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Development and validation of lactic acid starter cultures for enset

(*Ensete ventricosum*) fermentation

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ABSTRACT

In this study, lactic acid bacteria strains were screened for the development of (a) starter culture(s) specific for enset (*Ensete ventricosum*) fermentation. To this end, 158 isolates grown on de Man, Rogosa and Sharpe agar were obtained from fermenting enset and traditional starter material. They were screened based on their biochemical characteristics. Three promising strains, two *Lactobacillus plantarum* and one *Leuconostoc mesenteroides*, were selected and evaluated under field conditions. Aliquots of pulverized enset (without adding a liquid) were inoculated with one of the isolates at 3 log cfu/g and one uninoculated portion was used as control. Fermentation was performed in sauerkraut jars. Samples were taken on days 0, 7, 15, 30 and 75. On day 7 and onwards, the inoculated samples showed a larger pH reduction compared to the control. Enterobacteriaceae counts were reduced below the detectable level on day 7 and onwards. *Clostridium* spore counts were significantly lower for the inoculated than for the uninoculated samples on day 75. Inoculated enset showed a faster colonization of lactic acid bacteria than the uninoculated biomass. The three starters appeared to be suitable for enset fermentation. Further research is necessary to elucidate the proximate composition of enset fermented with these starters.

Keywords

Enset; fermentation; inoculation; lactic acid bacteria; starter culture

1. Introduction

Kocho is a starchy fermented food obtained from the mixture of scraped pseudostem pulp and pulverized corm of the enset (*Ensete ventricosum*) plant. It is widely consumed by millions

of Ethiopians and plays an important role in ensuring food security (Andeta et al., 2018b; Brandt et al., 1997). Kocho is a good source of starch (65%) and soluble sugars (9%), but it is low in protein ($< 4\%$) and fat ($< 1\%$). It has a moisture content above 62% (Bosha et al., 2016; Atlabachew & Chandravanshi, 2008). The sensory quality of kocho is often compromised due to the action of undesirable microbes (Andeta et al., 2018a; Birmeta et al., 2018). The lack of well-defined starter cultures and of controlled fermentation conditions are the main reasons for variability in the sensory and nutritional quality of kocho (Andeta et al., 2018b; Dibaba et al., 2018). Therefore, fermentation using a starter culture containing carefully selected lactic acid bacteria (LAB) with desirable functional properties has been consistently proposed (Andeta et al., 2019; Birmeta et al., 2018). The selection criteria for an ideal starter culture for enset fermentation are: a fast pH reduction (to inhibit growth of undesirable microorganisms), a reasonable acid tolerance (so that the starter is not impeded by auto-inhibition), growth possible at different temperatures (enset is grown and fermented in a temperature range between 10 and 30°C, corresponding to several heights above sea level where it occurs) and elevated NaCl concentrations (because in some regions salt is added at the start of fermentation) (Birmeta et al., 2018; Bosha et al., 2016; Saarisalo et al., 2007).

In our recent studies (Andeta et al., 2018a, 2018b) and in studies of others (Birmeta et al., 2018; Gashe, 1987), the dominance of *Lactobacillus* and *Leuconostoc* species during enset fermentation was reported. These LAB are likely important in enset fermentation because they can contribute to the sensory characteristics and preservative effects (Birmeta et al., 2018; Bosha et al., 2016). Likewise, the presence of yeasts in fermenting enset has also been reported and suggested to play a role in the fermentation (Andeta et al., 2018a; Dibaba et al., 2018). However, LAB outnumber yeasts throughout the fermentation and LAB starters are

hypothesized to colonize the fermentation more rapidly than yeasts (Andeta et al., 2019; Birmeta et al., 2018).

The aim of this study was to isolate and characterize LAB from fermenting enset and from traditional (and hence not standardized) starter material produced in local households, to select three promising candidates as starter culture and to ferment enset under field conditions with these potential starters. The fermentation was monitored with respect to pH, moisture content, microbial counts and bacterial community composition.

2. Materials and methods

2.1. Isolation of presumptive lactic acid bacteria from fermented enset and traditional starters

Fermented enset samples and traditional starter material were obtained during studies described previously by Andeta et al. (2018b, 2019). Samples were collected in a sterile beaker and LAB counts were determined in those studies on de Man, Rogosa and Sharpe (MRS) agar (Biokar Diagnostics, Beauvais, France) incubated at 30°C for 72 h. For the research described here, first 158 colonies were picked from the aforementioned MRS plates and individually streaked on the same medium. A single colony was picked from each plate, inoculated in MRS broth (Biokar Diagnostics) and incubated overnight at 30°C to obtain a purified culture. The cultures were preserved in a mixture of MRS broth and sterile glycerol (Loba Chemie, Mumbai, India) (50%/50%, v/v) at -18.

2.2. Characterization of the isolates

All isolates were subjected in the first place to Gram staining and assessed for catalase activity, spore formation and indole production according to methods described by Khedid et al.

(2009) and Saarisalo et al. (2007). Moreover, amylolytic activity of the strains was determined by streaking them onto MRS agar containing 2% soluble starch (Suvidhinath Laboratories, Vadodara, India) followed by incubation at 30°C for 72 h (Agati et al., 1998). After incubation, the plates were flooded with lugol solution and the appearance of a clear halo around the colonies was considered as a positive test (Dibaba et al., 2018).

For screening the ability to grow in an enset-based broth, fresh enset pseudostem sheath was obtained from the Chenchu district and was shredded into small pieces using a knife. Subsequently, it was mixed with distilled water (50% w/v) and blended using a 750-watt blender (PSAW, Haryana, India) for 3 minutes. The aliquot was filtered using Whatman 40 (Labsys, Bangalore, India) and sterilized at 120°C for 15 min after the addition of three drops of bromocresol purple indicator solution (Merck KGaA, Darmstadt, Germany). Then it was aseptically diluted (50% v/v) with sterile physiological saline (0.85% NaCl). Each isolate was inoculated into the prepared enset-based broth at a cell density of 4 log cfu/ml and incubated at 30°C for 48 h. Color change from brown to yellow and pH reduction below 4.20 was considered as a positive result. Afterwards, strains showing amylolytic activity and growth on enset-based broth in the aforementioned test were inoculated into MRS broth and incubated at different temperatures (10 and 45°C) to test their temperature tolerance, in different NaCl concentrations (2%, 4% and 6%, all at 30°C) to test their salt tolerance and at pH 4 (at 30°C) to test their acid tolerance. Growth was monitored by measuring OD values at 580 nm using a Spectrophotometer (Biospec-Mini, Duisburg, Germany) after 1, 2, 3, 8, 12 and 24 hours incubation. Eight strains that showed amylolytic activity, growth on enset-based broth, growth at different temperatures and different NaCl concentrations were selected for further

identification.

2.3. Identification of selected bacterial species by *pheS*, *rpoA* and 16S rRNA gene sequencing

The 16S rRNA gene was amplified and sequenced using Sanger sequencing, at the MacroGen sequencing service (Amsterdam, the Netherlands). Furthermore, the phenylalanyl-tRNA synthase alpha subunit (*pheS*) partial gene and the RNA polymerase alpha subunit (*rpoA*) partial gene were also sequenced for the identification of the isolates to the species level by the method described by Naser et al. (2005).

2.4. Enset fermentation using starters at laboratory scale

One enset plant (variety Gena) was processed (i.e. its pseudostem was harvested and chopped manually into pulp as described by Andeta et al. (2018a) and the pulp was homogenized by mixing. Then the mass was divided into nine equal parts. Eight aliquots were inoculated with one of the isolates at a cell density of 3 log cfu/g enset as described in section 2.5.1. Uninoculated enset was used as control. For each of the eight inoculated enset aliquots and for the control, nine 50 ml Falcon tubes were stuffed completely (48 g) and then closed to create (semi) anaerobic conditions in the same way as in enset fermentation in the field, and they were incubated at 30°C for 15 days. Per treatment, three falcon tube samples were withdrawn on days 1, 7 and 15 and analyzed for pH and presumptive LAB counts (MRS Agar, 30°C, 72 h).

2.5. Enset fermentation using starters in the field

2.5.1. Preparation of starter cultures for inoculation

Based on the lab scale fermentations, three strains were selected and tested in larger scale fermentation, being a *Lactobacillus plantarum* (further referred to as FX 15), another *Lactobacillus plantarum* strain referred to as SAM 6 and a *Leuconostoc mesenteroides* strain indicated as HM 53. To prepare cultures for inoculation of the fermentation in the field, a colony from an MRS plate was inoculated into 20 ml MRS broth and incubated at 30°C for 18 h to reach a final cell concentration of approximately 9 log cfu/ml. The cells were harvested by centrifugation at 3400 g for 15 min and then washed twice with sterile 0.9% saline as described by Xiong et al. (2014a). Then, the pellets were suspended in a sterile peptone physiological salt solution (0.85% NaCl, 0.1% peptone, Biokar Diagnostics). The LAB suspensions were adjusted by diluting to reach a final cell concentration of 6 log cfu/ml for starter inoculation.

2.5.2. Enset processing, inoculation and sampling

Six enset plants were obtained from the Chench district in the Gamo zone and processed in a traditional way as described previously by Andeta et al. (2018b). The processed enset mass was homogenized using disinfected equipment to obtain a total mass of 156 kg. This mass was divided into four equal portions of 39 kg. For fermentation using the prepared starter cultures, each of the three LAB strains was inoculated (3 log cfu/g of enset) into one portions while one portion was left uninoculated as a control. The inoculated enset aliquots were homogenized using a disinfected stainless-steel noodle serving spoon. Twelve sauerkraut jars with a volume of 10 l (Keuken Keramiek, Beegden, the Netherlands) were each filled with 13 kg of enset mass as described previously by Andeta et al. (2018b). Every jar contained two stone plates to apply pressure on the contents. The lid was placed in a water lock to maintain anaerobic conditions in the jar. Three jars were filled for each LAB strain as well as for the control, and all twelve jars were kept in the same room. The average environment temperature during the study period was

18.5°C. Sampling was carried out in a sterilized beaker using sterilized spoons on days 0, 7, 15, 30 and 75. A sample was taken at the surface (about 70 g), in the middle (about 70 g) and at the bottom (about 70 g), and then pooled to one sample. Afterwards, the fermenting enset mass was pressed to restore the anaerobic conditions. Physicochemical (pH and moisture content) and microbial dynamics were monitored on fresh samples as described in section 2.5.3. Metagenomic analyses were performed on freeze-dried samples (24 h, -50°C, Martin Christ freeze-drier, Alpha 1-4 LD plus, Osterode am Harz, Germany).

2.5.3. Monitoring of pH, moisture content and microbial counts during field fermentation

The pH and the moisture content of the samples was determined as described previously by Andeta et al. (2018b), using a pH meter (HQ40d; HACH, Düsseldorf, Germany) and the oven drying method, respectively. Microbial counts were performed on the pooled samples using the pour plate technique, as published previously by Andeta et al. (2018b). Plate Count Agar was used for total viable aerobic counts, Violet Red Bile Glucose Agar for Enterobacteriaceae, Oxytetracycline Glucose Agar for yeasts and molds and Reinforced *Clostridial* Agar for *Clostridium* spores (after a heat treatment performed as in Andeta et al. (2018b)). All media were obtained from Biokar Diagnostics.

2.5.4. Bacterial community composition during field fermentation

From each freeze-dried fermented sample, five grams were aseptically crushed and homogenized in 45 ml of sterile peptone physiological saline (0.85% NaCl, 0.1% peptone, Biokar Diagnostics) for 2 min using a Bagmixer® Stomacher (Interscience, Saint Nom, France). The homogenate was centrifuged at 16,000 g for 5 min. Total DNA was extracted from 0.25 g pellet using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Each sample was extracted twice and the replicates were combined

and diluted 10 times. The V4 region of the 16S rRNA gene was amplified by PCR, quantified and sequenced as in Andeta et al. (2018b). The same bioinformatics pipeline was followed as in the aforementioned paper, with the difference that in this study rarefaction was performed up to 11,688 sequences per sample. In addition, in this study sequences were grouped into species-level operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off using the algorithm described in Andeta et al. (2018b). The taxonomic origin of each OTU was determined with the SINTAX algorithm in USEARCH (Edgar, 2016), using 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 search (Altschul et al., 1990) against GenBank (Benson et al., 2008), retaining only type strains entries. Primarily, assignments were based on SINTAX results, but BLAST results were used when SINTAX assignment was inconclusive or produced assignment scores below 0.80 (Table S1, supporting information). Chao1 and Shannon-Wiener diversity indices were calculated for the microbial communities of inoculated and uninoculated samples using R package Phyloseq v1.16.2 (R-package, R Development Core Team 2013).

2.5.6. Statistical analysis of data obtained during and after field fermentation

All statistical analyses were performed using SPSS (IBM©SPSS Statistics v. 23, New York, USA). Differences in the pH, moisture content and microbial counts throughout the different sampling days and between the inoculated and uninoculated samples were analyzed by one-way ANOVA. For all analyses, multiple comparisons were performed using Turkey's HSD, while considering a significance level of 0.05.

3. Results and discussion

3.1. Isolation, characterization and pre-selection of the isolates

A total of 158 isolates were obtained from the colonies cultivated on MRS and originating from fermented enset and traditional starter material (Table S2, supporting information). Gram-positive, non-spore formers, catalase and indole negative isolates were considered to be plausible LAB and selected for further screening. Biochemical characteristics of the isolates are shown in Table 1. Of the 158 strains, the majority showed desirable biochemical characteristics (i.e. 154 were Gram-positive, 155 catalase-negative and 154 indole-negative). Part of the strains were able to utilize sucrose (57.56%), lactose (89.87%) and glucose (92.41%). However, only 5.06% and 11.76% of the strains were able to grow in enset-based broth and ferment starch, respectively. Amylolytic LAB (ALAB) can indeed be found in starch-based fermented foods (Oguntoyinbo & Narbad, 2015; Agati et al., 1998). Predominantly, *Lactobacillus*, *Lactococcus*, and *Streptococcus* genera are reported to produce lactic acid from starch (Petrova et al., 2013). Isolates with ALAB characteristics were reported in fermented enset previously (Dibaba et al., 2018). Based on their ability to utilize different substrates and to grow in enset-based broth, eight amylolytic strains were selected for further identification.

3.2. Identification of the eight pre-selected isolates by pheS, rpoA and 16S rRNA gene sequencing

The combined use of the 16S rRNA, *pheS* and *rpoA* gene sequences offers a reliable identification system for all species of the genus *Lactobacillus* and other closely related species (Naser et al., 2005, 2007). The eight isolates were identified up to species level (Table 2, Table S3-S5, supporting information). Among the eight isolates, seven isolates were identified as

Lactobacillus plantarum (FX 2, FX 15, FX 17, FX 36, SAM 6, SAM 7 and GM 69) and HM 53 was identified as *Leuconostoc mesenteroides*. These species were identified in fermented enset and traditional starter material in earlier research (Andeta et al., 2019, 2018a; Birmeta et al., 2018).

3.3. Growth rates at low pH, different salt concentrations and temperatures

The growth properties of the eight LAB strains isolated from fermented enset or traditional starter cultures in laboratory conditions are shown in Figure 1. The eight isolates were characterized for their ability to grow at low pH, at different temperatures and at elevated salt concentrations. Growth under all these conditions is to be preferred when selecting a starter, as previous research has shown that these conditions prevail during enset fermentation and/or are requirements for good starters (Ammor & Mayo, 2007; Gashe, 1987). Important differences in the ability of the selected strains to grow at pH 4 were observed (Figure 1A). Strain *Lactobacillus plantarum* FX 15 and FX 36 reached the highest cell densities at 24 h as measured at 580 nm ($OD = 1.12 \pm 0.00$, $OD = 1.11 \pm 0.02$, respectively). Significant differences were observed between 1 h and 3 h ($p = 0.000$) for *Lactobacillus plantarum* FX 15 and *Lactobacillus plantarum* SAM 6. The strain FX 15 showed a faster onset of growth than the other *Lactobacillus plantarum* strains. On the other hand, *Leuconostoc mesenteroides* HM 53 showed a significantly ($p = 0.000$) lower growth rate after 8 hours and onwards. A potential starter needs to survive and preferably even grow and metabolize during the entire enset fermentation process, where the pH can eventually reach levels as low as 4.0. Acid tolerance of LAB strains has already been reported in various fermented foods, including enset (Dibaba et al., 2018; Xiong et al., 2014b).

In addition, the eight isolates were tested for their ability to grow at different salt concentrations (2%, 4% and 6% NaCl (w/w), presented on Figures 1B, 1C and 1D, respectively) on MRS broth at 30°C. High osmotolerance is a requirement of LAB strains to be used as a starter cultures (Menconi et al., 2014). Osmotolerance is also an important characteristic for fermenting enset as well, since salt is being added to the fermenting enset in some enset growing regions, such as the Wolayita zone (Andeta et al., 2019). All isolates were able to grow at 2% with the exception of *Lactobacillus plantarum* GM 69, which showed poor growth. At 6% NaCl, the OD profiles varied substantially between strains, with FX 15, FX 17 and FX 36 showing the best result.

Regarding temperature requirements for growth, FX 2, FX 15, FX 17, FX 36 and SAM 6 yielded the highest cell density at 10°C at 24 h incubation (Figure 1E). Menconi et al. (2014) reported that a low temperature coincides with a low production of lactic acid in *Lactococcus* spp., but such disadvantage could be avoided by selecting only these strains that show sufficient growth at low temperature (Wouters et al., 2000). Strains FX 36, FX 17 and SAM 6 showed the highest cell density at 45°C (Figure 1F). The ability to grow at high temperature is also a desirable characteristic, as it is related to a high rate of lactic acid production (Ibourahema et al., 2012). On the basis these criteria, FX 2, SAM 7 and GM 69 strains were not suitable for selection as a starter culture.

3.4. Enset fermentation using starters under laboratory conditions

The pH and LAB counts observed during fermentation of enset in Falcon tubes without inoculation (control) and with the eight isolates selected so far are shown in Figure 2A and 2B, respectively. Throughout the fermentation, the pH values of all samples ranged from 5.52 to 4.26. Significant reductions ($p = 0.000$) were observed from day 1 to day 15 for all inoculated

samples as well as the control, while the pH was significantly lower ($p = 0.000$) using the starters compared to the control at day 15. SAM 6 showed the fastest pH reduction, which is an important criterion in starter selection. Figure 2B reveals that LAB counts generally increased in all samples, including the control. For inoculated samples, the counts included both endogenous LAB present in the enset at the start of the test as well as those in the inoculum. The largest count was observed on day 7 for the fermentation inoculated with FX 15 (9.38 ± 0.03 log cfu/g), but a non-significant ($p = 0.556$) decrease was seen on day 15. After 15 days of fermentation, starter FX 36 resulted in a statistically higher count compared to the other samples ($p < 0.004$).

Based on these results, three strains were selected for the field conditions. In the first place, *Lactobacillus plantarum* FX 15 was chosen due to its fast growth in the lab scale fermentation and *Lactobacillus plantarum* SAM 6 for its fast pH reduction. Although *Leuconostoc mesenteroides* HM 53 did not perform exceptionally well for these aspects, it was selected as well, because it was the only amylolytic strain not being *Lactobacillus plantarum*. *L. mesenteroides* has regularly been detected in fermenting enset and in traditional starter material before (Andeta et al., 2018b and 2019) and therefore it was concluded to be interesting to include this strain in the field fermentations.

3.5. Enset fermentation using starters in the field conditions

3.5.1. Physicochemical parameters

Figure 2C points out that the initial pH of all samples was around 5.6. As of day 7, the pH of the inoculated samples was significantly lower than the control ($p = 0.000$). At the end of the fermentation on day 75, the pH of all inoculated samples was generally 0.5 unit below that of the control, demonstrating the activity of the starters.

Overall, the moisture content (Figure 2D) declined during fermentation in the jars in the same way as in our previous study (Andeta et al., 2018b) from about 85% to about 77%. This can mainly be explained by the evaporation of water through the water lock. Statistical analyses showed no difference in the moisture content between the inoculated and uninoculated samples at day 0 ($p > 0.998$), indicating that inoculation did not affect the moisture content. At the end of the fermentation, the moisture content of the inoculated fermentations was significantly ($p < 0.04$) higher than that of the control. The temperature inside the fermenting mass ranged between 17.0 to 22.4°C (Figure S1), which is in line with a previously reported range of 16.0 to 20.5°C (Andeta et al., 2018b).

3.5.2. Microbial counts

Microbial counts measured during fermentation in jars are shown in Table 3. The total viable aerobic counts (TVC) were generally high and ranged between 7.40 to 8.18 log cfu/g for both inoculated and uninoculated samples. The results obtained for TVC were lower than the previously reported values in Andeta et al. (2018b), which generally ranged from 8.93 ± 0.35 to 9.21 ± 0.30 . This may be related to the lower temperature during the processing season in the current study. At day 7, TVC of inoculated samples showed significantly higher counts than the uninoculated samples ($p < 0.030$). However, at the final stages of the fermentation (day 75) no significant differences were observed between inoculated (FX 15, SAM 6, HM 53) and uninoculated samples ($p = 0.916$, $p = 0.277$ and $p = 0.257$, respectively).

LAB constituted a large fraction of the total microbial count on day 0 and onwards, for the inoculated samples but also for the control. On day 0, LAB exhibited an average count ranging between 7.31 to 7.39 for the inoculated samples and 5.42 ± 0.03 for the uninoculated sample. A significant difference was found between the inoculated (FX 15, SAM 6 and HM 53) and

uninoculated samples ($p = 0.000$) at day 0. Significant differences were not observed within the inoculated samples at day 0 ($p > 0.05$). At day 7, the LAB count reached a maximum in all fermentations, which also corresponds to our previous research (Andeta et al., 2018b). At day 75, LAB counts showed significant differences between FX 15 and SAM 6 ($p = 0.012$), and between FX 15 and HM 53 ($p = 0.009$). The Enterobacteriaceae counts were the highest at the start of the fermentation and ranged between 6.75 to 6.94. At day 0, no statistical differences were observed between the inoculated and uninoculated samples ($p = 0.226$). The count remained below the detection limit of 1.0 log cfu/g on day 7 and other subsequent sampling days for all samples. The acidic environment (Figure 2C) created by endogenously present or added LAB may have been unfavorable for the Enterobacteriaceae (Andeta et al., 2018b; Bosha et al., 2016).

A striking observation for the yeast and mold counts was that for the inoculated fermentations, the counts decreased slightly during the first 7 days with 0.16, 0.33 and 0.34 log cycles for FX 15, SAM 6 and HM 53, respectively, but then they showed a sudden (and statistically significant) drop to day 15 with 1.74, 1.91 and 1.77 log cycles for FX 15, SAM 6 and HM 53, respectively. For the rest of the fermentation, the counts remained between 2.50 and 2.77 log cfu/g. In contrast, the control showed a smaller but steady decrease in yeast and mold counts from 4.88 on day 0 to 3.96 log cfu/g on day 75. On day 7 and onwards, the yeast and mold count of all inoculated samples were significantly lower than from the control ($p = 0.000$). Hence, inoculating onset with LAB starters has the potential to inhibit the spoilage yeasts and molds (Birmeta et al., 2018; Gallo et al., 2018).

The average *Clostridium* spore counts on day 0 (2.96 to 3.00 log cfu/g) were generally lower compared to those reported in a previously study Andeta et al. (2018a); ranging from 4.02

to 6.05 log cfu/g). Significantly higher counts were observed for the uninoculated sample than for the inoculated samples on days 30 and 75 ($p = 0.000$, $p < 0.001$, respectively). It can be hypothesized that the faster pH reduction in the inoculated fermentations than in the control has resulted in a larger inhibition of the germination of the spores present in the initial enset biomass into vegetative cells and hence of the subsequent growth and sporulation of these cells. Similarly, Tabacco et al. (2009) reported that inoculation of fresh forage with *Lactobacillus buchneri* and *Lactobacillus plantarum* species reduced the risk of *Clostridium* outgrowth.

3.5.3. Bacterial community composition

Next generation sequencing targeting the V4 region of the 16S rRNA gene was used to characterize the bacterial community of all samples for the five sampling days. A total of 1,318 Operational Taxonomic Units (OTUs) were identified from all samples, belonging to more than 4 phyla (Figure 3). The Proteobacteria represented the most abundant phylum at day 0 for control as well as inoculated fermentations, but they gradually decreased in function of time. On day 75, the Firmicutes (37.02 – 50.96%) represented the most abundant phylum in all inoculated samples, which was comparable with the plate count as the LAB dominated the entire fermentation (Table 3). Again, Firmicutes were reported to be the most dominant phylum in comparable fermentation products, such as cereal foods (Oguntinyinbo & Narbad, 2015; Gabaza et al., 2019) and fermented vegetables (Yu et al., 2013). Proteobacteria were not detected in samples inoculated with FX 15, HM 53 and the control on day 75, and they occurred at a relatively high abundance (23.54 %) in the sample inoculated with SAM 6.

Focusing on a lower taxonomic level, the most abundant OTUs for the five sampling days are shown in Figure 4. Only bacterial OTUs with a relative abundance larger than 3% are

369 shown. OTUs 1 and 3, belonging to the genera *Buttiauxella* and *Leuconostoc*, were present in
 370 both inoculated and uninoculated samples for days 0, 7, 15 and 30. *Buttiauxella* sp. (64.70 –
 371 17.32%) were abundant on the early stages of the fermentation. The abundance decreased below
 372 the detection level on day 75 for all samples, except for SAM 6. At day 0, the sample inoculated
 373 with *Leuconostoc mesenteroides* (HM 53) showed a higher abundance (23.60%) of *Leuconostoc*
 374 sp. than the other samples (due to the inoculation). However, *Leuconostoc* (OTU 3) was not
 375 observed anymore on day 75, which was probably due to its poor acid tolerance (Xiong et al.,
 376 2014b). In addition, the fermentations inoculated with *L. plantarum* (FX 15 and SAM 6)
 377 harbored *Lactobacillus* sp. (OTU 2) on day 7 and onwards. This OTU was most abundant on
 378 day 15 for the two fermentations with *L. plantarum*, being 49.75 and 42.88%, respectively.
 379 However, this OTU was absent in fermentations inoculated with *L. mesenteroides* (HM 53) on
 380 day 7. OTU 2 also appeared on uninoculated samples on day 7 and onwards. Other OTUs
 381 belonging to LAB appeared as from day 7 in both inoculated and uninoculated samples,
 382 including OTUs belonging to *Lactobacillus* (OTU 8 and 20), but rather in a relatively low
 383 abundance (3.07 to 8.23%). Similarly, the sample inoculated with SAM 6 harbored *Weissella*
 384 sp. (OTU 6) with a relative abundance of 20.36% on day 75. Moreover, other OTUs belonging
 385 to *Lactobacillus* sp. (OTUs 16, 32 and 518) appeared in samples inoculated with HM 53 on day
 386 75. Similar dynamics have been reported in many fermented vegetables, in particular carrot
 387 juice (Wuyts et al., 2018) and kimchi (Park et al., 2012) fermentations, during which the LAB
 388 communities shift from less acid tolerant (e.g. *Leuconostoc*) to more acid tolerant species (e.g.
 389 *Lactobacillus*). Another particular observation is that OTUs assigned to the genus *Clostridium*
 390 (OTU 12 and 45) appeared on day 75 for the fermentation inoculated with FX 15 and for the

control, respectively. On day 75, *Bifidobacterium* (OTU 4) appeared on all samples, except SAM 6. Species of this genus are known as probiotics (Tham et al., 2012).

As shown in Table 4, the OTU richness for the inoculated samples ranged from 35 to 65 OTUs, with an average of 50.6 ± 10.3 OTUs per sample. The richness varied between 73 and 212 OTUs for the uninoculated samples. Statistical analysis showed no difference ($p > 0.05$) in the Chao1 index between inoculated and uninoculated samples on days 0, 7 and 15, indicating that inoculating enset biomass with LAB starters does not affect the bacterial species richness for the first 15 days. The coverage ranged between 36.4% and 80.5%, suggesting that in some cases there were unseen OTUs. Furthermore, the Shannon-Wiener diversity indices increased from day 0 to day 75. However, the diversity decreased from day 7 to day 15 for the uninoculated samples and increased at day 75. Moreover, the Shannon-Wiener indices of inoculated samples were significantly ($p = 0.001$) lower than the uninoculated samples at day 15 and onwards. Given that a lower Shannon-Wiener index indicates a lower species diversity (Di Bitetti, 2000), inoculated samples had the lowest microbial diversity. This can most probably be explained by the fact that the inoculated samples had lower pH values, which inhibits the growth of a number of microorganisms and hence reduces diversity (Gabaza et al., 2019).

4. Conclusions

In this study, 158 presumptive LAB strains were isolated from fermenting enset and traditional starter material (i.e. produced in local households) with the objective to select starter cultures. The isolates were characterized and screened for their ability to degrade starch, to use enset as carbon source, to grow at pH 4, at different temperatures and salt concentrations. Based on these criteria, three strains, being *Lactobacillus plantarum* FX 15, *Lactobacillus plantarum*

SAM 6 and *Leuconostoc mesenteroides* HM 53 were selected for fermentation under field conditions. All three strains reduced the pH faster and to a lower end value than the uninoculated fermentation. More importantly, SAM 6 appeared to be more suitable as a starter culture for ensilage fermentation, mainly due to its fast pH reduction and colonization of the ensilage mass. Next steps in the research consist of elucidation of the proximate composition of ensilage fermented with the three strains and further upscaling of the fermentation with involvement of the local farmers. This will also require the cultivation of the strains on a larger scale to provide sufficient inoculum.

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Figure captions

Figure 1: Growth curves for the eight pure LAB cultures in MRS broth for 24 h at 30°C (except for E and F). Spectrophotometric OD values are displayed in function of time at pH 4 (A) using broth supplemented with 2% NaCl (B), 4% NaCl (C), or 6% NaCl (D), and at 10°C (E) or 45°C (F). The error flags indicate standard deviations.

—■— FX 15 —●— FX 36 —■— HM 53 —▲— SAM 6 —■— FX 17 —■— FX 2 —■— GM 69 —■— SAM 7

Figure 2: pH (A) and LAB counts (B) during starter culture fermentation of enset in Falcon tubes (laboratory scale) incubated at 30°C for 15 days: eight strains and one control, and pH (C) and moisture contents (D) of enset fermented in sauerkraut jars in function of fermentation time in field conditions: three strains and one control.

—■— FX 15 —●— FX 36 —■— HM 53 —▲— SAM 6 —■— FX 17 —■— FX 2 —■— GM 69 —■— SAM 7 —■— Control

Figure 3: Relative abundance (%) of bacterial phyla during fermentation of enset with *Lactobacillus plantarum* (FX 15 and SAM 6) or *Leuconostoc mesenteroides* (HM 53) and for the uninoculated control sample. Phyla with a relative abundance below 3% are grouped together in “Other phyla”. Data are the mean of two sequenced extracts per sample (on days 0, 7, 15, 30 and 75).

Figure 4: Relative abundance (%) and dynamics of the bacterial community composition of fermenting enset without inoculation (control) and inoculated with *Lactobacillus plantarum* (FX 15 and SAM 6) or *Leuconostoc mesenteroides* (HM 53). OTUs with a

550 relative abundance below 3% are grouped together in “Other OTUs”. Data are the
551 mean of two sequenced extracts per sample (on days 0, 7, 15, 30 and 75).

Table 1

Biochemical properties of 158 MRS grown isolates obtained from fermented enset and traditional starters

Biochemical test	Desired characteristics	Number of isolates with desired characteristics
Gram staining	+	154 (97.47%)
Catalase test	-	155 (98.10%)
Spore formation	-	157 (99.37%)
Indole test	-	154 (97.47%)
Acid production from glucose	+	146 (92.41%)
Starch hydrolysis	+	17 (10.76%)
Sucrose utilization	+	91 (57.56%)
Lactose utilization	+	142 (89.87%)
Glucose utilization	+	146 (92.41%)
Growth in enset broth	+	8 (5.06%)

Table 2

Lactic acid bacteria species identified by *pheS*, *rpoA* and 16S rRNA gene sequencing

Isolates code	Species name	Source
FX 2	<i>Lactobacillus plantarum</i>	One day fermented enset
FX 15	<i>Lactobacillus plantarum</i>	One week fermented enset
FX 17	<i>Lactobacillus plantarum</i>	One week fermented enset
FX 36	<i>Lactobacillus plantarum</i>	One month fermented enset
HM 53	<i>Leuconostoc mesenteroides</i>	Enset traditional starter from Hadiya zone
GM 69	<i>Lactobacillus plantarum</i>	Enset traditional starter from Gurage zone
SAM 6	<i>Lactobacillus plantarum</i>	Enset traditional starter from Segen zone
SAM 7	<i>Lactobacillus plantarum</i>	Enset traditional starter from Segen zone

Table 3

Microbial counts during fermentation of onset in jars under field conditions

Inoculant	Microbial group	Microbial counts (log cfu/g)				
		Day 0	Day 7	Day 15	Day 30	Day 75
Control	Total viable aerobic counts	7.40 ± 0.44 ^{bA}	8.89 ± 0.03 ^{aC}	8.56 ± 0.19 ^{aA}	7.85 ± 0.13 ^{bA}	7.83 ± 0.00 ^{bA}
	Lactic acid bacteria	5.42 ± 0.03 ^{cB}	8.74 ± 0.06 ^{aC}	8.48 ± 0.15 ^{aA}	7.56 ± 0.08 ^{bA}	7.55 ± 0.16 ^{bAB}
	Enterobacteriaceae	6.75 ± 0.03 ^{aA}	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b
	Yeasts and molds	4.88 ± 0.02 ^{aA}	4.81 ± 0.04 ^{aA}	4.66 ± 0.02 ^{abA}	3.94 ± 0.66 ^{bcA}	3.69 ± 0.07 ^{cA}
	<i>Clostridium</i> spores	3.02 ± 0.03 ^{cA}	2.48 ± 0.06 ^{dA}	2.62 ± 0.01 ^{dA}	4.12 ± 0.04 ^{aA}	3.62 ± 0.13 ^{bA}
FX 15	Total viable aerobic counts	7.92 ± 0.22 ^{cA}	9.04 ± 0.05 ^{aB}	8.39 ± 0.03 ^{bA}	7.85 ± 0.03 ^{cA}	7.85 ± 0.02 ^{cA}
	Lactic acid bacteria	7.34 ± 0.04 ^{dA}	9.00 ± 0.01 ^{aB}	8.35 ± 0.01 ^{bAB}	7.74 ± 0.13 ^{cA}	7.45 ± 0.13 ^{dB}
	Enterobacteriaceae	6.80 ± 0.18 ^{aA}	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b
	Yeasts and molds	4.60 ± 0.04 ^{aA}	4.44 ± 0.06 ^{bB}	2.70 ± 0.01 ^{cB}	2.77 ± 0.06 ^{cB}	2.65 ± 0.04 ^{cB}
	<i>Clostridium</i> spores	2.96 ± 0.03 ^{aA}	2.61 ± 0.05 ^{abA}	2.45 ± 0.26 ^{bA}	2.36 ± 0.11 ^{bC}	2.69 ± 0.11 ^{abC}
SAM 6	Total viable aerobic counts	7.90 ± 0.06 ^{cA}	9.09 ± 0.03 ^{aB}	8.41 ± 0.04 ^{bA}	7.67 ± 0.07 ^{dAB}	7.78 ± 0.06 ^{cdA}
	Lactic acid bacteria	7.39 ± 0.06 ^{dA}	9.10 ± 0.01 ^{aA}	8.33 ± 0.01 ^{bAB}	7.27 ± 0.07 ^{eB}	7.70 ± 0.06 ^{cA}
	Enterobacteriaceae	6.90 ± 0.02 ^{aA}	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b
	Yeasts and molds	4.74 ± 0.10 ^{aA}	4.41 ± 0.11 ^{bB}	2.50 ± 0.05 ^{cC}	2.67 ± 0.04 ^{cB}	2.68 ± 0.02 ^{cB}
	<i>Clostridium</i> spores	2.94 ± 0.05 ^{aA}	2.65 ± 0.05 ^{bA}	2.32 ± 0.12 ^{cA}	3.04 ± 0.10 ^{aB}	2.61 ± 0.00 ^{bC}
HM 53	Total viable aerobic counts	8.18 ± 0.42 ^{bA}	9.35 ± 0.20 ^{aA}	8.27 ± 0.11 ^{bA}	7.59 ± 0.07 ^{cB}	7.78 ± 0.05 ^{bcA}
	Lactic acid bacteria	7.31 ± 0.04 ^{dA}	9.11 ± 0.01 ^{aA}	8.20 ± 0.12 ^{bB}	7.13 ± 0.06 ^{eB}	7.13 ± 0.06 ^{cA}
	Enterobacteriaceae	6.94 ± 0.09 ^{aA}	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b	<1.00 ± 0.00 ^b
	Yeasts and molds	4.67 ± 0.05 ^{aA}	4.33 ± 0.07 ^{bB}	2.56 ± 0.08 ^{cdC}	2.46 ± 0.06 ^{dB}	2.66 ± 0.01 ^{cB}
	<i>Clostridium</i> spores	3.00 ± 0.02 ^{aA}	2.39 ± 0.28 ^{bA}	2.39 ± 0.11 ^{bA}	3.02 ± 0.10 ^{aB}	3.16 ± 0.05 ^{aB}

Data are the mean of three samples, one from each jar per treatment ± standard deviation.

^{a,b,c,d} 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 4

Diversity indices for samples subjected to metagenomics analysis.

Inoculant	Sampling day	Diversity index			
		Observed richness	Chao1 ¹	Coverage (%) ²	Shannon – Wiener ³
Control (None)	0	212.0 ± 212.1 ^{aA}	282.1 ± 266.1 ^{aA}	71.5 ± 7.7 ^{aA}	2.1 ± 1.5 ^{aA}
	7	196.5 ± 207.2 ^{aA}	289 ± 330.9 ^{aA}	78.1 ± 8.8 ^{aA}	2.6 ± 0.5 ^{aA}
	15	50.0 ± 2.8 ^{aA}	78.4 ± 12.1 ^{aA}	64.2 ± 6.3 ^{aA}	2.0 ± 0.0 ^{aA}
	30	55.5 ± 10.6 ^{aA}	74.7 ± 19.3 ^{aB}	75.0 ± 5.2 ^{aA}	2.3 ± 0.1 ^{aA}
	75	73.0 ± 4.2 ^{aA}	83.9 ± 9.9 ^{aA}	87.3 ± 5.3 ^{aA}	2.5 ± 0.0 ^{aA}
FX 15	0	55.5 ± 9.2 ^{aA}	75.1 ± 19.9 ^{aA}	74.9 ± 7.7 ^{aA}	1.3 ± 0.0 ^{eA}
	7	39.5 ± 2.1 ^{aA}	51.5 ± 3.5 ^{aA}	76.7 ± 1.1 ^{aA}	1.6 ± 0.0 ^{dA}
	15	58.0 ± 7.1 ^{aA}	137.4 ± 48.6 ^{aA}	44.1 ± 10.4 ^{aA}	1.6 ± 0.0 ^{cC}
	30	57.5 ± 6.4 ^{aA}	84.3 ± 1.0 ^{aAB}	68.2 ± 6.7 ^{aA}	2.1 ± 0.0 ^{bB}
	75	39.5 ± 0.7 ^{aC}	58.2 ± 23.8 ^{aA}	74.3 ± 31.5 ^{aA}	2.3 ± 0.0 ^{aB}
SAM 6	0	65.0 ± 8.4 ^{aA}	104.8 ± 11.7 ^{abA}	61.9 ± 1.2 ^{abA}	1.3 ± 0.0 ^{aA}
	7	48.5 ± 2.1 ^{aA}	74.9 ± 1.1 ^{abA}	64.7 ± 1.9 ^{abA}	1.8 ± 0.0 ^{bA}
	15	56.0 ± 7.1 ^{aA}	78.9 ± 15.5 ^{abA}	71.4 ± 5.1 ^{abA}	1.8 ± 0.0 ^{bB}
	30	63.5 ± 3.5 ^{aA}	117.9 ± 11.5 ^{aAB}	54.3 ± 8.3 ^{bAB}	2.0 ± 0.0 ^{cB}
	75	55.0 ± 0.0 ^{aB}	68.9 ± 8.6 ^{bA}	80.5 ± 10.1 ^{aA}	2.3 ± 0.0 ^{dB}
HM 53	0	58.0 ± 12.7 ^{aA}	95.3 ± 11.3 ^{abA}	60.6 ± 6.2 ^{aA}	1.3 ± 0.0 ^{aA}
	7	44.0 ± 2.8 ^{aA}	72.7 ± 10.7 ^{bA}	61.5 ± 13.0 ^{aA}	1.6 ± 0.0 ^{bA}
	15	41.5 ± 0.7 ^{aA}	72.0 ± 12.7 ^{bA}	58.6 ± 11.3 ^{aA}	1.7 ± 0.0 ^{bC}
	30	49.0 ± 2.8 ^{aA}	134.7 ± 15.9 ^{aA}	36.4 ± 2.2 ^{aB}	1.8 ± 0.0 ^{bC}
	75	34.5 ± 3.5 ^{aC}	52.5 ± 9.2 ^{bA}	61.7 ± 4.8 ^{aA}	2.2 ± 0.0 ^{cB}

Data are the mean values of two sequenced extracts ± standard deviations. ¹Chao1 index: the total number of OTUs estimated for infinite sampling (Chao, 1984). ²Coverage was determined by using the equation (observed richness/Chao1 estimate) X 100. ³Shannon-Wiener index: index to characterize species diversity based on species richness as well as their relative abundance. A higher value means more diversity (Shannon, 1948). ^{a,b,c} Different superscripts within the same column and within the same treatment for the different sampling days indicate significant differences (p < 0.05). ^{A,B,C} Different superscripts within different treatments, but same sampling day indicate significant differences (p < 0.05).

Highlights

- In Ethiopia, enset is fermented into kocho which is consumed as daily bread
- Lactic acid bacteria were isolated from enset plants and artisanal starter material
- The amylolytic activity and growth of the isolates in enset extract were determined
- Potential starter cultures for enset fermentation were selected and identified
- They improved the pH reduction and microbial composition during field fermentation

Conflict of interest

The authors declare no conflict of interest.