieties. During fermentation, samples evolved to a more similar bacterial community composition. Finally, as from day 31 and continuing until day 60, samples from all varieties

had reached a comparable bacterial community composition, since the symbols are clustered together.

The bacterial profiles per variety during fermentation as determined by metagenetics are presented in Figures 6, 7 and 8 for Gena, Maze and Ketishe, respectively. Only bacterial OTUs in a relative abundance of at least 3% are shown (an overview of all bacterial OTUs is shown in Tables S2 and S3, Supporting Information). The most important similarities in the bacterial community of the three varieties on day 1 were in the first place, the presence of a bacterium assigned to the family of Enterobacteriaceae (OTU 2; 22.9, 28.2 and 17.7% relative abundance for Gena, Maze and Ketishe, respectively) and in the second place the clear presence of the lactic acid bacterium *Leuconostoc mesenteroides* subsp. *cremoris* (OTU 1; 29.7, 9.5 and 13.9%). Xiong et al. (2012) and Wouters et al. (2013) also described *L. mesenteroides* to be the dominant LAB in the initial phases of vegetable fermentation. Other OTUs belonging to the LAB and present on day 1 -be it not always in major abundancies- were assigned *Lactococcus lactis* subsp. *cremoris* (OTU 8; 29.7, 4.8 and 1.0%), *Enterococcus* sp. (OTU 5; 1.5, 3.8 and 2.0%), *Lactobacillus* sp. (OTU 7; 0.03, 6.2 and 0.2%) and *Weisella beninensis* (OTU 11; 0.2, 8.0 and 0.6%). In addition, the varieties Gena and Ketishe harboured the aerobic bacterium *Nocardiopsis* sp. (OTU 9; 14.2 and 24.6% respectively) but this OTU was absent in Maze. *Nocardiopsis* species are characterized by a wide ecological niche, including plants. On plants they produce many fibre degrading enzymes and α-amylases, which may be the case in enset too, as well as antibiotics and antifungal agents as a defence against plant pathogens (Bennur et al., 2015). An OTU identified as *Hafnia psychrotolerans* (OTU 17) was also present on all varieties (7.3, 7.7 and 4.1) this species was isolated from Lake water in China and it was negative for citrate utilization, lipase activity and α-glucosidase (Gu et al., 2015).. *Clostridium* (OTU 14) was absent (Ketishe) or only present in extremely low abundancies (Gena: 0.002%, Maze: 0.13%) on day 1. This is in

372 contrast to the substantial *Clostridium* spore counts on day 1 which were about 4 log cfu/g for
373 the three varieties. . Based on these counts, a reasonable abundancy of *Clostridium* in the
374 metagenetics-based data would be expected; however, under detection of bacterial endospore
375 counts in metagenetics data as explained by Filippidou et al .(2015); can cause discrepancy
376 between cultural and metagenetics results.
377
378 For the three varieties, differences between the profiles for days 1 and 7 (corresponding to the
379 first aerobic phase) on the one hand and the profiles for days 17, 31 and 60 (related to the
380 anaerobic second phase) can be observed. The transfer from the first to the second phase of
381 the fermenting enset implied for the three varieties an increase in the bacterial diversity, as
382 well as an elimination or a clear reduction in abundance of *L. mesenteroides* subsp. *cremoris*
383 (OTU 1), *Nocardiopsis sp.* (OTU 9) and the Enterobacteriaceae sp. (OTU 2). As shown in
384 Table 2, the species richness on day 1 was 63 ± 12, 58 ± 8 and 57 ± 22. On day 60, the
385 diversity was increased to 165 ± 17, 162 ± 6 and 162 ± 15, for Gena, Maze and Ketishe
386 varieties, respectively. Similarly, increasing Shannon–Wiener diversity indices were observed
387 from the 1stday (1.87 ± 0.31, 2.29 ± 0.41 and 2.22 ± 0.59) to the 60th day (3.36 ± 0.22, 3.30 ±
388 0.14 and 3.31 ± 0.29). In contrast to *L. mesenteroides*, the other LAB remained present
389 throughout the fermentation, such as e.g. *W. beninensis* (OTU 11). Moreover, new OTUs
390 belonging to the LAB appeared in the second phase, such as the facultatively anaerobic
391 *Lactobacillus concavus* in Gena (OTU 15; 11.3% on day 60) and the anaerobic
392 *Bifidobacterium minimum* in all three varieties (OTU 6; 3.8, 12.0 and 14.9% in Gena, Maze
393 and Ketishe on day 60). The disappearance of *Nocardiopsis* (OTU 8) in the second phase can
394 be explained by its oxygen requirements. Furthermore, although a decrease in abundance of
395 the Enterobacteriaceae sp. (OTU 2) from days 1 and 7 to day 60 was found for the three
396 varieties, its abundance was not completely reduced to zero at day 60 (9.0, 7.0 and 5.5% for

Gena, Maze and Ketishe). In the plate counts, no Enterobacteriaceae could be detected as from day 31. Probably, as Enterobacteriaceae were present at a high relative abundance in all three varieties on days 1 and 7, their DNA was still recovered in the metagenetic analysis at day 60, even though the cells were dead or at least not cultivable any more.

Another unequivocal observation from the metagenetic profiles is that the OTUs assigned to the genera *Dysgonomonas* (OTU 13) and *Clostridium* (OTU 14) and the species *Prevotella paludivivens* (OTU 3) were not or only in a very low abundance found in the first phase but in a consistent way (i.e. present in the three varieties on days 17, 31 and 60) in the second phase. They are all (facultatively or strictly) anaerobic. *Dysgonomonas* is related to human sources and is reported to cause gastroenteritis in immunocompromised persons (Murray et al. 2013). *P. paludivivens* was first isolated and described based on isolates from rice plant residues and the species is known to have xylanolytic activity (Ueki et al., 2007). Its abundance was especially high in enset produced from the Maze variety, with and abundance of 26.7% on day 31 and 16.0% on day 60. The higher abundance of *Clostridium* in the second phase (days 17, 31 and 60) than in the first (days 1 and 7) for all three varieties corresponded with *Clostridium* spore counts being higher in the second phase than in the first (although the counts for the second phase were not always statistically higher than those for the first phase). Gashe (1987) reported spore formers to reach fairly high numbers during the first 15 days of fermentation and to show active growth in the fermenting enset. As the genus *Clostridium* contains pathogenic species as well as spoilage organisms, further research is necessary to elucidate the food safety and sensorial attributes of fermented enset with respect to this genus and to develop fermentation practices that prevent *Clostridium* from colonizing the enset mass.

4. Conclusions

Processing of enset for human consumption in the Gamo highlands of Ethiopia is based on traditional knowledge of the people. Some of the processing steps and storage conditions differ among districts and even among individual villages. In a fermentation performed according to an average process and using erosas, a decrease in pH and an increase in titratable acidity were mainly seen in the first aerobic phase. This was likely due to the activity of acid producing microorganisms, mainly *Leuconostoc mesenteroides*. The second anaerobic stage of the fermentation was dominated by *Prevotella paludivivens, Lactobacillus* sp., *Enterococcus* sp. and *Bifidobacterium minimum.* Yeasts and moulds were also observed throughout the fermentation period.]*Clostridium* spores were present throughout the fermentation in high numbers, evoking questions on food safety and spoilage. Further research is needed to optimize the fermentation, in particular with respect to the moisture reduction before fermentation, food safety evaluation (identifying the *Clostridium* species involved in the fermentation) and the development of (a) standardized starter culture(s). Moreover, the role of yeasts throughout the fermentation process also needs further investigation.

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List of figure captions

Figure 1: Enset plant in the garden (A), traditional enset processing (B), bamboo basket or so-called 'erosa' filled with chopped enset and covered with dried enset leaves (C) and enset fermented for 60 days (D).

Figure 2: Survey results on the preferred variety for kocho and bulla preparation (A), the reasons for preferring a specific variety (B), the tools used to process the enset pseudostem and corm (C) and the indications used to establish the end of the fermentation (D). Results indicate percentages of the respondents indicating a specific answer.

Figure 3: Schematic diagram of the traditional enset fermentation process, compiled as an average process flow based on the survey.

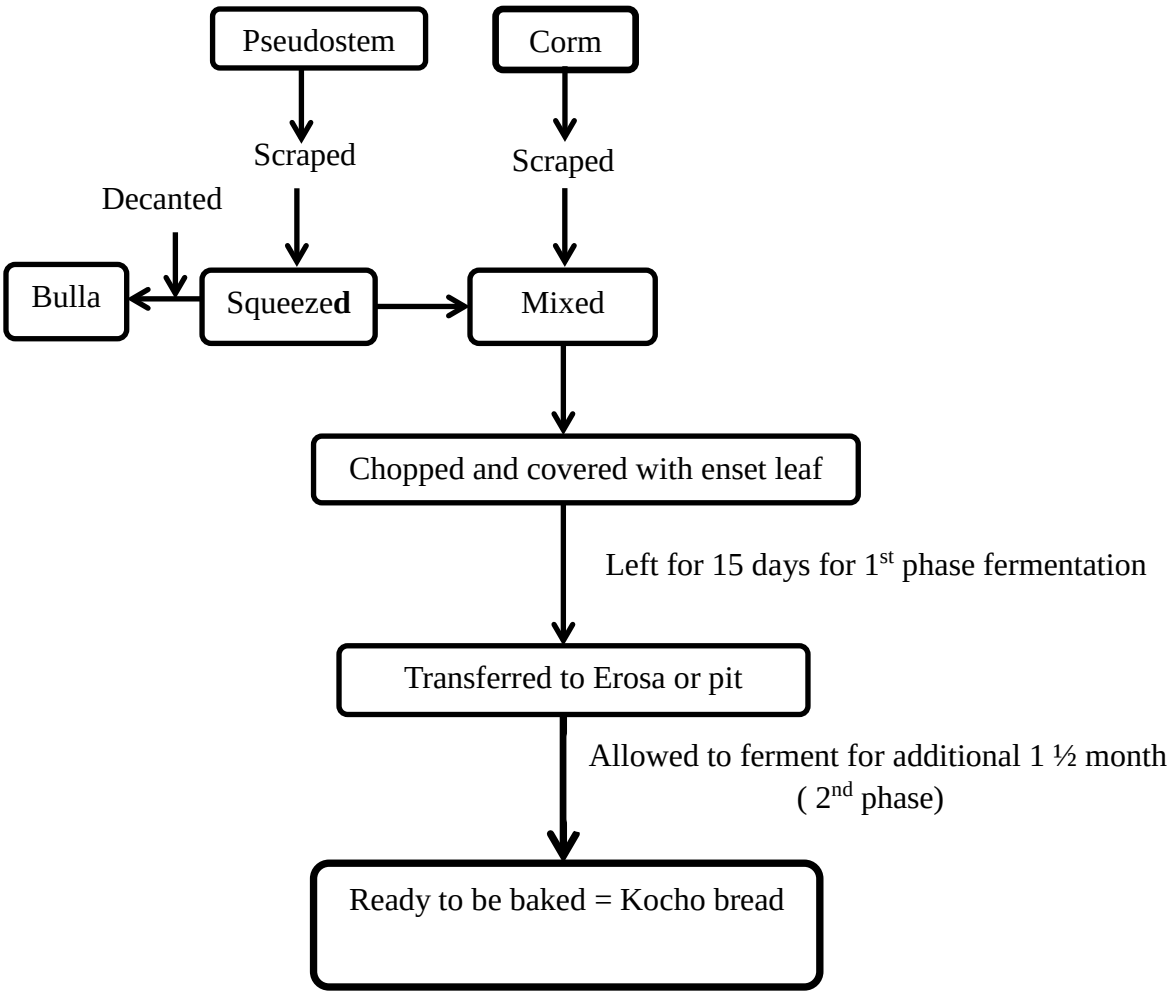
Figure 4: Temperature in the enset mass (A), moisture content (B), pH (C) and titratable acidity (D) in function of fermentation time (♦: variety Gena; ■: variety Maze; ▲: variety Ketishe).

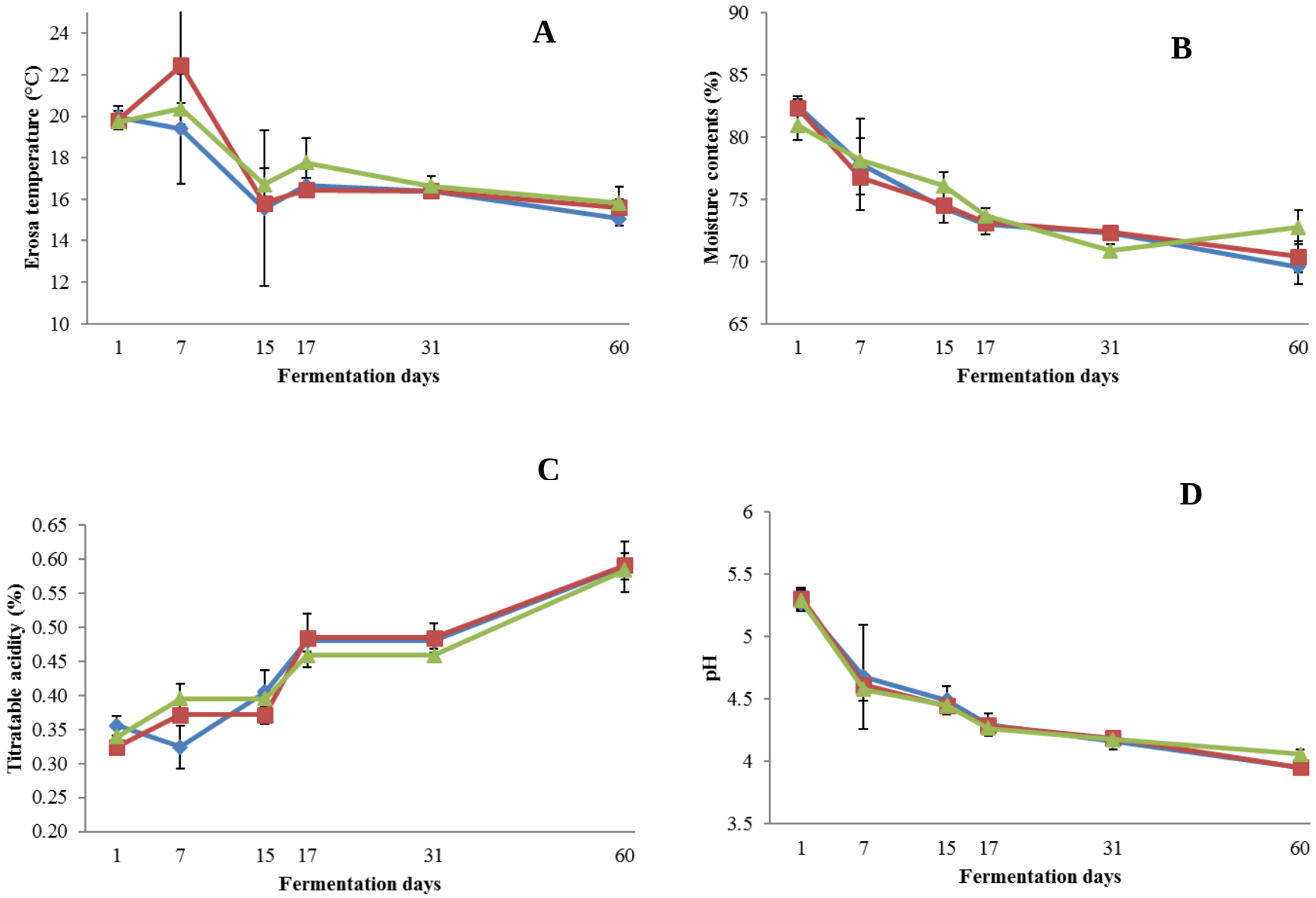
Figure 5: Non-metric multidimensional scaling ordinations composed of the bacterial community composition data (stress value of 0.079) from one (days 7, 17 and 31) or four (day 1 and 60) samples per fermentation day. Samples from the same variety are represented by the same colour (G = Gena, red; M = Maze, green; K = Ketishe, blue) and fermentation day is displayed as the number in the sample code. The distance between different points on the plot reflects their similarity level: the more similar the bacterial communities, the smaller the distance between the points.

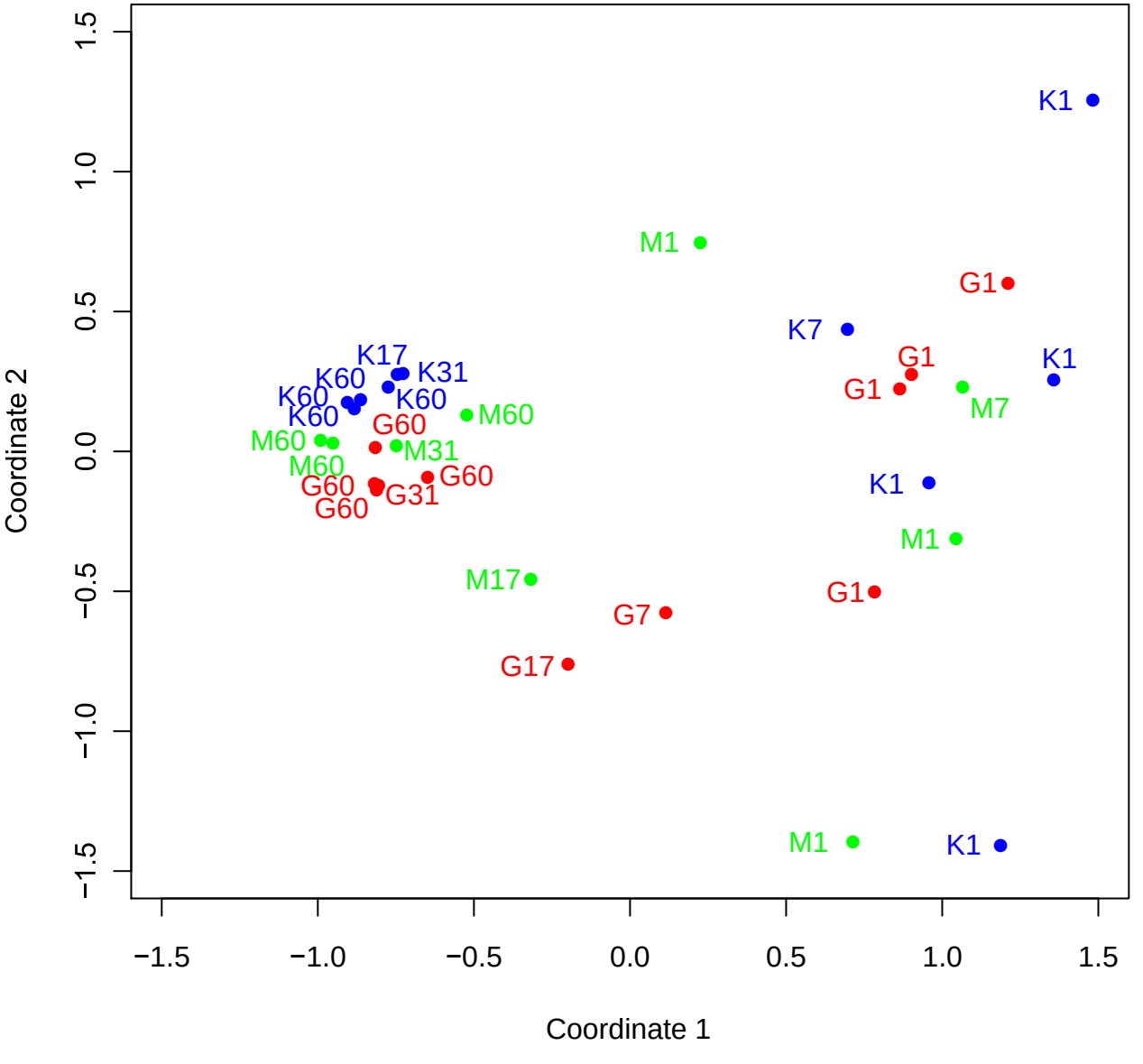
Figure 6: Relative abundance (%) of the bacterial community during enset fermentation of variety Gena for 60 days. OTUs with a relative abundance below 3% are grouped

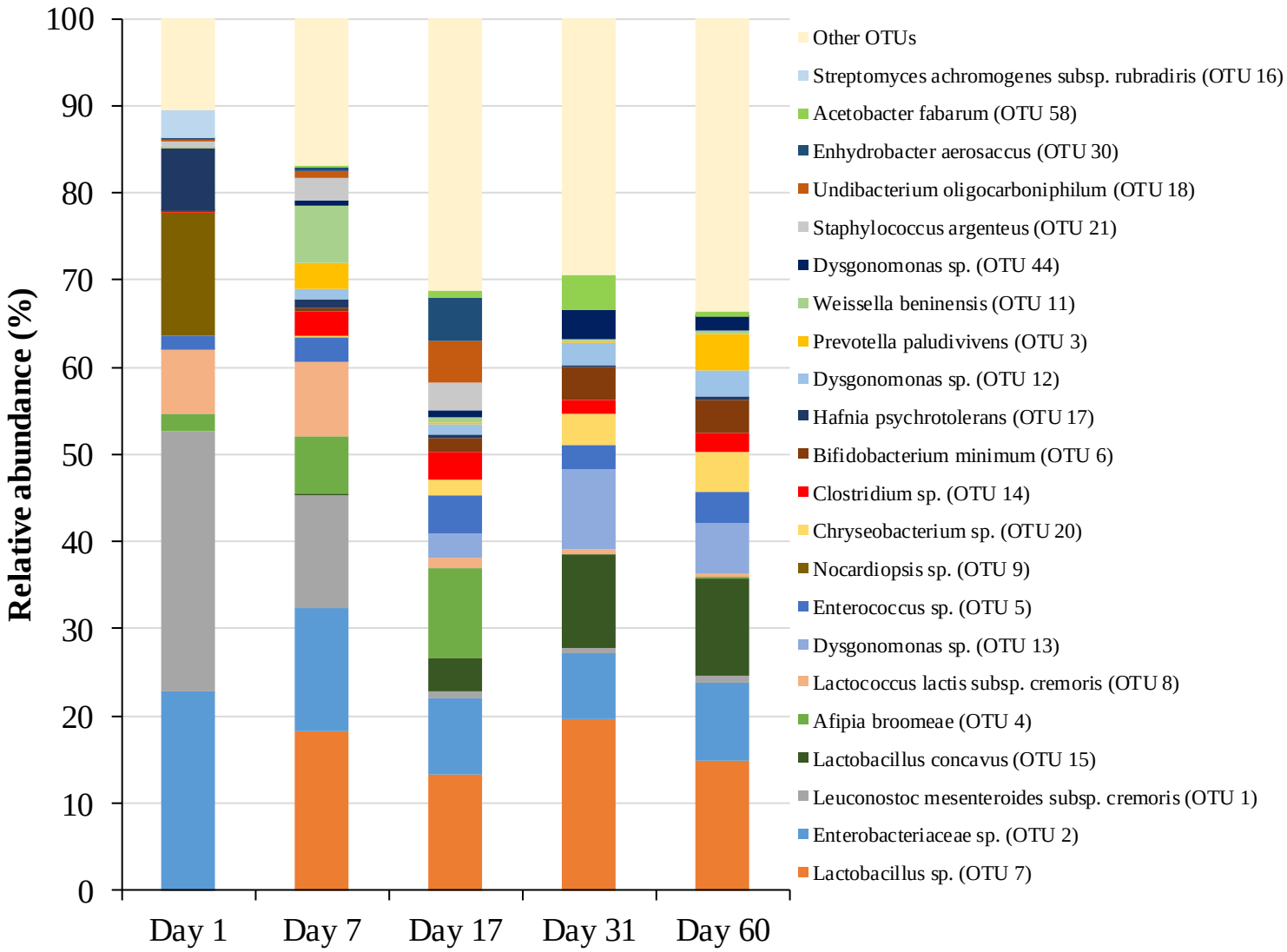
629 together in “Other OTUs”. Data are the mean of both replicates of one (day 7, 17 and
630 31) or four (day 1 and 60) samples.
631 Figure 7: Relative abundance (%) of the bacterial community during enset fermentation of
632 variety Maze variety for 60 days. OTUs with a relative abundance below 3% are
633 grouped together in “Other OTUs”. Data are the mean of both replicates of one (day
634 7, 17 and 31) or four (day 1 and 60) samples.
635 Figure 8: Relative abundance (%) of the bacterial community during enset fermentation of
636 variety Ketishe for 60 days. OTUs with a relative abundance below 3% are grouped
637 together in “Other OTUs”. Data are the mean of both replicates of one (day 7, 17 and
638 31) or four (day 1 and 60) samples.
639 Figure S1: Outside temperature during the study period

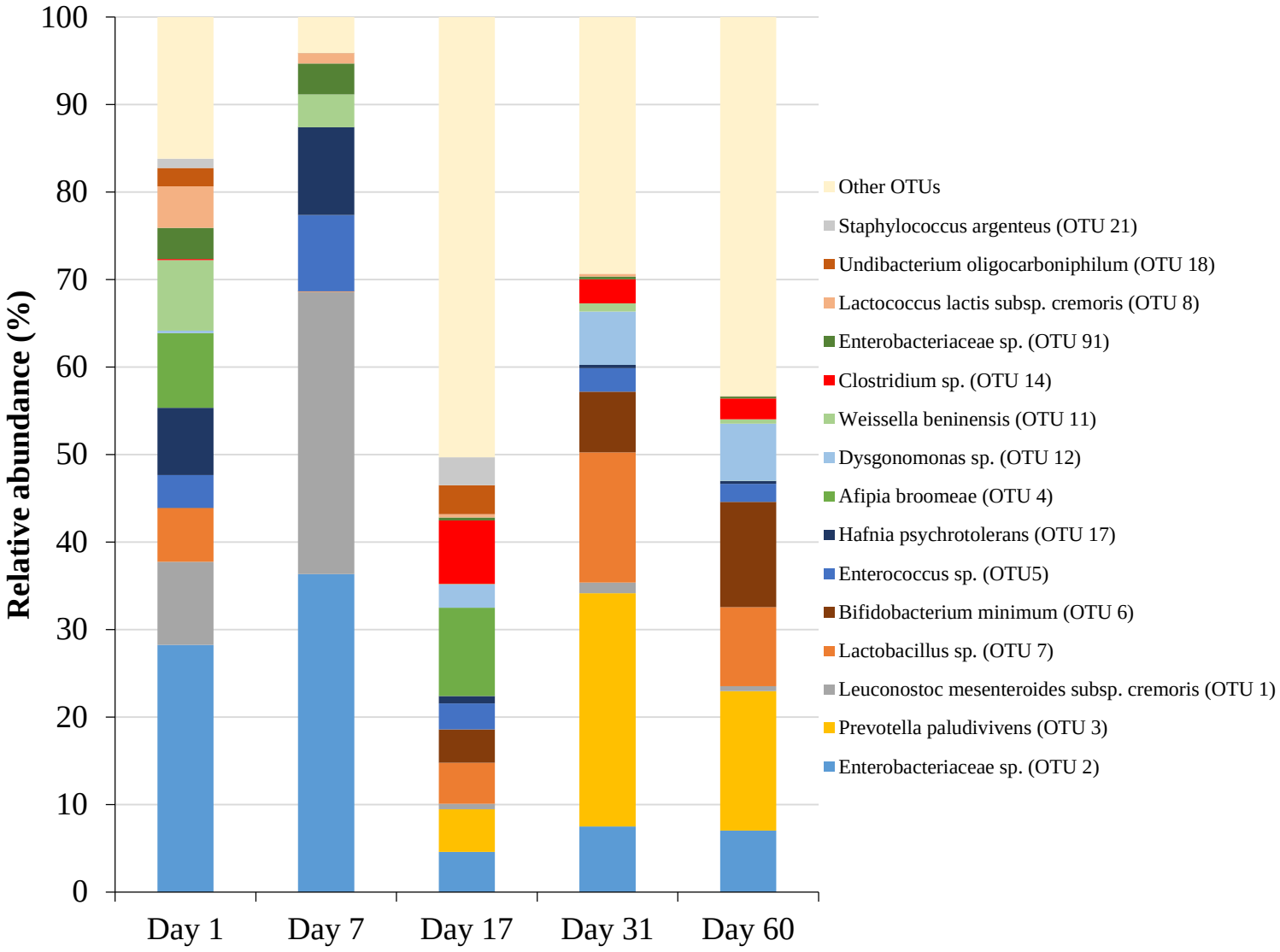

A
B
C
D


Pseudostem
Corm
Scraped
Scraped
Decanted
Bulla
Squeezed
Mixed
Chopped and covered with enset leaf
Left for 15 days for 1st phase fermentation
Transferred to Erosa or pit
Allowed to ferment for additional 1 ½ month
(2nd phase)
Ready to be baked = Kocho bread


A
Erosa temperature (°C)
24
22
20
18
16
14
12
10
1
7
15
17
31
60
Fermentation days
B
Moisture contents (%)
90
85
80
75
70
65
1
7
15
17
31
60
Fermentation days
C
Titratable acidity (%)
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
1
7
15
17
31
60
Fermentation days
D
pH
6
5.5
5
4.5
4
3.5
1
7
15
17
31
60
Fermentation days


K1
M1
G1
K7
G1
K1
K17
K31
K60
K60
K60
K60
M60
G1
M7
K60
G60
M60
M31
K1
M60
G60
G60
G31
M1
M17
G1
G7
G17
M1
K1
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5
Coordinate 2
Coordinate 1


Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Day 1
Day 7
Day 17
Day 31
Day 60
Other OTUs
Streptomyces achromogenes subsp. rubradiris (OTU 16)
Acetobacter fabarum (OTU 58)
Enhydrobacter aerosaccus (OTU 30)
Undibacterium oligocarboniphilum (OTU 18)
Staphylococcus argenteus (OTU 21)
Dysgonomonas sp. (OTU 44)
Weissella beninensis (OTU 11)
Prevotella paludivivens (OTU 3)
Dysgonomonas sp. (OTU 12)
Hafnia psychrotolerans (OTU 17)
Bifidobacterium minimum (OTU 6)
Clostridium sp. (OTU 14)
Chryseobacterium sp. (OTU 20)
Nocardiopsis sp. (OTU 9)
Enterococcus sp. (OTU 5)
Dysgonomonas sp. (OTU 13)
Lactococcus lactis subsp. cremoris (OTU 8)
Afipia broomeae (OTU 4)
Lactobacillus concavus (OTU 15)
Leuconostoc mesenteroides subsp. cremoris (OTU 1)
Enterobacteriaceae sp. (OTU 2)
Lactobacillus sp. (OTU 7)


Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Day 1
Day 7
Day 17
Day 31
Day 60
Other OTUs
Staphylococcus argenteus (OTU 21)
Undibacterium oligocarboniphilum (OTU 18)
Lactococcus lactis subsp. cremoris (OTU 8)
Enterobacteriaceae sp. (OTU 91)
Clostridium sp. (OTU 14)
Weissella beninensis (OTU 11)
Dysgonomonas sp. (OTU 12)
Afipia broomeae (OTU 4)
Hafnia psychrotolerans (OTU 17)
Enterococcus sp. (OTU5)
Bifidobacterium minimum (OTU 6)
Lactobacillus sp. (OTU 7)
Leuconostoc mesenteroides subsp. cremoris (OTU 1)
Prevotella paludivivens (OTU 3)
Enterobacteriaceae sp. (OTU 2)

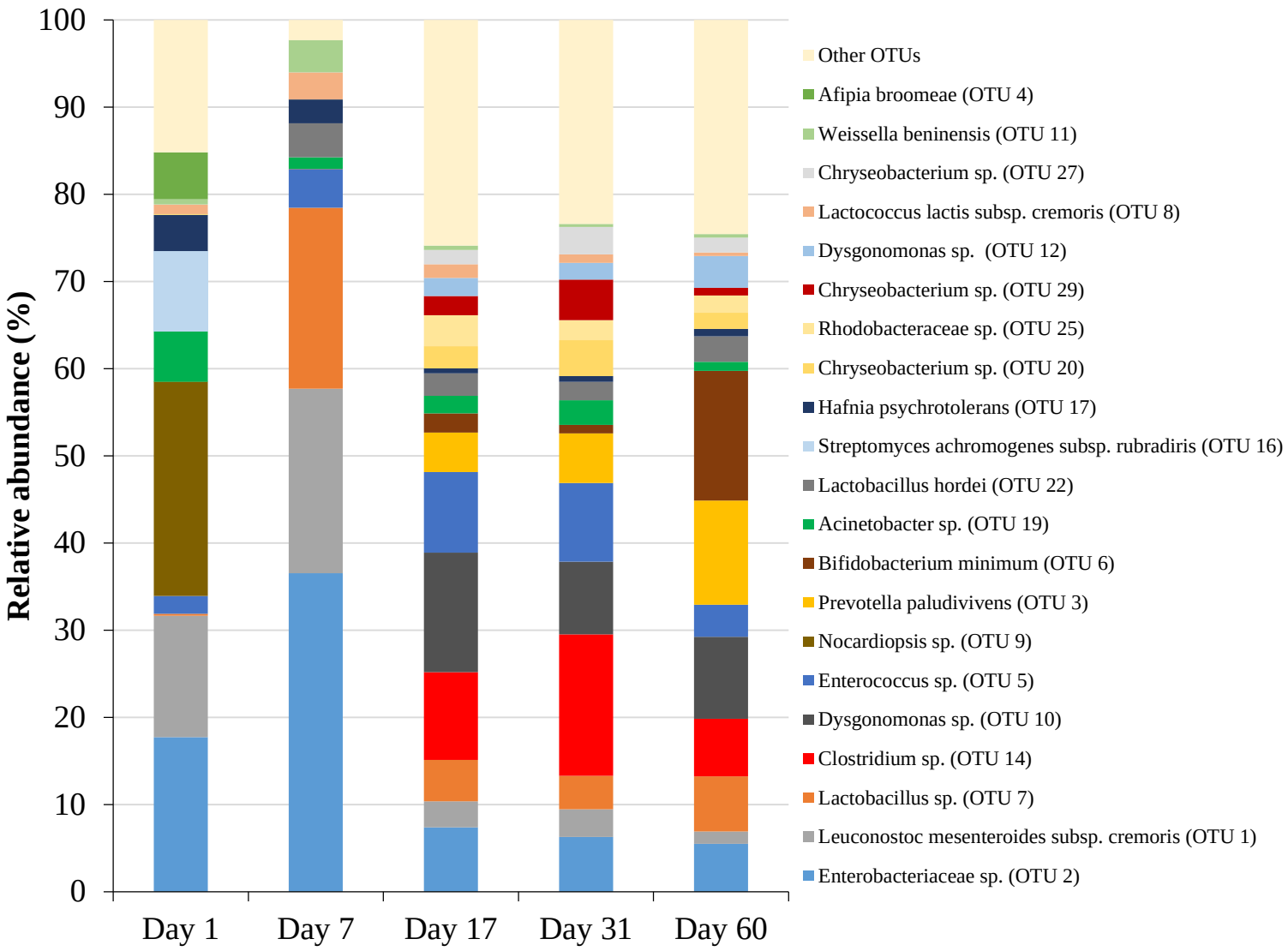

Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Day 1
Day 7
Day 17
Day 31
Day 60
Other OTUs
Afipia broomeae (OTU 4)
Weissella beninensis (OTU 11)
Chryseobacterium sp. (OTU 27)
Lactococcus lactis subsp. cremoris (OTU 8)
Dysgonomonas sp. (OTU 12)
Chryseobacterium sp. (OTU 29)
Rhodobacteraceae sp. (OTU 25)
Chryseobacterium sp. (OTU 20)
Hafnia psychrotolerans (OTU 17)
Streptomyces achromogenes subsp. rubradiris (OTU 16)
Lactobacillus hordei (OTU 22)
Acinetobacter sp. (OTU 19)
Bifidobacterium minimum (OTU 6)
Prevotella paludivivens (OTU 3)
Nocardiopsis sp. (OTU 9)
Enterococcus sp. (OTU 5)
Dysgonomonas sp. (OTU 10)
Clostridium sp. (OTU 14)
Lactobacillus sp. (OTU 7)
Leuconostoc mesenteroides subsp. cremoris (OTU 1)
Enterobacteriaceae sp. (OTU 2)

Table 3.1 Microbial counts of enset in erosas in function of fermentation time.

Variety	Microbial parameter	Microbial counts (log cfu/g)					
		Day 1	Day 7	Day 15	Day 17	Day 31	Day 60
Gena	Total viable count	8.16 ± 0.03^{aA}	8.58 ± 0.07^{bA}	9.63 ± 0.06^{cA}	9.66 ± 0.08^{cA}	9.12 ± 0.20^{dA}	8.30 ± 0.11^{abA}
	Lactic acid bacteria	6.17 ± 0.09^{aA}	8.13 ± 0.02^{bA}	8.99 ± 0.01^{cA}	9.58 ± 0.06^{dA}	9.19 ± 0.16^{cA}	8.07 ± 0.07^{bA}
	Enterobacteriaceae	7.98 ± 0.05^{aA}	5.15 ± 0.04^{bA}	4.65 ± 0.06^{cA}	3.19 ± 0.10^{dA}	$<1.0 \pm 0.00$	$<1.0 \pm 0.00$
	Yeasts and molds	4.76 ± 0.01^{aA}	6.15 ± 0.05^{bA}	6.67 ± 0.01^{cA}	3.92 ± 0.07^{dA}	5.03 ± 0.07^{eA}	3.57 ± 0.02^{fA}
	Clostridium spores	4.08 ± 0.07^{aA}	5.51 ± 0.02^{bA}	5.79 ± 0.02^{bcA}	7.78 ± 0.07^{dA}	6.41 ± 0.34^{eA}	5.67 ± 0.02^{bcA}
Maze	Total viable count	8.12 ± 0.12^{aA}	8.40 ± 0.03^{bA}	9.70 ± 0.11^{cA}	9.95 ± 0.03^{cB}	9.34 ± 0.17^{dA}	7.92 ± 0.05^{eB}
	Lactic acid bacteria	6.06 ± 0.05^{aA}	8.09 ± 0.07^{bA}	9.04 ± 0.00^{cB}	9.53 ± 0.05^{dA}	9.14 ± 0.17^{cA}	7.91 ± 0.05^{bA}
	Enterobacteriaceae	8.09 ± 0.03^{aB}	5.14 ± 0.02^{bA}	4.74 ± 0.14^{cA}	3.48 ± 0.05^{dB}	$<1.0 \pm 0.00$	$<1.0 \pm 0.00$
	Yeasts and molds	4.72 ± 0.02^{aA}	6.22 ± 0.09^{bA}	6.71 ± 0.04^{cA}	3.98 ± 0.07^{dA}	5.00 ± 0.02^{eA}	3.62 ± 0.02^{fB}
	Clostridium spores	4.02 ± 0.03^{aA}	5.57 ± 0.05^{bA}	5.84 ± 0.03^{cA}	7.83 ± 0.04^{dAB}	5.95 ± 0.03^{cA}	5.50 ± 0.09^{bB}
Ketishe	Total viable count	8.35 ± 0.05^{aB}	8.41 ± 0.13^{aA}	9.36 ± 0.27^{bA}	9.72 ± 0.01^{cA}	9.01 ± 0.11^{dA}	7.73 ± 0.04^{eC}
	Lactic acid bacteria	6.21 ± 0.05^{aA}	8.07 ± 0.08^{bA}	9.05 ± 0.03^{cB}	9.49 ± 0.04^{dA}	8.90 ± 0.16^{cA}	7.57 ± 0.15^{eB}
	Enterobacteriaceae	7.91 ± 0.06^{aA}	5.20 ± 0.11^{bA}	4.78 ± 0.20^{cA}	3.40 ± 0.06^{dB}	$<1.0 \pm 0.00$	$<1.0 \pm 0.00$
	Yeasts and molds	4.62 ± 0.03^{aB}	6.22 ± 0.16^{bA}	6.62 ± 0.11^{cA}	3.47 ± 0.02^{dB}	4.86 ± 0.02^{eB}	3.34 ± 0.03^{dC}
	Clostridium spores	4.02 ± 0.03^{aA}	5.49 ± 0.09^{bA}	5.78 ± 0.03^{cA}	7.93 ± 0.05^{dB}	6.05 ± 0.03^{eB}	6.05 ± 0.05^{fC}

Data are the mean of four samples, one from each erosa (four erosas per accession) ± standard deviation.

^{a,b,c,d,e,f} Different superscripts within the same row indicate significant differences ($p < 0.05$).

$^{A, B, C}$ Different superscripts per type of microbial count within the same column indicate significant differences ($p < 0.05$).

Table 2. Microbial community diversity indices from the enset during fermentation.[1]

Accession	Sample day	Diversity index			
		Observed richness	Chao1	Coverage (%)[2]	Shannon - Wiener
Gena	1[3]	63± 12[a]	93.75± 26.69[a]	71.40± 17.98[a,c]	1.87± 0.31[a]
	7	133 ± 11[a,b,c]	172.63 ± 16.29[b,c]	77.08 ± 0.72[a,b]	3.01 ± 0.13[b]
	15	125 ± 22[a,b,c]	126.38 ± 24.57[a,b]	98.70 ± 1.84[b]	3.63 ± 0.03[b]
	17	110 ± 3[b]	114.50 ± 7.07[a,b]	96.18 ± 3.47[a,b,c]	3.64 ± 0.11[b]
	31	150 ± 1[c]	187.84 ± 15.19[c]	80.15 ± 7.23[a,b,c]	3.25 ± 0.06[b]
	60	165 ± 17[c]	195.68 ± 19.90[c]	84.58 ± 4.92[c]	3.36 ± 0.22[b]
Maze	1[3]	58± 8[a]	65.31± 7.62[a]	89.75± 13.03[a]	2.29 ± 0.41[a]
	7[3,4]	44	68.00	64.71[a]	3.01
	17	138 ± 6[b]	138.55 ± 7.00[b]	99.25 ± 0.42[a]	4.04 ± 0.18[b]
	31	161 ± 17[c]	197.26 ± 42.13[a,b,c]	82.58 ± 9.03[a]	3.09 ± 0.08[b]
	60	162 ± 6[c]	197.86 ± 10.47[c]	82.27 ± 5.77[a]	3.30 ± 0.14[b]
Ketishe	1[3]	57 ± 22[a]	67.46 ± 25.06[a]	84.91 ± 10.63[a,b]	2.22 ± 0.59[a]
	7	52 ± 2[a,b]	78.50 ± 13.44[a,b]	66.35 ± 8.65[a,b]	1.83 ± 0.03[a]
	15	115 ± 6[b,c]	119.50 ± 6.36[b]	95.81 ± 0.22[a]	3.93 ± 0.13[b]
	17	153 ± 8[b,c,d]	195.33 ± 6.84[c]	78.30 ± 1.60[a,b]	3.49 ±0.05[b]
	31	141 ± 2[c]	177.81 ± 28.08[c]	80.11 ± 13.84[a,b]	3.35 ± 0.01[b]
	60	162 ± 15[d]	197.81 ± 13.40[c]	82.12 ± 5.40[b]	3.31 ± 0.29[b]

[1]Data are the mean of two sequenced extracts of one (day 7, 15, 17 and 31) or four (day 1 and 60) samples± standard deviation.[a,b,c,d]Different superscripts within the same column from the same accession indicate significant differences ($p < 0.05$). No significant differences were found between the different accessions for the same sample day.

[2]Coverage = (Observed richness/Chao1)*100.

[3]One or more samples were omitted for the calculation of the indices because of the low coverage (< 50%).

[4]Sample was omitted from post-hoc analysis because it concerns a single repetition.